

# **Lymphocytes and Cytokines in Ocular Fluids in Human Intraocular Inflammation**

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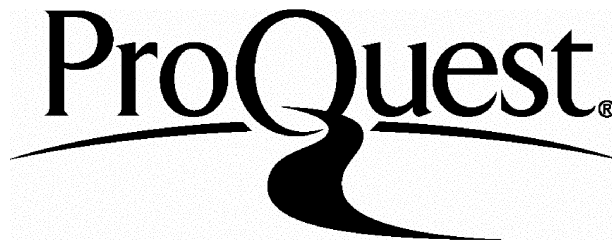
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## ABSTRACTS

Many forms of uveitis have an unknown aetiology but are believed to be due to dysregulation of the immune system either locally in the eye or as part of a systemic disorder. **Purpose:** To ascertain the cellular phenotypes and cytokine pattern in the AH of patients with uveitis and pattern of cytokine production by T cells infiltrating the intraocular compartment. **Methods:** The AH and PB of patients with different types of uveitis (n=59) and healthy controls (n=10) were characterised according to the cellular phenotypes by three colour flow cytometry, and levels of T cell-derived and related cytokines quantitated by ELISAs. T cell lines were obtained by expansion of T cells in the VH and PB of FHC and IU patients and the *ex vivo* cytokine production by these cell lines determined. **Results:** There was a selective elevation of CD4<sup>+</sup> T cells in the AH compared to PB in all types of uveitis with the exception of FHC, where there was a selective increase in CD8<sup>+</sup> T cells. There was a significantly lower level of regulatory cytokine IL-10 in the AH of uveitis compared to control patients. T cell lines derived from the VH of patients with FHC and IU showed high production of type-1 cytokines (IL-2, IFN $\gamma$ ) and low production of type-2 cytokine, IL-4 following stimulation with a non-specific T cell mitogen, phytohaemagglutinin-A (PHA). The production of regulatory cytokine IL-10 was also low in these lines. **Conclusions:** The results further confirm that Th 1 cells are important in the pathogenesis of uveitis and that the presence of high level of IL-10 in the AH appears to have a protective effects against ocular morbidity. This knowledge is useful in planning future immunotherapeutic strategies to avoid use steroids and thus avoiding its well-known side effects.

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## **List of Abbreviations**

AC:	Anterior Chamber
ACAID:	Anterior Chamber Associated Immune Deviation
AH:	Aqueous Humour:
AICD:	Activation Induced Cell Death
APC:	Antigen Presenting-Cell
ARN:	Acute Retinal Necrosis
AS:	Ankylosing Spondylitis
AUS:	Anterior Uveitis Associated with Systemic Diseases
CBG:	Cortisol-Binding-Globulin
CD:	Cluster Differentiation
CI:	Confidence Interval
CLMF:	Cytotoxic Lymphocyte Maturation Factor
CMO:	Cystoid Macular Oedema:
CNS:	Central Nervous System
CSF	Cerebrospinal fluid
CTL:	Cytolytic T Lymphocytes
DTH:	Delayed Type Hypersensitivity
ELISA	Enzyme-Linked-Immunosorbent-Assay

EAU:	Experimental Autoimmune Uveitis
FACS:	Fluorescein Activated Cytofluorometry
FHC:	Fuchs' Heterochromic Cyclitis
FITC:	Fluorescein Isothiocyanate
gld:	generalised lymphoproliferative disease
HLA:	Human Leucocyte Antigen
HSV-1:	Herpes Simplex Virus-1
IAU:	Idiopathic Anterior Uveitis
IBD:	Inflammatory Bowel Disease
IFN $\gamma$ :	Interferon-Gamma
IL-1:	Interleukin 1
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-2:	Interleukin 2
IL-4	Interleukin 4
IL-6	Interleukin 6
IPU:	Idiopathic Panuveitis
IRBP:	Interphotoreceptor retinoid-binding protein
JRA:	Juvenile Rheumatoid Arthritis
KP:	Keratic Precipitates:

LAK:	Lymphokine-Activated Killer cells
LGL:	Large Granular Lymphocytes
lpr:	lymphoproliferation
LU:	Localised Uveitis
MAF:	Macrophage-activating-factor
MHC:	Major Histocompatibility Complex
MS	Multiple Sclerosis
NGF	Nerve Growth Factor
NK:	Natural Killer
NKSF:	Natural Killer cell Stimulatory Factor
PB:	Peripheral Blood
PE:	Phycoerythrin
PerCP:	Peridin Chlorophyll Protein
PMN:	Polymorphonuclear leucocytes
PS:	Posterior Synechiae
SEM	Standard Error Of Mean
SU:	Uveitis associated with systemic disease
TCR:	T cell receptor
TGF- $\beta$ :	Transforming Growth Factor -Beta
TNF $\alpha$ :	Tumour Necrosis Factors

VH:	Vitreous Humour
VIP:	Vasoactive-Intestinal-Peptide

## **INTRODUCTIONS**

### **1.1. GENERAL IMMUNOLOGY**

The term immunity is derived from the Latin word *immunitas*, which referred to the exemption from various civic duties and legal prosecution offered to Roman senators during their term of office. This term was initially referred to as protection from disease, and more specifically, infectious disease. However it is now known that many of the mechanisms of resistance to infections are also involved in the individual's response to non-infectious foreign substances. Furthermore, mechanisms that normally protect individuals from infections and eliminate foreign substances are themselves capable of causing tissues injury and disease in some situations. Therefore a more complete and modern definition of immunity is a reaction to foreign substances, including microbes, as well as macromolecules such as proteins and polysaccharides, without implying a physiologic or pathologic consequence of such a reaction. The coordinated response to the introduction of foreign substances by the cells and molecules of the immune system is the immune response.

#### **1.1.1. Innate and Specific Immunity**

The immune system is divided into innate and specific immunity. Innate immunity provides the early line of defence against microbes. The principal components of the innate immunity are (1) physical and chemical barriers, such as epithelia and antimicrobial substances produced at epithelial surfaces; (2) blood proteins, including members of the complement system and other mediators of inflammation; and (3) phagocytic cells (neutrophils, macrophages) and other leucocytes, such as NK cells.



In contrast to innate immunity, adaptive or specific immunity is a more highly evolved defence mechanism. The essential distinguishing features of adaptive immunity are the very high degree of specificity and the development of memory, both of which contribute to the immunity that usually follows a first attack of an infectious disease and can also be induced by vaccination.

### **1.1.2. Lymphocytes**

These are cells that specifically recognise and respond to foreign antigens. They can be separated into T and B-lymphocytes. T lymphocytes originate in the bone marrow and migrate to the thymus where they undergo a process of selection to ensure tolerance to self-antigens, which are normal constituents of the body and develop the capacity to recognise and react with non-self antigens. This process is called T cell education. The identification of appropriate antigens is made possible by the presence of special recognition molecules on their surface, which are called T cell receptor (TCR). From the thymus, they travel through the blood stream to the periphery where they form the T cell repertoire.

The principal functions of T lymphocytes are to regulate immune responses to protein antigens and to serve as effector cells for the elimination of intracellular microbes. The main effector functions of T lymphocytes are cytokine production and cytotoxicity. These functions are generally attributed to separate T cell subpopulations, the helper and cytolytic (cytotoxic) T cells, expressing different surface markers being CD4 and CD8 respectively.

Helper T cells function as pivotal modulators of the immune response, helping B cells in the production of antibody and augmenting cell-mediated reactions through further recruitment of immunoreactive cells. Cytolytic T

lymphocytes (CTL) lyse cells that produce foreign antigens such as cells infected by viruses and other intracellular microbes.

B cells develop in the bone marrow and do not pass through the thymus, but directly enter the lymph node and the spleen through the blood stream and circulate in the lymphatic network. They will continue to circulate between secondary lymphoid tissues until they encounter an antigen that their receptor can recognise or they die.

### **1.1.3. Phagocytes**

These are of two basic kinds i.e. the monocytes/macrophages and polymorphonuclear granulocytes. The latter may be divided into neutrophils, basophils, and eosinophils. These three types of cells have distinct effector functions. The most numerous are the neutrophils, also called polymorphonuclear leucocytes (PMNs) which constitute the majority of the leucocytes in the bloodstream. They originate in the bone marrow and travel through the blood stream but unlike the lymphocytes they do not recirculate. Instead they make a one way journey to the tissues particularly areas of infection. On encountering a foreign material they will phagocytose and destroy it internally before they eventually die.

All the cells of mononuclear phagocyte system originate in the bone marrow and after maturation and activation can achieve varied morphologic forms. Monocytes are an incompletely differentiated cell type that first enters the PB after leaving the bone marrow. Once they settle in the tissues, these cells mature and become macrophages. Macrophages serve three important functions. Firstly they serve as antigen presenting cells (APCs) for T cells and secondly as

inflammatory effector cells with their phagocytic ability, and finally as regulators in other processes, such as fibrosis. In addition they produce cytokines that recruit other inflammatory cells to the site of immune response.

Dendritic cells are terminally differentiated bone marrow derived APCs. They are the most potent APCs for generating primary T cell dependent immune responses, and they are capable of initiating the activation of quiescent lymphocytes (Steinman, 1991). These cells travel from the bone marrow to fixed areas in the lymph node, spleen and tissues. Their function is to trap antigens circulating in the lymph, the blood and present it to the resident lymphocytes. They can also take up antigens from non-lymphoid tissue, migrate to lymphoid tissues and present the antigens to the lymphocytes that are able to bind to them by suitable receptors on their surface (Sting & Bergstressen, 1995)

## **1.2 T CELL SUBSETS**

Since their discovery more than a decade ago, CD4<sup>+</sup> T helper 1 (Th 1) and T helper 2 (Th 2) subsets have been implicated in the regulation of many immune responses. Th 1 and Th 2 patterns of cytokine production were originally described among mouse CD4<sup>+</sup> T cell clones ((Mosmann, et al., 1986; Cherwinski, et al., 1987) and later among human T cells (Del Prete, et al., 1991). Th 1 cells from mouse produce IL-2, IFN $\gamma$  and lymphotoxin also called TNF $\alpha$ , whereas Th 2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. Human Th 1 and Th 2 cells produce similar patterns, although the synthesis of IL-2, IL-6, IL-10 and IL-13 is not as tightly restricted to a single subset as in mouse T cells. Several other proteins are secreted both by Th 1 and Th 2 cells, including IL-13, TNF $\alpha$ , GM-CSF, and members of the chemokine families

Several factors, including the dose of antigen, the type of APC and the major histocompatibility complex (MHC) class II haplotype, influence the differentiation of naive CD4<sup>+</sup> T cells into specific Th subsets. However, the best-characterized factors affecting the development of Th subsets are cytokines themselves (Paul & Seder, 1994). For example, IFN $\gamma$  inhibits the differentiation and effector functions of Th2 cells, and can lead to a dominant Th1 response (Mosmann & Coffman, 1989). In addition the APC-derived cytokine IL-12 strongly drives the differentiation of Th1 cells ex vivo and in vivo, partly through its potent induction of IFN $\gamma$  production (Trinchieri, et al., 1992). In contrast, IL-4 strongly directs the development of Th2 cells, both ex vivo and in vivo and IL-10 inhibits cytokine synthesis by Th 1 cells (Fiorentino, et al., 1989). Therefore, reciprocal regulation occurs between the Th 1 and Th 2 cell subsets. This interaction and crossregulation may partly explain the strong biases towards Th 1 or Th 2 responses during many infections and autoimmune diseases in mice and humans (Tsicopoulos, et al., 1992).

Although initial expectations were that different APC types would selectively influence T cell differentiation, it is now known that dendritic cells, macrophages, and B cells are all capable of inducing differentiation of Th 1 or Th 2 cells in the presence of the appropriate cytokines (Beverly, et al., 1992; Mosmann, 1994; Sad & Mosmann, 1995).

The functions of Th 1 and Th 2 cells correlate well with their distinctive cytokines. Th 1 cells are involved in cell-mediated inflammatory reactions and several Th 1 cytokines activate cytotoxic and inflammatory functions (Mosmann & Coffman, 1989). Th 1 clones induce DTH reactions and IFN $\gamma$  is commonly

expressed at sites of DTH reactions (Tsicopoulos, et al., 1992). Th 2 cytokines facilitate antibody production, particularly Ig-E responses (Mosmann & Coffman, 1989), and also enhance eosinophil proliferation. Hence Th 2 responses are commonly associated with strong antibody and allergic responses.

The Th 1 and Th 2 patterns of cytokine secretion correspond to activated effector phenotypes generated during an immune response. They do not exist among naive T cells, or possibly even among long term memory cells. When first stimulated by antigen on APCs, naive CD4<sup>+</sup> T cells principally or entirely produce IL-2, and then differentiate into phenotypes that secrete other cytokines. Thus, Th 1 and Th 2 cells can both be derived from a single precursor cell.

Even though there has been many well-documented Th 1 and Th 2 responses, these are not the only cytokine pattern possible. T cells producing cytokines of both patterns have been called Th 0 cells (Mosmann & Coffman, 1989) and cells producing high amounts of TGF- $\beta$ 2 have been termed Th 3 (Chen, et al., 1994) which are thought to have regulatory role. At the single cell level among normal cells in short term culture, IL-4 and IL-5 are often co-expressed, and IL-4 and IFN $\gamma$  are normally exclusively expressed, and IFN $\gamma$  and IL-10 are co-expressed in some cells (Assenmacher, et al., 1994; Bucy, et al., 1995; Elson, et al., 1995; Openshaw, et al., 1995).

Although Th 1 and Th 2 cells are major sources of their respective cytokines many other cells within and outside the immune system also produce these cytokines. Natural killer cells (NK) produce IFN $\gamma$  and TNF $\alpha$ , and contribute to Th 1-like responses. Also IL-4 and other Th 2 cytokines are synthesised by mast cells, B cells and basophils (Seder, et al., 1991; Yoshimoto & Paul, 1994). In

addition IL-10 is produced by macrophages, keratinocytes, and cytotrophoblast cells in the placenta (Mosmann, 1994). Thus several cell types may contribute to an overall Th 1 or Th 2 cytokine pattern, and it has been suggested that these responses should be described as 'type 1' and 'type 2' (Salgame, et al., 1991a). Type 1 and type 2 cytokine patterns have now been implicated in several immune responses concerning infections, allergy and autoimmunity (Sher & Coffman, 1992; Liblau, et al., 1995).

The different subsets have also been demonstrated among the CD8<sup>+</sup> T cells. The strongest evidence for the existence of different subsets of CD8<sup>+</sup> T cells comes from the finding that CD8<sup>+</sup> T cell clones specific for *Mycobacterium leprae*, derived from the skin biopsies of patients with leprosy, could be divided into two subtypes according to their cytokine secretion profile. (Mosmann & Moore, 1991; Salgame, et al., 1991a). Type 1 CD8<sup>+</sup> T cell clones which were derived from patients with tuberculoid leprosy, were cytotoxic, secreted IFN $\gamma$ , but not IL-4, and were restricted to MHC class I. However type 2 CD8<sup>+</sup> T cell clones derived from patients with lepromatous leprosy, suppress the killing of *Mycobacterium leprae* by *Mycobacterium leprae* specific CD4<sup>+</sup> T cell clones by secretion of IL-4, IFN $\gamma$ , IL-4, IL-5, and IL-10, but not IL-6 and were restricted by MHC class II. Hence the term Tc 1 and Tc 2 refers to subsets of CD8<sup>+</sup> T cells producing type 1 and type 2 cytokines respectively. CD8<sup>+</sup> Tc 2 cells are as cytotoxic as Tc1 cells in some (Croft, et al., 1994), but not other (Romagnani, et al., 1994) systems. This may depend on cytokine exposure prior to the cytotoxicity assay (Sad, et al., 1995). Tc 1 and Tc 2 cells both kill mainly by a

$\text{Ca}^{2+}$  /perforin-dependent mechanism, and to a lesser extent via Fas (Carter & Dutton, 1995).

### **1.3. ANTIGEN RECOGNITION**

#### **1.3.1. T Cell Recognition of Antigen**

A fundamental aspect of antigen recognition by helper T cells and cytotoxic T lymphocytes (CTLs) is that any one T lymphocyte is restricted to recognising a peptide antigen only when it is complexed to a single allelic form of an MHC molecule. This phenomenon is called MHC restriction. MHC restriction differs between T cells subsets where  $\text{CD4}^+$  T cells are class II restricted whereas  $\text{CD8}^+$  T cells are class I restricted.

##### **1.3.1.1. Antigen Presenting Cells (APC)**

APCs are a heterogenous population of leucocytes with exquisite immunostimulatory capacity, although cells other than leucocytes such as endothelium and epithelial cells can 'acquire' the ability to present antigens when stimulated by cytokines. APCs are found mainly in the skin, lymph node, spleen and thymus. Historically the term APC has most often been used to describe cells that present antigen to  $\text{CD4}^+$  helper T cells. It is also appropriate to describe target cells of CTL lysis as APCs as well, since CTLs also recognise peptide-MHC complexes on the surface of these target cells.

APCs are often subclassified into 'professional' (classical) APCs, which constitutively express MHC class II molecules on their surface which includes, dendritic cells, macrophages, and B cells (Unanue & Allen, 1987; Weaver & Unanue, 1990) and 'non-professional' cells, which acquire MHC class II expression under certain circumstances (non-classical APCs) (Peters, et al.,

1996). Because of their phagocytic capability, macrophages are probably most effective at processing particulate antigens, such as whole bacteria. B cells are likely more effective at processing soluble antigens, which are internalised in endocytic vesicles.

The nature of the APC initially presenting the antigen may determine whether responsiveness or tolerance ensues (Unanue & Allen, 1987). Thus presentation by competent APCs such as dendritic cells or activated macrophages, which express high levels of MHC class II as well as co-stimulatory molecules, results in highly effective T cell activation leading to proliferation and differentiation of T lymphocytes. However if antigen is presented to T cells by a 'non-professional' APC that is unable to provide co-stimulation, the unresponsiveness results producing a state of immunological tolerance (Umetsu, et al., 1986). This tolerance is specific, only affecting T cells that respond to a particular antigen, and is known as clonal anergy.

Research over the years has convincingly shown that resting T cells require at least two signals for induction of cytokine gene expression and cell proliferation. Signal 1 can be produced by ligation of the TCR/CD3 complex with antigenic peptides bound to appropriate MHC molecule. This signal provides the antigen specificity. By contrast signal 2 also called 'co-stimulatory' signal the name given to interactions that do not involve antigen-specific receptors. These events may involve either CD28-CD80/86 or CD40/CD40L interactions.

#### **1.3.1.2. Antigen Processing**

Antigen processing involves the proteolytic degradation of these protein into peptides, the binding of these peptides to newly assembled MHC molecules, and



the display of the peptide-MHC complexes on the APC surface for potential recognition by T cells (Ashwell & Klausner, 1990; Germain, 1994).

Professional APCs, which includes macrophages, B-lymphocytes, and dendritic cells need to internalise extracellular proteins into endosomes for processing by the class II MHC pathway (Unanue, 1992). The peptides generated from extracellular proteins then bind to the class II MHC molecules, and the trimeric complex (class II MHC chains of peptide) moves to the surface of the cell (Neefjes & Ploegh, 1992). On the other hand cytosolic proteins, usually synthesised in the cells, such as viral proteins, enter the class I MHC pathway of antigen presentation (York & Rock, 1996).

These pathways of MHC-restricted antigen presentation ensure that most of the body's proteins are screened for the possible presence of foreign antigens. The pathways also ensure that proteins from extracellular microbes are likely to generate peptides bound to class II MHC molecules for recognition by CD4<sup>+</sup> helper T cells, while proteins encoded by intracellular microbes generate peptides bound to class I MHC molecules for recognition by CD8<sup>+</sup> CTLs. Hence both normal self-proteins and foreign proteins are displayed by MHC molecules for surveillance by T lymphocytes.

#### **1.3.1.3. Cell-Mediated-Immunity**

Different types of cell-mediated immune responses may result from T cell recognition of antigen. Firstly in DTH, antigen-activated T cells secrete cytokines, which have several effects. Some cytokines such as TNF $\alpha$  and TNF $\alpha$  activate venular endothelial cells to recruit monocytes and other leucocytes from the blood at the site of antigen challenge. Other cytokines such as IFN $\gamma$  convert

the monocytes into activated macrophages that serve to eliminate the source of antigen. T cells that mediate DTH are usually  $CD4^+$  Th 1 cells, but cytokines produced by  $CD8^+$  T cells can initiate the same reaction. Microbe-laden macrophages become activated by T cell derived cytokines, and the physiologic purpose of the response is to promote killing of the intracellular microbes residing in phagolysosomes of macrophages. Although DTH reactions are targeted by T cells to be selective for invading organisms, they are still injurious to normal host tissues.

The second cell-mediated immune response is CTL responses to intracellular infections of non-phagocytic cells, such as viral infection, or infection by bacteria such as *Listeria monocytogenes*. (Doherty, et al., 1990). In addition CTLs are involved in acute allograft rejections and rejection of tumour cells.

CTL immune response begins with antigen-activated  $CD8^+$  T cells differentiating into functional CTLs following antigen recognition on target cells expressing specific antigen MHC complexes. CTLs are not fully differentiated when they exit from the thymus. Hence CTLs develop or differentiate from pre-CTLs. Pre-CTLs are T cells that are committed to the CTL lineage, have undergone T cell education in the thymus, and are already specific for a particular foreign antigen. These cells express CD3-associated  $\alpha\beta$  T cell receptors (TCRs) and CD8, but they lack cytolytic function. It is quite accurate to consider  $CD8^+$  T cells as CTLs as most  $CD8^+$  T cells are pre-CTLs. Pre-CTLs do not require a special microenvironment for differentiation and can develop within the infected or foreign tissue. The differentiation of pre-CTL into CTL

requires at least two separate kinds of signals. The first is specific recognition of antigen on a target cell, and the second signal may be provided either by co-stimulators expressed on professional APCs or by cytokines such as IL-2, IFN $\gamma$ , produced by helper T cells and IL-12 which is produced by macrophages.

There are two independent pathways mediating T cell cytotoxicity. These are the exocytosis of perforin containing granules on cognate target cells and the engagement of Fas on cognate or neighbouring target cells (Kagi, et al., 1994a; Kagi, et al., 1994b; Lowin, et al., 1994). In addition to these two effector mechanisms CTLs share with other effector T cells the capacity to transcribe and secrete cytokines and other proteins upon activation mostly IFN $\gamma$ , TNF $\alpha$ , and to a lesser degree IL-2.

The third cell-mediated-immunity is the initial response to viral infections where NK cells serve to eradicate infected cells prior to the appearance of specific CTLs. NK cells, stimulated by cytokines from antigen activated CD4<sup>+</sup> T cells, differentiate into lymphokine-activated killer cells (LAK). The fourth and final cell-mediated response is in helminthic and arthropod infection and in allergic inflammation, where antigen activated CD4<sup>+</sup> Th 2 cells secrete cytokines that activate mast cells and recruit and activate basophils and eosinophils.

## **1.4. CYTOKINES**

### **1.4.1. General Properties of Cytokines**

Cytokines are a family of protein mediators of both innate and specific immunity, which are produced during the effector phases of innate and specific immunity and function to mediate and regulate immune and inflammatory

responses. Their secretions are brief and self-limited and these mediators are not stored as preformed molecules. The synthesis of cytokines is initiated by new gene transcription and once synthesised, cytokines are usually rapidly secreted resulting in a burst of cytokine release as needed. Many individual cytokines are produced by multiple diverse cell types and they act upon many different cell types, a property called pleiotropism. They also often have multiple different effects on the same target cell, which occur simultaneously or at different time frames. In addition cytokine actions are often redundant where many functions originally attributed to one cytokine proved to be shared properties of several different cytokines. One important property of cytokine is the ability of one cytokine to influence the synthesis and action of another cytokine. This may lead to positive and negative regulatory mechanisms for immune and inflammatory responses. Cytokines also like other polypeptide hormones initiate their action by binding to specific receptors on the surface of target cells. The relevant target cell may be the same cell that secretes the cytokine (autocrine action), a nearby cell (paracrine action), or like true hormones, a distant cell that is stimulated via cytokines that have been secreted into circulation (endocrine action).

Receptors for cytokines often show very high affinity for their ligands and this results in very small quantities of cytokines being able to elicit a biologic effect. The expression of these receptors is regulated by specific signals which maybe another cytokine or even the same cytokine that binds to the receptor, permitting positive amplification or negative feedback.

#### **1.4.2. Tumour Necrosis Factor Alpha (TNF $\alpha$ )**

TNF $\alpha$  has a molecular mass of 17 kD and was purified and sequenced, (Aggarwal, et al., 1985), and its encoding gene cloned (Wang, et al., 1985) in the mid-80s. Since then, several biological properties of the cytokine have been demonstrated, in addition to the induction of cachexia and lysis of tumour cells that originally led to its identification. The major source of TNF $\alpha$  is activated monocytes/macrophages. TNF $\alpha$  effects are transmitted via crosslinking of the membrane-bound receptor molecules TNF receptor I (TNFRI, p55) and TNFRII (p75) (Bazzoni & Beutler, 1996). Soluble TNFR p55/p75 are naturally occurring TNF $\alpha$  inhibitors (Olsson, et al.,).

TNF $\alpha$  has a wide range of activities and affects the function of multiple target cells (Beutler & Cerami, 1987). TNF $\alpha$  is a major mediator of cell adhesion and chemotaxis and largely contributes to the regulation of cell migration during inflammation. Its effects closely resemble IL-1 and may in part be mediated by IL-1. Hence TNF $\alpha$  induces IL-1 from inflammatory cells. Other major effects include the triggering of lipid mediators of inflammation, induction of reactive oxygen radical, and the production and release of pro-inflammatory enzymes from cells (Beutler & Cerami, 1989). In addition TNF $\alpha$  stimulates mononuclear phagocytes and other cell types to secrete chemokines that contribute to leucocyte recruitment. When present in high concentration in blood TNF $\alpha$  acts as an endocrine hormone with the accompanying effects of wasting disease (cachexia) and bone resorption (Beutler & Cerami, 1987). In addition it is an endogenous pyrogen and stimulates hepatocytes to produce acute phase proteins.

Intravitreal injections of microgram amounts of TNF $\alpha$  result in protein leakage and mononuclear cell infiltration. However several features suggest that TNF $\alpha$  work indirectly through the stimulation of other chemical mediators. First the onset of TNF $\alpha$  induced infiltration is slightly delayed (24 to 48 hours) and longer lasting compared to that induced by injection of other cytokines. The infiltrate consists of greater density of monocytes and less neutrophils (Rosenbaum, et al., 1988; Fleisher, et al., 1990). Second intracorneal injection of TNF $\alpha$  induces IL-8 production by stromal keratocytes (Elner, et al., 1991). Pharmacologic blockade of the effects of intravitreal TNF $\alpha$  has been attempted with cyclooxygenase inhibitors or PAF antagonists. Treatment with both antagonist was most effective whereby there was a decrease in the vascular permeability without profound effect on cell infiltrate. TNF $\alpha$  induces inflammation indirectly via the activation of lipid mediators (Fleisher, et al., 1991).

Although the detection of TNF $\alpha$  in ocular fluids has been difficult, it has been detected by cytoplasmic immunostaining within infiltrating lymphocytes obtained from vitrectomy specimens in eye with AIDS-associated retinitis (Hofman & Hinton, 1992).

#### **1.4.3. Interleukin 1 (IL-1)**

This cytokine is present in two forms, IL-1 $\alpha$  and IL-1 $\beta$  (each of approximately 17 kD), together with TNF $\alpha$  are the prototype proinflammatory cytokines being one of the major cytokines that participate in the inflammatory responses (Dower, 1991). It was first defined as a polypeptide derived from mononuclear phagocytes that act as a costimulator of T cell activation. However it is now clear

that the principal function of IL-1, similar to that of TNF $\alpha$ , at low concentration, is a mediator of local inflammation. Many cell types produce IL-1 however the main source of IL-1 is activated mononuclear phagocytes (Dinarello & Wolff, 1993). Its production by mononuclear phagocytes can be triggered by bacterial products such as LPS, and by TNF $\alpha$ , and by IL-1 itself, and by contact with CD4<sup>+</sup> T cells. Both TNF $\alpha$  and IL-1 can be found in the circulation following bacterial sepsis where they can act as endocrine hormones.

Direct injection of IL-1 into the eye produces altered vascular permeability and acute cellular infiltration of the uvea and retina with neutrophils, and later with monocytes (Rosenbaum, et al., 1987; Kulkarni & Mancino, 1993). The inflammatory response resolves within several days. IL-1 is also a potent stimulator of ocular prostaglandin and platelet activating factor (PAF) activation (Kulkarni & Mancino, 1993). Intravitreal injection with neutralising antibody to IL-1 can block its effects up to 6 hours after cytokine injection, suggesting that the early inflammatory effects of IL-1 are the consequence of direct actions of the cytokine (Martiney, et al., 1990).

Recently naturally occurring inhibitors of IL-1 have been identified i.e. IL-1 receptor antagonist (IL-1 ra), (Dinarello & Wolff, 1993) which is structurally homologous to IL-1 and binds to IL-1 receptor but biologically inactive. Protection from the infiltration and protein leakage can be demonstrated with intravitreal injection of the IL-1 ra or soluble IL-1 only when the eye is pre-treated and not if this antagonist are injected after the onset of the intraocular inflammation (Rosenbaum & Boney, 1991). The antagonist had little effect, however, upon LPS induced uveitis suggesting that other cytokines in addition to

IL-1 are contributing to inflammation. Interestingly some of the effects of intravitreally injected IL-1 can be blocked by pharmacologic inhibitors of PAFs and prostaglandins (Rubin & Rosenbaum, 1988).

#### **1.4.4. Interleukin 6 (IL-6)**

IL-6 is a cytokine of approximately 26 kD that is synthesised by mononuclear phagocytes, vascular endothelial cells, fibroblasts, and other cells in response to IL-1, and to a lesser extent  $\text{TNF}\alpha$ . It can also be made by some activated T cells. IL-6 is involved in the regulation of immune responses, the acute phase response and haematopoiesis (Akira, et al.,) IL-6 causes hepatocytes to synthesise several plasma proteins such as fibrinogen that contribute to acute phase response. IL-6 also serves as a growth factor for activated B cells late in the sequence of B cell differentiation. Intravitreal injection of IL-6 produces altered vascular permeability and severe uveal and retinal infiltration with PMNs (Hoekzema, et al., 1991; Hoekzema, et al., 1992). However repeated injections with IL-6 result in desensitisation and unresponsiveness (Hoekzema, et al., 1990). AH levels of IL-6 correlate with clinical inflammation in LPS-induced uveitis is detected at high concentration in many forms of human uveitis, especially FHC (Murray, et al., 1990a; Murray, et al., 1990b). It is also an important mediator in other intraocular diseases, such as proliferative vitreoretinopathy (Limb, et al., 1991).

#### **1.4.5. Interleukin-12 (IL-12)**

IL-12 was originally identified as a macrophage-derived activator of NK cell cytolytic function, although it is now appreciated to be a potent inducer of  $\text{IFN}\gamma$  production by T cells as well as NK cells. Active IL-12 exists as a disulphide-linked heterodimer of 35 kD (p35) and 40 kD (p40) subunits. Many cells appear



to synthesise p35 but p40 synthesis is largely restricted to activated mononuclear phagocytes and dendritic cells. (Trinchieri, et al., 1996). Early in the infection, NK and T cells produce IFN $\gamma$ , which contributes to phagocytic cell activation and inflammation. In addition IL-12 contributes to optimal IFN $\gamma$  production and to proliferation of differentiated Th 1 cells in response to antigen. The early preference expressed in the immune response depends on the balance between IL-12, which favours Th 1 response and IL-4, which favours Th 2 responses. IL-12 is classified as a mediator of innate immunity because it links between macrophage activation by microbes to the development of NK cell effector functions.

#### **1.4.6 Interleukin-10 (IL-10)**

IL-10 is an 18kD cytokine produced by activated macrophages, some lymphocytes, and some non-lymphocytic cell types (e.g. keratinocytes). Interleukin 10 (IL-10) was originally characterized as a factor generated by mouse T helper 2 (Th2) cells that inhibits cytokine synthesis by Th1 cells (Fiorentino, et al., 1991). Subsequently, IL-10 was shown to downregulate the synthesis of a broad spectrum of proinflammatory cytokines by monocytes/macrophages (Moore, et al., 1993) and neutrophils (Casatella, et al., 1993), and to promote the release of the IL-1 receptor antagonist (IL-1ra) by these cells (Casatella, et al., 1994). Such *ex vivo* data led to the proposal that IL-10 might inhibit inflammatory processes mediated by Th1 cells *in vivo*. Indeed, systemic administration of IL-10 in rodents suppresses delayed-type hypersensitivity experimental autoimmune encephalomyelitis and T-cell-mediated inflammatory bowel disease (Powrie, et al., 1994).

IL-10 also reduces expression of class II MHC molecules and expression of costimulators, e.g. B7-1 and B7-2. The net effect is to inhibit both innate and T cell mediated specific immune inflammation. In addition, IL-10 likely contributes to regulating proliferation and differentiation of B cells, mast cells, and thymocytes (Moore, et al., 1993).

#### **1.4.7. Interleukin-2 (IL-2)**

This is the first of the series of lymphocytotropic hormones to be recognised and completely characterised, and is the principal cytokine responsible for progression of T lymphocytes from the G1 to S phase of the cell cycle (Smith, 1988). IL-2 is produced by CD4<sup>+</sup> T cells and in lesser quantities by CD8<sup>+</sup> T cells. Normally, IL-2 is transcribed, synthesised, and secreted by T cells upon activation by antigens. Secreted IL-2 is a 14 to 17 kD glycoprotein encoded by a single gene on chromosome 4 in humans. The action of IL-2 on T cells is mediated by binding to IL-2 receptor proteins (Theze, et al., 1996). IL-2 function both in autocrine and paracrine manner and is the major growth factor for T lymphocytes. The quantity of IL-2 synthesised by CD4<sup>+</sup> T cells is an important determinant of the magnitude of T cell-dependent immune responses. This cytokine also stimulates the synthesis of other T cell-derived cytokines such as IFN $\gamma$  and TNF $\alpha$ .

However IL-2 may act a death factor for antigen activated T cells, promoting activation induced cell death (AICD) or apoptotic cell death following stimulation of previously activated T cells through TCR/CD3 complex that is an important mechanism in immune regulation. It appears paradoxical for IL-2 to function both as growth factor and a death factor, but these actions occur on T

cells at different stages of activation and the overall effect of IL-2 also depend on the presence of other signals (e.g. Fas-ligand expression for activation-induced-cell-death). High concentrations of IL-2 enhance the expression of Fas-ligand on antigen-stimulated T cells and the development of sensitivity to Fas-mediated apoptosis. Thus IL-2 is both a growth factor for T cells and a feedback regulator of T cell responses (Kroemer, et al., 1991).

#### **1.4.8. Interleukin-4 (IL-4)**

IL-4 was initially identified as a helper T cell-derived cytokine of approximately 20 kD that stimulated the proliferation of mouse B cells in the presence of anti-Ig antibody and caused an enlargement of resting B cells and increased expression of class II MHC molecules. It is now known that the main physiologic function of IL-4 is as a regulator of Ig-E and mast cell/eosinophil-mediated immune reactions (Kuhn, et al., 1991). The principal sources of IL-4 are CD4<sup>+</sup> T lymphocytes specifically of the Th 2 subset (Paul, 1991). Activated mast cells and basophils, as well as some CD8<sup>+</sup> T cells are also capable of producing IL-4. Currently IL-4 production is used as a criterion for classifying CD4<sup>+</sup> T cells into this subset. (Cohen, 1995)

In the production of Ig-E IL-4 is the principal cytokine that stimulates switching of B cells to this heavy chain isotype (Rothman, 1993). Since Ig-E is the principal mediator of immediate hypersensitivity (allergic) reactions, an enhanced production of IL-4 is believed to be central to the development of allergies. IL-4 is a growth and differentiation factor for T cells, in particular for cells of the Th 2 subset. It promotes the development of Th 2 cells from naive T

cells stimulated with antigen and it also functions as an autocrine growth factor for differentiated Th 2 cells further promoting expansion of this subset.

#### **1.4.9. Interferon-gamma (IFN $\gamma$ )**

IFN $\gamma$  is a homodimeric glycoprotein containing two 21 to 24 kD subunits. It is produced by activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells, and by NK cells. Its transcription is directly initiated as a consequence of antigen activation and is enhanced by IL-2 and IL-12. IFN $\gamma$  produced by NK cell may function as a mediator of innate immunity and contribute to septic shock. It has important roles in immune regulation by acting on mononuclear phagocytes, T lymphocytes, B cells, NK cells, neutrophils, vascular endothelial cells, and some tissue cells.

IFN $\gamma$  is the principal cytokine that causes functional changes in mononuclear phagocytes rendering them more efficient in their phagocytic and APC function (Meltzer & Nacy, 1989). In addition IFN $\gamma$  increases class I MHC expression and causes a wide variety of cells to express class II MHC on a variety of cell types such as conjunctival fibroblasts (Harrison, et al., 1990). IFN $\gamma$  has a crucial role in promoting differentiation of CD4<sup>+</sup> T cells to the Th 1 subset and inhibit the proliferation of Th 2 cell in mice. These effects may be mediated by activating mononuclear phagocytes to secrete IL-12 and T cells to express functional IL-12 receptors (Trinchieri, 1995). In addition IFN $\gamma$  is also one of the cytokines required for the maturation of CD8<sup>+</sup> cytolytic T cell (CTLs).

One other effect that is important in inflammation is the effect on vascular endothelial cells, where IFN $\gamma$  promotes CD4<sup>+</sup> T lymphocyte adhesion and morphologic alterations that facilitate lymphocyte extravasation. The net effect of these varied activities of IFN $\gamma$  is to promote macrophage-rich

inflammatory reactions, while inhibiting Ig-E dependent eosinophil-rich reactions.

#### **1.4.10. Transforming Growth Factor Beta (TGF- $\beta$ )**

TGF- $\beta$  is a family of closely related molecules, encoded by distinct genes, commonly designated TGF- $\beta$ 1, TGF- $\beta$ 2, TGF- $\beta$ 3, TGF- $\beta$ 4 and TGF- $\beta$ 5 that has fundamental roles in cell growth and differentiation (Sporn, et al., 1987). As the name suggests, TGF- $\beta$  was initially defined by its ability to transform the phenotype of non-neoplastic cells in culture. Most mammalian cells including macrophages and lymphocytes (Sporn, et al., 1987), have the capacity to produce TGF- $\beta$ , and virtually all cell types express high affinity receptors for this cytokine (Sporn, et al., 1987).

Because of the many number of cells producing TGF- $\beta$  and the extensive biological actions, there is a great need for adequate control of its activity. This is achieved by its unique biology i.e. TGF- $\beta$  is secreted as a biologically inactive precursor (latent TGF- $\beta$ ) that must be activated extracellularly into the bioactive “mature” molecule although it is not known how lymphocytes achieve this, macrophages utilise at least two distinct cell surface receptors and plasmin for this conversion (Sporn, et al., 1987).

Wahl et al suggested that TGF- $\beta$  might be an important inflammatory mediator (Wahl, et al., 1989). When injected into inflamed joints, TGF- $\beta$  can exacerbate arthritis (Alkin, et al., 1990). However its immunosuppressive and antiinflammatory activities have been the focus of more recent studies (Kehrl, et al., 1991). Ex vivo, TGF- $\beta$  acts as a potent inhibitor of both T and B cell activation (Nathan & Sporn, 1991). It has also been shown to inhibit cytokine

production and lymphocyte proliferation by various culture systems for lymphocytes (Espevik, et al., 1987). It prevents macrophage activation and is a potent inhibitor of macrophage function (Tsunawaki, et al., 1988). The ultimate biologic role of TGF- $\beta$  is may be to downregulate inflammatory responses that involve T cells as immune effector cells.

In addition treatment of macrophages with TGF- $\beta$  alters the APC cell function of resident APCs in the ocular compartment (Wilbanks & Streilein, 1992a) contributing to immune-privilege. In addition, the level of active TGF- $\beta$  is decreased in the secondary or AH of inflamed eyes (Taylor, et al., 1992a) further suggesting its predominant role in immune-privilege.

#### **1.4.11. Interleukin-8 (IL-8) and the Chemokines**

IL-8 belongs to a family of polypeptide mediators called chemokines. Chemokines comprise a large family of structurally homologous cytokines, approximately 8 to 10 kD in size that share the ability to stimulate leucocyte motility (chemokinesis) and directed movement (chemotaxis). The name 'chemokines' is a contraction of chemotactic cytokines. IL-8 is primarily a potent chemotactic factor for PMNs and to a lesser extent, other granulocytes and lymphocytes. Most cells produce IL-8 in response to other exogenous signals (Colditz, et al., 1989; Holtkamp, et al., 1998). IL-8 and other chemokines have been detected in the AH of patients with acute anterior uveitis (Verma, et al., 1997). Levels of serum IL-8 are currently being investigated as an indicator of possible systemic disease in uveitis. Recently it has been shown that human retinal pigment epithelial (RPE) cells secrete IL-6 and IL-8 in a polarised fashion towards the choroid which may prevent damage to the adjacent fragile retinal

tissue (Holtkamp, et al., 1998). RPE cells are considered an important source of chemokines during uveitis and in *ex vivo* studies have shown This maybe one of the mechanism whereby these RPE cells limit the site and extent of posterior segment inflammation.

## **1.5. AUTOIMMUNITY**

Autoimmunity is the result of loss of self-tolerance against one's own, or autologous antigens called autoimmunity.

### **1.5.1. Mechanisms of self tolerance**

Unresponsiveness to self-antigens, or self-tolerance, is maintained by mechanisms that actively prevent the maturation or stimulation of potentially reactive self-reactive lymphocytes. This is an actively acquired process, in which self reactive lymphocytes are either prevented from becoming functionally responsive to self antigens or are inactivated after the encounter with these antigens (Kruisbeek & Amsen, 1996). This can occur at various stages of lymphocyte development and activation.

Central tolerance is the induction of tolerance in generative lymphoid organs as a consequence of immature self-reactive lymphocytes recognising self-antigens. In the periphery, mature T cells undergo selection to achieve peripheral tolerance. Those cells that recognize self-antigens are first activated then die by apoptosis (via AICD), leading to clonal deletion (Webb, et al., 1990). Another mechanism of- tolerance is clonal anergy, or functional inactivation without cell death. This occurs when mature T lymphocytes encounter self-antigens presented by co-stimulator deficient, resting APCs. Both deletion and anergy are induced by the binding of antigens to specific receptors. In addition functional

unresponsiveness may also be due to the induction of regulatory T cells that suppress the activation and effector functions of mature self-reactive lymphocytes.

### **1.5.2. Mechanisms of autoimmunity**

Autoimmunity develops secondary to multiple interacting factors that collectively lead to failure or breakdown of self-tolerance. The immunologic mechanism that may lead to autoimmunity include abnormalities in lymphocyte selection, mechanisms that overcome peripheral tolerance, polyclonal lymphocyte stimulation (antigen independent stimulation of self-reactive lymphocytes), and cross reactions between foreign and self antigens.

Tissue inflammation may cause structural alterations in self-antigens, and the formation of new determinants capable of inducing autoimmune reactions. Inflammation also can result in macrophage activation by locally produced cytokines, and if these cytokines stimulate the expression of co-stimulatory-stimulators, the result may be loss of peripheral tolerance leading to autoimmunity.

## **1.6. OCULAR IMMUNOLOGY**

### **1.6.1. Regional Immunology of the Eye**

The concept of regional immunity is based on the idea that distinct organs and tissues are represented by a unique range of pathogens to which each is especially vulnerable. Many immune effectors eliminate pathogens by nonspecific inflammatory mechanisms where the pathogens reside. Consequently, release of enzymes and mediators by the inflammatory cells has the potential to injure “innocent bystander” cells and structures in the vicinity.



This is a potential threat to organs such as the eye, in which the physiologic function requires that a clear visual axis be maintained.

The general immunological mechanisms are modified for different tissues such as the eye and the skin. They display differential vulnerability to the injurious side effects of immune-mediated protection. Therefore the immune responses generated are subjected to modifications by the tissue that qualitatively and quantitatively mould them. This then promotes the generation of a selected subset of immune mediators that are consistent with, and not deleterious to the physiologic function of the tissue.

There are microanatomic features of the eye that are unique and partially account for the unusual immunity observed in this organ. The ocular surface covered by the conjunctiva is part of the mucosal immune system. Induction and expression of immunity at this surface resembles that found at other mucosal surfaces. In contrast, the intraocular compartments are neither integral parts of the mucosal immune system, nor relatives of the other major regional system, the skin associated lymphoid tissues. Instead the tissues and fluid filled spaces within the eye comprise a unique, perhaps self-contained and integrated, set of interacting molecules and cells that communicate with the rest of the body in highly distinctive ways.

### **1.6.2. Ocular Immune Privilege**

The study of the ocular immune environment using animal models have given great insight into this milieu. Initially it was thought that the eye was a privileged site since it was somehow 'ignored' by the immune system. However it is now clear that this concept of privileged site is more of an active process. Other

immune-privileged sites includes the brain, ovary, testis, pregnant uterus, and hamster cheek pouch. Ocular immune-privilege can be defined as follows: foreign tissues placed in the AC, vitreous cavity, the subretinal space and the corneal stroma experience extended (even indefinite) survival compared with similar tissues placed subcutaneously (a conventional site) (Streilein, 1995)

There are several features unique to the eye that contributes to immune-privilege. First, the integrity of the blood-ocular-barrier which severely restricts the inflow of blood-borne molecules and cells into the eye, hence preventing specific and non-specific mediators of immunogenic inflammation from gaining access to the eye. Second, the virtual absence of lymphatics combined with the bulk flow of AH through the trabecular meshwork into the canal of Schlemm (and thus into the venous circulation) ensures that antigenic material that escapes the eye does so almost exclusively by the blood route. As a result, the spleen rather than draining lymph nodes act as the primary lymphoid organs.

Third, the bone-marrow derived cell with morphological and surface phenotypic characteristics typical of professional APC are present and strategically placed within the tissues of the eye, namely the iris, ciliary body and retina (Williamson, et al., 1989; McMenamin, et al., 1992). However the antigen presentation by these cells result in a 'deviant' type of immunity where the antigens introduced into the eye are captured by distinctive local antigen-presenting cells that migrate via the blood to the spleen. At that site, they generate a stereotypic systemic immune response that is deficient in CD4<sup>+</sup> T cells that mediate delayed hypersensitivity and that help B cells to secrete complement-fixing antibodies, yet replete with CD8<sup>+</sup> T cells that function as

cytotoxic cells and as regulatory cells. This response is termed AC associated immune deviation (ACAID). Because intraocular inflammation is deleterious to vision, ACAID and immune privilege are considered to be adaptations that enable the eye to benefit from immune protection against pathogens without suffering blindness from immune injury (Streilein, et al., 1997).

Fourth, ocular fluids contain a unique spectrum of factors (cytokines, neuropeptides, growth factors) that suppress immunogenic inflammation and complement activation (Streilein, et al., 1992). AH has been found to contain physiologically relevant concentrations in addition to of TGF- $\beta$ , of alpha-melanocyte-stimulating-hormone ((-MSH), vasoactive-intestinal-peptide (VIP) and calcitonin-gene-related peptide. Furthermore the AH is grossly deficient in cortisol binding globulin (CBG) (Knisely, et al., 1994). Hence AH contains biologically relevant concentrations of glucocorticoids and that CBG is relatively absent so that glucocorticoids present are largely free contributing to the immunosuppressive microenvironment.

The fifth factor is the existence of intraocular mechanisms that downregulate the immune response, e.g. the downregulatory capabilities of Müller cells (Caspi, et al., 1987). Isolated cultures of these cells will downregulate proliferative responses of autoaggressive T cells. In addition when specific Müller cell poisons are utilised, inflammatory disease in the eyes of animals so treated becomes worse (Chan, et al., 1991). This role is in contrast to that of RPE cells, which can act as an APC to autoaggressive cells (Percopo, et al., 1990). The RPE has many characteristics to support its role in interacting with the immune system. Firstly it possesses macrophage-like characteristics and

secondly it constitutively bears class I molecules on its cell surface and can express class II when stimulated (Chan, et al., 1986), and finally it produces IL-6 (Planck, et al., 1992).

Finally, recently, an additional participant in immune-privilege was identified with the observation that the eye and the testis constitutively express Fas ligand (FasL) (Li, et al., 1997; Xerri, et al., 1997). In the eye, FasL is found on a number of important structures, where it promotes the death of invading Fas<sup>+</sup> cells during a viral infection. Fas is related to members of the TNF $\alpha$  /NGF receptor family and is widely distributed on lymphoid and nonlymphoid cells.

Constitutive expression of FasL has been shown in strategic locations, including the cornea, retina, iris and ciliary body (Griffith, et al., 1995) i.e. surrounding the entire organ. This ligand is expressed in areas that comprise the blood-ocular-barrier and in locations where there is a chance for interaction between ocular tissue and inflammatory cells. In the retina, FasL is expressed on retinal pigment epithelial cells and the photoreceptors, where it may have a crucial role in protecting vision. FasL present on the iris and ciliary body, the vascular areas in the eye, can kill cells entering the blood vessels. Interestingly in the cornea, FasL is expressed both on the epithelium and endothelium. This is important as it will enable the control of inflammatory cells entering either from the conjunctiva or AC. Soluble FasL has recently been demonstrated in humans (Tanaka, et al., 1996). Hence it is possible that the AH might contain soluble FasL as another means of T kill invading lymphoid cells. Furthermore the confined space of the AC would be an ideal place for this soluble molecule to function.

The crucial role of FasL to immune-privilege is observed when an inflammatory insult occurs at which point, the eye does not simply act as a barrier preventing entry of dangerous cells, rather it traps and kills the invading lymphoid cells that could be dangerous to organ integrity. This confirms that FasL expression in the eye is a major factor contributing to immune-privilege as its disruption leads to serious consequences.

#### **1.6.2.1. ACAID**

Immune-privilege depends in part upon the induction of ACAID, which occurs when antigen is placed within any of several intraocular compartments. The strategic location of inhibitory cells in the tissues that line the AC of the eye raises the possibility that these cells may play a role in the phenomenon of immunological privilege that is characteristic of this site. Wilbanks et al have demonstrated that these parenchymal iris/ciliary cells secrete TGF- $\beta$  locally and into the AH which endows resident, mature macrophages ACAID-inducing capabilities. These APCs were able to migrate out of an antigen-bearing eye and activate regulatory T cells within the spleen (Wilbanks & Streilein, 1992a). Importantly, the ACAID-inducing effect is achieved when conventional, extraocular APCs are exposed exposure vivo to AH or culture supernatants from iris and ciliary body cells.

The role of cytokines in ACAID was investigated by Li et al (Li, et al., 1996). The cytokine pattern of antigen-pulsed spleen cells from mice primed in the AC was determined. The results indicated that CD4<sup>+</sup> spleen cells from hosts primed in the AC with antigens that induce ACAID produced significant quantities of interleukin-10 (IL-10) but insignificant levels of IL-2, IL-4, IFN $\gamma$ .

In contrast, hosts primed in the AC with antigens that do not induce ACAID, but instead elicit normal DTH, displayed cytokine patterns indicative of a Th 1 response where significant quantities of IL-2 and IFN $\gamma$  were produced while IL-4 and IL-10 secretion was insignificantly different from normal controls. The immunological phenotype of the AC-primed hosts could be altered by systemic treatment with antibodies against either a Th 1 cytokine (IFN $\gamma$ ) or a Th 2 cytokine (IL-10). Hosts treated with anti-IL-10 antibody and subsequently primed in the AC with ACAID-inducing antigens developed normal DTH responses, while hosts treated with anti-IFN $\gamma$  antibody and primed in the AC with antigens that normally produce positive DTH responses failed to develop positive DTH. These observations collectively support the proposition that immune privilege in the AC of the eye is due to the selective activation of a Th 2 population that cross-regulates Th 1 responses (Li, et al., 1996).

ACAID has been characterised as being distinct from the conventional immune response in a number of ways. First, except for highly immunogenic antigens, ACAID in response to AC-injected antigens is extremely long lasting. It has been demonstrated experimentally to persist in mice for at least 6 months (Bando, et al., 1993). Second when antigen is injected simultaneously into the AC and into the subcutaneous space, ACAID is routinely induced, rather than conventional immunity which indicates that ACAID is dominant to conventional immunity and strongly suggests that both are highly regulated. Third, injection of an antigen into the AC of mice presensitised conventionally to the same antigen also results in ACAID, implying that the regulatory mechanisms of ACAID can

even suppress preformed memory and effector T cells that mediate delayed hypersensitivity (Kosiewicz, et al., 1994).

Early experiments in the rat model of ACAID clearly revealed the need of an intact camero-splenic axis in the generation of ACAID (Kaplan & Streilein, 1978; Streilein & Niederkorn, 1981). Enucleation of the antigen-containing eye (even as late as 5 days after AC injection) aborts ACAID. On the other hand, removal of the spleen- prior to AC injection or as late as 5 days thereafter also prevents ACAID induction (Niederkorn & Streilein, 1982; Streilein, 1995). These observations suggest that antigenic signals must escape the eye and are received by the spleen during ACAID induction (Wilbanks & Streilein, 1991; Wilbanks & Streilein, 1992b).

Eventhough ACAID has been demonstrated in animal experiments it is apparent that ACAID might not be wholly protective for T cell mediated responses in humans since conditions such as sarcoidosis can affect all ocular components. Clearly selective tissue immunomodulatory mechanisms can be bypassed if the microenvironmental conditions are changed appropriately.

### **1.6.3. Immunologic Basis of Uveitis**

There has been numerous works to ascertain the immunologic mechanisms in uveitis. However, studies during the past few decades indicate that, a T cell infiltrate, T cell sensitization to retinal antigens and expression of major histocompatibility complex (MHC) class II antigens are associated with this process. Several lines of indirect evidence have indicated this important role of cell-mediated-immunity in pathogenic mechanism of uveitis. Firstly T cells have been shown to accumulate in inflammatory sites of uveitic eyes by immunohistochemical examinations. This is further strengthened by the

demonstration of high proportions of CD4<sup>+</sup> T cells in the Dalen-Fuchs nodules at the early phase of sympathetic ophthalmia, similar to the finding in rat eyes with experimental autoimmune uveitis (EAU) (Chan, 1985; Chan, et al., 1985) while CD8<sup>+</sup> T cells were more numerous at a later phase (Chan, 1986).

Secondly the effector molecules for these T cells, namely IFN $\gamma$  and IL-2 had been detected at the inflammatory sites. Hooks et al (Hooks, et al., 1988) have shown that IL-2 and IFN $\gamma$ , are present in the human eye during inflammatory and autoimmune diseases. This was associated with a lymphocyte infiltrate, predominantly of T cell origin, and with the expression of MHC class II antigens on both the infiltrating cells and ocular resident cells, that is, retinal pigment epithelial cells and retinal vascular endothelial cells. In addition, ex vivo studies demonstrate that IFN $\gamma$  can enhance the expression of the HLA-DR determinant on both of these cell types.

Thirdly, the animal model experimental autoimmune uveitis (EAU), that serves as a model for certain uveitic conditions in man, is mediated by T cells, where studies have shown that only activated CD4<sup>+</sup> ocular antigen-specific T cell lines can adoptively transfer EAU to naive syngeneic recipients (Caspi, et al., 1986). These T cells are found to be highly restricted in their interaction with APCs (Mochizuki, et al., 1985). In addition the uveitogenic T cells were shown to belong to the Th 1 subset of CD4<sup>+</sup> lymphocytes (Rizzo, et al., 1996). Moreover animals deficient in T cell immunity are not susceptible to the induction of EAU (Salinas-Carmona, et al., 1982). Finally lymphocytes isolated from vitrectomy specimens have been shown to proliferate ex vivo to the retinal S-Ag (Nussenblatt, et al., 1984). In addition patient's circulating lymphocytes



have manifested *ex vivo* proliferative responses to the S-Ag and IRBP (Nussenblatt, et al., 1984; Doekes, et al., 1987; de Smet, et al., 1990). When fragments of both IRBP and S-Antigen are placed into culture with lymphocytes of uveitis patients proliferative response are induced (de Smet, et al., 1990). This recognition, routinely determined by the proliferative response of PB lymphocytes *ex vivo*, is considered the equivalent of the *in vivo* anamnestic response. A more direct evidence of the role of T cells in uveitis is the therapeutic benefit of cyclosporin A (a well-known inhibitor of T cell activation) on many types of human intraocular inflammatory conditions (Nussenblatt, et al., 1983a).

#### **1.6.3.1. Experimental autoimmune uveitis (EAU)**

A large number of experiments have been made over the past two decades using three uveitogenic antigens: the retinal S-antigen, the interphotoreceptor retinoid-binding protein (IRBP), and rhodopsin. Immunisation at a site far from the globe with these antigens will lead to a bilateral uveitis, which has been called EAU. These proteins are organ-specific, being also found only in the pineal gland.

In EAU the disease affects mainly the posterior segment, but will often have an anterior segment inflammatory component. In some species and depending on the antigen used, the disease can be recurrent (Caspi, et al., 1990). There will be inflammatory cells in the AC and sometimes a deposition of fibrin. This is often seen in the human situation in the more severe presentation of anterior uveitis. The disease process in the posterior segment simulates the clinical appearance of the human condition as well, with inflammatory infiltrates and haemorrhages in the retina. A fluorescein angiogram of the retinal

vasculature of nonhuman primates with EAU will demonstrate evidence of a 'retinal vasculitis', with late staining of the retinal blood vessels and leakage of dye, which are changes seen in the retinae of humans with posterior uveitis.

The histologic pattern of EAU will clearly vary to some degree depending on the species, the antigen and the amount injected. However in all cases the dominant feature of the disease in the posterior segment is the infiltration of T cells. In rats, the disease appears to be acute, and the neutrophils are noted early in the process (Gery, et al., 1986). On the other hand in non-human primates and in mice, the disease has a more chronic appearance, with granuloma formation (Nussenblatt, et al., 1981; Hirose, et al., 1986). Cells will infiltrate the retina and choroid in large numbers. Ultimately there will be destruction of the retina (from which comes IRBP, S-Antigen and rhodopsin). Adhesion molecules (LFA-1, VLA-4, and Mac-1) have been detected on the lymphoid cells in eyes with EAU, and their counter ligands ICAM-1, ICAM-2, VCAM-1 and ELAM-1 have been seen on endothelial cells (Whitcup, 1994). Class II antigens have been detected on ocular cells in animals with EAU (Fujikawa, et al., 1987). Interestingly mast cells rich in the uvea, appear to play an important role in the ocular immune response. It has been noted that the number of mast cells in the choroid correlates well to rat susceptibility to EAU (Mochizuki, et al., 1984), and that there is degranulation of mast cells within the eye one to two days before the onset of clinically evident EAU (Li, et al., 1992) to suggest that anterior mast cells participate in the immunopathogenesis of EAU and may influence the genetic susceptibility to EAU. Hence it has been speculated that mast cells open the 'gates' for the lymphocytes by altering the

usually impermeable retinal vasculature (Mochizuki, et al., 1984; Li, et al., 1992).

It is well known that uveitogenic effector T cells have a Th1-like phenotype (high IFN $\gamma$ , low IL-4), and genetic susceptibility to EAU is associated with an elevated Th1 response. Jones et al have demonstrated that the ability to produce IFN $\gamma$  is necessary for the development of EAU by immunising IFN $\gamma$  knock out (GKO) mice with the uveitogenic protein interphotoreceptor retinoid binding protein (IRBP) and characterising the associated immunologic responses. These GKO mice developed EAU comparable in severity and incidence to that of their wild-type littermates. However, the cytokine profile in their uveitic eyes as well as the cytokines produced by primed lymph node cells in response to IRBP showed a distinct profile ie. one of undiminished TNF $\alpha$  and elevated IL-5, IL-6, IL-10, and lymphotoxin (but not IL-4) responses. The inflammatory infiltrate in GKO eyes contained an excess of granulocytes and IL-5- and IL-6-producing cells, but uveitic GKO mice did not up-regulate inducible nitric oxide synthetase. In addition these GKO mice had enhanced lymphocyte proliferation and delayed-type hypersensitivity responses to IRBP. Histology of the delayed-type hypersensitivity lesion in GKO had superimposed elements of an allergic-like response. Anti-IRBP Ab isotypes of GKO mice showed a reduction of IgG2a, but no enhancement of IgG1. Comparison of responses in +/+ and +/- wild-type mice revealed some limited evidence of a gene-dose effect. It was concluded that IFN $\gamma$  is not required for priming of pathogenic T cells or for effecting the retinal damage and photoreceptor loss typical of EAU.

However, what appears to be a grossly similar disease is caused in the GKO by a deviant type of effector response (Jones, et al., 1997)

### **1.7. EXPERIMENTAL APPROACHES TO TREATMENT**

The experimental model of posterior uveitis in the Lewis rat, EAU is a useful model for assessment of new drugs or new approaches to therapy. Attempts have been made to prevent disease occurring after immunisation and to decrease the intensity of the inflammatory process once it has begun.

Treatment with monoclonal antibodies against the CD4 molecule (Atalla, et al., 1990) class II MHC molecules (Ia) (Wetzig, et al., 1988) or with retinal S-Ag has been successful in preventing or delaying the development of uveitis in rats. A suppressor T cell line has reduced the intensity of EAU (Caspi, et al., 1988), and vaccination of with ocular-antigen-specific T cells has protected against disease induction (Beraud, et al., 1990)

A large proportion of T cells infiltrating the eye in EAU is activated T cells. Selective immunosuppression directed against activated T cells has been attempted in EAU using a chimeric recombinant protein (IL-2-PE40) composed of IL-2 fused to a modified *Pseudomonas* exotoxin lacking its cell-recognition domain (Roberge, et al., 1989). There was a greater reduction in the incidence and severity of EAU in IL-2-PE-40 treated rats than in controls, but the treatment produced severe side effects. Interestingly feeding rats with retinal S-Ag prevented induction of EAU (Nussenblatt, et al., 1990), and it is thought that this form of therapy induced specific suppressor T cells.

### 1.8. CYCLOSPORIN A

Cyclosporin A differs in action from the older immunosuppressive drugs. Its unique immunomodulatory properties were first observed by Jean Borel (Borel & Ryffel, 1986) in that it is specific for activated T lymphocytes. It appears to block the activation of genes in T cells (Cross, et al., 1989). The T cell most affected seems to be the helper T cell subset. The recruitment of helper T cells into the draining lymph node of a site of S-Ag immunisation is markedly diminished with cyclosporin A administration (Nussenblatt, et al., 1996b). By inhibiting T cell secretion of cytokines such as IFN $\gamma$  and IL-2 cyclosporin A administration results in a decrease in inflammatory activity (Nussenblatt, et al., 1983a).

Cyclosporin A is effective in ocular inflammatory disease when used alone at 10mg/kg per day, but the permanent renal damage it causes precludes its use at this dose (Palestine, et al., 1986). At 5 mg/kg per day the side effects are less severe but the drug becomes ineffective on its own in the initial management of posterior uveitis and has to be given with systemic steroids. At 5 mg/kg per day cyclosporin A are usually given more commonly as a twice-daily regimen to avoid large spikes of in serum cyclosporin A levels, which may predispose to renal toxicity. The duration of treatment with cyclosporin A is an open-ended question. It appears that an immunologically tolerant state is not induced with this medication, and therefore an extended therapeutic course is indicated in most patients. Reduction and termination of cyclosporin A therapy can be associated with a rebound increase in inflammatory activity (Binder, et al., 1987).

### **1.9. VITREOUS HUMOUR (VH)**

This refers to the virtually acellular viscous fluid behind the lens and the iris. The vitreous of the normal human eye weighs approximately 4 g and occupies a volume of almost 4 mls. The precise weight and volume vary with the age and the size of the eye. The vitreous body is spherical, with a depression in the anterior surface, the patellar fossa, corresponding to the posterior surface of the crystalline lens. The vitreous body can be divided into two zones or regions. The more peripheral zone, the cortical vitreous, encases the medullary vitreous. The cortical vitreous consists of a relatively more condensed, fibrillar vitreous. Although the cortical vitreous represents only 2% of the total vitreous volume, it is the metabolic centre of the vitreous body, since it contains the hyalocytes (Balazs, et al.). Another connective tissue cell, the fibrocyte, is also found in the cortical vitreous. The majority of the vitreous body, the medullary vitreous, is essentially a cell-free mixture of collagens and hyaluronic acid existing either in a gel or a liquid state depending on the age, refraction, and condition of the eye. It can be infiltrated by inflammatory cells in intraocular inflammation.

### **1.10. AQUEOUS HUMOUR (AH)**

The AH is a transparent, colourless solution continuously formed from plasma by the epithelial cells of the ciliary processes. It is secreted into the posterior chamber from where it passes through the pupil into the AC and is drained at the AC angle. Most of the aqueous drains into the venous circulation via the trabecular meshwork, Schlemm's canal, scleral collector channels, and aqueous

and episcleral veins; the remainder drains into the orbit via the interstices of the ciliary muscle, the suprachoroidal space, and the sclera.

### **1.11 UVEITIS**

Uveitis, or intraocular inflammatory disease is an old term that has been qualified with additional term such as iritis, cyclitis, and iridocyclitis since the beginning of the last century (Duke-Elder, 1966). While the term initially indicated that the centre of the inflammatory response was located in the uvea, it now simply indicates that there is an intraocular inflammatory response. Hence all layers of the eye may be involved by inflammation, although the uvea refers to the blood vessel coat of the eye, including the iris, ciliary body, and choroid. Because the uvea is the vascular coat of the eye and is supplied by the systemic circulation, systemic diseases and local diseases affecting the blood vessels can affect local or contiguous parts of the eye.

Intraocular inflammatory disease is further characterised by the anatomic position of the disease. The classification of uveitis often is related to the anatomic structure involved. Iritis refers to inflammation of the iris. If the ciliary body is also involved, the condition is called iridocyclitis. Choroiditis refers to inflammation primarily in the choroid. Because these tissues are contiguous with the retina, the condition may also be called chorioretinitis or, if the retina is the primary component, retinochoroiditis.

The causes of uveitis include infections by bacteria, protozoa, viruses, and spirochetes, associated with a systemic disorder such as ankylosing spondylitis (AS) and sarcoidosis, or maybe due to a purely ocular event with a putative autoimmune component such as birdshot retinochoroidopathy. With

experience, dividing the disease processes into acute and chronic phases helps with the diagnostic and therapeutic evaluations. Chronic uveitis refers to inflammation that has existed for more than 12 weeks (Bloch-Michel & Nussenblatt, 1987).

#### **1.11.1. Non-Infectious Uveitis**

The ocular manifestations differ according to the anatomical involvement, nature of onset of disease and the type of inflammation. It can be divided into four major categories comprising anterior, intermediate, posterior and panuveitis, based on the principal anatomic site of inflammation, using a classification scheme proposed by IUSG (Bloch-Michel & Nussenblatt, 1987). The majority of uveitis cases are idiopathic in nature but a substantial proportion is associated with systemic diseases such as Behçet's disease, juvenile RA, sarcoidosis and HLA-B27 related conditions.

##### **1.11.1.1. Anterior Uveitis**

Anterior uveitis refers to disease predominantly limited to the anterior segment of the eye. Other terms used in the literature for anterior uveitis are iritis, iridocyclitis, and anterior cyclitis. The symptoms of acute anterior uveitis include ocular redness, pain, blurred vision and photophobia. However patients with chronic low-grade inflammation may be asymptomatic. The pain of iritis is commonly due to ciliary spasm. Since the ciliary body is innervated by the trigeminal nerve, pain may radiate to the whole area distributed by this nerve, including the periorbital region and the eye itself. The signs of anterior uveitis include ciliary injection, pupillary constriction and keratic precipitates (KPs) in addition to cells and flare in the AC which represent extravasated inflammatory



cells and protein into the AH, respectively. Cells in the AH usually indicate active inflammation in the iris and ciliary body. These cells vary from tiny grains to three or four times this magnitude. Larger cells probably represent swollen macrophages or clumps of lymphocytes, whereas small cells may be individual lymphocytes. Studies of AC cells show that lymphocytes and monocytes predominate. Even in hypopyon, a sign of severe anterior uveitis, (layering of leucocytes in the AC), a predominance of lymphocytes with minimal neutrophils were observed. This tends to occur in HLA-B27 related uveitis and anterior uveitis of Behçet's disease.

KPs are clusters of inflammatory cells deposited on the inner surface of the cornea. The precipitated cells are often found inferiorly on the cornea, in a linear vertical formation or in a triangular formation with the apex above. This inferior corneal distribution results from the convection current in the AC that rises along the warm iris and falls along the cool cornea. The mechanisms appear to involve the expression of cell adhesion molecules that are upregulated in the presence of inflammatory cytokine such as IL-1 (Whitcup, et al., 1992). The cells that precipitate represent the composition of those in the AH. Thus, chronic inflammatory cells such as macrophages, lymphocytes, and plasma cells predominate over polymorphonuclear leukocytes. Clinically, these clumps of cells may be small, medium, large, and coalescent. Large, white KP often termed greasy or mutton fat usually represents clusters of epithelioid cells and mononuclear macrophages that approach 1 mm in diameter.

Posterior synechiae (PS) occurs in severe untreated and persistent anterior uveitis. This is the adhesion of the iris to the structure posterior to it,

being the lens in phakic patients and the anterior vitreous phase in aphakic eyes. If untreated, posterior synechiae form between the pupil margin and the lens, fixing the pupil in a moderately miotic position. If the posterior synechiae encompass 360 degrees of the pupil, AH flow into the AC is blocked; resulting in the iris bowing forward, a condition called iris bombé. The resulting closed AC angle causes an acute rise in intraocular pressure leading to pupillary block glaucoma. Hence the remedy and prevention of this sequelae is one of the main aim in the treatment of acute anterior uveitis to prevent this complication which may lead to significant ocular morbidity.

Primary immune-related processes are usually treated with antiinflammatory agents. Corticosteroids are most commonly used for eye disease, and they can be given topically, in the form of periocular injections, or administered systemically through oral or intravenous routes. The rationale is to treat the inflammatory components to eradicate the disease promptly and reduce intraocular scarring.

#### **1.11.1.1.1. Idiopathic Anterior Uveitis**

In a clinical setting, whenever patients present with anterior uveitis, a thorough medical history and an ocular and physical examination performed usually failed to disclose any association with any defined clinical syndrome in almost 50% of cases. This form of anterior uveitis is referred to as idiopathic anterior uveitis (IAU). They not only lack systemic disease association, but also cannot have the HLA-B27 haplotype, which is also associated with anterior segment inflammatory disease. Patients with IAU tend to have non-granulomatous inflammation and respond well to topical corticosteroid therapy.

#### 1.11.1.1.2. HLA-B27- Associated Anterior Uveitis

Anterior uveitis associated with HLA-B27 has frequent associations with systemic diseases which includes, ankylosing spondylitis (AS), Reiter syndrome, juvenile RA (JRA), inflammatory bowel disease (IBD), Whipple's disease, and psoriatic arthritis (Wakefield, et al., 1991). However many patients with HLA-B27 haplotype and anterior uveitis have no systemic illness.

The majority of patients with HLA-B27-associated anterior uveitis have no obvious precipitating event. However there are findings that link gram-negative bacteria to HLA-B27 associated diseases. This includes the triggering of Reiter's syndrome by infection with gram-negative bacteria such as *Salmonella* species (Simon, et al., 1981). In addition Taurog et al observed that HLA-B27 transgenic rats raised in a germ-free environment develop less joint disease (Taurog, et al., 1993). Also, endotoxin from gram-negative bacterial cell wall was found to induce iritis (Kogiso, et al., 1992) in experimental animals. An additional finding linking infective causes to HLA-B27 related disease is the variety of its cross reactivity with gram-negative bacteria (Scofield, et al., 1993).

The underlying mechanism for the association of HLA-B27 with disease remain unknown (Kingsley & Panayi, 1992). A recent theory to explain the association between HLA-B27 and spondyloarthropathies is that HLA-B27-derived peptides may be presented by self MHC class II to CD4<sup>+</sup> arthritogenic T cells, tolerance having been broken by mimic peptides from the triggering bacteria (Sieper & Braun, 1995).

Interestingly, Wildner and Thureau (Wildner & Thureau, 1994) have described the immunological features of a peptide from the polymorphic region of HLA class I antigens associated with uveitis, B27PD. This peptide was able to induce uveitis when injected into rats, and lymphocytes from these animals show a strong cross-reactivity with a uveitogenic peptide of retinal soluble antigen (S-Ag). These cross-reactive immune responses to retinal peptide and the peptide from disease associated HLA types were seen in both animal models and humans. In addition the HLA-derived peptide B27PD can induce tolerance to S-Ag and IRBP, when administered orally. Hence these workers have shown for the first time a direct immunological link between HLA class I antigen and an organ-specific autoantigen, where the class I antigen itself is presented as a peptide and, thereby, cross-reactive with the respective peptide from the ocular autoantigen.

#### **1.11.1.1.3. Fuchs' Heterochromic Cyclitis (FHC)**

FHC is a form of localised chronic uveitis. It is usually unilateral, chronic, and eventually results in a change in the colour of the iris and cataract. The cause of this disorder is unclear and many studies have suggested it to be an immune-related process (Murray, et al., 1990b). The classic presentation usually involves minimal symptoms and the typical ocular findings are that of a low-grade cell and flare response with stellate, white, KPs involving the entire endothelial surface in contrast to other anterior uveitis where the KPs are distributed in the inferior portion of the corneal endothelium. Occasionally, floaters (caused by a cellular reaction in the vitreous) are the presenting symptom.

The chronicity of the process affects the pigment epithelium of the iris, resulting in patchy transillumination defects and a lighter coloured iris than in the opposite eye. Although PS does not develop in an unoperated eye glaucoma and cataract formation are long-term complications of the disease. FHC is an interesting uveitic entity to the ophthalmologist because of its entirely different clinical course, being a chronic inflammation that does not subside with the usual treatment of topical corticosteroids

#### **1.11.1.2. Intermediate Uveitis (IU)**

IU does not refer to any distinct clinicopathologic entity. The diagnosis is made when there is inflammation involving primarily the vitreous and peripheral retina as recommended by IUSG. (Bloch-Michel & Nussenblatt, 1987) and is accompanied by minimal or no anterior or chorioretinal inflammatory. The term IU is used regardless of whether the inflammatory disorders are idiopathic or associated with systemic disease such as sarcoidosis and MS. Aggregates of inflammatory cells are frequently seen in the inferior vitreous and are termed vitreous snowballs. These inflammatory cells also accumulate as debris along the pars plana and ora serrata and are called snowbanks. A mild anterior uveitis often coexists and cystoid macular oedema (CMO) is a frequent finding.

#### **1.11.1.3. Posterior Uveitis**

Any condition that affects the deeper tissues of the eye, including the vitreous gel, retina, choroid, posterior sclera, and optic nerve, is referred to as posterior uveitis. Toxoplasmosis, a condition caused by an obligate intracellular parasite, is one of the more common forms of posterior uveitis. As there are many causes of posterior uveitis, it has been classified as being predominantly a retinitis or

choroiditis and as a focal or multifocal disease (Nussenblatt, et al., 1996a). Despite the difference in clinical presentation, most forms of posterior uveitis have four cardinal features: 1) inflammatory cells and/or granulomata within the vitreous, 2) focal chorioretinal infiltrates, 3) retinal vasculitis, and 4) macular oedema.

#### **1.11.1.4. Panuveitis**

This is reserved for diseases that involve all segments of the eye, typically with a sight threatening complications. The common causes of panuveitis are syphilis, sarcoidosis, Vogt-Koyanagi-Harada-syndrome, infectious endophthalmitis, and Behçet's disease. Hence the clinical manifestations is a mixture of anterior and posterior segment inflammation.

#### **1.11.2. Behçet's disease**

This condition worth a special discussion by itself as it is one of the most aggressive uveitis and is one of the important causes of blindness among the uveitides. The ocular manifestations of Behçet's disease denote serious implications for the patients. Although the disease can affect the anterior and posterior segment of the eye separately, the majority of the patients have panuveitis or posterior uveitis. Only a small proportion, have isolated anterior uveitis. Behçet's disease is characterised by recurrent explosive attacks of intraocular inflammation. An anterior uveitis is seen frequently with an associated hypopyon in about a third of cases (Mishima, et al., 1979). A characteristic feature of the hypopyon in Behçet's disease is that it shifts with gravity as the patient changes head positions. The hypopyon resolves spontaneously without sequelae. PS, iris atrophy, and peripheral anterior

synechiae may develop during the course of repeated ocular inflammatory attacks.

Retinal disease is the most serious complication of Behçet's disease (Sakamoto, et al., 1995). It is the recurrent retinal vasoocclusive episodes that ultimately cause irreversible alterations and serious visual impairment. The retinal vasculitis may involve both arteries and veins. Behcet's ocular disease can be severe, and irreversible damage can easily lead to blindness if the condition is not diagnosed or if it remains untreated.

### **1.11.3. Sarcoidosis**

This is a multisystem granulomatous disease that can affect almost every organ in the body. The most common organs involved in sarcoidosis are the lung, thoracic lymph nodes and skin. Anterior uveitis is the most common ocular manifestation and occurs in almost two thirds of patients with ocular sarcoidosis. About 53 to 60 % of patients with sarcoidosis will have a chronic granulomatous uveitis (James, et al., 1976; Obenauf, et al., 1978; Jabs & Johns, 1986). Inflammation of the posterior segment is less common but more visually disabling than anterior segment involvement (Silver & Messner, 1994). It occurs in 6% to 33% of patients with sarcoidosis (James, et al., 1976; Obenauf, et al., 1978; Jabs & Johns, 1986). Chronic cystoid macular oedema is usually the cause of decreased visual acuity in patients with posterior uveitis and sarcoidosis.

### 1.12 RESEARCH HYPOTHESIS

Cell-mediated-immunity is an important mechanism in the pathogenesis of immune mediated uveitis. T cells are actively recruited into the eye and via their effector molecules, the cytokines contribute significantly to the intraocular inflammation. Knowledge of the effector cells and cytokine pattern in the intraocular fluids may help dissect the immune mechanism in the intraocular microenvironment. The different effector cells and cytokines may explain the differing clinical profiles and response to treatment in the different types of uveitis.

### 1.13. AIM OF STUDY

- To ascertain the predominant cellular phenotypes and the cytokine pattern in the AH of patients with different types of uveitis.
- To investigate any relationship between clinical manifestations and the level of proinflammatory and downregulatory cytokines.
- To compare and contrast the cytokine pattern and cellular phenotypes between two contrasting localised anterior uveitis: FHC vs IAU and between localised uveitis (LU) and uveitis associated with systemic disease (SU).
- To investigate the *ex vivo* cytokine production by the T cells infiltrating the intraocular compartment in uveitis.





## **CHAPTER 2**

### **Materials And Methods**

#### **2.1. PATIENT CLASSIFICATION**

Patients with uveitis presenting to the clinic were characterised on clinical grounds, with additional investigations to exclude systemic disease as required. The groups of patients identified were 10 FHC, 18 idiopathic anterior uveitis (IAU), 10 anterior uveitis associated with systemic disease (AUS), 7 idiopathic panuveitis (IPU), 5 panuveitis as part of Behçet's disease, 7 panuveitis as part of sarcoidosis, 1 patient with intraocular toxoplasmosis, 1 patient with intraocular B cell lymphoma making a total of 59 patients. IAU and IPU were defined as anterior uveitis and panuveitis respectively with no evidence of systemic disease clinically or after appropriate investigations. AUS was defined as patients with anterior uveitis related to systemic problems such as Reiter's syndrome (1), JCA (2), MS (1), AS (5) and Psoriasis (1). All patients with FHC had classic clinical features.

#### **2.2. AQUEOUS HUMOUR (AH) SAMPLING PROCEDURE**

AH samples were obtained from the eyes of two groups of patients i.e. uveitis patients and patients undergoing cataract surgery that has no history of uveitis and the eyes were uninflamed. Ethical approval was obtained and informed consent given by the patient for both paracentesis and venesection.

##### **2.2.1. Paracentesis of uveitis patients**

AC paracentesis was carried out using a 30G needle on a 1-ml insulin syringe. Topical amethocaine was instilled to anaesthetise the ocular surface. The patients were put on the slit lamp and instructed to position their eyes in primary position

and cautioned not to make any ocular movements. A 30G needle on a one-ml syringe was used to enter the AC via the temporal limb approach. The needle was gently pushed and rolled at the same time to allow a steady entry into the AC. Approximately 100 to 200  $\mu$ l of AH was obtained from the AC and there was no recorded incidence of any trauma to the iris or lens. The AH obtained was immediately allocated into three Eppendorf tubes precoated with EDTA to avoid cell clumping. These samples were centrifuged for 5 minutes at 300g at 4°C. The supernatants were aspirated and kept at -70°C for subsequent ELISAs. 5 mls of PB (PB) samples were taken from these patients immediately after paracentesis.

#### **2.2.2. Paracentesis of control patients.**

This procedure was performed at the beginning of surgery. A partial grooved limbal incision was made and the AC entered using a 23G needle. The AH was aspirated and immediately treated as above. Extreme measures were taken to prevent any blood from entering the AC. Any samples that were stained with blood were excluded to ensure that no contaminating cytokines from the systemic circulation enter the AC.

### **2.3. VITREOUS HUMOUR (VH) SAMPLING PROCEDURE**

The VH were obtained during therapeutic vitrectomies of patients with FHC (a different group of patients distinct from those whose AH were obtained by paracentesis) and IU. Prior to any infusion of fluid into the vitreous cavity, neat VH was syringed out as the vitreous gel was cut with the vitreous cutter. The volumes of VH obtained ranged from 500 to 1500  $\mu$ l. Some of the samples were obtained during diagnostic vitreous tap from patients suspected of having

intraocular lymphoma. These are usually small in volumes ranging from 100 to 500  $\mu$ l.

#### **2.4. ELISA**

Cytokines in the AH, VH and cell culture supernatants of VH-derived T cell lines were quantitated by ELISA (R&D Systems Europe, Abingdon, Oxon, UK). The ELISA that had been used is a solid phase sandwich Enzyme Linked Immunosorbent Assay. It employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for the cytokine tested has been coated onto the wells of the microtiter plate. The cytokine present in the standard and samples was bound to the well at the first incubation by the immobilised antibody. The unbound protein was washed away and an enzyme-linked polyclonal antibody specific for the cytokine measured was added to the well to sandwich the cytokine immobilised during the first incubation. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and colour developed in proportion to the amount of the cytokine bound in the first step. The colour development was stopped and the optical density measured.

A standard curve was prepared, plotting the mean optical density (OD) from duplicate wells after subtracting the background versus the concentration of the cytokine in the standard wells using SlideWrite for windows version 2.1. An equation was obtained for the curve and the concentrations of the unknown samples were calculated using the equation by the SlideWrite programme (Figure 2.1).

On the day of the analysis the samples were thawed and diluted to avoid the inhibitory effect of proteins in the AH and VH. The optimum dilution was determined prior to ELISA being performed on some of the actual AH samples by spiking method. A known concentration of the different recombinant cytokines were put into some of the samples which were then diluted from two to twenty times. The amount of each recombinant cytokine to be added was determined by obtaining a standard curve for the cytokine. A line was then drawn at 50% of the optical density of the standard curve and the concentration of cytokine for spiking determined. The samples were then diluted and known concentrations of the cytokines were added to the samples. ELISAs were then performed and the measured levels of cytokines were compared with the actual concentration of the recombinant cytokines (Figure 2.1). The optimum dilution was determined by taking the dilution that gave the maximum yield for the given cytokines. The optimum dilution varied between 5 to 10 times for the various cytokines. As the volume of the samples was small averaging about 50 to 100  $\mu$ l the dilution was advantageous in obtaining sufficient quantity of samples for duplicate wells of the ELISA. However the maximum volume obtained was adequate for the cytokine measurement of only one or two ELISAs. Sequential ELISAs overcame this in some of the samples (Verna, et al., 1998).

In this method, after the first step of the ELISA, i.e. the step where the cytokine measured bind to the immobilised antibody in the microtiter plate, the samples were aspirated instead of being thrown away. These are then transferred to the next ELISA plate measuring other cytokines. The unbound substances were then sequentially removed and transferred to the next ELISA plate. Since

the assays were specific for both natural cytokines and the recombinant human cytokines there were no measurable cross reactivity.

The concentration of the various cytokines detected were in pg/ml with the following minimum detection levels as determined by the manufacturers (R&D, IL-2 > 6.0 pg/ml, IL-4 > 3.0 pg/ml, IL-10 > 1.5 pg/ml, IL-12 > 3.0 pg/ml, IFN $\gamma$  > 3.0 pg/ml, TGF $\beta$ 2 > 2.0 pg/ml and TNF $\alpha$  > 2.0pg/ml).

## **2.5. TISSUE CULTURE**

### **2.5.1. VH samples**

Undiluted VH (ranged from 200 $\mu$ l-1000 $\mu$ l) was collected during vitrectomy and was immediately spun at 4°C at 400g for 10 minutes. The supernatants were aliquoted and kept at -70°C for subsequent ELISAs. The cell pellets were washed twice with human medium comprising RPMI 1640-Dutch modification supplemented with 10% heat-inactivated AB<sup>+</sup> human serum (Sigma, Poole, Dorset, UK) with gentamicin at 50  $\mu$ g/ml (Sigma), 200 $\mu$ M of L- glutamine (Sigma), 1mM sodium pyruvate (Sigma) and 1 in 100 dilution of MEM- non-essential amino acid (Sigma).

The cell pellet was washed with complete human medium twice before being transferred to a 24-well plate (Costar, Bucks, UK) containing 1000 $\mu$ l complete human medium containing 20 units/ml of Lymphocult-T which is human IL-2 (Biotest, Birmingham, UK) that is useful in enabling continuous *ex vivo* cultivation of normal activated T cells. Phytohaemagglutinin (PHA) (Sigma) was added at 1 $\mu$ g/ml and the cells incubated in humidified 5% CO<sub>2</sub> in air at 37° C. An additional 1 ml of human medium containing IL-2 was added after three days. After a week irradiated (3000 rad) autologous feeders were added with

PHA and the human medium and IL-2 was replaced every three days. This cycle was repeated for all the lines until  $2 \times 10^5$  cells were obtained. Almost all the samples required 4-5 restimulations to expand to this number. There were a significant number of samples where the cells did not grow well in the initial stage when the samples were not washed with complete human medium prior to culturing them. Samples, which are of small volume being less than 300  $\mu$ l, do not grow if the cell pellets were immediately plated into a 2-ml volume in 24 well plate. These pellets were then plated into a smaller U- bottom 200 $\mu$ l plate. After one or two weeks when there was enough number of cells growing in the plate these are then transferred to the 24 well plates and treated as the larger samples.

### **2.5.2. Peripheral Blood Samples**

20 mls fresh blood were obtained from the patients simultaneously with the VH samples and the lymphocytes isolated by density centrifugation using Ficoll-Hypaque (Boyum, 1974) (Pharmacia, St.Albans, Hertfordshire, UK) and cultured as for the vitreous at  $2 \times 10^5$ /ml. The remaining fresh PB monocytes (PBMC) were frozen in liquid nitrogen for later use as autologous feeder cells. The PB lymphocytes that were kept in culture in each cycle was approximately  $2 \times 10^5$  in 25 mls making a total of 5 million cells. As the numbers increased with each cycle, the excess cells were frozen. The PB lymphocytes were expanded in similar number of restimulations as the vitreous lymphocytes. This is to ensure that the PB-derived T cell lines underwent the same number of cycles and treatment as the VH-derived T cell lines.

## **2.6. PHA STIMULATION OF VH-DERIVED T CELL LINES.**

This was performed when enough number of cells approximating  $2 \times 10^5$  were obtained from the T cell lines. On the day of experiment, equal number of cells from VH and PB T cell lines were aliquoted into two separate wells at  $1 \times 10^5$  /ml in 2 mls in the 24 well (Costar) plate. Equal number of autologous feeders (prepared by irradiation of PB derived T cell lines) were added in addition to PHA without the addition of IL-2 as was usually done during the expansion process of T cell lines. A third well containing feeder cells alone was prepared to act as a negative control. 200  $\mu$ l of supernatants was harvested from all the three wells at 24, 48 and 72 hours after stimulation with PHA. These were then stored at  $-70^\circ$  Celsius for subsequent ELISA for IL-2, IL-4, IFN $\gamma$ , and IL-10.

## **2.7. FLOW CYTOMETRY ANALYSIS**

Three colour immunofluorescence was analysed using the Becton Dickinson (BD) flow cytometer equipped with a 15 mW argon laser and filter settings for FITC (Fluorescein Isothiocyanate) (530 nm), PE (Phycoerythrin) (585 nm), and PerCP (Peridin Chlorophyll Protein) emitting in the deep red ( $>650$  nm) was used.

### **2.7.1. Freshly isolated AH cells**

The cell pellets obtained after centrifugation were aliquoted into three separate tubes and washed twice with phosphate buffered saline (PBS, containing 0.1 % sodium azide and filtered through 0.45  $\mu$ l filter prior to use). The cells were then resuspended in a final volume of 15-20  $\mu$ l PBS. Triple staining was performed in the three tubes using only two different combinations in addition to a negative control tube due to small number of cells in the samples; CD3/CD14/CD19 and

CD4/CD8/CD25 with directly conjugated labelled mAbs and one tube with isotype-matched control antibodies (BD) (Table 2.1). In brief, cells were incubated with mAbs for 45-60 minutes in the dark at 4°C. Cells were then washed twice with PBS (Sigma) and resuspended in 15 to 20 µl PBS after which 500µl of 1% paraformaldehyde in PBS was added to fix the cells. Cells were then stored in the dark at 4°C for a minimum period of 60 minutes prior to data acquisition. In addition three colour flow cytometry was performed on the AH of two patients with FHC to ascertain the percentage of CD3<sup>-</sup> / CD8<sup>+</sup> /CD16<sup>+</sup> cells (NK cells) in the AH of these patients.

### **2.7.2. Fresh PB leucocytes.**

In parallel, 100µl of anticoagulated blood was added to 4 mls of lysis buffer (FACS lysis solution from Becton Dickinson Immunocytometry Systems). This was then allowed to stand for 10-20 minutes at room temperature, to allow complete lysis of the red blood cells. The washing procedure and staining were performed as described above for the AH. To properly set up and compensate the flow cytometer, these additional samples were prepared from the PB; unstained cells, cells stained with FITC reagents only; cells stained with the PE reagent only; and cells stained with the PerCP reagent only.

### **Acquisition of cells**

The prepared cell suspension was run through the flow cytometer, observing the forward and right angle scatter dot plot. The lymphocyte population was identified in the forward and right angle light scatter signals. A lymphocyte scattering gate (R1) was drawn around the lymphocyte population.



Spectral compensation was performed because even though FITC, PE, and PerCP emit light at distinct wavelengths when excited by laser light at 488 nm there is a slight overlap in their emission spectra. In addition, FL1, FL2 and FL3 detectors do not detect FITC, PE and PerCP emissions exclusively. Hence the amount of spectral overlapping was corrected using electronic compensation network. In order to confirm that compensation was optimised, cells, which have been singly stained with each fluorochrome-labeled antibody, were run in addition to singly, stained cells.

Firstly the unstained cells were run and the light scatter gate was set around the lymphocyte population. FL1, FL2 and FL3 were adjusted so that the unstained population appears in the lower left-hand corner of the fluorescence display. Next, the singly stained FITC cells were run and while monitoring the FL1 (green) versus FL2 (red-orange) dot display, the FL2-%FL1 compensation level was set so that the FL1 positive population was horizontally aligned with the FL1 negative population. Subsequently the singly stained PE cells were run. Again while monitoring the FL1 vs FL2 dot display the FL1-% FL2 was optimised so that the FL2-positive population is vertically aligned with the FL2-negative population. Then while monitoring the FL3 (red) versus FL2 dot display, the FL3-%FL2 compensation level was optimised so that the FL2 positive population was vertically aligned with the FL2 negative population. Finally, the singly stained PerCP cells were run and the FL2-%FL3 compensation level was set at zero while the FL3 vs FL2 dot display was monitored ensuring that PerCP fluorescence was not detected on the FL2 channel.

The cells were then acquired and the data stored for analysis. An additional gate apart from R1 was drawn i.e. R2. This is for the analysis of percentage expression among all leucocytes. At least 2000 cells in the AH and minimum of 5000 cells in the blood were analysed using Lysis II software. Some of the samples were not included, as there were too few cells to allow good acquisition of cells.

### **2.7.3. VH-derived T cell lines**

When sufficient number of T cells were obtained following expansions with PHA, potential cytokine production by these cells was investigated by stimulating with PHA without IL-2 supplement. After 72 hours of stimulation of T cells with PHA, the dead autologous feeder cells were removed by centrifuging with Ficoll-Hypaque and the viable cells eluted and subsequently washed with RPMI. The viable cells were then washed twice in PBS containing 0.2% sodium azide (Sigma) and 0.2% Bovine Serum Albumin (Sigma) and the cell pellets resuspended in 30 $\mu$ l volume and the appropriate monoclonal antibodies added. The cells were phenotyped according to the following surface markers CD3<sup>+</sup>(PE), CD4<sup>+</sup>(PerCP) and CD8<sup>+</sup>(FITC). The percentages of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the T cell lines were determined by the percentage of CD4 and CD8 staining of the CD3<sup>+</sup> cells.

# Standard Curve for TGF β2 6/3/95

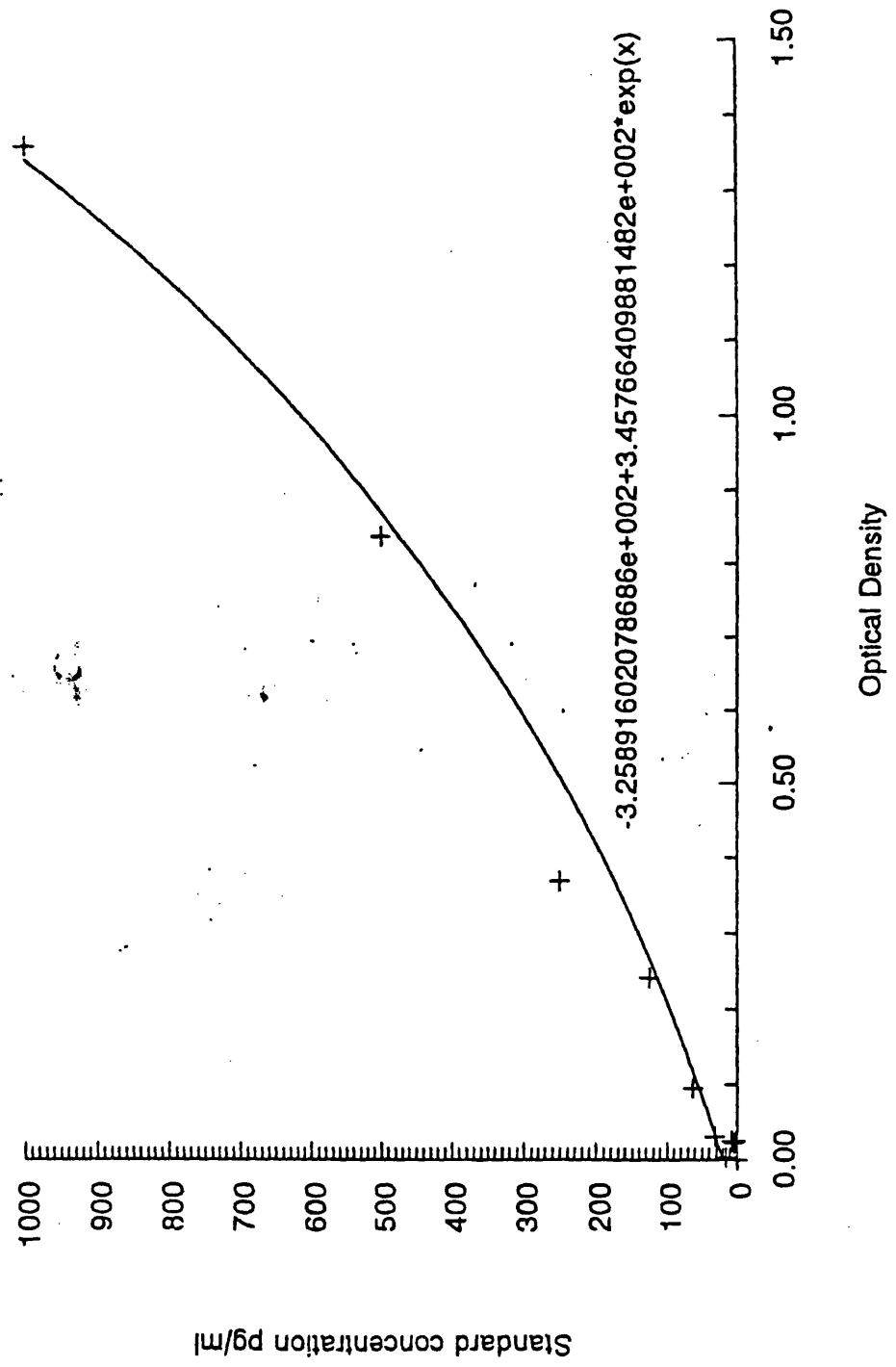


Figure 2.1

**Table 2.1. Monoclonal antibodies used during three-colour flow cytometry**

Antibody	CD	Isotype	Fluochrome	Predominant
Specificity				Reactivity
IgG1		IgG1	FITC	
IgG1		IgG1	PE	
IgG1		IgG1	PerCP	
Leu-4	CD3	IgG1	PE	Pan T cell
Leu-M3	CD14	IgG2b	FITC	monocyte/macrophage
Leu-12	CD19	IgG1	PerCP	B cell
Leu-3a	CD4	IgG2b	PerCP	T helper/inducer
Leu-2a	CD8	IgG1	FITC	T cytotoxic/suppressor
IL-2 receptor	CD25	IgG1	PE	Activated T cells

All antibodies were purchased from Becton Dickinson Immunocytometry system, Oxford UK.

## **CHAPTER 3**

### **CYTOKINE PROFILE IN AH IN DIFFERENT TYPES OF UVEITIS AND CONTROL PATIENTS.**

#### **3.1. INTRODUCTION**

Uveitis may be restricted to the anterior segment, involve the anterior segment and anterior vitreous, or predominantly affect the posterior segment with or without signs of inflammation within the AC. The varying manifestations of uveitis may represent the spectrum of intraocular inflammation in response to different insults be it infectious or non-infectious. The spectrum can vary from uveitis that are acute in nature with explosive recurrent attacks like Behçet's disease to the chronic low grade uveitis as in FHC. PS or adherence of the iris to structures posterior to it develops in all severe untreated anterior uveitis. This may result in pupillary block and occlusion of the angle by forward movement of the peripheral iris leading to pupillary block glaucoma with significant ocular morbidity. Interestingly this complication does not develop in-patients with FHC despite the ongoing chronic inflammation. These differences in clinical manifestations may be due to different triggering or effector mechanisms in the various types of uveitis. The former is difficult to ascertain in human diseases as opposed to animal models of uveitis. However the latter could be dissected by studying the effector molecules produced during active uveitis such as cytokines.

The important role of T cells in uveitis has been well demonstrated by several workers (Chan, 1985; Chan, et al., 1985; Nussenblatt & Scher, 1985; Lightman & Chan, 1989; Lightman & Chan, 1990). The pathogenic mechanism is believed to be due to cell-mediated immunity with the predominant role of T cells

and other accessory cells of the immune system. Apart from the T cells, which are specific effectors of immunity, other cells play their role in the amplification of immune response leading to uveitis. In some form of cell mediated immunity, antigen-specific T cells directly perform the effector function, as when CTLs lyse specific target cells; in other types, antigen-activated T cells secrete cytokines which are soluble mediators of innate and specific immunity that recruit and activate effector cells that are not specific for the antigen, such as macrophages and NK cells.

In general cytokines are synthesised in response to inflammatory or antigenic stimuli and act locally, in an autocrine or paracrine fashion by binding to high affinity receptors on target cells. Cytokines mediate many of the effector functions of cells that produce them and are the principal mechanisms by which various immune and inflammatory cell population communicate with each other. Hence by means of cytokine secretion T cells stimulate the function and focus the activity of non-specific effector cells of innate immunity, thereby converting these cells into agents of specific immunity.

Cytokines, which can be regarded as biologic response modifiers, can be classified into three groups according to their principal actions (Abbas, et al., 1996). The first group consist of those cytokines that mediate innate immunity and includes anti-viral cytokines (e.g. type 1 interferons, IL-12, IL-15), proinflammatory cytokines (TNF, IL-1, IL-6, and chemokines) and regulatory cytokines (e.g.IL-10). The predominant cellular source of these molecules is mononuclear phagocytes.

The second group of cytokine is derived from antigen-stimulated T lymphocytes and serves to intensify, focus and specialise inflammatory reactions of specific immunity. This group includes IL-2, the principal T cell growth factor, IL-4, the principal switch factor for Ig-E, currently known as a major regulator of Ig-E and mast cell/eosinophil mediated immune reaction, and TGF $\beta$  which inhibits lymphocyte response and function as “anti-cytokine” by its antagonising effect on proinflammatory cytokines. Another T lymphocyte derived cytokine is IFN $\gamma$ , the principal activator of mononuclear phagocytes in addition to TNF $\beta$ , an activator of neutrophils, and IL-5 an activator of eosinophils.

CD4<sup>+</sup> T cells may differentiate into specialised effector Th 1 that secrete IFN $\gamma$  and TNF $\beta$ , which favours cell-mediated-immunity, or into Th 2 cells that secrete IL-4, and IL-5, which favours Ig-E and eosinophil mediated immunity. The pattern of differentiation may be influenced by cytokines produced in the innate response or early in the specific immune response (Trinchieri, 1995). Although Th 1 and Th 2 cells are major sources of their respective cytokines, many other cells within and outside the immune system also produce these cytokines. Subsets of T cells, CD4<sup>+</sup> and CD8<sup>+</sup> T cells can all secrete Th 1 or Th 2 like cytokine patterns. NK cells produce IFN $\gamma$  and TNF $\alpha$ , and contribute to Th 1 like responses (Trinchieri, et al., 1992). IL-10 is produced by macrophages, keratinocytes and cytotrophoblast cells in the placenta. Since several cell types may contribute to an overall Th 1 or Th 2 cytokine pattern, the term type1 (for cell mediated immunity) and type 2 (for humoral immunity) is preferred.

The third group of cytokine consists of cytokines derived from marrow stromal cells and T cells, which stimulate the growth of bone marrow progenitors, thereby providing a source of additional inflammatory leucocytes. These are collectively called colony-stimulating-factors. Hence the cytokines serve many functions and provide links between specific and innate immunity.

Many previous studies have quantitated various cytokines in the AH (Hoekzema, et al., 1990; Murray, et al., 1990a; Murray, et al., 1990b; Hoekzema, et al., 1991; de Boer, et al., 1992) and in this study more cytokines which regulate both innate (IL-12, IL-10,  $\text{TNF}\alpha$ ) and specific immunity (IL-2, IL-4,  $\text{IFN}\gamma$ ,  $\text{TGF}\beta$ ) have been quantitated. The levels of these cytokines in the AH of uveitis patients are compared with levels in AH of control patients. In addition the cytokine pattern in these AH was determined to ascertain any correlation between the clinical manifestations and the cytokine levels.

### **3.2. STATISTICAL ANALYSIS.**

Non parametric Mann-Whitney-U test was used to compare the cytokine level between uveitis and control patients. In this test the median is used in the ascertaining significant differences between the groups being compared. The Chi square test was used to compare association between detection of IL-10 and presence of PS. Pearson correlation was used to calculate correlation between the different cytokines in the AH.

### **3.3. RESULTS**

A total of 59 AH samples from patients with a variety of different types of uveitis and 10 control AH samples were analysed for the presence of various cytokines. The number of patients in each group were 10 FHC patients, 18 IAU,



10 AUS, 7 IPU, 5 panuveitis as part of Behçet's disease, 7 panuveitis as part of sarcoidosis, 1 patient with intraocular toxoplasmosis and 1 patient with intraocular B cell lymphoma (Table 3.1 and Table 3.2). Because of the limited volume of AH available, it was not possible to measure all the cytokines in each sample. The number of samples tested for each cytokine are shown (Table 3.3). The mean and range of cytokines detected is also shown (Table 3.4). The levels of cytokines were defined as 'high' (when the levels are more than 75<sup>th</sup> centile of levels of control AH) for (IL-4 > 2.1 pg/ml, IL-10 > 14.2 pg/, IL-12 > 14.5 pg/ml, IFN $\gamma$  > 91.8 pg/ml and TGF- $\beta$ 2 > 549.8 pg/ml) and 'low' when the level is lower than the 75<sup>th</sup> centile of levels of control AH.

Both type 1 and type 2 cytokines were detected in the AH of uveitis patients with predominance of type 1 cytokines. This predominance is suggested by the higher proportion of patients with detection of 'high' level of IL-12 (46.3%) and IFN $\gamma$  (31.1%) than 'high' level of IL-4 (11.1%) and IL-10 (11.1%). Interestingly a lower proportion of uveitis patients has a 'high' level of TGF $\beta$  (20.5%). A striking finding is the significantly higher level of IL-10 in control AH ( $107.9 \pm 33.1$  pg/ml; mean  $\pm$  s.e.m) compared to AH ( $49.4 \pm 12.1$ ) of uveitis patients ( $p < 0.05$ ) (Fig.3.1).

In addition IL-2 was not detected in the AH of any of the 10 control patients. However it was detected in five of the uveitis patients. The highest level of IL-2 (247.0 pg/ml) was in a patient with anterior uveitis as part of systemic disease MS (MS). In this patient there was a marked AC inflammation and extensive PS. The level of IFN $\gamma$  however was 'low' in the AH of this patient. In

addition none of the type 2 cytokines IL-4 and IL-10 was detected in the AH of this patient.

The level of TGF $\beta$ 2 was higher in AH of control patients ( $457.5 \pm 52.6$ ) compared to AH of uveitis patients ( $375.9 \pm 29.2$ ). However this was not statistically significant. There is a positive association between the detection of IL-10 and the absence of PS ( $p = 0.029$ ) by chi square test. In addition the level of IL-10 was highest in a patient with FHC (274 pg/ml). This cytokine was detected in the highest proportion of patients with FHC (7 out of 10 AH) compared to other types of uveitis and interestingly the levels of IL-10 detected were 'high'.

In the patient with intraocular lymphoma the level of IL-10 in the AH was exceedingly high with a level of 1021 pg/ml. In addition the level of TNF $\alpha$  was the highest among all the AH with a value of 167.2 pg/ml in this pt. This proinflammatory cytokine however was not detected in any of the AH from control or FHC patients.

The level of IL-12 was all in the 'low' category in patients with FHC. However there was no apparent association between IL-12 level and PS formation in patients with other types of uveitis. The level of IL-12 was highest in the AH of a patient with Behçet's disease who showed a marked AC reaction with hypopyon and extensive PS. In addition in a patient with ocular toxoplasmosis, the level of IFN $\gamma$  was highest with a value of 470.18 pg/ml. IL-10 was not detected in the AH of this patient.

Another interesting finding is the positive correlation between TGF- $\beta$ 2 and IL-12 ( $r = 0.60$ ,  $n = 39$ ) and TGF- $\beta$ 2 and IFN $\gamma$  ( $r = 0.65$ ,  $n = 39$ ) in the AH of uveitis patients.

### 3.4. DISCUSSION

Cytokines are produced during the activation and effector phases of innate and specific immunity and serve to mediate and regulate immune and inflammatory response. In this study the cytokines quantitated have revealed a mixture of both type 1 and type 2 cytokines. However there is a predominance of type 1 cytokines in the AH of uveitis patient compared to type 2. This is inferred from the fact that there were a higher percentage of uveitis patients with 'high' (defined as being more than 75<sup>th</sup> centile of values from control AH) level of IL-12 and IFN $\gamma$  compared to IL-4 and IL-10. This is in agreement with a recent study by Murray et al where the mRNA expression of IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-10, IL-12, IFN $\gamma$ , TGF- $\beta$  were analysed in the AH obtained during cataract surgery of uveitis patients (Murray, et al., 1997). It was shown that there was a predominant Th 1 polarisation with expression of IL-2 and IFN $\gamma$  despite the absence of IL-12. In contrast, in this study IL-12 was detected in the AH and the mean level of all the type 1 cytokines (IL-2, IFN $\gamma$ , IL-12) responsible for cell mediated immunity was higher in uveitis compared to control AH even though it was not statistically significant. The crucial difference between this study and that by Murray et al was that the AH obtained from the latter study was from uveitis patients whose eyes were presumably inactive by systemic immunosuppressive therapy in some while in the former study the AH were obtained from patients with active uveitis. The detection of these cytokines,

which are the effector molecules of T cells (except for IL-12) and cell-mediated-immunity further, confirms the role of T cells in the pathogenesis of uveitis.

IL-10 was significantly higher in the control AH compared to uveitis patients. This cytokine which was initially called cytokine synthesis inhibitory factor serves two main functions. The first function is to inhibit the production of other cytokines (TNF $\alpha$ , IL-1, chemokines and IL-12) by macrophages thereby exerting its antiinflammatory action. The second function is to inhibit the accessory cell function of macrophages in T cell activation. This effect is due to reduced expression of class II MHC and reduced expression of costimulators, e.g. B7-1 and B7-2 on these cells which are essential co-stimulators for these cells to function as competent APCs. The net effect of these actions is to inhibit both innate and T cell mediated specific immune inflammation. This is responsible for its downregulatory role in inflammation. Another significant role of IL-10 is its contribution to maintaining immune-privilege. Li et al (Li, et al., 1996) has demonstrated that immune privilege in the AC of the eye is due to the selective activation of a Th2 population that cross-regulates Th1 responses in mice. This may be reflected in the AH of uveitis patients, which contain significantly lower level of IL-10. Similarly the mRNA expression of IL-10 was detected in only three out of twenty-four uveitic AH in a previous study (Murray, et al., 1997).

Among the uveitis patients the highest level and detection rate of IL-10 was in FHC patients. This is a very interesting observation as FHC is a chronic uveitis with low-grade inflammation. As cell-mediated-immunity is the most likely effector mechanism of inflammation in uveitis, the 'high' IL-10 may

account for the minimal inflammation in FHC due to its downregulatory role in inflammation.

The absence of PS is a characteristic feature of FHC in contrast to other types of anterior uveitis. PS is usually a sequelae of untreated acute anterior uveitis. However it also occurs in chronic uveitis of children with juvenile RA (JRA). The adhesion of the iris to structures posterior to itself does involve some amount fibrosis, which is the final outcome in inflammation especially in DTH where the ultimate effector cells are activated macrophages. The effects of these activated macrophage derived cytokines and growth factors occur in two phases. Acutely,  $\text{TNF}\alpha$ , IL-1 and macrophage-derived chemokines augment inflammatory reactions initiated by T cells. Chronically these same cytokines also stimulate fibroblast proliferation and collagen production. If we infer the same sequence of events in the inflammation of the anterior segment of the eye, then well established PS could have resulted from the chronic effects of the macrophage derived cytokines. Since IL-10 has antagonistic effect on the effector function of macrophages (Moore, et al., 1993), PS could be 'prevented' from occurring in eyes of patients with FHC despite the ongoing chronic inflammation in these eyes.

The positive association between PS and the detection of IL-10 in AH of patients with anterior uveitis other than FHC further suggests that IL-10 may have a protective role in PS formation. The protection could be a direct effect of IL-10 as a result of inhibition of synthesis of proinflammatory cytokines such as  $\text{TNF}\alpha$ , IL-1, chemokines and IL-12 by macrophages (Fiorentino, et al., 1991) making it a potent suppressor of the effector functions of macrophages, T cells,

and NK cells (Moore, et al., 1993). Alternatively this could be a coincidental finding as the lowered inflammatory reaction in those ACs as a result of IL-10 effects, is not severe enough to cause PS.

Similar association between presence of PS and IL-12, a cytokine favouring cell-mediated-immunity was ascertained by chi-square test but no association could be observed between detection of 'high' level of IL-12 and presence of PS in the eyes of those patients. However an important observation is that all the AH of FHC patients contained 'low' (less than 14.5 pg/ml; 75<sup>th</sup> centile of control AH) level of IL-12. This may be in favour of the role of IL-12 in PS formation if PS is believed to be a sequelae of DTH. IL-12 induces cytokine production, primarily of IFN $\gamma$  from NK and T cells (Trinchieri, et al., 1996), acts as growth factor for activated (Trinchieri, 1993) NK and T cells, and favours cytotoxic T lymphocyte generation in other words favouring cell-mediated-immunity (Romagnani, 1995). It also favours the differentiation of Th 1 which in turn secrete IFN $\gamma$  that is the principal macrophage-activating factor (MAF). IFN $\gamma$  also activates macrophages to produce IL-12 and T cells to express IL-12 receptors. This provides an amplification mechanism for a type 1 or cell mediated immunity that may result in ocular morbidity. Hence the low IL-12 in FHC may contribute to protection from PS in addition to high IL-10 level.

The proinflammatory cytokine TNF $\alpha$  was not detected in the control AH which is not an unexpected finding. The non-detection of this cytokine in the AH of some uveitis patients such as FHC, IPU and Behçet's disease may have different implications. In benign uveitis such as FHC the non-detection may reflect a true absence of the cytokine and may contribute to the benign nature of

this condition since this cytokine is a major mediator of cell adhesion and chemotaxis with significant contribution to the regulation of cell migration during inflammation (Beutler & Cerami, 1989). In florid ocular inflammation such as Behçet's disease and IPU its absence may indicate increased consumption during the initial inflammatory stage as it is an important mediator in metabolic and immunological responses and can be considered as one of the early, critical mediators in inflammation.

IL-2, originally called T cell growth factor, is the principal cytokine responsible for progression of T lymphocytes from the G1 to S phase of the cell cycle (Smith, 1988). This cytokine was not detected in any of the control AH. Normally IL-2 is transcribed, synthesised, and secreted by T cells only upon activation by antigens. Hence in the control AH where there is no inflammation, the absence of activated T cells may explain the non-detection of IL-2. However IL-2 was detected in only 5 of the 45 uveitis AH tested. This can be due to the fact that the secretion of IL-2 as other cytokines is usually transient, and as it functions as an autocrine growth factor, this cytokine was most probably consumed as soon as it was secreted. This may reflect a high turnover of T cell activation and proliferation in these uveitic eyes resulting in high consumption of this cytokine.

Interestingly in the patient with anterior uveitis related to MS with marked AC reaction the level of IL-2 was highest. As the quantity of this cytokine synthesised by CD4<sup>+</sup> T cells is an important determinant of the magnitude of T cell dependent immune response, (Smith, 1988) the high level may reflect the dominant T cell dependent response during active anterior

uveitis. Also the increased cellular infiltration as seen by the marked AC reaction in this patient suggests that the cells are actively involved in the inflammation and not passively extravasated into the AC as a result of a breakdown in the blood-aqueous-barrier.

Transforming growth factor  $\beta$  (TGF $\beta$ ) is a powerful inhibitor of T and B cell proliferation. Cells of the immune system (T cells and monocytes) synthesise mainly TGF $\beta$ 1, but certain anatomic sites such as CNS may contain high level of TGF $\beta$ 3 and TGF $\beta$ 2 is the isoform of TGF $\beta$  that is most commonly found in the eye. These cytokines are present and act to suppress local immunity. TGF $\beta$ 2 in the eye has been recognised to be contributing to ACAID. Work by Wilbanks et al (Wilbanks, et al., 1992) suggests that macrophages that reside in the iris and ciliary body can migrate out of an antigen-bearing eye and activate regulatory T cells within the spleen. Their results reveal that the parenchymal iris/ciliary cells secrete a soluble factor(s) (later identified as TGF- $\beta$ ) locally and into the AH which endows resident, mature macrophages with ACAID-inducing capabilities. This cytokine is now believed to be secreted by Th 3 cells apart from other cells (Chen, et al., 1994). Thus, CD4<sup>+</sup> cells that primarily produce TGF- $\beta$  appear to be a unique T-cell subset that includes mucosal helper T-cell function and downregulatory properties for Th1 and other immune cells. In contrast to Th1 and Th2 cells, these cells provide help for IgA production and primarily secrete TGF $\beta$  (Chen, et al., 1994).

In this study the level of TGF- $\beta$ 2 was higher in the AH of control patients compared to uveitis patients even though it was not statistically significant. This is consistent with its contribution to immune-privilege. In addition among the



uveitis patients, highest level of TGF- $\beta$ 2 was found in FHC patients. Its ability to inhibit cytokine production and lymphocyte proliferation in addition to being a potent inhibitor of macrophage function may contribute to the low grade and benign inflammation in FHC patients.

An interesting finding is the positive correlation between TGF- $\beta$ 2 and IL-12 and TGF- $\beta$ 2 and IFN $\gamma$  in the AH of uveitis patients. This suggests that there are interactions between these cytokines in the AH. The increase of type 1 cytokines may upregulate the expression of regulatory TGF- $\beta$  reflecting the attempt by the intraocular microenvironment to downmodulate immune activation.

Apart from the above general observation between control and uveitis patient as a group there are individual cases where the AH may demonstrate *in vivo* relationship between cytokines and correlation with clinical manifestation. In a patient with Behçet's disease the levels of type 1 cytokines IFN $\gamma$  and IL-12 were very high and there was profound AC inflammation with hypopyon, when the massive amount of inflammatory cells settle down inferiorly in the AC. Interestingly there was extensive PS in this eye, which is consistent with our above postulate that this cytokine may contribute to PS formation. In addition, no IL-10 was detected in this AH implying antagonistic action by the type 1 cytokine towards its production. Another interesting finding in this AH is the exceedingly high level of TGF- $\beta$ 2, which also was the highest among all the patients including control patients. The high TGF- $\beta$ 2 could be produced by the Th 3 cells that are known to inhibit the action of both Th 1 and Th 2 cells

resulting in downregulatory action in an attempt to counteract the severe inflammation.

Another case that illustrates some *in vivo* picture of cytokine production is in the patient with intraocular toxoplasmosis where IFN $\gamma$  was detected at a very high level of 470.18 pg/ml whereas IL-10 was not detected. This is consistent with the fact that this cytokine is important in promoting macrophage-rich inflammatory reaction providing protection against intracellular infection. Previous studies of mice have implicated natural killer (NK) cells as mediators of protective activity against *Toxoplasma gondii* through their production of IFN $\gamma$ . The production of IL-10 by mice infected with *Toxoplasma gondii* has been implicated in the suppression of lymphocyte proliferation observed during acute toxoplasmosis, as well as susceptibility to infection with this parasite (Hunter, et al., 1994; Neyer, et al., 1997). Hence in this patient the high IFN $\gamma$  production may reflect the attempt by ocular inflammatory cells to combat the infection by *Toxoplasma gondii*.

Finally in one interesting case of intraocular B cell lymphoma, the level of IL-10 was very high. The role of IL-10 detection in the VH in the diagnosis of intraocular and CNS lymphoma is increasingly recognised (Whitcup, et al., 1997). Recently it was shown that both IL-13 and IL-10 genes are expressed in B-cell lymphomas (Emilie, et al., 1997). However this is the first time that the detection of IL-10 in AH of intraocular lymphoma is documented. Interestingly PS was present in this patient and there was marked AC reaction. It may suggest that the IL-10 produced is not functionally the same as in other situation. This defective function of IL-10 is further suggested by the detection of high level of

TNF $\alpha$  when the production of this cytokine by macrophages is normally inhibited by IL-10.

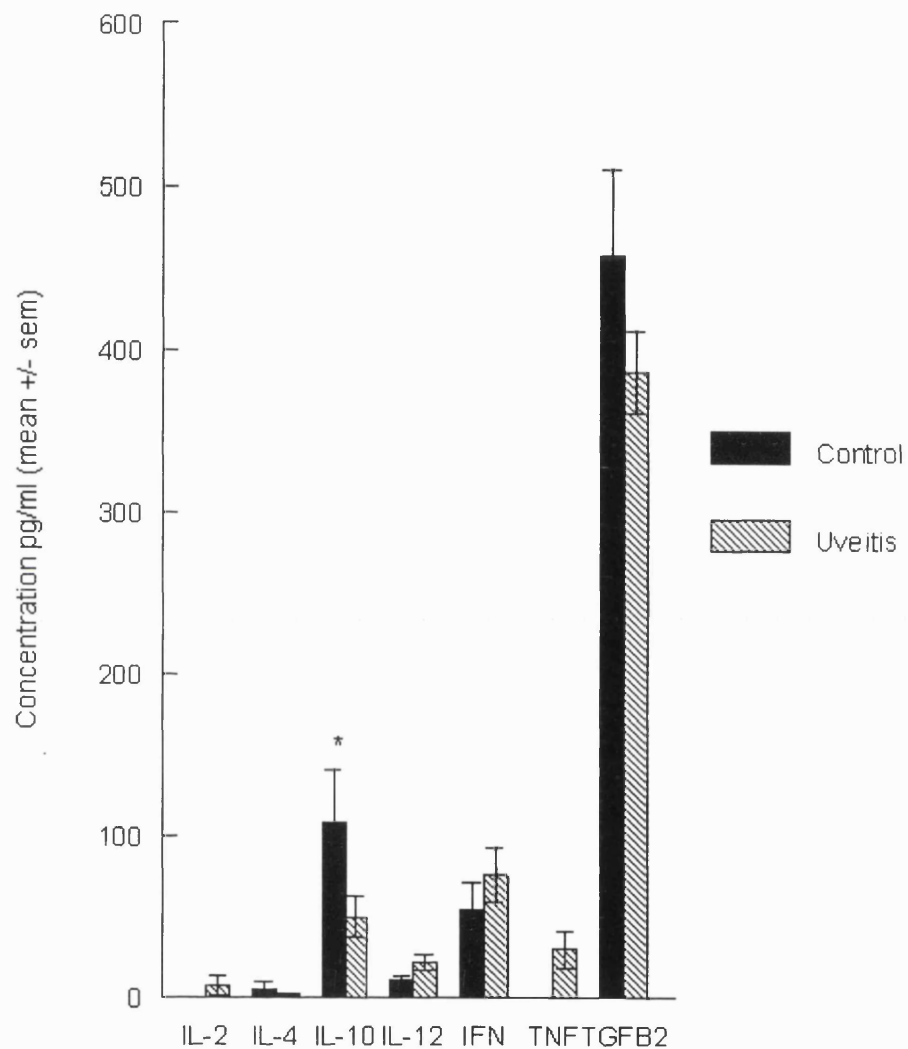
These facts above suggests that the cytokines detected in the AH do reflect the immune dysregulation occurring in the intraocular microenvironment of the eye. The significant correlation between the antagonistic cytokines may reflect the attempt by the ocular immune system to maintain equilibrium. The T cell cytokines and other related cytokines detected and the presence of relationship between the cytokines and the clinical picture of the patients is consistent with the fact that these cytokines detected in the AH are important in the pathogenesis of uveitis.

The presence of other non-T cell cytokines produced by accessory cells suggests that there are interactions between the activated T cells and the accessory cells in an attempt to amplify and focus the immune response. The presence of these cells are necessary for the full physiologic activation of the T cells, as they serve in processing of antigen and providing stimuli or costimulators in the activation of T cells. What would appear to be some downregulatory role of IL-10 and its possible role in protection from PS formation has opened up another realm of therapeutic potential, i.e. the use of topical IL-10 in the treatment of severe anterior uveitis, hence avoiding the well known side effects of topical steroids.

The detection of the various T cell and its related cytokines in the AH of patients with intraocular inflammation has demonstrated the various possible interactions between these inflammatory mediators. A knowledge of the cellular phenotypes present in the AH will further dissect the immunological mechanisms

in these eyes. In addition, comparing the cytokine and leucocyte profiles of AH between patients with localised anterior uveitis but with differing clinical profile i.e. FHC and IAU and uveitis localised to the eye (LU) and uveitis as part of a systemic disease (SU) regardless of the anatomical involvement will further increase the understanding in the immune dysregulation in these eyes which would be useful when planning certain immunotherapeutic modalities. The possibilities of using cytokines rather than steroids to downregulate inflammation will be a major breakthrough as the ill effects of steroids could be avoided.

### Comparison of cytokines in the AH of Control vs Uveitis



\*  $p < 0.05$

Figure 3.1. This illustrates the significantly higher IL-10 in the AH of control population compared to uveitis patients ( $p < 0.05$ ). It also demonstrates the absence of TNF $\alpha$  in control AH and the relatively higher TGF- $\beta$ 2 in control AH compared to AH of uveitis patients.

**Table 3.1 Details of uveitis patients**

No	Age	Gender	Race	AC activity	Treatment	Diagnosis
1	31	male	white	1+	none	FHC
2	38	male	white	1+	none	FHC
3	30	female	white	1+	none	FHC
4	18	male	black	1+	none	FHC
5	30	female	white	1+	none	FHC
6	69	female	white	1+	none	FHC
7	22	female	white	1+	none	FHC
8	21	male	white	1+	none	FHC
9	20	female	white	1+	none	FHC
10	28	female	white	1+	none	FHC
11	45	male	white	1+	TS	CIAU
12	66	female	white	1+	TS	CIAU
13	38	female	white	3+	TS	AIAU
14	37	male	white	3+	TS	AIAU
15	43	male	white	2+	TS	AIAU
16	47	female	white	3+	TS	AIAU
17	56	male	white	3+	TS	CAIU
18	47	male	Asian	1+	TS	CAIU
19	53	male	white	1+	TS	CIAU
20	35	male	white	2+	TS	AIAU
21	43	female	black	2+	TS	AIAU
22	21	female	Asian	3+	TS	AIAU
23	39	male	white	2+	TS	AIAU
24	40	male	white	2+	TS	AIAU
25	44	male	white	2+	TS	AIAU
26	54	female	white	2+	TS	AIAU
27	59	male	white	1+	TS	AIAU
28	43	female	Asian	2+	TS	AIAU
29	45	female	Asian	1+	TS	IPU
30	31	female	white	3+	TS	IPU
31	11	male	white	1+	P-10	IPU
32	27	male	Asian	2+	TS	IPU
33	41	female	black	1+	P-10	IPU
34	54	male	white	1+	TS	IPU
35	35	male	black	3+	TS	IPU
36	35	male	white	no AC activity	P-40	Behçet's disease
37	26	male	Asian	3+	P-15,csa-200,aza-150	Behçet's disease
38	35	male	white	4+-hypopyon	aza-150	Behçet's disease
39	34	male	white	1+	P-15	Behçet's disease
40	36	male	white	1+	none	Behçet's disease
41	35	female	black	2+	TS,P-7.5	PU-sarcoidosis
42	45	female	black	2+	TS,P-7.5	PU-sarcoidosis
43	34	female	Asian	2+	none	PU-sarcoidosis
44	43	female	black	1+	TS	PU-sarcoidosis
45	48	female	Asian	1+	TS	PU-sarcoidosis
46	48	female	Asian	2+	TS	PU-sarcoidosis
47	49	male	black	2+	TS,P-7.5	PU-sarcoidosis
48	33	male	black	3+	TS	AUS-RS-acute

49	53 female	white	2+	none	AUS-AS-acute
50	45 male	white	2+	none	AUS-AS-acute
51	33 male	white	2+	none	AUS-AS-acute
52	33 male	white	1+	none	AUS-AS-acute
53	54 male	white	3+	none	AUS-AS-acute
54	33 male	white	no AC activity	none	AUS-JCA-chronic
55	28 female	white	1+	TS	AUS-JCA-chronic
56	37 female	white	1+	TS,P-40	AUS-MS-acute
57	61 female	white	1+	none	AUS-Psoriasis-acute
58	39 female	white	1+	TS,P-15	B-cell lymphoma
59	23 male	black	2+	TS	Toxoplasmosis

**Key- PU-panuveitis**

IPU-idiopathic panuveitis

P-number- Prednisolone dosage in mg  
dosage

TS-topical steroids

aza-number- azathioprine dosage in mg

csa-number- cyclosporin A dosage in mg

AIAU-acute idiopathic anterior uveitis

CIAU-chronic idiopathic anterior uveitis

RS-Reiter's syndrome

AS-ankylosing uveitis

**Table 3.2. Details of control patients**

No	Age	Gender	Race	AC activity	Treatment	Diagnosis
1	73	male	white	nil	nil	cataract
2	73	male	white	nil	nil	cataract
3	65	male	white	nil	nil	cataract
4	78	female	white	nil	nil	cataract
5	55	male	white	nil	nil	cataract
6	75	male	Asian	nil	nil	cataract
7	25	male	Asian	nil	nil	keratoconus
8	75	male	white	nil	nil	cataract
9	66	male	black	nil	nil	cataract
10	48	male	Asian	nil	nil	cataract

		CYTOKINES MEASURED IN THE AH							
		Phenotypes	IL-2	IL-4	IL-10	IL-12	IFN- $\gamma$	TGF- $\beta$ 2	TNF- $\alpha$
<b>DIAGNOSIS</b>									
<b>control AH</b>		not done	10	10	10	6	5	10	10
n=10									
<b>uveitic AH</b>									
FHC			10	10	10	10	10	10	10
n=10									
IAU	}	LU	18	10	6	6	6	6	6
n=18									
IPU			1	5	3	3	3	3	3
n=7									
Behçet's disease	}	SU	3	5	5	5	5	5	5
n=5									
Sarcoidosis			5	7	7	7	7	7	not done
n=7									
AUS	}		5	6	6	6	6	6	not done
n=10									
Toxoplasmosis			not done	1	1	1	1	1	not done
n=1									
Intraocular lymphoma			1	1	1	1	1	1	1
n=1									
Total tested in abnormal AH			43 45*		39	39	39	39	25
<b>Key</b>									
LU- uveitis localised to the eye									
SU- uveitis associated with systemic disease									

\*IL-2 was measured in more samples (n=45) than other cytokines (n=39) as it

was the first cytokine to be quantitated, hence more samples were available.

Sequential ELISA was performed after IL-2 were quantitated hence overcoming

the problem of adequate sample volume



**Table 3.4. The mean and range of cytokines detected in the AH of control and uveitis patients**

<b>DISEASE GROUP</b>	<b>IL-2 range pg/ml</b>	<b>IL-4 range pg/ml</b>	<b>IFN-gamma range pg/ml</b>	<b>TGF-beta2 range pg/ml</b>	<b>TNF-alpha range pg/ml</b>	<b>IL-10 range pg/ml</b>	<b>IL-12 range pg/ml</b>
<b>CONTROL</b>	not detected	4--39	14--175	308--498	not detected n=2	17--144	0--15
<b>mean,n=10</b>	not detected	4.87	53.45	457.54	not detected	107.90	10.04
<b>FHC</b>	0--4.8	0--25	60--180	313--768	not detected	126--274*	0--7
<b>mean,n=9</b>	0.53	2.82	64.6	423.2	not detected	84.9	2.81
<b>IAU</b>	0--7.2	0--35	21--104	355--509	0--167	6--118	8--75
<b>mean, n=9</b>	0.55	1.95	30.21	340.88	29.58	35.78	22.24
<b>IPU</b>	not detected	0--11	0--119	303--498	not detected	34--88	17--23
<b>mean, n=3</b>	not detected	1.61	88.48	320.83	not detected	21.84	18.5
<b>AUS</b>	7--247	0--4.3	14--381	340--526	0--59	58-124	0--141
<b>mean, n=8</b>	93.86	0.86	85.43	385.43	29.4	60.63	42.96
<b>BD</b>	not detected	0--4	14--587	360--902	not detected	39--97	0--108
<b>mean, n=3</b>	not detected	2.15	239.6	412.33	not detected	59.43	41.7
<b>SD</b>	not detected	0--11	0--42	339--450	64-167	0--118	0--5.6
<b>mean, n=7</b>	not detected	2.26	30.24	372.61	120.12	33.82	2.23

**\* - highest level detected**

## **CHAPTER 4**

### **Characterisation Of T Cells And Cytokines In The Aqueous Humour (AH) In Patients With Fuchs' Heterochromic Cyclitis (FHC) And Idiopathic Anterior Uveitis (IAU)**

#### **4.1. INTRODUCTION**

Anterior uveitis is the most common form of uveitis accounting for 75% of all uveitis cases with an annual incidence of 8 cases per 100,000 population (Vedor, et al., 1984; Wakefield, et al., 1986; Rothova, et al., 1987) and consists of inflammation predominantly in the AC and the ciliary body. It can be part of some well-defined clinical syndromes eg. sarcoidosis or Behçet's disease or a localised ocular disorder as in IAU and FHC. Even though both FHC and IAU are both localised ocular diseases they are strikingly different in their manifestations.

FHC is characterised by a white eye with a chronic, low-grade anterior uveitis with widely scattered small non-pigmented keratic precipitates, a variable degree of iris atrophy and depigmentation of the iris. In contrast, IAU presents with either acute or chronic inflammation in which keratic precipitates tend to be distributed mainly in the inferior half of the corneal endothelium. Keratic precipitates are comprised of macrophages or other leucocytes, which are deposited over the posterior layer of the cornea ie. the endothelium. One other contrasting feature between FHC and IAU is the response to topical steroids. Topical steroids do not help reduce inflammation in FHC whereas in IAU they have a significant effect. The explanation of this differing response to topical

steroids has not been found, nor why FHC has such a benign chronic inflammatory course.

Many workers have investigated the histopathology of FHC and other types of chronic uveitis from iris biopsies taken at the time of cataract surgery and despite the use of light microscopy (Redslob & Brini, 1957; Goldberg, et al., 1965; Loewenfeld & Thompson, 1973) and electron microscopy (Wobmann, 1976; Melamed, et al., 1978; Mc Cartney, et al., 1986) no difference in the pathology of FHC compared to other types of uveitis has been identified. The overall picture is that of a chronic inflammation with mainly lymphocytes and plasma cell infiltration in addition to some eosinophils and mast cells. An immunohistochemical analysis of the iris samples obtained by peripheral iridectomy at the time of cataract or glaucoma surgery in patients with FHC and other types of uveitis did not detect any specific immunohistological differences (Murray, et al., 1990c). Hence it has been the general consensus that it is impossible to differentiate FHC from other types of chronic iridocyclitis on histopathologic and immunohistologic changes seen in the iris.

The differing clinical profile between FHC and other types of chronic uveitis despite similar histological changes suggests that there may be some differences in the functional characteristics of the infiltrating cells influencing the outcome of disease in these entities. The similar cellular types present structurally in these eyes with different clinical features may imply that the cells are functionally different. Several workers have investigated the lymphocyte subsets in the PB of FHC and other types of uveitis (Nussenblatt, et al., 1983b; Murray, et al., 1984b; Murray, et al., 1984a; Deschenes, et al., 1988). However

in uveitis occurring without systemic involvement, immunological changes occurring within the eye may not be reflected in the PB. The aim of this study was to compare the cellular phenotypes in AH and PB of patients with FHC and IAU and the cytokine profile present in the AH in these two clinically distinct types of anterior uveitis. The cellular phenotypes were quantitated simultaneously in the PB to enable any selective increase of any of the cellular phenotypes present in the AH to be interpreted.

#### **4.2. STATISTICAL ANALYSIS**

Data are presented as mean (standard deviation; s.d.); median and 90% confidence interval (CI 90). The non-parametric Mann-Whitney U test was performed comparing the cellular phenotypes between FHC and IAU. The Chi-square test was performed to compare the proportion of patients with 'high' and 'low' level of the various cytokines between the two clinical entities after stratification of the data using the 75th centile of the pooled data from the two groups of patients as the cut-off point. P value of less than 0.05 was considered significant in both statistical tests. Pearson correlation coefficient was used to ascertain the strength of association between the level of cytokines and the cellular phenotypes in the AH in both patient groups. Minitab 10.5 for Windows software was used for statistical analysis.

#### **4.3. RESULTS**

##### **Cellular phenotypes**

The AH and PB of 10 FHC (age range-18-69 years, mean-30.7) and 18 IAU (age range- 21-67 years, mean - 45) patients were analysed. In addition the PB of 12 healthy volunteers were analysed as a comparison. There was no significant

difference in the cellular phenotypes of the PB between the patients and the control patients.

There was predominance of T cells (CD3) compared to B cells (CD19) in the AH of all the patients. The percentage expression of CD3 was significantly higher in the AH compared to PB in both FHC and IAU, ( $p=0.042$  and  $p=0.015$  respectively). CD4 expression in the AH of the IAU patients  $\{55.7\% \pm 24.2$  (mean  $\pm$  s.d.);  $60.3\%$  (45.5-66.0) median (CI 90)} was significantly higher than in the AH of FHC patients  $\{29.4\% \pm 18.0; 28.4\%$  (18.2-44.5), ( $P=0.01$ ) (Table 4.1). In addition there was a selective increase in the CD4 expression in the AH  $\{55.7\% \pm 24.2; 60.3\%$  (45.5-66.0)} compared to PB  $\{29.5 \pm 15.8; 33.5\%$  (18.2-39.8),  $p=0.005$ } only in IAU and not in FHC. Similarly the levels of double positive CD4/CD25 was only significantly raised in the AH  $\{5.8\% \pm 3.7; 5.9\%$  (4.2-7.3)} compared to PB  $\{0.9\% \pm 1.3; 0.2\%$  (0.3-1.4)  $P=0.0001$ } in IAU but not in FHC.

Interestingly the percentages of CD8<sup>+</sup> T cells in the AH were significantly higher in FHC  $\{46.8\% \pm 16.6; 47\%$  (37.7-57.4)} compared to IAU  $\{21.9\% \pm 14.3; 19\%$  (8.6-28.3)  $p=0.003$ }. In addition CD8 expression in the AH  $\{46.8\% \pm 16.6; 47\%$  (37.7-57.4)} was significantly higher than PB  $\{24.5 \pm 11.2\%; 27.3\%$  (15.8-33.1),  $p=0.005$ ) in FHC suggesting a selective increase in CD8<sup>+</sup> T cells in the AH in this patient group. The high percentage of CD8 expression is illustrated in Fig. 4.1. The percentage expression of cellular phenotypes in the PB is illustrated in Fig 4.2. The selective increase of CD8 expression is not seen in IAU. The percentage of CD3<sup>+</sup>/CD8<sup>+</sup>/CD16<sup>+</sup> in the AH of the two FHC patients was  $0.25\% \pm 0.07; 0.25\%$  (0-0.6).

The expression of CD19 (a marker for B cells) was very significantly lower in the AH compared to PB in both FHC ( $p=0.003$ ) and IAU ( $p=0.00001$ ) patients. The expression of CD14 (a marker for monocyte / macrophage) was significantly higher in the AH in IAU  $\{9.0 \% \pm 5.8; 8.8 \text{ } 5 (3.7-14.2)\}$  compared to FHC  $\{2.2 \% \pm 3.0; 1.1\% (0.4-4.0) p=0.015\}$ . In addition CD14 expression in the AH in FHC is significantly lower when compared to PB  $\{5.4 \% \pm 5; 5.1 \% (2.6-8.2) p=0.02\}$ .

### **Cytokines**

The levels of the following cytokines were measured in pg/ml in the AH of both patient groups: IL-4, IL-10, IL-12 and IFN $\gamma$  (Table 4.2). The cytokine results were stratified to 'high' and 'low' value using the 75th centile as a reference point (Table 4.3). A chi-square test was performed comparing the proportion of patients with 'high' level of the cytokines between FHC and IAU. The proportion of patients with 'high' level of IFN  $\gamma$  was higher in FHC compared to IAU,  $p=0.02$ . However the proportion of patients with 'high' level of IL-12 was significantly higher in IAU compared to FHC,  $p=0.02$ . There was no significant difference in the proportion of patients with 'high' level of IL-10 in the AH between FHC and IAU. IL-4 was not detected in either patient group. There was a positive correlation between CD8 expression and the level of IFN $\gamma$  in the AH of patients with FHC with a correlation coefficient, ( $r = 0.68, n = 10$ ).

### **4.4. DISCUSSION**

The results of this study demonstrate significant differences in cellular phenotypes and cytokines between the AH of patients with FHC and IAU. This is in complete contrast to the findings in iris biopsies which showed no specific

histological changes (Redslob & Brini, 1957; Geogiardes, 1964; Goldberg, et al., 1965; Loewenfeld & Thompson, 1973; Wobmann, 1976; Melamed, et al., 1978; Mc Cartney, et al., 1986; Murray, et al., 1990c). The importance of T cells in the pathogenesis of anterior uveitis (Nussenblatt, et al., 1983b; Murray, et al., 1984b; Murray, et al., 1984a; Deschenes, et al., 1988) is further confirmed by their predominance in the AH in both patient groups.

Comparison of AH and PB revealed a markedly high level of CD4<sup>+</sup> T cells in the AH of IAU implying a predominant role for these cells in the pathogenesis. The importance of these cells in the pathogenesis of other ocular and non-ocular autoimmune diseases has been reported (Cush & Lipsky, 1988; De Berardinis, et al., 1988; De Maria, et al., 1990; Lightman & Chan, 1990; Feron, et al., 1992). The percentage of CD4<sup>+</sup> T cells in the AH was significantly higher in IAU compared to FHC. In addition, the percentage of activated CD4<sup>+</sup> T cells was significantly higher in the AH compared to PB in IAU but not in FHC. Hence, their predominance in the AH of IAU patients compared to FHC patients may explain the more aggressive clinical course and worse outcome of this disease in comparison to FHC.

The selective elevation of CD8<sup>+</sup> T cells in FHC is interesting. In this study it has not been possible to confirm whether these CD8<sup>+</sup> T cells have a cytotoxic or suppressor function, because flow cytometry analysis defines phenotype and not the function of these cells. Apart from the well known subdivision of CD4<sup>+</sup> helper T cells into Th 1 and Th 2 based on their cytokine production, CD8<sup>+</sup> T cells have also been shown to be sub-divided on the basis of cytokine expression, many producing a spectrum of cytokines including Th 1 and

Th 2 like cytokines (Salgame, et al., 1989; Salgame, et al., 1991a; Salgame, et al., 1991b; Bloom, et al., 1992; Romagnani, et al., 1994). It is possible that the CD8<sup>+</sup> T cells which are predominant in the AH of FHC patients may be producing both IFN $\gamma$  and IL-10 which are detected at a higher level in the AH of FHC compared to IAU even though it was only statistically significant in the former. CD8<sup>+</sup> T cells have been shown to produce both of these cytokines in a study by Hoiden and Moller (Hoiden & Moller, 1996). The possibility of the CD8<sup>+</sup> T cells being NK cells (CD3<sup>-</sup>/CD8<sup>+</sup>/CD16<sup>+</sup>) has been excluded by flow cytometry of AH from other FHC patients in whom only a very small percentage of these cells has been found.

The lack of response of the ocular inflammation in FHC to topical steroids is an intriguing feature of this condition. Systemic steroids have long been known to alter immune responses by affecting cellular traffic and function, and have been shown to cause a reduction in the percentage of CD8<sup>+</sup> T cells in the blood and cerebrospinal fluid (Durelli, et al., 1991; Pountain, et al., 1993a; Pountain, et al., 1993b). None of the patients in the study were on systemic steroids but some of the IAU patients were on topical steroids. Topical steroids are not known to affect the lymphocyte subset percentages unlike the systemic steroids, which are known to have such an effect. The predominance of CD8<sup>+</sup> T cells in the AH of FHC patients may imply the possibility of a viral aetiology as CD8<sup>+</sup> T cells or CTLs are effector cells for killing virally infected cells (Doherty, et al., 1990). However using PCR with specific oligonucleotide primers, Epstein-Barr, cytomegalovirus, herpes simplex and varicella-zoster virus was not



detected in the AH of 20 FHC patients (Mitchell, et al., 1996) This of course does not rule out a viral aetiology as it could be associated with other viruses.

The immune response is affected by steroids via its suppressive effect on a variety of cell types including activated macrophages, interference with APC function and a reduction in the expression of MHC antigen. The macrophage, being an important APC, is found to be significantly lower in the AH of FHC compared to IAU as evidenced by the lower CD14 expression. Both the macrophages and CD4<sup>+</sup> T cells are significantly lower in the AH of FHC in contrast to IAU where there is a selective increase of CD4<sup>+</sup> T cells in the AH compared to PB. These findings might explain the steroid nonresponsiveness of FHC as the helper T cells and macrophages, which are the predominant target cells of the corticosteroids, appear to have less important roles compared to IAU.

There are interesting findings in the cytokine levels detected in the AH of patients with FHC and IAU. IL-10 initially known as the cytokine synthesis inhibitory factor now regarded as anti-inflammatory cytokine was detected at a 'high' level in higher number of patients in FHC compared to IAU but this was not statistically significant.

IL-12 was also detected in the AH of the patients. In this study there was significantly higher proportion of FHC patients with 'low' level of IL-12 compared to IAU patients. This is consistent with the significantly low percentage of macrophages which is one of the main producer of this cytokine, in the AH of FHC. This low level of IL-12 may result in reduced cell mediated immunity which could be an additional factor contributing to the low-grade inflammation in FHC patients.

The relationship between IL-10 and IL-12 is interesting. It has been demonstrated by *ex vivo* and *in vivo* studies that IL-10 downregulates IL-12 and paradoxically IL-12 primes T cells for high production of IL-10 (Trinchieri, et al., 1992). This provides a negative feedback mechanism for IL-12 production. In FHC the ratio of mean IL-10 to IL-12 was 21:1 compared to IAU where the ratio was 1: 1. The combined lower level of IL-12 and higher level of IL-10 may contribute to reduce cell-mediated immunity and might explain the low-grade inflammation.

Another interesting finding in the cytokine assay is the significantly higher proportion of patients with FHC with 'high' level of IFN $\gamma$ , which is the strongest activator of macrophages compared to IAU. In addition there was a positive correlation between percentage of CD8<sup>+</sup> T cells and the detected level of IFN $\gamma$  in the AH of patients with FHC, which is consistent with the fact that CD8<sup>+</sup> T cell is one of the main producers of IFN $\gamma$  apart from NK and Th 1 cells. As mentioned above, IL-10 is known to inhibit IFN $\gamma$  production by Th 1 cells resulting in its downregulatory effect on inflammation. It is then possible that there is a balance between these 'protective' and 'damaging' cytokines respectively in the AH of these patients. This is well illustrated in the ratio of mean IL-10 to IFN $\gamma$ , which is 1:1 in FHC and 0.6:1 in IAU. This balance in FHC then results in a relatively benign microenvironment and could account for the benign clinical course and chronicity of this interesting ocular entity in contrast to IAU.

IL-4 was not detected in the AH samples of both patient groups. It is well known that in cytokine assays many factors can interfere with its detection. The

Dot plot histogram of three colour flow cytometry of the AH of a patient with FHC.

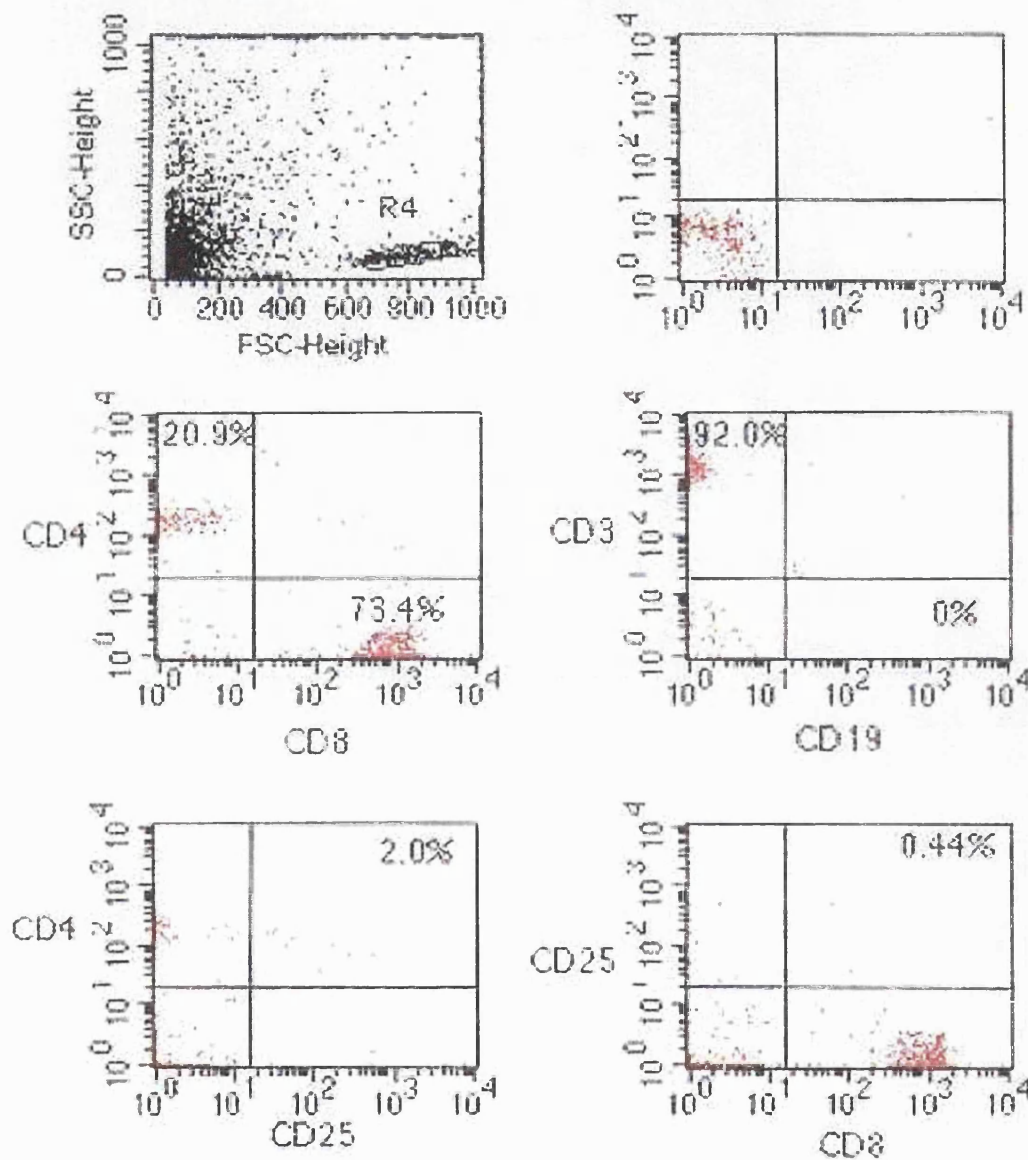


Figure 4.1. A three colour flow cytometry of AH of a patient with FHC showing a very high percentage of T cells (CD3) compared to B cells (CD19). The CD8 T cell subset is predominant compared to CD4 subset in contrast to the percentage expression in PB in Figure 4.2. This illustrates the selective increase in CD8<sup>+</sup> T cells in the AH of this patient with FHC. R4 refers to lymphocyte gate.

assay may be complicated by the presence of inhibitors such as soluble cytokine receptors or receptor antagonists or even by cytokine autoantibodies. However the non-detection may mean that the cytokine is being consumed as soon as it is being produced. There is also the possibility that the cytokine may be degraded by proteases or other substances present in the AH. Alternatively the level might be too low to be detected which would suggest that this cytokine plays little role if any in the pathogenesis of uveitis. This also suggests that B cells have a negligible effect on the ocular inflammation in consistence with the low expression of CD19 in the AH in all the patients. One plausible reason for the non detection of IL-4 in the AH of all the patients is, its production may be downregulated by IFN $\gamma$  which are known to inhibit the differentiation and effector functions of Th 2 lymphocytes leading to a dominant Th 1 function which could account for the inflammation in both ocular conditions.

In conclusion the cellular phenotypes and cytokine profile in the AH in FHC and IAU are different. FHC is a distinct entity from IAU both clinically and immunologically in that CD8<sup>+</sup> T cells appear to have an important role in its pathogenesis. The lower level of IL-12 and the balance between IL-10 and IFN $\gamma$  in the AH in FHC could account for the low-grade ocular inflammation in this intriguing entity.

**Dot plot histogram of three colour flow cytometry of the AH of a patient with FHC.**

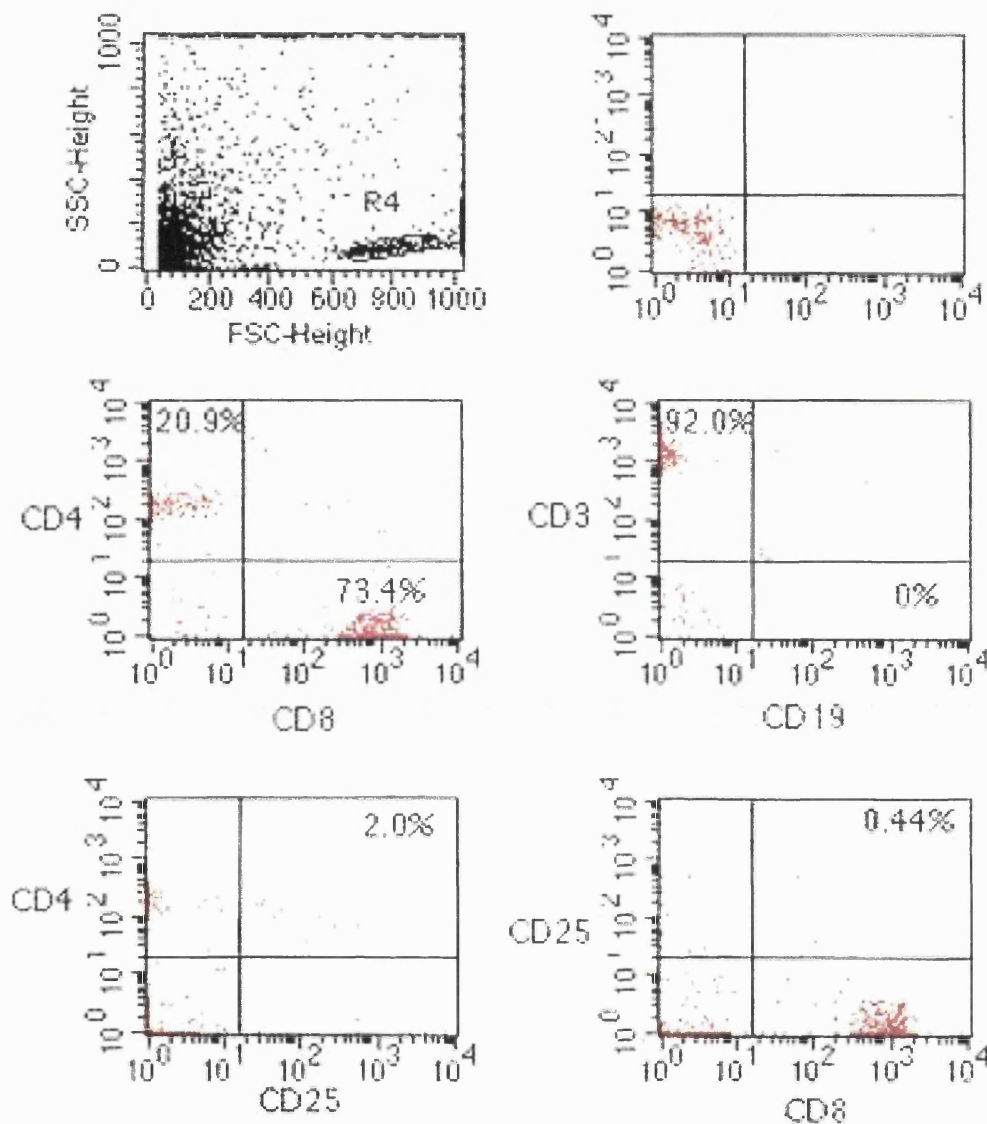


Figure 4.1. A three colour flow cytometry of AH of a patient with FHC showing a very high percentage of T cells (CD3) compared to B cells (CD19). The CD8 T cell subset is predominant compared to CD4 subset in contrast to the percentage expression in PB in Figure 4.2. This illustrates the selective increase in CD8<sup>+</sup> T cells in the AH of this FHC patient. R4 refers to lymphocyte gate.

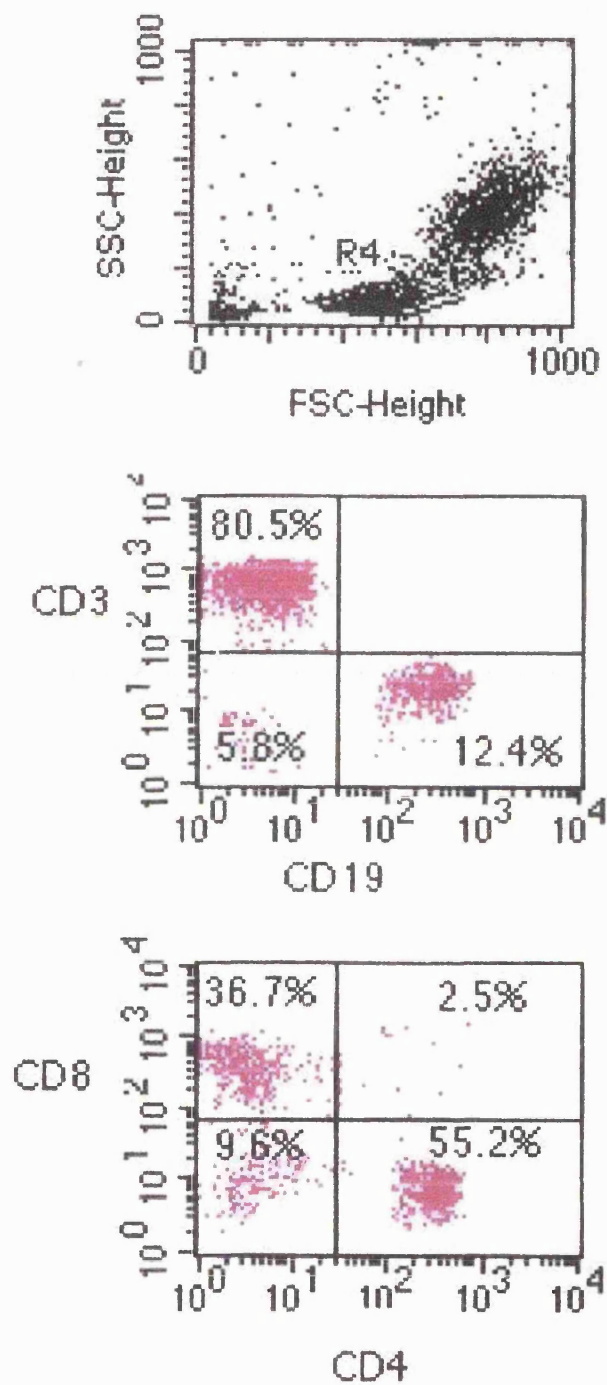


Figure 4.2. There is predominance of CD3 expression compared to CD19. In addition there is predominance of CD3<sup>+</sup>/CD4<sup>+</sup> compared to CD3<sup>+</sup>/CD8<sup>+</sup>. R4 refers to lymphocyte gate.

**Table 4.1** Percentage expression of cellular phenotypes in the aqueous humour (AH) and peripheral blood (PB) in patients with FHC and IAU.

Diagnosis	% Phenotypes	CD3	CD4	CD4/CD25	CD8	CD8/CD25	CD19	CD14
FHC n=10	AH mean (s.d.)	75.9 (19.8)*	29.4 (18.0)	4.1 (7.6)	●●46.8 (16.6)**	0.8 (1.0)	1.2 (1.6)**	2.2 (3.0)* ●
	median	82	28.4	1.9	47	0.6	0	1.1
	CI 90	63.6-88.1	18.2-40.5	0.0-8.7	36.5-57.0	0.2-1.4	0.2-2.2	0.4-4.1
	PB mean (s.d.)	53.1 (27.1)	36.9 (14.9)	0.9 (0.7)	24.5 (11.2)	0.1 (0.2)	7.6 (4.1)	5.6 (2.9)
	median	64.7	31	0.9	27.3	0	8.7	5
	CI 90	36.3-69.9	17.7-36.2	0.5-1.3	17.5-31.4	0-0.2	5.0-10.2	3.8-7.4
IAU n=18	AH mean (s.d.)	76.6 (24.7)*	●55.71 (24.2)**	5.8 (3.7)***	21.9 (14.3)	0.3 (0.7)	1.0 (1.5)****	9.0 (10.2)
	median	87	60.3	5.9	19	0	0	8.8
	CI 90	66.1-87.0	45.5-66.0	4.2-7.3	15.8-28.0	0.0-0.6	0.3-1.6	4.7-13.3
	PB mean (s.d.)	59.4 (20.8)	29.5 (15.8)	0.9 (1.3)	20.6 (8.3)	0.02 (0.1)	8.3 (4.6)	5.4 (5.5)
	median	63.8	33.5	0.2	26.4	0	8.7	5.1
	CI 90	50.6-68.2	22.8-36.2	0.3-1.4	22.1-29.2	0.0-0.1	6.4-10.3	3.1-7.7

Non parametric Mann Whitney test was used to compare the percentage expression (i) between the AH and PB in each patient group (\*) and (ii) in the AH in between the two patient groups (●).

\* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001, \*\*\*\* p < 0.0001

● p < 0.05, ●● p < 0.01.

**Table 4.2 Levels of cytokines detected in the AH of patients with FHC and IAU.**

Diagnosis	Cytokines pg/ml	IL-4	IL-10	IL-12	IFN $\gamma$
FHC	mean (s.d.)	not detected	84.9 (98.6)	4.0 (2.9)	77.7 (72.3)
	median		0.0	4.1	89.3
	**CI 90		13.0-135.2	1.6-5.8	29.4-126.3
IAU	mean (s.d.)	not detected	23.1 (44.0)	22.2 (22.7)	41.8 (39.2)
	median		0.0	19.2	25.8
	**CI 90		0-53.0	8.1-36.3	17.5-66.1

\*\*CI 90 - 90 % confidence interval



**Table 4.3.** Levels of cytokines in the AH stratified into two groups.

<b>Cytokine</b> <b>Diagnosis</b>	<b>IL-10</b>		<b>IL-12</b>		<b>IFN<math>\gamma</math></b>	
	<b>FHC</b>	<b>IAU</b>	<b>FHC</b>	<b>IAU</b>	<b>FHC</b>	<b>IAU</b>
<b>**High</b>	4	1	0*	4	4*	0
<b>**Low</b>	5	8	9	5	5	9
<b>Percent.of High</b>	44.4%	11.1%	0.0%	44.4%	44.4%	0.0%

Statistical analysis was made using the chi-square test. Significant differences were obtained between FHC and IAU patients when IL-12 (\*p= 0.02) and IFN $\gamma$  (\*p= 0.02) level were compared. No statistically significant difference was obtained for IL-10.

**\*\*High-** indicating levels higher than 75th centile of the pooled data of FHC and IAU.

**\*\*Low -** indicating levels lower than 75th centile of the pooled data of FHC and IAU.

IL-10 - 75th centile- 108.5

IL-12 - 75th centile- 20.62

IFN $\gamma$  - 75th centile- 111.0

## CHAPTER 5

### **Characterisation of Cellular Phenotypes and Cytokine Profile in the Aqueous Humour and Peripheral Blood of Patients with Localised Uveitis (LU) And Uveitis Associated with Systemic Disease (SU)**

#### **5.1. INTRODUCTION**

Uveitis can occur in a clinical setting as a localised ocular disorder (LU) or as part of a systemic disease (SU) regardless of the anatomical involvement of the intraocular inflammation. Anterior uveitis can be an isolated anterior segment inflammation or part of a diffuse inflammation of the eye. Although in anterior uveitis control of the inflammation is usually straightforward, associated complications such as glaucoma (Panek, et al., 1990) and cataract may result in severe visual loss (Wolf, et al., 1987). The anterior uveitis in patients with either LU or part of a SU such as sarcoidosis and Behcet disease, which are two systemic diseases most frequently, associated with anterior uveitis, may be similar in appearance. In addition in Behçet's disease the anterior uveitis may be severe as in HLA-B27 related uveitis where hypopyon may be present as a result of the severe anterior uveitis (Nussenblatt, 1997). However on many occasions the anterior uveitis syndromes are similar in appearance and there are no gross distinguishing features to differentiate between these clinical entities. There are instances when an apparently localised uveitis manifests systemic symptoms years later.

The important role of T cells in the pathogenesis of several systemic autoimmune diseases such as RA (Ridderstad, et al., 1992) MS (Merelli, et al.,

1991; Scolozzi, et al., 1992; Dufour, et al., 1993; Frequin, et al., 1993) IDDM (Fowell, et al., 1995) and autoimmune thyroid disease (Ohashi, et al., 1991; Covas, et al., 1992; de Carli, et al., 1993) are well established. The predominant role of CD4<sup>+</sup> T cell is a shared feature in two autoimmune thyroid diseases i.e. Grave's disease and Hashimoto's thyroiditis (Ohashi, et al., 1991) where an increase in CD4<sup>+</sup> T cells occurs during immune systemic activation in patients with hyperthyroid Grave's disease, Hashimoto's thyroiditis and the thyrotoxic phase of subacute thyroiditis. However the activated CD8<sup>+</sup> cells in Grave's disease are induced by antithyroidal therapy suggesting a role of this subset of T cell in disease remission. In another study by Sakasutme et al CD8<sup>+</sup> T cells were demonstrated to be inhibiting IFN $\gamma$  secretion by the CD4<sup>+</sup> T cells (Sakatsume, et al., 1990) which may explain its increase in disease remission.

The above observations demonstrated the important roles of T cells and the subsets in the disease pathogenesis and remission of various autoimmune diseases. There is evidence to suggest that in these organ specific autoimmune diseases the site of activation of T cells are in the target organs themselves. T cells however are known to produce cytokines, which will recruit more inflammatory cells responsible for the damage in the target organs. Hence a study of the cellular phenotypes in the biological fluid of the target organ is a feasible method to dissect the immunological mechanisms taking place.

In the previous chapter a comparison of the cellular phenotypes in the AC in eyes with two contrasting LU; the more benign FHC and the more aggressive IAU was made. CD4<sup>+</sup> T cells are the predominant cell types in the AH of patients with IAU in contrast to FHC where CD8<sup>+</sup> T cells predominated

(Muhaya, et al., 1998). In this study the leucocytes in the AH and PB of patients with LU and SU are compared and the cytokine profiles analysed with the aim of studying the difference in the immunoregulation in the ocular compartment.

## **5.2. STATISTICAL ANALYSIS.**

The non-parametric Mann-Whitney-U test was used to compare the difference between the mean percentage expression of cellular phenotypes and cytokine level. Pearson correlation coefficient was calculated to illustrate the strength of association between the percentage expression of cellular phenotypes and the cytokine level

## **5.3. RESULTS**

The cellular phenotypes of AH sample from 19 LU (which includes IAU and IPU) and 13 SU (which includes AUS, Behçet's disease and sarcoidosis) patients were analysed. The cytokine profiles of AH samples from 9 LU and 18 SU patients were determined. In addition the cytokine profiles from AH of 10 control patients were examined. The reason for the discrepancy in the number of samples analysed for cellular phenotypes and cytokine profiles is because in some of the samples where the supernatants were analysed, there were not enough cells in them to allow good acquisition during flow cytometry. In addition, the cytokine measurements were not done in all of the LU patients due to inadequate volume of the samples.

There was a significantly higher percentage of CD3 expression in the AH of LU ( $82.3 \pm 3.5$ ; mean  $\pm$  s.e.m.) compared to PB ( $58.8 \pm 4.0$ ),  $p < 0.0001$  (Fig 5.1). This was not the case with SU where there was no selective increase in percentage of CD3 expression in the AH. In addition the percentage expression

of CD3 of AH in SU ( $64.8 \pm 8.3$ ) was also lower than LU. Similarly there was a selective increase in the percentage expression of CD4<sup>+</sup> T cells in the AH compared to PB ( $56.9 \pm 3.9$  vs  $30.1 \pm 2.9$ ),  $p < 0.001$  in LU but not in SU ( $42.7 \pm 6.6$  vs  $31.1 \pm 4.5$ ). This was not observed in percentage expression of CD8 (Figure 5.2). In addition, the percentage of activated CD4<sup>+</sup> T cells was significantly higher in the AH ( $4.8 \pm 0.8$ ) compared to PB ( $0.9 \pm 0.2$ ) in LU but not in SU where the percentage expression in the AH was  $2.7 \pm 0.9$  and the percentage expression in the PB was  $1.3 \pm 0.6$  (Figure 5.3). Interestingly there was a significantly lower CD19 expression (the marker for B cells) in AH ( $1.3 \pm 0.34$ ) compared to PB ( $5.2 \pm 1.1$ ) in LU with a p value less than 0.001, but not in SU where the percentage expression in the AH ( $6.9 \pm 3.1$ ) and PB ( $6.4 \pm 1.5$ ) was very similar to each other (Figure 5.1). Also the percentage expression of CD14, a marker for macrophage was significantly higher in the AH of LU ( $11.0 \pm 2.5$ ) compared to AH of SU ( $4.3 \pm 1.4$ ),  $p < 0.001$  (Figure 5.4). An illustration of the predominance of CD4<sup>+</sup> T cells in the AH is shown in the figure showing the dot plot diagram of flow cytometry taken from the AH of a patient with sarcoidosis (Figure 5.5).

With regards to the cytokine level, there was a lower level of IL-10 in the AH of LU ( $31.8 \pm 16.2$ ) compared to SU ( $43.2 \pm 22.5$ ) even though it was not statistically significant. However these levels of IL-10 are significantly lower in both conditions compared to control AH ( $107.9 \pm 33.1$ ),  $p < 0.05$  (Figure 5.6). This was also the case with TGF- $\beta$ 2 level where it was also lower in LU ( $387.8 \pm 19.2$ ) compared to SU ( $437.9 \pm 30.2$ ) but again not statistically significant.

Similarly the level of IFN $\gamma$  was also lower in LU ( $49.0 \pm 20.0$ ) compared to SU ( $87.2 \pm 34.7$ ) even though it was not statistically significant.

There were some interesting correlations between the paired data of cellular phenotypes and cytokine levels in the AH of these patients. There was a strong positive correlation between IFN $\gamma$  and IL-12 in LU ( $n = 9$ ,  $r = 0.85$ ) but not in SU ( $n = 18$ ). A similar positive correlation was seen between IL-10 and TGF- $\beta$ 2 in LU ( $n = 9$ ,  $r = 0.77$ ) but not in SU ( $n = 18$ ). On the contrary there was a negative correlation between the two antagonistic cytokines TGF- $\beta$ 2 and IFN $\gamma$  in LU ( $n = 9$ ,  $r = -0.77$ ) but this was not observed in SU. Similarly a negative correlation existed between IFN $\gamma$  and IL-10 ( $n = 9$ ,  $r = -0.65$ ) in LU but not in SU.

Another striking finding is the positive correlation between percentage expression of CD8 and level of IFN $\gamma$  ( $n = 5$ ,  $r = 0.72$ ) in LU but in SU the correlation was negative ( $n = 5$ ,  $r = -0.66$ ). Also there was a positive correlation between level of IL-10 and percentage expression of CD8<sup>+</sup> in SU ( $n = 5$ ,  $r = 0.82$ ). This was not seen in LU. The CD4/CD8 ratio of the mean percentage of cellular expression was higher in the AH than PB in both LU (1.77 vs 1.23) and SU (1.87 vs 1.25).

#### **5.4. DISCUSSIONS**

Both clinically and experimentally, the separation of uveitis affecting only the anterior segment from that affecting the posterior segment has a sound pathogenetic basis. The similar anterior and posterior segment manifestations in various causes of uveitis are familiar to practising ophthalmologists. Investigations of the various forms of endogenous posterior uveitis using animal models of autoimmune uveitis (EAU) have revealed that this condition can be

induced by a variety of retinal antigens and that each antigen has been shown to induce different forms of EAU. This depends on such factors such as dose of antigen, species and strains of animal model, and the type(s) of adjuvant used. Interestingly within each model a similar spectrum of uveoretinal responses can be induced by each antigen. This suggests that the pathogenic mechanisms are probably similar. In addition, if these models of EAU are analogous to human form of posterior uveitis disease, then each clinical entity within this apparently heterogeneous group of clinical posterior uveitis syndromes may represent one aspect of a general organ-specific uveoretinal response to autoantigens (Forrester, et al., 1990a). This is the observation in human uveitis where the anterior segment manifestation is the same regardless of the initial disease process except for some distinct entities as FHC. In addition some granulomatous illness may manifest as granulomatous uveitis as sarcoidosis.

It is conceivable then that uveitis is the final common pathway or response by the eye faced with an antigenic challenge whether it is endogenous or exogenous. An important point to note is that animal models may not be representative of human uveitis as these animals are vaccinated with a known antigen whereas in human uveitis there is still uncertainty about the factors triggering the onset and recurrence of uveitis.

In this study however we have tried to compare the immune dysregulation in the intraocular microenvironment in uveitis cases classified not according to anatomical involvement but whether it was associated with any systemic diseases (SU) or an isolated ocular involvement (LU) by comparing the

percentage expression of cells infiltrating the AC and cytokine level in the AH between these two groups of patients.

There are interesting variations in the cellular phenotypes in the AH between patients with LU compared to SU. The T cells are significantly higher in the AH compared to PB in LU patients but not in SU patients indicating that there is a selective recruitment of T cells into the intraocular compartment in LU but not in SU. This further confirms that T cells are important in the pathogenesis of uveitis and not B cells as the percentage of B cell is significantly lower in the AH of LU compared to PB as has been confirmed by other workers (Deschenes, et al., 1986; Deschenes, et al., 1988; Hooks, et al., 1988). This is in contrast to other studies by Belfort and co-workers where equal number of T cells were found in the AH and PB (Belfort, et al., 1982). The relatively higher CD4/CD8 ratio in AH compared to PB in both conditions further suggests the important role of CD4<sup>+</sup> T cells in the pathogenesis of uveitis.

The T cell subset that was predominant was CD4<sup>+</sup> T cells both in LU and SU compared to CD8<sup>+</sup> T cell subset. However it was only in LU that there was a significantly higher CD4<sup>+</sup> in AH compared to PB. Similarly the activated form of CD4<sup>+</sup> was also significantly higher in the AH of LU patients. This confirms the predominant role of CD4<sup>+</sup> in the pathogenesis of LU. The finding of activated T cells in the AH may suggest that antigen specific T lymphocyte act as initiators of the inflammatory process. Studies carried out in humans (De Maria, et al., 1990) and in experimental animals (Carding, et al., 1993) suggest that lymphocytes infiltrating inflamed tissues comprise a mixture of antigen-specific



and antigen non-specific activated T cells. The sites of activation of these T cells are still uncertain at the moment whether it is in the eye or the periphery.

There are evidences to suggest altered systemic immune response in patients with idiopathic or what we regard here as LU. These are supported by the following investigations. Kauhner et al (Kauhner, et al., 1983) found elevated systemic immunoglobulin levels in idiopathic uveitis whereas Snyder et al (Snyder, et al., 1979) observed elevated IgG levels and anti ganglioside antibodies in pars planitis. On the other hand Nussenblatt et al (Nussenblatt, et al., 1983b) reported increased suppressor T lymphocytes in patients with posterior uveitis while Murray and Rahi (Murray & Rahi, 1985) as well as Grabner et al (Grabner, et al., 1980) observed a deficit of suppressor cell function in idiopathic uveitis. Deschenes et al (Deschenes, et al., 1988) demonstrated activated T cells in AH, VH and PB of uveitis patients whereby the level of T cell activation correlated with clinically assessed disease activity. This leads to the suggestion that some subgroups of idiopathic uveitis may be in fact systemic diseases with ocular manifestations only.

In this study however the significant findings are mainly in LU patient where there is a predominance of activated CD4<sup>+</sup> T cells and relatively lower levels of cytokines responsible for immune-privilege such as IL-10 and TGF- $\beta$ 2 (Wilbanks, et al., 1992) in the AH compared to SU. This suggests that the immune dysregulation are more severe and localised to the ocular microenvironment rather than the periphery. The immunological privilege of the AC of the eye is due, at least in part, to ACAID that is a selective antigen-specific down-regulation of delayed-type hypersensitivity (DTH) and a normal

induction of antibody responses. This has been shown to be mediated by antigen-specific regulatory T cells that secrete TGF- $\beta$  in an autocrine fashion and suppress effector functions of pathogenic CD4<sup>+</sup> T cells (Streilein, et al., 1997). At that site, they generate a stereotypic systemic immune response that is deficient in CD4<sup>+</sup> T cells that mediate delayed hypersensitivity and that help B cells to secrete complement-fixing antibodies, yet replete with CD8<sup>+</sup> T cells that function as cytotoxic cells and as regulatory cells. The fact that there is a predominance of CD4<sup>+</sup> in the AH suggest that the immune-privilege is compromised hence leading to immunogenic inflammation.

Generation of autoreactive CD4<sup>+</sup> cells requires the processing and presentation of autoantigen by antigen presenting cells (APC) in combination with MHC Class II antigen. Efficient presentation of antigen to T cells has also been shown to depend on accessory molecules of adhesion such as intercellular adhesion molecule-1 (ICAM-1) and leukocyte function-associated antigen-1 (LFA-1). Aberrant expression of Class II antigens by local tissue cells has been suggested as a possible mechanism in autoimmune processes. Several ocular cells express Class II antigens during inflammation, while other cells such as Muller cells inhibit antigen presentation *ex vivo*. Forrester et al (Forrester, et al., 1990b) have shown that retinal pigment epithelial cells (constitutively) and endothelial cells (after induction) express ICAM-1 and that CD4<sup>+</sup> lymphocyte adhesion to these cells is inhibited by antibodies to ICAM-1. Accessory molecules may therefore be important, not only in local presentation of antigen but in recruitment of circulating autoreactive cells to the eye since these cells represent the site of the blood-retinal barrier.

There is now the question of the site of activation of these T cells. In many organ specific autoimmune diseases the site of activation is in the target organ itself. In immune mediated uveitis the site of activation of T cells could either be in the periphery or in the eye itself. Two aspects of lymphocyte activation are important in order to allow the small number of cells that respond to any one antigen to perform the many functions that lead to elimination of the antigen. First, immunisation and antigen recognition trigger numerous amplification mechanisms that rapidly increase the number of cells that respond to the antigen. Second, lymphocytes efficiently home to sites of antigen entry and persistence. Whether such homing occurs in the eye is still in great doubt and there is to date no known addressin (to emphasise their role in organ specific lymphocyte homing) or homing receptors for eyes as there are for the peripheral lymphoid organs (for recirculation of lymphocytes) and mucosal tissues such as Peyer's patches,

The prerequisite of T cell activation is antigen presentation by competent APCs with the necessary co-stimulatory molecules. Antigen presentation may occur in the eye and there are resident ocular cells which are known to express MHC class I and II (Forrester, et al., 1990b; Streilein, et al., 1990). Following antigen (be it foreign or endogenous) presentation by resident APCs the antigen specific T cells secrete cytokines which then recruit more effector cells such as macrophages which in turn produce cytokines leading to amplification of the immune response. This may be consistent with the significantly higher CD14, which is the macrophage marker expression in the AH of LU compared to SU.

Cytokines released locally by the activated T cells may play a role in tissue damage in uveitis. In this study the cytokines responsible for mediating innate immunity; IL-10 and IL-12 and cytokines that mediate specific immunity i.e. IFN $\gamma$  and TGF- $\beta$ 2 have been quantitated in the AH. As mentioned both IL-10 and TGF- $\beta$ 2 both of which are downregulatory in nature, with important contributions to immune-privilege (Wilbanks, et al., 1992; Li, et al., 1996) of the eye, are relatively lower in the AH of LU compared to SU. In addition there are interesting correlations between the levels of these cytokines. The strong positive correlations between (IL-10 and TGF- $\beta$ 2) and (IFN $\gamma$  and IL-12) in AH of LU suggests that one upregulate the other. Similarly there is a strong inverse correlation between cytokines with antagonistic roles such as IFN $\gamma$  and IL-10 and also between IFN $\gamma$  and TGF- $\beta$ 2. This again illustrates the *in vivo* cytokine regulation in the AH which suggesting it to be an excellent source reflecting the immune environment just as CSF and synovial fluid are to the pathogenesis of MS and RA. Interestingly in SU no similar correlation was illustrated.

IFN $\gamma$  was detected in majority of AH of uveitis patients. The availability of large amounts of IFN $\gamma$  might influence the ocular microenvironment in many ways. For example, IFN $\gamma$  can activate tissue macrophages to release a wide spectrum of cytokines and inflammatory molecules (Nacy & Maltzer, 1991). Furthermore IFN $\gamma$  up-regulates the surface expression of HLA-class I and class II antigens, thus enhancing the APC capabilities of various cell types. (Wong, et al., 1983). Finally IFN $\gamma$  may favour the differentiation of Th 1 type cells and, consequently inhibit the differentiation of Th 2 type cells (Gajewski & Fitch, 1988).

An interesting finding is the positive correlation between IFN $\gamma$  level and CD8<sup>+</sup> T cell percentage in the AH of LU patients. This is consistent with a previous report of AH of FHC patients (Muhaya, et al., 1998) whereby a similar correlation occurred between CD8<sup>+</sup> and IFN $\gamma$ . The fact that the CD8<sup>+</sup> T cell is one of the main sources of IFN $\gamma$  is well known (Paliard, et al., 1988) and this correlation support the notion that these T cells are effectors cells and not merely detected in the ocular fluid because they are not retained in the tissues and not contributing to the tissue pathology. However there was a negative correlation between IFN $\gamma$  and CD8<sup>+</sup> but positive correlation between CD8<sup>+</sup> and IL-10 in SU. There is a possibility that the nature of CD8<sup>+</sup> T cells in the two entities is different. It is possible is that these CD8<sup>+</sup> T cells are regulatory or suppressor in nature by their secretion of IL-10 since this downregulatory cytokine is relatively higher in the AH of SU compared to LU although this is not statistically significant.

As a conclusion, despite the similar clinical appearance of uveitis between some patients with LU and SU, the immune dysregulation in LU appears to be localised to the eye in contrast to SU where the infiltrating cells are likely to be passively extravasated as a result of systemic immune dysregulation. This knowledge is beneficial in planning therapeutic modalities where immune intervention can be applied locally resulting in a more focused effect with minimal systemic side effects.

### Comparison of lymphocyte subsets in AH and PB in SU vs LU (R1)

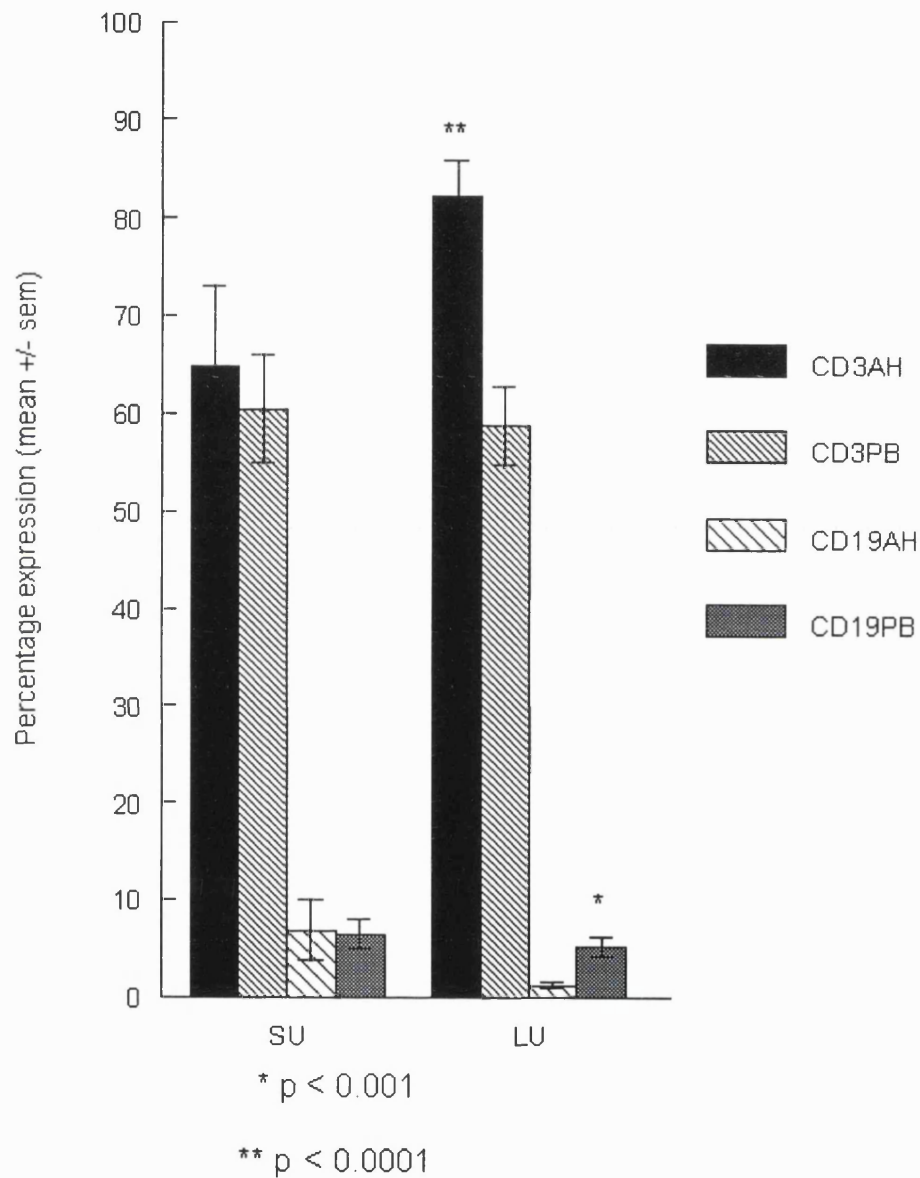
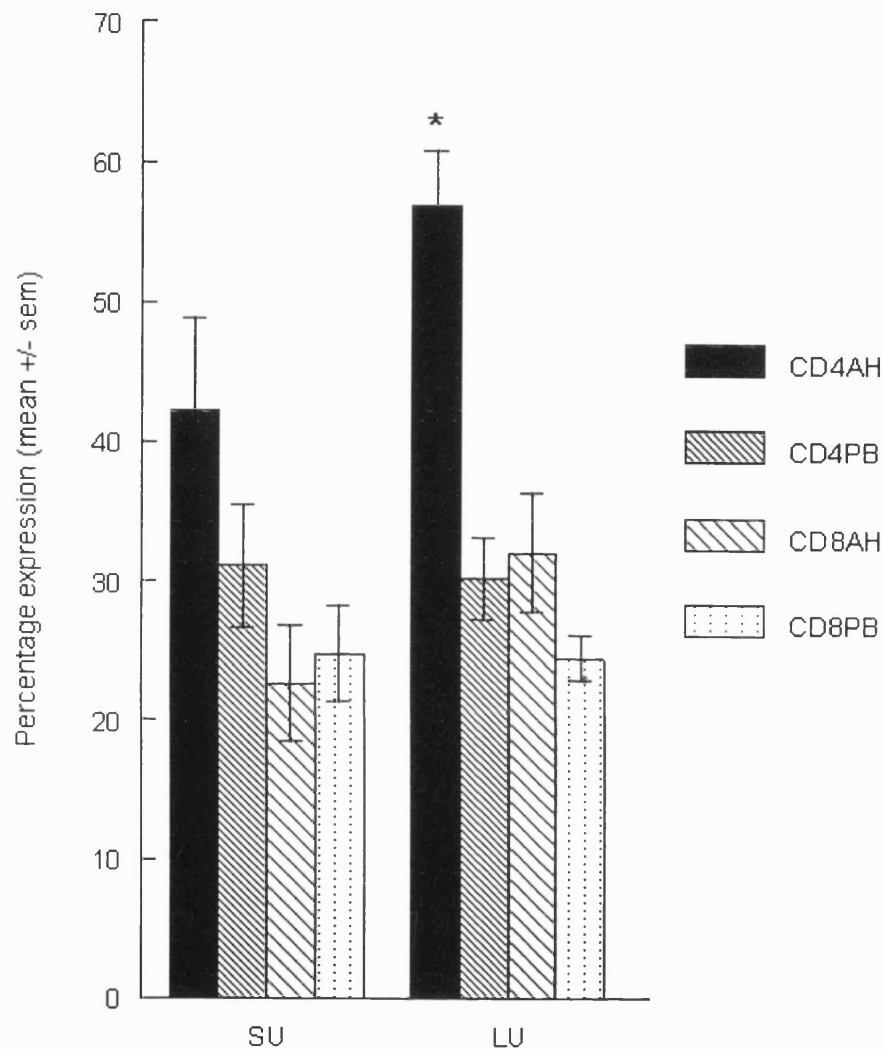


Figure 5.1. This illustrates the selective increase of CD3 ( $p < 0.0001$ ) and selective reduction of CD19 ( $p < 0.001$ ) in the AH of LU. These changes are not evident in SU. R1 refers to lymphocyte gate.

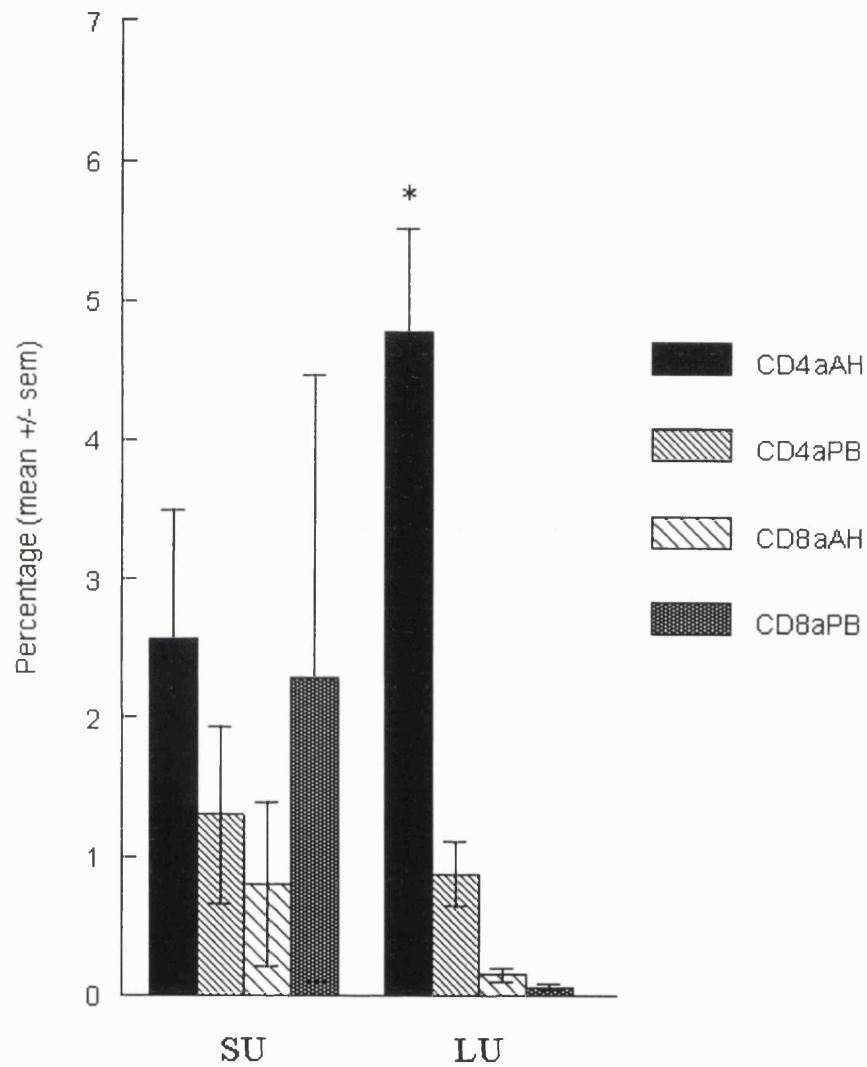
Comparison of expression of CD4 and CD8  
in AH and PB of SU versus LU



\*  $p < 0.001$  by Mann Whitney-U test

Figure 5.2. This illustrates the significantly higher percentage of CD4<sup>+</sup> T cells in the AH of LU but not LU (p, 0.001). There was no similar selective increase in the expression of CD8.

### Comparison of percentage of activated T cells in AH of SU vs LU



\*  $p < 0.001$  by Mann Whitney-U test

Figure 5.3. This figure illustrates the significantly higher activated CD4<sup>+</sup> T cells in the AH compared to PB ( $p < 0.001$ ) in LU but not in SU.



**Comparison of cellular phenotypes (R2)  
in AH and PB SU vs LU**

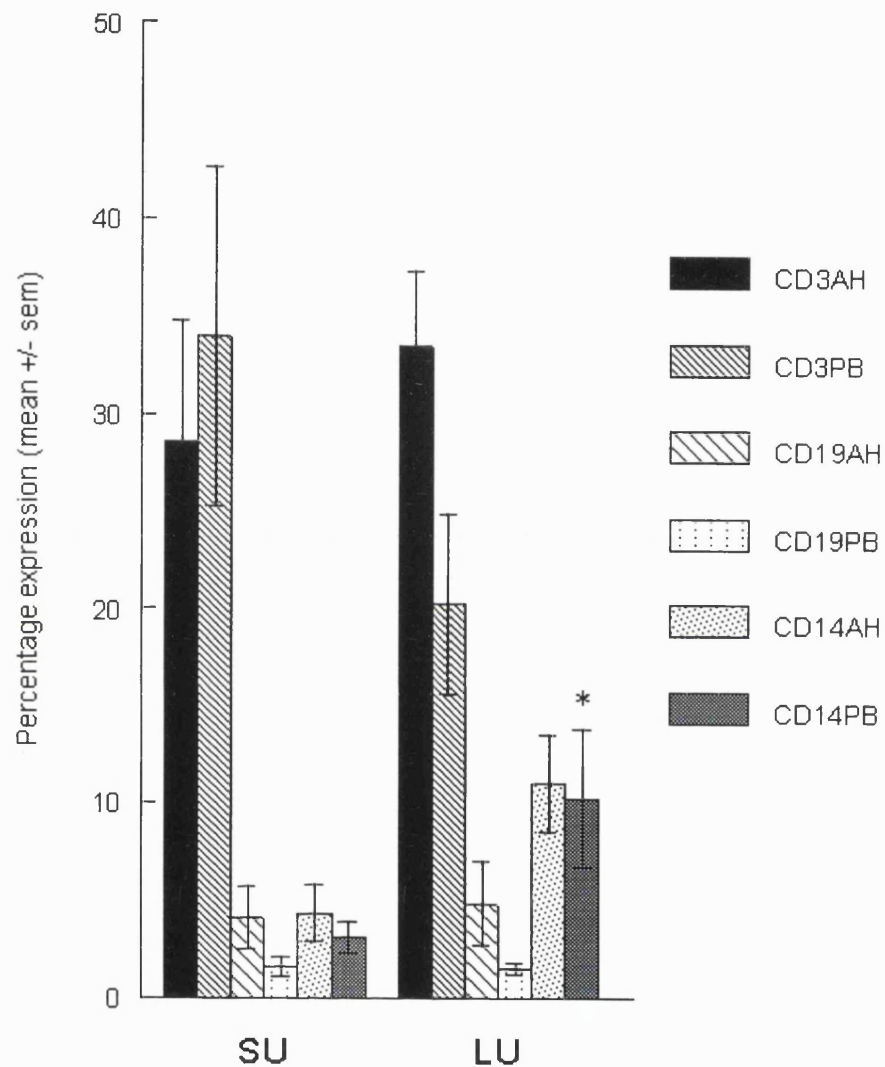


Figure 5.4. This illustrates the predominance of T cells in comparison to other leucocytes in the AH of both SU and LU patients. In addition the expression of CD14 is significantly higher in the AH of LU compared to SU ( $p < 0.001$ ).

Dot plot histogram of three colour flow cytometry of the AH of a patient with sarcoidosis

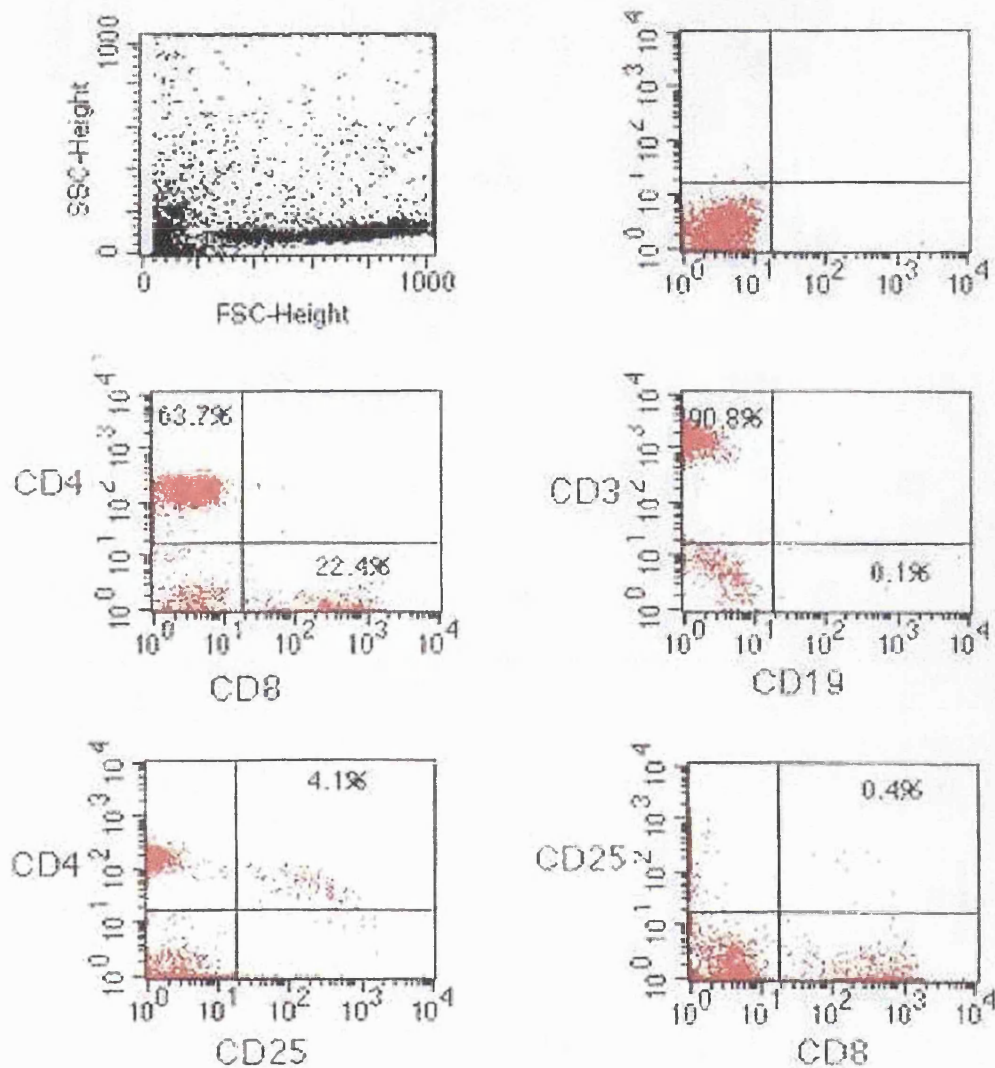


Figure 5.5. This illustrates the predominance of T cells in the AH of a patient with ocular sarcoidosis. It also illustrates the predominance of CD4<sup>+</sup> T cells compared to CD8<sup>+</sup> T cells in the AH. There are more activated CD4<sup>+</sup> T cells (CD4/CD25) than activated CD8<sup>+</sup> T cells (CD8/CD25) in the AH of this patient.

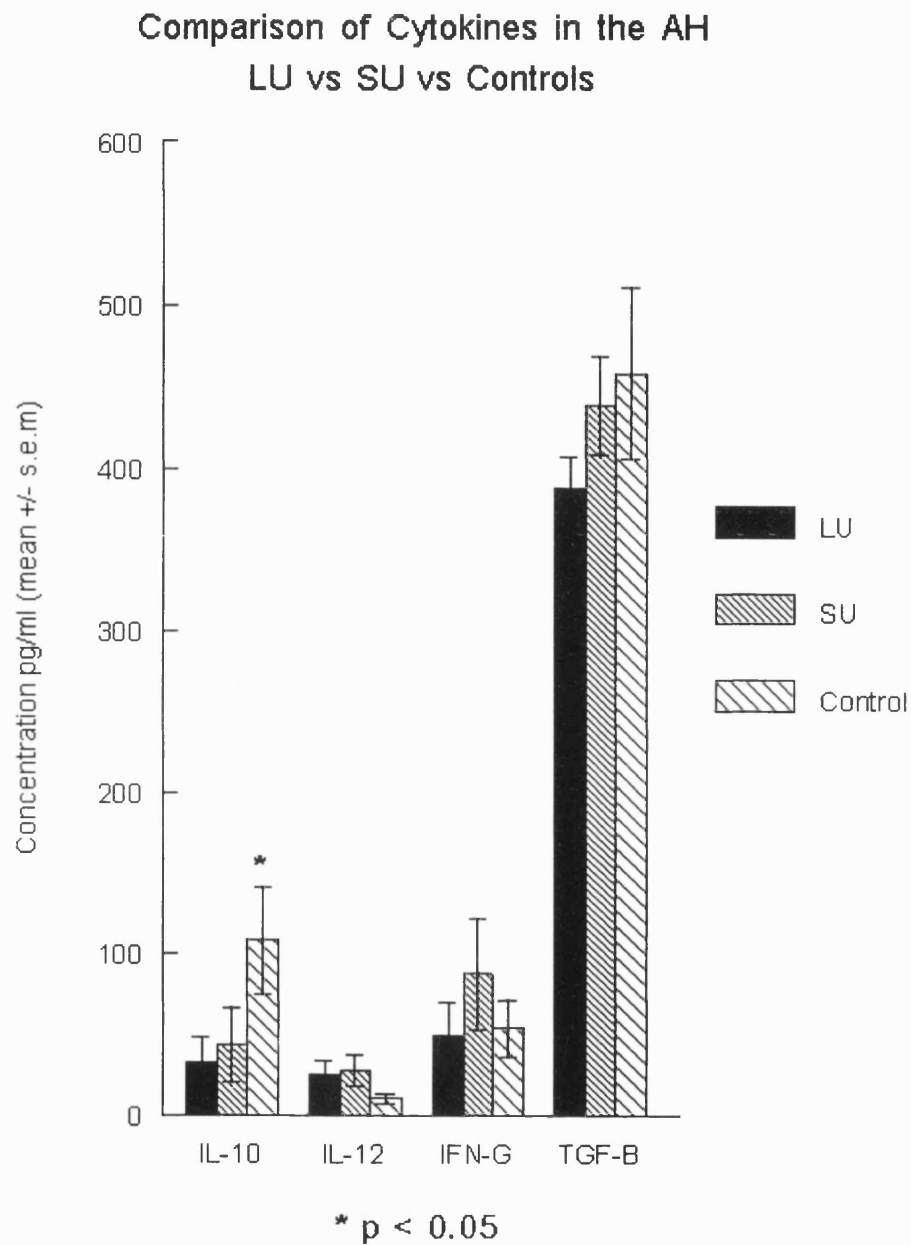


Figure 5.6 This illustrates the significantly higher IL-10 in the AH of control compared to LU and SU ( $p < 0.05$ ). In addition the level of IL-10 and TGF- $\beta$  are lower in LU compared to SU, but not statistically significant ( $p > 0.05$ ).

## **CHAPTER 6**

### **Characterisation of Phenotype and Cytokine Profiles of T Cell Lines Derived from VH in Ocular Inflammation in Man.**

#### **6.1. INTRODUCTION**

IU and Fuchs' Heterochromic iridocyclitis (FHC) are two chronic uveitis entities. IU refers to intraocular inflammation involving predominantly the vitreous and the peripheral retina. FHC as the name implies involves inflammation of the iris and ciliary body. Most patients with IU present with blurred vision and/or floaters as a result of vitritis ie. cellular infiltration of the VH and retinal oedema. In contrast FHC may initially go unnoticed by the patient who may present to the ophthalmologist several years later with complications of chronic inflammation ie. cataract and glaucoma.

The vitreous is infiltrated by inflammatory cells in both intermediate uveitis and FHC. Several workers have characterised intermediate uveitis and FHC immunologically. In both there were T cells in the VH (Davis, et al., 1992; Nolle & Eckardt, 1992). There are significant differences in the clinical course between FHC and intermediate uveitis in that retinal oedema, particularly in the macula area which is a major cause of visual loss, occurs infrequently in intermediate uveitis (Welch, et al., 1960; Kimura & Hogan, 1963; Smith & Godfrey, 1976) but is rare in FHC occurring only after cataract surgery (Liesegang, 1982; Jones, 1993). FHC is therefore a benign chronic uveitis in contrast to intermediate uveitis, which is a disease with a higher ocular

morbidity. An additional intriguing difference is the vitritis, which responds well to steroid in intermediate uveitis whereas it has no effect in FHC.

It has been widely accepted that T cells play a predominant role in uveitis (Chan, et al., 1985; Nussenblatt & Scher, 1985; Lightman & Chan, 1989). We have demonstrated that the predominant cellular phenotypes in the AH of 10 patients with FHC was  $CD8^+$  T cells and that minimal B cell were present (Muhaya, et al., 1998). On the contrary the predominant phenotypes in the AH of patients with IAU, a more aggressive anterior uveitis was  $CD4^+$  T cells. In this study also T cells comprise 90 % of the cells infiltrating the AH of two patients with IU.

The classification of T helper cells into Th 1 and Th 2 based on their cytokine production pattern are well established (Mosmann, et al., 1986; Cher & Mossman, 1987; Mosmann & Coffman, 1989; Sher & Coffman, 1989; Romagnani, 1991). More recently, cells other than  $CD4^+$  T cells including  $CD8^+$  T cells, monocytes, NK cells, B cells, eosinophils, mast cells, basophils and other cells have been shown to be capable of producing “Th 1” associated (type 1) and “Th 2” associated (type 2) cytokines (Sad, et al., 1995). Type 1 cytokines include IL-2,  $IFN\gamma$ , IL-12 and  $TNF\beta$ , while type-2 cytokines include IL-4, IL-5, IL-10, and IL-13. In humans however it has been shown that IL-10 is produced by both Th 1 and Th 2 type cells (Del Prete, et al., 1993). In general type-1 cytokines favour the development of cell mediated immunity whereas type-2 cytokines favour a humoral immune response.

The T cells infiltrating the vitreous in both FHC and IU are presumably activated and are likely to be at least in part responsible for the pathology in the

ocular structures. The importance of the VH in the pathology of intermediate uveitis is demonstrated by the fact that vitrectomy performed for complications of intermediate uveitis has been advocated as potentially having an anti-inflammatory effect in patients who failed to respond to periocular corticosteroids or cryotherapy (Mieler, et al., 1988).

Since T cells mediate their effects via cytokines it is highly relevant to determine which cytokines these cells produce, as these are likely to be important in the disease processes. However our previous attempt to isolate T cells from the small vitreous samples yielded very small numbers of cells making their characterisation and cytokine profile difficult to undertake. This problem has been largely overcome by establishing mitogen driven T cell lines. In this study the T cell population from the VH of patients with FHC and IU was isolated and expanded with PHA, a T cell mitogen. Cytokine analysis of the VH and the potential production of cytokines by these T cell lines after non-specific stimulation obtained has highlighted the differences in the immunopathological process between these two disease entities. In addition the cytokine profiles of the fresh VH were determined.

## **6.2. STATISTICAL ANALYSIS**

The differences between the two patient groups were analysed using the non-parametric Mann-Whitney U test. P values of less than 0.05 were considered significant.

## **6.3. RESULTS**

VH was initially obtained from 10 classical FHC and 16 idiopathic IU patients including 2 patients with pars planitis, a subtype of IU. However T cell lines

were successfully derived from only 4 FHC (age 35-45, mean age 38) and 10 IU including the 2 pars planitis patients (age 31-68, mean 48). There were two reasons for failure of expansion of T cells from other VH samples. Firstly the small starting volume of the vitreous samples and secondly the initial failure to wash the VH samples adequately with complete human medium.

The indications for the vitrectomy for the FHC patients were troublesome floaters due to the vitreous debris and in the IU patients ranged from vitreous biopsy to exclude masquerade syndromes to vitrectomy to control inflammatory process. None of the IU patients were on disease modifying drugs at the time of sampling even though four of them had been treated with systemic steroids previously. None of the FHC patients were ever treated with steroids.

#### **Cytokine level in fresh vitreous supernatants**

IL-10 was detected in two out of four FHC patients with a mean level of  $8.88 \pm 15.94$  pg/ml (mean  $\pm$  standard deviation) pg/ml and in 5 out of 10 IU patients ( $22.3 \pm 48.0$  pg/ml). There was no significant difference in the IL-10 level between FHC and IU patients. IL-2, IL-4 and IFN $\gamma$  were not detected in the VH supernatants in either patient group.

#### **T cell line phenotypes**

There was a predominance of CD8<sup>+</sup> T cells in all the lines (Table 6.1). Interestingly the percentage of CD8<sup>+</sup> T cells was significantly higher in the cultures derived from VH of FHC ( $77.8 \pm 9.2$ ) patient compared to those derived from VH of IU ( $64.6 \pm 8.3$ ) patients,  $p = 0.04$ . In contrast there was significantly higher percentage of CD4<sup>+</sup> T cells in the VH-derived from IU ( $32.0 \pm 8.6$ ) compared to FHC ( $19.2 \pm 8.9$ ),  $p = 0.04$ .

**Cytokines in culture supernatants in T cell lines from VH and PB following non-specific stimulation with PHA**

The criteria for defining the Th subtypes, Th 1, Th 2, Th 0, were the detection limits of the IFN $\gamma$  and IL-4 ELISAs (Cohen, 1995): Th 1 > 120 pg/ml IFN $\gamma$  and < 40 pg/ml IL-4, Th 2 > 40 pg/ml IL-4 and <120 pg/ml IFN $\gamma$ , and Th 0 > 120 pg/ml IFN $\gamma$  and > 40 pg/ml IL-4. According to the above criteria all the cells were of Th 1 like subtype, producing IFN $\gamma$  of > 120 pg/ml and IL-4 production of < 40 pg/ml. The CD8<sup>+</sup> T cells can be considered as Tc 1 cells based on similar criteria.

All the T cell lines derived from the VH of both groups of patients produced significantly higher levels of IL-2, IL-10 and IFN $\gamma$  as compared to PB lines (Figure 6.1 and Figure 6.2) at one of the time points. In the entire T cell lines IL-2 level peaked at 24 hours. The IL-2 level was statistically significantly higher in the VH-derived T cell lines in IU as compared to FHC at 48 hours ( $1810 \pm 220$  vs  $518.1 \pm 94.2$  pg/ml,  $p = 0.009$ ), (Table 6.2). IL-4 was detected at relatively low levels (< 40pg/ml) in all the lines (Figure 6.1 and 6.2). There was no significant difference in the level of IL-4 between VH-derived T cell lines and PB-derived T cell lines in both entities. IL-10 was higher in the VH-derived T cell lines compared to PB-derived T cell lines at 48 ( $p = 0.03$ ) and 72 hours ( $p = 0.04$ ) in FHC and at 72 hours in IU ( $p = 0.04$ ), (Table 6.2). The level of IL-10 was highest at 48 hours after stimulation in both FHC and IU, being significantly higher in the VH lines in FHC ( $237.2 \pm 150.8$  pg/ml) compared to IU lines ( $60.9 \pm 36.1$  pg/ml), ( $p = 0.03$ ) (Figure 6.3).



#### 6.4. DISCUSSION

In this study we have quantitated the cytokines present in the fresh VH reflecting the *in vivo* environment and the *ex vivo* cytokine production by T cell lines derived from the vitreous and the PB of patients with FHC and IU. The comparison between these two situations is important as *in vivo* the T cells are influenced by multiple factors affecting their effector functions and only reflects one time point.

In the fresh vitreous supernatants, IL-10, an immunoregulatory cytokine mainly produced by monocytes and T cells known to inhibit the expression of inflammatory and haematopoietic cytokines as well as its own expression (Casatella, et al., 1993; Moore, et al., 1993) was detected in only half the number of patients in each group. In addition none of the T cell derived cytokines IL-2, IL-4 or IFN $\gamma$  were detected. The failure to detect these cytokines may reflect a balance between the various cytokines involved in the pathogenesis, which may be due to the fact that VH was taken at one time point from patients whose eyes were stable clinically.

The predominant phenotype after prolonged culture with multiple passages of expansion with PHA of all the lines was predominantly CD3<sup>+</sup>CD8<sup>+</sup>. However in IU the percentage of CD4<sup>+</sup> was significantly higher than FHC. The PB lymphocytes, which contain more CD4<sup>+</sup> T cells at the initial phase, however were found to have higher percentages of CD8<sup>+</sup> T cells after longer periods of culture. Similar phenotyping was done on some of the cultured vitreous lymphocytes and a similar predominance of CD8<sup>+</sup> T cells was observed with time. This suggests that the culture conditions are benefiting the CD8<sup>+</sup> T cells

more than CD4<sup>+</sup> T cells or that the CD8<sup>+</sup> T cells are more responsive to PHA compared to CD4<sup>+</sup> T cells.

An interesting finding is the selective high cytokine production by VH-derived T cell lines compared to the PB-derived T cell lines of all the cytokines except IL-4 in both patient groups. The profoundly higher level of cytokine production by a similar number of cells from the vitreous compared to PB suggests larger proportion of cytokine producing cells in the VH.

Interestingly in this study the VH-derived T cell lines were capable of producing massive levels of IFN $\gamma$  and IL-2, type-1 cytokines both of which were not detected in any of the fresh VH supernatant. This could be due to the presence of inhibitory factors such as TGF- $\beta$  (Cousins, et al., 1991a; Streilein, et al., 1992),  $\alpha$ -MSH (Taylor, et al., 1992b) and neuropeptides (Ferguson, et al., 1995) which are immunosuppressive in nature. These factors may inhibit the cells from producing type-1 cytokines responsible for cell mediated immunity. In the absence of these factors, in *ex vivo* their capability to produce these cytokines was made apparent. The evidence for the presence of these inhibitory factors is the poor proliferation of the T cells when the cell pellets were not washed with human medium.

T cell lines derived from VH of IU patients produced significantly higher level of IL-2 (cytokine produced by activated T cells acts in an autocrine manner and is a growth factor for T, B and NK cells) compared to VH-derived T cell lines of FHC patients. As the production of IL-2 is an important determinant of the magnitude of T cell dependent response (Smith, 1988), the higher production VH-derived T cell lines from IU patients may be consistent with the more

aggressive clinical course of IU patients compared to FHC patients. As IL-2 is produced by activated T cells, it may suggest that the T cells isolated from IU patients are more activated to start with compared to T cells isolated from FHC patients. However no phenotyping was done for markers of T cell activation such as CD25 in these lines as they have been in culture for quite a few weeks hence the activation markers may not reflect the true initial state of activation of these T cells. In order to ascertain this, these cells should be phenotyped prior to culture but as the sample is scarce and the success of growth is determined by the starting number of cells this was not done as the priority was to get a good culture of T cell lines.

We suggest that activated Th 1 cells play a predominant role in the pathogenesis of IU based on the above findings. This is a significant finding as the inflammation in IU responds well to steroids, which are known to block the expression and action of most cytokines. Moreover cyclosporin A that prevents transcription of IL-2 and other cytokines at the cellular level (Cross, et al., 1989) is usually effective in controlling the inflammation in IU (Nussenblatt, et al., 1983a; Towler, et al., 1990; Lightman, 1991).

The source of IL-2 could be both either CD4<sup>+</sup> and CD8<sup>+</sup> T cells. However in intracytoplasmic staining study CD4<sup>+</sup> T cells have been shown to be the major producer of IL-2 compared to CD8<sup>+</sup> T cells (Andersson, et al., 1990). The percentage of CD4<sup>+</sup> T cells being a more important source of IL-2, was significantly higher in the T cell lines derived from the VH of IU patients.

The levels of IFN $\gamma$  and IL-10 in the T cell culture supernatant from VH in FHC patients were significantly higher compared to culture supernatant of IU

patients. This may appear paradoxical as the antagonistic role of these two cytokines are well established (Cousins, et al., 1991b; de Waal Malefyt, et al., 1993; Bai, et al., 1997). However as CD8<sup>+</sup> T cells (the percentage of which are significantly higher in FHC) have been shown to produce both IFN $\gamma$  and IL-10 (Hoiden & Moller, 1996), these two cytokines may be produced by these cells leading to higher IFN $\gamma$  and IL-10 in FHC. Similarly the levels of IFN $\gamma$  and IL-10 were significantly higher in the AH (Muhaya, et al., 1998) in FHC compared to IAU, a more aggressive form of anterior uveitis. The greater production of IL-10 by the T cell lines derived from patients may reflect the *in vivo* behaviour of these cells in AH of FHC patients and may account for the benign clinical features of FHC as IL-10 is known to be an important downregulator of macrophage functions and suppresses production of pro-inflammatory cytokines by activated monocytes and macrophages (Fiorentino, et al., 1991; Enk, et al., 1993). IL-10 is also able to counteract the macrophage activation by IFN $\gamma$  hence leading to equilibrium accounting for the chronicity of this condition.

IL-10 has been shown to be chemotactic to CD8<sup>+</sup> T cells (Jinquan, et al., 1993). This may result in CD8<sup>+</sup> cells coming in following IL-10 production by macrophages and CD4<sup>+</sup> T cells. It is uncertain whether this contributes in any way to the anti-inflammatory action of IL-10. The important role of IL-10 in FHC, which is suggested by these results, may explain the steroid non-responsiveness of uveitis in FHC. Steroids have been shown to increase IL-10 protein and mRNA expression in the serum in MS patients with acute relapse by Gayo et al (unpublished data). In addition *ex vivo* steroids were shown to increase IL-10 secretion, increase intracellular IL-10 mean fluorescent intensity

and the percentage of IL-10 expressing cells. These data suggest that the beneficial effect of steroids in the control of disease activity may be due to their capacity to increase the spontaneous expression of IL-10. Hence in FHC as IL-10 is already produced at a relatively high level with its anti-inflammatory effect the further immunosuppressive effects of steroid are not apparent.

In view of its ability to inhibit production of proinflammatory cytokines involved in acute and chronic inflammatory processes, IL-10 is being studied in an array of conditions such as RA (van Roon, et al., 1996), Crohn's disease (van Deventer, et al., 1997) and inflammatory bowel disease (Kuhn, et al., 1993; Powrie, et al., 1994). Recently in-patients with moderate RA, IL-10 administration was well tolerated and was associated with improvement in clinical and inflammatory markers of disease (Grint, unpublished data). Slow release devices are available for intraocular use (Martin, et al., 1994) and could potentially allow chronic administration of downregulatory cytokines within the eye.

IL-4, a type-2 cytokine in human T cells was minimally produced by the cells despite the isolation from the possible inhibitory factors in the vitreous. The predominant type-1 cytokines produced by the cell lines suggests that the T cells infiltrating the eye may have been polarised from the beginning resulting in a homogenous cell population producing type-1 cytokines. The other possibility for the minimal production of IL-4 is the antagonistic effect of IFN $\gamma$  on IL-4 production.

In conclusion the T cells infiltrating the VH in FHC and IU patients are of the Th 1 and Tc1 responsible for cell mediated immunity accounting for the

intraocular inflammation in these two entities. Both IFN $\gamma$  and IL-10 however were produced in higher quantity by the T cells derived from VH of FHC patients possibly being produced by CD8<sup>+</sup> T cells which were predominant in vitreous T cell lines accounting for the more benign clinical course of this chronic uveitis.

**Table 6.1**

Cellular phenotypes	CD4(VH)	CD4 (PB)	CD8 (VH)	CD8 (PB)
FHC	19.2 ± 8.9	27.5 ± 6.3	77.8 ± 9.2*	68.2 ± 7.1
IU	32.0 ± 8.6*	27.0 ± 6.8	64.6 ± 8.3	70.6 ± 9.0

This illustrates the predominance of CD8<sup>+</sup> T cells in the T cell lines derived from both VH and PB. There is a significantly higher percentage of CD4<sup>+</sup> T cells in the VH-derived T cell lines from IU patients compared to VH-derived T cell lines from FHC patients, \* (p = 0.04). Conversely there is a significantly higher percentage of CD8<sup>+</sup> T cells in the VH derived T cell lines from FHC compared to IU patients, \* (p= 0.04).

Table 6.2. Level of cytokine secretion by T cell lines derived from VH humour (VH) and peripheral blood (PB) of patients with FHC and IU.

Cytokine Level pg/ml		IL-2		IL-4		IL-10		IFN $\gamma$	
FHC		VH	PB	VH	PB	VH	PB	VH	PB
24		1358 $\pm$ 793**	293.8 $\pm$ 100.5	19.05 $\pm$ 19.9	12.92 $\pm$ 14.79	107.4 $\pm$ 98.4	43.6 $\pm$ 25.5	•1367 $\pm$ 332**	278.8 $\pm$ 87.7
48		518.1 $\pm$ 188.5*	114.5 $\pm$ 77.5	13.04 $\pm$ 14.59	19.2 $\pm$ 21.9	•237.2 $\pm$ 80.9*	26.6 $\pm$ 26.7	947 $\pm$ 458*	441 $\pm$ 265
72		194.0 $\pm$ 156.9*	37.9 $\pm$ 28.4	11.86 $\pm$ 13.2	12.33 $\pm$ 14.0	65.48 $\pm$ 12.36*	25.1 $\pm$ 12.76	605.8 $\pm$ 141.3*	171.3 $\pm$ 65
IU									
24		1874 $\pm$ 318*	919 $\pm$ 744	21.26 $\pm$ 12.95	17.06 $\pm$ 14.02	55.7 $\pm$ 13.4	17.88 $\pm$ 16.24	912 $\pm$ 303	617 $\pm$ 772
48		••1810 $\pm$ 622**	453 $\pm$ 341	21.76 $\pm$ 14.92	14.41 $\pm$ 9.37	60.9 $\pm$ 36.1	34.4 $\pm$ 52.4	951 $\pm$ 405	604 $\pm$ 671
72		1307 $\pm$ 825*	194.9 $\pm$ 175.4	16.79 $\pm$ 10.69	19.9 $\pm$ 12.69	60 $\pm$ 29.4*	11.18 $\pm$ 9.37	859 $\pm$ 378*	345 $\pm$ 291

Non- parametric Mann-Whitney test was used to compare the level of cytokine secretion by T cell lines (i) between the VH and PB in each patient group (\*) and (ii) in the VH in between the two patient groups (•).

\* p < 0.05, \*\* p < 0.01

• p < 0.05, •• p < 0.01.



### Higher cytokine production by VH-derived vs PB-derived T cell lines in FHC

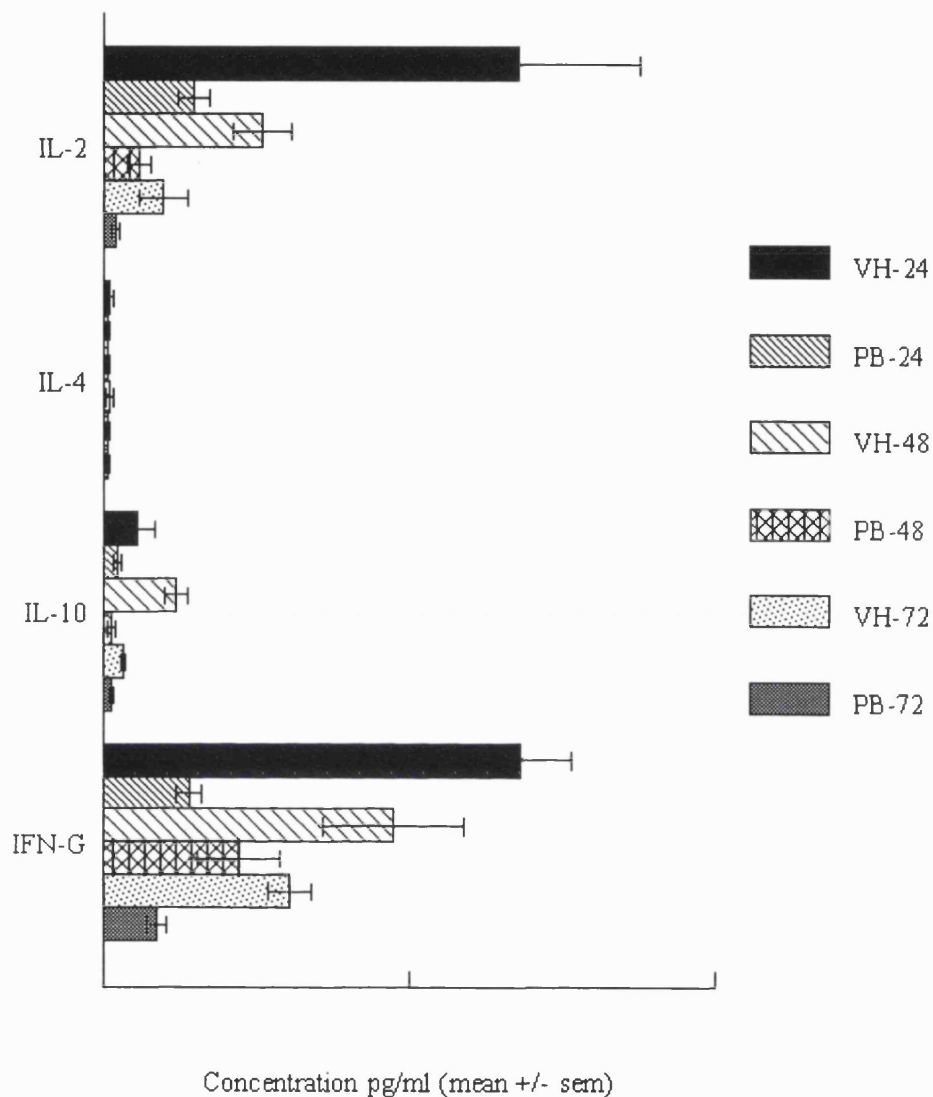


Figure 6.1. This illustrates the pattern of cytokine production by VH-derived T cell lines from patients with FHC. There is predominant production of type 1 cytokines IL-2 and IFN $\gamma$  and minimal production of type 2 cytokines IL-4 and IL-10. It also demonstrates the higher production by VH-derived T cell lines compared to PB-derived T cell lines of all the cytokines except IL-4.

### Higher cytokine production by VH-derived vs PB-derived T cell lines in IU

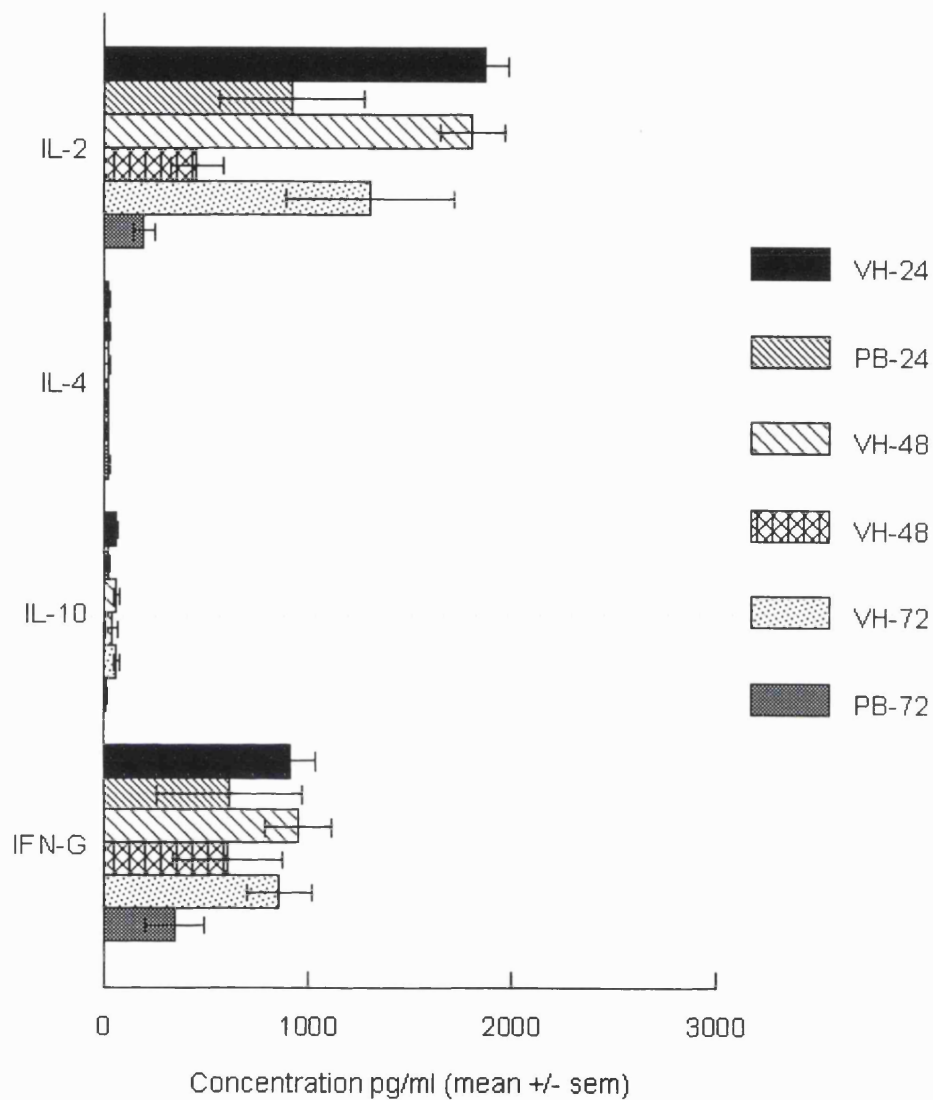
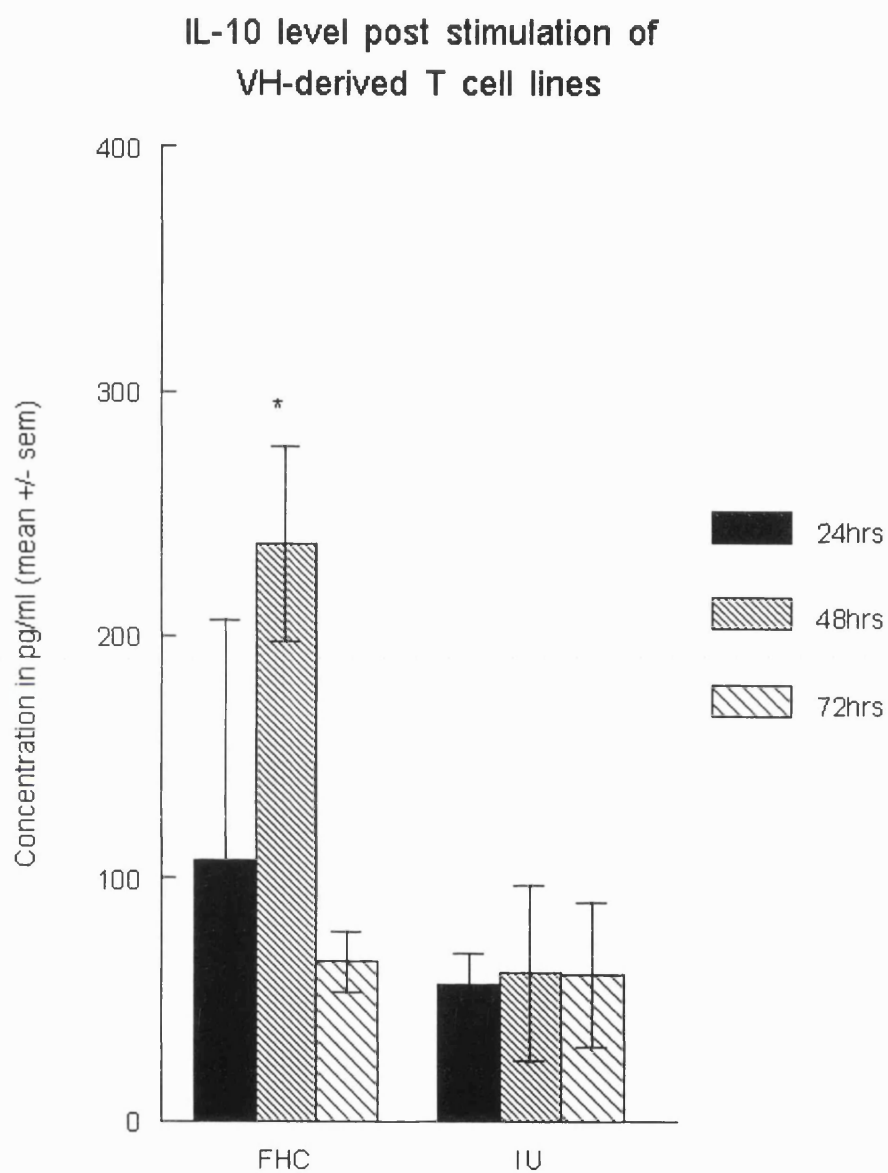


Figure 6.2. This illustrates the pattern of cytokine production by VH-derived T cell lines from patients with IU. There is predominant production of type 1 cytokines IL-2 and IFN $\gamma$  and minimal production of type 2 cytokines IL-4 and IL-10. It also demonstrates the higher production by VH-derived T cell lines compared to PB-derived T cell lines of all the cytokines except IL-4.



\*  $p < 0.05$  by MannWhitney-U test

Figure 6.3. This illustrates the significantly higher production of IL-10 by VH-derived T cell lines from patients with FHC compared to VH-derived T cell lines from patients with IU after 48 hours of stimulation with PHA ( $p < 0.05$ ).

## CHAPTER 7

### GENERAL DISCUSSIONS AND CONCLUSIONS

Advances in immunology have provided us with new insights about the ocular inflammatory response. Still, the aetiology and pathophysiology of many uveitides remain unclear. Factors such as the blood-ocular barrier, sequestration of retinal antigens, local immunomodulators in the AH, and ACAID interact and render the eye an immunologically "privileged" site. On the other hand, HLA interactions, the immunopathology of hypersensitivity reactions, T cell mediated disease, and autoimmunity are associated pathogenic mechanisms of immune-related ocular disease.

Uveitis is a term, which encompasses many clinical syndromes, which would appear to be discrete entities. It may occur in isolation, or be associated with systemic disease (example Behçet's disease and sarcoidosis) and can be classified anatomically as anterior, posterior, intermediate uveitis and panuveitis. The different representations of different clinical types of uveitis have always been an intriguing phenomenon to the practising ophthalmologist. The different anatomical involvement for instance is interesting. What is the determining factor that limits the inflammation to say only the anterior segment of the eye? There are no differentiating clinical features in the anterior segment between uveitis localised to the eyes and those, which are part of systemic syndromes such as sarcoidosis and Behçet's disease. Even though sarcoidosis are more often associated with granulomatous iridocyclitis it may present with non-granulomatous anterior uveitis. There are entities like FHC, where there is inflammation of the anterior segment associated with some vitreous infiltration

where the intraocular inflammation is benign and do not respond to topical steroids. In addition PS, which occurs in almost all untreated uveitis, do not develop preoperatively in FHC. At the other extreme is uveitis as part of Behçet's disease where the intraocular inflammation is florid and patients present with severe bouts of recurrent inflammation consequently ending up in visual loss.

In order to dissect the immunological mechanisms taking place in the intraocular microenvironment the cytokine profiles and cellular phenotypes of the intraocular fluids, AH and VH have been analysed. Both proinflammatory ( $\text{IFN}\gamma$ ,  $\text{TNF}\alpha$  and IL-12) and immune response-down-regulating ( $\text{TGF-}\beta 2$  and IL-10) cytokine have been quantitated in the AH.

Several comparisons have been performed between the various types of uveitis to further understand the pathogenesis of ocular disease. Initially we have compared the cytokine profiles of the AH between uveitis and control patients. In addition we have compared the cytokines and cellular phenotypes in the AH of inflamed eyes in patients with uveitis patients in two ways; the first comparison being between FHC (benign ocular manifestation) and IAU (more aggressive anterior uveitis) where the inflammation is predominantly in the anterior segment and only localised to the eye with no evidence of systemic involvement; the second comparison is between another group of patients where the inflammation is localised to the eye against patients with intraocular inflammation as part of a systemic disease regardless of the anatomical location of the uveitis.

The cytokine profiles detected in the AH of the patients in this study appear to reflect the immunopathologic mechanisms. There are lower levels of cytokines responsible for immune-privilege such as TGF- $\beta$ 2 and IL-10 (Streilein, et al., 1997) in the AH of uveitis patients compared to AH of control patients. Both 'type 1' and 'type 2' cytokines were detected in the AH of patients with uveitis suggesting that it is the imbalance of the cytokines that contribute to the immunopathology rather than overproduction of only 'type-1' or 'type-2' cytokines. Since cytokines are both pleiotropic and redundant and work in a network, it was not possible to study them in isolation. Hence it was more useful to study the pattern of the cytokine production rather than individual cytokines.

IL-2 a Th 1 cytokine was not detected or detected at very low levels in most AH which could be due to it being used up as it has both autocrine and paracrine action that suggests a rapid turnover of this cytokine. The low level detected may be sufficient for its action as receptors for cytokines often show very high affinity for their ligands. As a consequence only very small quantities of a cytokine need be produced to occupy receptors and elicit a biologic effect.

Th 1 defining cytokine IFN $\gamma$ , on the other hand was detected at a higher level in the AH of uveitis patients compared with control patients. Its antagonistic action against TGF- $\beta$ 2, an immunosuppressive cytokine with crucial role in ACAID has been demonstrated by Cousins et al (Cousins, et al., 1991b). This antagonistic action of IFN $\gamma$  and its ability to recruit APCs from the blood and activate resident APC precursors makes this cytokine an important player in pathogenesis of uveitis.

IL-12, a non T cell cytokine also considered as a 'type1' for its role as a potent inducer of IFN $\gamma$  production by T cells as well as NK cells was also detected in the AH of the patients. A positive correlation was observed between IL-12 and IFN $\gamma$  levels in AH of patients with LU suggesting possible interactions between IL-12 and IFN $\gamma$  production in the ocular fluid. The highest level of IL-12 was detected in the AH of patients with Behçet's disease. This is consistent with the aggressive intraocular inflammation in this entity as this cytokine promotes cell-mediated-immune response. On the contrary, the level of IL-12 was low in patients with FHC that is the mildest form of intraocular inflammation in our group of patients. This further confirms the important role of IL-12 in promoting cell-mediated-immunity responsible for the immunogenic inflammation in uveitis. This is in keeping with the result of an animal study where (Sun, et al., 1997) it was shown that genetic susceptibility to EAU was dependent on a Th1 antigen-specific response (high IFN $\gamma$  and IL-12p40) in the draining lymph nodes.

The cytokine IL-10 which suppresses monocyte and Th 1 cell functions was predominant in the AH of control patients and FHC compared to other types of uveitis. The important role of this cytokine in immune-privilege was demonstrated by Li et al (Li, et al., 1996). It has been shown (Navikas & Link, 1996) that there is upregulation of TGF- $\beta$ 2 and IL-10 production systemically at the cellular level in remissions in MS. Similarly recombinant human IL-10 was shown to suppress clinical experimental autoimmune neuritis (EAN), a CD4<sup>+</sup> T cell-mediated monophasic inflammatory disorder of the peripheral nervous system and that this suppression was associated with downregulation of Th 1

responses and macrophage function and upregulated Th 2 responses (Bai, et al., 1997) suggesting that IL-10 may be a negative regulator for DTH. These findings showing the antiinflammatory effect of IL-10 is consistent with the higher level of this cytokine in AH of both control patients and patients with the mildest form of uveitis, FHC. Interestingly the downregulatory role of IL-10 in EAU has been demonstrated recently where systemic neutralisation of IL-10 during the expression phase of EAU resulted in elevated disease score suggesting that endogenous IL-10 limits expression of EAU and may play a role in the natural resolution of disease (Rizzo, et al., 1998).

The effect of systemic IL-10 on the DTH reaction (induration, with oedema and granulocytic infiltration) induced by injection of Th1 clones into mouse footpads have been investigated and the significant correlation between the inhibition of footpad swelling and cytokine production suggested that the effect of IL-10 on DTH may be mediated through suppression of cytokine synthesis (Li, et al., 1994). The apparent protective role of IL-10 to PS formation in eyes with anterior uveitis was shown in chapter 3. We have suggested PS to occur as a sequeale of DTH, which has been shown to be prevented by IL-10 hence accounting for the protective role against PS formation.

The relationship between IL-10 and IL-12 has been demonstrated by *ex vivo* and *in vivo* studies where IL-10 downregulates IL-12 and that IL-12 paradoxically primes T cells for high production of IL-10 (Trinchieri, 1993). This provides a negative feedback mechanism for IL-12 production. This relationship was seen in some of the AH where in samples where IL-12 was high the IL-10 was not detectable and vice versa.



IL-4, a type 2 cytokine, was not detected in the majority of AH samples of the patients with uveitis. However the level was relatively higher in the AH of control patients compared to uveitis patients. One plausible reason for the non detection of IL-4 in the AH of the majority of all the patients is, its production may be downregulated by IFN $\gamma$  which is relatively higher in uveitis compared to control AH. The ability of IFN $\gamma$  to inhibit the differentiation and effector functions of Th 2 lymphocytes leading to a dominant Th 1 function could account for the intraocular inflammation.

The first comparison of cytokines and cellular phenotypes between the group of uveitis patients has answered the question of the reason for the more benign intraocular inflammation and ocular morbidity in localised uveitis, FHC compared to IAU. It has been revealed that the CD8<sup>+</sup> T cells are the predominant phenotype in the AH of FHC in contrast to IAU where the CD4<sup>+</sup> T cells are the predominant phenotype. These CD8<sup>+</sup> T cells appear to be producing both IL-10 and IFN $\gamma$  although intracellular staining will be required to confirm this.

The predominance of CD8<sup>+</sup> T cells in the AH of FHC patients is really interesting. The immune-privilege of the eye is due, at least in part, to a selective antigen-specific down regulation of DTH and a normal induction of antibody responses or ACAID. In this phenomenon which has been demonstrated in animal studies, following an encounter with an antigen the local APC, migrate to the spleen where they then generate a stereotypic systemic immune response that is deficient in CD4<sup>+</sup> T cells that mediate DTH, yet replete with CD8<sup>+</sup> T cells that function as cytotoxic and regulatory cells. ACAID is believed to be mediated by antigen-specific regulatory cells that secrete TGF- $\beta$ 2 in an autocrine fashion and

suppress effector functions of immunogenic CD4<sup>+</sup> T cells. Assuming that ACAID does exist in humans, the predominance of CD8<sup>+</sup> T cells in AH of FHC may suggest that ACAID is not as compromised in FHC as in other types of uveitis where there is predominance of CD4<sup>+</sup>, presumably Th 1, that are effector cells for cell-mediated-immunity. The role of IL-10 in ACAID was confirmed in IL-10 knockout mice. In addition, Ag-pulsed APC from IL-10 knockout mice were unable to induce ACAID following *ex vivo* treatment with TGF- $\beta$ . Thus, TGF- $\beta$  predisposes ocular APC to secrete IL-10 during Ag processing. This, in turn, directs the immune response away from a Th1 pathway and toward a Th2-like response in which DTH is suppressed (D'Orazio & Niederkorn, 1998).

IL-10 was detected at a higher level in the AH of patients with FHC compared to IAU which may account for the more benign inflammation. In addition higher levels of IL-12 were found in a higher proportion of patients with IAU compared to FHC. Since this is an important cytokine for the development of Th 1 and cell mediated immunity this is consistent with the more aggressive inflammation in IAU compared to FHC. A good balance between 'type-1' and 'type-2' cytokines was well demonstrated in FHC resulting in a benign but a chronic ongoing inflammation in the eyes of these patients. The important observation of steroid non-responsiveness of FHC could be accounted for by the high IL-10. As one of the mechanisms of anti-inflammatory of steroids is the induction of IL-10 it fails to make a significant difference in the already relatively high IL-10 in the AH of these patients.

The second comparison of the cellular phenotypes and cytokines in the AH and PB between patient with LU and SU was aimed at studying the

compromise in the immunoregulation in the target organ, being the eye in these conditions. In diseases thought to have an autoimmune aetiology, such as RA, examination of the target organ tissue revealed a totally different pattern of immune cell involvement from that suggested by examination of the PB. The selective increase of CD3<sup>+</sup> i.e. T cells in the AH implies that T cells are actively recruited to the ocular microenvironment. The T cell subset that is increased is CD4<sup>+</sup> which, are the T helper cells that have a central role in many immune responses. The CD8<sup>+</sup> T cells are not increased significantly in either LU or SU.

Early flow cytometry studies comparing AH, VH and PB from non-infectious uveitis patients detected mainly T cells in the AH (Deschenes, et al., 1986; Deschenes, et al., 1988) but normal ratios of PB lymphocytes. Previous flow cytometric studies in which intraocular T cells from AH and VH have been examined for expression of activation markers have reported increased levels of T cell activation (Deschenes, et al., 1988; Muhaya, et al., 1998). Activated T lymphocytes play an important role in the pathogenesis of posterior uveitis with an increase in activated T cells in PB lymphocytes and in intraocular fluids has been reported in-patients with various forms of uveitis, in particular among the CD4<sup>+</sup> T cells in posterior uveitis (Feron, et al., 1992).

In the present study both the CD4<sup>+</sup> T cells and the activated form of these cells are significantly higher in the AH compared to PB of LU but not in SU. This suggests that these activated CD4<sup>+</sup> play an important role in the pathogenesis of LU. Our previous study has shown that there is a significant increase in the circulating CD4/CD25 in the PB of uveitis as part of systemic disease (Behçet's disease) compared to uveitis being localised to the eyes (Feron,

et al., 1992). This suggests that in localised uveitis the immune dysregulation leading to a compromise in immune-privilege is limited to the eye. The lower level of antiinflammatory cytokines IL-10 and TGF- $\beta$ 2 in AH of patients with LU compared to SU further suggests this hypothesis.

A similar situation regarding the T cells occurs in the joints. A simultaneous analysis of PB and synovial lymphocyte subsets of RA, a non-organ specific autoimmune disorder patients and locally active osteoarthritis patients revealed an increase of HLA DR<sup>+</sup> and IL-2R<sup>+</sup> T cells in the PB of RA patients compared to PB lymphocytes of osteoarthritis patients. The synovial fluid lymphocytes of both RA and osteoarthritis patients revealed an increase in CD3<sup>+</sup>, CD8<sup>+</sup>, HLA DR<sup>+</sup> and IL-2R<sup>+</sup> T cells. The increase of HLA DR<sup>+</sup> and IL-2R<sup>+</sup> T cells in the PB of RA patients but not osteoarthritis patients implies that in osteoarthritis, a localised joint disorder, the T cell system seems not to be activated in PB in opposition to RA patients (Kuryliszyn Moskal, 1995). In MS the CSF lymphocytes of MS patients contain significantly higher number of IL-2R<sup>+</sup> T cells than PB lymphocytes of the same patients or the CSF of patients with non-inflammatory neurological disease (Tournier Lasserre, et al., 1987). This demonstrated that T cell activation occurs in the CNS but not in the peripheral lymphoid tissue, which is similar to the situation in the present study. Another possibility is that activated cells have trafficked from the periphery to the site of inflammation.

CD19, which is a B cell marker, was significantly lower in LU but not SU. This may reflect the selection in the lymphocytes being recruited to the eye. The B cells being important in humoral type of immunity are not actively

recruited into the eye. Similarly the CD19 expression in the synovial fluid is significantly lower compared to the PB of both RA and osteoarthritis patients suggesting the little role if any of B cells in the pathogenesis of these joint disorders (Kuryliszyn Moskal, 1995).

The information obtained from the characterisation of the T cells and cytokines in the AH reflects the in vivo situation of these eyes with uveitis. The ocular microenvironment however does have immunoregulatory effects to the antigens and inflammatory cells infiltrating the eye via its soluble immunosuppressive factors, including TGF- $\beta$ 2, MSH, and vasoactive-intestinal peptide. These factors are largely the secretory products of parenchymal cells of the iris and ciliary body. As a result the levels of cytokines detected were low and some of the cytokines were not detectable especially IL-4 and IL-2 in the AH. Whether the cells are prevented from secreting these cytokines or whether they do not have the potential to secrete them was investigated by growing these T cells isolated from the VH of patients with FHC and IU, two types of intraocular inflammation where the vitreous cavity is infiltrated by cells. The cells were harvested from the VH rather than AH, as larger volumes of VH were obtainable during surgery hence yielding higher number of cells for culture.

There was a slightly different detection rate of cytokines between fresh AH and VH. A higher proportion of AH samples was positive for cytokines compared to vitreous. The most likely reason is that the AH was taken during active inflammation of the eyes when there are inflammatory cells in the AC. On the other hand the VH samples were taken from patients during surgery when the inflammation was well controlled which is an important prerequisite to surgery.

As cytokines are produced during the activation and effector phases of innate and specific immunity, and their secretion is a brief self-limited event, it is more likely to be detected in eyes with significant inflammation. Another explanation for the lower detection rate in the VH compared to AH is the fact that the AH is more fluid in nature resulting in a more free movement of cells leading to a higher turnover of cells. There may then be more activated cells with more cytokine production ability, whereas the VH, being a gel like structure is less of a sump for all the soluble mediators of inflammation. However as the infiltrating cells are trapped in the gel network, the chances of isolating them are better.

To ascertain the potential of cytokine production by these T cells the PHA expanded T cell lines from patients with FHC and IU were stimulated and the cytokines secreted quantitated. In contrast to the non-detection of IL-2, IL-4, and IFN $\gamma$  from the fresh VH, the T cell lines produced massive amounts of IL-2 and IFN $\gamma$ . IL-4 and IL-10 on the other hand were secreted in lower quantities. This suggests that the T cells infiltrating the vitreous were polarised from the beginning and were producing the type-1 cytokines responsible for the intraocular damage especially in IU where the intraocular morbidity is greater as compared to FHC. This is consistent with the higher IL-2 production by the T cell lines derived from VH of IU patients compared with FHC patients. The source of IL-2 from the T cell lines could be both either CD4<sup>+</sup> and CD8<sup>+</sup> T cells. However using intracytoplasmic staining, CD4<sup>+</sup> T cells have been shown to be the major producer of IL-2 compared to CD8<sup>+</sup> T cells (Andersson, et al., 1990). The percentage of CD4<sup>+</sup> T cells being a more important source of IL-2, was significantly higher in the T cell lines derived from the VH of IU patients.

The levels of both IFN $\gamma$  and IL-10 in the T cell culture supernatants from VH-derived T cell lines from FHC patients were significantly higher compared to those from IU patients. This may appear paradoxical, as the antagonistic role of these two cytokines is well established (Bai, et al., 1997). However these two cytokines have been shown to be produced by CD8<sup>+</sup> T cells (Hoiden & Moller, 1996). The predominance of CD8<sup>+</sup> T cells over CD4<sup>+</sup> T cells may contribute to the higher IFN $\gamma$  and IL-10 in the supernatants of VH-derived T cell lines from FHC compared to IU patients.

The greater production of IL-10 by the VH-derived T cell lines derived from patients with FHC is consistent with the relatively higher level in the fresh AH and may account for the benign clinical features of FHC as IL-10 is known to be an important downregulator of macrophage functions. IL-10 suppresses production of pro-inflammatory cytokines by activated monocytes and macrophages (Fiorentino, et al., 1991; Enk, et al., 1993) and perhaps was able to counteract the macrophage activation by IFN $\gamma$ .

IL-10 has been shown to be chemotactic to CD8<sup>+</sup> T cells (Jinquan, et al., 1993). Hence following IL-10 production by macrophages and CD4<sup>+</sup> T cells there may be an influx of CD8<sup>+</sup> cells. This may be the reason for the predominant CD8<sup>+</sup> T cells in the AH of FHC patients where the level of IL-10 is relatively high compared to other types of uveitis. It is uncertain whether this chemotactic activity towards CD8<sup>+</sup> contribute in anyway to the anti-inflammatory action of IL-10.

An important point to consider is that the cells isolated from the ocular fluid ie. AH and VH may not reflect those in the tissues. There is a possibility

that these cells are the ones not retained in the tissues hence not representative of the effector cells in the pathogenesis. However as tissue biopsy is difficult to obtain from human eyes, isolation of cells from the ocular fluid is the best approach available. In sarcoidosis a multisystem disorder characterised by the development of non-caseating, epithelioid granulomas affecting mainly the lungs analysis of the bronchoalveolar lavage (BAL) have been informative on the disease pathogenesis. The basic abnormality is widely believed to be due to imbalance of the T helper/suppressor cell balance where a high CD4/CD8 ratio is diagnostic in this condition and activated CD4<sup>+</sup> T cells are found to be important in the pathogenesis of sarcoidosis. As in our VH-derived T cell lines, the T cell lines derived from BAL by Kawakami (Kawakami, et al., 1995) et al displayed Th1 like profiles.

Even though we can infer from our study that these T cells are responsible for the pathogenesis of uveitis a study that will allow exact localisation of the T cell phenotype producing a particular cytokine would be ideal. This can be achieved by intracellular staining of the T cells isolated from the ocular fluids. Knowledge of the exact cellular phenotypes producing the various cytokines will be extremely beneficial as the candidate cytokine responsible for the main role in the pathogenesis can then be blocked.

The therapy of immune-mediated disorder is aimed at reducing immune responses and the attendant inflammation. The mainstay of treatment is anti-inflammatory drugs particularly corticosteroids which are limited by its side effects hence leading to the search for a more specific agents aimed at the effector phases of the pathologic immune responses. Current immunotherapies



which are being designed for the treatment of chronic inflammatory and allergic diseases either aim to block inflammatory mediators (example. leukotriene inhibitors, anti-cytokine antibodies), compete with cytokine receptor binding i.e. at the effector phase of the immune response.

In view of its ability to inhibit production of proinflammatory cytokines involved in acute and chronic inflammatory processes, IL-10 is being studied in an array of conditions such as RA (van Roon, et al., 1996), Crohn's disease (van Deventer, et al., 1997) and inflammatory bowel disease (Kuhn, et al., 1993; Powrie, et al., 1994). Recently in patients with moderate RA, IL-10 administration was well tolerated and was associated with improvement in clinical and inflammatory markers of disease (Grint, unpublished data). A future therapeutic use that can be derived from the information in this study is the use of IL-10 since its presence appears to be associated with minimal inflammation in FHC and apparent protective role from PS formation. Slow release devices are available for intraocular use (Martin, et al., 1994) and could potentially allow chronic administration of downregulatory cytokines within the eye. Using this kind of approach, IL-10 would be a suitable cytokine for long term administration to treat intraocular inflammatory disease.

Apart from intracellular staining another future study that will be useful is the detection of another family of cytokine, which are the chemokines. These molecules are likely to be involved in chronic intraocular T cell recruitment (Taub, et al., 1996) and have been detected in the AH of patients with acute anterior uveitis (Verma, et al., 1997). The detection of these chemokines within the AH in different forms of uveitis may explain the selective recruitment of

different cell types to the eye. This might point to a particular important chemokines, which could be blocked as an alternative to non-specific therapy of antiinflammatory agents.

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