Immunoregulation of Acquired Ocular Immunobullous Disease

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Abstract

Ocular Mucous Membrane Pemphigoid (OcMMP) is a blinding immunobullous disease, characterised by auto-antibody driven conjunctival inflammation and scarring. My hypothesis was that *progressive fibrosis in OcMMP, occurring in the apparent absence of clinical inflammation, was driven by underlying inflammatory processes.*

I observed that in OcMMP, progressive scarring did occur in the apparent absence of clinically identifiable inflammation and I was able to improve clinical documentation by developing and validating an objective Fornix Depth Measurer (FDM) for assessment of scarring.

I optimised non-invasive Ocular Surface Impression Cytology (OSIC) combined with flow cytometry to characterise conjunctival leukocytes. I found that CD8$\alpha\beta$+ effector memory, cytotoxic, mucosal-homing T cells were the dominant population in health. This population was unaltered with age but CD4+ T cells, capable of producing IFN-γ, increased.

In OcMMP, the conjunctiva was characterised by decreased CD8+ lymphocytes and an elevation in $CD45^{INT}$CD11b+CD16+CD14- neutrophils. Although neutrophils correlated with clinical inflammation, they were even present in the absence of identifiable conjunctivitis. This elevation was associated with progression of scarring assessed by FDM, even in the clinically *Non-inflamed* eye.

These findings confirmed my hypothesis and provide a platform for quantifying neutrophils as a biomarker of sub-clinical inflammation and their role in the scarring process.

Conjunctival Inflammation: a historical perspective

"Hyperaemia of the conjunctiva may result from many causes, as the irritation produced by cold wind, dust, or irritating vapours, such as tobacco smoke; overwork of the eyes, actual or relative to their capabilities...

The amount of injection varies; in the worst cases, the whole of the ocular conjunctiva presents a network, with very irregular meshes, formed by dilated and tortuous vessel; these can be moved over the surface of the eye by rubbing the lid over them, and they can be emptied by slight pressure; in both respects, as well as not being especially marked in the circumcorneal zone, differing from the dilated vessels seen in inflammation of the cornea or iris...

In slighter cases there are very few dilated vessels, and in in some they only become visible after prolonged use of the eyes, or after exposure to wind, etc."

Robert Brudenell Carter and William Adams Frost.
Affections of the eyelids, lacrymal apparatus, and conjunctiva.
In *Ophthalmic Surgery*.
London, Cassell & Co.1887 *pp*117-118

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List of abbreviations used

Abbreviation	
Ab	Antibody
APC	Allophycocyanin
BOSU	The British Ophthalmological Surveillance Unit
BP	Bullous Pemphigoid
BMEC	The Birmingham and Midland Eye Centre
BMZ	Basement Membrane Zone
CMV	Cytomegalovirus
CRF	Clinical Record Form
DC	Dendritic Cell
DIF	Direct Immunofluorescence
DN	Double Negative (CD8β-CD4-) T cells
EALT	Eye-Associated Lymphoid Tissue
EB	Epidermolysis Bullosa
EOD	Early Onset Disease (OcMMP)
EBV	Epstein Barr Virus
EstD	Established Disease (OcMMP)
FDM	Fornix Depth Measurer
GALT	Gut Associated Lymphoid Tissue
GEE	Generalised Estimating Equation
HLA	Human Leukocyte Antigen
HSV	Herpes Simplex Virus
IEL	Intra-Epithelial Lymphocytes
Ig	Immunoglobulin
IFN-γ	Interferon gamma
IIF	Indirect Immunofluorescence
IOSD	Inflammation in Ocular Surface Disease
MALT	Mucosa Associated Lymphoid Tissue
MEH	Moorfields Eye Hospital
MHC	Major Histocompatability Complexes
MMP	Mucous Membrane Pemphigoid
NK	Natural Killer (cell)

Oc	Ocular
OS	Ocular Surface
OSD	Ocular Surface Disease
OSIC	Ocular Surface Impresion Cytology
PAMPs	Pathogen-Associated Molecular Patterns
PCC	Progressive Cicatricial Conjunctivitis
PDGF	Platelet Derived Growth Factor
PE	Phycoerythrin
PMMA	Polymethylmethacrylate
PSS	Primary Sjögrens Syndrome
ROS	Reactive Oxygen Species
SLE	Systemic Lupus Erythematosis
SJS	Stevens-Johnson Syndrome
TCR	T-cell Receptor
TEN	Toxic Epidermal Necrolysis
TGF-β	Transforming Growth Factor-β
TLR	Toll-Like Receptor
TNF-α	Tumour Necrosis Factor-α
VA	Visual Acuity

Chapter 1

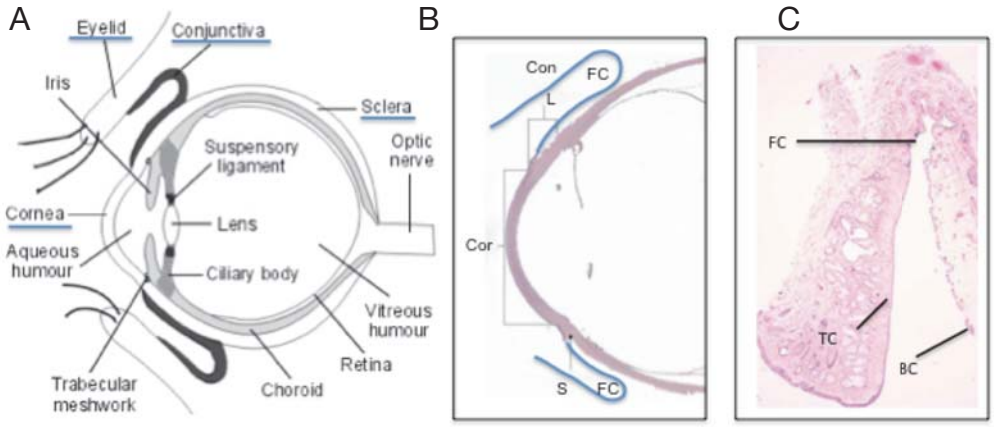
General Introduction

1.1 The Ocular Surface

Vision is sustained by transparent ocular tissues such as the cornea and the lens and optically clear media within the eye including aqueous and vitreous. Nowhere is this threatened more than at the ocular surface, which represents the interface between the eyeball and its surroundings. Maintaining a clear cornea and thus optical clarity relies upon the ocular surface as a whole. The ocular surface (**Figure 1.1**) consists of:

(i) The corneal epithelium and underlying anterior stroma

(ii) The conjunctiva (a delicate protective mucosa that lines the back surface of the eyelids and the sclera) (Knop and Knop, 2007).

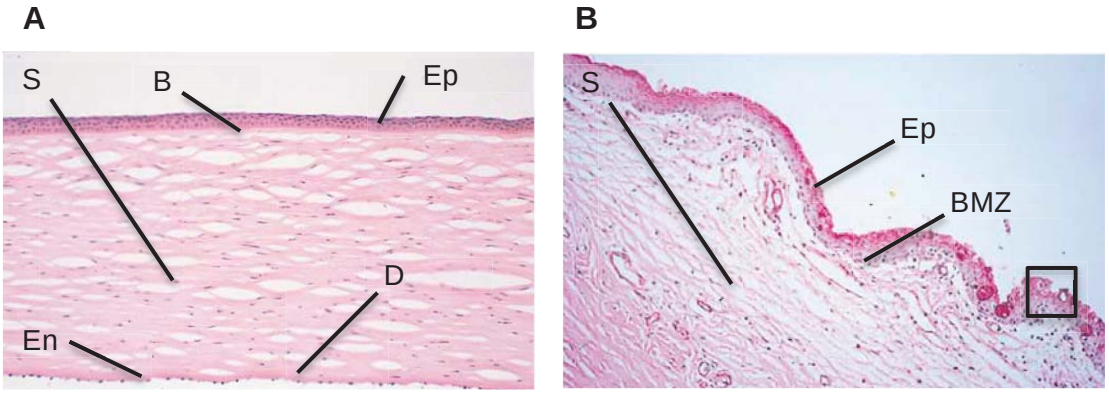


Adapted from Yanoff and Fine. Ocular Pathology. Mosby 2002 (Yanoff and Fine, 2002).

Figure 1.1: The Ocular Surface. Saggital representation of the eye demonstrating the important components of the ocular surface, underlined in panel A. Panel B shows a histological section of the cornea (Cor), sclera (S) and their interface - the corneoscleral limbus (L). The conjunctiva (drawn in blue, Con) is closely attached to the sclera 3mm posterior to the limbus and fuses with the cornea at the conjunctival limbus (1mm anterior to the corneoscleral limbus). Panel C demonstrates the forniceal conjunctiva (FC), a continuation of the bulbar (BC) and tarsal conjunctiva (TC), forming a pocket separating the two surfaces.

Embryologically, the corneal and conjunctival epithelia are derived from surface ectoderm while the stroma arises from the second wave of neural crest derived mesenchymal cell migration (from the optic cup) by the seventh week. Collagen deposition, organisation and sensory innervation continue until the 6-7th month.

The avascular cornea is an imperfect sphere measuring 9-11mm vertically and 11-12mm horizontally. Its thickness is variable, measuring 1100μm at its limbus, 650μm at its periphery and thinning to 520μm centrally (American Academy of Ophthalmology., 2006). Its central zone is a five-layered structure composed of a 5-7 cell deep stratified, squamous non-keratinised epithelium; Bowman's layer (underlying the epithelial basement membrane); stroma (with parallel bundles of predominantly Type I collagen arranged to optimise transparency); Descemet's layer (a basement membrane) and cellular monolayer: the endothelium (**Figure 1.2**).



A: Adapted from Yanoff and Fine. Ocular Pathology. Mosby 2002 (Yanoff and Fine, 2002).

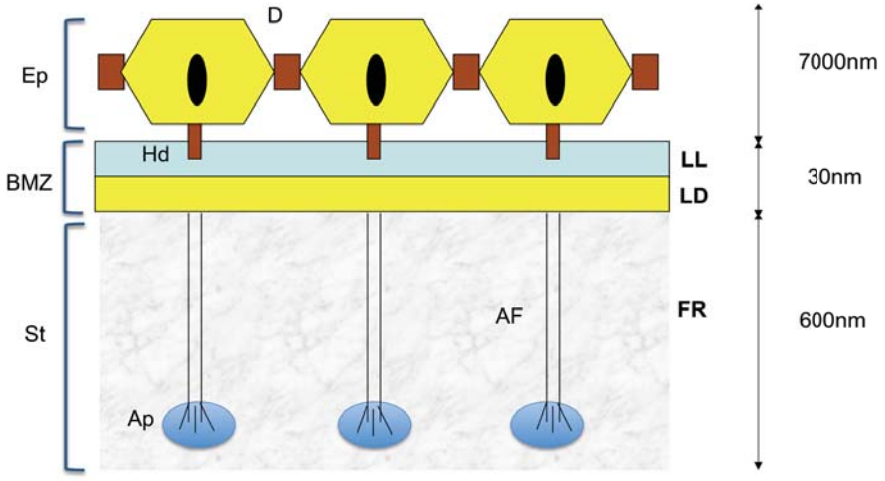
Figure 1.2: **Histology of the cornea and conjunctiva**. The five layers of the cornea are demonstrated in panel A (Ep: Epithelium; B: Bowman's layer; S: Stroma; D: Descemet's layer and En: Endothelium). The stroma occupies 90% of the thickness. The conjunctival epithelial goblet cells are boxed in panel B. The vascularised stroma can be seen underneath the basement membrane zone (BMZ). The conjunctiva consists of tarsal (palpebral) and bulbar components (lining the inner surface of the eyelids and globe of the eye respectively), and connected via the fornices (singular fornix) and is continuous with the corneal epithelium at the limbus (**Figure 1.1).** The lower fornix (the natural pocket formed between the bulbar and tarsal conjunctiva) depth is variable and appears to progressively shorten with age *(Schwab et al., 1992)*. There is no detailed epidemiological data relating to the depth of the upper fornix.

Due to the absence of a blood supply, nutrients are derived from tears and the aqueous humour. The avascularity also influences wound healing, which is achieved by activation of stromal fibroblasts and the laying down of fresh collagen. Corneal epithelial regeneration is derived from a rich stem cell niche at the limbus (Pellegrini et al., 1999). Destruction of the limbal epithelial stem cells has important implications for the maintenance of corneal clarity, as corneal epithelial cells become repopulated from the conjunctival stem cells, resulting in loss of corneal clarity.

Mucosa or mucous membranes are mucous tissue linings composed of a specialised epithelium and an underlying lamina propria. The epithelial surfaces are non-keratinised and secrete a viscous (mucous) fluid. The conjunctiva is histologically a mucous membrane composed of stratified epithelium, a basement membrane zone (divided in to a lamina lucida, lamina densa and fibro-reticularis), and an underlying stroma divided in to loose connective tissue (termed the lamina propria) and a deeper vascularised layer (overlying the episclera) (Pflugfelder et al., 2004). The 2-3 layer stratified epithelium is squamous in its tarsal and columnar in its bulbar part and can be up to 7 layers. The conjunctiva contains accessory lacrimal tissue and goblet cells, which contribute to formation of the inner tear film layer through the production of mucin. Unlike corneal stem cells, conjunctival stem cells are uniformly distributed throughout the conjunctiva, but probably more densely populated in the fornices (Pellegrini et al., 1999). The tarsal and bulbar conjunctiva derives their blood supply from the medial and lateral palpebral arteries (from the lacrimal and ophthalmic arteries respectively) and the limbal conjunctiva from the anterior ciliary arteries (from muscular branches of the ophthalmic artery). The cornea and bulbar conjunctiva are

both innervated by the long ciliary nerves (branches of the nasociliary division of the ophthalmic nerve).

The ocular surface epithelial basement membrane zone (BMZ), termed Descemet's layer in the cornea, like other BMZs are composed of an extra-cellular matrix of proteins, which bind the epithelium to the underlying stroma. The BMZ can be divided in to the lamina lucida, lamina densa and underlying fibro-reticularis (**Figure 1.3**). Epithelial cells (bound to each other by desmosomes) are attached to the underlying lamina lucida by hemi-desmosomes. The lamina lucida and lamina densa (thought to be less dense in mucosa) contain extra-cellular proteins, primarily Type IV collagen (Type I, V and VI collagen are found in the corneal stroma), together with the cross-shaped glycoprotein Laminin while the less densely packed lamina lucida also contain glycosaminoglycans such as Heparan Sulphate (Elder, 1997a, Forrester, 2008, Challacombe et al., 2001). Integrins are hetero-dimeric receptors found within extra-cellular matrix and also act as signalling proteins in immune responses (discussed in **section 1.3.2**). They contain an α and a β chain e.g. α_1:β_1 which bind laminins in the BMZ. This not only contributes to the cell matrix, but also plays a role in trans-membrane signalling (Forrester, 2008, Humphries, 2000). The fibro-reticularis contains collagen bundles (primarily collagen VII) called anchoring filaments, which attach the BMZ to sub-basal dense plates termed anchoring plaques (again composed of Type IV collagen and Laminin) via anchoring fibrils.



Adapted from Elder. Cicatrising conjunctivitis. Karger. (Elder, 1997a)

Figure 1.3: The Basement Membrane Zone (BMZ). Epithelial (Ep) cells are held together by desmosomes (D) and anchored to the underlying lamina lucida (LL) of the BMZ by hemi-desmosomes (HD). The LL and LD are composed of Type IV collagen and Laminin, which in turn are attached to the underlying fibro-reticularis (FR) of the anterior stroma (St) via anchoring filaments (composed of Type VII collagen) to anchoring plaques (AP).

1.2 The Lacrimal Functional Unit

The ocular surface is protected by the ability to close the eyelids, facilitating lubrication and distribution of the tear film. Numerous glands found on the ocular surface maintain the tear film structure. Of critical importance is the lacrimal functional unit that comprises the lacrimal gland, conjunctiva, cornea, eyelids and sensory innervations, which together are essential for optical clarity (Zierhut et al., 2002, Stern et al., 2010). The sponge-like tear film itself is a complex, broadly divided in to an outer lipid layer, an aqueous layer and a mucin layer.

The outer lipid layer of the tear film is created by Meibomian glands (embedded in the tarsal plates underlying the tarsal conjunctiva) and glands of Zeiss (found in the eyelid margins opening in to the eyelash follicles). They help prevent evaporative

loss of the other components of the tear film. The principal oily components include wax and sterol esters which are non-polar lipids which act as a lubricant and as a water barrier, while polar lipids act as a base for the non-polar lipids and have surfactant properties, helping to spread other lipid components of the tear film (McCulley and Shine, 2003).

The lacrimal glands are bilobed structures found in the superotemporal region of the orbits. They are exocrine glands, lined by acinar cells, producing a serous secretion draining in to the superior fornices. Supported by the accessory glands of Krause and Wolfring (residing in the conjunctival fornices), they form the aqueous layer of the tear film. This layer also contains secretory immunoglobulin A (IgA); antimicrobial lysozymes and lactoferrin; lipocalin (maintaining surface lipid spreading), electrolytes such as Na^+, K^+, Cl^-, HCO^{3-}, Mg^{2+} and Ca^{2+} and cytokines such as IL-1, IL-6, IL-8, IL-10 and TNF-α (Zierhut et al., 2002, Nakamura et al., 1998, Sonoda et al., 2006, Tiffany, 2003).

Finally, the inner mucous layer is produced by the conjunctival goblet cells, by producing O-glycosylated serine and threonine residue glycoproteins called mucins (Guzman-Aranguez and Argueso, 2010). They render the hydrophobic surface of the ocular tissues hydrophilic (by its surfactant properties), thereby increasing wettability and increased retention of the aqueous component. This glycocalyx bridges the outer layers to the corneal and conjunctival surfaces and therefore helps overall lubrication. It is thought there may be a role for ‘circulating’ mucins, which act as a debris removal system, the principal mucin being MUC5AC (Gipson, 2004, Tiffany, 2003). Mucins may also play a role in anti-adhesion, signalling and pathogen binding as well

as stabilising the tear film (Govindarajan and Gipson, 2010). Murine studies have indicated that goblet cells homeostasis is regulated by IL-13, secreted by NKT cells in the case of the conjunctiva (De Paiva et al., 2011).

The tear film also supplies nutrients, glucose and oxygen to the cornea, maintains immune regulation of the ocular surface and washes away unwanted debris. Tears are cleared via drainage through small puncti (found in the medial limit of each eyelid margin) into a canalicular system, in turn draining into lacrimal sacs and out through nasolacrimal ducts into the nose. Disruption of any of the components of the tear film plays an important role in dry eye problems.

Turnover of tears is regulated by the parasympathetic supply to the lacrimal glands (via secretomotor branches of the lacrimal nerve). Basal secretion is 1.2μl/min and can be massively increased by mechanical or psychological stimuli in the presence of a healthy lacrimal functional unit.

1.3 Immunoregulation of the ocular surface

Immunoregulation refers to the "*tolerance and modulatory mechanisms responsible for tightly controlling the innate and adaptive immune response to limit bystander tissue damage after stress and/or microbial assaults*" (Stern et al., 2010). This is especially true in maintaining a healthy and optically clear ocular surface. Before considering immunoregulation in this specialised environment, we will consider general principles relating to the immune system, how these have adapted in relation to mucosal environments and finally in the ocular surface itself.

1.3.1 Principles of immunity and inflammation

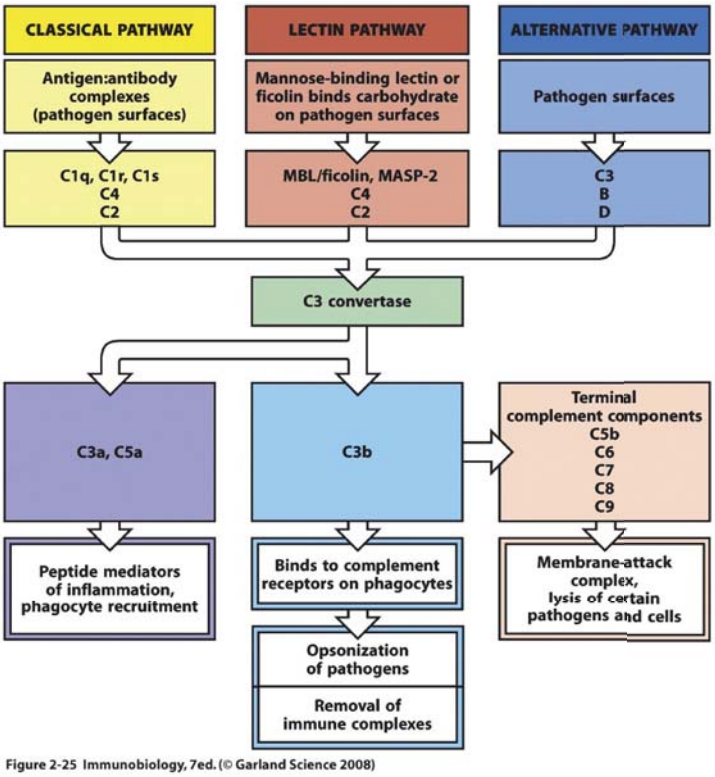
The immune system provides an intricate means of protecting host species against pathogens and removing cancerous cells. Historically, immunity has been divided into the innate and adaptive systems. The innate system provides a rapid or non-specific means of dealing with attack, while the adaptive immune system offers a specific response based on immunological memory.

Innate Immunity

Innate immunity includes the natural barriers of the body e.g. the skin or mucosal surfaces, the ability to clear microorganisms through fluids such as the tear film, and recruiting cells that are capable of mounting an immediate response to neutralise or destroy invading pathogens if epithelial surfaces are breached. These include granulocytic cells termed polymorphonuclear cells and include neutrophils, eosinophils, basophils and mast cells; macrophages and dendritic cells (DCs), derived from monocytes and Natural Killer (NK) cells, derived from lymphoid precursors.

Cellular components of the innate immune system are aided by the complement system. This is a highly conserved organisation of plasma peptides that is able to recognise molecules found on the surface of pathogens such as bacteria. Three pathways exist including the alternative pathway (an innate response), the classical pathway (an adaptive response, discussed later) and the lectin pathway (Ricklin et al., 2010). They work by inducing a cleavage cascade by enzymatic conversion of one complement protein to another e.g. in the alternative pathway C3 is converted to

C3a, C3b or C5b by C3 convertase following recognition of pathogen. C3a can recruit phagocytic cells such as neutrophils and macrophages; C3b binds to phagocytes and promotes engulfment of micro-organisms in a process termed opsonisation and C5b forms a complex with C6, 7 and 8 termed the Membrane-Attack Complex resulting in a pore-formation in the micro-organism cell wall (Sjoberg et al., 2009). An overview of the complement system is shown in **Figure 1.4**.



From *Janeway' s Immunobiology* 2008. (Murphy et al.)

Figure 1.4 The complement system. A summary diagram showing the three principle pathways of the complement cascade. The classical pathway can be activated by antibody while the alternative pathway is part of the innate immune system. The lectin pathway is initiated by recognition of foreign carbohydrate sequences. Phagocytic recruitment and activation including is a common end-point.

Neutrophils are considered primary host responder cells, capable of detecting pathogens and are normally recruited rapidly from peripheral blood by a number of mechanisms, and which serve to activate them. These include the complement cascade (e.g. via C3 activation of the mannose-binding lectin receptors or the alternative pathways), pattern recognition via Toll-like receptors (TLRs; which activate phagocytes by recognising peptide sequences called Pathogen-Associated Molecular Patterns [PAMPs] e.g. dsDNA by TLR3 or lipopolysaccharide by TLR4) or by cytokines and chemokines e.g. Interleukin-6 (IL-6), CXCL7 and CXCL8 produced by activated macrophages (Sadik et al., 2011, Williams et al., 2011).

Macrophages also contribute to the innate response through phagocytosis of dying/dead cells, removal of opsonised cells by the compliment cascade and the release of cytokines such as Tumour Necrosis Factor-α (TNF-α). TNF-α helps contain local inflammation but together with other inflammatory cytokines produced such as IL-1 and IL-6, systemic release can induce an acute phase response resulting in elevated temperature, activate acute phase proteins (such as C-reactive proteins) and mobilise neutrophils from the bone marrow.

Eosinophils and basophils help destroy parasitic worms by the release of proteases or histamine respectively in response to Immunoglobulin-E (IgE). Natural Killer (NK) cells have an important role in destruction of virally infected or tumour cells through the release of cytotoxic granules onto the infected cell surface (Caligiuri, 2008). The acute responses to foreign attack precede a specific immune response termed adaptive immunity.

Adaptive Immunity

The major leukocytes involved in adaptive immunity are T-lymphocytes (derived form the thymus) and B-lymphocytes (arising in the bone marrow). The primary immune response in adaptive immunity relies on afferent and efferent arms and are characterised by antigen presentation to lymphocytes in lymphoid structures (the afferent response), T-lymphocyte activation and T:B cell interaction resulting in specific antibody production by B-cells (the efferent or humoral response).

DCs have an important role in that they are able to bridge the innate and adaptive immune responses by phagocytosing foreign microorganisms and interacting with other immune cells. They are therefore termed professional antigen presenting cells. They can reside within the lamina propria and have projections through the basement membrane zone to the epithelium e.g. Langerhans cells in skin. Antigens processed by the engulfment and breakdown of pathogens are transferred to secondary lymphoid structures such as draining lymph nodes, or in the case of mucosal surfaces, Mucosa Associated Lymphoid Tissue (MALT) e.g. Peyer's patches in the small intestine. It is here that antigen presentation to lymphocytes facilitates an adaptive immune response (Sheridan and Lefrancois, 2011).

Professional antigen presenting cells such as DCs and macrophages, having come in to contact with foreign antigen, present peptides in conjunction with Major Histocompatability Complexes (MHCs) and signal T lymphocytes via the T-cell receptor (TCR - a part of the immunoglobulin family), the common leukocyte antigen (CD45) and co-stimulatory molecules (e.g. CD3 and CD28). MHCs (also called HLA

or Human Leukocyte Antigen, located on chromosome 6) are divided in to Class I (present on all nucleated cells) or Class II (presented on professional antigen presenting cells such as DCs and macrophages). MHC class I present antigen and stimulate CD8+ T cells via TCR, CD8 and co-stimulatory molecules while MHC class II signals CD4+ T cells. Following antigen exposure, T cells undergo a massive expansion in their numbers and the ability to recognise specific antigen, the principle of immunological memory (Kim and Williams, 2010, Sheridan and Lefrancois, 2011).

The ability of T cells to recognise antigen presented to them as foreign is determined by the principle of central tolerance i.e. self-reactive T cells are removed during maturation in the thymus. Additional mechanisms include anergy, where the absence of co-stimulatory molecules during antigen presenting cell interaction with T-cells in the periphery results in the removal of potentially auto-reactive T cells and by T regulatory cells and Type b Intra-Epithelial Lymphocytes (IEL).

T cell maturation is based on the type of antigen presented by antigen presenting cells so that precursor T cells (Th0 cells) are polarised to become a specific T cell subtype via the cytokine signal generated at the immunological synapse e.g. Th1 responses, involved in cell-mediated responses by stimulating T and NK cells, are induced by IL-12 production by DCs in response to engulfing intra-cellular pathogens such as viruses and PAMPs. T cells (expressing common CD3+ marker) can be sub-classified by the presence of CD8 or CD4, their TCR ($\alpha\beta$ or $\gamma\delta$) and further classified by their role (effector or regulatory) based on the production of cytokines such as IL-17 in the promotion of inflammatory responses or IL-10 in the context of regulation (Annunziato et al., 2007, Zhou et al., 2009).

CD8+ cells, conventionally cytotoxic (Tc cells), have a primary function to kill cells infected with viruses or having undergone malignant transformation. This is achieved by the production of cytolytic granules such as granzyme B and perforin and through Interferon-γ (IFN-γ) production (Cheroutre and Madakamutil, 2005) although there are unusual population that produce IL-17 termed Tc17 cells and others showing regulatory properties, more commonly in the mucosa. By contrast, CD4+ cells are broadly divided on the basis of their helper responses (Kondo et al., 2009, Koch et al., 2008). Th1 responses are characterised by cytokines such as Interferon-γ (IFN-γ) and the transcription factor T-bet and induce cell mediated responses that kill infected cells through activation of macrophages and NK cells; Th2 by IL-4, IL-5 and IL-13 (the transcription factor is GATA-3) which activate B cells; Th17 by IL-17 and the transcription factor RORγt that are implicated in inflammatory responses including anti-fungal and intercellular bacteria roles, and T-regulatory cells (T^{REG}) by CD25+ and the transcription factor FoxP3+ that serve to dampen/regulate immune responses (Cope et al., 2011, van Wijk and Cheroutre, 2009)

B cells are generated in the bone marrow and are typified by their ability to produce antibodies (immunoglobulins; Ig) and immature B cells producing IgM travel to lymphoid structures. Once Th2 (helper CD4 cells) have been activated they are able to form T:B cell interactions. B cells act as professional antigen presenting cells and engulf free antigens in draining lymph nodes, and when they come in to contact with cognate T cells primed by the same antigen, they are able to form a complex which stimulates the differentiation of the B cell when combined with co-stimulatory molecule CD40 (which binds to CD154) (Neron et al., 2011). Cytokines produced by

the Th2 cell e.g. IL-4, 5 and 6 assist this process. Activation of B cells results in proliferation and maturation and the formation of plasma cells.

The immunoglobulins produced by B cells (and latterly plasma cells following affinity maturation and induction of memory) include IgG, A, M, E and D and characterise the adaptive immune response by neutralising virus and helping to promote rapid responses to known antigens through the recruitment of other immune cells – both from the innate and adaptive 'efferent' arms. The classical complement pathway can also be initiated by the adaptive immune response through antibody:antigen complexes on pathogens, which in turn convert C1q to C4 and C2 to give C4b2a and this becomes the C3 convertase. This occurs at the site in which it is initiated.

Historical distinction between innate and adaptive responses have become blurred through greater understanding of cellular interactions, cell plasticity and the diversity of responses seen in specialist sites. Antigen-antibody complexes may also serve to initiate innate responses e.g. the classical complement pathway which can lead to the recruitment of inflammatory cells (Janeway, 2005). Neutrophils can also be activated by Fc receptors that can bind for example Immunoglobulin (Ig) G1 and IgA, and this assists target recognition.

Inflammation and Autoimmunity

The acute inflammatory response, initiated by foreign antigen and/or tissue damage results in a signalling cascade which draws innate cells rapidly, with neutrophils able to extravasate in to tissue spaces from the peripheral blood compartment, assisted

by tethering to the endothelial wall and enhanced vascular permeability. This process is facilitated by PECAM-1-dependent $\alpha_{6:}\beta_1$ integrin up-regulation on transmigrating neutrophils (Sadik et al., 2011) and leakage of the endothelium, resulting in tissue swelling, redness and local temperature rise, and pain associated with the release of histamine – the cardinal features of inflammation described by Celsus as tumor, rubor, calor and dolor. This results in further recruitment of inflammatory cells including macrophages and NK cells that aid in the phagocytic process and lymphocytes which act as bystanders pending adaptive responses.

Neutrophils exert their effect by engulfing pathogens and subjecting them to reactive oxygen species (ROS) such as H_2O_2 and O_{2-} and utilising destructive azurophilic granules e.g. elastases and proteinases (Kessenbrock et al., 2008). These proteins have potential to induce severe local tissue damage as well as eradicating pathogens. Neutrophils may also contribute to sites of inflammation by enzymatic cleavage by proteinase-3 of pro-cytokines including TNF-α (Wright et al., 2010). Following this acute response, their retention within tissue has historically been considered short lived, preceding a more chronic inflammatory response. Recent evidence however suggests that they may play a role in modifying adaptive responses by cytokine release, migration to lymph nodes or by scavenging inflammatory cytokines and chemokines (Mantovani et al., 2011).

Chronic inflammation takes place when inadequate clearance of antigen occurs. In the case of infection this may result from failure to mount initial response or evasive mechanisms by the pathogen such as antigenic variation or drift in the case of influenza virus or intra-cellular localisation in the case of *Mycobacteria tuberculosis*.

Autoimmunity may also result in chronic inflammation. Autoimmunity arises due to the aberrant recognition of self-antigen as foreign. Numerous mechanisms can result in this situation and numerous diseases have an autoimmune basis including for example Sjögren's Syndrome (Ramos-Casals et al., 2010a).

Diseases that are antibody-mediated have been described as Type II hypersensitivity disorders. The mechanisms by which autoimmunity arises are diverse and include failure of central tolerance or anergy, failure of regulatory lymphocyte populations, molecular mimicry (where a pathogen produces antigen which is mistaken for self), polyclonal B cell direct activation of B cells by e.g. bacterial endotoxins), MHC mutations, superantigen activation (multiple T cell activation by e.g. Staphylococcus by direct binding to MHC) and bystander activation (where anergised T cells yet to be cleared are activated by the up regulation of co-stimulatory molecules during infections). They can be classified as organ specific or systemic based on the source of their antigens (Hoyne, 2011).

1.3.2 Mucosal Immunology

Mucosal surfaces, comprise the 'wet surfaces' of the body such as the ocular surface, the nasopharynx, the mouth, the respiratory and gastro-intestinal tracts (including the oesophagus, stomach, small and large intestines). They have adapted their role as a 'first line of defence' in order to undertake specialised roles such as acting as specialist transport systems e.g. gas exchange or the absorption of nutrients but without the additional protection afforded by keratinisation, as in the skin (Lawson et al., 2011).

Infections pose a major challenge to the mucosal immune system and many human infections such as *M. tuberculosis*, Human Immunodeficiency Virus (HIV) and measles breach the immune system through mucosal surfaces. Vulnerability of the mucous membranes to infections is compensated for by the specially adapted MALT. In the gastro-intestinal tract these are termed Gut-Associated Lymphoid Tissue (GALT) and include tonsils and Peyer's patches in the small intestine. The surface epithelium of Peyer's patches contain microfold (M) cells and lack villi and goblet cells, characteristic of the surrounding epithelium. M cells are adapted to traffic pathogenic antigen to the underlying lymphoid structure, containing both B and T cell areas (Sheridan and Lefrancois, 2011).

Local antibody production takes place in the mucosa by plasma cells in the lamina propria and MALT. Immunoglobulin A is important at mucosal sites because it is secretory, it has an ability to form dimers and interact with Fc receptors on phagocytes (Woof and Russell, 2011). There are two isotypes of secretory IgA, IgA1 and IgA2 (with a ratio of 3:2) and class switching from naïve B cells to IgA secreting lymphocytes is regulated by Transforming Growth Factor-β (TGF-β) within MALT. IgA dimers are attached to J chains and are transported across the epithelium by transcytosis following binding to poly-Ig receptor at the base of the epithelium. IgA is the dominant secretory antibody involved in mucosal immunity and assist antigen presentation through microfold (M) cells in the gut lumen (van Wijk and Cheroutre, 2009).

Leukocytes within the mucosal epithelium are primarily lymphocytes and are termed Intra-Epithelial Lymphocytes (IEL). An abundance of IEL are found in the small bowel

(10-20 per 100 villus) and (unlike peripheral blood) are predominantly $CD8^+$ cells with one T cell for every 10 epithelial cells (Hayday et al., 2001, MacDonald et al., 2011). Two subtypes have been defined in mucosal tissues (Types a and b) (**Table 1.1**). Type a IEL are 'conventional' TCR α:β CD8 α:β cells. Type b IEL are distinct from the majority of T cells found in blood (Jarry et al., 1990) and are characterised by expression of unusual T Cell Receptors (TCR) such as TCR γ:δ CD8 α:α and TCR α:β CD8 α:α subsets. TCR γ:δ CD4 CD8 'double-negative' cells are also found at levels >10% in murine small intestine. These numbers appear to be higher in the large than the small intestine and may reflect a requirement in response to bacterial load.

Type A IEL	**Type B IEL**
TCRαβ+CD8αβ+	TCRαβ+ CD8αα+
	TCRγδ+ CD8αα+
	TCRγδ+ Double Negative (DN)

Table 1.1: **Intra-Epithelial lymphocyte (IEL) subsets**. Two groups have been identified including 'conventional' TCRαβ+CD8αβ+ cells (Type a IEL) and others, expressing less common TCRγδ, CD8αα or no CD4/CD8 markers (Type b IEL).

Type a IEL bind conventional MHC class I and II are thought to have a protective role including protection against Cytomegalovirus and *Toxoplasma* (Hayday et al., 2001). Type b IEL are distinct in that the TCR bind to unconventional MHC class 1B molecules and thymus leukaemia (TL) antigen. Type b IEL are thought to represent an interface between the innate and adaptive immune response and are implicated in the repair of damaged mucosa. TCR $\gamma\delta^{-/-}$ mice have heightened cutaneous

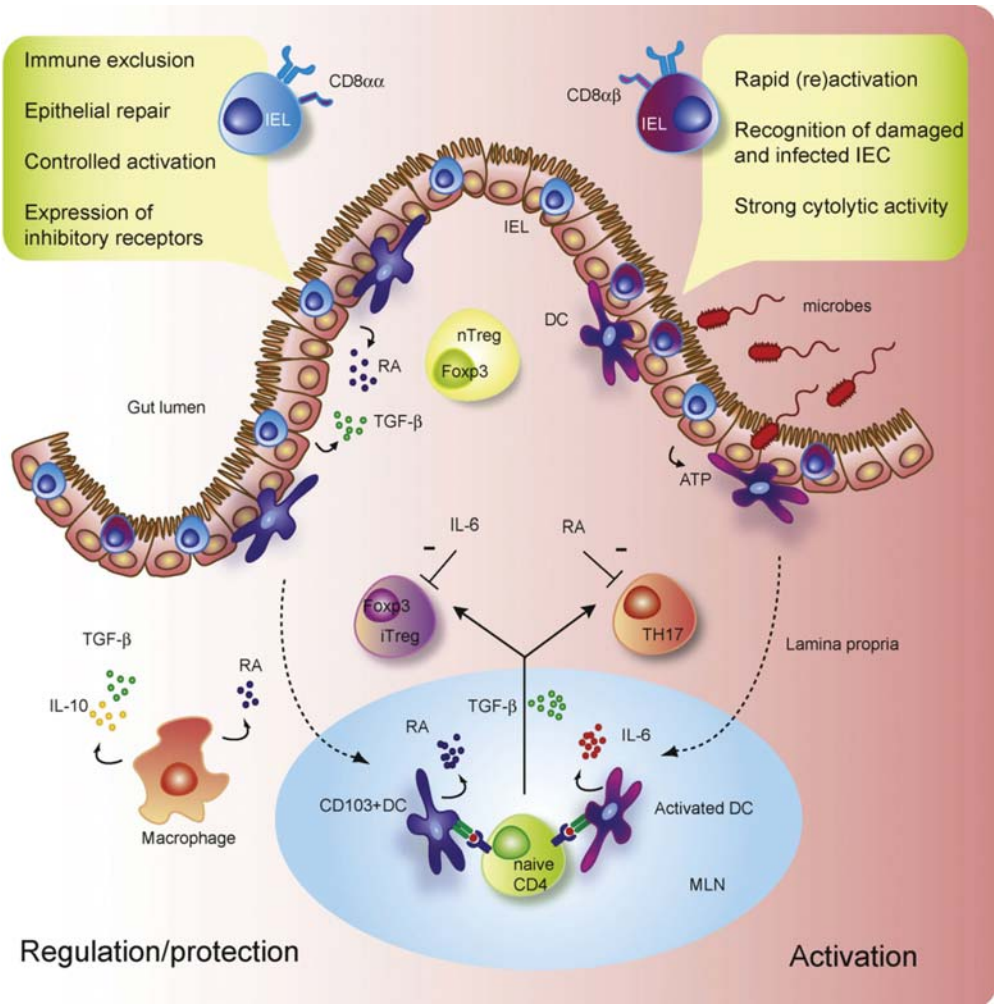
inflammation as a result of α:β T-cell responses suggesting a protective role in maintaining epithelial integrity (Hayday et al., 2001, O'Brien et al., 2009). It is thought that they act as activated Th-1 skewed effectors cells. IEL require activation by additional cytokines and in their resting state a number of anti-proliferative genes are expressed e.g. Btg1 and 2. Therefore additional steps are required *in vivo* to fully induce activation. This has been termed 'activated but resting' (Hayday et al., 2001).

At mucosal sites, repeated exposure to antigen may account for an altered role for epithelial CD8+ lymphocytes, some of which may be regulatory in nature (Koch et al., 2008, Xystrakis et al., 2004). A population of regulatory CD8+ cells have been identified as expressing the CD103 (α_E) integrin and these are thought have suppressive effects, mediated by IL-10 (Uss et al., 2006).

CD4+ cells are the more dominant T cell type in the underlying lamina propria. In the gut these have been identified as inflammatory Th17 and Th22 cells, counteracted by T^{REGs} and play an important role in the pathogenesis of inflammatory bowel diseases (van Wijk and Cheroutre, 2009), in particular T^{REGs} play an important role, mediated by IL-10, in preventing excessive inflammation at mucosal interfaces such as the gut and the lung (Rubtsov et al., 2008). IL-22 secreting cells play an important role in mucosal barrier function and disruption has been identified in inflammatory bowel diseases (Sonnenberg et al., 2011). Both Th17 and T regulatory cell differentiation requires TGF-β (produced by epithelial cells and CD103+ DCs) and an imbalance between inflammation and regulation between these cell population may contribute to autoimmune diseases (Horwitz et al., 2003, Stockinger et al., 2007, Coombes et al., 2007). Of interest, the mechanism may include the production of IL-17 by cells such

as monocytes, NK cells and γδ T-cells, which in turn promote an inflammatory infiltrate including neutrophils. Failure to resolve this may result in an increase in local TGF-β production resulting in the promotion of both Th17 an T^{REG} populations (Stockinger et al., 2007).

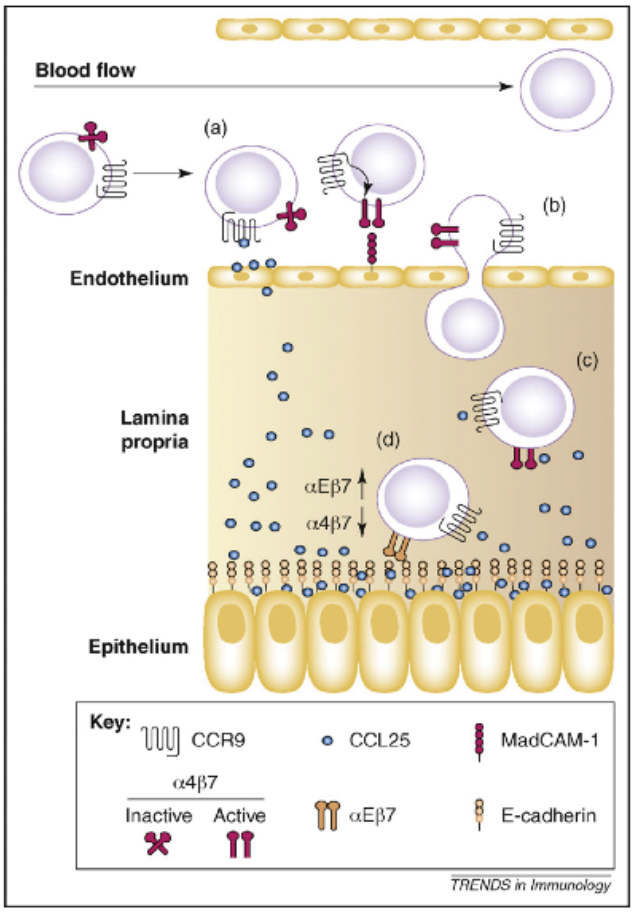
A summary of mucosal IEL in the gut is shown in **Figure 1.5.**



From Van Wijk *Seminars in Immunology* (van Wijk and Cheroutre, 2009)

Figure 1.5. Mucosal T cell regulation and activation. In the gut microenvironment, Type a IEL help destroy pathogens that have breached the epithelial barriers while Type b IEL such as CD8αα+ T cells play a role in repair and regulation. The epithelial cells (IECs) help induce Retinoic Acid production (RA) by DCs, which assist in gut-homing receptor induction by CD4+ T cells when primed in the mesenteric lymph nodes (MNs). CD103+ DCs under the influence of IL-10 and TGF-b induce FoxP3+ T REGs whereas activated DCs promote inflammatory Th17 lymphocytes via IL-6.

Circulating T cells are recruited to mucosal tissue such as the gut by expression of the integrin α_4:β_7 (which binds to MadCam-1 on lamina propria vascular endothelium) and the chemokine receptor CCR9 (**Figure 1.6**). A switch to α_E:β_7 integrin expression on these mucosal homing T cells directs lymphocytes within the lamina propria to bind to E-cadherin on epithelial cells (Cepek et al., 1994). This is mediated by the expression of TGF-β on epithelial cells.



From Agace 2008. *Trends Immunol (Agace, 2008)*

Figure 1.6: **T cell recruitment to the small intestine**. Expression of the chemokine receptor CCR9 and the integrin α_4:β_7 bind to vascular endothelial CCL25 and MadCam-1 respectively. Once recruited to the lamina propria, up regulation of α_E:β_7 takes place with T cell attraction to E-cadherin expressed on epithelial surfaces.

A hallmark of adaptive immunity is memory, or the ability to recognise previously encountered foreign peptides. Antigen experience, taking place in a mucosal draining lymph nodes or MALT results in an alteration in cell surface marker expression including a switch from a high (RA) to a low molecular weight isoform (RO) of the CD45 common leukocyte antigen marker (**Table 1.2**) (Akbar et al., 1988). Effector memory 'primed' cells are able to extravasate into peripheral mucosal tissues (Gupta et al., 2004). CD8+ CD103+ (α_E:β_7) mucosal cells have also been identified as being CD45RA- CCR7- (CCR7 being a lymph node homing marker). Local environmental education in mucosal lymphoid tissue has been shown to induce the expression of CCR9 and α_4:β_7 on T cell subsets although alternative routes of immunisation can also induce mucosal-homing such as subcutaneous yellow-fever vaccination in humans (Agace, 2008, Masopust et al., 2010).

Naïve	**Central Memory**	**Effector Memory**	**Effector Memory RA (Revertants)**
CD3+	CD3+	CD3+	CD3+
CD45RA+	CD45RA-	CD45RA-	CD45RA+
CD45RO-	CD45RO+	CD45RO+	CD45RO-
CD28+	CD28+	CD28-	CD28-
CCR7+	CCR7+	CCR7-	CCR7-
IL-2 expression	IL-2 expression	IL-2, IFN-γ, IL-4 or IL-5 expression	IL-2 expression +/- IFN-γ

Table 1.2: **T cell memory status**. T cell memory can be defined by the expression of numerous markers including high and low molecular weight isoforms of the Tyrosine Phosphatase CD45RO.

The majority of the tissue specific CD8+ populations are antigen experienced (CD45RO+). Our understanding of memory status is further defined by the loss of the lymph-node homing marker CCR7 as the cell is antigen primed and changes form a central to an effector memory phenotype (CCR7-CD45RA-). A significant proportion of CCR7-CD45RA+ 'revertant' CD8+ T cells are antigen experienced and are able to produce IFN-γ (Faint et al., 2001). This is thought to be driven by chronic viral infection such as Cytomegalovirus and in the periphery there is an accumulation of viral specific cells and CMV is described as the most immune-dominant peptide in humans (CMV) (Moss and Khan, 2004). Another herpes virus, Epstein Barr Virus (EBV) can also act as immunodominant peptide, long after the primary infection (Hislop et al., 2001).

1.3.3 Ocular Surface Immunity

Ocular surface immunity is regulated by the lacrimal functional unit and immune cells located on the ocular surface. Like other mucosal surfaces, the absence of a keratinised epithelium increases vulnerability to attack. This is particularly true of the optically clear cornea; even more susceptible because it is avascular (precluding rapid recruitment of cells as in other tissues) and has few lymphoid cells under normal conditions (Knop and Knop, 2007). The cornea therefore relies on components of the tear film and the surrounding conjunctiva for immunological protection (Knop and Knop, 2005b).

It has become clear that like other mucosal surfaces, the ocular surface has a form of MALT termed Eye Associated Lymphoid Tissue (EALT) (Knop and Knop, 2003, Knop

and Knop, 2005a, Knop and Knop, 2005b). These are found in the lamina propria of the conjunctiva and like the lacrimal gland contribute to the production of IgA to the tear film (Knop et al., 2008). Local antibody production by plasma cells is an important component of the lacrimal functional unit and the maintenance of a healthy ocular surface. As discussed in **section 1.2**, the tear film also contains soluble factor including cytokines such as IL-1, IL-6, IL-8, TNF-α and IL-10 and growth factors such as TGF-β and Epidermal Growth Factor (EGF) (Zierhut et al., 2002, Nakamura et al., 1998, Sonoda et al., 2006). TGF-β has been demonstrated in healthy tears (Gupta et al., 1996). Whether TGF-β is derived from the epithelium and plays a role in switching homing markers such as $\alpha_4{:}\beta_7$ to $\alpha_E{:}\beta_7$ or influences Th17 or T^{REG} induction is unknown.

The conjunctiva, like other mucosal surfaces, contains leukocyte populations including lymphoid cells called intra-epithelial lymphocytes (IEL) (Allansmith et al., 1978, Knop and Knop, 2007, Hayday et al., 2001, Knop and Knop, 2005b). Ocular surface immune cells are derived from the rich conjunctival vasculature and components of the tear film also contribute to ocular surface immune regulation.

Resident conjunctival cells identified in the normal human conjunctiva include abundant populations of CD3+ cells, primarily CD8+ cells in the epithelium (with a CD4:CD8 ratio of 0.3) and CD4+ in the lamina propria (CD4:CD8 ratio 1.3) (Sacks et al., 1986, Hingorani et al., 1997). The majority of epithelial T cells are CD45RO+ or 'antigen experienced'. Few TCR$\gamma\delta$ cells, B or plasma cells have been identified in the epithelium but a population of CD20+ (B cells) and plasma cells can be found in the lamina propria. In non-inflammatory conditions, populations of macrophages and

dendritic cells are abundant in the epithelium and lamina propria. Natural Killer cells and neutrophils are scarce and are confined to the lamina propria (Knop and Knop, 2007).

Our knowledge of the role of IEL within the conjunctiva is currently limited. It is unclear which subtypes (Type a or b) exist, what their precise memory status is, what cytokines they produce and whether there is any indication of regulatory properties. Mucosal IEL CD8+ recognition of powerful immune-dominant peptides such as CMV in the conjunctiva is undetermined. The human conjunctiva is dominated by bacterial commensals such as *Staphylococcus*, *Streptococci*, *Moraxella* and *Diphtheroids* (Forrester, 2008). Acute bacterial conjunctivitis in adults is largely dominated by the same pathogens in the immunocompetent, while viral conjunctivitis largely relates to adenovirus, molluscum contagiosum and Herpes Simplex Type 1 (Denniston and Murray, 2009, Hovding, 2008). Herpes Simplex has far greater morbidity as a cause of keratitis rather than conjunctivitis and like other herpes viruses, CD8+ T cells play an important role in maintaining latency of the virus in the trigeminal ganglion (Divito et al., 2006).

The gut has been described as having continuous low-grade inflammation (MacDonald et al., 2011). It may therefore be the case that the phenotype of resident IEL on the ocular surface are different to other mucosal sites, not least because of their different antigen exposure. It is known that in dry eye disease, there is an elevation in conjunctival IFN-γ and IL-17 producing T cells, and there appears to be a defective suppressor function by T regulatory cells on Th17 cells (Chauhan et al., 2009). The effector function, memory status and antigen recognition of conjunctival T

cells in health, in particular the CD8+ population, therefore warrants further investigation.

1.4 Ageing changes and the immune system

The implications of ageing changes on the immune system has attracted attention as a greater understanding of immunosenescence is gained (Haynes and Maue, 2009). The immune system alters with age and an accumulation of memory cells is observed including an increased frequency of CD45RO+/RA- T cells (Utsuyama M, 2009) and in keeping with the strongly immune-dominant effects of CMV discussed, a greater proportion of the T cell repertoire is committed to recognising this virus with increasing age (Khan et al., 2004). Furthermore, a process of thymic involution is thought to contribute to an exhaustion of naïve T cells, and this has been demonstrated in thymectomised young people undergoing cardiac surgery (Sauce et al., 2009).

The innate immune system can also undergo changes with age. Neutrophils for example, maintain their numbers with increasing age but there is evidence of a diminished phagocytic capacity (Shaw et al., 2010). Natural killer cells are believed to have a diminished function with increasing age (Shaw et al., 2010, Panda et al., 2009). Of interest, is the observation that the severity of ocular surface infections such as microbial or herpetic keratitis is clinically worse in the elderly (van der Meulen et al., 2008) and Mucous Membrane Pemphigoid (Foster, 1986, Chan, 2001) typically affect people in later life. Some studies have demonstrated an increased prevalence of dry eye problems with age (McCarty et al., 1998, Moss et al., 2000,

Stern et al., 2010). Despite this, little is known about age-related changes in the leukocyte populations within the ocular surface (Gwynn et al., 1993).

Changes in major peripheral blood populations are demonstrated in **Table 1.3**.

Cell population	**Age-related changes**	
	Males	**Females**
Leukocytes numbers	No change	No change
Lymphocytes numbers	No change	No change
Monocyte numbers	No change	No change
Neutrophil numbers	No change	No change
T cell numbers	No change	No change
CD8+ numbers	Decrease	Decrease
CD4+ numbers	No change	No change
CD45RO+ cells	Increased trend	Increase
CD4:CD8 ratio	Increase	Increase
B cells	Decrease	Decreased trend
NK cells	Increased trend	Increase

Adapted from Utsuyama. Handbook of immunosenescence (Utsuyama M, 2009)

Table 1.3 Major leukocyte population changes in peripheral blood with age.

1.5 Cicatricial Conjunctivitis

A delicate balance of the lacrimal functional unit exists on the ocular surface that can be disrupted by a number of processes including dry eye states and conjunctivitis. Conjunctivitis (inflammation of the conjunctiva) may arise from a number of insults including infection, trauma (mechanical, chemical or thermal injuries), oculocutaneous diseases (including immunobullous disease and atopic kerato-conjunctivitis), other systemic diseases, drug reactions and topical therapy such as the use of anti-glaucoma medication (**Table 1.4**) (Bernauer et al., 1997). These triggers can lead to a process of scarring termed cicatrisation.

The cicatricial conjunctivitides are a group of diseases usually characterised by scar formation (cicatrisation) in response to conjunctival inflammation (**Table 1.4**). Chronic inflammation causes disruption to the delicate micro-environment of the ocular surface (Zierhut et al., 2002) (Pflugfelder and Stern, 2009). Importantly, some of these conditions have the capacity to progress, as the stimulus for inflammation is not temporary or potentially removable e.g. a chemical injury or infection. They are clinically important as they may contribute to progressive cicatricial conjunctivitis (PCC), characterised by chronic progressive scar formation (fibrosis) in response to conjunctival inflammation affecting the deeper layers of the conjunctiva. Some of these are mediated by anti-basement membrane zone (BMZ) antibodies (Bernauer et al., 1997).

Aetiology		Progressive Scarring	Immunobullous Disease
Physical:			
	Heat		
	Ionising radiation		
	Chemical		
Infection:			
	Trachoma		
	Membranous conjunctivitis (e.g. Streptococcus and Adenovirus)		
Oculocutaneous disorders:			
	Mucous Membrane Pemphigoid (MMP)	Yes	Yes
	Bullous Pemphigoid (BP)	Yes	Yes
	Linear IgA disease	Yes	Yes
	Dermatitis herpetiformis		Yes
	Pemphigus	Yes	Yes
	Systemic Lupus Erythematosis		
	Epidermolysis bullosa (EB)	Yes	Yes
	Stevens-Johnson Syndrome (SJS) and Toxic Epidermal Necrolysis (TEN)	Yes +	Yes +
	Lichen planus		
	Chronic atopic keratoconjunctivitis		
Other associated systemic disorders:			
	Rosacea		
	Sjögren's Syndrome		
	Inflammatory bowel disease		
	Graft-versus-host disease		
	Immune complex diseases		
	Paraneoplastic syndromes	Yes	
	Drug-induced	Yes	
	Sarcoid		
	Porphyria		
Topical (pseudopemphigoid)	Antiglaucoma medication		

+ A subset may develop autoantibody-positive progressive scarring akin to MMP

Adapted from Wright 1986 (Wright, 1986) and Bernauer et al 1997 (Bernauer et al., 1997)

Table 1.4: **Summary table of causes of cicatrising conjunctivitis**.

PCC causes severe damage to the ocular surface through destruction of the conjunctival goblet cells, scarring of the lacrimal and accessory glands and lid margin disorganisation and disruption of the oily component of the tear film. This in turn leads to ocular surface inflammation (Baudouin, 2001), keratinisation and limbal stem cell destruction. Parallel to these alterations, the conjunctival fibrosis results in obliteration of the conjunctival fornices and the scarring process results in entropion formation (eyelid deformity) and lash trauma to the ocular surface, exacerbating the inflammatory process (Bernauer et al., 1997, Wright, 1986). This results in ocular surface destruction, notably limbal stem cell loss with resultant opacification and neovascularisation of the optically clear corneal surface, through a process of conjunctivalisation and/or keratinisation. This epithelium is vulnerable to breakdown, which in turn leads to a higher risk of infective conjunctivitis and keratitis, promoting a vicious cycle resulting in eventual blindness.

Dry eye is a dominant feature of cicatrising conjunctivitis. Dry eye has been described as a '*multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface. It is accompanied by increased osmolarity of the tear film and inflammation of the ocular surface*' (2007). Although dry eye symptoms are relatively common, pathological causes of dry eye (synonymous with keratoconjunctivitis sicca) are multiple and include age-related changes discussed previously (2007). A relatively common cause of dry eye problems is Sjögren's syndrome, an autoimmune condition characterised also by dry mouth (Ramos-Casals et al., 2010b). In the absence of a related autoimmune condition it is termed Primary Sjögren's Syndrome (PSS), its prevalence is 0-5-3% and includes an increase risk of

lymphoma (Ramos-Casals et al., 2010b, Kassan and Moutsopoulos, 2004, Helmick et al., 2008).

Although the initiating antigen is undetermined, Anti-Ro and La antibodies are directed against peptides within the lacrimal glands, resulting in a CD4+ T-cell and B-cell infiltration of the gland (Stern et al., 2010, Pflugfelder et al., 2009). This causes a reduction of the aqueous tear component and hyperosmotic inflammatory ocular surface changes, with consequent conjunctival goblet cell loss and squamous metaplasia. Murine models of experimental dry eye, based on a desiccating stress environment, have revealed the importance of antigen presenting cells in initiating and maintaining the recruitment of auto reactive T cells (Schaumburg et al., 2011). A CD4+ infiltrate has been recognised in the conjunctiva of patients with PSS (Stern et al., 2002), and evidence points to the importance of the role of Th17 subsets in the pathogenesis of the disease with IL-17 detected in the tears of patients with PSS (De Paiva et al., 2009).

Some of the diseases outlined in **Table 1.4** have an underlying immunobullous component characterised by autoimmune mediated blistering of cutaneous surfaces including the conjunctiva. Although the principal ocular problems in PSS are dry eye there is a recognised cicatricial element. With the exception of Paraneoplastic and Drug-Induced causes of cicatricial conjunctivitis however, PSS is considered a slowly progressive cicatricial conjunctivitis (Bernauer et al., 1997), unlike many of the acquired ocular immunobullous diseases.

1.6 Acquired Ocular Immunobullous Disease

Acquired immunobullous diseases that have potential ocular involvement include Mucous Membrane (formerly Ocular Cicatricial) Pemphigoid (MMP), Bullous Pemphigoid (BP), linear IgA disease, dermatitis herpetiformis, Pemphigus and bullous systemic lupus erythematous (Wong and Chua, 2002). Their annual incidence is thought to be 10.4 per million in Western populations (Bernard et al., 1995). MMP is the most common of the true autoimmune diseases in this group with an incidence study in France demonstrating it was the most common to affect the ocular surface (Bernard et al., 1995, Wojnarowska and Briggaman, 1990).

Epidermolysis bullosa is an inherited immunobullous disease. Reaction patterns, including exogenous triggers in the case of Stevens-Johnson Syndrome (SJS) and its severe immediate manifestation Toxic Epidermal Necrolysis (TEN) also have the potential to cause mucocutaneous blistering including PCC (Wojnarowska and Briggaman, 1990). Historically SJS-TEN were thought to be temporal and therefore failed to progress once the initial acute phase had resolved. Evidence has demonstrated that a significant proportion can go on to develop severe ocular sequelae after the systemic disease has resolved (Foster et al., 1988, De Rojas et al., 2007). Furthermore, this may result in the development of an MMP phenotype, with indistinguishable clinical and immunohistological features to MMP (Chan et al., 1991).

Although these diseases have the potential to cause PCC, with the exception of SJS-TEN, ocular involvement is relatively uncommon. The British Ophthalmological Surveillance Unit (BOSU) was established to determine the incidence of rare

diseases in the United Kingdom (Stanford, 1997). A recent study attempted to determine the incidence of cicatrising conjunctivitis in the United Kingdom (Radford *et al*. The Incidence of Cicatrising Conjunctival Disorders in the UK. IOVS 2011; 52: E-Abstract 1136). The overall incidence was found to be 1.3 per million with Mucous Membrane Pemphigoid confirmed as the commonest cause of PCC (0.8/million), followed by SJS-TEN (0.2/million). Other causes had a combined incidence of 0.2/million and included primarily immunobullous disorders such as Linear IgA disease, drug toxicity and ocular surface squamous neoplasia but also oculocutaneous disorders such as lichen planus and atopic conjunctivitis and other associated conditions including ocular rosacea, graft versus host disease, Adenovirus and PSS.

1.7 Stevens-Johnson Syndrome and Toxic Epidermal Necrolysis

Stevens-Johnson Syndrome (SJS) and Toxic Epidermal Necrolysis (TEN) are reaction patterns that have the potential to cause a life-threatening mucocutaneous blistering disease (Wojnarowska and Briggaman, 1990, Bastuji-Garin et al., 1993). TEN is a severe manifestation of SJS (Schopf et al., 1991, Bastuji-Garin et al., 1993) which can be classified systemically by scales such as the SCORTEN (Bastuji-Garin et al., 2000). It is a devastating disorder characterised by sloughing of epithelial surfaces associated with a mortality rate of up to 90% (Schopf et al., 1991) that can be predicted by the severity of SCORTEN (Bastuji-Garin et al., 2000). Major systemic problems simulate severe burns injury and include severe dehydration, renal failure and secondary infection.

The underlying aetiology is usually attributed to pharmacological agents including antibiotics (in particular sulphonamides but also penicillin, cephalosporins and tetracyclines), Non-Steroidal Anti-Inflammatories (NSAIDs) and anticonvulsants and less commonly to infections such as *Mycoplasma*, although the exact cause often remains unknown (Roujeau et al., 1995, French, 2006, Pereira et al., 2007). Drugs can activate T cells by behaving as haptens, fusing endogenous proteins following metabolic breakdown to form antigens, or by direct interaction with the MHC molecule and TCR, resulting in activation of the T cell (Pereira et al., 2007).

Ocular complications include severe dry eye, ocular surface epithelial breakdown associated with sloughing of the muco-cutaneous junction, and infection. Long-term sequelae are related to conjunctival scarring and subsequent ocular surface failure that may persist and progress after resolution of systemic disease (Foster et al., 1988, De Rojas et al., 2007). While little is known about the ocular surface inflammatory process, keratinocyte death in cutaneous SJS-TEN is induced by blister fluid granulysin secreted by CD8+ cytotoxic T cells and CD56+ Natural Killer cells (Chung et al., 2008). There is evidence for the presence of CD8+ cytotoxic T cells in the cornea during the acute stages of TEN, (Williams et al., 2007) but detailed knowledge of the cellular profile during the acute and chronic stages of the disease in the conjunctival mucosa is unknown. Furthermore, how acute ocular SJS-TEN converts to a chronic condition in some patients remains unknown.

The distinction between acute and chronic ocular disease is not clear. Although the systemic disease can be expected to resolve within days or weeks, there may be a variable ocular picture. A period of 2-6 weeks has been suggested as acute ocular

SJS-TEN while chronic disease has been defined as greater than 12 months (Power et al., 1995, Sotozono et al., 2007, Fu et al., 2010). There is no agreed definition of acute and chronic however and these definitions leaves a large 'sub-acute period' between 6 and 52 weeks.

Predicting ocular surface outcome in disease is difficult although an estimate of mortality may be predicted by utilising either the scoring system SCORTEN (Bastuji-Garin et al., 2000) or elevated serum granulysin for those with early drug-induced skin reactions (Fujita et al., 2011). Although conjunctival epithelial HLA-DR has been suggested as a biomarker for dry eye (Baudouin et al., 2002), no biomarker exists for the ocular surface in SJS-TEN and SCORTEN has been shown not to predict ocular sequelae (Morales et al., 2010).

The approach to managing systemic disease has classically been supportive and approaches including the use of intravenous immunoglobulin (ivIg) and systemic steroids have been controversial because of fears regarding systemic infection (Halebian and Shires, 1989), although recent data has indicated a role in improving visual outcome (Araki et al., 2009) and early, aggressive intervention by ophthalmologists may help prevent blinding sequelae of the disease (Fu et al., 2010). Intensive topical lubrication, steroids and autologous serum drops may all play a role in the management of dry eye and ocular surface inflammation, (Aragona et al., 2002, Noble et al., 2004, Sotozono et al., 2009) but critically early use of amniotic membrane transplantation for acute SJS-TEN has been advocated to minimise ocular morbidity (John et al., 2002, Shay et al., 2010, Gregory, 2011).

1.8 Mucous Membrane Pemphigoid (MMP)

1.8.1 Aetiology and Pathogenesis of MMP

The first international consensus statement defines MMP as "*a group of putative autoimmune, chronic inflammatory, subepithelial blistering diseases predominantly affecting the mucous membranes that is characterized by linear deposition of IgG, IgA, or C3 along the epithelial basement zone (BMZ)*" (Chan et al., 2002).

The aetiology of OcMMP remains unclear but it is a Type II hypersensitivity disorder. Genetically susceptible individuals, exposed to an unknown environmental trigger, develop autoantibodies that are directed against the basement membrane zone (BMZ). In turn this leads to the deposition of complement and an inflammatory infiltrate leading to fibrosis (cicatrisation) along the BMZ (**Figure 1.7**).

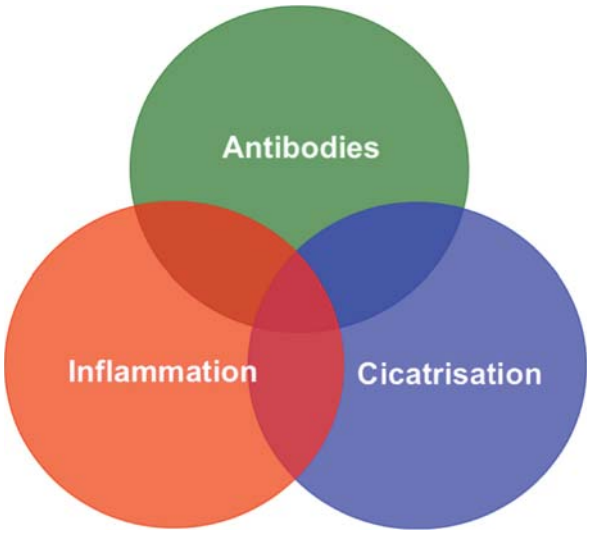


Figure 1.7: **Venn diagram showing the overlap between autoimmunity, inflammation and fibrosis (cicatrisation).**

1.8.2 Antibodies in MMP

The precipitant for anti-BMZ antibodies is currently unknown although the HLA-DQB1*0301 allele is more common in MMP patients, and has been linked to T cell recognition of BMZ antigens, resulting in B cells producing BMZ-specific auto-antibodies (Setterfield et al., 2001, Zakka et al., 2011). This association was also found in association with Bullous Pemphigoid (BP) (Delgado et al 1996). A linear deposition of anti-BMZ antibodies is seen in immunofluorescent studies (**Figure 1.8**).

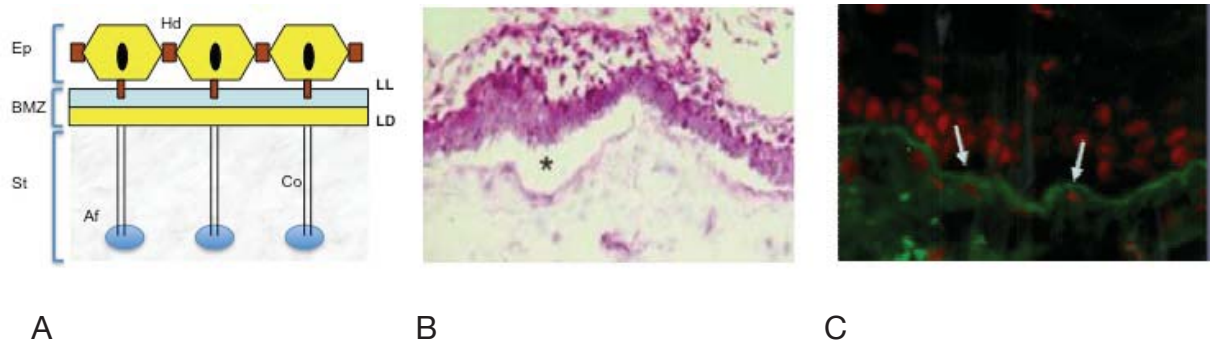


Figure 1.8: **Immunofluorescence in MMP**. Panel A is a schematic representation of the conjunctival epithelium (Ep); Basement Membrane Zone (BMZ) including its lamina lucida (LL) and lamina densa (LD); and the Stroma (St). Desmosomes (Dm) hold epithelial cells together and hemidesmosomes (Hd) anchor the epithelial cells to the underlying matrix. The BMZ is anchored to the underlying stroma by collagen fibres (Co) and anchoring filaments (Af). Panels B and C show sequential histological sections demonstrating subepithelial blistering (*****) of the conjunctival BMZ and linear staining by anti-BMZ antibodies (arrowed) on direct immunofluorescence (DIF).

Numerous BMZ antigens have been identified as targets for immunoglobulins in MMP (Challacombe et al., 2001, Chan et al., 2002) (**Figure 1.3, Table 1.5**). The principal targets associated with MMP are β4 (Zakka et al., 2011) and BP180 within the hemidesmosomes but the major disruption has been identified as taking place in the lamina lucida (Challacombe et al., 2001, Wozniak and Kowalewski, 2005). The passive transfer of anti-mouse Laminin 5 antibodies (found in up to 5% of patients with MMP) raised in rabbits induces murine subepidermal blisters in the neonatal

period (Lazarova et al., 1996). Furthermore, this results in increased circulating anti-BMZ antibodies in BALB/c mice, independent of C3 complement (another hallmark of the disease) (Lazarova et al., 2001). The results of these studies should be interpreted with some caution in the context of ocular disease however, as the primary outcome was evidence of dermal blistering, a minor feature in human disease, and no clinical evidence of ocular pathology was assessed. Indeed, while microscopic evidence of blistering takes place this is not seen at a clinical level.

Antigen/Epitope	Location and Characteristic
Epiligrin (subunit of Laminin 5)	Ligand for $\alpha 6\beta 4$ integrin
Bullous Pemphigoid Antigen 1 (BP1) or BP230	Role in hemidesmosomal assembly and formation. Located in inner plate of hemidesmosomes (Hd)
Bullous Pemphigoid Antigen 2 (BP2) or BP180	Transmembrane molecule and part of Hd anchoring filament complex
$\alpha 6$ integrin	Heterodimeric molecules associated with Hd
$\beta 4$ integrin	Heterodimeric molecules associated with Hd
Laminin 5	Adhesion molecule associated with anchoring filaments in the lamina lucida and densa
Collagen VII	Fibro-reticularis
45kDa protein	Implicated in pure ocular disease (identity unclear)
168kDa protein	Implicated in pure oral disease (identity unclear)

From (Challacombe et al., 2001, Chan et al., 2002)

Table 1.5: **BMZ antigens/epitopes targeted in MMP**

A perilesional biopsy of affected tissue (e.g. conjunctiva or oral mucosa) for Direct Immunofluorescent (DIF) evidence of linear IgG, IgA or C3 along the BMZ supports clinical evidence of MMP. IgG1 and IgG4 have been identified as the dominant isotypes, the former associated with complement deposition (Bernard et al., 1991). Histological exclusion of malignancy is required specifically for similar cases before making the diagnosis. The Consensus statement declared that immunofluorescent evidence of anti-BMZ antibodies was a mandatory requirement for the diagnosis of

MMP. However, there is a large subset (14-40%) in whom DIF is negative (Thorne et al., 2004, Bernauer et al., 1994), these patients have an identical clinical disease phenotype and progression pattern, to those patients who are DIF positive (Saw, 2008). Failure to recognise the existence of this subset can result in delays in diagnosis and treatment in those who potentially have severe blinding disease. Furthermore, the linear deposition along the BMZ by antibodies (**Figure 1.8**) is also seen in BP, linear IgA disease and Epidermolysis Bullosa (EB) (Chan et al., 2002)

Indirect Immunofluorescence (IIF) can also be employed to detect circulating anti-BMZ antibodies (Ab) in MMP. Although there is evidence of circulating anti-BMZ Ab binding to (healthy) conjunctiva correlating to conjunctival DIF in ocMMP (Leonard et al., 1988), difficulty in handling and lack of availability of local tissue confines substrate for IIF to skin or monkey oesophagus (Wojnarowska and Briggaman, 1990). Sensitivity is increased however by exposure of antigens in the basement membrane by splitting skin tissue with salt (Woodley et al., 1983).

Although circulating anti-BMZ Ab correlate with disease activity (Setterfield et al., 1999), detection by IIF using salt-split skin can be insensitive and as low as 50% (Chan, 2001). Furthermore, the technique is not tissue specific and there is no correlation between circulating antibodies and prognosis (Chan et al., 2002). There are also some ocular patients who are IIF positive and DIF negative, indicating that a more sensitive IIF test could not only provide an improved diagnostic tool for MMP, but also inform the Consensus criteria where IIF is not an absolute requirement (Chan et al., 2002).

1.8.3 Inflammation in MMP

The inflammatory process in OcMMP is manifest clinically as conjunctival inflammation. The detailed cellular infiltrate and inflammatory microenvironment in MMP, particularly in clinically involved and seemingly uninvolved mucous membranes is not fully understood and little progress has taken place in recent years (Bernauer et al., 1993a, Rice and Foster, 1990, Sacks et al., 1989). Although numerous *in vitro* and *ex vivo* studies have been undertaken, a major limitation is access to tissue due to the possibility of precipitating or aggravating disease (Chan et al., 2002). Material is often restricted to surplus conjunctiva from diagnostic biopsies and cellular phenotyping and quantification has been limited to histological sections with immunohistochemistry.

In the presence of clinically defined minimal inflammation, there is evidence for a predominantly T cell infiltration of the conjunctival stroma (with a CD4:CD8 ratio of 0.5) (Bernauer et al., 1993a, Rice and Foster, 1990). In severe conjunctival inflammation however there is a 3-fold increase in neutrophils and dendritic cells compared to controls, a 2-fold increase in macrophages and an altered CD4:CD8 ratio to 1.0 (Bernauer et al., 1993a, Rice and Foster, 1990).

Neutrophils were found to be the dominant infiltrate in the conjunctival epithelium in OcMMP compared to healthy controls. Furthermore, the neutrophil infiltrate increased with the degree of conjunctival inflammation with fewer cells seen in chronic, uninflamed eyes, but more than in healthy participants (Bernauer et al., 1993a). Another smaller study also demonstrated the presence of neutrophils in clinically white eyes (n=5/6) (Rice and Foster, 1990).

As outlined in **section 1.3.1**, neutrophils normally exert an immediate innate response. Neutrophil removal relies, among other factors, on apoptosis and other phagocytic cells such as macrophages (Bratton and Henson, 2011). Given the short life span of neutrophils, a process is required to maintain turnover albeit at a lower level in more chronic states. In other chronic inflammatory diseases, such as the joint in Rheumatoid Arthritis, a persistent infiltrate with neutrophils is evident (Wright et al., 2010). Autoantibodies are able to activate the classical complement cascade, which in turn recruit neutrophils.

Activated neutrophils not only destroy pathogens via their azurophilic granules but also have the potential to cause collateral tissue damage. They also have a role in modulating immune response and contribute to an alteration in their own removal through apoptosis, but also release pro-inflammatory cytokines such as TNF-α, promoting an inflammatory cellular infiltrate (Witko-Sarsat et al., 2011). Like many auto-inflammatory and autoimmune diseases though, the trigger in OcMMP has not been established. It is possible that the presence of a persistent inflammatory infiltrate therefore contributes to the on-going damage seen in OcMMP.

Our understanding of the site of production and role of inflammatory cytokines in the pathogenesis is also incomplete. Elevation of IL-1, Th_1 cytokines such as IL-2, Th_2 cytokines such as IL-4 and IL-5, inflammatory cytokines such as TNFα and a reduction in IL-6 have been demonstrated (Bernauer et al., 1993b, Lee et al., 1993, Caproni et al., 2003, Letko et al., 2002, Saw et al., 2009a) (**Figure 1.9**) but data has been confined to circulating serum or tissue sections (Bernauer et al., 1993b, Razzaque et al., 2004, Razzaque et al., 2003a). Longitudinal research has therefore

been extremely challenging. This has been compounded by a paucity of animal models. In order to quantify inflammation in humans, alternative non- invasive tissue assessment is required.

1.8.4 Cicatrisation in MMP

Inflammation results in the deposition of sub-conjunctival matrix collagen and progressive cicatrisation. The mechanism underlying this process remains unresolved (Elder et al., 1997, Razzaque et al., 2003b). Pro-fibrotic cytokines involved in wound healing such as TGF-β, Connective Tissue Growth Factor (CTGF) and Platelet Derived Growth Factor (PDGF) are elevated in MMP (Bernauer et al., 1993b). TGF-β's role as a growth factor, stimulating collagen deposition by fibroblasts, is thought to be maximal during the acute stages of diseases. Furthermore TGF-β mRNA is up regulated in conjunctival fibroblasts during acute inflammation in MMP (Elder, 1997b).

There is also an increase in Heat Shock Protein 47 (HSP 47), a cytokine implicated in fibrogenesis and increase in Macrophage Migration Inhibitory Factor (MIF) (Razzaque et al., 2003a, Razzaque et al., 2004). More recently IL-13 has been demonstrated as a pro-fibrotic cytokine found in the conjunctiva of individuals with OcMMP. Produced by T cells, it has been demonstrated to induce collagen contraction and the authors suggest that this cytokine may contribute to cross-talk with fibroblast by up-regulation of co-stimulatory molecules CD80, CD40, and CD154 on fibroblasts in culture (Saw et al., 2009b).

As yet the pro-fibrotic cytokines exact role in the maintenance or resolution of the condition are unknown. If the scarring or fibrotic process is maximal during acute inflammation, it is not surprising that historically it was thought that only a small group of patients demonstrated progression of disease in an apparently quiescent eye (Elder, 1997b). Other studies have shown however that while progression can occur in 50% of patients in the presence of mild or moderate inflammation, 50% progressed without clinically apparent conjunctival inflammation (Saw et al., 2008). This has been termed ‘white inflammation’ (Elder, 1997b).

Indeed, the cellular studies undertaken previously have shown a cellular infiltrate in the presence of minimal inflammation (Bernauer et al., 1993a, Sacks et al., 1989). Does this suggest that clinically identifiable conjunctival inflammation does not correlate with true underlying inflammation? There is evidence from other diseases that neutrophils may play a role in the fibrotic process, including pulmonary fibrosis and cryptogenic fibrosing alveolitis (CFA) (Chua et al., 2007, Hara et al., 2009, Obayashi et al., 1997).

Understanding of the pathogenesis of OcMMP is currently incomplete. A summary diagram based on current understanding is shown in **Figure 1.9.**

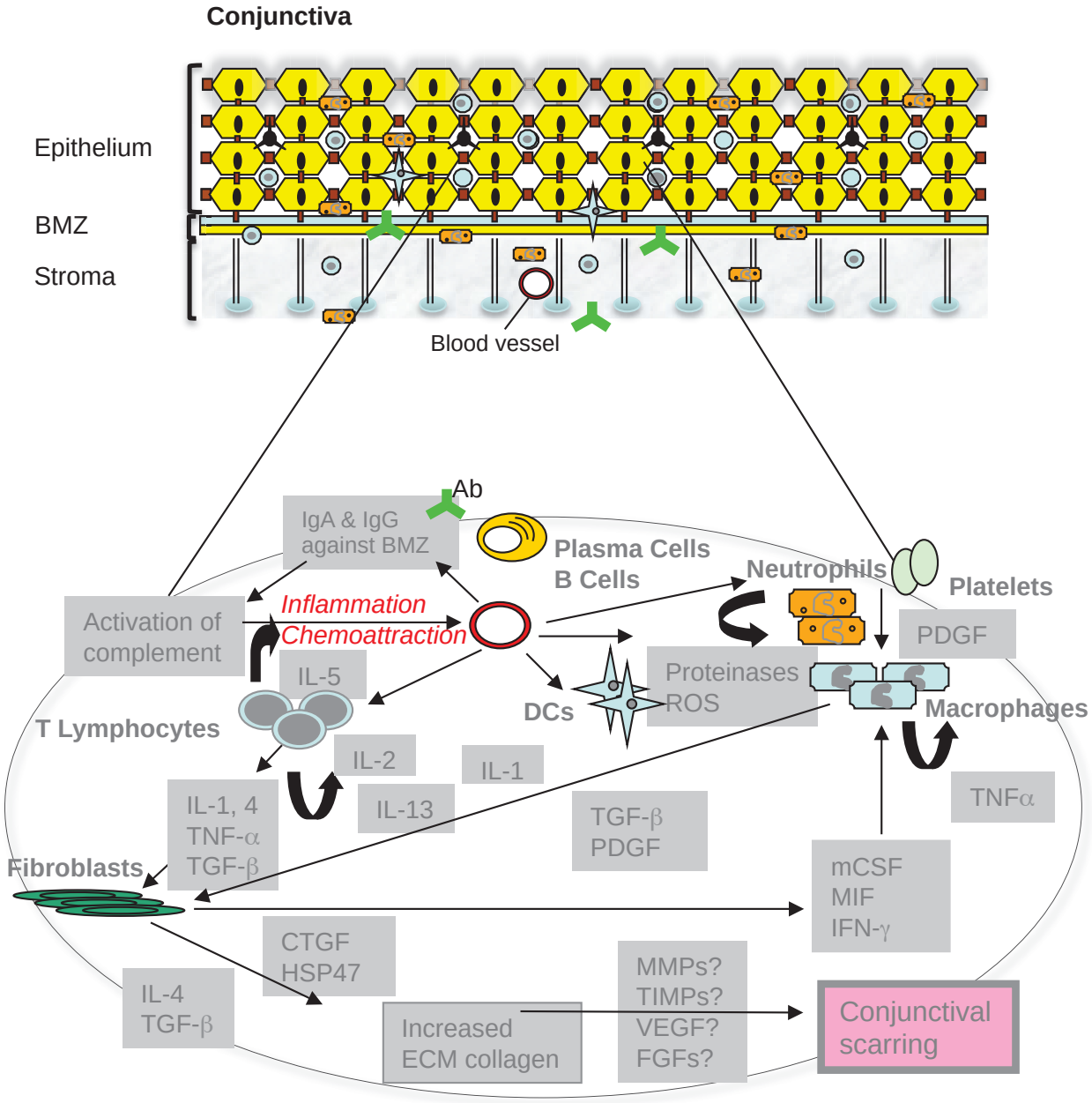


Figure 1.9: **Summary of understanding of the pathogenesis of ocMMP.** This cartoon illustrates how anti-BMZ antibodies (Ab) are directed against the conjunctival BMZ. This leads to inflammation and chemoattraction including the recruitment of neutrophils (which may release proteinases and reactive oxygen species (ROS), macrophages, platelets, dendritic cells and lymphocytes (B cells and T cells although the phenotypes are poorly defined) and plasma cells. An increased expression of cytokines IL-1, IL-2, IL-4, IL-5 and TNFα are thought to increase the inflammatory response, recruit T and B lymphocytes and activate macrophages and fibroblasts. An elevation of pro-fibrotic Transforming Growth Factor-β (TGF-β), Connective Tissue Growth Factor (CTGF), IL-13 and Platelet Derived Growth Factor (PDGF) contribute to the deposition of extracellular matrix (ECM) collagen and scarring. Heat Shock Protein 47 (HSP47) and Macrophage Inhibitory Factor (MIF) have also been implicated in the fibrotic process, while IL-13 derived from T-cells induce collagen contraction and potential fibroblast-T cell cross-talk. The roles of Matrix Metalloproteinases (MMPs), their inhibitors (TIMPs), Vascular Endothelial Growth Factor (VEGF) and Fibroblast Growth Factors (FGFs) have yet to be defined.

1.8.5 Clinical Features and Challenges in MMP

Although the skin is involved in MMP, this is primarily a disease affecting the mucous membranes with only a mild cutaneous phenotype. The oral mucosa is the most commonly affected site and the nasal, pharyngeal, anogenital, laryngeal and oesophageal surfaces are affected in descending order. Involvement of the oesophagus and airways are potentially life threatening and are therefore defined as 'high risk' (Chan et al., 2002). Whereas symptoms of disease activity are common in oral disease, the scarring sequelae seen in other tissues are not (Higgins et al., 2006) (Mobini et al., 1998). Development of ocular disease in those with pure oral involvement can occur at a later date and in one series 37% of patients developed both ocular and oral disease (Higgins et al., 2006).

The ocular (conjunctival) surface is the second most common site to be involved. The ocular phenotype of MMP (OcMMP) is a bilateral sight-threatening disorder characterised by clinical conjunctival inflammation, progressive conjunctival fibrosis (cicatrisation, manifest in part as adhesions called symblephara) and eventual surface failure sequelae including corneal vascularisation and scarring. This process leads to destruction of goblet cells resulting in severe dry eyes and cicatricial entropion causing mechanical abrasion of the cornea. Ocular features are therefore regarded as "high risk" (Chan et al., 2002).

MMP usually presents between 30 and 90 years of age, with a peak age of onset after 70 years (Foster, 1986, Mondino and Brown, 1981, Chan et al., 2002) with disease progression being more aggressive in younger patients (Rauz et al., 2005a).

The disease is also associated with other autoimmune diseases such as Rheumatoid Arthritis. Symptoms of disease in the early stages are those of non-specific chronic conjunctivitis and may include conjunctival irritation, hyperaemia and discharge (Wright, 1986). The course of ocular disease is variable and determining disease activity and progression represents a major challenge (Mondino and Brown, 1983). Patients are often diagnosed at an advanced stage of disease when signs are abundant (Miserocchi et al., 2002, Rogers et al., 1982) but the duration of symptoms at presentation is variable, ranging from 1 month to 25 years (Rauz et al., 2005a, Thorne et al., 2004, Thorne et al., 2008, Saw et al., 2008). There is also evidence that disease progression is more rapid in the context of manifest conjunctival inflammation (Mondino et al., 1979).

In the United Kingdom (UK) streams of referral are to tertiary services specialising in the management of OcMMP from other ophthalmologists, other specialists caring for patients with MMP or directly from the primary care service such as general (family) practitioners and optometrists. There is limited information regarding the differences in duration of symptoms, disease activity, stage and progression in those who present to specialised services.

As in SJS-TEN, the distinction between acute and chronic disease has not been fully established. Bernauer and colleagues suggested that acute disease was less than 12 months duration (Bernauer et al., 1993a) although the actual duration of symptoms before medical intervention may actually be far longer than this, not least because of the non-specific nature of symptoms.

1.8.6 Clinical Assessment of Disease Activity in OcMMP

At present, disease activity is based upon the extent of clinically identifiable conjunctival inflammation (1) absent, (2) mild, (3) moderate, (4) severe (which includes inflammation in all 4 quadrants, limbitis and conjunctival ulceration) (Elder, 1997c) (**Figure 1.10**). Limbitis is associated with a greater risk of progression (Elder et al., 1996).

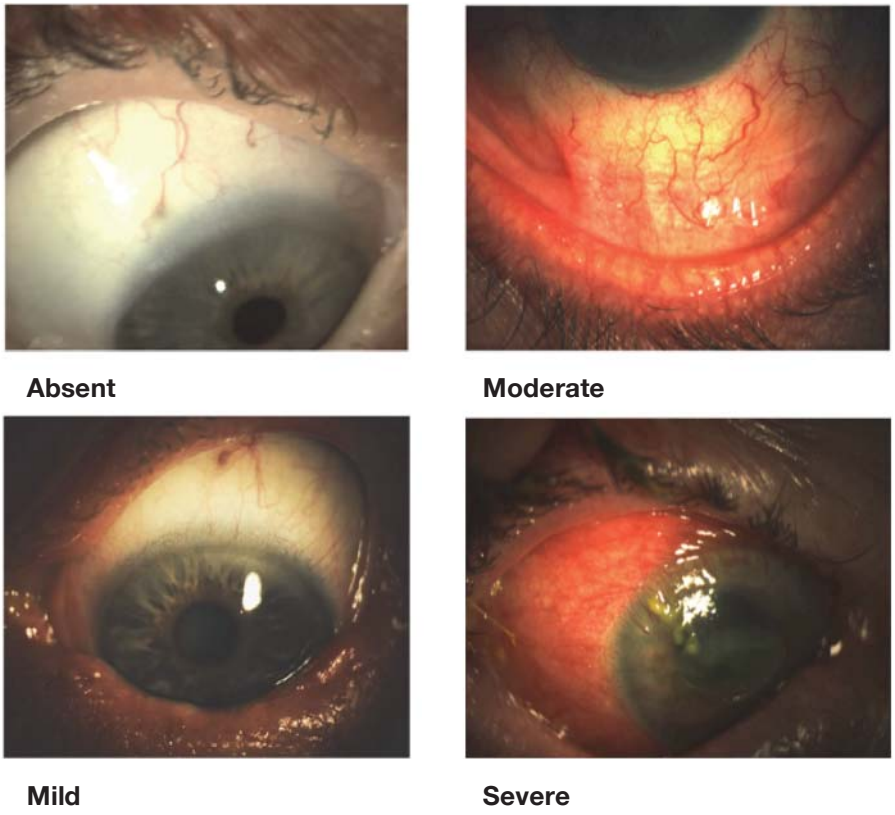


Figure 1.10: **Clinical disease activity in OcMMP.** This is determined by clinically identifiable conjunctival inflammation. The picture denoting severe clinical inflammation also demonstrates limbitis, associated with a greater risk of progression.

1.8.7 Clinical Assessment of Disease Damage in OcMMP

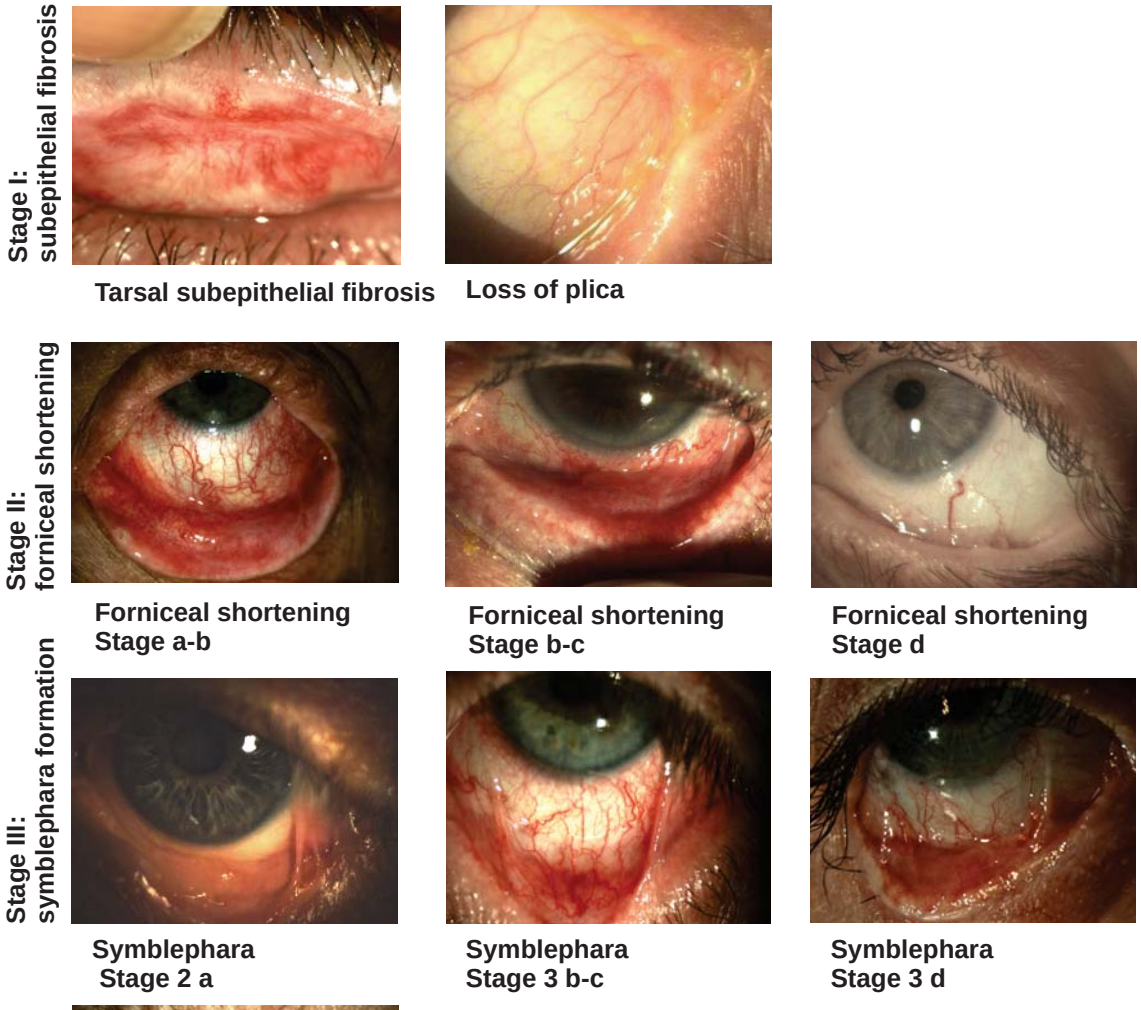
Fibrotic damage is based either on the extent of conjunctival scarring or the presence of symblephara. Different staging systems have been described including the ocular staging described by Mondino and Brown ([I] 0%-25%, [II] 25%-50%, [III] 50%-75%; [IV] 75%-100% loss of inferior fornix) (Mondino and Brown, 1981) and Foster ([I] subconjunctival scarring and fibrosis, [II] fornix foreshortening of any degree, [III] presence of any degree of symblepharon [IV] end-stage cicatricial pemphigoid) (Foster, 1986). Conjunctival blisters are rarely seen in ocular disease (Saw et al., 2009b).

Sequential documentation of forniceal foreshortening is important in assessing stage and progression of disease (defined as increase in either Mondino or Foster stage) (Tauber et al., 1992, Mondino and Brown, 1981, Foster, 1986). Clinical documentation presents challenges including reliance on the subjective evaluation of subepithelial fibrosis, extent of symblepharon formation and grading of percentage shrinkage of the lower fornix (Foster, 1986, Mondino and Brown, 1983). A modification encompassing both systems, was described by Tauber and colleagues, who proposed that counting the number of and percentage horizontal obliteration of the lower fornix by symblepharon could potentially improve detection of disease progression (**Figure 1.11**). (Tauber et al., 1992). This scale, while limited by its lack of a numerical score, has the advantage of detecting more subtle changes compared to the 4-point ordinal scales described by Mondino and Foster in isolation. In order for the scales, in particular the proposed staging described by Tauber, to be effective then accurate documentation in the clinical setting is vital.

Lower fornix shortening is frequently documented either subjectively or semi-objectively by utilising a slit-light beam; whereas the degree of upper forniceal obliteration is seldom quantified. Measurement of the fornices using devices such as the '*fornicometer'* have been described, but their current design limits upper fornix measurement (Schwab et al., 1992, Kawakita et al., 2008). Specifically, the depth and curvature of the upper fornix dictates the requirement of a FDM that is sufficiently long and curved to enable comfortable assessment of the upper fornix. An FDM however offers the possibility of measuring fornix shrinkage in mm, allowing greater flexibility in determining progression compared to categorising change by order (0-25%, 25-50% etc.) and potential to measure symblephara.

Other problems associated with current documentation strategies relate to confining activity and damage indices to the conjunctiva when many of the problems relate to dry eye, entropion formation, limitation of ocular movements and the final, potentially blinding outcome of reduction in clarity or destruction of the optically clear cornea.

Quantification of corneal damage has been attempted in the context of SJS-TEN. A scoring system for chronic SJS-TEN (defined as >12 months since disease onset) was developed by Sotozono and colleagues (Sotozono et al., 2007). This took the form of a 39-point scale, assessing criteria involving the eyelid, conjunctiva, tear film and cornea. In particular the assessment of damage to the cornea including neovascularisation (by extent and central encroachment) and evidence of corneal opacification (scarring, conjunctivalisation and keratinisation) was considered. This study has shown how enumerating corneal damage is possible in the context of PCC.



Staging	Characteristics	Description
I	Subconjunctival scarring & fibrosis	
II	Fornix foreshortening	% loss of inferior vertical fornix depth
a		0-25%
b		25-50%
c		50-75%
d		75-100%
III	Presence of symblephara	% horizontal involvement of inferior fornix
a		0-25%
b		25-50%
c		50-75%
d		75-100%
IV	Ankyloblepharon, frozen globe	

Figure 1.11: Disease damage according to Tauber's proposed staging systems (Tauber *et al* 1992). This incorporates the staging systems of Mondino and Foster and quantifies the percentage horizontal involvement by and number of symblephara.

1.8.8 Therapeutic Intervention in OcMMP

The aim of therapy in OcMMP is to remove correctable factors that cause ocular surface inflammation (including tear substitution for dry eyes, prevention of keratitis, surgical correction of trichiasis and cicatricial entropion) and initiation of systemic immunosuppression to control inflammation secondary to disease and leading to fibrosis; prophylaxis of corneal exposure and ulceration; improvement in vision (through contact lens use, cataract surgery, corneal transplantation, ocular surface reconstruction and keratoprosthesis) (Saw and Dart, 2008).

Although topical corticosteroids can improve comfort they do not prevent cicatrisation (Foster, 1986). Therefore, in all but mild disease, 75% of patients require immunosuppression to control inflammation, limit disease progression and facilitate surgery (Elder et al., 1996). Without systemic immunosuppression, the disease progresses to conjunctival scarring and blindness (Miserocchi et al., 2002). Progression by Mondino staging occurred in 40-50% of stage I, 62-75% stage II and 73-77% stage III disease in the absence of systemic immunosuppression in one study (Mondino and Brown, 1983, Mondino, 1990).

The approach to systemic immunosuppression can be summarised by a stepladder approach, dependent on disease severity, outlined in **Figure 1.12** (Rauz et al., 2005a, Saw et al., 2008). Although systemic immunosuppression can limit progression, halting the disease completely can be a challenge. Disease progression can occur despite systemic immunosuppression in 25-75% of patients (by Mondino staging) (Mondino and Brown, 1983, Mondino, 1990, Elder et al., 1996, Saw et al., 2008) and 10-20% (according to Foster staging) (Miserocchi et al., 2002, Thorne et

al., 2008). Control of clinically identifiable conjunctival inflammation is achieved in 70-90% patients (Miserocchi et al., 2002, Thorne et al., 2008) but unfortunately, there is also a disease subset that either is completely refractory to conventional immuno-suppression or relapses despite initial success (Thorne et al., 2008).

Alternatively the primary use of the alkylating agent Cyclophosphamide has been advocated (Thorne et al., 2008) with good success but the use of Cyclophosphamide is limited because of the risk of haemorrhagic cystitis and neoplastic changes in the bladder (Jabs et al., 2000). A few isolated case reports indicate that 'biological' agents, such as rituximab (anti-CD20) or infliximab (anti-TNFα), may be beneficial in some of these patients, but as randomised trials are lacking, funding for such treatment in the United Kingdom prohibits regular use (Segura et al., 2007, Canizares et al., 2006, Heffernan and Bentley, 2006, John et al., 2007, Taverna et al., 2007, Ross et al., 2009).

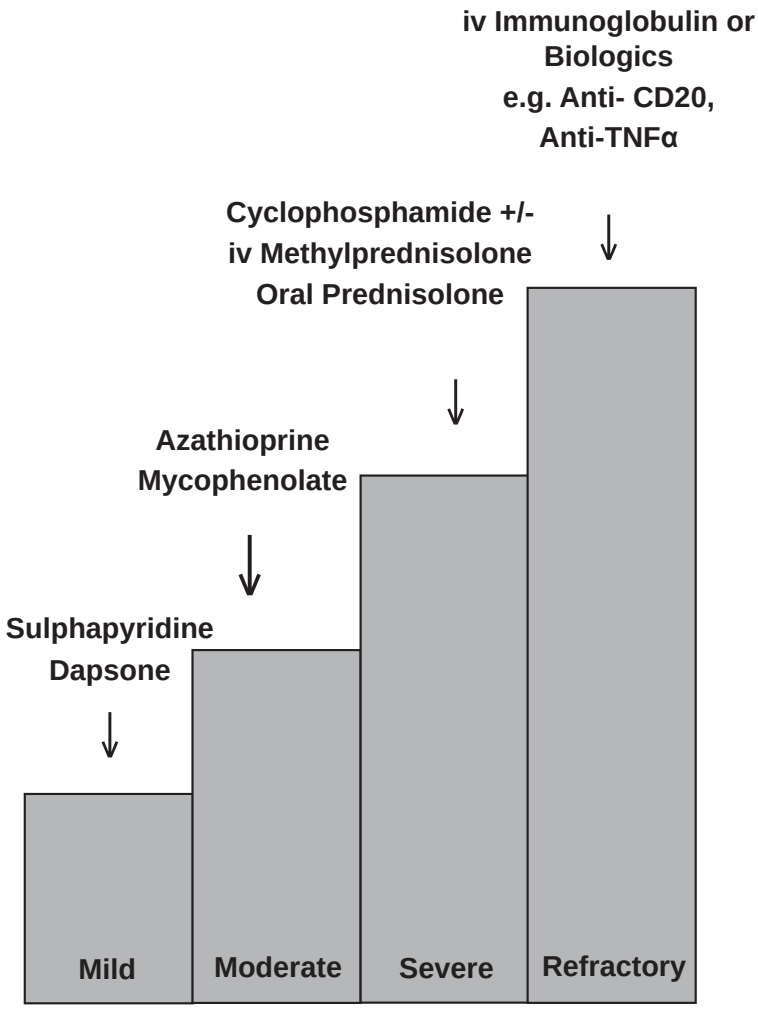


Figure 1.12: Immunosuppressive Strategies for OcMMP (based on Rauz *et al* Ophthalmology 2005). Step-up treatment with agents having the fewest side effects were used for ocular mucous membrane pemphigoid. Disease activity (mild, moderate or severe) is used to guide therapy. Dapsone (25-50mg twice a day), Sulphapyridine (500mg twice a day) was used for mild inflammation; Azathioprine (1-2.5mg/kg/per day) or Mycophenolate mofetil (500-1000mg twice a day if intolerant to Azathioprine) were added or substituted for recalcitrant disease. Severe inflammatory disease was treated with Cyclophosphamide (1-2mg/kg/per day) and adjuvant Prednisolone (1mg/kg/per day with or without a maximum of 3 supplementary loading doses of 1g intravenous Methylprednisolone preceding oral therapy) for up to 3 months until the optimal effects of Cyclophosphamide had taken effect. Refractory disease is managed through intravenous immunoglobulin or biologics e.g. anti-CD 20 (Rituximab) or anti TNF-α therapy.

1.9 Detecting Cellular Changes in the Ocular Surface

Gaining access to mucosal tissue in non-ocular or oral sites present challenges because of the need to employ endoscopic techniques in order to lavage fluid or biopsy. This is clearly evident in the case of the gastro-intestinal and respiratory tracts. For human translational work, surplus diagnostic tissue has therefore been an invaluable source of material. Despite the clear advantage of tissue access however, as in OcMMP, repeated sampling is precluded because of the risk of precipitating further scarring. This has certainly limited our ability to take samples for research purposes. Two developments have offered a potential means of non-invasively determining human cell populations in both health and disease – ocular surface impression cytology (OSIC) and multi-colour flow cytometry.

OSIC is a technique that has been available since the late 1970s. First described by Egbert *et al*, the ability to use cellulose acetate filters to collect bulbar conjunctival surface cells allowed fixation, staining and examination under a microscope (Egbert et al., 1977), and it has been employed since for studying ocular surface disease (Nelson, 1988).

OSIC is a technique that facilitates the removal of 2-3 layers of superficial cells although visualisation of the basal epithelium through multiple impression of the same area can be achieved (Singh et al., 2005). Conjunctival OSIC has been used for monitoring ocular surface health in contact lens wearers and evaluating ocular surface neoplasia (Knop and Brewitt, 1992, Nolan et al., 1994, Tole et al., 2001). Perhaps its widest application is the use of the technique for the quantification of dry eye severity through determination of goblet cell density and changes to the epithelial

cell morphology (Nelson, 1988, Tseng, 1985, Adams et al., 1988). OSIC has also been used to determine analysis of the inflammatory microenvironment in related conditions such as Sjögren's syndrome and other dry eye states (Baudouin et al., 2002).

Flow cytometry is a technique that allows the quantification and characterisation of cell populations based on their size and granularity and by employing fluorochrome-conjugated monoclonal antibodies to cell-surface or intra-cellular peptides. It utilises a high-speed cell analyser and lasers: (e.g. 488nm, 635nm and 405 nm wavelengths) which in turn excite cells passing through the column of a fluidic system at high speed. As cells pass through a sheath flow, lasers excite the fluorochromes conjugated to the labelled cells and the light emitted are detected by photomultiplier tubes. Cells can be detected by either their size or granularity (termed forward and side scatter) and/or by the emission of the cellular fluorochromes (Loken and Stall, 1982, Maftah et al., 1993).

Baudouin and colleagues pioneered the use of flow cytometry on conjunctival epithelial cells recovered by OSIC (Baudouin et al., 1997). The superior unexposed bulbar conjunctiva has been advocated as the most suitable location for conjunctival OSIC, in particular because of variation in inflammation in exposed areas of the ocular surface (Brignole-Baudouin et al., 2004). They describe a simple means of recovering cells from the impression membrane by using a pipette tip to gently agitate cells from membranes stored in preservative.

Flow cytometry technology has evolved rapidly in the last 15 years and multi-colour panels can be designed to accommodate numerous cell surface markers (Perfetto et al., 2004). This allows a more detailed phenotype to be explored both in terms of cell surface marking and also intra-cellular staining for cytokines and other proteins.

By combining multiple markers, T cells for example, can be discriminated not only by the presence of CD3, CD4 and CD8 but according to the T cell receptor subtype e.g. TCR$\alpha\beta$ or TCR$\gamma\delta$, the presence or absence of common markers through CD56 (NKT cells express CD3 and CD56) and whether they are antigen experienced by the presence of CD45RO.

Innate cell markers such as CD14 (LPS receptor present on myelomonocytic cells such as macrophages), CD16 (FcγRIII receptor present on neutrophils, macrophages and Natural Killer (NK) cells) can be combined with additional markers e.g. CD11b (present on myeloid cells and NK cells) in discriminating granulocytes such as neutrophils from e.g. basophils and eosinophils (Gopinath and Nutman, 1997). The pan-leukocyte marker CD45, can also be used to discriminate lymphocytes, monocytes and eosinophils by the presence of $CD45^{HIGH}$ expression compared to $CD45^{INTERMEDIATE\ (INT)}$ expression seen on neutrophils (Stelzer et al., 1993, Kern et al., 2000). This affords greater flexibility and accuracy in characterising the conjunctival epithelial leukocyte populations – in both health and disease.

1.10 My hypothesis and its context

The most common acquired ocular immunobullous disease is OcMMP. The binding of autoantibodies to the BMZ and the deposition of complement, with associated inflammatory infiltration is recognised, but there is no means of non-invasively detecting this process. Clinicians currently rely upon subjective clinical assessment of disease activity to monitor clinically identifiable inflammation and to guide immunosuppressive therapy. These issues are compounded by the further lack of understanding of healthy ocular surface immune regulation and the ability to accurately quantify the conjunctival scarring process. Unfortunately the disease may progress in the absence of conjunctivitis leaving the clinician in an invidious position and wondering whether a self-perpetuating fibrotic process occurs, or in fact that sub-clinical inflammation does take place and results in conjunctival fibrosis.

My hypothesis therefore is that *progressive fibrosis in OcMMP, occurring in the apparent absence of clinical inflammation, is driven by underlying inflammatory processes.*

1.11 Aims of the thesis

The aim of my thesis is to determine the detailed cellular profile in OcMMP by non-invasive conjunctival OSIC and multi-colour flow cytometry by undertaking (i) cross-sectional and (ii) longitudinal comparisons with healthy volunteers and those with other PCC and determine the relationship between clinical parameters and cellular profiles, in particular the inflammatory profile in a clinically quiescent eye.

1.12 Objectives for the thesis

In order to address my hypothesis I have sought to undertake three principle objectives during my thesis:

Part 1: Measuring clinical parameters in acquired ocular immunobullous disease

1. Defining the clinical phenotype of patients with OcMMP
2. Determining an objective method for recording the conjunctival cicatricial process
3. Determining an optimal system for accurately phenotyping disease

Part 2: Characterising the healthy conjunctival epithelial leukocyte population

1. Optimisation of mucous membrane cellular profiles detection by OSIC
2. Defining resident conjunctival leukocyte populations
3. Age-related changes in the healthy human conjunctival epithelium
4. Effector function of the dominant conjunctival epithelial CD8+ T cell population

Part 3: Characterising the conjunctival epithelial leukocyte population in acquired ocular immunobullous disease

Determine whether inflammatory cellular profiles differ in

1. Stevens-Johnson Syndrome (SJS) and Toxic Epidermal Necrolysis (TEN)
2. Ocular Mucous Membrane Pemphigoid (OcMMP) in clinically inflamed and uninflamed eyes

Chapter 2

Materials and Methods

2.1 Clinical Studies

Details of the recruitment of patients with PCC such as MMP, SJS-TEN and PSS and healthy participants are outlined in this chapter. Study-specific details are outlined in the relevant chapters (**3, 4** and **5**).

2.1.2 Ethical Approval

Clinical data collection and patient sampling were undertaken following ethical approval in accordance with the Declaration of Helsinki (London MREC: National Survey Of Cicatrising Conjunctivitis and Stevens−Johnson Syndrome/Toxic Epidermal Necrolysis In The UK **07/MRE02/41** and Birmingham East, North and Solihull Ethics Committee: Inflammation in Ocular Surface Disease **IOSD 08/H1206/165** (UKCRN number 7448).

2.1.3 Study Recruitment

Patients with disease (MMP, SJS-TEN or PSS) were identified either at first presentation to the ocular surface disease (OSD) clinic (Birmingham and Midland Eye Centre - BMEC) or from existing cohorts (BMEC; University Hospital Birmingham – UHB and Moorfields Eye Hospital – MEH) utilising electronic medical databases.

Diagnosis of OcMMP was based on clinical findings characteristic for the disease, namely progressive conjunctival cicatrisation in the absence of other causes of

conjunctival scarring. If patients did not have a previous positive tissue biopsy, a confirmatory perilesional conjunctival and/or oral mucosal biopsy for direct immunofluorescence was undertaken. A positive result was defined as linear deposition of IgG, A or complement (C3) along the basement membrane zone (Chan et al., 2002). If typical clinical characteristics were evident, a negative result did not exclude the diagnosis (Saw et al., 2008, Ahmed et al., 2004, Bernauer et al., 1994, Tauber, 2008) because of the recognition of a subgroup of ocular patients who have ocular features consistent with OcMMP but have a negative biopsy (Bernauer et al., 1994, Thorne et al., 2004). In accordance with the first international consensus, a positive indirect immunofluorescence was not an essential requirement for diagnosis (Chan et al., 2002).

Diagnosis of SJS and TEN was based on the criteria described by Bastuji-Garin and made by a consultant dermatologist. Patients were classified as having SJS if the maximal percentage of denuded skin was below 10%; SJS/TEN overlap syndrome between 10 and 30%; and TEN above 30% (Bastuji-Garin et al., 1993).

Diagnosis of PSS was based on the revised American–European Consensus Group (AECG) criteria (Vitali et al., 2002). Briefly, this included a requirement to fulfil 4 out of 6 criteria if items IV or VI were positive or the presence of any 3 of the 4 objective criteria items (III, IV, V, VI) where I: Ocular symptoms, II: Oral symptoms, III: ocular signs, IV: Histopathology, V: Salivary gland involvement and VI: presence of serum Anti Ro (SSA) or La (SSB) antibodies. Ocular symptoms included (i) Daily, persistent, troublesome dry eyes for more than 3 months (ii) Recurrent sensation of sand or gravel in the eyes (iii) Use of tear substitutes more than 3 times a day. Ocular signs

included objective evidence of ocular involvement defined as a positive result for at least one of the following two tests: (i) Schirmer's I test, performed without anaesthesia (<5 mm in 5 minutes) or (ii) Corneal Rose Bengal/Lissamine Green staining.

Healthy volunteers were defined as those with no history or clinical evidence of ocular, systemic inflammatory or autoimmune disease (including dry eye) (Behrens et al., 2006, 2007), contact-lens wear, previous ocular surgery, cataract surgery within 3 months (unless stated - see **section 5.2.1**) or use of topical ophthalmic medication.

2.1.4 Clinical Documentation

The details of the clinical parameters recorded are outlined in **Chapters 3** and **5**. Where clinical examination took place, routine and validated assessments took place. Specific scoring systems are outlined within individual chapters. LogMAR visual acuity was assessed and tables converted to Snellen. Visual acuity (VA) was classified as good (6/6–6/18), or in accordance with the WHO definitions of 'visual impairment' (6/18–6/60), 'severe visual impairment' (6/60) and 'blind' (3/60). Details of the development of the Clinical Record Form (CRF) employed are outlined in **Chapter 3**.

2.1.5 Databases

A bespoke Filemaker Pro database was created (GPW and PJT) in order to store clinical linked-anonymised data in an electronic case record form (eCRF) (Filemaker

Pro v9.0, Santa Clara, California). At the time of recruitment, patient details and samples were given a linked-anonymised code. Details of patient research codes were stored on a database on the N3 secure Sandwell and West Birmingham NHS Trust SAN server. The anonymised eCRF data is stored on the University of Birmingham Active Directory Server. Only anonymised data was shared between the two databases and only members of the clinical care had access to codes relating to the patients.

2.1.6 Clinical Sampling

Collection of conjunctival cells was undertaken with autoclaved synthetic membranes divided in two semi-circles (measuring 13 x 6.5mm^2 each) (Brignole-Baudouin et al., 2004). Millipore isopore polycarbonate (0.4µm membranes) (Fisher, Leicester, UK) or Supor 200 polyethersulfone filters (0.2µm membranes) (VWR, Lutterworth, UK) were applied following instillation of 0.4% Oxybuprocaine (as a topical anaesthetic). Conjunctival Ocular Surface Impression Cytology (OSIC) was performed with up to four semi-circle membranes per eye (equivalent to 2 full impressions) from the superior unexposed bulbar conjunctiva for 5-10 seconds using a sterile technique (Brignole-Baudouin et al., 2004, Brignole et al., 2000) and before the application of topical fluorescein drops for clinical assessment. Membranes were removed and placed in 1.5ml of RPMI 1640 (Sigma-Aldrich, Dorset, UK) with 10% Heat Inactivated Foetal Calf Serum (Sigma-Aldrich) in a sterile 5ml universal container.

2.2 *Ex vivo* experimentation

General details of experimentation techniques are described in this chapter. Specific adaptations are described in the relevant results chapters.

2.2.1 Haematoxylin and Eosin staining of OSIC membranes

Identification of cells retained on impression cytology membranes were undertaken by Haematoxylin and Eosin staining. Briefly, membranes were fixed for 1 minute in 95% methanol/5% glacial acetic acid and then rinsed in tap water. Staining with haematoxylin for 30 seconds followed by rinsing in tap water, incubation in tap water for 1 minute followed by a further rinse in tap water. Counterstaining with 1% aqueous Eosin for 5 seconds preceded dehydration through 2 stages of alcohol (70% for 30 seconds and 100% for 30 seconds). Membranes were cleared through 2 changes of Histoclear (National Diagnostics, Hessle, UK) for 1 minute each and mounted face up with neutral mounting medium (DPX) (Clin-Tech Ltd., Surrey, UK) and a coverslip.

Photography was undertaken with an Olympus BH1 light microscope and captured with PictureFrame 2.1 software (Optronics, California, USA 2004).

2.2.2 Cell Recovery from OSIC

Conjunctival epithelial cells collected by OSIC were recovered by gentle agitation with a sterile 1ml P1000 Gilson pipette tip for 1 minute unless stated. Cell

suspensions were transferred to a 1.5ml Eppendorf tube and centrifuged (400g for 5 minutes). The supernatant was discarded, leaving 10 µl, which was re-suspended in a further 90 µl RPMI:10% HIFCS (to a total 100 µl) unless stated. Cells were placed in 96 well plates for flow-cytometric analysis.

2.2.3 Preparation of Lysed Peripheral Blood

Peripheral blood was collected in EDTA tubes and aliquoted at a volume of 1ml, centrifuged and re-suspended in 1:10 dilution of filter-sterilised red cell lysis buffer (8.29g NH_4Cl (Sigma-Aldrich), 1g $KHCO_3$ (Sigma-Aldrich) and 37.2 mg EDTA (Sigma-Aldrich) per litre dH20). After 5 minutes at room temperature, the suspension was diluted with up to 15ml RPMI to block further lysis. Following centrifugation, the pellet was re-suspended in Phosphate Buffered Solution (PBS; Oxoid, Cambridge, UK) at a concentration of $1x10^7$ cells/ml and aliquoted at a volume of 100µl in to individual wells.

2.2.4 Flow Cytometry

Flow cytometry was undertaken with a Dako Cyan ADP High Performance flow cytometer (Beckman Coulter, High Wycombe, UK). Multi-colour cytometry compensation was performed using cells or compensation beads individually stained with each fluorochrome conjugated-antibody. Compensation circumvents spectral overlap by adjusting for false positives from other fluorochromes. This is achieved by setting compensation levels for one fluorochrome at a level commensurate with the background fluorescence of another e.g. PE-Cy7 anti-CD56 vs. PerCP Cy5.5 anti-

CD4, the fluorescence intensity for the positive population of CD56 stained beads/cells is adjusted so that it is at the same median fluorescence as background (for the PerCP Cy5.5 channel).

2.2.5 Antibody Cell Surface Marker Staining

To characterise the cellular profile of the ocular surface, multi-colour flow cytometry panels were developed. These were titrated to determine the optimal concentrations. Each panel was applied to cells recovered from conjunctival impression cytology or from peripheral blood.

100µl of cells were placed in to 96-well plates (with a cell count per well ranging from $2x10^5$—$1x10^6$ for PBMCs) or 20µl of positive and negative compensation beads. Cells were centrifuged for 4 minutes at 400g at 4°C, the supernatant removed and the 96-well plate gently vortexed. Cells were stained with surface marker antibodies (made up in 50µl at appropriate dilutions) and incubated on ice in the dark for 20 minutes. 100µl of FACS buffer (PBS and 0.5% Bovine Serum Albumin (BSA; Sigma-Aldrich) was added to each well prior to further centrifugation and removal of supernatant. Cells were re-suspended in 295µl of FACS buffer and 5µl counting beads (CALTAG/Invitrogen, Paisley, UK) (1002 beads/ µl) buffer prior to analysis. For dead cell exclusion, 30µl Sytox blue (Invitrogen, Paisley, UK) was added at a dilution of 1/800 to the FACS tubes and incubated for 5 minutes prior to running on the flow cytometer.

For healthy volunteers and patients with disease (**Chapters 4** and **5**) cell-surface fluorochrome-labelled monoclonal antibodies were employed in two panels; Panel 1 mouse anti-human CD45RO (FITC), γδTCR (Phycoerythrin, PE), CD4 (PerCP Cy5.5), CD45 (Allophycocyanin, APC), CD3 (AlexaFluor 780) (Ebioscience, Hatfield, UK); CD8α (Pacific Orange) (Invitrogen, Paisley, UK); CD8β (PE Texas Red) (Beckman Coulter); CD56 (PE Cy7) (Biolegend, Cambridge, UK) and Panel 2 mouse anti-human CD16 (FITC), CD45 (Allophycocyanin), CD14 (AlexaFluor 780) (Ebioscience); CD20 (Pacific Orange), CD19 (PE Texas Red) (Invitrogen); CD138 (PerCP Cy5.5) (BD, Oxford, UK) and CD11b (PE Cy7) (Biolegend). These were titrated to determine the optimal concentrations.

Appropriate panels were applied to cells recovered from conjunctival OSIC or peripheral blood.

2.2.6 Intracellular Cytokine and Cytolytic Protein Staining

For cytokine assays, conjunctival and lysed peripheral blood cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) and Ionomycin (Sigma-Aldrich). Briefly, cells were incubated in 200μl containing PMA (250ng/ml), Ionomycin (250ng/ml) and Brefeldin A (Sigma-Aldrich) 2ug/ml for 3 hours at 37°C, 5% CO_2. For cytolytic proteins

A Live Dead fixable yellow dye (Invitrogen) was used to discriminate dead cells. Cells were suspended 100μl of 1:1000 Dye:DMSO for 30 minutes on ice in the dark.

For cytokine assays and cytolytic protein studies, surface marker antibodies in this panel were suspended in Fixation medium A (Fix & Perm, Invitrogen) under the same conditions as described for *Antibody Cell Surface Marker Staining* (Section **2.2.5**). Intracellular antibodies and cytolytic protein antibodies were suspended in Permeabalisation medium B (Fix & Perm, Invitrogen) on ice in the dark for 20 minutes before centrifugation and re-suspension as described.

2.2.7 Transcription Factor Staining

For the transcription factor FoxP3 cell surface marker staining was as described in **2.2.5**. An eBioscience staining buffer kit was employed for intra-cellular staining. Briefly, cells were suspended in Fix buffer on ice in the dark for 30 minutes before washing in PBS, fixing in Perm buffer and staining with rat serum and anti-FoxP3 antibody.

2.2.8 Viral peptide MHC-I Tetramer Staining

Conjunctival and matched peripheral blood cells were incubated with pooled CMV or EBV peptide-MHC-Class I tetramers conjugated to APC as previously described by Khan et al (Khan et al., 2004). For donors of known CMV/EBV response and HLA type, the following HLA restriction/peptide sequence were employed: CMV - HLA-A1 VTE, HLA-A1 YSE, HLA-A2 NLV, HLA-B7 RPH, HLA-B8 ELK, HLA-B8 QIK and EBV - HLA-A2 GLC, HLA-A2 YVL, HLA-B8 RAK and HLA-B8 FLR). A full table is listed in **Table 2.1**. Briefly, cells were incubated at room temperature for 20 minutes with pooled CMV or EBV tetramers prior to cell surface staining with anti-CD45

(Allophycocyanin), CD3 (AlexaFluor 780), CD8β (PE Texas Red) and CD56 (PE Cy7) (Biolegend) as described above (**2.2.5**).

Viral Antigen	HLA restriction	Peptide Sequence	Sequence location
CMV			
IE-1	B8	QIKVRVDMV	88 –96
	B8	ELKRKMIYM	199 –207
pp50	A1	VTEHDTLLY	245–253
	A1	YSEHPTFTSQY	363–373
pp65	A2	NLVPMVATV	495–503
	B7	RPHERNGFTVL	265–275
EBV lytic cycle			
BMFL1	A2	GLCTLVAML	280 –288
BMRF1	A2	YVLDHLIVV	109 –117
BZLF1	B8	RAKFKQLL	190 –197
EBV latent cycle			
EBNA3A	B8	FLRGRAYGL	325–333

Adapted from Khan *et al* 2004 (Khan et al., 2004)

Table 2.1 Table of MHC Class 1 restricted peptides used.

2.3 Statistical Analysis

Statistical analysis was undertaken using SPSS 16.0, 18.0 for Macintosh and 14.0, 19.0 for Windows (SPSS, Chicago, Illinois 2006, 2010), Excel for Macintosh (Microsoft, Washington 2010) and Prism version 5.0 for Macintosh (GraphPad Software, California 2008).

Non-parametric continuous comparisons were undertaken with the Mann Whitney U test for two groups, Kruskal-Wallis test and Dunn's multiple comparison for multiple groups, Wilcoxon signed rank method (for paired analysis) and correlations by Spearman's correlation. Discrete variable were compared by Fishers exact test, McNemar's test (for paired analysis), Jonckheere-Terpstra test and Kendall's Tau b for rank correlations. Generalised Estimating Equations were employed where linear correlations were unknown. Agreement was determined by the Bland-Altman test.

Statistical guidance was sought from Dr Peter Nightingale, Statistician, Wellcome Trust Clinical Research Facility, Queen Elizabeth Hospital, University Hospitals Birmingham NHS Foundation Trust, Birmingham, B15 2TT.

Chapter 3

Measuring Clinical Parameters in Acquired Ocular Immunobullous Disease

3.1 Introduction

The challenge in acquired ocular immunobullous diseases such as OcMMP and SJS-TEN is not only to manage multiple problems arising from ocular surface inflammation, but to correctly identify individuals who are at risk of accelerating disease activity and progression. For this reason, OcMMP has been referred to as an ‘‘*evil curse’’* (Hossain, 2011). Elder *et al* coined the term “*white inflammation*” which referred to an occult fibrotic process in OcMMP driven by inflammation in the absence of clinical manifest aspects of inflammation (Elder, 1997b). In addition, the constellation of clinical signs affecting the eyelid, ocular surface and tear film can potentially overwhelm the clinician resulting in documentation in which key features indicative of progression may be overlooked.

In this chapter, I have sought to define the clinical phenotype of patients with OcMMP presenting to tertiary centres in the UK, determine an optimal means of measuring disease damage objectively and a system for accurately recording disease phenotype in the clinical setting.

3.1.1 Defining the clinical phenotype of patients with OcMMP

In the UK, streams of hospital referral are primarily to tertiary care services specialising in the management of ocular surface disease and OcMMP and originate chiefly from ophthalmologists practicing in secondary care. The duration of symptoms is variable at presentation (Rauz et al., 2005a, Thorne et al., 2004, Thorne et al., 2008, Saw et al., 2008). The literature offers a confusing picture related to disease duration, with disease progression believed to be more rapid in the context of

manifest conjunctival inflammation (Mondino et al., 1979) yet patients are often diagnosed at an advanced stage of disease when signs are abundant (Miserocchi et al., 2002, Rogers et al., 1982). Although the possibility of a referral bias of patients with a more severe phenotype is recognised (Thorne et al., 2008), there is limited information regarding the clinical features of patients with early or established disease who present to specialised services, whether these patients require continued tertiary care, or are discharged back to their referring unit.

The demographic profile and duration of symptoms at presentation including extra-ocular features and immunohistological profile of disease, current levels of clinical documentation, disease activity, damage and progression rates and clinical intervention at tertiary referral centres was undertaken.

3.1.2 Determining an objective method for recording the conjunctival cicatricial process

Forniceal measurement by measurer Fornix Depth Measurers (FDM), have been previously outlined, but their design restricts accurate upper fornix measurements (Schwab et al., 1992, Kawakita et al., 2008). Specifically, the depth and curvature of the upper fornix dictates the requirement that the ideal FDM is sufficiently long and curved to enable comfortable and accurate assessment of the upper fornix. A modification of the Schwab FDM was designed at MEH, for the purpose of a clinical trial. However this FDM was made ‘in-house’ and a re-design was required in order to develop a bespoke comfortable prototype that could provide an accurate tool for improving forniceal sac and horizontal fibrosis documentation in the outpatient clinic

setting, and for the purposes of clinical evaluation during my thesis, with potential to enable consistent and comparable assessment of disease stage.

3.1.3 Determining an optimal system for accurately phenotyping disease

In BMEC, like other tertiary centres, the 'proposed' scoring system described by Tauber (Tauber et al., 1992) has not routinely been documented, precluded because of an absence of an objective method for scoring and measuring disease damage. The 'proposed' system incorporates the Mondino (Mondino and Brown, 1981) and Foster (Foster, 1986) systems. The advantage of combining both systems is that the scale allows for a simpler, and it is argued, a more sensitive system of recording damage (Tauber et al., 1992), but this scale requires documentation of both the number and involvement of symblephara. The mechanism for objectively enumerating these parameters were not described in the original system however (Tauber et al., 1992).

While conjunctival fibrotic changes, as discussed in **Chapter 1.7.3,** could historically be considered *primary outcome measures* of disease damage, they are not the terminal event as far as vision is concerned. Corneal scarring including vascularisation ultimately results in the sight-threatening manifestations of disease (Elder, 1997c, Saw and Dart, 2008). Therefore, a means of recording corneal changes systematically in the clinic setting was required. The process for creating a clinical record form (CRF) for this purpose is described.

3.2 Methods

3.2.1 Defining the clinical phenotype of patients with OcMMP

Study population

Retrospective case note review involved two individuals (GPW and CR) with transfer of linked-anonymised data to secure databases (**Chapter 2**). Fifty-four consecutive patients with a documented history of OcMMP referred to dedicated ocular surface disease (OSD) clinics at MEH and BMEC (Cohort 1), were identified from electronic databases and followed for 24 months.

Patients were stratified according to duration of symptoms, where symptoms were defined as redness, tearing, burning, decreased vision or foreign body sensation (Ahmed et al., 2004). The frequency distribution of the duration of disease defined two groups straddling either side of the median (1460 days (4 years)) (**Figure 3.1**). Group1 (n=26, 51 eyes) consisted of patients with <1095 days (<3 years) history and was termed the “early-onset” disease (EOD) group, whereas Group 2 (n=24, 48 eyes) comprised patients with > 1825 days (>5 years) history and was termed the “established” disease (EstD) group. Four patients had duration of symptoms that fell on the median (4 years), and these patients were excluded from further analysis.

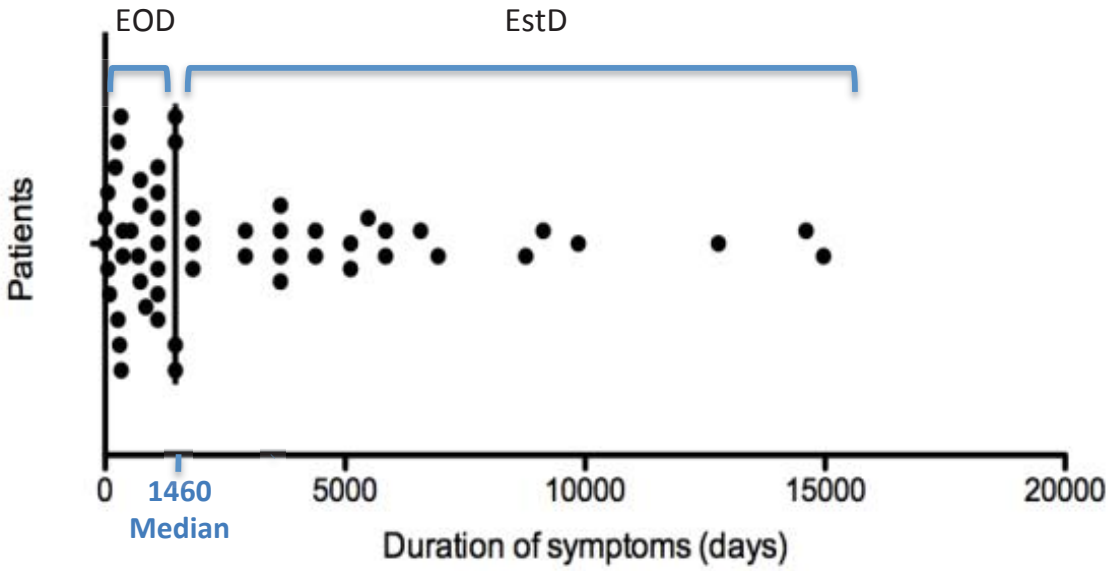


Figure 3.1: Symptom duration of patients presenting with OcMMP. The frequency distribution of the 54 patients referred to our specialist tertiary hospitals according to duration of ocular symptoms of MMP (days) at presentation to the specialist centres. Two groups are apparent: those with a duration of symptoms clustering under the median duration of symptoms of 1460 days termed the early onset of disease (EOD) group, and those with a duration of symptoms >1460 days indicating more established disease (EstD). The 4 patients symptomatic for 1460 days were excluded from further analysis.

Study Design

Data were captured at presentation, immediately following first consultation, and at 12 months and 24 months after first presentation. Visual acuity categories were as described in **2.1.4**. Disease activity was based upon the degree of conjunctival inflammation: absent, mild, moderate or severe (including inflammation in all 4 quadrants, limbitis and/or conjunctival ulceration) (**Figure 1.10**) (Elder, 1997c).

Stage of disease and progression was determined by utilising the staging systems described by Mondino and Brown ([I] 0%-25%, [II] 25%-50%, [III] 50%-75%; [IV] 75%-100% loss of inferior fornix) (Mondino and Brown, 1981) and Foster ([I] subconjunctival scarring and fibrosis, [II] fornix foreshortening of any degree, [III] presence of any degree of symblepharon [IV] end-stage cicatricial pemphigoid) (Foster, 1986). Progression was defined as an advance in either Mondino or Foster staging criteria. Immunosuppression strategies employed a 'step-ladder' approach as previously described (**Figure 1.6**) (Rauz et al., 2005a, Saw et al., 2008). Information regarding surgical intervention was also recorded.

Statistical Analysis

Statistical analysis was by SPSS 16.0 for Macintosh and 14.0 for Windows (SPSS, Chicago, Illinois 2006), and Prism version 5.0 for Macintosh (GraphPad Software, California 2008) using Fishers Exact test, McNemar's test, and Kendall's Tau b for rank correlations. Continuous variables were analysed by nonparametric tests (Mann-Whitney U test). Data were collected on all eyes and comparisons were

undertaken between the worst affected eye for cross-sectional analysis of inflammation/fibrosis and the better-seeing eye for visual acuity. When determining disease progression, comparisons were undertaken between patients (either one or both eyes). In order to determine if changes seen at differing time points were significant rather than as a result of a change in the cohort (e.g. due to patients being discharged back to the referring hospital or missing data), longitudinal analysis of the same eye was undertaken. Due to the referral of patients back to the originating physician, the sample sizes at the three time points differ and percentages rather than absolute counts are therefore reported.

3.2.2 Determining an objective method for recording the conjunctival cicatricial process

3.2.2.1 Design of a bespoke Fornix Depth Measurer (FDM; BMEC Version 1)

A polymethylmethacrylate (PMMA) FDM (BMEC Version 1) was designed using industry-standard jewellery computer software (GPW/SR/SE/PC) (CAD v5, 3Design, Brussieu, France 2007). The virtual model was then exported as an .STL file into a program to set the cutting parameters for the milling machine (Modela Player v4, Roland DG, Shizuoka, Japan 2002) (**Figure 3.2a**). The virtual model was cut with a machine to a precision of 2μm/step, and increments expressed at 2mm intervals. The BMEC v1 FDM measured 25mm x 5mm and was biconcave to ensure ease of insertion and comfort (**Figure 3.2b**).

Patients

An ethically approved evaluation of the bespoke FDM (BMEC v1) was undertaken through a prospective, masked, independent assessment of central lower fornix depth by two observers (GPW, TS). The validation of the FDM (BMEC v1) was conducted on a heterogeneous group of patients comprising of clinically normal and abnormal conjunctival fornices presenting to BMEC. This heterogeneity enabled a wide range of fornix depths to be tested with the bespoke FDM.

The cohort consisted of 51 eyes of 26 patients with a median age of 64 years (range 42-100 years) of whom 65% (17/26) of patients (33/51 eyes) had an identifiable cause of cicatrising conjunctivitis (OcMMP, 10; Dry Eyes, 5 (including 3 with Sjögren's syndrome); Stevens-Johnson Syndrome (SJS), 2) and 35% (18/51) had no evidence of conjunctival scarring (Age-Related Macular Degeneration (ARMD), 4; Uveitis, 3; Peripheral Ulcerative Sclero-keratitis, 2) (Cohort 2).

Following instillation of one drop of 0.4% Oxybuprocaine hydrochloride, patients were asked to look in the opposite direction to the placement of the FDM (BMEC v1) (upper fornix, downgaze; lower fornix, upgaze) and the central conjunctival fornix was measured to the eyelid margin, defined as the posterior lip of the meibomian gland orifice (**Figure 3.2c**). All FDM (BMEC v1) readings were in triplicate with the first measurement taken used for inter-observer comparison. A subjective estimation of lower fornix conjunctival shrinkage was also performed (NB a subjective assessment of the upper fornix is not possible). Patients were also asked about their tolerance to the FDM.

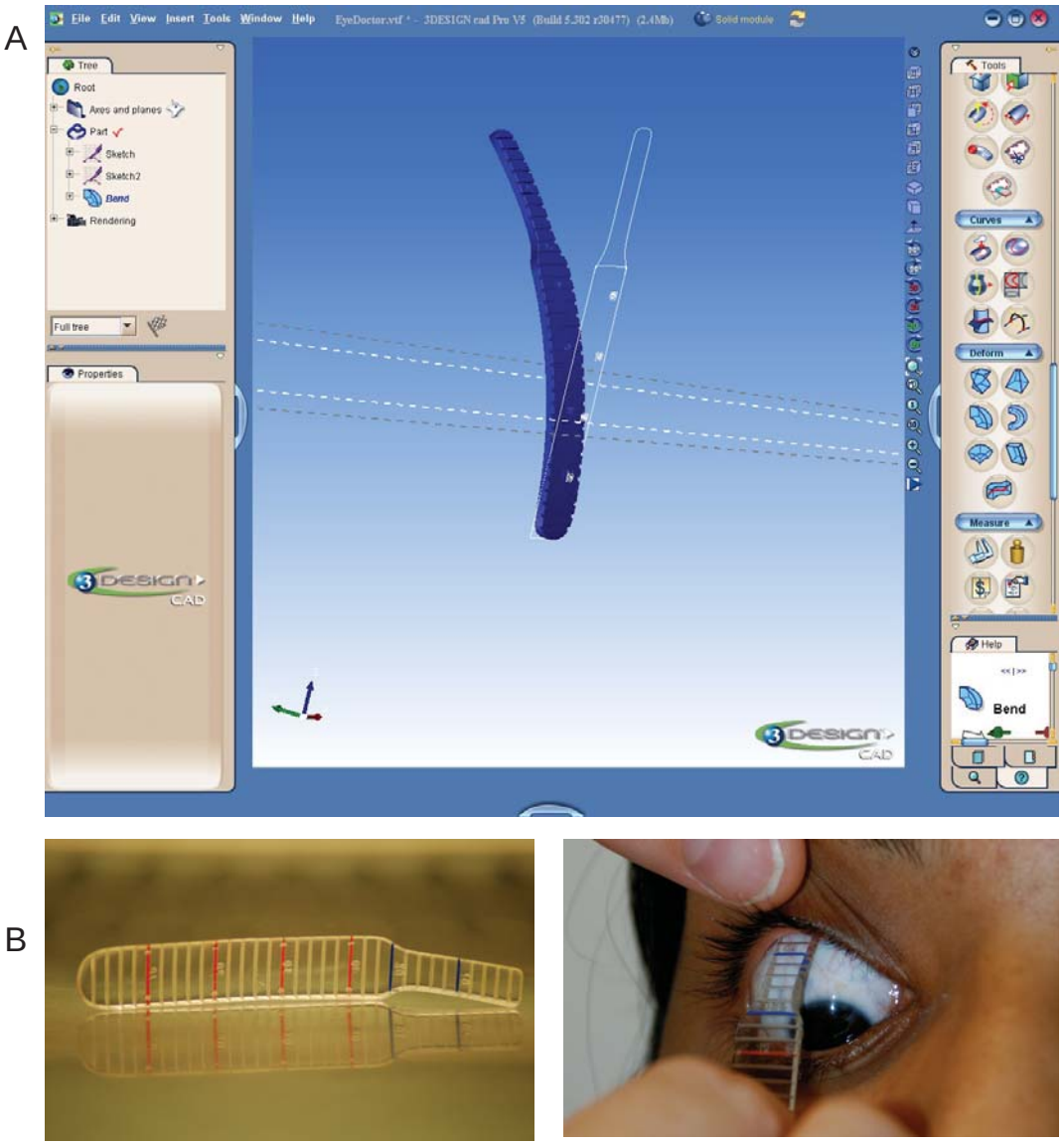


Figure 3.2: FDM (BMEC v1) Construction. A PMMA FDM was constructed using industry-standard jewellery software and machinery. A screenshot of the 3design prototype is shown in the top panel (panel A). The final prototype is illustrated in the left panel (panel B). Increments are expressed at 2mm intervals to a precision of 2μm/step and the FDM was heat moulded to a biconcave shape for comfort. The FDM (BMEC v1) was applied following instillation of one drop of 0.4% Oxybuprocaine hydrochloride (panel C). Patients were asked to look in the opposite direction to the placement of the FDM and the central conjunctival fornix was measured to the eyelid margin, defined as the posterior lip of the meibomian gland orifice.

Calculations and Statistical Analyses

The percentage loss of lower fornix for both methods of measurement (subjective and objective) was calculated using the equation:

(Fornix Depth (FD) age – FDM measurement)/FD age x 100=% loss of fornix

A correction factor for age was implemented as the lower forniceal depth is known to progressively shorten with age (Schwab et al., 1992). The "FD age" values were derived from published age-specific lower fornix depths in normal eyes detailed in **Table 3.1** (Schwab et al., 1992). For example, for a patient aged 80 years with a lower fornix measured at 6mm, the calculated percentage shrinkage is as follows:
(10.2-6)/10.2 x 100=41.2%

Age	Mean Depth of normal lower fornix in mm ('100%')	10% of normal lower fornix in mm
40-49	11.9	1.19
50-59	11.3	1.13
60-69	11.0	1.10
70-79	10.6	1.06
80+	10.2	1.02

Adapted from Schwab *et al* Ophthalmology 1992 (Schwab et al., 1992)

Table 3.1: **Age specific normal values for the conjunctival lower fornix**.

Intra- and inter-observer agreement was assessed using Bland-Altman plots of differences in measurements versus mean measurements using Excel for Macintosh (Microsoft Office 2008) (Bland and Altman, 1986, Bunce, 2009). The mean difference in observations and the 95% limits of agreement (the mean difference ±2 standard

deviations) (Bland and Altman, 1986) were calculated using SPSS v 16.0 for Macintosh (SPSS, Chicago, Illinois 2007). A continuity correction was applied to the 95% limits of agreement, to take account of the fact that Bland and Altman plots assume that the variables measured are continuous.

A 10% threshold or 'tolerance' was chosen as an allowance for intra-observer variation. Agreement was also evaluated by determining the percentage of observations that agreed to within the 10% 'allowance' for both observers.

3.2.2.2 Enhancement of the FDM for enumerating symblephara (FDM; BMEC Version 2)

A further adaptation of the FDM was undertaken (BMEC Version 2). This was to facilitate enumeration of the number and extent of symblephara (Tauber et al., 1992). In order to consistently measure the number and extent of symblephara we elected to re-design the FDM in order to measure the distance from medial to lateral canthus inferiorly and superiorly (**Figure 3.3A and B**).

It was anticipated that the BMEC v1 FDM could be adapted to facilitate fornix measurement and allow inter-canthal measurement, without the requirement for two separate FDMs. The 20mm 'handle' (**Figure 3.2A,B**) from BMEC v1 FDM was therefore incorporated in to a new FDM (BMEC v2) with a longer 50mm inter-canthal measurer (**Figure 3.3C and D**). The precise inter-canthal distance is unknown but two considerations were taken in to account during the re-design (i) curvature of the globe and (ii) length. The natural curvature of the eye means that assessing the presence of symblephara could not be achieved with a straight-line measurement

(**Figure 3.3A and B**). We therefore utilised the curvatures employed to fit scleral contact lenses employed at BMEC and MEH (recommended by scleral contact lens practitioners Paul Cottrell (PC) and Ken Pullum; 2008 personal communications; Innovative Sclerals Scleral Lens Fitting Manual, 2008).

The elongated FDM was re-designed as described in **3.2.2.1** (GPW/SR/SE/PC) but narrowed to 5mm diameter for comfort and to aid measurement. The longer measurer was moulded around a cylinder measuring 13.5mm in diameter, giving a bi-concave shape with a shorter handle identical to the original FDM, validated as outlined above (**Figure 3.3C and D**). This allowed measurement from medial to lateral canthi along the eyelid margin of the lower and upper fornix (**Figure 3.3E and F**).

40 eyes of 20 individuals were assessed by two independent observers (IK and AG, observers 3 and 4 respectively) in a cohort of healthy volunteers (median age 42 [range 20-78 years]; 9 females; 20 Asian) for the purpose of determining intra- and inter-observer variability in objective measurement of the inter-canthal curved distances as described in **3.2.2.1**.

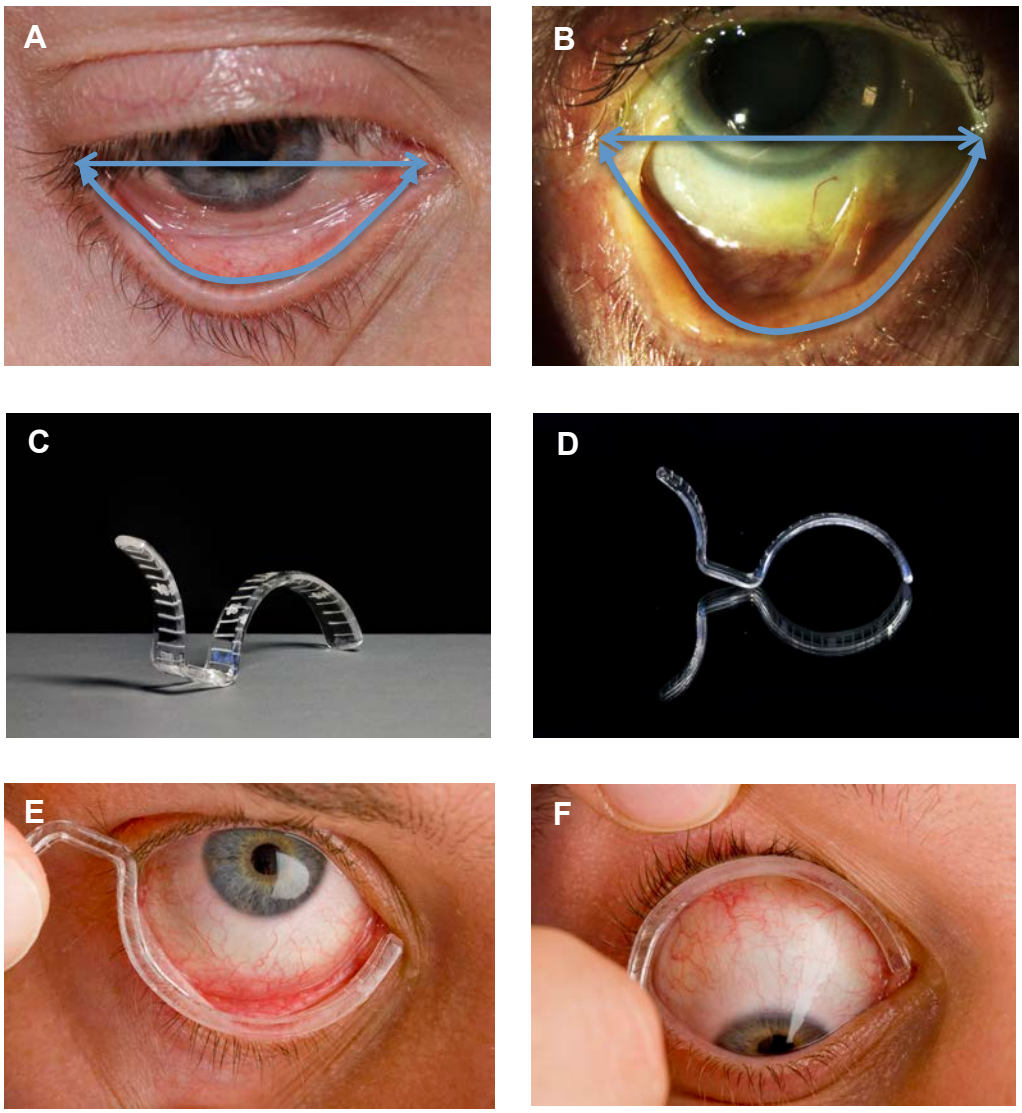


Figure 3.3: Development of BMEC FDM v2. Measurement of symblephara extent and number depends on the ability to enumerate objectively (A). Currently, no accepted system exists for this purpose. We therefore chose to measure the curved distance from canthus to canthus inferiorly (lower) and superiorly (upper), thereby circumventing an under-estimation taken with a straight-line measurement (A and B) using an enhanced FDM (BMEC v2). The symblephara can be measured and subtracted from the total inter-canthal distance. Figures C and D illustrate the short (conventional FDM for lower and upper fornix measurement) and the curved, extended arm for inter-canthal measurement, demonstrated in E and F. Pictures courtesy of Jonas Brane, Sandwell and West Birmingham Hospitals NHS Trust.

3.2.3 Determining an optimal system for accurately phenotyping disease

In order to calculate primary outcome measures of conjunctival fibrosis, based upon the proposed system described by Tauber, measurement of upper and lower fornix depth, number and extent of symblephara between the medial and lateral canthi and the inter-canthal distance were incorporated in to a Clinical Record Form (CRF) (Tauber et al., 1992, Mondino and Brown, 1981, Foster, 1986).

In the absence of validated secondary measurements of disease damage to the cornea, a literature search was undertaken to determine alternative scales employed for PCC using PubMed by GPW/SR/PT. The scoring system described by Sotozono and colleagues was identified as the most relevant system that could be adapted for this purpose (see **Chapter 1.7.3**). Individual relevant scales were trialled at the OSD clinic at BMEC. The following ordinal scales are employed in the Sotozono system for SJS-TEN:

(i) Corneal neovascularisation is quantified by extent as none, to the periphery, to the pupil margin or involvement of central cornea
(ii) Corneal opacification is quantified as none, partial obscuration of iris details, iris details poorly seen with pupil margins just visible and complete obscuration of iris & pupil details.
(iii) Corneal conjunctivalisation as none, < ¼, ¼ to ½, > ½
(iv) Corneal keratinisation as none, < ¼, ¼ to ½, > ½

The captured data also included currently used and accepted scoring system for clinical conjunctival inflammation in OcMMP described by Elder *et al* (Elder, 1997c).

The division of conjunctival and limbal hyperaemia in to quadrants was agreed in collaboration with Prof. John Dart (Consultant Ophthalmologist at MEH) in order to achieve consistency between BMEC and MEH and provide a platform for future validation studies. Although not considered primary or secondary outcome measurements during subsequent longitudinal assessment undertaken during this thesis (see **Chapter 5**), indices relevant to the tear film, eyelids, conjunctiva and sclera were also considered for completion including those described in the Delphi report on dry eyes; eyelid and corneal changes described for the chronic ocular manifestations of SJS and scleritis using standardised criteria for punctal involvement, (Sotozono et al., 2007, Behrens et al., 2006, 2007, Denniston and Murray, 2009). The presence of additional scales for filaments, lid margin disease, peripheral ulcerative keratitis (PUK), conjunctival mucous, keratinisation and corneal ulceration were defined arbitrarily and are subject to on-going validation.

Symptoms and legends used for clinical drawings were based on the scales used at MEH (Elder, 1997c) and BMEC (unpublished). Oral involvement was based on the scale described by Setterfield *et al* (Setterfield et al., 1998). This was used as platform for improving documentation of oral disease and referral/liaison with oral medicine colleagues specialising in MMP and agreed with Mr John Hamburger (JH), Senior Lecturer and Consultant Oral Physician at the University of Birmingham School of Dentistry. The CRF incorporated demographic (age, gender, race), therapeutic and clinical information outlined in the ‘Consensus’ statement (Chan et al., 2002) to aid general clinical documentation.

3.3 Results

3.3.1 Defining the clinical phenotype of patients with OcMMP

The patterns of referral to tertiary centres and duration of symptoms

The geographical origin of our patient cohort is illustrated in **Figure 3.4** and illustrates the distribution of patients presenting to BMEC and MEH (Cohort 1).

The EOD group was younger than the EstD group (62(32-82) versus 69(39-91) years (median, range) (p=0.02)). 19 patients (37 eyes) EOD and 16 EstD patients (32 eyes) were followed for 24 months. Of the 15 patients not reviewed at 24 months, 1 patient in the EstD group died before 12 months follow up and 14 (28%) were referred back to the referring hospital. Of these, 11 had no clinically detectable inflammation at their last visit before discharge from the tertiary centre, two had mild inflammation which continued to be monitored and treated at the local referring hospital and one patient repeatedly failed to attend for follow up despite recall. The remaining cohort (EOD 19; EstD 16) consisted of patients with more severe ocular disease.

Figure 3.4: Geographical distribution of referrals for OcMMP. Map containing Ordnance Survey data (© Crown copyright and database right 2010) showing the combined geographical distribution of referrals (▼) to the two tertiary referral hospitals: Moorfields Eye Hospital, London, UK (circled, L) and the Birmingham and Midland Eye Centre, Birmingham, UK (circled, B). The furthest referral was for Newquay, Cornwall to Moorfields (238 miles).

Extra-ocular features and histological profile of disease

Extra-ocular Features

Extra-ocular mucocutaneous involvement was present in 52% (26/50) of patients at presentation (62% (16/26) of the EOD group; 42% (10/24) of EstD group; p=0.257; **Table 3.2**). A total of 18% (9/50) patients had a history of skin involvement and this was more frequently reported in the EstD group (29.2% (7/24)) than the EOD group (7.7% (2/26)). Conversely, oral involvement was more common in the EOD group (57.7% (15/26)) compared with 20.8% (5/24) in the EstD group (p=0.01).

Biopsies

A total of 87.2% (34/39) of patients who underwent a biopsy, were direct immunofluorescence (DIF) positive. By contrast, indirect immunofluorescence studies were positive in only 34.8% (8/23) of tested individuals, all of whom were also DIF positive (**Table 3.2**). Although five (12.8%) patients were biopsy negative, these patients had clinical features consistent with OcMMP in the absence of other causes of progressive conjunctival scarring. Ten patients in total did not undergo a conjunctival biopsy: seven patients were of advanced age (> 80 years) with co-morbidities where systemic immunosuppression was contraindicated; and the remaining three patients had end-stage disease (defined as Mondino/Foster stage 4) where the sensitivity of a positive DIF conjunctival biopsy is low due to physical destruction of the basement membrane zone architecture (Bernauer et al., 1994).

	All patients	Early-onset disease	Established disease	p value
Total no. of patients	50	26	24	-
Total no. of eyes	99	51	48	-
Male: Female (% Female)	23:27 (54)	11:15 (58)	12:12 (50)	-
Median age (yrs) [Range]	67 (32-91)	62 (32-82)	69 (39-91)	**p=0.02**
Median duration of symptoms (yrs) [Range]	3 (0-41)	1.5 (0-3)	14 (5-41)	**p<0.001**
Patient follow-up (eyes)				
12 months	43 (85)	23 (45)	20 (40)	-
24 months	35 (69)	19 (37)	16 (32)	-
No of patients discharged back to referring hospital ¶				
Total number discharged	14	7	7	-
12 months follow-up	4	1	3	-
24 months follow-up	10	6	4	-
Biopsy *				
DIF +ve	87.2% (34/39)	92% (23/25)	78.6% (11/14)	p=0.33
IIF +ve †	34.8% (8/23)	42.9 % (6/14)	22.2% (2/9)	p=0.4
Extraocular mucocutaneous involvement				
All mucocutaneous tissues	52% (26/50)	62% (16/26)	42% (10/24)	p=0.26
Skin	18% (9/50)	7.7% (2/26)	29.2% (7/24)	-
Oral	40% (20/50)	57.7% (15/26)	20.8% (5/24)	**p=0.01**
Visual Acuity ‡				
Normal: 6/6 ->6/18	80.6% (29/36)	95.2% (20/21)	60% (9/15)	**p<0.01**
Visual impairment: <6/18- 6/60	8.3% (3/36)	4.8% (1/21)	13.3% (2/15)	
Severe visual impairment: 6/60-3/60	0% (0/36)	0% (0/21)	0% (0/15)	
Blind: <3/60	11.1% (4/36)	0% (0/21)	26.7% (4/15)	
‡*Excluded due to other causes*	28% (14/50)	19% (5/26)	38% (9/24)	

Table 3.2: Patient demographics and characteristics in OcMMP. The Early-Onset Disease (EOD) group consisted of a younger cohort of patients with increased frequency of oral MMP. Direct immunofluorescence (DIF) and Indirect immunofluorescence (IIF) refer to the proportion of patients who demonstrated the linear deposition of immunoglobulin G, A or complement (C3) along the basement membrane zone (BMZ) or had measurable titres of immunoglobulin in the serum respectively.

¶ Follow-up: one patient from the Established Disease (EstD) group died before 12 months FU, and one from the EstD group failed to attend between 12 and 24 months and was referred back to their local hospital for continuing care.

* Ten patients in total did not undergo a conjunctival biopsy (7 patients with advanced age (> 80 years) and immunosuppression was systemically contraindicated and the remaining 3 patients had end stage disease (defined as Mondino/Foster stage 4)). Data were missing for one individual and this patient was excluded from analysis.

† All patients who were IIF were also DIF positive. There were no patients who were IIF positive in the absence of positive DIF studies.

‡ VA represents a comparison of visual acuity in the better-seeing eye, after exclusion of other causes of reduced vision such as cataract, glaucoma, age-related macular degeneration and diabetic retinopathy (n=14, EOD 5; EstD 9).

Disease activity, damage and progression

Disease Activity

Information regarding conjunctival inflammation was documented in 95.0% (94/99) eyes at presentation, 90.6% (77/85) eyes by 12 months and 97.1% (67/69) at 24 months follow up.

At presentation, 53% (50/94) of all eyes had clinical evidence of conjunctival inflammation where the majority (76% (38/50 eyes)) were in the EOD group ($p<0.001$) when comparing the worst affected eye (**Figure 3.5A**). Patients with moderate / severe inflammation were also more likely to have EOD. By 12 months follow up (**Figure 3.5A**), inflammation had resolved in 78% (60/77) of all eyes (EOD=83% (35/42) versus EstD=71% (25/35), $p=0.917$) and there were no patients with residual severe conjunctival inflammation. These data were endorsed by McNemar's longitudinal analysis, showing a significant reduction in inflammation in the EOD ($p<0.001$) compared to the EstD group ($p=1.0$).

A recalcitrant group of patients with persistent inflammation not responsive or only partially responsive to treatment was identified in 29.9% (20/67) of eyes examined at 24 months. Interestingly, the persistence of inflammation was independent of group phenotype (EOD=27% (10/37) versus EstD=33.3% (10/30), $p=0.967$; **Figure 3.3A**).

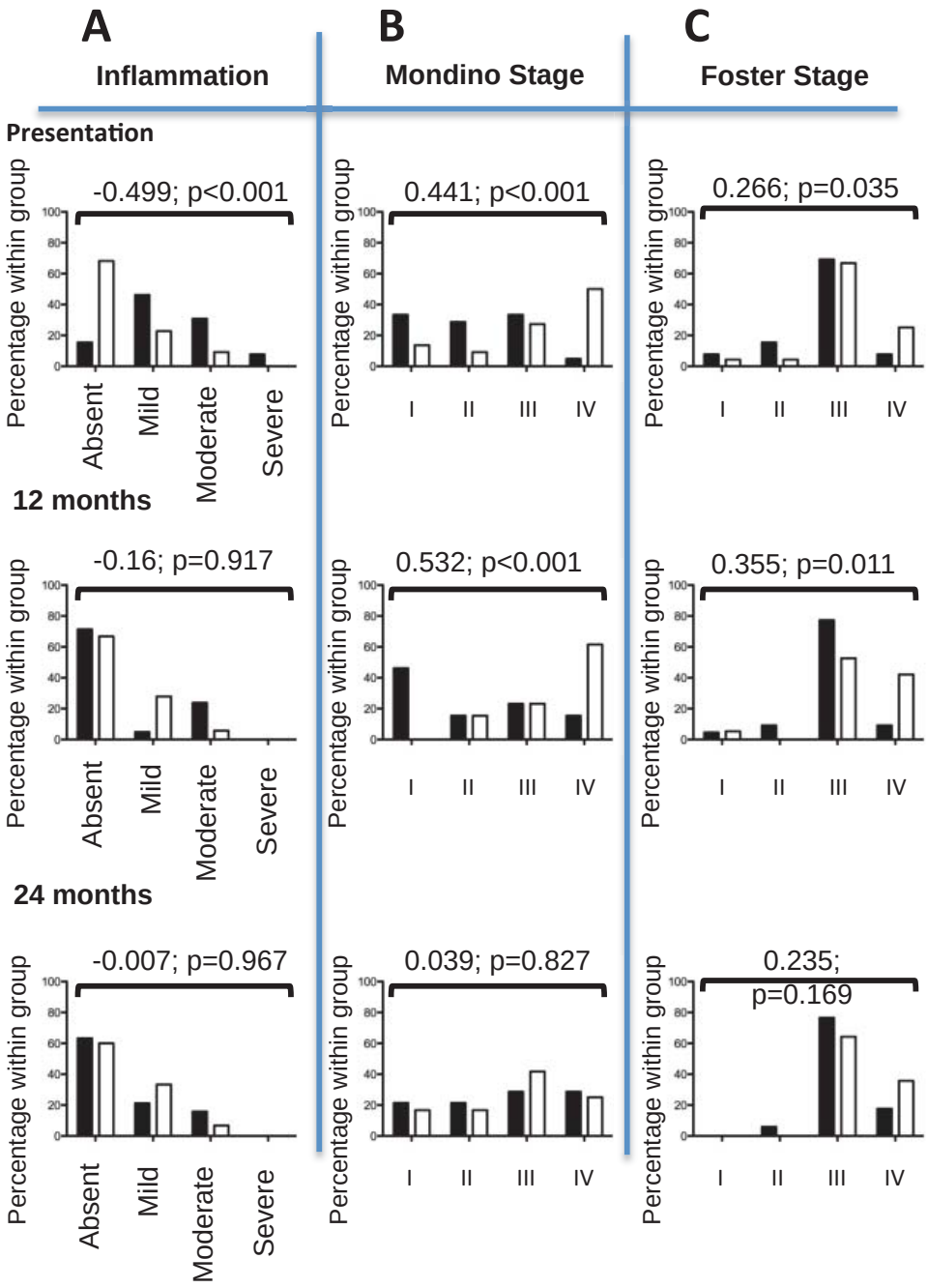


Figure 3.5: **Cross-sectional analysis of clinically detected conjunctival inflammation (A) and ocular staging using Mondino (B) and Foster (C) systems in the worst eye at presentation, 12 months and 24 months follow-up in the EOD (■) and EstD groups (□).** Differences in the extent of conjunctival inflammation and stage of disease were compared between the two groups by rank correlation using Kendal's tau b. At 12 months follow-up, inflammation had resolved in the majority of eyes within both groups, and there were no patients with severe inflammation. By 24 months, 30% of the remaining patients at the tertiary centres had residual inflammation not responsive to treatment. Note that patients in the EstD had more advanced stage of disease compared with the EOD throughout the follow-up period, but there was no difference in the progression rate (worsening of clinical stage of disease) between the two groups. **NB** 14 patients had been referred back to their original hospital by 24 months and 1 had died. These patients have been excluded from the analysis thereby accounting for the apparent increase in the percentage of patients at stage 1 and decrease in the percentage of patients at stage 4 disease during the 12 and 24 months according to the Mondino staging system

Disease Damage

Foster staging of conjunctival fibrosis was more consistently documented at presentation, 12 and 24 months follow up compared with the Mondino staging system. Foster stage was documented in 98.0% (97/99); 90.6% (77/85 eyes) and 87.0% (60/69) eyes versus Mondino stage in 83.4% (83/99); 60% (51/85) and 72.5% (50/69) eyes at presentation, 12 and 24 months respectively. Assessment of the staging system described by Tauber was not possible because of the absence of information regarding the extent of symblephara involvement.

Eyes in the EstD group had more severe conjunctival fibrosis at presentation (Figure 3.3B and 3.3C) gauged by staging systems described by both Mondino (Stage IV: EOD=5%(2/40) versus EstD=39.5%(17/45), $p<0.001$) and Foster (Stage IV: EOD=3.9%(2/51) versus EstD=13%(16/46), $p<0.035$). At 12 months, the EstD group demonstrated significantly advanced stage of disease, irrespective of staging system used (**Figure 3.5B and C**), despite a total of 20.8% of all eyes having progressed according to both Mondino and Foster systems. By 24 months, there was no significant difference in the stage of disease in both groups according to either staging systems (**Figure 3.5B and C**).

There was no significant difference when comparing progression (defined by worsening of clinical stage of disease in at least one eye) amongst patients in both groups neither between presentation and 12 months (Mondino: EOD=33.3%(4/12) versus EstD=23.1%(3/13), $p=0.67$; Foster: EOD=18.2% (4/22) versus EstD=38.9% (7/18) $p=0.173$), nor during the subsequent 12 to 24 months follow-up period

(Mondino: EOD=53.8%(4/22) versus EstD=16.7%(2/12), p=0.10; Foster: EOD=23.5% (4/17) versus EstD=28.6% (4/14) p=1.0) (Fishers exact test).

Disease Progression and the Presence of Conjunctival *Inflammation*

Differences in the rates of progression (defined by an advance in Mondino or Foster staging) were stratified according to the presence or absence of clinically identifiable conjunctival inflammation in at least one eye. There was no significant difference observed between the first and second 12 months follow-up periods (data not shown). Moreover, despite the absence of clinically detectable inflammation, progression of disease occurred in 42% of patients according to the Mondino system (**Figure 3.6A, left panel**), and 16% and 38% of patients according to the Foster (**Figure 3.6A, right panel**) system for each of the 12 months follow-up periods, respectively.

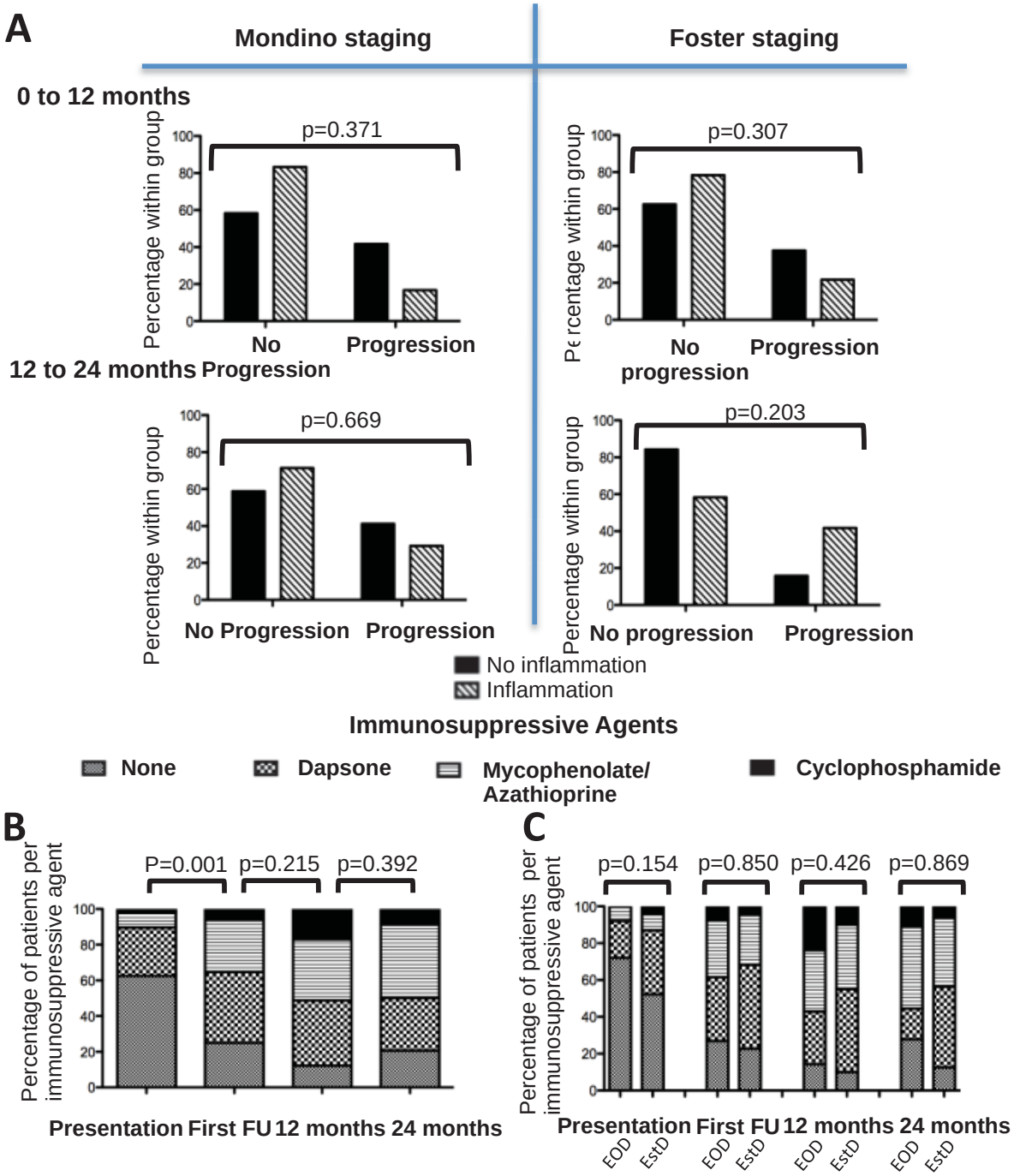


Figure 3.6: Progression rates and immunosuppression. Progression rates, defined by worsening of either Mondino or Foster clinical staging of MMP, in the presence or absence of clinically detectable conjunctival inflammation are shown in the upper composite Panel A. Note there was no significant difference in progression between eyes with clinically detectable inflammation or those that were seemingly uninflamed (Fishers exact test). The percentage of patients requiring immunosuppression at presentation, following the first follow up (FU) clinic visit, 12 months and 24 months follow-up time points are shown in the lower panels B and C. Immunosuppression strategies were ranked according to the hierarchy described by Rauz et al (Ophthalmology 2005). Overall, a significant initiation or escalation in 'strategic-step' was required at the first FU visit (Panel B; McNemar's test), but this did not significantly differ when the Early Onset (EoD) and Established Disease (EstD) groups were compared (Panel C; Kendal's tau b). By 12 months follow-up, 5 patients stabilised on immunosuppression and were discharged back to their originating hospitals, and similarly a further 10 between the 12 and 24 months follow-up.

Therapeutic Intervention at tertiary referral centre

Surgical Intervention

At initial presentation to the specialist units, 32% (16/50) of patients had previously undergone eyelid or fornix reconstructive surgery by the referring hospital with the majority of cases being performed in the EstD group (p<0.01) (Table 3.3). Although both groups required oculoplastic surgical intervention at the tertiary hospitals, this did not differ between the two groups, nor during the first and second 12 months follow-up periods.

Surgical intervention	Total	Early-onset disease	Established disease	p value
Presentation				
Eyelid/fornix surgery	32% (16/50)	11.5% (3/26)	54.2% (13/24)	**p<0.01**
Corneal intervention	2% (1/50)	0% (0/26)	4.2% (1/24)	NS (p=0.480)
Cataract surgery	20% (10/50)	23.1% (6/26)	16.7% (4/24)	NS (p=0.728)
Glaucoma surgery	4% (2/50)	7.7% (2/26)	0% (0/24)	NS (p=0.491)
0 to 12 months				
Eyelid/fornix surgery	27.9% (12/43)	34.8% (8/23)	20% (4/20)	NS (p=0.327)
Corneal intervention	7% (3/43)	4.3% (1/23)	10% (2/20)	NS (p=0.590)
Cataract surgery	7% (3/43)	0% (0/23)	15% (3/20)	NS (p=0.092)
Glaucoma surgery	2.3% (1/43)	0% (0/23)	5% (1/20)	NS (p=0.465)
12 to 24 months				
Eyelid/fornix surgery	25.7% (9/35)	31.6% (6/19)	18.8% (3/16)	NS (p=0.460)
Corneal intervention	5.7% (2/35)	5.3% (1/19)	6.3% (1/16)	NS (p=1.0)
Cataract surgery	11.4% (4/35)	5.3% (1/19)	18.8% (3/16)	NS (p=0.312)
Glaucoma surgery	0% (0/35)	0% (0/19)	0% (0/16)	-

Table 3.3 Surgical intervention in OcMMP. Figures in both groups at presentation, between presentation and 12 months and between 12 and 24 months follow up periods are shown. Differences were compared by the Fishers exact test.

Immunosuppression Strategies

40% (20/50) of all patients were on immunosuppression at the time of referral. After first consultation, 36% (18/50) required initiation and 12% (6/50) a switch to a more potent immuno-modulatory treatment representing a significant overall ‘step-up’ on the step ladder approach (p=0.001) (**Figure 3.6B**). During the first 12 months follow-up period, a further 30% (13/43) of patients required 'step-up' treatment (p=0.215) (Figure 3.4B) equating to a total of 88% (38/43) of patients requiring initiation or changes in immunosuppression at presentation or during the first year of follow-up. By 24 months, immunosuppression could be withdrawn in only one patient but no further escalation in therapy was required. 28% patients had stabilised and were discharged to the referring unit for immunosuppression monitoring (**Table 3.2**). There was no statistical difference between the requirement for immunosuppression for each of the patient groups at each of the time points (**Figure 3.6C**).

The most commonly used drug by the referring unit was Dapsone (26% (n=13)) followed by either Azathioprine or Mycophenolate (8% (n=4)) with only one patient on Cyclophosphamide (2% (n=1)). The majority of these patients were commenced on Azathioprine or Mycophenolate 10% (n=5) or switched to these drugs from Dapsone 10% (n=5). Two further patients required oral Cyclophosphamide to control inflammation. By 12 months, an additional seven patients had initiated Cyclophosphamide therapy and this was either because of the presence of exuberant inflammation (n=3) not adequately responding to less potent agents (Two requiring i.v. Methylprednisolone) or there was a requirement for an increase in immuno-modulation prior to ocular or eyelid reconstructive surgery. Resolution of

inflammation occurred in two patients who were “stepped-down” to less potent agents. By 24 months, oral Cyclophosphamide was withdrawn in three patients (due to completing the maximum safe duration of therapy of approximately 14 months i.e. a cumulative dose (oral or i.v.) of <20g. The majority (40%) of patients were maintained on either Mycophenolate or Azathioprine. There was no statistical difference in the immunosuppressive agents used between the EOD and EstD groups. Intravenous immunoglobulin or biological agents were not administered during the course of this study.

3.3.2 Optimising a system for objectively measuring the cicatricial process

3.3.2.1 FDM (BMEC Version 1)

Fornix Depth

A cohort of 51 eyes of 26 patients were evaluated (Cohort 2). 100% of patients had >1 assessments of their fornices.

Lower Fornix Assessment

Intra-observer variation

Triplicate measurements of FDM (BMEC v1) readings of the same anatomical position by each observer (central lower fornix) demonstrated exact agreement of 86% (42/49) and 89% (41/46) of measurements within observer 1 and observer 2 respectively. When allowing for 1mm 'tolerance' (approximating to 10% of the normal lower fornix, see **Table 3.1**), 100% of intra-observer observations fell within 1mm for both observers.

Inter-observer variation

Inter-observer variation between the subjective and objective measurements of the central lower fornix by the two observers was also assessed. Assessment of the lower fornix shrinkage was expressed as a percentage for both subjective and objective estimations, the latter using the age correction factor described in the methods (Schwab et al., 1992).

The mean difference in calculated percentage fornix depth using measurements obtained from the FDM (BMEC v1) by observer 1 and 2 was 1.19% and with a continuity correction, the 95% limits of agreement (±2SD) were narrower for inter-observer objective (FDM) measurements versus those obtained subjectively (-15 and + 20%) (**Figure 3.7**). The inter-observer agreement within the 10% allowance (i.e. approx. ±1mm) of total lower fornix depth was 86% (44/51) (**Figure 3.8**).

In contrast, the inter-observer mean difference in subjective estimation of percentage fornix depth was -1.86% and with a continuity correction, the 95% limits of agreements (±2 Standard Deviations, SD) were between -30 and +25% (**Figure 3.7**). Only 63% (32/51) of the subjective measurements taken by the two observers agreed to within a 10% allowance of total lower fornix depth (**Figure 3.8**).

These data highlight that the FDM (BMEC v1) afforded greater consistency in fornix depth measurement by each observer (intra-observer variation), and between observers (inter-observer variation).

Upper Fornix Assessment

Intra-observer variation

Triplicate measurements (to assess intra-observer variation) of FDM (BMEC v1) readings of the same anatomical position of the central upper fornix by each observer demonstrated minimal variation (i.e. identical objective measurements) in 88% (45/51) and 70% (33/47) of measurements by observer 1 and observer 2 respectively. There are no data regarding normal upper fornix depth in the published literature,

precluding calculations for age-based corrections and percentage fornix depth foreshortening and tolerance threshold.

Inter-observer variation

Inter-observer variation of the upper fornix showed a mean difference in fornix depth measurement using the FDM (BMEC v1) for observer 1 and 2 of 0.57mm with 95% agreement (± 2SD) of -2 and + 3mm (**Figure 3.9**). The absence of normal upper fornix values precludes evaluation of the 10% allowance, however 84.3% (43/51) of upper fornix measurements were within 1SD of the mean difference of 0.57mm (+2 to -1mm of the mean with a continuity correction).

Patient comfort and tolerance

The FDM (BMEC v1) was well tolerated by patients with only a few patients (3) experiencing mild discomfort with upper fornix measurement, despite repeated measurements. None of these patients reported prolonged discomfort or pain.

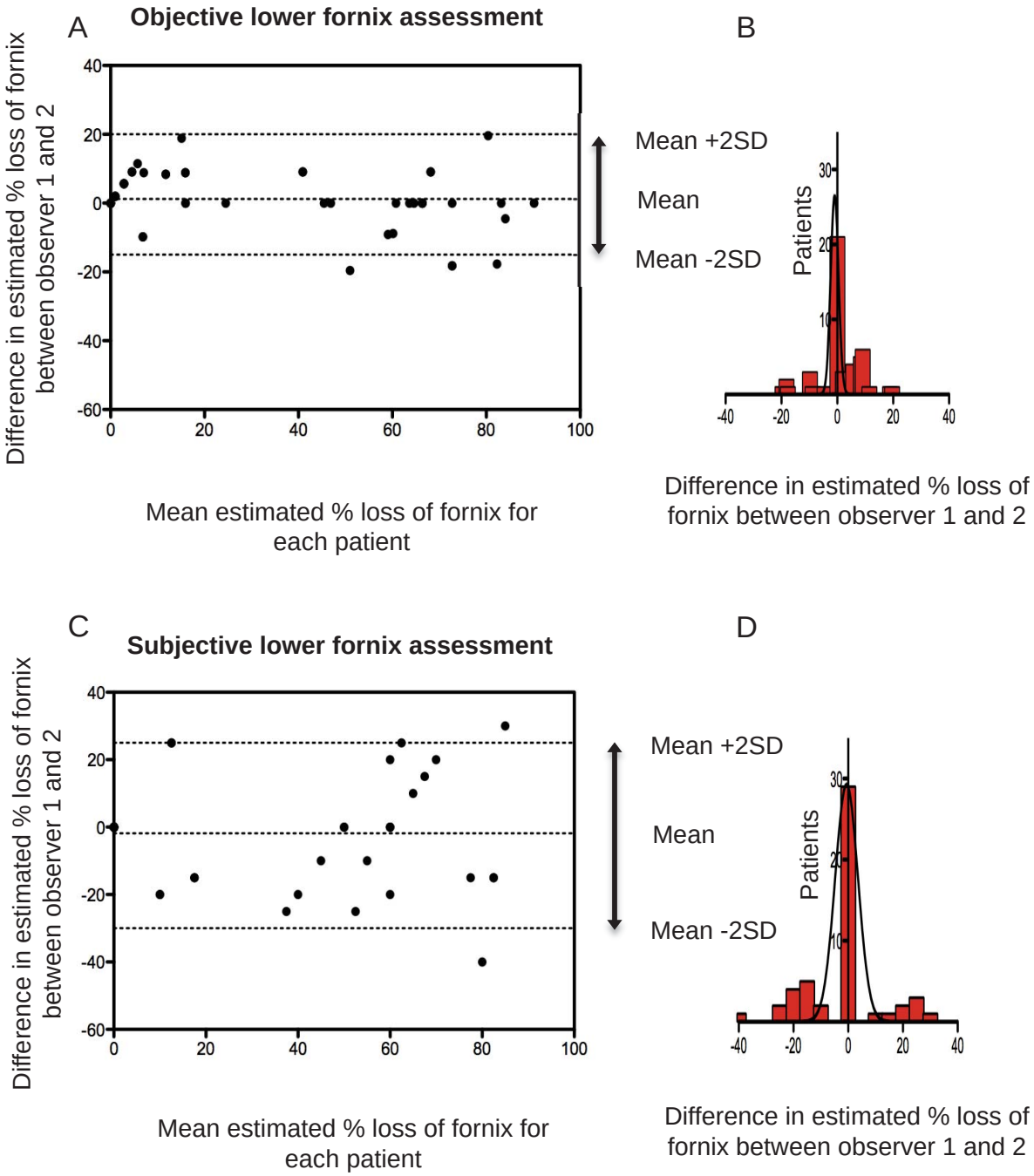
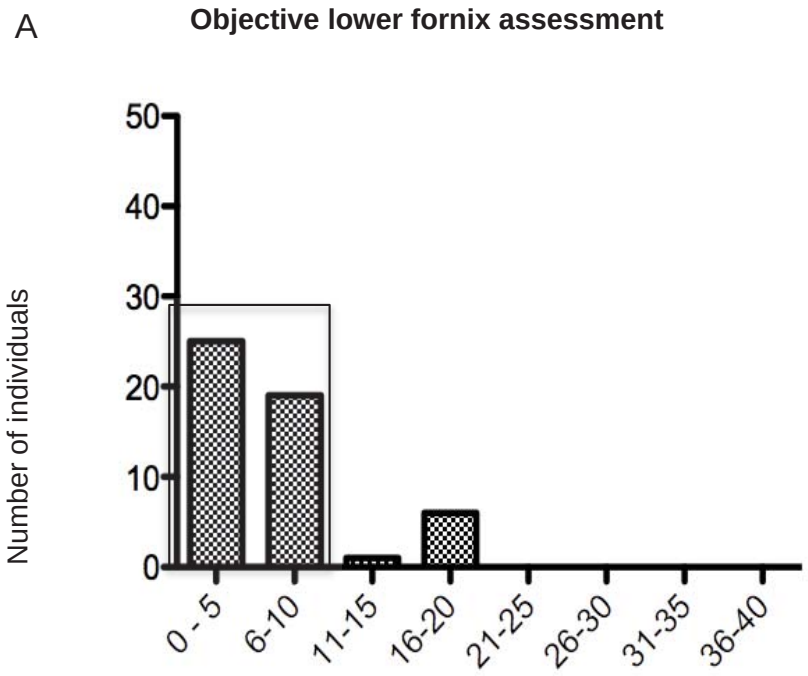


Figure 3.7: Lower fornix assessment using an FDM. Bland-Altman plots showing inter observer variation in lower fornix assessment (left panels; A objective and C subjective). Some data points are identical and therefore overlay each other on the figures. The % difference in assessment between observer 1 and 2 is plotted against the mean % loss of fornix for each eye using the BMEC FDM v1. If there was a completely normal fornix this is represented as 0% loss of fornix on the x axis. Note the increase in the 95% limits of agreement (±2SD) for subjective assessment (arrowed), demonstrated also by the histograms (right panels, B and D).



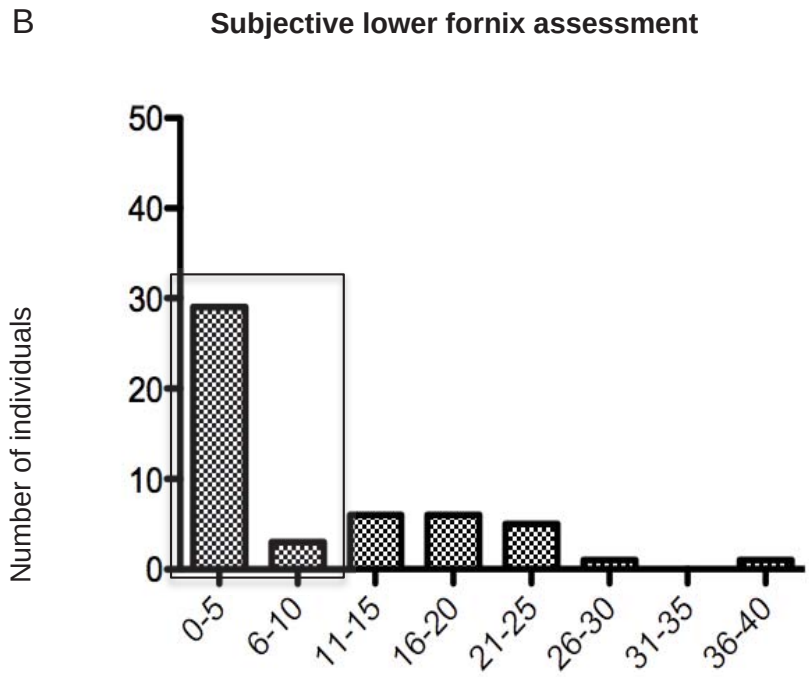


Figure 3.8: Difference in objective (FDM) and subjective assessment of lower fornix. The histograms demonstrate the difference in objective and subjective lower fornix assessment between observer 1 and 2 using the BMEC FDM v1. The number of individuals which agree to within a 10% 'allowance' are boxed and are higher for objective measurements A 86% (44/51), compared to subjective measurement, B, 63% (32/51).

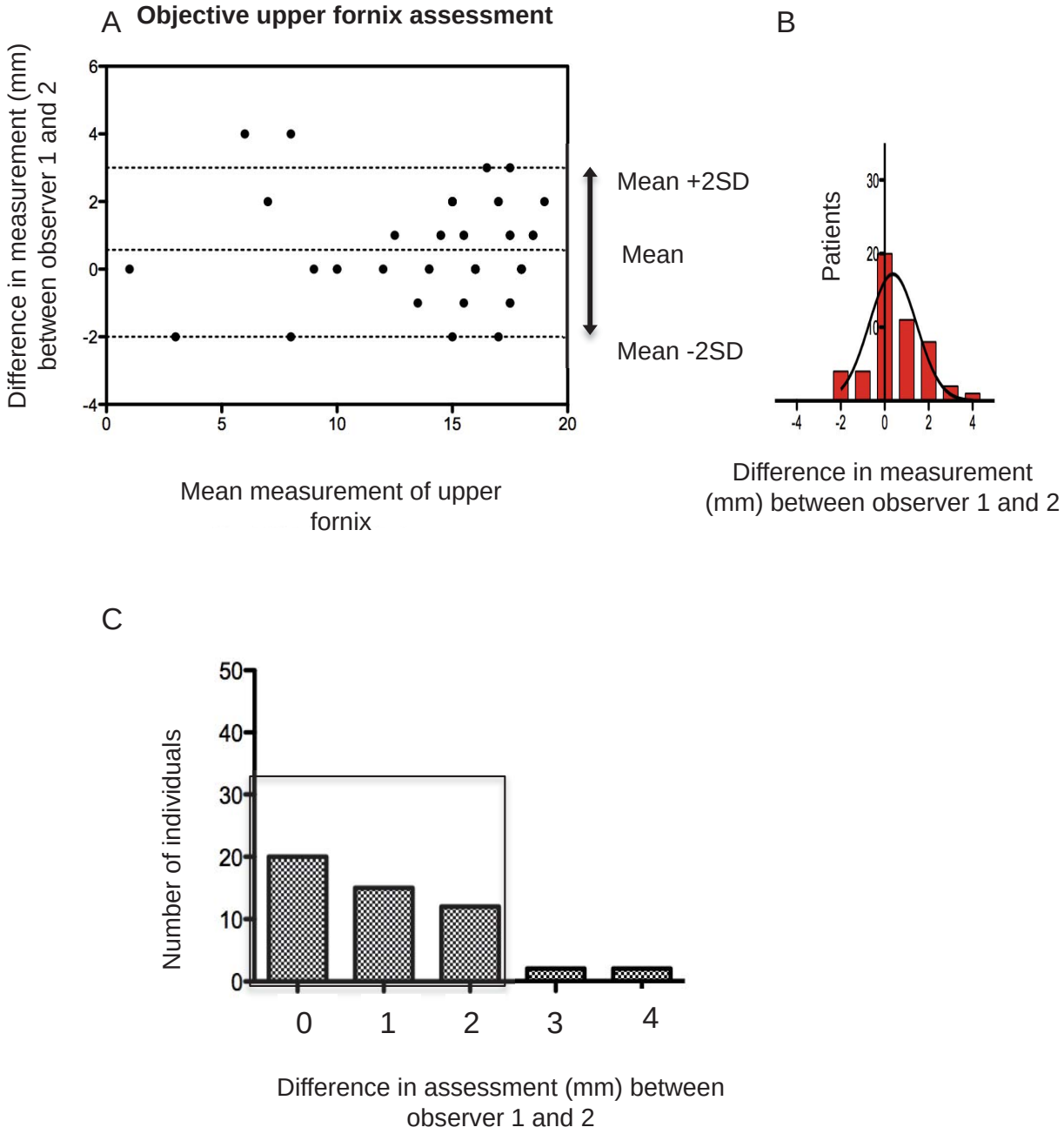


Figure 3.9: The FDM as a tool for assessing the upper fornix. The Bland-Altman plot (left upper panel, A) shows inter observer variation in upper fornix assessment. As there are no defined limits for the upper fornix, the calculations are in mm. The mm difference in assessment between observer 1 and 2 is plotted against mean mm measurement for each patient using the BMEC FDM v1. The 95% limits of agreement are vertically arrowed and represented also by the histogram (right upper panel B). The lower histogram (panel C) demonstrates the difference in upper fornix assessment between observer 1 and 2; the 10% allowance cannot be calculated in the absence of normal upper fornix values but 71% (36/51) of measurements were within 1mm and 92% (47/51) of observations were within 2mm of each other.

3.3.2.2 FDM (BMEC Version 2)

Triplicate measurements (to assess intra-observer variation) of FDM (BMEC v2) readings of the lower inter-canthal distance same location by each observer demonstrated no variation (i.e. identical objective measurement) in 53% (21/40) and 73% (29/40) of measurements by observer 3 and observer 4 respectively. Intra-observer variation of upper inter-canthal distance demonstrated no variation (i.e. identical objective measurement) in 63% (25/40) and 80% (32/40) of measurements by observer 3 and observer 4 respectively. If allowing for 1mm difference ('tolerance'), 100% of intra-observer readings were within 1mm. Like upper fornix depth, there are no data regarding inter-canthal distances in the literature, preventing calculations for age-based corrections.

Inter-observer variation of the lower inter-canthal distance showed a mean difference in fornix depth measurement using the BEMC v2 FDM for observer 3 and 4 of -1.6mm. The 95% limits of agreement (±2SD) were between -3.5 and + 0mm (**Figure 3.10A**). 88% (35/40) of lower fornix measurements were within 1SD of the mean difference of -1.6 mm (-3 to -1 mm of the mean with a continuity correction). Inter-observer variation of the upper inter-canthal distance showed a mean difference in fornix depth measurement using the BMEC v2 FDM for observer 3 and 4 of -1.3mm. The 95% limits of agreement (±2SD) were between -3 and + 1mm (**Figure 3.10B***).* 100% (40/40) of upper fornix measurements were within 1SD of the mean difference of -1.3 mm (-2 to 0 mm of the mean with a continuity correction).

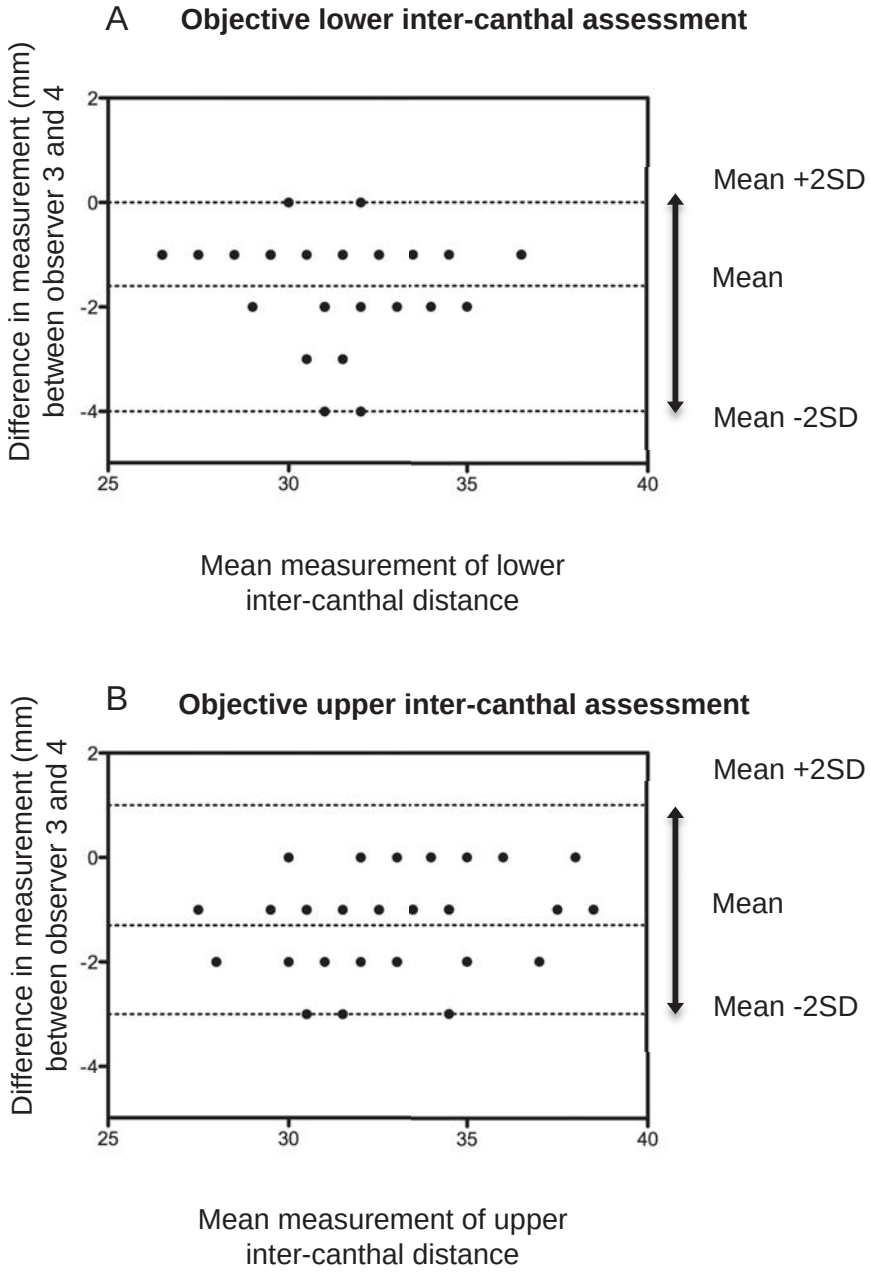


Figure 3.10: Inter-canthal agreement using an adapted FDM. The Bland-Altman plot (upper panel, A) shows inter observer variation in lower inter-canthal assessment using BMEC v2 FDM. The mm difference in assessment between observer 1 and 2 is plotted against mean mm measurement for each patient. The 95% limits of agreement are vertically arrowed. The lower Bland-Altman plot (lower Panel B) represents the upper inter-canthal assessment.

3.3.3 Optimisation of clinical documentation and assessment

A summary of the parameters agreed for the CRF are shown in **Table 3.4** and the full CRF can be found in **Appendix 7.1**. The CRFs were initially trialled in the OSD clinic at BMEC by experienced anterior segment specialists (SR/TS). In particular, the Sotozono scales were scrutinised to determine their practicality for quantifying the extent of corneal neovascularisation opacification, conjunctivalisation and keratinisation.

The development of the CRF, combined with the FDM outlined in 3.3.2 allowed the Tauber scale for the lower fornix to be calculated by the percentage of lower fornix shrinkage (**3.2.2.1**), percentage lower canthal involvement by symblephara (mm)/inter-canthal distance (mm) (denoted by horizontal arrows in the CRF) and number of symblephara.

During initial trialling of the secondary outcome measures (corneal neovascularisation, opacification, conjunctivalisation and keratinisation) it was determined that the neovascularisation should be expanded to reflect the degrees of involvement by clock hours e.g. to order to reflect early but extensive peripheral vascularisation in some individuals or a single central blood vessel. Furthermore, conjunctivalisation and keratinisation were changed from none, < ¼, ¼ to ½, > ½ to none, <⅓, ⅓-⅔, >⅔ as this was deemed to be a more incremental ordinal scale. Opacification was also changed to reflect this scale. The format of the CRF was also developed so that a consistent scale in a 'checklist' format of clinical parameters could be transferred to the linked-anonymised database. Full-scale validation, requiring internationally agreed consensus, was beyond the scope of this thesis.

Tear Film

Present None	Reduced Yes	Absent	.....	Break up time (s) Tear meniscus Filaments present	..... Present None	Reduced Yes	Absent	
Normal	<10	<5	<2	Schirmer's test in mm	Normal	<10	<5	<2

Lids and Lashes

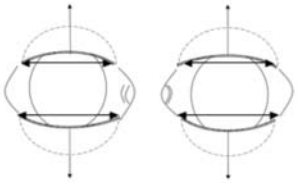
Normal	Anterior	Posterior	Metaplasia or lid notch	Lid Margin Disease	Normal	Anterior	Posterior	Metaplasia or lid notch
Normal	Lash metaplasia	Trichiasis	Entropion	Entropion	Normal	Lash metaplasia	Trichiasis	Entropion
Open	Small	Occluded	Plugged	Upper puncti	Open	Small	Occluded	Plugged
Open	Small	Occluded	Plugged	Lower puncti	Open	Small	Occluded	Plugged
Absent	Mild	Moderate	Severe	Lagophthalmos	Absent	Mild	Moderate	Severe

Conjunctiva/Limbitis

None (0)	Mild (1)	Mod (2)	Sev (3)	Inflammation (score each quadrant)	None (0)	Mild (1)	Mod (2)	Sev (3)
			No of bulbar quadrants of ulceration	Conjunctival ulceration	No of bulbar quadrants of ulceration			
None (0)	Mild (i)	Mod (ii)	Sev (iii) or ischaemia	Limbitis (score each inner quadrant)	None (0)	Mild (i)	Mod (ii)	Sev (iii) or ischaemia
None	<3'	3-9'	>9'	Peripheral ulcerative keratitis (PUK)	None	<3'	3-9'	>9'
None	<50%	>50%	Perforation	PUK depth	None	<50%	>50%	Perforation
None	Mild	Moderate	Pseudo-membrane	Conjunctival mucous	None	Mild	Moderate	Pseudo-membrane
None	Mild	Severe		Keratinisation	None	Mild	Severe	

Upper Fornix Assessment

		.....	Horizontal obliteration by fibrosis (mm)	.....			
		.....	Central Depth (mm)	.....			
None	Involvement in mm	No.	Horizontal involvement by symblephara	None	Involvement in mm	No	



Lower Fornix Assessment

		.....	Central Depth (mm)	.....			
		.....	Horizontal obliteration by fibrosis (mm)	.....			
None	Involvement in mm	No.	Horizontal involvement by symblephara	None	Involvement in mm	No.	
None	Ankyloblepharon Limitation	Frozen globe	Limitation in motility	None	Ankyloblepharon Limitation	Frozen globe	

Cornea

Present	Diminished	Absent		Sensation	Present	Diminished	Absent	
None	<1/3	1/3 to 2/3	>2/3	Punctate Keratopathy	None	<1/3	1/3 to 2/3	>2/3
None	<1/3	1/3 to 2/3	>2/3	Conjunctivalisation	None	<1/3	1/3 to 2/3	>2/3
None	<1/3	1/3 to 2/3	>2/3	Keratinisation	None	<1/3	1/3 to 2/3	>2/3
None	<3'	3-9'	>9'	Neovascularisation - total clock hours	None	<3'	3-9'	>9'
None	Periphery	Pupil margin	Central 4 mm	Neovascularisation - Encroachment to visual axis	None	Periphery	Pupil margin	Central 4 mm
None	<1/3	1/3 to 2/3	>2/3	Opacification /Scarring	None	<1/3	1/3 to 2/3	>2/3
None	<1/3	1/3 to 2/3	>2/3	Central corneal ulcer	None	<1/3	1/3 to 2/3	>2/3
None	<50%	>50%	Perforation	Central ulcer depth	None	<50%	>50%	Perforation

Sclera

None	Diffuse/ Nodular	Necrotising	Necrotising inflamed	Anterior scleritis	None	Diffuse/ Nodular	Necrotising	Necrotising inflamed
None	Present			Posterior scleritis	None	Present		

Table 3.4: Examination assessment scores developed for the Clinical Record From (CRF). Primary Outcome Measures for OcMMP were derived from the ability to determine forniceal fornix depth, symblephara number and mm of involvement (primary outcome measures) and corneal neovascularisation, opacification, conjunctivalisation and keratinisation (secondary outcome measures)

3.4 Discussion

3.4.1 Defining the clinical phenotype of patients with OcMMP

In the retrospective study undertaken at BMEC and MEH, two disease phenotypes of OcMMP were statistically defined: (i) those with early onset disease (EOD) who were characterised as having less advanced disease stage but significantly greater conjunctival inflammation, and (ii) those patients with established disease (EstD) who had less clinically identifiable inflammation but more advanced stage of disease. Although 40% of the patient cohort were on existing systemic immunosuppression, the majority of patents required initiation or escalation in systemic immunosuppression following either their first clinic visit, or during the first year of follow up in order to control inflammation, facilitate corrective eyelid surgery or prevent further progression in already advanced disease states. Despite these measures, 20.8% of eyes demonstrated disease progression during the first 12 months and another 20.8% between 12 and 24 months; and this progression was independent of the EOD or EstD clinical phenotypes.

Could the advanced stages seen in the established group therefore be explained simply by longer duration of disease? For the patients to have progressed, the working hypothesis outlined (**Section 1.9**) is that the disease may have been driven by underlying inflammatory processes, even if this was not clinically detectable. These results reinforce the observation that OcMMP may progress at any stage of disease (Mondino and Brown, 1981, Elder et al., 1996) and more importantly, progression rates amongst eyes that are clinically inflamed and those that are not, do not differ. These data endorse previously reported literature (Mondino and Brown,

1983, Saw et al., 2008, Elder, 1997b) and possibly signify a molecular, fibrotic process independent of inflammation which can be seen clinically.

Accurately documenting progression presents difficulties, not least because of the sensitivity of the currently employed staging systems. The increased sensitivity of the Mondino staging system (Elder et al., 1996, Saw et al., 2008) may reflect the ability to discriminate the stage of disease in both groups at presentation and 12 month, but documentation was superior for Foster staging system. The staging systems currently employed also rely upon subjective assessment of conjunctival fibrosis and obliteration, indicating that judgment of progression is open to individual interpretation. This was reflected in a patient documented as stage 4 at 12 months being documented at stage 2 at 24 months. Information regarding horizontal obliteration of the fornix by symblephara was also not routinely documented, precluding employment of the proposed staging system described by Tauber (Tauber et al., 1992). There is also no standardised method of documenting disease progression by percentage obliteration in the upper fornix, although the disease is clearly not confined to the inferior conjunctival surface.

The difficulty lies not only in identifying early disease and determining which patients may progress, but also recognising when this is happening. ‘Activity’ and ‘damage’ indices have been validated and accepted for a number of autoimmune conditions including Systemic Lupus Erythematosis (SLE) and Primary Sjögren’s Syndrome (PSS) (Yee et al., 2007, Gladman et al., 2000, Bowman et al., 2007, Barry et al., 2008). These indices facilitate not only comparison of clinical cohorts worldwide, but also inform clinical trials specifically those targeting therapeutic intervention. As such,

clearer strategies for discriminating MMP disease 'activity' and 'damage' are necessary to afford a uniform language and understanding when describing OcMMP phenotypes, before molecular targeting and evaluation of novel therapeutic approaches through randomised controlled trials can be considered. The employment of an agreed CRF as described in **Section 3.3.3** may help make these distinctions.

These difficulties in determining activity and progression highlight the challenge in directing appropriate therapy. The issue of suboptimal therapeutic immuno-modulation of disease course has been widely described (Foster et al., 1982, Mondino and Brown, 1983, Saw et al., 2008, Thorne et al., 2008, Tauber et al., 1991, Miserocchi et al., 2002). A recalcitrant group of patients with persistent mild or moderate inflammation is highlighted, in keeping with the findings of others (Saw et al., 2008, Thorne et al., 2008, Rauz et al., 2005a). Unfortunately, there is also a disease subset that either is completely refractory to conventional immunosuppression or relapses despite initial success (Thorne et al., 2008). A few isolated case reports indicate that 'biologic' agents such as rituximab (anti-CD20) or infliximab (anti-TNFα) may be beneficial in some of these patients, but as randomised trials are lacking, funding for such treatment in the UK prohibits regular use (Segura et al., 2007, Canizares et al., 2006, Heffernan and Bentley, 2006, John et al., 2007, Taverna et al., 2007, Ross et al., 2009). These data re-emphasise the fact that the pathogenesis of OcMMP is not resolved and strengthen the case for further study of clinically involved and seemingly uninvolved mucous membranes (Bernauer et al., 1993a, Rice and Foster, 1990, Sacks et al., 1989).

The reasons for a delay in presentation in the EstD group are not clear. It is possible that the clinical features or the severity of the disease may not have been recognised until late, as early symptoms may have been insidious and non-specific (Foster et al., 1982, Bernauer, 1997). A variable duration of disease and course have been described by others (Thorne et al., 2008), but the true definition of early disease in the context of OcMMP is not known. This may well lie under the 3 year duration of symptoms statistically defined in our cohort, and this is particularly relevant if disease activity or progression is initially either subtle or sub-clinical. Experience in other more common autoimmune diseases such as Rheumatoid Arthritis, point to the clinician actively pursuing identification of early disease to enable early therapeutic intervention, in order to limit tissue damage (Sheppard et al., 2008). In the light of the potential for disease progression in both early or late onset OcMMP disease forms, irrespective of whether inflammation is clinically detected or not, it may be prudent for ophthalmologists to take precedence from the rheumatological concepts for capturing early disease, and adopt a similar approach.

Many of our patients travelled long distances to our centres and this may represent a barrier to early tertiary-care of this rare disease, resulting in initiation of suboptimal immunomodulation and/or surgery. Stringent efforts to identify features characteristic of OcMMP are necessary in order to avoid missing an early diagnosis. Where local diagnosis or management is not possible, or where the identification of high risk features including severe/ refractory inflammation or evidence of progression is manifest, prompt referral of cases with OcMMP to specialised tertiary centres is essential for optimisation of immunosuppression aimed at limiting long-term tissue damage.

Whilst this study is limited by its retrospective nature and bias in the study population towards a more severe clinical phenotype, a high proportion (48%) of patients required initiation or a switch to more potent immunosuppression following referral to our tertiary centres. Additionally, 28% of patients were eventually returned to their local referring unit for monitoring after stabilisation of disease. Most importantly however, up to 42% of patients (irrespective of disease phenotype, early or late) continued to demonstrate progressive conjunctival scarring in the absence of clinically detectable inflammation. A greater understanding of disease pathology is required to facilitate earlier recognition of disease, improved activity and damage scores, and more accurate therapeutic targeting, specifically for patients recalcitrant to existing immunomodulatory therapy.

3.1.2 Determining an objective method for recording the conjunctival cicatricial process

Determining progression of disease in PCC is a challenging aspect of patient management, as demonstrated in the retrospective analysis of patients at BMEC and MEH. As outlined, this relies upon accurate documentation of disease, in particular conjunctiva shrinkage of the fornices. Subjective assessment of fornix foreshortening is inconsistent and variable, which led to the development of the FDM to enable objective FD measurement in our clinic. The use of such devices have been previously described for the assessment of scarring in cicatrising conjunctivitis, (Schwab et al., 1992, Barabino et al., 2003, Kawakita et al., 2008), but are not commercially available.

It was apparent that there was a need to design a custom-made FDM that was comfortable, accurate and demonstrated low intra- and inter-observer variability. Both v1 and v2 of the FDMs were designed and made using industry-standard software and machinery taking in to account the curvature of the globe, whilst assessing the upper fornix depth. The BMEC v1 and v2 FDMs demonstrated low intra- and inter-observer variability enabling repeatable and reproducible measurements of both upper and lower fornix depths and inter-canthal distance. This represents an important advantage in facilitating both accurate documentation and ensuring robust clinical documentation of disease stage takes place.

The FDM has the potential to measure upper fornix depth, currently not routinely employed in clinical assessment, nor taken into account during ocular staging systems which calculate percentage obliteration of the fornix (Mondino and Brown, 1981, Tauber et al., 1992). The cicatricial process is not confined to the lower fornix and the sight threatening sequelae including subtarsal fibrosis, upper lid entropion with subsequent lash trauma are clinically apparent. Certainly this is reflected in the staging system described by Foster, where fornix shortening or symblepharon of any degree is taken in to account (Foster, 1986). The potential for decreased sensitivity in relying on this system alone have been discussed and illustrate an obvious advantage of using the FDM.

The larger depth of the upper fornix and difficulty of access explains the omission of detailed upper fornix pathology in currently recognised scoring systems (Tauber et al., 1992, Mondino and Brown, 1981). Perhaps not surprisingly therefore, there appears to be little data in the ophthalmic literature regarding the anthropology and normal

depth of the upper fornix. Kawakita and colleagues have recently discussed the use of a non-curved 150mm x 2mm FDM in Japanese patients with Stevens-Johnson syndrome and healthy volunteers (Kawakita et al., 2008). They found that the mean supero-temporal and supero-nasal upper fornix depths were 14.1mm ±2.5mm in normal Japanese individuals. My findings demonstrate a median central upper fornix depth of 16mm, even in the presence of recognised causes of cicatricial conjunctivitis. Direct comparison is not possible due to the central location of measurement in this study, the inclusion of different ethnic groups and the small numbers employed in both studies. However, the central upper fornix depth in other healthy populations (Caucasian, Afro-Caribbean etc.) may be smaller or larger and clearly illustrates the need for a population-based study of the normal age-based upper fornix depths to facilitate percentage shrinkage calculations in cicatricial conjunctivitides. This has been highlighted in the context of other diseases affecting the size of the upper fornix such as the giant fornix syndrome (Rose, 2005). These pieces of data illustrate the need for population-based studies of the normal age-based upper fornix depths to facilitate percentage shrinkage calculations in conjunctival scarring diseases; and these are is currently being undertaken at BMEC and MEH.

The custom-designed FDM is well tolerated by patients with only 3 patients experiencing mild discomfort during assessment of the upper fornix. The FDM demonstrates low intra- and inter-observer variability enabling repeatable and reproducible measurements of lower fornix depths. The custom-design of an FDM using industry-standard jewellery software and machinery and curved to fit for the globe provides an accurate and comfortable means of assessing lower fornix depth. Furthermore, it has potential to measure upper fornix depth, currently not routinely

employed in clinical practice. By adapting the longer ‘arm’ of the FDM, allowing for the natural curvature of the globe, the FDM could be utilised to measure the inter-canthal distance. This has the advantage of facilitating assessment of the degree and number of symblephara, currently not documented in our clinics. The use of an objective mm scale allows a simpler assessment at baseline and follow-up of lower and upper fornix depth and the degree of symblephara.

3.4.3 Determining an optimal system for accurately phenotyping disease

In the retrospective series evaluated, current clinical practices of documentation were evaluated. Documentation of clinical activity (conjunctival inflammation) was 95% at presentation and damage according to the Foster staging 98% indicating that these scales were documented consistently in the outpatient setting. Unfortunately the Mondino staging system was documented only 84% in the retrospective analysis undertaken. Furthermore, Tauber staging was not possible because of the absence of information relating to symblephara extent.

The CRF takes the form of a clinical ‘free text’ sheet with accompanying checklist for parameters relating to the tear film, the eyelids, the conjunctiva, the fornices, the cornea and the sclera. It has been designed to facilitate documentation and is complimented by objective assessment by the use of the FDM. It is hoped that it will enable a robust, consistent means of documenting damage scales that have been validated in the context of OcMMP such as those described by Mondino, Foster and Tauber, but also offer the potential for further elucidation of sight-threatening corneal damage (Mondino and Brown, 1981, Foster, 1986, Tauber et al., 1992).

As stated, a full validation of corneal and indeed other damage indices was beyond the scope of this thesis. In order to undertake this, an internationally agreed consensus would be required, incorporating an expert panel to oversee an agreed list of clinical parameters, followed by validation and determination of inter-observer agreement. Whereas the complexity of this task has been highlighted in the case of the internationally agreed uveitis classification (Jabs et al., 2005, Kempen et al., 2008, Deschenes et al., 2008), our group is currently establishing a platform to allow the next stage of developing this tool for PCC. These data have helped inform the nature of the clinical phenotype, confirmed the presence of ‘white inflammation’ amongst our cohort, and facilitated a better system for measuring and documenting the scarring process in OcMMP and other causes of PCC.

Chapter 4

Characterising the healthy conjunctival epithelial leukocyte population

4.1 Introduction

In order to understand the inflammatory changes on the ocular surface during immune-mediated disease, a detailed knowledge of the healthy human conjunctival epithelial leukocyte compartment is required. Our current understanding is based on conjunctival sections combined with immunohistochemical analysis, the major leukocyte populations being predominantly T lymphocytes (Hingorani et al., 1997).

Flow cytometry has been employed widely and contributed to our understanding of immune processes, not least in the immunology of the gut both in animal models and human tissue (Agace, 2008). OSIC combined with flow cytometric analysis has been undertaken in the context of the conjunctiva, employing two-colour flow cytometry in dry eye disease on cells transferred in fixative (Baudouin et al., 1992, Baudouin et al., 1997). *Ex vivo* analyses of the healthy ocular surface cell populations through flow cytometry including the exploitation of multi-colour panels has not previously been undertaken however. This chapter outlines how this approach was optimised and utilised to determine leukocytes in health, ageing and how dominant T cells behave.

4.1.1 Optimisation of mucous membrane cellular profiles detection by OSIC

Before characterising healthy conjunctival epithelial leukocyte populations the following practicalities were considered:

1. How could conjunctival leukocytes be collected?
2. How they were transported and recovered?
3. How leukocytes were discriminated from other cells e.g. epithelial cells?

Of the two membranes commonly used for OSIC, the isopore polycarbonate membrane (Millipore) is attractive because of its commercial availability in a pre-sterilised form (mounted on a transparent cylinder), facilitating easy use in the clinical environment. By contrast, circular polyethersulfone membranes (Supor) (13mm diameter) are available in unsterilised packages that require division and autoclaving for application with forceps. However, more efficient cell recovery has been reported with the Supor filter combined with transport in fixative and recovery with gentle agitation using a pipette tip (Brignole-Baudouin et al., 2004).

When undertaking OSIC of the conjunctiva, all cell types are recovered including epithelium, goblet cells and intra-epithelial leukocytes (Brignole-Baudouin et al., 2004). In order to discriminate leukocytes a pan-leukocyte marker such as CD45 can be employed (Baudouin et al., 1997). It was important to determine the brightest signal that would allow accurate distinction of leukocytes from other cells e.g. neutrophils, which have a less bright expression of CD45 than lymphocytes and monocytes (Stelzer et al., 1993).

4.1.2 Defining resident conjunctival leukocyte populations

As outlined in **Chapter 1**, the precise composition of leukocytes in the healthy conjunctiva has not been undertaken. Multi-colour flow cytometry offers an opportunity to discriminate cell populations by using multiple markers e.g. CD3 and CD56 for T cells, NK and NKT cells or T cells based on TCR subtype e.g. TCR$\alpha\beta$ or $\gamma\delta$. In this chapter resident conjunctival epithelial leukocyte populations were defined.

4.1.3 Age-related changes in the healthy human conjunctival epithelium

Little is known about age-related changes in the leukocyte populations within the ocular surface (Gwynn et al., 1993). This study utilised OSIC in combination with multi-colour flow cytometry not only to provide a detailed characterisation of the frequency of leukocyte subsets in the healthy human conjunctival epithelium, but whether these altered with age. This has the potential to offer clues about natural ageing changes and vulnerability to age-related diseases affecting the ocular surface.

4.1.4 Effector Function of the dominant conjunctival epithelial CD8+ T cells

The effector function of dominant conjunctival epithelial T cells is unknown. Antigen experienced (CD45RO+) cells have been identified in the conjunctiva, but precise memory status of CD8+ population is unknown. At mucosal sites, effector function of CD8+ T cells may differ from a conventional cytotoxic phenotype and repeated exposure to antigen may account for an altered role for epithelial CD8+ lymphocytes, some of which may be regulatory in nature (Koch et al., 2008, Xystrakis et al., 2004). CD8+ T cells play an important role in regulating chronic viral infection such as CMV and the use of MHC restricted tetramers in HLA-typed individuals has demonstrated that 1-2% of CD8+ T cells recognise viral antigens such as pp65 and IE-1 and the aggregate response could account for 50% of the total CD8+ repertoire (Moss and Khan, 2004, Faint et al., 2001). Before phenotyping conjunctival epithelial leukocytes in MMP and how these may alter with disease activity or progression, it is important therefore to establish if and how the CD8+ T cells dominate the conjunctiva in health before characterising any potential change in the context of disease.

4.2 Methods

4.2.1 Optimisation of mucous membrane cellular profiles detection

Determining an optimal OSIC membrane

Cell recovery comparisons were undertaken as described in **section 2.1.6.** Application of an OSIC membrane on a healthy participant is shown in **Figure 4.1**.

Cells were collected from healthy volunteers (n=5) (median age 70 [30-88]; 3 male; 4 White European, 1 Asian) (Cohort 4) to establish whether events could be detected with both filters. Cells were recovered by agitation with a pipette tip for 30 minutes as described in **section 2.2.2**. Unlabelled cells were run through a flow cytometer as described in **2.2.4**. Where low events were detected by flow cytometry, haematoxylin and eosin staining of the membrane was undertaken as described in **2.2.1** to determine if there were residual cells on the membrane.

Figure 4.1 Application of an OSIC membrane. Colour photograph demonstrating the application of a Supor membrane to the healthy ocular surface of the superior bulbar conjunctiva.

Identification of leukocyte populations by CD45+ pan-leukocyte marker

Cells recovered by OSIC were stained with anti-CD45+ pan leukocyte markers in order to discriminate leukocyte populations. Two commercially available anti-CD45 antibodies were compared; Phycoerythrin (PE) anti-CD45 and Allophycocyanin (APC) anti-CD45 (Ebioscience). Staining procedures were as described in **2.2.5**.

Increasing the efficiency of cellular recovery

In order to determine whether cellular recovery by pipette tip agitation could be expedited, agitation of cells recovered with a pipette were compared at conventional (30 minute) and rapid (1 minute) time frames.

4.2.2 Defining resident conjunctival leukocyte populations and age-related changes in the healthy human conjunctival epithelium

Study Subjects. Two separate cohorts were evaluated:

Cohort 5 (**Figures 5.5-5.7**): OSIC of right and left eyes of 30 healthy participants (median age 61 years [21- 83]) together with matched peripheral blood were collected. 20/30 individuals were White (European) and 10/30 were Asian (defined according to the ethnic demographic categories employed in the UK census 2011). Fifteen were male and fifteen were female.

Cohort 6 (**Figure 5.8**): OSIC of right and left eyes were collected and pooled for each of 10 healthy volunteers (5 young; median age 24 [23-33] and 5 older; median age 66 [65-83], p=0.01) in order to evaluate conjunctival T cell cytokine production. All were White (European) with five males and five females. Samples were pooled in this cohort in order to maximize the yield of cells for cytokine staining.

Sample collection and recovery

OSIC sampling and cellular recovery were as described in section **2.1.6**. OSIC with sterile Supor filters were used to collect superficial conjunctival cells. Following cyto-centrifuge, the majority of the supernatant was discarded, which was re-suspended in RPMI:10% HIFCS to a total volume of 200µl (Cohort 5) or 100µl (Cohort 6). 100µl of cells were placed into each well of a 96-well plate for flow-cytometric analysis. Peripheral blood was centrifuged and re-suspended in 1:10 dilution of filter-sterilised red cell lysis buffer (8.29g NH_4Cl, 1g $KHCO_3$ and 37.2 mg EDTA per litre dH20) as described in in section **2.2.3**.

Flow cytometry was undertaken as described in section **2.2.4**.

To characterise the cellular profile of the conjunctival ocular surface, nine colour flow cytometry panels were employed as described in **2.2.5**.

Procedures for intracellular cytokine assays (Cohort 6) were as described in **2.2.7**. An additional panel was utilised to determine cytokine expression by T cell subsets: mouse anti-human IFN-γ (efluor 450) (Ebioscience), IL-17 (FITC) (Ebioscience) and

rat anti-human IL-10 (Phycoerythrin) (Biolegend) combined with cell surface marker antibodies to CD4 (PerCP Cy5.5), CD45 (Allophycocyanin), CD3 (AlexaFluor 780) (Ebioscience), CD8β (PE Texas Red) (Beckman Coulter) and CD56 (PE Cy7) (Biolegend).

4.2.3 Effector Function of the dominant conjunctival epithelial CD8+ T cells

Study Subjects. Two separate cohorts were evaluated:

Cohort 7 (**Figure 5.9-5.10**): OSIC of right and left eyes were collected and pooled for each of 17 healthy participants (median age 32 [21-81]; 10 males; 16 White European, 1 Asian) in order to evaluate memory and homing status and phenotypic markers of conjunctival epithelial CD8+ T cells.

Cohort 8 (**Figure 5.11**): OSIC of right and left eyes were collected and pooled for each of 10 healthy volunteers (median age 33 [25-52]; 6 male; 10 White European) of known HLA typed individuals in order to evaluate viral MHC class tetramer staining.

Samples were pooled in both cohorts in order to maximise the yield of cells for cytokine staining. The right and left eye samples together with matched peripheral blood were collected.

Sample collection was as described in **2.2.3**.

The majority of the supernatant was discarded, which was re-suspended in RPMI:10% HIFCS to a total volume of 100µl. Cells were placed into each well of a 96-well plate for flow-cytometric analysis as previously described.

The protocol for flow cytometric analysis was as described in **2.2.4**.

Surface staining was as described in section **2.2.5**. For determination of homing and memory markers (Cohort 7), a re-configured panel was designed and included mouse anti-human leukocyte markers CD45 (Allophycocyanin or Phycoerythrin), CD3 (AlexaFluor 780) (Ebioscience, Hatfield, UK); CD8α (Pacific Orange) (Invitrogen, Paisley, UK) or CD8β (PE Texas Red) (Beckman Coulter); CD56 (PE Cy7) (Biolegend, Cambridge, UK); Memory markers including CD45RO (FITC), CD45RA (PE Texas Red) (Beckman Coulter, UK), CCR7 (FITC) (R & D Systems, UK) and the homing markers α_E (CD103) (FITC) (DAKO, UK) and β7 (PE Cy5) (BD, UK). These were titrated to determine the optimal concentrations.

Intracellular Cytokine Staining, Cytolytic Proteins and Transcription Factors

Procedures were as described in **2.2.6** and **2.2.7**, including the use of a Live Dead fixable yellow dye (Invitrogen) Cytokine expression was determined by a panel including mouse anti-human IFN-γ (efluor 450) (Ebioscience), IL-17 (FITC) (Ebioscience), and rat anti-human IL-10 (Phycoerythrin) (Biolegend) or IL-22 (Ebioscience). These were combined with surface marker antibodies anti-CD45 (Allophycocyanin or Phycoerythrin), CD3 (AlexaFluor 780), CD8β (PE Texas Red) and CD56 (PE Cy7) (Biolegend) following stimulation of cells.

Mouse anti-human Granzyme B (PE) (BD, UK) or Perforin (PE) (Ancell, Bingham, UK) were combined with anti-CD45 (Allophycocyanin), CD3 (AlexaFluor 780), CD8β (PE Texas Red) and CD56 (PE Cy7) (Biolegend) without stimulation of cells.

Rat anti-human FoxP3 (PE) (Ebioscience) was combined with anti-CD45 (Allophycocyanin), CD3 (AlexaFluor 780), CD8β (PE Texas Red) and CD56 (PE Cy7) (Biolegend) without stimulation of cells.

Details of viral peptide MHC-I tetramer staining is described in **2.2.8**. Tetramers were prepared in advance and donated as a gift by AP and HL. CMV or EBV tetramers were pooled in order to maximise T-cell interaction in conjunctival OSIC or peripheral blood cells with known viral antigens derived from HLA typed individuals, known to respond to antigens outlined in **Table 2.1**.

Statistical Analysis

Non-parametric comparisons were undertaken with the Mann Whitney U test, Wilcoxon signed rank test and correlations by Spearman's correlation using Prism version 5.0 for Macintosh (GraphPad Software, California 2008).

4.3 Results

4.3.1 Optimisation of mucous membrane cellular profiles detection

Conjunctival OSIC and Cellular Recovery

Cell recovery using the Supor filter detected a median of 5 x 10^4 [range 100-100,000] events/filter paper (n=5). By contrast, cell recovery with Millipore filters following collection, suspension and agitation yielded no cells (n=4) or 1 x 10^3 events/filter paper (n=1). Few cells were identifiable by H & E staining of the residual membrane following agitation, indicating that the Millipore collection yield was poor (**Figure 4.2**).

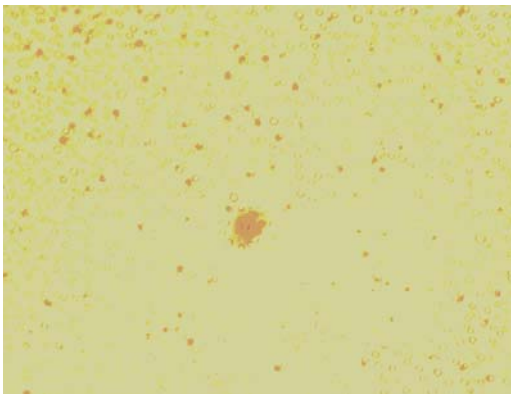


Figure 4.2: **Haematoxylin & Eosin staining of Millipore filter**. Representative H & E section (10x magnification) of a Millipore filter following conjunctival impression, suspension in RPMI and agitation of cells with a capillary tip. Note the scanty residual cells (1 demonstrated in this section) on the membrane despite agitation. The poor yield from agitation implies that initial collection of cells with this filter is poor, limiting its value in cell collection for the purpose of flow cytometric analysis.

Identification of tissue leukocyte populations by flow cytometry

In order to discriminate leukocyte populations from epithelial cells in conjunctival OSIC specimens using Supor filters, a pan-CD45 leukocyte marker was employed. **Figure 4.3** demonstrates surface marker staining of whole blood and conjunctival impressions taken from a healthy volunteer 'spiked' with 5μl of whole blood

(*1x10^{7}cells/ml*) in order to replicate the presence of neutrophils (which would not be expected in healthy human conjunctival epithelium). Two antibodies were compared to determine the brightness of surface staining and ability to discriminate subsets of cells (PE anti CD45 and APC CD45). Granulocytes (including neutrophils) have a high side scatter (SS) and when using the APC anti-CD45 antibody, the brightness of this antibody was greater than for PE (Regions R5 and R6 respectively), seen on the x-axis of the whole blood staining. This presents a problem for the 'spiked' impression as $CD45^{LOW}$ cells (predominantly epithelial cells from the conjunctival OSIC), cannot easily be discriminated from $CD45^{INT}$ (neutrophils) when using the PE anti-CD45 antibody. Back gating these populations (R5 and R6) on to a forward scatter (FS) and SS profile demonstrate a higher proportion of cells with a high SS, consistent with neutrophils, when using the APC-anti CD45 antibody. This was the antibody used for comparisons between healthy volunteers and patients with disease where the presence of neutrophils was anticipated.

A period of 30 minutes of agitation of cells from the impression filter has previously been described (Brignole-Baudouin et al., 2004). **Figure 4.4** demonstrates that the number of lymphocytes derived from 1 minute of agitation were greater than for re-suspension of the impression filter and agitation for 30 minutes after running both samples until completion. The forward and side scatter profile was also different for 30-minute agitation those observed for 1 minute. It is possible that prolonged agitation disrupts the filter membrane itself, contributing to the events detected by the flow cytometer.

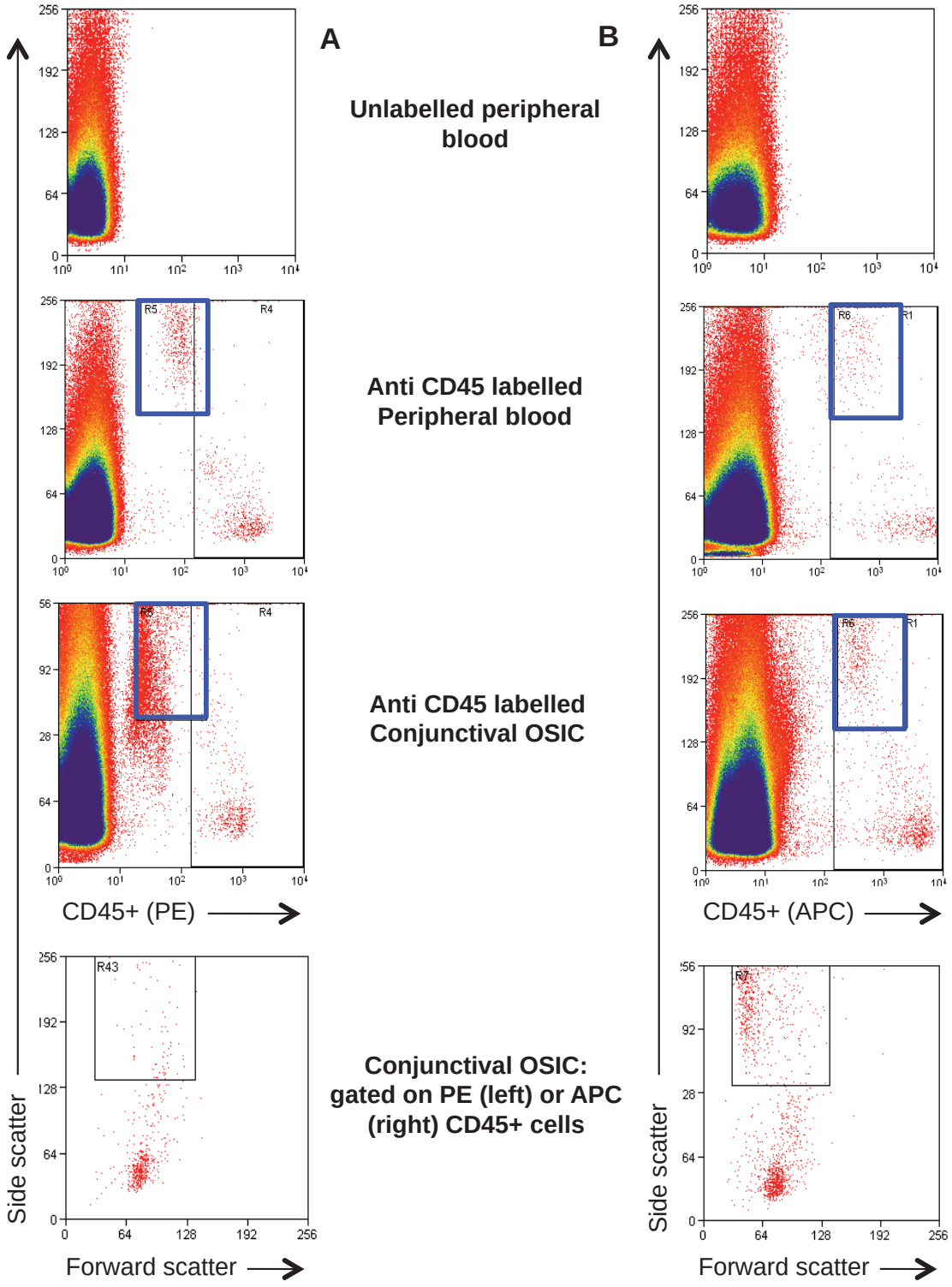


Figure 4.3: Determination of efficacy of Phycoerythrin (PE) and Allophycocyanin (APC) anti-CD45 labelling of leukocyte populations. The side scatter (SS) and staining intensity of $CD45^{HIGH}$ leukocytes can be compared between the two fluorochromes (regions R4 and R1 respectively for PE [Panel A] and APC [Panel B]). The SS and fluorescence profiles of conjunctival Ocular Surface Impression Cytology (OSIC) spiked with 5ul whole blood (as a 'positive' control') allow comparison of cells that have a SS profile consistent with neutrophils (regions **R5** and **R6**). Back-gating for CD45+ high cells (regions R4 and R1) allows discrimination of epithelial cells which are not masked by $CD45^{INT}$(intermediate) cells (demonstrated in R5 for PE). These cells had a scatter profile consistent with neutrophils, seen on regions R43 and R7 respectively.

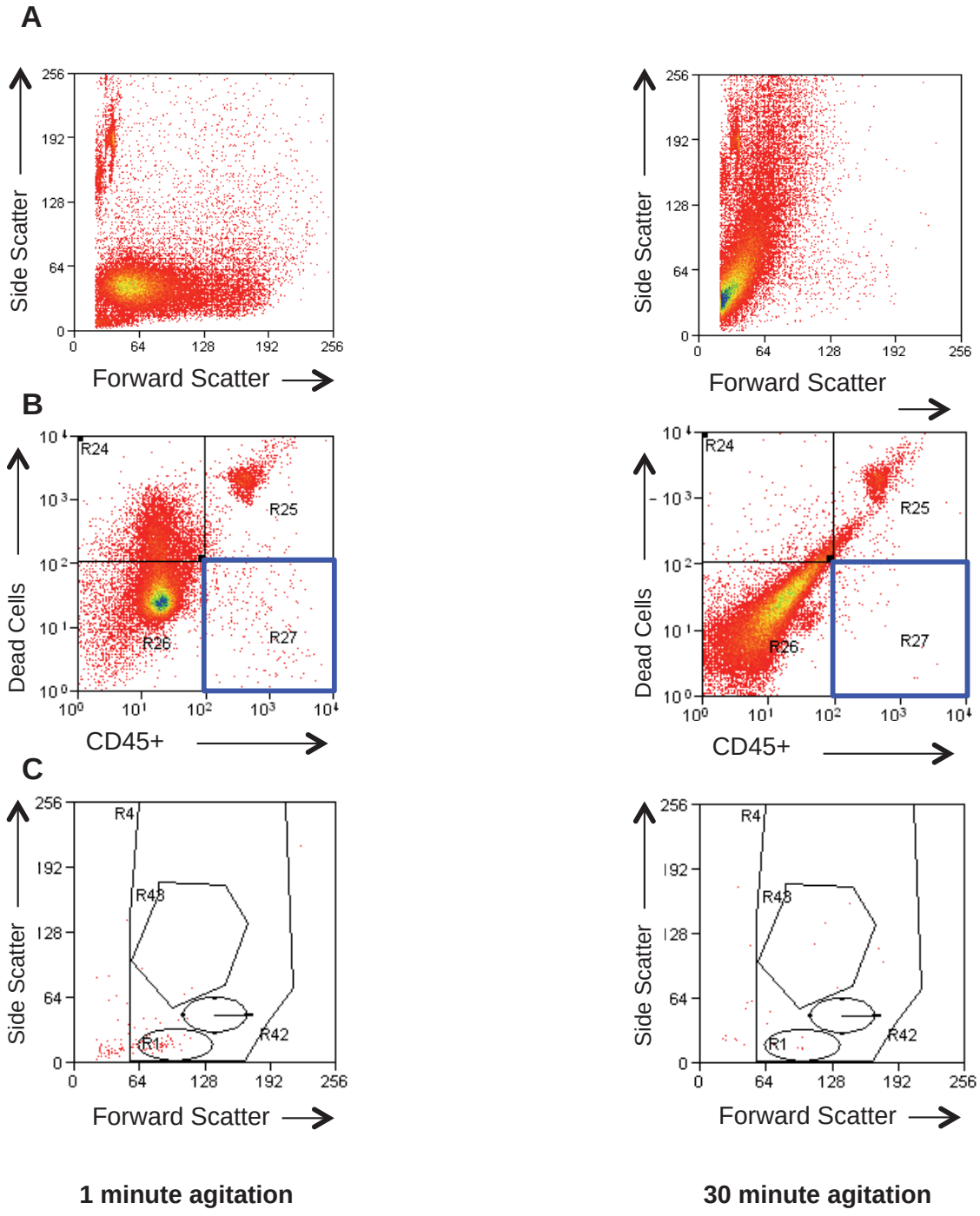


Figure 4.4: Demonstration of cellular recovery from conjunctival Ocular Surface Impression Cytology (OSIC) at 1 minute agitation (left panels) versus 30 minutes agitation (right panels). Cells were recovered from media following 1 minute agitation, centrifuged and re-suspended in 100ul of media prior to antibody labelling. The residual impression filters were then re-suspended and agitated for 30 minutes before processing (Panel B). Upper Panels A show the unlabelled forward (FS) and side scatter (SS). Back gating of live cells (blue boxed, panel C) on to a FS v SS profile are shown in Panel C. Note the abnormal scatter profile following 30 minute agitation and diminished yield of lymphocytes from the 30 minute agitation.

4.3.2 Defining resident conjunctival leukocyte populations

Conjunctival OSIC and matched peripheral blood samples taken from healthy subjects (Cohort 5) demonstrated that while the light scatter profile for peripheral blood clearly delineated each leukocyte population (**Figure 4.5A**), it was not possible to make this discrimination from OSIC (**Figure 4.5D**). This was overcome by gating on APC labelled CD45+ live cells, which permitted demonstration of lymphocytes as the dominant leukocyte population in the conjunctival epithelium (**Figure 4.5E,F**). This approach also clarified the identity of the leukocyte populations found in peripheral blood (**Figure 4.5B,C**).

Leukocyte populations were therefore defined as being live CD45+ve cells derived from conjunctival impressions specimens from the superior bulbar conjunctiva, transferred immediately to RPMI:HIFCS and agitated for 1 minute before preparation and staining.

There were no differences in the number of leukocytes between the right and left eyes ($p=0.23$; Wilcoxon signed rank test) and right and left eye leukocyte numbers were highly correlated ($r=0.72$; $p<0.0001$). Therefore, the mean of right and left eyes for each subject was calculated (i.e. right and left eyes per individual subject were considered to be experimental duplicates). CD45+ live cells accounted for a median 834 [range 60-17635] of total events. Of the cohort of 30 subjects (median age 61 years [21-83]), the dominant conjunctival leukocyte population were lymphocytes (median 89% of the total lymphocyte, monocyte and neutrophil counts [32-99]) as defined by their forward and side scatter profiles; 9% [0-34] were monocytes and <1% [0-66] were neutrophils. This compared to 52%[18-75], 5%[3-28] and 42%[19-76] respectively in matched peripheral blood.

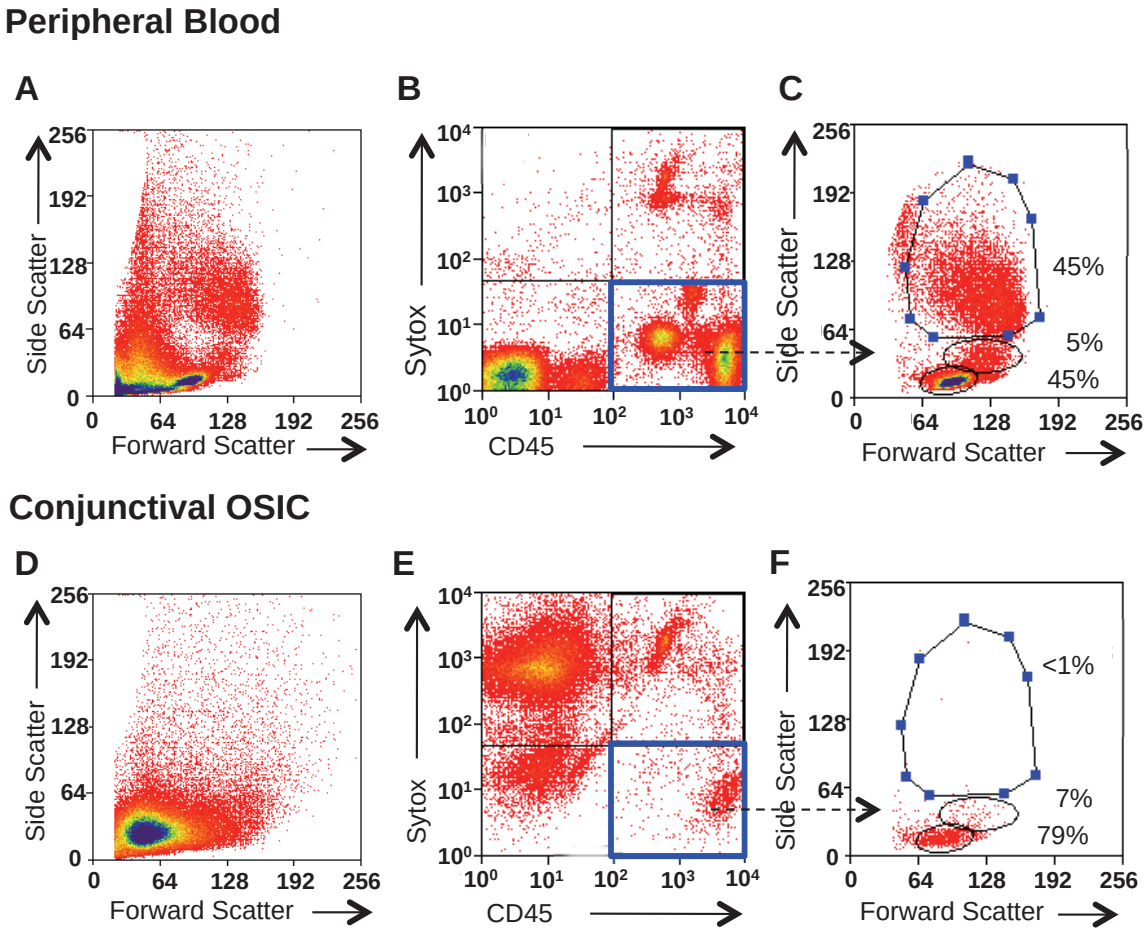


Figure 4.5: Lymphocytes are the dominant conjunctival epithelial leukocyte population. Representative plots of a subject demonstrating the gating strategy used to identify conjunctival leukocytes. Panels A and D show the forward and side scatter profiles for peripheral blood and conjunctival Ocular Surface Impression Cytology (OSIC) respectively. Live leukocytes were identified by gating for CD45+ cells that were negative for the dead cell exclusion dye Sytox blue (lower right box in panel B and E) and back-gated to show the forward and side scatter profiles of the CD45+ live cells (panels C and F). Percentages are shown for the representative subjects (Granulocytes 45% and <1%; Monocytes 5 and 7%; Lymphocytes 45 and 79% respectively).

T and NK cell subsets were defined by the expression of CD3 and CD56 (**Figure 4.6**). T cells (CD3+CD56-) dominated in both conjunctival OSIC (69% [47-90]) and in matched peripheral blood (74% [57-84]), and these were >98% TCR$\gamma\delta$-(TCR$\alpha\beta$+) in both (**Figure 4.6A,B**). T cells were further characterised by the expression of the CD4, CD8α and CD8β cell surface co-receptors (**Figure 4.6A,B).** Unlike in the peripheral blood where the dominant T cell population was CD4+ (**Figure 4.6D;** 69% [45-91]), the dominant population from the conjunctival impression was CD8$\alpha\beta$+ (**Figure 4.4C:** 80% [37-100]). The majority of CD4+ and CD8$\alpha\beta$+ T cells were CD45RO+ in the conjunctival epithelium (100%[0-100] and 94%[55-100] respectively), higher than the proportion of antigen experienced populations in blood (61%[0-90] and 56%[20-86] respectively). CD4-CD8$\alpha\beta$- (DN) T cells accounted for only 7.3% [0.7-22] and 3.5% [0.4-26] of conjunctival and peripheral blood T cells, respectively (**Figure 4.6E**). Whilst CD8$\alpha\alpha$+ cells formed the minority of T cells, these were significantly higher in the conjunctiva than in peripheral blood (**Figure 4.6F**; 2.6% [0-12.5] versus 1.4% [0.1-4.4] ($p<0.001$)).

NK cells represented 7%[0-20] of conjunctival epithelial lymphocytes compared with 9%[0-22] in peripheral blood. There was also a greater proportion of NKT (CD3+CD56+) cells (conjunctiva 6%[0-17] versus peripheral blood 2%[0-6] ($p>0.05$)) but fewer CD19+CD20+ B cells (3%[1-45] versus 9%[3-23], respectively, ($p<0.0001$)).

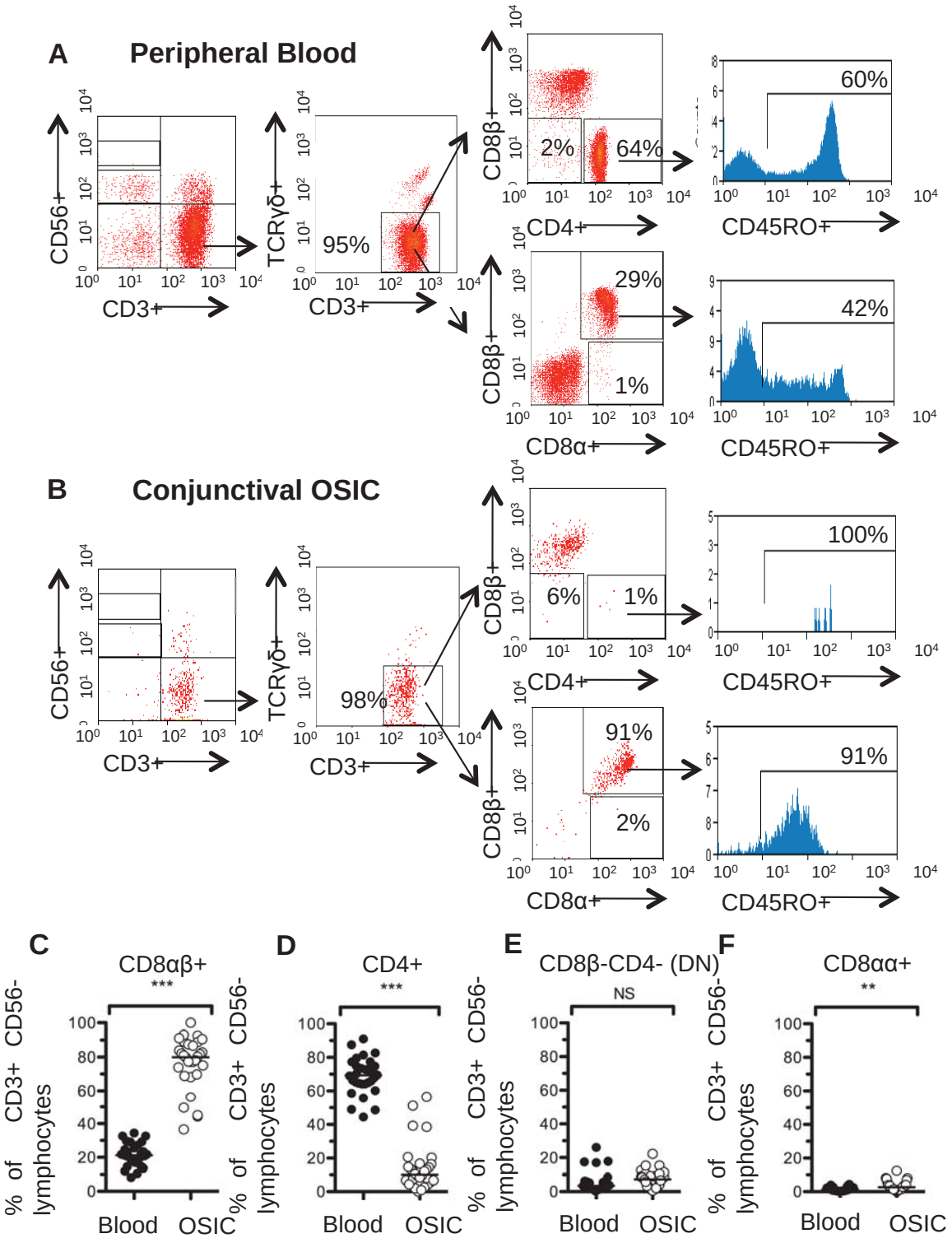


Figure 4.6: TCRαβ+ CD8αβ+ T cells are the dominant population of lymphocytes in the conjunctival epithelium. Representative scatter profile of leukocyte populations derived from matched peripheral blood and conjunctival ocular surface impression cytology (OSIC) from a healthy subject. Data is shown for a representative subject, gated on CD45+ live cells for peripheral blood (A) and conjunctival OSIC (B). CD45RO staining is shown for CD3+CD56-TCRgd- gated CD4+CD8b- (top panel) and CD8ab+ (bottom panel) cells. Percentages of CD3+CD56- lymphocytes are shown for the representative subject (n=30). Statistical comparisons between peripheral blood and conjunctival impression populations of CD8αβ+, CD4+, CD8αα+ and TCRαβ+CD4-CD8αβ- (double negative; DN) T cells (C-F) were undertaken by the Mann-Whitney U test (NS: Not significant [p>0.05], **P < 0.01, ***P < 0.001).

4.3.3 Age Related Changes In Leukocyte Populations In The Healthy Human Conjunctival Epithelium

Having determined the normal leukocyte populations in Cohort 5, the age spectrum within this cohort allowed us to determine if age-related changes occurred in these leukocyte populations (**Table 4.1**). Analysis of peripheral blood monocyte, neutrophil and lymphocyte frequencies showed that the only change was a decrease with age in lymphocytes calculated as a percentage of total leukocytes (**Table 4.1**). This was not observed in cells recovered from the conjunctival epithelium. By contrast, conjunctival cells showed an increase in the numbers of lymphocytes and monocytes (but not neutrophils), resulting in a significant increase in the total number of leukocytes (**Table 4.1**). This demonstrates that the primary observation of lymphocytes being the dominant population is maintained with age, but alterations in the composition of the lymphocytes occurred.

Within the conjunctival epithelial lymphocyte population there was an age-related decrease in the proportion of T cells (**Figure 4.7A; Table 4.2**), compensated for by an increase in the percentage of NK cells (**Table 4.2**). The dominant CD8$\alpha\beta$+ cell population remained unchanged in the conjunctival epithelium with age but decreased in peripheral blood (**Figure 4.7B, Table 4.2**). Conversely, the absolute number of CD4+ cells significantly increased in the conjunctiva but remained unchanged in peripheral blood (**Figure 4.7E, Table 4.2**). This resulted in proportional changes in CD8$\alpha\beta$+ and CD4+ lymphocytes (**Figure 4.7C and F Table 2**) with a consequent increase in the CD4:CD8 ratio with age (**Table 4.2**). In addition, the CD45RO+ memory population increased in the peripheral blood with age, a change that was not seen in conjunctival OSIC (**Figure 4.7D and G, Table 4.2**), reflecting the

high proportion of antigen experienced CD8+ (93%) and CD4+ cells (100%) present in the conjunctival epithelium from a young age.

Cell population	Age correlation		r value (p value[1])	
	Peripheral Blood	Conjunctival OSIC	Peripheral Blood	Conjunctival OSIC
Leukocytes numbers	No change	Increase	0.05 (NS)	0.43 (0.02)
Lymphocytes numbers	No change	Increase	-0.23 (0.03)	0.38 (0.04)
Lymphocytes (% of leukocytes)	Decrease	No change	-0.4 (0.03)	-0.08 (NS)
Monocyte numbers	No change	Increase	0.01 (NS)	0.45 (0.01)
Monocytes (% of leukocytes)	No change	No change	-0.01 (NS)	0.34 (NS)
Neutrophil numbers	No change	No change	0.20 (NS)	0.33 (NS)
Neutrophils (% of leukocytes)	No change	No change	0.34 (NS)	-0.09 (NS)

[1]Spearman's correlation: NS: Not significant [p>0.05],

Table 4.1. Ageing changes in leukocyte populations in peripheral blood and conjunctiva.

Cell population	Age correlation		r value (p value[1])	
	Peripheral Blood	Conjunctival OSIC	Peripheral Blood	Conjunctival OSIC
T cells (% of lymphocytes)	No change	Decrease	-0.18 (NS)	-0.45 (0.01)
CD8αβ+ numbers	Decrease	No change	-0.5 (<0.01)	0.3 (NS)
CD8αβ+ (% of T cells)	No change	Decrease	0.31 (NS)	-0.49 (<0.01)
CD45RO+ cells (% of CD8αβ+ cells)	Increase	No change	0.53 (<0.01)	0.13 (NS)
CD4+ numbers	No change	Increase	-0.1 (NS)	0.61 (<0.001)
CD4+ (% of T cells)	Increase	Increase	0.52 (<0.01)	0.52 (<0.01)
CD45RO+ cells (% of CD4+ cells)	Increase	No change	0.53 (<0.01)	0.27 (NS)
CD4:CD8 ratio	Increase	Increase	0.41 (0.03)	0.49 (<0.01)
B cells (% of lymphocytes)	No change	No change	0.18 (NS)	-0.08 (NS)
NK cells (% lymphocytes)	No change	Increase	0.24 (NS)	0.4 (0.03)
NKT cells (% lymphocytes)	No change	No change	-0.05 (NS)	0.15 (NS)

[1]Spearman's correlation: NS: Not significant [p>0.05],

Table 4.2. Changes in lymphocyte populations in peripheral blood and conjunctiva with age.

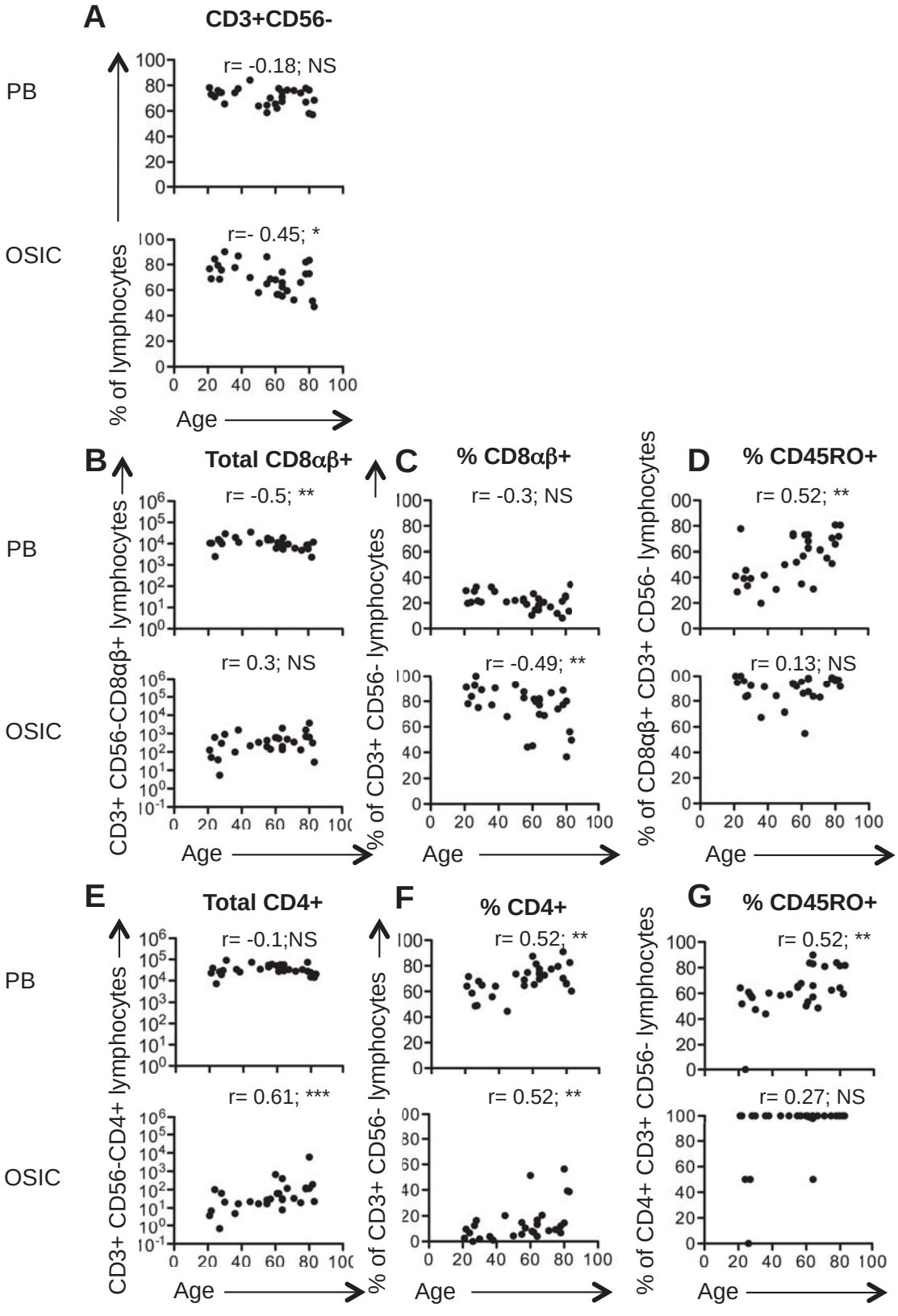


Figure 4.7: Changes in T cell subsets and memory populations in peripheral blood and conjunctiva with age. Changes in the T cell populations in peripheral blood (PB) and conjunctival ocular surface impression cytology (OSIC) for CD3+CD56- lymphocytes (A), as well as CD8αβ+ and CD4+ subsets (B/C, E/F) with their respective CD45RO frequencies (D, G). Statistical analysis was undertaken by Spearman's correlation (NS: Not significant [p>0.05], *P < 0.05, **P < 0.01) (n=30).

In light of the increase in conjunctival CD4+ T cells with age, further analysis of their phenotype was undertaken (Cohort 6). Conjunctival CD4+ T cells of a cohort of 5 additional healthy younger (<35 years) and 5 older subjects (>65 years) were characterised for the expression of IFNγ, IL-17 and IL-10 following stimulation with PMA/Ionomycin (**Figure 4.8A**). The CD4+ population as a percentage of T cells was significantly elevated in the older age group (19%[11-52) vs. those <35 years] (4%[2-13]; p=0.02), confirming the observation from the larger cohort of 30 individuals (Cohort 1) (**Figure 4.8B**).

18%[14-48] of conjunctival CD4+ T cells were capable of expressing IFN-γ, 3.5%[0-22] IL-17 and 0%[0-4] IL-10. The absolute number of CD4+ T cells able to secrete IFN-γ was significantly elevated with age (<35 years, 7[4-39] vs. >65 years, 43[20-145];p=0.03) while the percentage of IFN-γ+ CD4+ remained unchanged (17%[14-35] vs. 18%[16-48],p=NS, respectively) (**Figure 4.8C**). Changes in IL-17 and IL-10 producing CD4+ T cells were not observed with age (**Figure 4.8C**).

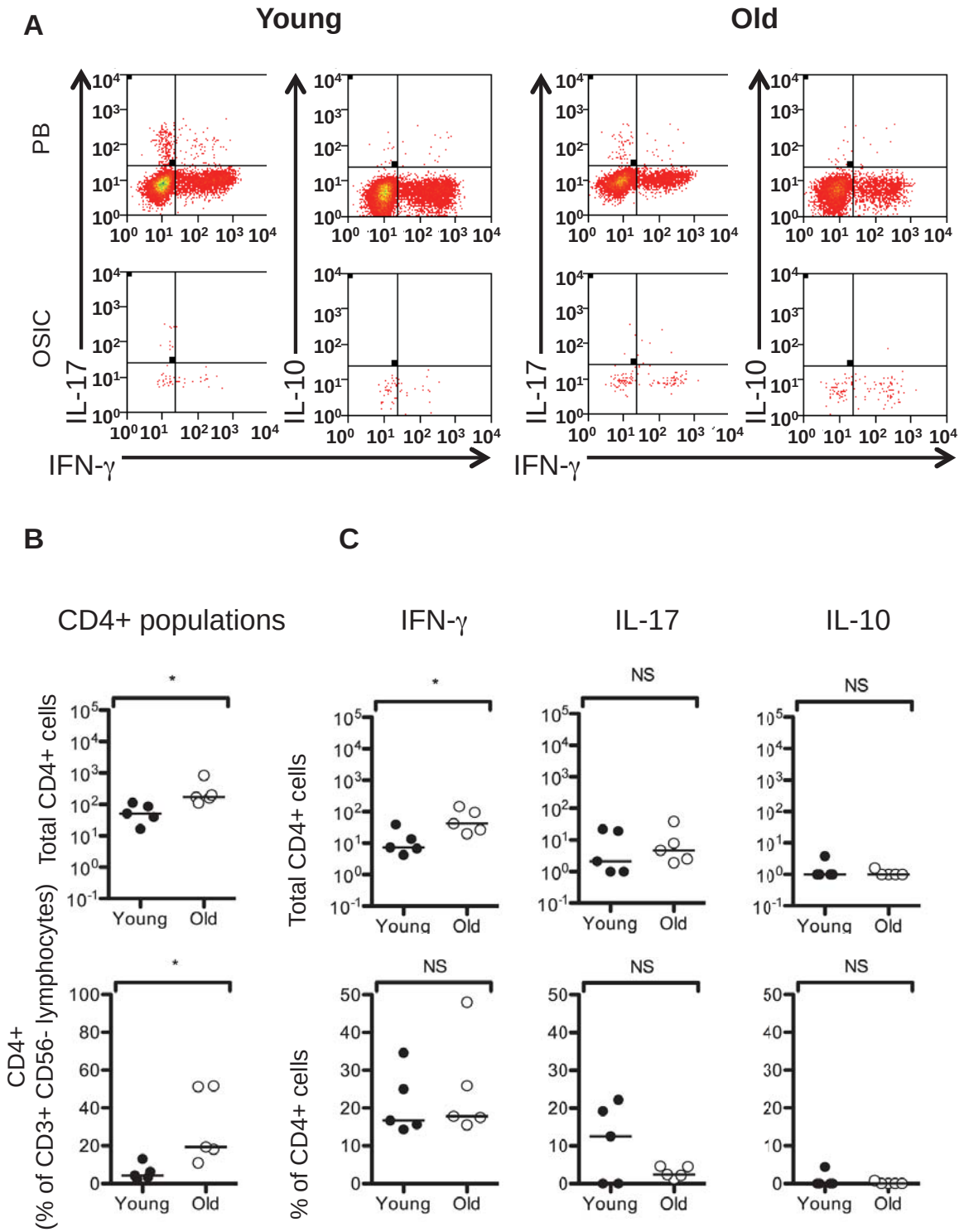


Figure 4.8: IFN-γ producing TCRαβ+ CD4+ T cells increase with age while IL-17 producing cells are maintained. Expression of IFNγ, IL-17 and IL-10 in the T cell populations in stimulated peripheral blood (PB) and conjunctival ocular surface impression cytology (OSIC) for CD45+CD3+CD56-CD4+ live lymphocytes (A). Representative figures from a subjects aged <35 and >65 years are shown. The number and percentage of CD4+ T cells are shown for this cohort (B), as well as the number and percentages of cytokine-secreting cells (C). Statistical comparisons were undertaken by the Mann-Whitney U test (NS: Not significant [p>0.05], *P < 0.05).

4.3.4 Effector Function of the dominant conjunctival epithelial CD8+ T cells

Although there is an elevation in CD4+ cells in the conjunctival IEL compartment with age, the CD8+ T cell population remains unaltered. In order to characterise these dominant and stable cells in more detail, the memory status, homing marker expression, phenotypic markers and antigen recognition of CD8+ T-cells were determined (Cohorts 7 and 8). 85%[59-94] of conjunctival T cells were CD8+ (**Figure 4.9A**), in keeping with the normal values demonstrated in **4.3.2**.

Memory and Homing

Mucosal homing cells were predicted to have switched to the expression of a epithelial homing marker CD103(α_E). 99%[92-100%] of conjunctival T cells in this cohort expressed the integrin $\alpha_E\beta_7$ (CD103) compared to 1.8% [0.7-2.3] in peripheral blood (p=0.005) (**Figure 4.9B, 1D**) (n=6). Conversely, 0.1%[0-2] of conjunctival CD8+ T cells were $\alpha_E\beta_{7-}$ ($\alpha_4\beta_7$+) compared to 56%[21-78] in peripheral blood (p=0.005).

The majority of conjunctival epithelial cells were previously identified as being antigen experienced (CD45RO+). 88% [62-100] of the conjunctival epithelial CD8+ population were in fact CD45RA-(CD45RO+) CCR7- effector memory (EM) cells compared to 43%[18-61] in peripheral blood (**Figure 4.9 C, E**) (n=7). Although there was no difference in the proportion of central memory cells between the conjunctiva and peripheral blood, these formed a minor population of CD45RO+ (RA-) cells (2%[0-28] vs. 1%[0.5-6]; p=0.6). The dominant CD45RO+ population was reflected in a significantly lower proportion of naïve cells (CD45RA+CCR7+) in the conjunctiva

(0.2%[0-3]) compared to peripheral blood (36%[1-58]) ($p<0.05$) (**Figure 4.9E**). There were also a significantly lower proportion of effector memory RA 'revertant' CD8+ T cells in the conjunctiva (6%[0-21] vs. 28%[5-56]; $p<0.05$). The dominant antigen experienced CD8+ T cell population is therefore composed primarily of EM cells and were less likely to have reverted to a CD45RA+ CCR7-phenotype, as seen in peripheral blood.

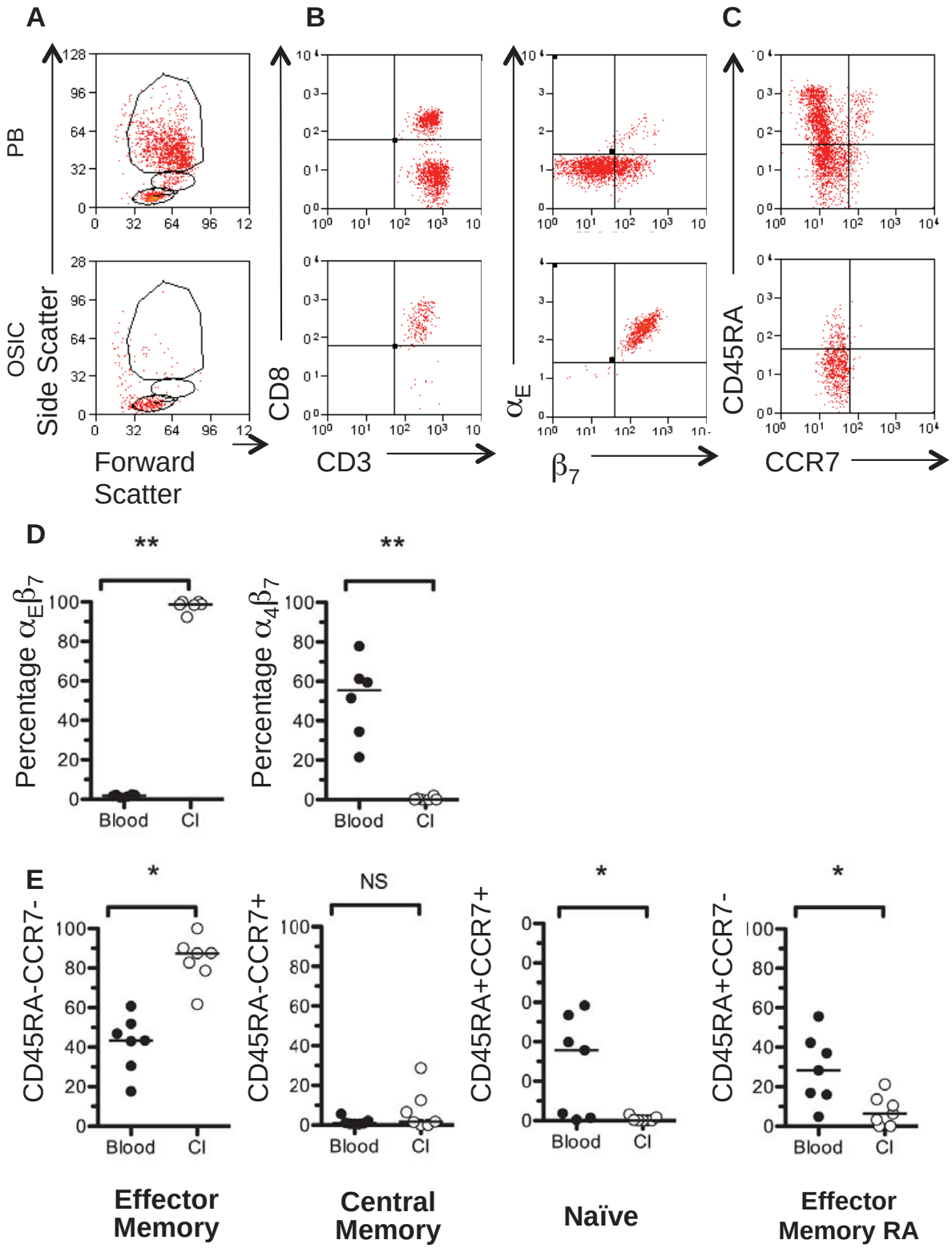


Figure 4.9: Conjunctival CD8+ lymphocytes are mucosal homing, effector memory T cells. The gating strategy for determining CD8+ T cell populations from conjunctival ocular surface impressions and accompanying matched peripheral blood samples are shown in Panel A. Representative plots for mucosal homing integrin expression and memory status is shown for CD3-CD8+CD56- live lymphocytes (Panels B and C) Significant differences in homing (Panel D) and memory status (Panel E) between conjunctival epithelial leukocyte and peripheral blood populations (n=6). Statistical analysis was undertaken by the Wilcoxon signed rank test (Key: NS: Not significant (p>0.05), *p=0.01 to 0.05; **p=0.001 to 0.01).

Phenotypic Markers of conjunctival epithelial CD8+ T cells

36%[24-80] of antigen experienced conjunctival CD8+ T cells were capable of expressing IFN-γ, compared to 34%[20-64] in peripheral blood (p=0.84). Although there was no significant difference in the Median Fluorescence Intensity (MFI) of IFN-γ in the conjunctiva 78[54-112] compared to peripheral blood 134[50-184] (p=0.15), the trend indicated that this was lower in the conjunctival population (**Figure 4.10 A, 4.11**). Of the conjunctival memory CD8+ T cells, 55[34-68]% produced Granzyme B compared to 38%[29-49] in peripheral blood (p=0.03) (**Figure 4.10 A, 4.11**). The MFI however was significantly lower among conjunctival CD8+ T cells; 39[28-47] vs. 100[76-149] (p=0.03). This demonstrates a cytotoxic phenotype among resident conjunctival epithelial CD8+ T cells, but with lower expression of Granzyme B despite a relatively higher proportion of cells. Expression of Perforin, also capable of inducing cellular apoptosis, was variable in unstimulated cells with a median of 0%[0-13] among conjunctival cells compared to 9%[5-10] in peripheral blood (n=3). A representative flow cytometric plot of Perforin expression by peripheral blood NK cells is demonstrated for comparative purposes (**Figure 4.10B, 4.11**).

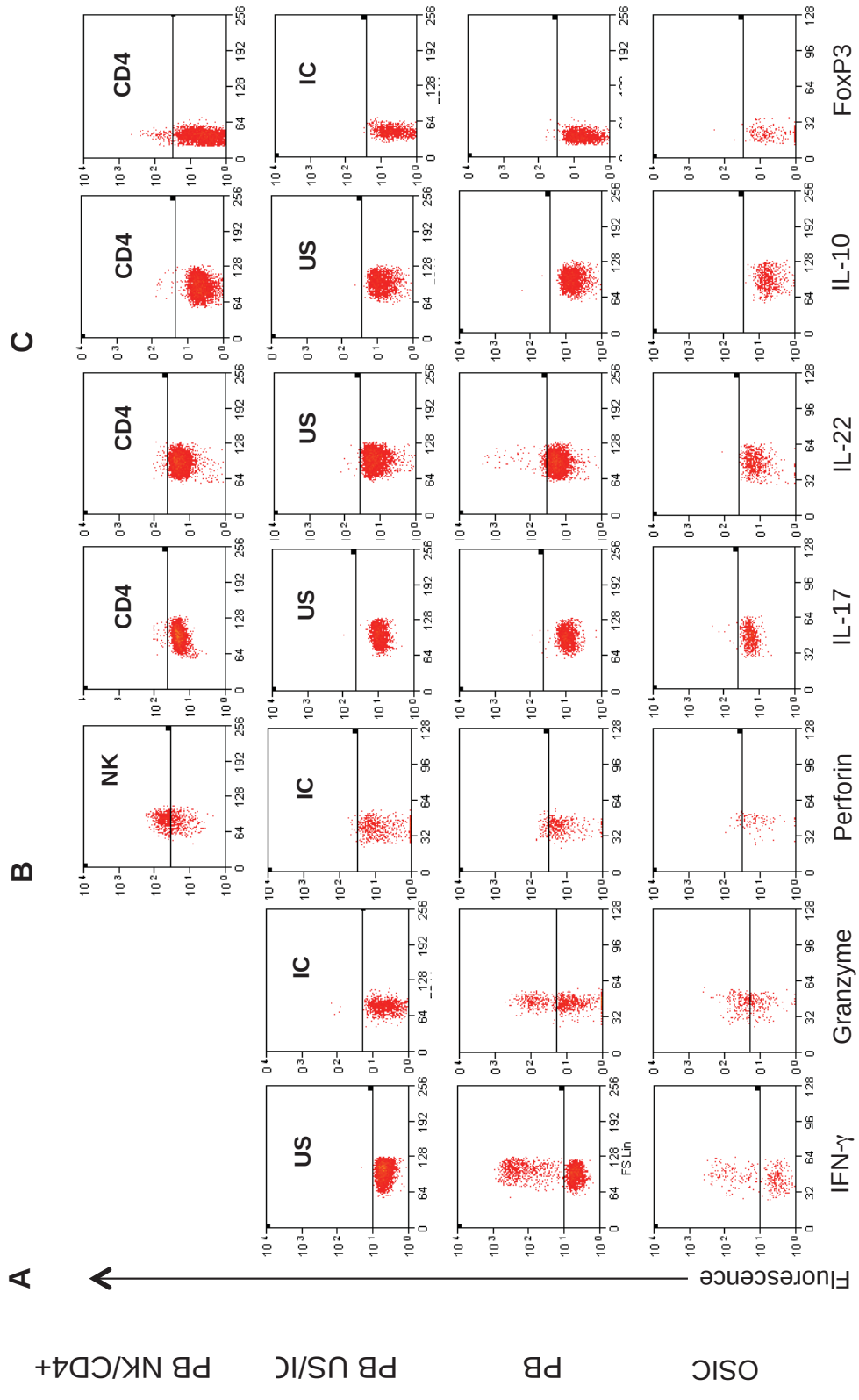


Figure 4.10: Conjunctival epithelial CD8+ lymphocytes display a cytotoxic phenotype, producing IFN-γ, and Granzyme B. Representative plots showing the cytokine, apoptotic protein or transcription factor expression of CD3+CD56-CD8+ live lymphocytes among conjunctival leukocytes, matched peripheral blood (PB) cells and unstimulated (US) or Isotype Controls (IC) of PB cells (Panels A, B and C). The upper rows, where appropriate, show relevant NK or CD4+ expression as controls.

A minor but relatively higher percentage of conjunctival CD8+ T cells produced IL-17 (2%[1-7)] compared to peripheral blood cells (0.3%[0.2-0.4;(p=0.03), indicating a potential population of Tc17 cells in the ocular surface mucosa. There was no difference in the proportion of CD8+ T cells producing IL-22 (1.4%[0-3] vs. 1%[0-3]; p=0.56). Representative plots demonstrating peripheral blood CD4 expression of IL-17, 10 and 22 are shown in **Figures 4.10B** and **4.11**. There was little evidence for regulatory CD8+ population in the superficial conjunctival epithelium. No conjunctival CD8+ T cells were capable of producing IL-10, compared to 0.4%[0.2-0.6] of peripheral blood cells (p=0.03). No differences were also found in the proportion of cells capable of expressing FoxP3 (0.6%[0-1.6] vs. 0.6%[0.2-1.2]; p=1.0).

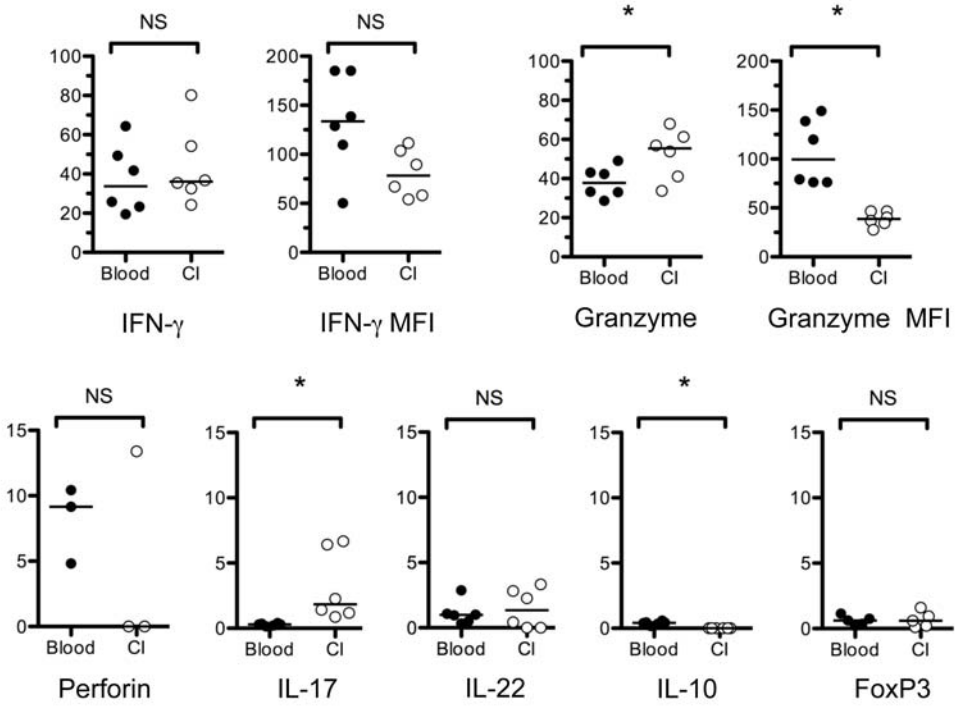


Figure 4.11: Conjunctival epithelial CD8+ Granzyme B+ cells are significantly higher in the conjunctival epithelium compared to peripheral blood but their Median Fluorescence Intensity (MFI) is lower. Comparative analysis of effector function of conjunctival (CI) and peripheral blood CD8+ T cells are shown. Comparisons were between % of CD8+ T cells or the MFI. Statistical analysis was undertaken by the Wilcoxon signed rank test (Key: NS: Not significant (p>0.05), *p=0.01 to 0.05).

Viral antigen recognition by conjunctival epithelial CD8+ T cells

Given the predominant CD45RA-CCR7- CD8+ population, it was anticipated that the CD8+ population may contain cells that recognise the herpetic viruses CMV and/or EBV. By pooling tetramers of known viral antigens including CMV - HLA-A1 VTE, HLA-A1 YSE, HLA-A2 NLV, HLA-B7 RPH, HLA-B8 ELK, HLA-B8 QIK and EBV - HLA-A2 GLC, HLA-A2 YVL, HLA-B8 RAK and HLA-B8 FLR in a cohort of HLA-typed volunteers, known to respond to >1 antigen (Cohort 8) we were able to determine percentage of CD8+ T cells that were able to recognise respective viruses from conjunctival OSIC and peripheral blood. 0.4%[0-7.9] of conjunctival CD8+ cells recognised CMV antigens and 0.6%[0-1.9] EBV antigens (n=10), reflecting peripheral blood frequencies; 1%[0.3-6.7]% and 2%[0-4] respectively (r=0.95;p=0.0001) (**Figure 4.12 A, B and C**). There were also no differences between the frequencies of CMV+ and EBV+ CD8+ T cells in the conjunctiva (p=0.62) or in the peripheral blood p=0.25).

EBV+ CD8+ T cells were predominantly Effector Memory (CD45RA-CCR7-) in the conjunctiva compared to peripheral blood (75%[0-100] vs. 44%[0-75]; p=0.008), with a higher proportion of CD45RA+CCR7- ‘revertant’ CMV+ or EBV+ cells in peripheral blood (32%[0-88] vs. 0%[0-78]; p=0.004) (**Figure 4.12D**).

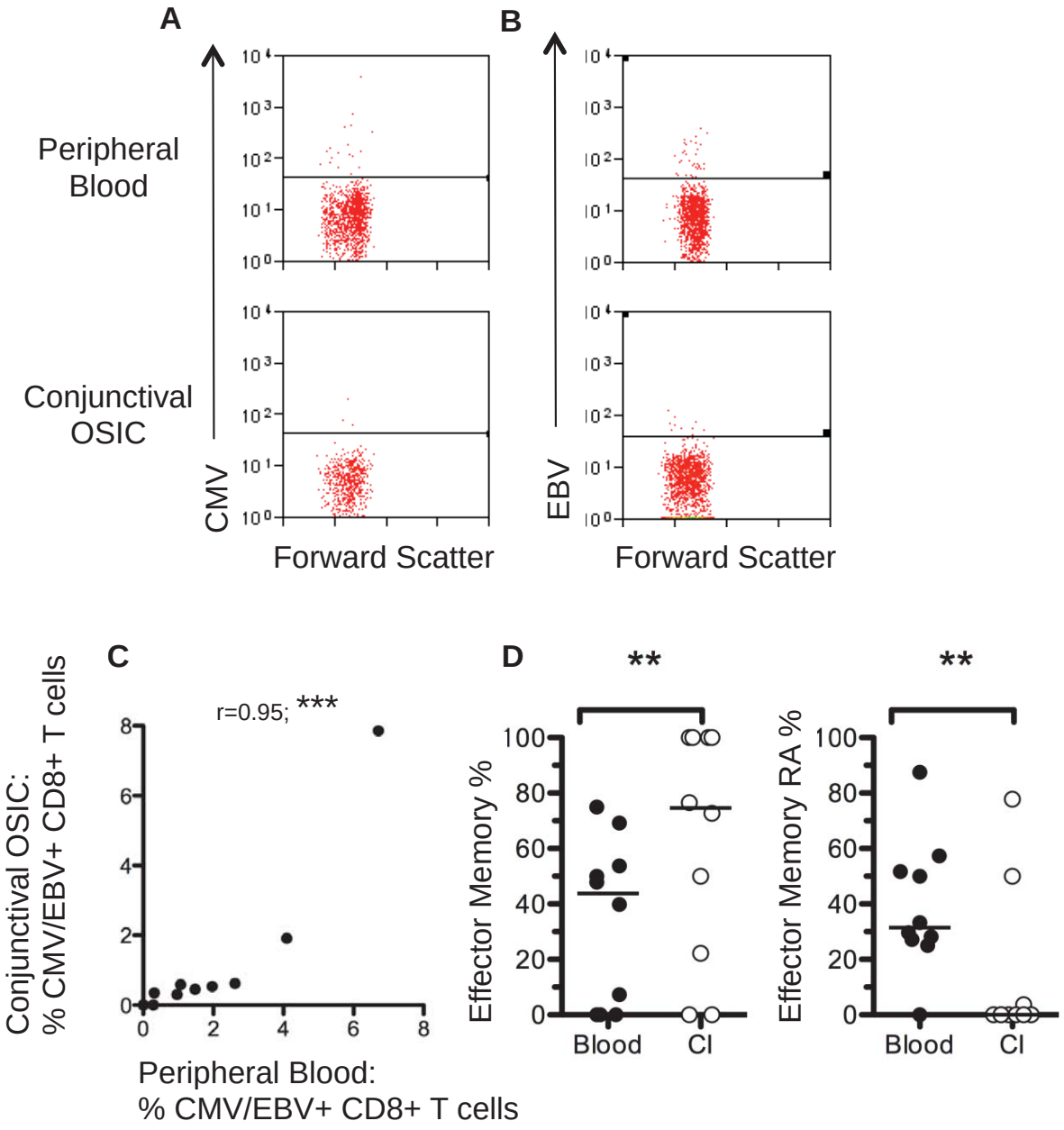


Figure 4.12: Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) recognition among conjunctival epithelial CD8+ T cells reflects that of peripheral blood. Conjunctival CD8+ T cells were stained with pooled CMV or EBV peptide MHC-Class I restricted tetramers conjugated to APC (CMV: HLA-A1 VTE, HLA-A1 YSE, HLA-A2 NLV, HLA-B7 RPH, HLA-B7 NLV, HLA-B8 ELK HLA-B8 QIK and EBV: A2 GLC, HLA-A2 YVC, HLA-B7 RAG, HLA-B7 FLR, HLA-B8 RAK HLA-B8 FLR). Representative plots for CMV+ and EBV+ CD3+CD56-CD8+ T cells are shown in Panels A and B respectively. Correlation between percentage CMV+ or EBV+ CD8+ T cells in peripheral blood and conjunctiva are shown in Panel C. Differences between % Effector Memory and Effector Memory RA populations in peripheral blood and conjunctiva are shown in Panel D. Statistical analysis was undertaken by Spearman's correlation or Wilcoxon signed rank test (Key: NS: Not significant ($p>0.05$), *$p=0.01$ to 0.05; **$p=0.001$ to 0.01; *** $p<0.001$).

4.4 Discussion

4.4.1 Optimisation of mucous membrane cellular profiles detection

The ability to take OSICs from healthy participants and patients with PCC such as OcMMP has major advantages in facilitating:

(i) A non-invasive means of collecting ocular surface cells

(ii) A consistent method for longitudinally sampling patients

(iii) A means of interrogating cell populations in an *ex vivo* manner

Conjunctival OSIC has been employed in studying ocular surface disease for over 30 years (Nelson, 1988). First described by Egbert *et al*, the ability to use cellulose acetate filters to collect bulbar conjunctival surface cells allowed fixation, staining and examination under a microscope (Egbert et al., 1977). The technique usually facilitates the removal of 2-3 layers of superficial cells although visualisation of the basal epithelium through multiple impression of the same area can be achieved (Singh et al., 2005). Conjunctival OSIC has been used for monitoring OS health in contact lens wearers and evaluating ocular surface neoplasia (Knop and Brewitt, 1992, Nolan et al., 1994, Tole et al., 2001). Perhaps its widest application is the use of the technique for the quantification of dry eye severity through determination of goblet cell density and changes to the epithelial cell morphology (Nelson, 1988, Tseng, 1985, Adams et al., 1988). The data from my experiments now suggest that using a Supor filter for OSIC, RPMI as a transport medium and agitating cells for 1 minute to recover them allows the discrimination of conjunctival epithelial leukocytes when combined with an APC anti-CD45 antibody.

4.4.2. Defining resident conjunctival leukocyte populations and age-related changes in the healthy human conjunctival epithelium

OSIC combined with multicolour flow cytometric analysis enables a comprehensive characterisation of the conjunctival epithelial leukocytes superficial to the basement membrane zone. In this study, this methodology has afforded a detailed phenotyping of conjunctival leukocytes in a cohort of healthy participants and how these change with age. The dominant conjunctival epithelial leukocyte population were confirmed as CD8+ but the precise subtype was found to be CD3+CD56-TCR$\alpha\beta$+CD8$\alpha\beta$- lymphocytes. Interestingly, although this population remained unchanged with age, there was an increase in the conjunctival epithelial CD4+ population resulting in an alteration in the CD4:CD8 ratio. Furthermore, 14-48% of conjunctival epithelial CD4+ cells were capable of producing IFN-γ and 0-22% were capable of producing IL-17. This was maintained in older subjects and given the increase in absolute numbers of CD4+ cells with age, this resulted in a substantial increase in the number of pro-inflammatory conjunctival CD4+ T cells.

The role of conjunctival IEL is unresolved. In other mucosal tissues, two subtypes of IEL have been defined - Type a (TCR$\alpha\beta$+CD8$\alpha\beta$+) ‘conventional’ and Type b (TCR$\alpha\beta$+ CD8$\alpha\alpha$+, TCR$\gamma\delta$+ CD8$\alpha\alpha$+ and TCR$\gamma\delta$+ Double Negative (DN)) IEL, with differing roles in effector function and regulation (Hayday et al., 2001). This study suggests that the dominant IEL population in the human conjunctiva is ‘conventional’ (Type a) TCR$\alpha\beta$+CD8$\alpha\beta$+ with less than 1% TCR$\gamma\delta$+. Whether TCR$\gamma\delta$+ cells have a relatively minor role to play in conjunctival epithelial biology, or are confined to below

the BMZ within the substantia propria (which we were unable to sample using OSIC) remains unknown.

Tissue based immunohistochemical analyses identified CD45RO+ cells (75-100%) in the bulbar conjunctiva (Hingorani et al., 1997), but those studies were limited as precise T cell subsets were not quantified. These data showed that antigen experience (defined by the expression of CD45RO) was evident in all CD4+ and almost all (median 94%) of CD8αβ+ conjunctival epithelial T cells, whereas a significant increase in the CD8αβ+ and CD4+ CD45RO+ T cell population was observed in peripheral blood, in keeping with the findings of others (Saule et al., 2006, Utsuyama M, 2009). The predominance of CD45RO+ lymphocytes in the conjunctival epithelium is expected and consistent with the preferential recruitment of memory cells into mucosal tissues.

The major alteration in IEL was an increase in the number and percentage of CD4+ cells with age. This resulted in a reduction in the percentage of the dominant conjunctival CD8αβ+ population and an increase in the CD4:CD8 ratio. By contrast, there was a decrease in the number of CD8αβ+ T cells in blood, although this too resulted in an increase in the CD4:CD8 ratio, as previously reported by Utsuyama *et al* (Utsuyama M, 2009) (see **Table 1.3**).

As the proportion of T cells decreased in the conjunctival epithelium with increasing age, both the proportion and number of NK cells, increased. Although this observation has been previously described in peripheral blood (Borrego et al., 1999) (and there was an observed trend in this cohort) this is the first time those changes

have been defined in the conjunctiva. Whether this represents an accumulation of NK cells in the ocular surface reflecting immune senescence, or a direct response to a specific change in ocular surface antigen exposure, remains unknown.

It is clear that the changes in IEL populations seen in this cohort, in particular the increased TCR$\alpha\beta$+ CD4+ T cell population, have implications for age-matching when undertaking comparisons with disease populations, specifically in relation to infective or immune-mediated processes affecting the ocular surface including PCC such as MMP. Dry-eye problems increase with age (Draper et al., 1999) including dry eye disease (Moss et al., 2000, McCarty et al., 1998). Although changes to the lacrimal acinar gland have been attributed to age-related ocular surface dryness (Draper et al., 1999), dry eye syndromes (including Sjögren's syndrome and non-Sjögren's syndrome related dry eye) are thought to have an underlying inflammatory and autoimmune component.

Intriguingly, elevations of CD4+ T cells in both humans and in animal models of dry-eye have been identified (Stern et al., 2010, De Paiva et al., 2010), but the contribution of elevated conjunctival intraepithelial CD4+ cells to a pro-inflammatory state is not known and may offer clues to dry-eye vulnerability amongst older subjects. The absolute number of CD4+ T cells able to secrete Interferon-γ was significantly elevated with age and IL-17 producers were maintained with age in this study. An elevation of IFN-γ and IL-17 producing cells has been identified in the conjunctiva in murine models of dry eye (De Paiva et al., 2009). Moreover, an increase in these cytokines is seen in tears of human subjects with dry eye disease (De Paiva et al., 2009). Whether age-associated accumulation of CD4+ cells

predisposes to dry eye problems by an increased number of IFN-γ and IL-17 secreting cells or whether an alteration in function occurs under dry eye conditions in humans, remains to be defined.

In murine models there appears to be a defective suppressor function by T regulatory cells on Th17 cells (Chauhan et al., 2009). Few conjunctival CD4+ T cells were capable of producing IL-10 in this study, and no changes were observed with age. The expression of the transcription factor FoxP3 was seen in approximately 2% of CD3+ conjunctival T cells (data not shown). This suggests that in the healthy conjunctival epithelium, there is not a significant population of CD4+ T cells with an IL-10+ or FoxP3+ regulatory phenotype. An increase in the stromal CD4+ population has also been identified from histological conjunctival sections (Bernauer et al., 1993a) taken from patients with MMP (Chan et al., 2002, Foster, 1986, Liesegang, 2008), that typically affects older patient populations (although disease activity and progression is worse in younger patients) (Rauz et al., 2005b).

The possibility of utilising a non-invasive sampling technique such as OSIC to characterise changes in supra-basement membrane structures of the ocular mucosa in the context of infectious and non-infectious disease affords an attractive method for both cross-sectional and longitudinal research into human ocular surface inflammatory disease. The importance of ageing on conjunctival leukocyte profiles in disease states is yet to be elucidated, but has implications in forming comparative healthy control cohorts, indicating that age-matching is essential.

4.4.3 Effector Function of the dominant conjunctival epithelial CD8+ T cells

The majority of conjunctival epithelial CD8+ T cells are mucosal homing $\alpha_E\beta_7$+ Effector Memory T cells, capable of secreting IFN-γ and Granzyme B. There was a minor population of IL-17 secreting CD8+ T cells, higher than in peripheral blood. There were also no IL-10 secreting CD8+ T cells and <1% of cells expressed the transcription factor FoxP3.

Circulating T cells are recruited to mucosal tissue such as the gut by expression of the integrin α_4:β_7 (which binds to MadCam-1 on lamina propria vascular endothelium) and the chemokine receptor CCR9 (Svensson et al., 2002). It is suggested that a switch to (CD103+)α_E:β_7 integrin expression on these mucosal homing T cells directs lymphocytes within the lamina propria to bind to E-cadherin on epithelial cells (Cepek et al., 1993, Cepek et al., 1994), although there is evidence that the β_7 integrin may play as an important role in recruiting the cells from the lamina propria (the counterpart to the conjunctival stroma) in the small intestine. Switching from α_4 to α_E is mediated by the expression of TGF-β expression on epithelial cells and E-cadherin expression has been identified on normal human conjunctival epithelial cells (Scott et al., 1997). It is not know whether the recruitment to the epithelium is wholly CD103+ dependent. In the gut, α_E:β_7 is thought to play an adhesive role and as such the actual recruitment across the basement membrane zone (BMZ) may occur through a different signalling cascade. It has been suggested that this trafficking may be facilitated by BMZ pores (Chan et al., 2008).

When T-cells are primed In mucosal tissue, either in draining lymph nodes or MALT, the process of becoming antigen experienced conforms an alteration in the CD45 common leukocyte antigen marker from a high (RA) to a low molecular weight isoform (RO) (Faint et al., 2001, Akbar et al., 1988). The process of antigen experience in MALT or in draining lymph nodes also results in CCR9 and α_4:β_7 expression on T cell subsets, however non-mucosal routes of immunisation (for example the skin) can result in mucosal-homing (Agace, 2008, Masopust et al., 2010). Effector memory 'primed' cells are able to home to peripheral mucosal tissues and CD8+ CD103+ (α_E:β_7) mucosal cells have also previously been identified as CD45RA-CCR7- (Gupta et al., 2004, Koch et al., 2008). The dominant TCR$\alpha\beta$+CD8$\alpha\beta$+ sub-population of our cohort were defined as being effector memory cells and CD103+.

Effector memory cells typically secrete IFN-γ upon stimulation (Gupta et al., 2004), yet there is evidence of a regulatory/suppressive phenotype among cells that express CD103+ (α_E:β_7) (Koch et al., 2008). The possible role of regulatory CD8+ cells has attracted attention and suppression of T-cell proliferation in mixed lymphocyte culture via a cell-to-cell contact-dependent mechanism by alloantigen-induced CD8+CD103+(α_E+) T cells has been reported (Uss et al., 2006). CD8+CD28- (non-antigen specific) T regulatory cells do not depend on CD80 and CD86 (expressed on TLR dependent antigen presenting cells) and suppression is thought to be mediated by IL10/TGF-β or direct inhibition of DCs. Additionally CD103+ have been demonstrated to secrete IL-10, not IFN-γ, and maintain their phenotype after re-stimulation with alloantigen (Uss et al., 2006). In human peripheral blood CD103+ CD8+ T cells, in keeping with their regulatory phenotype, do not produce Granzyme

B or Perforin (Koch et al., 2008). The conjunctival epithelial CD103+ mucosal homing population therefore, and in contrast to peripheral blood, have a cytotoxic phenotype – producing IFN-γ upon stimulation and have the capacity to produce Granzyme B (in significantly higher proportions than in peripheral blood). Granzyme B is a cytolytic granular protein that induces apoptosis in conjunction with pore-formation induced by the release of Perforin. Granzyme B production is found in 20% or peripheral blood at any time and suggests recent activation of the effector memory CD8+ cells (Nowacki et al., 2007).

There was no evidence in this study that conjunctival epithelial CD8+ T cells had a regulatory phenotype – there was no IL-10 production and a minor population expressed the transcription FoxP3. A small but significant proportion also produced IL-17. Tc17 cells have been previously identified in peripheral blood (Kondo et al., 2009) and that these cells are CD103+ (Yen et al., 2009). The functional role of these cells on the ocular surface is unknown, but have been implicated in skin lesions in psoriasis (Ortega et al., 2009).

The role for a cytotoxic CD103+ effector memory CD8+ T cell population residing in the healthy conjunctival epithelium may be for viral host defence. In human peripheral blood, the CD45RA+CCR7- CD8+ pool demonstrates an age-related commitment to the recognition of Cytomegalovirus (CMV) (Khan et al., 2002). Failure to clear CMV, and another herpetic virus Epstein Barr Virus (EBV), results in clonal expansion of EM CD8+ cells that recognize these viruses. Type a IEL have been shown to be effective in clearing virus from the gut mucosa, as demonstrated by increased cytotoxicity in mice infected with lymphocytic choriomeningitis virus

(LCMV) by CD8αβ+ compared to CD8αα+ T cells (Muller et al., 2000). We were able to demonstrate that conjunctival CD8+ CMV and EBV, resulting in increased clonal expansion of memory T cells including CD45RA+CCR7-, antigen recognition reflect those of peripheral blood. The presence of mucosal homing EBV- and CMV-specific cells on the conjunctival epithelium suggests that though there is no preferential recruitment to this peripheral tissue, the conjunctival CD8+ T cell pool might provide protection against a broad range of viruses. Further characterisation of viral antigen recognition implicated in human ocular surface disease such as Herpes Simplex Virus may offer further clues to why there is such a limited repertoire of CD8+ T cells found in the human conjunctival epithelium. It may be that rapid recruitment of other leukocytes in the context of bacterial or fungal insult may take place to assist this conserved population of T cells as a front-line defence of the ocular surface.

These data suggest that recently activated conventional CD8αβ+ T cells are effector memory and have a cytolytic function. Although these cells are able to recognize latent herpetic viruses such as CMV and EBV, further evaluation may reveal a commitment to an ocular surface pathogen such as HSV or a more promiscuous recruitment based on multiple differing viral antigens.

Collectively these data have afforded a greater insight in to the dominant cell populations found in healthy conjunctival epithelium and how they change with age. More importantly, it has established a means of defining how these populations or others may alter in the context of disease.

Chapter 5

Characterising the conjunctival epithelial leukocyte populations in acquired ocular immunobullous disease

5.1 Introduction

Acquired ocular immunobullous diseases including SJS-TEN and OcMMP can result in ocular surface failure, emanating from persistent conjunctival inflammation, cicatrisation and eventually corneal scarring and neovascularisation as discussed in **sections 1.6**-**1.8**. This problem is compounded by disease progression in the apparent absence of clinically identifiable inflammation.

Whereas little is known about the ocular surface inflammatory process, keratinocyte death in cutaneous SJS-TEN is induced by blister fluid granulysin secreted by CD8+ T cells and NK cells (Chung et al., 2008) while OcMMP is an autoimmune disease (Chan et al., 2002). The detailed cellular characterisation of ocular disease in the conjunctival mucosa has not been clarified for either disease or how this may change with time. Furthermore, the ability to undertake longitudinal characterisation of leukocyte populations has been limited by the inability to non-invasively sample the ocular surface. This is also the case in OcMMP and the aim of this study was therefore to determine:

1. The conjunctival epithelial cellular infiltrate in SJS-TEN and OcMMP
2. How this infiltrate correlates with disease activity and damage
3. Whether inflammatory infiltrates are maintained over time
4. Whether conjunctival inflammatory infiltrates can predict disease progression in OcMMP and specifically in the context of a clinically uninflamed eye

5.1.1 Stevens-Johnson Syndrome (SJS) and Toxic Epidermal Necrolysis (TEN)

SJS-TEN is an uncommon condition but with potentially devastating ocular surface consequences. The age of presentation, whilst variable, tends to be younger than in MMP (Schopf et al., 1991, Pereira et al., 2007), reported as 28 years in one recent series (Wetter and Camilleri, 2010) and 40 years [5-100] in a recent BOSU study (Radford *et al*. The Incidence of Cicatrising Conjunctival Disorders in the UK. IOVS 2011; 52: E-Abstract 1136). The initial, rapid granulysin mediated blistering seen in SJS-TEN carries a potentially fatal outcome. Ocular involvement, can result in devastation of the ocular surface through conjunctival inflammation, symblephara formation and corneal and conjunctival ulceration. Although the disease carries a high mortality, the disease is considered temporal. Systemic features would therefore be expected to resolve. This is not the case with ocular disease however (Foster et al., 1988, De Rojas et al., 2007).

The underlying aetiology of SJS-TEN and OcMMP differ and also manifest in very different ways. SJS-TEN is characterised by widespread desquamation of skin and mucosal surface, but varying in its severity (Bastuji-Garin et al., 1993). This may or may not be associated with ocular involvement. By contrast, OcMMP typically has a more insidious onset. Although the skin may be involved in MMP, the symptoms and signs are less severe, and confined to the head and trunk (Chan et al., 2002). As illustrated in **Chapter 3**, the median duration of ocular symptoms in a cohort of patients with OcMMP was 4 years. Systemic SJS-TEN can occur rapidly, within hours or days, and ocular involvement can occur in a very short space of time (Gueudry et al., 2009). A recent BOSU study also revealed that there was a significantly longer duration of symptoms between ocular SJS-TEN and OcMMP

before presentation (7 days vs. 225 days;p<0.001) (Radford *et al*. The Incidence of Cicatrising Conjunctival Disorders in the UK. IOVS 2011; 52: E-Abstract 1136).

The distinction between the acute and chronic stages of ocular SJS-TEN is a moot point (Fu et al., 2010). Power and colleagues suggested that this period was up to 6 weeks after onset (Power et al., 1995). By 6 weeks the systemic disease may have completely resolved and in this respect it could be argued that this period is too long to be truly considered as the acute phase. Conversely, the only definition suggested for chronic ocular SJS-TEN is greater than 12 months (Sotozono et al., 2007). There is therefore a discord between the acute systemic and chronic ocular disease. The process by which this switch occurs is not established, and understanding how the initial disease alters to one that has a phenotype resembling OcMMP is not clear. There may be important similarities or indeed differences in the ocular surface cell populations of SJS-TEN in the acute or chronic phases, which may help us to understand how this disease can contribute to the scarring process and behave like OcMMP in some individuals.

In this chapter I have sought to identify inflammatory cellular infiltrates in patients in the acute and chronic phases of SJS-TEN. This provided an important preliminary study of a potentially younger group of patients with an acquired immunobullous disease, before undertaking a more comprehensive assessment of cell changes in the more common OcMMP.

5.1.2 Ocular Mucous Membrane Pemphigoid (OcMMP)

The underlying disease process in OcMMP is driven by autoantibodies against the basement membrane zone (Chan et al., 2002). This in turn leads to the complement activation and the recruitment of an inflammatory infiltrate including neutrophils, CD4+ T cells and macrophages (Bernauer et al., 1993a, Rice and Foster, 1990). The CD4+ infiltrate is thought to be Th2 mediated, leading to activation of TGF-β and IL-13 (Elder, 1997b, Saw et al., 2009b), but the epithelium is dominated by neutrophils (Bernauer et al., 1993a, Rice and Foster, 1990).

The conjunctival cellular infiltrate has been identified from histological sections but repeat sampling has been precluded because of the risk of exacerbating scarring. Conjunctival OSIC has been previously utilised in OcMMP. Nelson found that there were abnormal epithelial cells and the absence of conjunctival goblet cells in 6 eyes of 4 patients with MMP when applying a cellulose acetate filter (Nelson, 1982). This apparent decrease in goblet cell density was later confirmed in both MMP and SJS with a bulbar conjunctiva goblet cell loss of 96-99% (Nelson and Wright, 1984). To date however, the characterisation and quantification of the cellular infiltrate by OSIC in OcMMP at different stages of disease activity has not been undertaken.

By using non-invasive OSIC combined with multi-colour flow cytometry we were able to establish that conjunctival epithelial leukocytes are dominated by TCRαβ+CD8αβ+ T cells (**Chapter 4**) and this population remains unaltered with age in a healthy cohort of volunteers while the CD4+ population increases. The aim of this study was to characterise infiltrative cellular profiles within the conjunctival epithelium during

acute and chronic SJS-TEN and OcMMP using OSIC and multi-colour flow cytometry. It is hoped that this could potentially provide a means of objectively monitoring disease activity and predicting outcome at a sub-clinical level.

5.2 Methods

5.2.1 SJS-TEN Study

Study subjects

Over a 12-month period, 10 consecutive patients presenting with SJS-TEN (Cohort 9) to a tertiary OSD service (BMEC) were recruited and followed for one year. Comparisons were made with a cohort of healthy, age-matched controls (n=21) (median age 50 [range 21-64 years]; 11 females; 16 White European, 5 Asian) (Cohort 10). There was no significant difference in age.

Systemic steroids can induce granulocytosis (Bishop et al., 1968). In anticipation of our SJS-TEN cohort requiring topical steroids during the course of the study, the effects on conjunctival neutrophils we determined by sampling an additional cohort of healthy participants (n=7) by OSIC before and after routine cataract surgery (median age 74 [range 57-84 years]; 4 females; 6 White European, 1 Asian) (Cohort 11). This facilitated a comparison of neutrophils numbers with and without the application of topical preservative free Dexamethasone in an uninflamed eye. The repeat samples were taken if the following criteria were met (i) Cataract surgery was uncomplicated (ii) There was no evidence of ocular surface or intra-ocular surface inflammation at 4 week review (iii) Individuals were using topical non-preserved Dexamethasone 0.1% QID since the time of surgery.

Disease Grading and Staging

Data were collected at presentation and at 12 months follow-up. Chronic disease was defined as persistence of ocular disease for >12 months after disease-onset (Sotozono et al., 2007). Visual acuity was categorised as described in **section 2.1.4**. Disease activity was based upon the extent of conjunctival inflammation: absent, mild, moderate or severe (severe defined as being inflamed in all 4 quadrants, the presence of limbitis and/or conjunctival ulceration) (**Figure 1.10**) (Elder, 1997c). Staging of disease was determined by the staging described by Power *et al* (Absent, Mild, Moderate and Severe) (Power et al., 1995) and Sotozono *et al* (Score:1-39) (Sotozono et al., 2007) while quantification of lower and upper conjunctival fornix shrinkage using an FDM and number of symblephara were also measured. Information regarding therapeutic and surgical intervention was recorded.

Sample collection and laboratory analysis

Details of conjunctival OSIC sampling and peripheral blood collection are described in **sections 2.1.6** and **section 2.2.3**. Details of *ex vivo* experimentation are fully described in **sections 2.2.4-2.2.5**.

Statistical analysis

Non-parametric comparisons were undertaken with the Mann Whitney U test, Wilcoxon signed rank test and correlations by Spearman's correlation using Prism version 5.0 for Macintosh (GraphPad Software, California 2008). Data were collected on all eyes and comparisons were undertaken between the worst affected eye in patients and the arbitrarily the right eye in healthy participants for cross-sectional analysis. Longitudinal analysis of the same eye was undertaken.

5.2.2 OcMMP Study

Study subjects

Sixty patients with OcMMP were recruited from patients presenting to the OSD clinic at BMEC over a 24 months period and followed for 12 months (Cohort 12). Comparisons were made with a group of healthy, age-matched controls (n=21) (median age 64 [range 45-83 years]; 12 females; 14 White European, 7 Asian) (cohort 13) and a positive disease group with an autoimmune disease associated with slowly progressive PCC. Patients with Primary Sjögrens Syndrome (n=19) (1 Male, 18 Female; Median age 64 ([range 56-79);18 White European, 1 Asian) (Cohort 14) were recruited as the positive group from a tertiary multi-disciplinary clinic (UHB) (as a positive control group). There was no significant difference in age.

Disease Grading and Staging

Diagnosis of OcMMP was as described in section **2.1.3**. Data were collected at 0, 3, 6, 9 and at 12 months follow-up. Visual acuity was categorised as described (**section 2.1.4**). Disease activity was based upon the extent of conjunctival inflammation: absent, mild, moderate or severe (severe defined as being inflamed in all 4 quadrants, the presence of limbitis and/or conjunctival ulceration) (**Figure 1.10**) (Elder, 1997c). Outcome measures were as defined in **Chapter 3**:

Primary outcome measures for disease stage:

1. Tauber's proposed staging system combined with the use of the FDM (BEMC v2) for determining percentage shrinkage of the lower fornix (Tauber et al., 1992) (**Figure 1.11**, **Table 3.1**)
2. Conjunctival shrinkage in mm defined by using the validated FDM (BEMC v2) for the lower and upper fornix (**Chapter 3.1.3**).

Secondary outcome measures for disease stage:

1. Extent of corneal neovascularisation by clock hours of involvement: None, <3°, 3-6° or >9°
2. Corneal neovascularisation encroachment: None, periphery, pupil margin or central cornea
3. Corneal opacification: None, <1/3, 1/3 to 2/3, >2/3
4. Corneal conjunctivalisation: None, <1/3, 1/3 to 2/3, >2/3
4. Corneal keratinisation: None, <1/3, 1/3 to 2/3, >2/3

based upon a modification of the grading systems described for chronic SJS-TEN by Sotozono (Sotozono et al., 2007) and validated for use in the CRF (**Chapter 3**).

Progression was defined as an advance in Tauber staging, an increase in forniceal shrinkage (1mm or greater) for primary outcome measures, an increase in neovascularisation (either clock hours or encroachment) and an increase in corneal opacification (opacification, conjunctivalisation or keratinisation) for secondary outcome measures.

Immunosuppression strategies used a 'step–ladder' approach as previously described (**Figure 1.12**). Information regarding surgical and therapeutic intervention was also recorded.

Sample collection and laboratory analysis

Both eyes were clinically phenotyped and sampled. Sample collection and laboratory analysis is described in **sections 2.1.6** and **2.2**.

Statistical analysis

Data were collected on all eyes and comparisons were undertaken between the worst affected eye for cross-sectional analysis of inflammation and the better-seeing eye for VA. Cross-sectional comparisons were undertaken by non-parametric Mann-Whitney U test for two groups, the Kruskal-Wallis test with Dunn's post hoc analysis when comparing continuous variables e.g. cell numbers or percentage for both eyes. The Jonckheere-Terpstra test was used for ordered alternatives.

Data for both eyes were used for progression, as outcomes could be different for each eye for an individual. Initial comparisons were undertaken by non-parametric Mann-Whitney U test for two groups. If differences using the Mann-Whitney U test were significant, Generalised Estimating Equations (GEEs) were employed when using data for both eyes in order to determine correlation and establish whether progression data for both eyes were significant whilst taking the effects of correlation into consideration. Normality was assessed by using a Kolmogorov-Smirnoff test and data was log transformed where necessary. Differences in progression were determined by the Wald chi-square test.

Differences in repeated measures were also determined by GEE between the right and the left eyes and overall difference over time was determined by the Huynh-Feldt test. Differences in progression between Non-inflamed eyes were undertaken by the Fishers-exact test or Jonckheere-Terpstra test for ordered alternatives.

Significance was defined as $p<0.05$.

5.3 Results

5.3.1 SJS-TEN study

Demographic Information

Of 10 patients (median age 44 [range 18-67 years], 8 females) (Cohort 9), seven where of White European decent, 2 were Black and 1 was Asian. Six individuals presented within 12 months of disease onset which for the purposes of this study we defined as “acute” [<12 months after disease-onset] and four with chronic [12 months after disease-onset]) disease. Five patients were categorised as TEN and all but one patient had a SCORTEN of 1 (**Table 5.1**). Drugs were the most common causative agent (n=7). Visual acuity was found to be unchanged during the study period with 7/10 patients having a VA of 6/18 or better at presentation. All patients were using non-preserved topical lubricants and 9/10 required topical non-preserved steroids (Dexamethasone 0.1% in 7/9).

Disease Activity and Staging

Clinically identifiable inflammation of the conjunctiva was deemed to be absent or mild in 9/10 patients (18/20 eyes) (**Table 5.2**). At presentation, 9/10 had severe disease according to the grading system described by Power and colleagues (Power et al., 1995) whereas the scoring grade was milder according to the staging described by Sotozono and colleagues *(Sotozono et al., 2007)* (**Table 5.2**) (median score of 8/39 [range 1-21]). Objective evidence of fornix shrinkage using a Fornix Depth Measurer was seen in 9/10 subjects, and this inversely correlated with duration of disease ($p<0.05$).

At 12 months follow-up, all patients reviewed had mild conjunctival inflammation: all had severe disease according to Power's classification (indicating that one had evidence of disease progression) and the median Sotozono score remained unchanged (8/39 [range 1-21]) although 3 patients had progressed according to this scale. Only one patient each had evidence of lower and upper fornix shrinkage during the study period (**Table 5.2**).

Patient	Ethnicity	Age	Gender	Disease Severity	Precipitant	Days post onset at 0 months assessment *	Topical steroids during study period	Other steroids/ Immuno-suppression during study period	Surgery during study
1	White	18	Female	TEN	Amoxicillin	114 (Subacute)	Yes	Pulse iv methyl-prednisolone	Phaco/ AMG
2	Black	41	Female	SJS	Mycoplasma	168 (Subacute)	Yes	None	None
3	White	67	Female	SJS	Trimethoprim	16 (Acute)	Yes	None	None
4	White	59	Female	TEN	Phenytoin	9178 (Chronic)	None	Azathioprine	None
5	White	27	Male	SJS	Unknown	9 (Acute)	Yes	None	None
6	Black	20	Male	SJS	Carba-mazepine	1 (Acute)	Yes	None	None
7	White	48	Female	TEN	Unknown	3657 (Chronic)	Yes	None	None
8	White	37	Female	TEN	Penicillin	592 (Chronic)	Yes	None	None
9	Asian	57	Female	SJS	Unknown	2047 (Chronic)	Yes	None	None
10	White	46	Female	TEN	Carba-mazepine	25 (Acute)	Yes	Subtarsal Triamcinolone	AMG

*Acute defined as <365 days post onset (sub-acute defined as 30-364 days); Chronic defined as >365 days post onset (Power et al 2005; Sotozono et al 2007 Ophthalmology)
AMG = Amniotic Membrane Grafting
iv=Intravenous
Phaco=Phacoemulsification cataract extraction

Table 5.1. Demographic information, aetiology and therapy during the SJS-TEN study.

	Conjunctival inflammation[+1]		Disease Stage (Power[+2])		Disease Stage (Sotozono: out of 39[+3])		Lower Fornix				Upper Fornix			
							Symblephara: mm horizontal involvement of fornix (number)[+4]		Fornix Depth in mm (shrinkage[+5])		Symblephara: mm horizontal involvement of fornix (number)		Fornix Depth in mm[+4]	
Time point	**0**	**12**	**0**	**12**	**0**	**12**	**0**	**12**	**0**	**12**	**0**	**12**	**0**	**12**
1	Mild	Mild	Severe	Severe	7	4	3(1)	3(1)	8 (Yes)	6 (Yes)	0 (0)	0 (0)	16	15
2	None	Mild	Severe	Severe	8	10	10(2)	10(2)	8 (Yes)	8 (Yes)	0 (0)	0 (0)	16	15
3	Mild	Mild	Severe	Severe	9	10	10(3)	16(3)	4 (Yes)	3 (Yes)	0 (0)	4 (1)	16	16
4	None	Mild	Severe	Severe	12	17	12(3)	18(2)	2 (Yes)	2 (Yes)	8 (2)	12 (2)	4	8
5	Mild	-	Mild	-	1	-	0(0)	-	10 (Yes)	-	0 (0)	0 (0)	18	-
6	None	Mild	Severe	Severe	3	3	4(1)	2(1)	12 (No)	12 (No)	0 (0)	0 (0)	20	20
7	Mild	Mild	Severe	Severe	21	21	8(3)	8(3)	0 (Yes)	0 (Yes)	4 (2)	4 (2)	10	10
8	None	Mild	Severe	Severe	5	5	0(0)	0(0)	6 (Yes)	4 (Yes)	0 (0)	0 (0)	16	17
9	Mild	Mild	Mild	Severe	5	5	0(0)	2(1)	9 (Yes)	10 (Yes)	0 (0)	0 (0)	18	18
10	Moderate	Mild	Severe	Severe	8	8	6(1)	10(2)	8 (Yes)	8 (Yes)	0 (0)	0 (0)	18	16

+Worst (Right) Eye

Scoring references:

1. Elder *et al* Cicatrising Conjunctivitis (Elder, 1997c)
2. Power *et al*. Ophthalmology (Power et al., 1995)
3. Sotozono *et al*. Ophthalmology (Sotozono et al., 2007)
4. Tauber *et al.* Cornea (Tauber et al., 1992)
5. Schwab *et al*. Ophthalmology (Schwab et al., 1992)

Table 5.2. Disease activity and staging at 0 and 12 months during the SJS-TEN study. All patients had a SCORTEN of 1 (with the exception of patient 10 who had a score of 2). None had an episode of scleritis during the study period. Conjunctival inflammation and staging systems were assessed at 0 and 12 months. 9/10 patients were reviewed at 12 months (1 individual declined follow up).

Conjunctival Epithelial Leukocyte Populations

Gating strategies to characterise cellular populations are shown in **Figure 5.1**. There were no differences in the number of conjunctival epithelial leukocytes (p=0.15) or lymphocytes in SJS-TEN compared with healthy controls (median 337[range 35-6383] vs. 488[10-3026], p=0.75). There was also no difference between the total number of CD3+CD56- T cells, or of the number of cells for T cell subsets including TCR$\alpha\beta$+CD3+CD8$\alpha\beta$+ cells (**Figure 5.2**). A reduction in the percentage of TCR$\alpha\beta$+CD3+CD8$\alpha\beta$+ T cells was however seen (57% vs. 80%;p<0.01), with a corresponding elevation of TCR$\alpha\beta$+CD3+CD8β-CD4- (Double Negative) T cells (23% vs. 7%;p=0.11). No differences were seen in the TCR$\alpha\beta$+CD3+CD4+ (9% vs. 9%;p=0.67) or TCR$\alpha\beta$+CD3+CD8$\alpha\alpha$+ T cell subsets (1% vs. 3%;p=0.16), or in other lymphocyte subsets including NK cells (7.5% vs. 5%;p=0.45) (**Figure 5.2**), NKT cells (2% vs. 5.5%;p=0.28) or CD19+CD20+ B cells (3% vs. 3%;p=0.72).

Granulocytes were further defined as $CD45^{INT}$CD11b+CD16+CD14- cells. This gating strategy discriminates neutrophils from other granulocytes (such as basophils and eosinophils) and monocytes (Stelzer et al., 1993, Gopinath and Nutman, 1997), so that the $CD45^{INT}$CD11b+CD16+CD14- granulocyte population would be comprised primarily of neutrophils. A significant increase in the number and percentage of $CD45^{INT}$CD11b+CD16+CD14- neutrophils (186 vs. 3.4, p<0.01;31% vs. 0.8%, p<0.001) were seen in individuals with SJS-TEN (**Figure 5.2**). At presentation however, the elevation in neutrophils was found to inversely correlate with disease duration (r=-071,p=0.03) (**Figure 5.3A**). This suggests that neutrophil levels were highest in the acute stages of disease. Of interest, this elevation was maintained over the study period ((p=1.0) indicating a persistent elevation in this inflammatory

infiltrate (**Figure 5.3B**). This was confirmed when comparing the number and percentage of neutrophils between patients with SJS-TEN at 12 months with the same cohort of healthy participants (247 vs. 3.4, p<0.01;15% vs. 0.8%, p<0.001).

As the majority of our patients were prescribed topical Dexamethasone, it was important to determine the influence of this synthetic glucocorticoid on conjunctival neutrophils. This was particularly important given the elevation in neutrophils seen. A cohort of 7 healthy volunteers were therefore sampled before and after cataract surgery in order to test the effect of Dexamethasone on healthy participants. There was no significant difference in the $CD45^{INT}$CD11b+CD16+CD14- neutrophil populations before and after the use of topical treatment (13 vs. 0, p=0.06;1.7% vs. 0%, p=0.13), although the trend suggested a reduction in the numbers present on the occur surface (**Figure 5.4**). The use of topical Dexamethasone in the majority of the SJS-TEN cohort is unlikely therefore to have contributed to an elevation in ocular surface granulocytes.

The total number and percentage of monocytes was elevated in SJS-TEN (185 vs. 21, p=0.02;12% vs. 6%, p=0.02) and this was attributable to an increase in the number of CD14+CD16- monocytes (140 vs. 6, p=0.03). No change in the total leukocyte (p=0.82), or lymphocyte populations (p=0.57) was observed during the study period and CD8αβ+ T cells (p=0.57) and NK cells populations also remained unaltered (p=1.0) (**Figure 5.3B**).

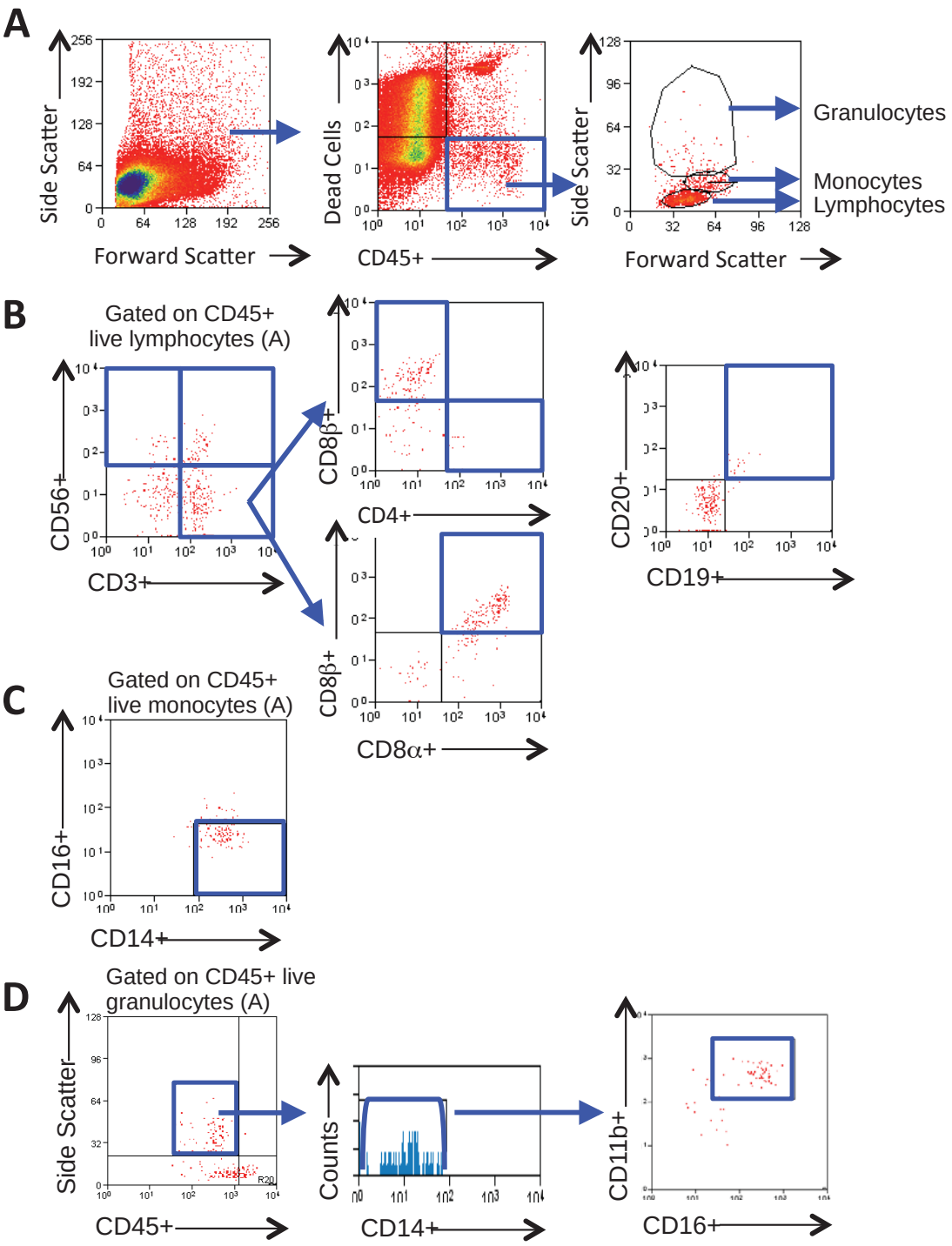


Figure 5.1. Gating strategy to determine cellular populations in disease using OSIC. Representative plots of a subject with SJS-TEN demonstrating the gating strategy used to identify conjunctival leukocytes. Live leukocytes populations (lymphocytes, monocytes and granulocytes) were identified in conjunctival cells by gating for CD45+ cells that were negative for the dead cell exclusion dye Sytox blue and back-gated to show the forward and side scatter profiles of the CD45+ live cells (Panel A). Lymphocyte subsets were identified according to the expression of CD3 and CD56 in one panel (to discriminate T cells [CD3+CD56-], Natural Killer Cells [CD56+CD3-], NKT cells [CD3+CD56+]) and B cells [CD19+CD20+] (Panel B) (CD8+ cell populations shown are TCRαβ+). Monocytes were further characterized by the presence of CD14 and CD16 (Panel C). Neutrophils were defined as $CD45^{INT}$CD14-CD11b+CD16+ granulocytes (Panel D). Cell populations of interest are boxed (blue).

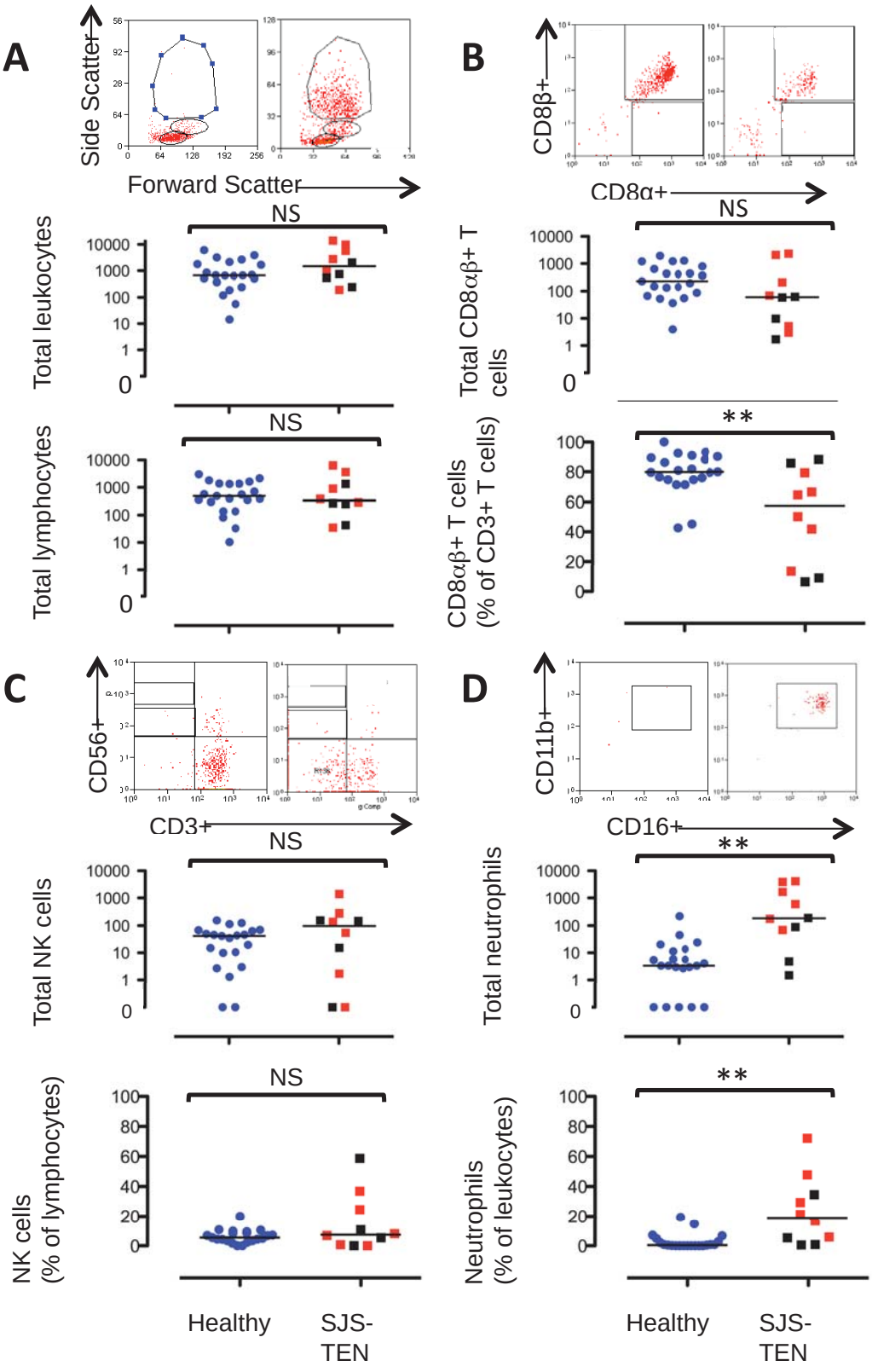


Figure 5.2. The dominant cellular infiltrate in the conjunctival epithelium in SJS/TEN is characterised by an increase CD45^{INT}CD11b+ CD16+ CD14- neutrophils. 21 healthy subjects were compared with 10 patients with SJS-TEN (6 patients in the acute stages ■ and 4 in the chronic stages of disease ■). Representative flow cytometry plots are shown. Statistical comparisons between healthy and SJS-TEN conjunctival leukocyte (Panel A), lymphocyte (A), CD8αβ+ (B), NK (CD56+CD3-) and CD45 ^{INT}CD11b+CD16+CD14- neutrophil (D) populations were undertaken by the Mann-Whitney U test (NS: Not significant [p>0.05], **p < 0.01).

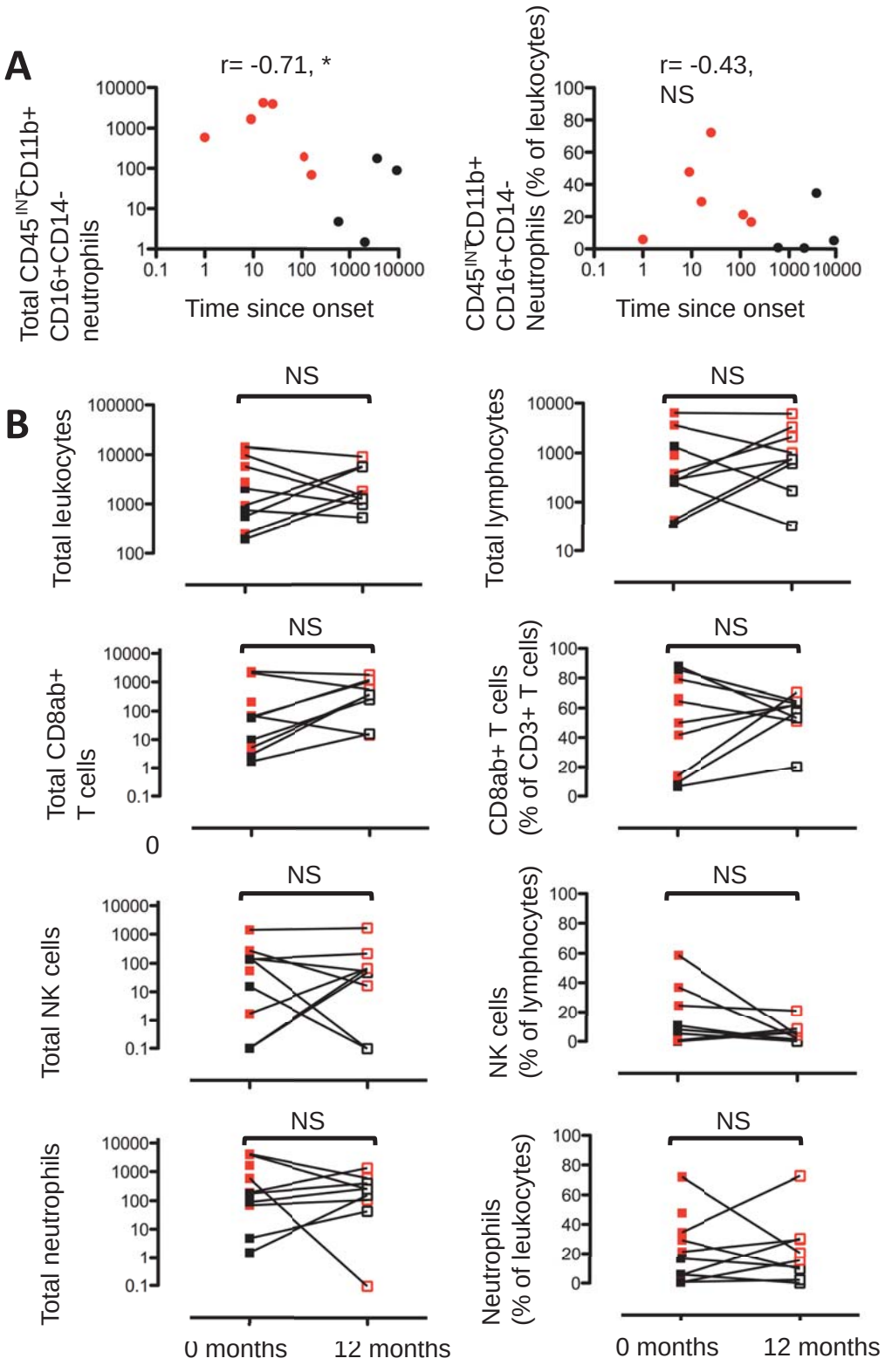


Figure 5.3. CD45^{INT}CD11b+CD16+CD14- neutrophils are inversely correlated with disease duration but persist with time. CD45^{INT}11b+CD16+CD14- neutrophil populations were correlated with disease duration at presentation (0 months; Panel B) using Spearman's correlation (NS: Not significant [p>0.05]; *p< 0.05). 9/10 patients with SJS-TEN (6 patients in the acute stages ■ and 4 in the chronic stages of systemic disease■) were sampled at 0 and 12 months. Paired analysis of SJS-TEN conjunctival leukocyte, lymphocyte, CD8ab+, Natural Killer (CD56+CD3-) and CD11b+CD16+ neutrophil populations at 0 (closed squares) and 12 months (open squares) were undertaken by the Wilcoxon signed rank test (NS: Not significant [p>0.05]) (Panel A).

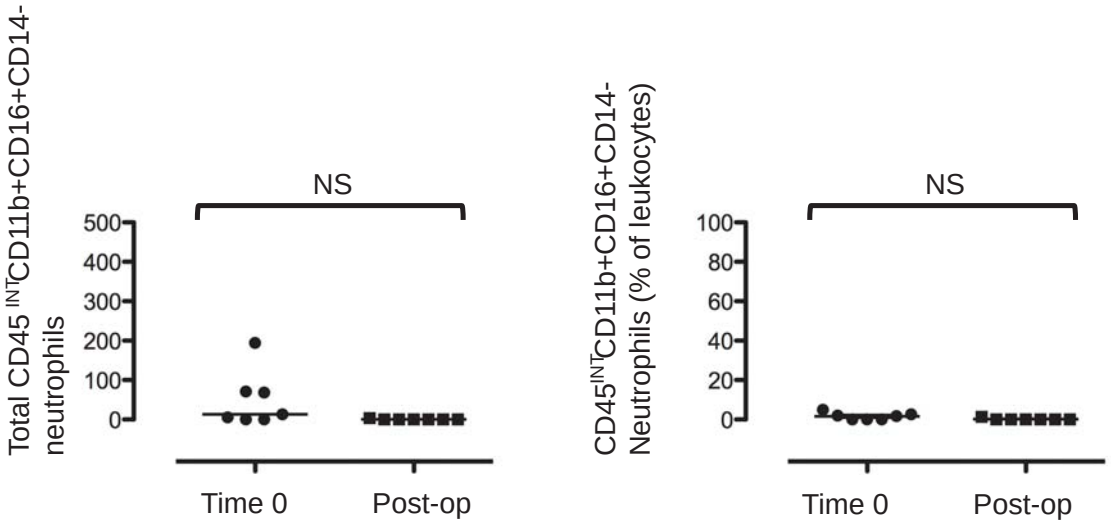


Figure 5.4. Epithelial neutrophil populations are unaltered by topical Dexamethasone. Neutrophils (defined as $CD45^{INT}$CD11b+CD16+CD14- live granulocytes) are shown as absolute numbers and percentage of leukocytes before and after the use of topical steroids (preservative free Dexamethasone 0.1%). Individuals had conjunctival OSIC before and after uncomplicated cataract surgery. Repeated samples were taken at 4 weeks post-op and in the absence of ocular surface and intra-ocular inflammation (NS: Not significant [p>0.05]).

5.3.2 OcMMP study

Demographic information, histological profile of disease and extra-ocular features in OcMMP

The conjunctival epithelium in a younger cohort of patients with SJS-TEN was shown to have significant elevation of $CD45^{INT}$CD11b+CD16+CD14- neutrophils. This alteration in neutrophils was also maintained over the course of the 12-month study period. Although this cohort was of a modest size, it has offered an insight to the inflammatory infiltrate in one type of acquired ocular immunobullous disease. The next step was to determine leukocyte changes in a larger cohort of patients with OcMMP.

Of the sixty patients with OcMMP approached, 57 were recruited to the study. The median age was 72[range 49-97 years]; 30 females (54%); 55 White European, 1 Asian and 1 Afro-Caribbean.

The majority, 70%(40/57), of patients were biopsy positive OcMMP. Thirteen were biopsy negative and four did not undergo biopsy; 3 were greater than 95 years and not considered for immunosuppression and 1 patient declined a conjunctival biopsy. Forty two per cent (24/57) had evidence of extra-ocular involvement, of whom 40% had oral MMP. Demographic information is summarised in **Table 5.3**.

	All patients
Total no. of patients	57
Total no. of eyes	114
Male: Female (% Female)	27:30 (53%)
Median age (yrs) [Range]	72(48-97)
Median duration of symptoms (Days) [Range]	930(30-14600)
No of patients sampled longitudinally for 12 months ¶	35
Biopsy *	
DIF +ve	70% (40/57)
IIF +ve †	30% (17/57)
Extra ocular mucocutaneous involvement	
All mucocutaneous tissues	42% (24/57)
Skin	4% (2/57)
Oral	40% (23/57)
Visual Acuity ‡	
Normal: 6/6 ->6/18	91% (43/47)
Visual impairment: <6/18- 6/60	4% (2/47)
Severe visual impairment: 6/60-3/60	0% (0/47)
Blind: <3/60	4% (2/47)
‡*Excluded due to other causes*	18% (10/57)

Table 5.3 Patient demographics and characteristics in the OcMMP study. Direct immunofluorescence (DIF) and Indirect immunofluorescence (IIF) refer to the proportion of patients who demonstrated the linear deposition of immunoglobulin G, A or complement (C3) along the basement membrane zone (BMZ) or had measurable titres of immunoglobulin in the serum respectively.

¶ Follow-up: 35 patients were followed longitudinally and underwent clinical phenotyping and OSIC sampling at 0, 3, 6, 9 and 12 months. Of the 22 who did not undergo longitudinal assessment, 6 declined longitudinal sampling, 4 did not attend for repeated sampling, 3 died and 9 could not be followed up for longitudinal sampling as they were sampled within 12 months of the end of the study.

***** Four patients in total did not undergo a conjunctival biopsy (3 patients with advanced age (> 95 years) and immunosuppression was systemically contraindicated and the remaining 1 patients declined a confirmatory biopsy.

† All patients who were IIF were also DIF positive. There were no patients who were IIF positive in the absence of positive DIF studies.

‡ VA represents a comparison of visual acuity in the **better-seeing eye**, after exclusion of other causes of reduced vision such as cataract, glaucoma, uveitis and optic neuritis (n=10).

Disease Activity and Damage at Presentation

Conjunctival inflammation was present in 65% (37/57) individuals with 35%(20/57) patients having no clinically identifiable conjunctival inflammation at presentation. Details of the severity of inflammation are outlined in **Table 5.4**

Disease Activity	Right Eyes	Left Eyes	All Eyes
Conjunctival Inflammation			
Absent	40% (23/57)	42% (24/57)	41% (47/114)
Mild	39% (22/57)	35% (20/57)	37% (42/114)
Moderate	16% (9/57)	19% (11/57)	17.5% (20/114)
Severe	5% (3/57)	3.5% (2/57)	4% (5/114)

Table 5.4 Disease Activity – Clinically identifiable conjunctival inflammation in the OcMMP study. Conjunctival inflammation was graded by the scale described by Elder *et al (Elder, 1997c)*.

Table 5.5 summarises the primary damage indices derived from clinical data recorded in CRFs. Tauber scores for the right and left eyes are shown together with Mondino and Foster scores for comparative reasons (documentation was complete with the exception of one individual where the Tauber score could not be calculated).

Primary scarring measurements for scarring included upper fornix depth, lower fornix depth and the scale described by Tauber et al (Tauber et al., 1992) (**Table 5.5**). Median upper fornix depth was 14mm[0-22], the number of upper fornix symblephara was 0[0-4], symblephara involvement was 0mm[0-15]. In the absence of anthropological information about the normal upper fornix depth, Tauber scale cannot be calculated. Median lower fornix depth was 4mm[0-13] the number of lower fornix

symblephara was 2[0-6], with symblephara involvement 6mm[0-30]. Forty three per cent eyes (49/114) were Mondino stage III/IV, reflecting the median lower fornix depth of 4mm. Ninety six per cent of eyes (110/114) were Foster stage III/IV. The Tauber scale's ability to stratify disease further by the extent of symblephara is reflected by the distribution within Tauber stage III.

Secondary scarring measures were also quantified from CRFs and reveal that 41% of eyes (47/114) had evidence of at least 3° (clock hours) of corneal neovascularisation (36% of eyes (41/114) had encroachment to the periphery or greater). Opacification was seen in 33% (38/114) whereas conjunctivalisation was evident in 27% (31/114) and keratinisation in 10% (11/114).

Disease Damage	Right eyes	Left eyes	All eyes
Upper Fornix			
Depth in mm (median[range])	14mm[0-22]	13mm[0-22]	14mm[0-22]
Symblephara number (median[range])	0[0-3]	0[0-4]	0[0-4]
Symblephara involvement (median[range])	0[0-15]	0[0-15]	0[0-15]
Lower Fornix			
Depth in mm (median[range])	4mm[0-13]	4mm[0-12]	4mm[0-13]
Symblephara number (median[range])	2[0-6]	2[0-5]	2[0-6]
Symblephara involvement (median[range])	8[0-30]	6[0-30]	6[0-30]
Mondino			
I	12% (7/57)	12% (7/57)	12% (14/114)
II	26% (15/57)	26% (15/57)	26% (30/114)
III	35% (20/57)	39% (22/57)	37% (21/114)
IV	26% (15/57)	23% (13/57)	25% (28/114)
Foster			
I	1.5% (1/57)	1.5% (1/57)	1.5% (2/114)
II	10.5% (6/57)	10.5% (6/57)	10.5% (12/114)
III	65% (37/57)	61% (35/57)	63% (72/114)
IV	23% (13/57)	26% (15/57)	24% (28/114)
Tauber			
I	1.5% (1/57)	1.5% (1/57)	1.5% (2/114)
IIa	3% (2/57)	5% (3/57)	4% (5/114)
IIb	1.5% (1/57)	1.5% (1/57)	1.5% (2/114)
IIc	4.5% (3/57)	3% (2/57)	4% (5/114)
IId	0% (0/57)	0% (0/57)	0% (0/114)
IIIa	26% (15/57)	26% (15/57)	26% (30/114)
IIIb	28% (16/57)	25% (14/57)	26% (30/114)
IIIc	7% (4/57)	7% (4/57)	7% (8/114)
IIId	1.5% (1/57)	0% (0/57)	1% (1/114)
IV	23% (13/57)	28% (16/57)	25% (29/114)

Table 5.5 Disease Damage: Primary measures of scarring at presentation in the OcMMP study. Stratification of patients by upper and lower fornix scarring. Primary measures were considered to be upper fornix depth, lower fornix depth and lower fornix Tauber scoring (Tauber et al., 1992) (Mondino and Foster scales are shown for comparative purposes) (Mondino and Brown, 1981, Foster, 1986).

Disease Damage	Right eyes	Left eyes	All eyes
Cornea			
Neovascularisation (clock hours)			
None	54% (31/57)	60% (34/57)	57% (65/114)
<3	5% (3/57)	10.5% (6/57)	8% (9/114)
3-9	7% (4/57)	5% (3/57)	6% (7/114)
>9	32% (18/57)	23% (13/57)	27% (31/114)
Neovascularisation (encroachment)			
None	60% (34/57)	*65% (37/57)*	62% (71/114)
Periphery	28% (16/57)	18% (10/57)	23% (26/114)
Pupil margin	3.5% (2/57)	*10.5% (6/57)*	7% (8/114)
Central	7% (4/57))	5% (3/57)	6% (7/114)
Opacification			
None	65% (37/57)	65% (37/57)	65% (74/114)
<1/3	18% (10/57)	18% (10/57)	18% (20/114)
1/3 to 2/3	7% (4/57)	10.5% (6/57)	9% (10/114)
>2/3	9% (5/57)	5% (3/57)	7% (8/114)
Conjunctivalisation			
None	70% (40/57)	72% (41/57)	71% (81/114)
<1/3	10.5% (6/57)	10.5% (6/57)	10.5% (12/114)
1/3 to 2/3	9% (5/57)	10.5% (6/57)	10% (11/114)
>2/3	9% (5/57)	5% (3/57)	7% (8/114)
Keratinisation			
None	86% (49/57)	91% (52/57)	89% (101/114)
<1/3	5% (3/57)	0% (0/57)	2.5% (3/114)
1/3 to 2/3	1.5% (1/57)	1.5% (1/57)	1.5% (2/114)
>2/3	5% (3/57)	5% (3/57)	5% (6/114)

Table 5.6 Disease Damage: Secondary measures of scarring at presentation in the OcMMP study. Stratification of patients by corneal scarring. Secondary measures were considered to be corneal neovascularisation (clock hours of involvement and encroachment), opacification, conjunctivalisation and keratinisation (all measured by the extent of corneal involvement).

Surgical and Therapeutic Intervention

The details of surgical and therapeutic intervention are summarised in **Table 5.7**. The most common intervention was eyelid/fornix intervention at presentation and during the course of the study. The majority of patents required topical steroids and almost all of the cohort required topical preservative free lubrication. Fifty six per cent (30/57) were on some form of immunosuppression at presentation and this had increase as a proportion to 72% (25/35) by 12 months.

	intervention	**Presentation**		**0 to 12 months**	
Surgery					
	Eyelid/fornix surgery	35% (20/57)		20% (7/35)	
	Corneal intervention	3.5% (2/57)		0% (0/35)	
	Cataract surgery	12% (7/57)		3% (1/35)	
Topical Therapy					
	PF Lubrication	86% (49/57)		97% (34/35)	
	PF Steroid*	54% (31/57)		52% (18/35)	
Systemic Immunosuppression			**Step Up**		**Step Up**
			16% (9/57)		20% (7/35)
	Oral Prednisolone	14% (8/57)		14% (5/35)	
	Dapsone	16% (9/57)		6% (2/35)	
	Azathioprine/ Mycophenolate	23% (13/57)		34% (12/35)	
	Cyclophosphamide	2% (1/57)		9% (3/35)	
	Biologics (Rituximab)	2% (1/57)		3% (1/35)	

* **Dexamethasone was the topical steroid used in 55% (17/31) at presentation and 67% (12/18) between 0 and 12 months**

Table 5.7 Therapeutic Intervention including surgery, topical therapy and systemic immunosuppression in the OcMMP study. A step up in therapy was considered to be an escalation of treatment according to the validated step-ladder described by Rauz et al (Rauz et al., 2005a); **Figure 1.12**.

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Conjunctival epithelial cell populations in OcMMP

Cross-Sectional Comparison at Presentation:

OcMMP vs. healthy controls vs. positive disease controls

Comparisons between the conjunctival leukocyte populations in the worst inflamed eye in the cohort with OcMMP, a healthy group (right eyes) and those with Primary Sjögrens Syndrome (PSS) (right eyes) revealed that there was no difference in the total number of leukocytes (including the total number of lymphocytes, monocytes and neutrophils) between groups (**Table 5.8**). Gating strategies were as described in **Figure 5.1**.

Comparisons between individual populations revealed no difference between the total numbers of lymphocytes (median[range] Healthy(H):684[73-9775] vs. PSS(P):849[70-7013] vs. OcMMP(M):772[23-6059];p=0.2) or monocytes (H:107[2-1949] vs. P:224[2-1945] vs. M:229[0-10763];p=0.12) although the lymphocytes percentage was lower in the OcMMP cohort (**Table 5.8** and **Figure 5.5**). This was accounted for by a significantly higher number and percentage of neutrophils, compared to healthy participants and those with PSS (n; H:5.8[0-151] vs. P:0.8[0-109] vs. M:109[1-197799];p<0.0001 and %; H:0.8[0-38] vs. P:0.2[0-4] vs. M:18[0.1-86.7];p<0.0001 respectively) (**Table 5.8** and **Figure 5.5**).

Individual lymphocyte subset comparisons demonstrated that there were less T cells (CD3+CD56- live cells) as a total number and percentage of lymphocytes in the conjunctiva of patients with MMP (**Table 5.9**). In addition, the dominant CD8$\alpha\beta$+ population was significantly lower in patients with MMP (H:445 vs. P:104 vs. M:371;

p=0.0001), resulting in an increased percentage of CD4+ cells as a proportion of T cells and subsequently an elevation in the CD4:CD8 ratio in OcMMP compared to healthy controls (0.15 vs. 0.4 vs. 0.5; p=0.003) (**Figure 5.6**). This alteration was greater despite an expected elevation in CD4+ T cells in PSS (Stern et al., 2002). An elevation in the percentage of CD8αα+ and Double Negative (CD8αβ-CD4-) T cells was also observed.

No changes in the B cell or NK populations were seen, but a reduction in the NKT cell population was seen in MMP compared to healthy controls (**Table 5.9**).

Cell population	Healthy	PSS	MMP	Kruskal-Wallis	Dunn's post hoc
CD45+ live leukocytes	870.9	1459	1987	0.44 (NS)	NS
Leukocytes subsets					
Total lymphocyte, monocytes & neutrophils	766.3	1041	1504	0.53 (NS)	NS
Lymphocytes numbers	684	849	772	0.20 (NS)	NS
Lymphocytes % of leukocytes	73.0	65.1	35.6	**<0.0001**	H v M ***, P v M ***
Monocyte numbers	107	204	229	0.12 (NS)	NS
Monocytes % of leukocytes	7.00	15.0	10.7	0.12 (NS)	NS
Neutrophil numbers	5.80	1.80	109	**<0.0001**	H v M ***, P v M ***
Neutrophils % of leukocytes	0.800	0.200	18.1	**<0.0001**	H v M ***, P v M ***
CD11b+ MFI	374	0.0	588	**0.002**	P v M **

Table 5.8. Conjunctival leukocyte populations in healthy volunteers, PSS and OcMMP. Comparisons were undertaken by comparing the most inflamed eye in patients with MMP (M) vs. the right eye of healthy participants (H) and patients with PSS (P). Median values are shown and comparisons were undertaken by the Kruskal-Wallis test with Dunn's post hoc analysis test (Key: NS: Not significant, *p=0.01 to 0.05; **p=0.001 to 0.01; *** p<0.001).

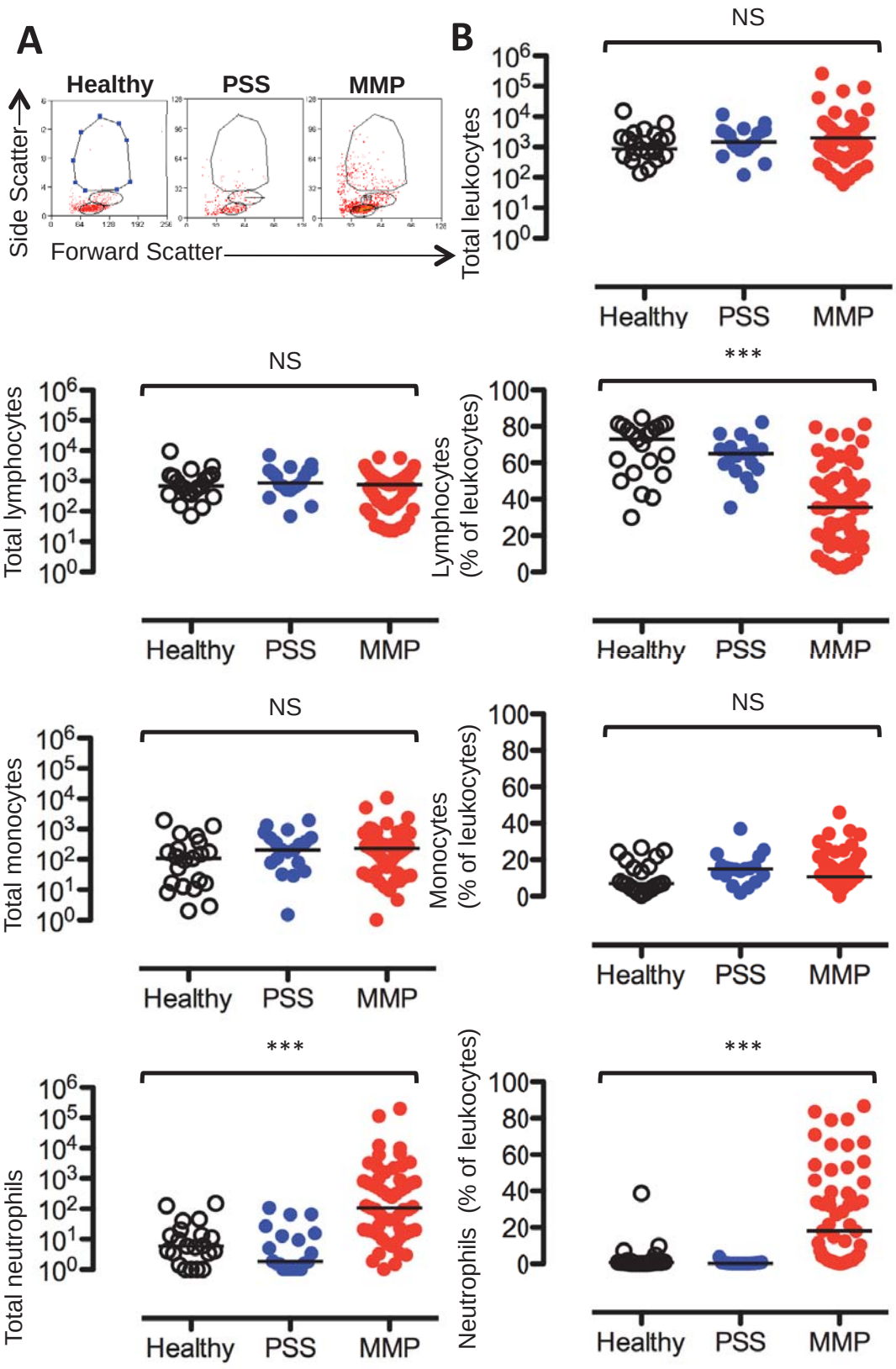

Figure 5.5: Ocular Mucous Membrane Pemphigoid is characterised by an elevation in conjunctival epithelial CD45^{INT}CD11b+CD16+CD14- neutrophils. Comparisons were undertaken by comparing the most inflamed eye in patients with MMP vs. the right eye of healthy individuals and patients with PSS (Flow cytometric plots are shown in Panel A). Three group comparisons were undertaken by the Kruskal-Wallis test (Panel B) (Key: NS: Not significant, *p=0.01 to 0.05; **p=0.001 to 0.01; *** p<0.001).

Cell population	Healthy	PSS	MMP	Kruskal-Wallis	Dunn's post hoc
T cells					
T cell numbers	436	686	312	**0.005**	P v M **
T cells (% of lymphocytes)	69.9	70.8	53.7	**<0.0001**	H v M**, P v M***
CD8αβ+ numbers	371	445	124	**0.0001**	H v M **, P v M ***
CD8αβ+ % of T cells	76.0	61.5	50.0	**0.0008**	H v M***
CD8αβ+ CD45RO+ %	92.7	96.1	94.6	0.10 (NS)	NS
CD4+ numbers	38.2	199	50.3	0.05 (NS)	NS
CD4+ % of T cells	10.7	25.2	24.5	**0.02**	H v M*
CD4+CD45RO+ %	100	100	100	0.88 (NS)	NS
CD4:CD8 ratio	0.150	0.400	0.500	**0.003**	H v M**
DN numbers	13.9	52.9	34.0	0.07 (NS)	NS
DN% of T cells	5.40	7.90	17.5	**0.0006**	H v M***
CD8αα+ numbers	8.00	20.1	12.6	0.17 (NS)	NS
CD8αα+ % of T cells	1.80	4.60	7.05	**0.01**	H v M**
B cell numbers	18.5	43.2	17.6	0.32 (NS)	NS
B cell % of lymphocytes	2.50	2.70	4.40	0.4 (NS)	NS
NK cell numbers	46.5	60.2	49.5	0.48 (NS)	NS
NK cells % lymphocytes	6.90	5.60	7.90	0.62 (NS)	NS
NKT cell numbers	31.5	43.9	18.6	**0.02**	P v M*
NKT cells % lymphocytes	5.10	6.30	2.90	0.14 (NS)	NS

Table 5.9 Conjunctival lymphocyte populations in healthy volunteers, PSS and OcMMP. Comparisons were undertaken by comparing the most inflamed eye in patients with MMP (M) vs. the right eye of healthy participants (H) and patients with PSS (P). Median values are shown and comparisons were undertaken by the Kruskal-Wallis test with Dunn's post hoc analysis test (Key: NS: Not significant, *p=0.01 to 0.05; **p=0.001 to 0.01; *** p<0.001).

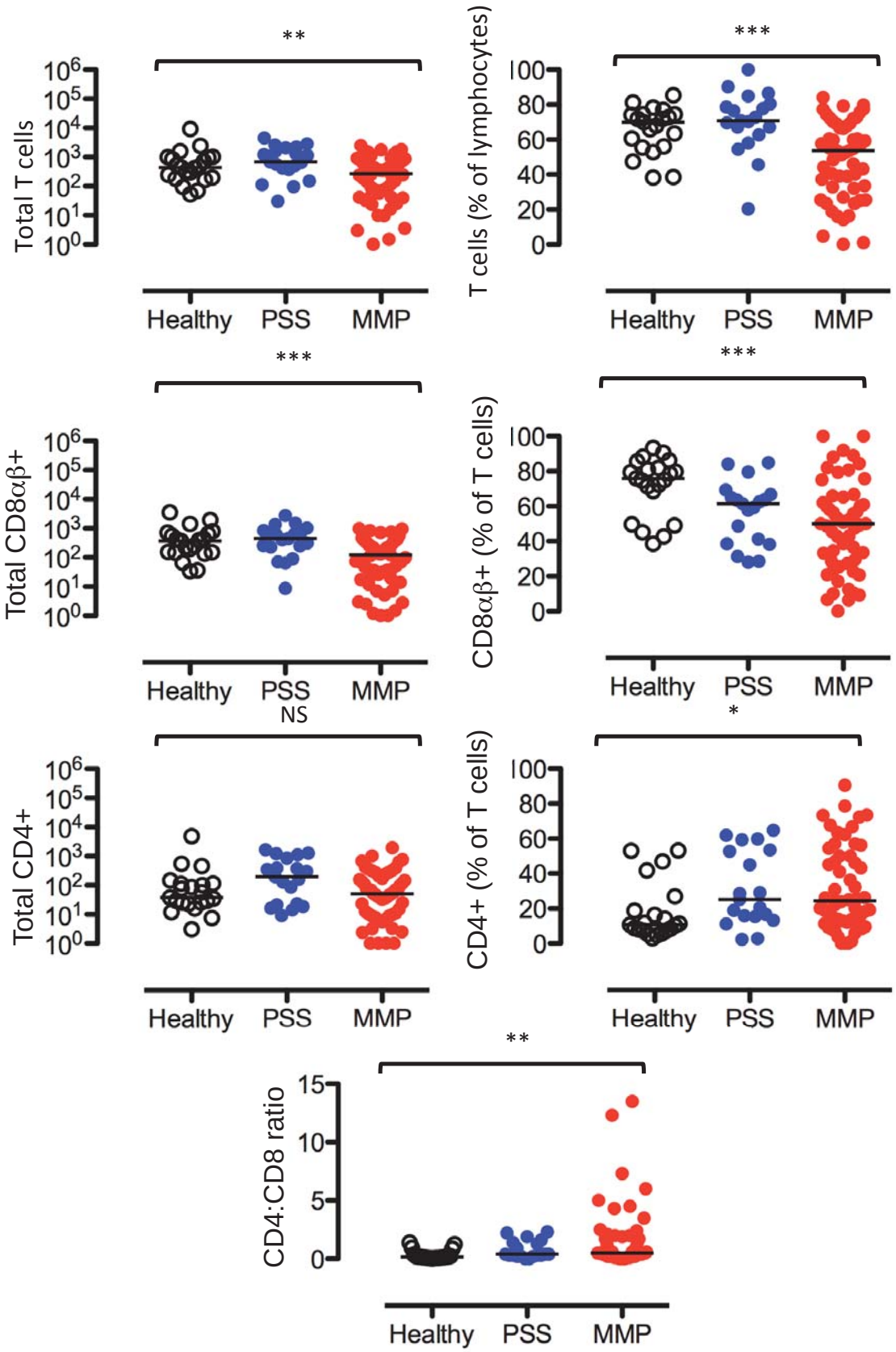

Figure 5.6: Ocular Mucous Membrane Pemphigoid is characterised by a reduction in the dominant CD8αβ+ T cell population and an elevation in the CD4:CD8 ratio. Comparisons were undertaken by comparing the most inflamed eye in patients with MMP vs. the right eye of healthy individuals and patients with PSS. Three group comparisons were undertaken by the Kruskal-Wallis test (Key: NS: Not significant, *p=0.01 to 0.05; **p=0.001 to 0.01; *** p<0.001).

No differences in the total leukocyte populations were observed in the peripheral blood of patients with MMP, healthy volunteers and those with PSS (153,838 cells/ml blood vs. 155052 vs. 188391; p=0.68). Lymphocytes (n(%);59,408(37%) vs. 73,188(44) vs. 53,874(35);p=0.2), neutrophils (n(%);74,786(52%) vs. 93,421(45) vs. 76,314(49);p=0.78) and monocyte populations were also unaltered.

Some alterations in the subsets of lymphocytes and monocytes were evident. The total $CD14^{INT}CD16+$ monocyte population was lower in MMP compare to PSS (n(%);961(9.8%) vs. 833(9.1) vs. 1533(18.6);p=0.006). The proportion of T cells populations were significantly lower in MMP compared to PSS (65.8% vs. 70.1 vs. 75;p=0.01). Antigen experienced (CD45RO+) CD4+ T cells were also higher in MMP (81%) v Healthy (65%) vs. PSS (70.1) (p=0.01). No difference in the CD8$\alpha\beta$+ T cell (7602 vs. 9205 vs. 11,067;p=0.2) or CD4+ (25,227 vs. 35,209 vs. 20,220;p=0.06) population was seen however.

Cross-Sectional Comparison at Presentation:

Biopsy Positive vs. Biopsy Negative OcMMP

There was no difference in the total number/percentage of leukocytes (1579 vs. 2595;p=0.18), monocytes (194 vs. 289;p=0.51) and neutrophils (102 vs. 239;p=0.94) between biopsy positive (n=40) and negative (n=17) MMP patients. The total lymphocyte (543 vs. 1080; p=0.048) and T cell numbers (including CD8$\alpha\beta$+, CD4+, DN and CD8$\alpha\alpha$+) (238 vs. 630; p=0.006) were lower in the biopsy positive group. The percentage of lymphocytes (35 vs. 41%; p=0.36), T cells (54 vs. 54%; p=0.18) and CD4:CD8 ratio (0.5 vs. 0.6; p=0.6) however were not different and no other

changes were seen. There was no statistically significant difference in the duration of symptoms between the biopsy positive and negative groups to account for differences in lymphocytes (730[30-14600] vs. 1009[180-9125]; p=0.12).

Cross-Sectional Comparison at Presentation:

The inflammatory cellular infiltrate and clinically identifiable conjunctival inflammation

The number and percentage of conjunctival epithelial $CD45^{INT}$CD11b+CD16+CD14- neutrophils correlated with the severity of conjunctival inflammation; Healthy 5.8(0.8%) vs. Non-inflamed MMP 44.3(7.9%) vs. Mildly inflamed MMP 94.3(7.3%) vs. Moderately/Severely inflamed 1912(42.3%) ($p<0.0001$), shown in **Figure 5.7A,B**. However, when comparing the Non-inflamed conjunctival neutrophil population, this was still significantly higher than when compared to healthy controls 44.3(7.9) vs. 5.8(0.8);p=0.02 and p=0.004 (**Figure 5.7C)**. This suggests that neutrophils are present, even in the absence of clinically manifest conjunctivitis.

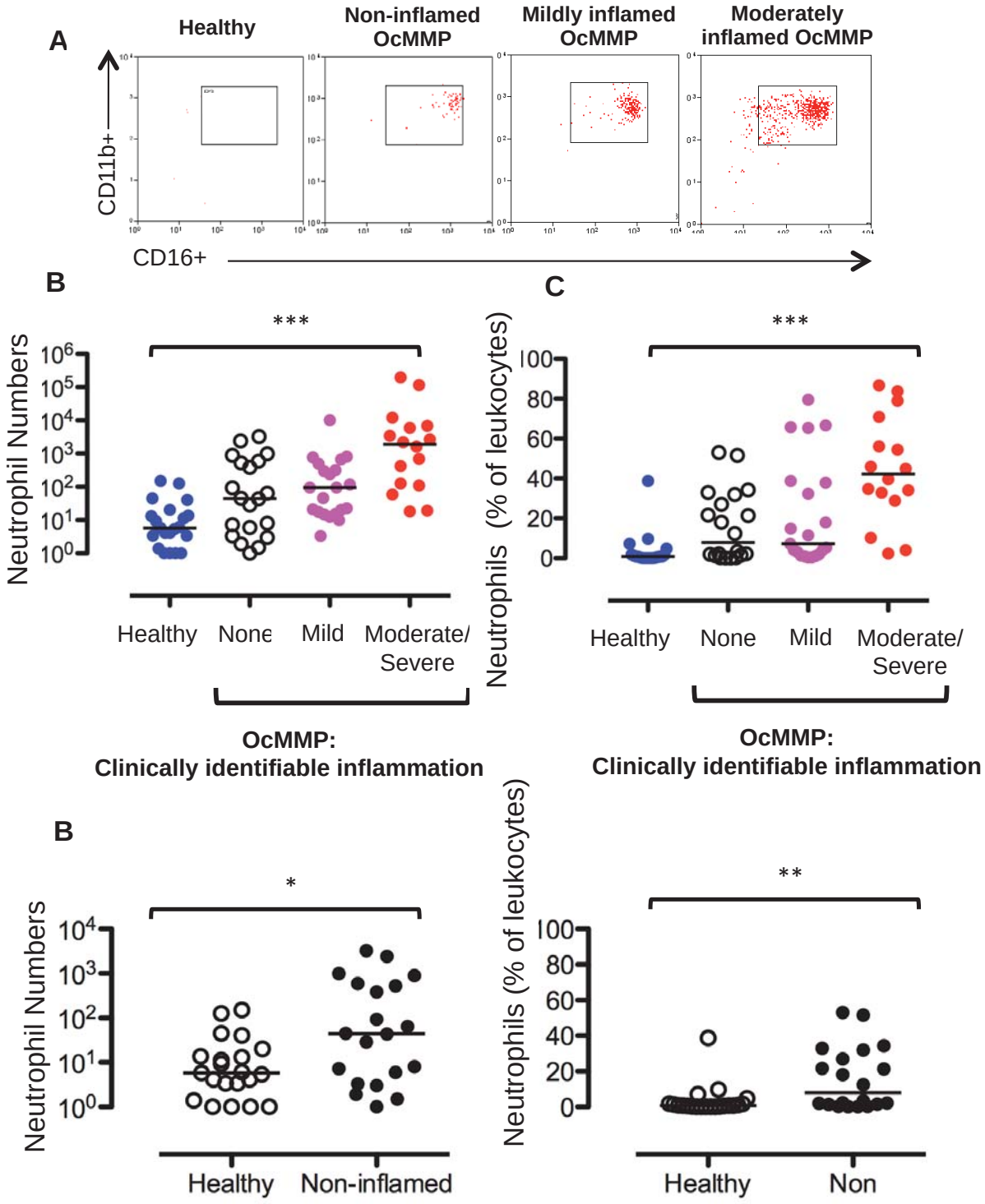

Figure 5.7 The severity of clinically identifiable conjunctival inflammation correlates with the number of epithelial CD45^{INT}CD11b+CD16+CD14- neutrophils but these cells are present even in clinically uninflamed eyes. Panel A shows representative flow plots for CD45^{INT}CD11b+CD16+CD14- live granulocytes for a healthy individual, patients with OcMMP with a clinically uninflamed, mildly inflamed and moderately inflamed eye. The number and percentages of neutrophils were correlated with the degree of conjunctival inflammation and healthy individuals (Panel B). Healthy and clinically uninflamed individuals were also compared (Panel C). Comparisons were undertaken by the Jonckheere-Terpstra test and the Mann-Whitney U test (Key: NS: Not significant, *p=0.01 to 0.05; **p=0.001 to 0.01; *** p<0.001).

Longitudinal Comparisons:

The inflammatory cellular infiltrate and disease progression

Table 5.10 shows the progression rates for the primary and secondary measures of scarring. For comparison, the median depth [range], symblephara numbers and extent for the upper fornices are also shown.

Disease Damage		Right eyes	Left eyes	All eyes
Upper Fornix				
	Depth in mm (median[range])	14mm[0-19]	12mm[0-19]	12mm[0-19]
	Symblephara number (median[range])	0[0-3]	1[0-2]	1[0-3]
	Symblephara involvement (median[range])	0[0-15]	0[0-30]	2[0-30]
	Central Depth	49%(17/35)	46%(16/35)	47%(33/70)
Lower Fornix				
	Depth in mm (median[range])	4mm[0-8]	3mm[0-8]	3mm[0-8]
	Symblephara number (median[range])	2[0-6]	3[1-5]	3[0-6]
	Symblephara involvement (median[range])	12[0-30]	10[3-26]	12[0-30]
	Central Depth	51%(18/35)	54%(19/35)	53%(37/70)
	Tauber¶	63%(22/35)	71%(25/35)	67%(47/70)
Cornea				
	Neovascularisation	26%(9/35)	29%(10/35)	27%(19/70)
	Opacification*	26%(9/35)	26%(9/35)	26%(18/70)

¶ One patient did not have Tauber scoring documented at 12 months

* Progression of neovascularisation was defined as an escalation of degrees clock hours or encroachment Progression of opacification of was defined as an escalation of opacification, conjunctivalisation or keratinisation

Table 5.10 Disease progression by primary and secondary outcome measures in the OcMMP study.

Primary outcome measures:

The median upper fornix depth had contracted in 47% of eyes, resulting in a median depth of 12mm. A median of 1 symblephara involving 2mm of the upper fornix was seen at 12 months follow up. For the lower fornix, there had been progression of scarring (measured by central depth) in 53% of eyes, resulting in a median depth of 3mm. The number and extent of symblephara had also progressed (median 3 symblephara; 12mm of lower fornix involvement). Tauber scoring had increased in 67% of eyes at 12 months.

In light of the significant elevation in neutrophils, reduction in CD8$\alpha\beta$+ T cells and alteration in CD4:CD8 ratio when comparing MMP to other diseases, comparisons among cell populations at presentation and disease progression were determined.

Neutrophil numbers and percentage were significantly elevated in the group that progressed compared to the group that did no progress according to lower fornix shrinkage (n;143[2-36,636] vs. 27[1-5910];p=0.004 and %;10[0.1-74] vs. 3.7[0-71];p=0.04) (**Figure 5.8**). In light of the significant changes seen in the elevation of neutrophils (total numbers and percentage) a generalised estimating equation (GEE) was undertaken to account for the effects of correlation between the right and left eyes. A 'moderate' (see fc value) degree of correlation was observed between the right and the left eyes (correlation matrix fc=0.376). The number of neutrophils was found to be 128 in the eyes that progressed vs. 39 ($p<0.001$) in the non-progressed group, confirming the test statistic using the Mann-Whitney U test. The percentage of neutrophils was also significantly higher among eyes that progressed using the GEE (fc=0.605; 10% vs. 5%;p=0.02).

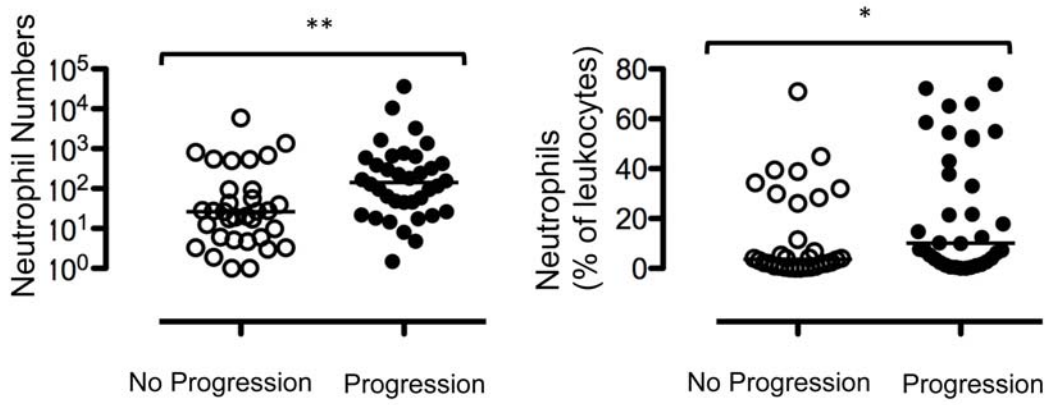

Figure 5.8 Neutrophil numbers and percentage are significantly higher among eyes that develop lower fornix shrinkage in OcMMP. Comparisons between the $CD45^{INT}$CD11b+CD16+CD14- neutrophils numbers and percentage of leukocytes was undertaken by the Mann-Whitney U test (Key: NS: Not significant, *p=0.01 to 0.05; **p=0.001 to 0.01).

The levels of CD8αβ+ T cells and the CD4:CD8 ratio were no different among those with or without evidence of lower fornix shrinkage (n(%);139(39%) vs. 97(45);p=0.75 and 0.6 vs. 0.4;p=0.32 respectively).

There was no significant difference in the number/percentage of neutrophils among eyes that progressed vs. those that did not progress according to upper fornix depth (n(%);49(4) vs. 69(12%);p=0.56). This was also the case for the CD8αβ+ T cell population (n(%);124(27) vs. 138(28%);p=0.8) and CD4:CD8 ratio (0.5 vs. 0.6;p=0.98). This indicated that inflammatory infiltrate with neutrophils, a reduction in CD8αβ+ T cell populations and an alteration in the CD4:CD8 ratio did not predict upper fornix shrinkage during this study period.

When assessing progression by Tauber staging, there was no significant difference in the number/percentage of neutrophils among eyes that progressed (n(%);49(6%)

vs. 41(4);p=0.27). This was also the case for the CD8αβ+ T cell population (n(%);114(40%) vs. 144(52);p=0.36) and CD4:CD8 ratio (0.6 vs. 0.2;p=0.08). This indicated that inflammatory infiltrate with neutrophils and a reduction in CD8αβ+ T cell populations also did not predict progression by Tauber stging in this cohort. There was a trend towards an elevation in the CD4:CD8 ratio but this was not statistically significant.

In summary, elevation of $CD45^{INT}$CD11b+CD16+CD14- neutrophils at presentation was seen in eyes that went on to develop lower fornix shrinkage, as measured with an FDM.

Secondary outcome measures:

Corneal neovascularisation had increased in 27% and opacification had progressed in 26% of eyes by 12 months.

When measuring inflammatory cell changes in eyes that demonstrated progression according to secondary outcome measures, no significant differences were observed. Neutrophil numbers and percentage (n(%);42(4%) vs. 64(7);p=0.25), CD8αβ+ (n(%);19(41%) vs. 139(45);p=0.23) and CD4:8 ratio (0.9 vs. 0.5;p=0.34) were not different among eyes that had evidence of established corneal vessels. No significant differences were also seen between the neutrophil numbers or percentage among those who did or did not have evidence of corneal opacification (n(%);64(28%) vs. 46(4);p=0.18). This was also the case for the CD8αβ+ (n(%);109(37%) vs. 140(45);p=0.5) and CD4:CD8 ratio (0.8 vs. 04.;p=0.08).

Longitudinal Comparisons:

The inflammatory cellular infiltrate and time

Given that the elevation of conjunctival epithelial neutrophils seen in patients with OcMMP at presentation (**Figure 5.5**) was more likely to result in lower fornix depth shrinkage (**Figure 5.8**), changes over time were determined. In order to verify if this inflammatory infiltrate was maintained over time, a GEE was employed. Neutrophil numbers at 0, 3, 6, 9 and 12 months were unaltered with no significant difference seen over this period ($p=0.46$) and no change was observed in neutrophils percentage ($p=0.76$) (**Figure 5.9**). This suggests that the inflammatory neutrophil infiltrate is maintained in OcMMP, signifying that the elevation demonstrated at time 0 continued during the course of the study.

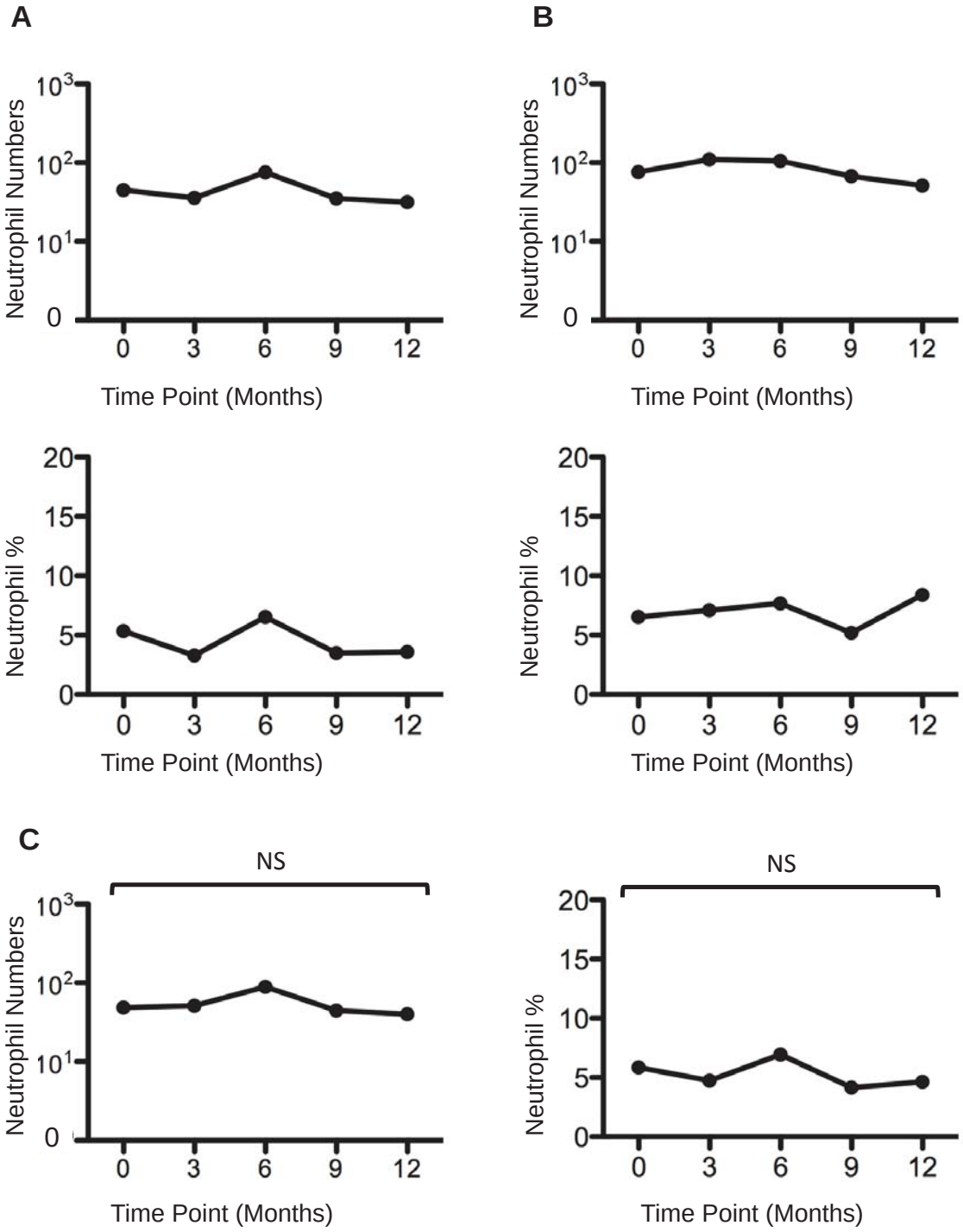

Figure 5.9 Elevated CD45^{INT}CD11b+CD16+CD14- neutrophils in OcMMP are maintained over time, indicating a persistent inflammatory infiltrate. The median neutrophil numbers and percentage are shown for the right eyes (Panel A) and left eyes (Panel B). Changes over time were calculated by using a generalised estimating equation (GEE) at 0, 3, 6, 9 and 12 months follow up for both eyes (Panel C) (Key: NS: Not significant, p>0.05).

Longitudinal Comparisons:

The inflammatory cellular infiltrate and disease progression in clinically uninflamed eyes

Having determined that an inflammatory infiltrate with $CD45^{INT}$CD11b+CD16+CD14- neutrophils was present in patients with OcMMP and that this infiltrate was higher even among those without evidence of inflammation on clinical examination, it was important to determine whether this elevation could predict progression in this challenging group of patients. Sub-group analysis of progression rates among clinically uninflamed eyes (patients with uninflamed eyes followed up at 12 months; n=14) was therefore undertaken in order to establish whether an inflammatory neutrophil infiltrate could predict lower fornix shrinkage.

First, the change in lower fornix depth (ΔFDM) correlated with the number of neutrophils seen in the right eyes of patients with OcMMP (p=0.035; Jonckheere-Terpstra test) (**Figure 5.10**). Second, an alternative means of addressing this question was undertaken by comparing progression among the right eyes of those with an elevated neutrophil count against those without an elevation of $CD45^{INT}$CD11b+CD16+CD14- granulocytes. An elevation in neutrophils was defined by using the median number of neutrophils (44) determined in the uninflamed group at cross sectional analysis (**Figure 5.7**). A count of >44 neutrophils was therefore considered elevated and those with <44 neutrophils were deemed not to be elevated. Progression rates were found to be significantly higher among those with a higher neutrophil infiltrate (p=0.02; Fishers exact test).

These data indicate that progression is more likely with an elevation in the number of conjunctival epithelial neutrophils. Neutrophil percentages did not correlate with ΔFDM or determine progression by fornix shrinkage (p=0.85; p=0.52).

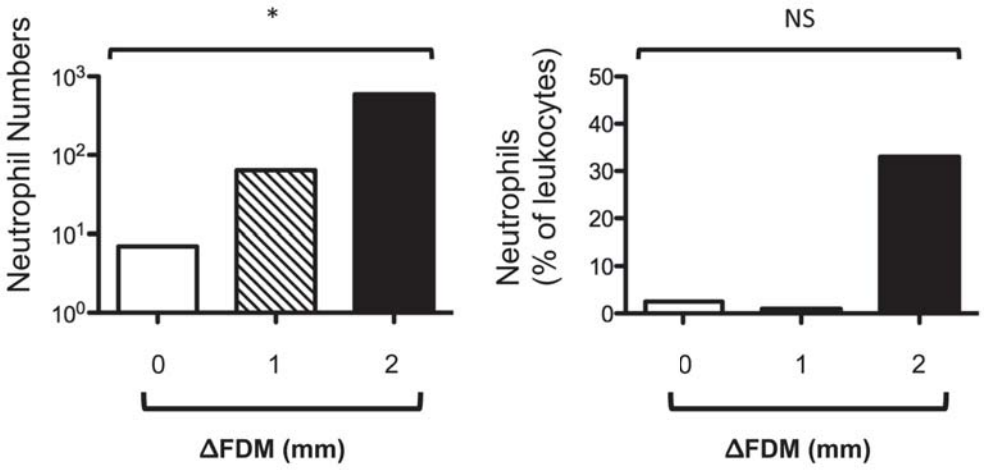

Figure 5.10 In clinically uninflamed eyes, the number of CD45^{INT}CD11b+CD16+CD14- neutrophils correlates with the degree of central lower fornix shrinkage in OcMMP. The number and percentage of neutrophils were compared by ΔFDM (The change in central lower fornix measured in mm between 0 and 12 months). Comparisons were undertaken by the Jonckheere-Terpstra test (Key: NS: Not significant, *p=0.01 to 0.05).

5.4 Discussion

Characterisation of inflammatory cells in ocular surface disease started with the application of immunocytochemistry to conjunctival OSIC (Pflugfelder et al., 1990, Baudouin et al., 1992). This preceded the use of flow cytometry in OSIC, a powerful tool for quantitative cellular and cytokine analysis (Kellar and Iannone, 2002, Baudouin et al., 1997). This has been used to demonstrate the increased expression of HLA-DR in dry eye states, and the presence of leukocytes (CD45+ cells) in the ocular surface (Brignole et al., 2000, Baudouin et al., 2002, Barabino et al., 2010).

In this study, I have demonstrated by non-invasive OSIC how in two forms of acquired ocular immunobullous diseases, SJS-TEN and OCMMP, there was an epithelial infiltrate with granulocytes composed primarily of $CD45^{INT}$CD11b+CD16+CD14- neutrophils. Moreover, the persistent elevation of these cells was demonstrated in both diseases and in the case of OcMMP their elevation was associated with disease progression, even in the absence of clinically identifiable inflammation. This may offer a new, non-invasive and previously impossible way of quantifying sub-clinical inflammation.

5.4.1 SJS-TEN study

The initial insult in acute SJS-TEN has been identified as a cell-mediated phenomenon regulated by granulysin produced by CD8+ T-cells (a type of NK+ cell) resulting in severe disruption of epithelial surfaces including the skin (Chung et al., 2008). Although these studies did not evaluate ocular disease, CD8+ T cells have

been identified in the cornea of a patient with TEN (Williams et al., 2007) and the healthy human conjunctival epithelium is dominated by a population of CD8+ T cells (**Chapter 4**), (Hingorani et al., 1997).

The idea that ocular SJS/TEN was temporal and did not progress once the initial acute phase has resolved, has been superseded by the understanding that many will develop chronic disease, requiring long-term specialist care including systemic immunosuppression in severe cases (De Rojas et al., 2007). The precise definition of acute and chronic ocular SJS-TEN has not been universally agreed. A cut-off of 12 months to define chronic disease was used as described by Sotozono and colleagues (Sotozono et al., 2007). The very acute stages of disease are probably better reflected by the 2-6 weeks described by Power and colleagues (Power et al., 1995, Fu et al., 2010), and while 4/6 'acute' patients were <30 days of disease onset in this study, 2/6 were greater than 30 days (one 114 and the other 168 days) and were therefore defined as 'sub-acute'.

Discriminating acute and chronic ocular features of SJS-TEN are challenging because of the absence of a universally agreed grading system or indeed one that discriminates markers of activity and damage. These limitations meant that two current scoring systems to classify acute and chronic disease respectively were employed (Power et al., 1995, Sotozono et al., 2007). The staging system described by Sotozono and colleagues is a more detailed system, but may present challenges in the very acute stages of severe disease e.g. ascertaining meibomian gland secretion by manual expression due to the friable nature of eyelid skin in severe acute disease. Nonetheless, the other parameters allow for a systematic

determination of eyelid, conjunctival and corneal disease severity. The median score according to the Sotozono system of 8/39 in our patients suggests less severe disease than the 9/10 patients having severe disease according to the system described by Power. The increased weighting given to the presence of symblephara in the Power scale may account for this discrepancy, and for this reason we used objective evidence of conjunctival scarring by employing formal fornix depth assessment.

The median lower fornix depth was 8 [0-12]mm indicating that the severity of scarring may not be as severe as the Power scoring system would indicate through the presence of symblephara alone. In addition, there was little evidence of disease progression in this cohort, although this was limited to a follow-up of 12 months only and the cohort size may also have been too small to demonstrate significant differences. Fu and colleagues have recently made the argument for a more universal scoring system (Fu et al., 2010), and predicting outcome as well as monitoring disease activity will undoubtedly be facilitated by a consistent and easily applied grading system.

In this cohort the CD8+ and NK+ populations were not significantly elevated compared to healthy controls and that this remained unaltered over time. This finding may be explained by the fact that the damage induced is thought to be mediated by the soluble factor granulysin, explaining how disease severity may be out of keeping with the degree of inflammatory cellular infiltrate by cytotoxic cells as release of granulysin is not dependent on recruitment of additional CD8+ T cells and Natural Killer cells (Quinn et al., 2005). Early vacuolation of the cornea has been seen in the

very early stages of ocular TEN in the absence of a lymphocytic infiltration (Williams et al., 2007). In addition, the healthy conjunctival epithelium is dominated by CD8+ cells, with a proportion of NK cells also residing in this layer (**Chapter 4**), so a major alteration in the context of disease may not be surprising.

Both monocyte and $CD45^{INT}$CD11b+CD16+CD14- neutrophils were elevated in the context of SJS-TEN in the acute and chronic stages of disease together with an elevation TCR$\alpha\beta$+CD3+CD8β-CD4- (Double Negative) T cells. The elevation in neutrophils was inversely correlated with the duration of disease indicating a higher proportion in those with acute disease. During the study period of 12 months however, the elevation of neutrophils was maintained with time, indicating a persistent inflammatory response. In a cohort of volunteers undergoing cataract surgery, the use of topical Dexamethasone (which most of our SJS-TEN patients were prescribed) did not result in an elevation of neutrophils. In fact, although this was not significant different, the trend suggested a reduction in these cells. These data would suggest that alterations in neutrophils seen in this study cannot be attributed to the use of topical steroids, where systemic administration of Prednisolone and Cortisol are known to cause a systemic granulocytosis (Bishop et al., 1968).

It is unclear whether the presence of a persistently elevated neutrophil infiltrate represents enhanced recruitment or contributes to scarring in the context of PCC such as SJS-TEN. The role for innate immune processes however has attracted greater attention including the presence of TLR-3 polymorphisms in a cohort of Japanese patients with SJS-TEN (Ueta et al., 2007). Disruption of the innate immune

responses in genetically susceptible individuals has been postulated as one reason for abnormal drug reactions in some individuals and a predisposition to SJS (Ueta and Kinoshita, 2010).

These data highlight that in ocular SJS-TEN, an inflammatory infiltrate with CD^{INT}CD11b+CD16+CD14- neutrophils is present even in mildly involved or clinically quiescent conjunctival mucosa and are maintained indicating the persistence of clinically occult inflammation. There was also a reduction in the percentage of CD8$\alpha\beta$+ cells in the superficial conjunctival epithelium during acute and chronic disease and an elevation in 'double negative' T cells. TCR$\gamma\delta$+ double negative T cells, considered to be a type b IEL, have a regulatory phenotype (Hayday et al., 2001) and it is unclear whether an inflammatory environment in ocular SJS-TEN or the reduction in resident CD8+ T cells results in the recruitment of these cells.

OSIC recovers cells from the superficial layers of the epithelium due to its non-invasive nature, but cannot access the deeper layers including the stroma. OSIC has been used to identify a reduction in conjunctival goblet cells on the conjunctiva of patients with TEN (Lopez-Garcia et al., 2011), but it has not been combined with cell surface markers to quantify inflammatory cellular infiltrates. The potential to study the presence of neutrophils in early SJS-TEN by OSIC is attractive, not least because of its non-invasive methodology, and it also affords the opportunity to undertake longitudinal sampling. In the absence of both a consistent clinical scoring system and biomarker of ocular disease, this technique combined with multi-colour flow cytometry warrants further investigation in the context of SJS-TEN. This may allow us to identify a cohort of patients with acute SJS-TEN and explore the potential role of

neutrophils in developing chronic inflammation, and its effects on local ocular sequelae. It is also intriguing that in a PCC with a very different aetiology to the autoimmune disease OcMMP, that a persistent elevation of these innate cells has been identified.

5.4.2 OcMMP study

This is the first longitudinal *ex vivo* demonstration of cellular infiltrates in the conjunctiva in OcMMP. These data confirm that an inflammatory cellular infiltrate occurs in the conjunctival epithelium in MMP compared to healthy participants and differs from patients with PSS. An elevation of neutrophils is evident (akin to that demonstrated in SJS-TEN), combined with a reduction in the dominant CD8$\alpha\beta$+ T cell population and resultant alteration in the CD4:CD8 ratio.

The OcMMP cohort in this prospective study (Cohort 12) had a duration of symptoms similar to the EOD group (Cohort 1) (930 days). The levels of conjunctival inflammation were similar (65% vs. 76%) but as previously discussed (**Chapter 3**) the rates of progression can be variable and depend on the accuracy of measurement. This study provided the opportunity to utilise the objective FDM and calculate the Tauber staging system and perhaps it was not surprising therefore that were able to detect progression as high as 53 and 67% of eyes respectively. It also re-emphasises the concern that sub-clinical inflammation is taking place.

Elevation in inflammatory cellular infiltrates has previously been identified from histological sections of conjunctiva of patients with OcMMP (Bernauer et al., 1993a,

Rice and Foster, 1990, Sacks et al., 1989). In the presence of clinically defined minimal inflammation, this was found to be a predominantly T cell infiltration of the conjunctiva and neutrophils in the epithelium (with a CD4:CD8 ratio of 0.5) (Bernauer et al., 1993a, Rice and Foster, 1990). In severe conjunctival inflammation however the CD4:CD8 ratio increases to 1.0 (Bernauer et al., 1993a, Rice and Foster, 1990). This alteration has previously been shown to arise form an elevation in CD4+ T cells, demonstrated also in PSS (Stern et al., 2002, Bernauer et al., 1993a). In the case of PSS, both murine models and human studies have led to a more comprehensive phenotyping of CD4+ subsets, demonstrating the importance of a Th17 phenotype in dry eye disease (Stern et al., 2010, De Paiva et al., 2010). Although there was a trend toward elevated CD4+ T cells in MMP and PSS in this study (this did not reach significance), the major influence on the alteration in CD4:CD8 ratio was a reduction in CD8$\alpha\beta$+ T cells. More recently a small cohort of patients with MMP had OSIC applied to their conjunctiva, also demonstrating an altered CD4:CD8 ratio. Characterisation of neutrophils was not undertaken however (Rolando *et al.* Immune Response of the Ocular Surface in Ocular Cicatricial Pemphigoid Patients; IOVS 2010; 51: E-Abstract 2365.).

The implications of a decrease in the dominant CD8$\alpha\beta$+ lymphocyte population are not clear. Whether this represents an alteration in the effector function of these cells or simply reflects abnormal architecture of the ocular surface secondary to scarring remains to be determined. A similar finding was identified in the CD8% in patients with SJS-TEN although direct comparisons were precluded because of the significant age difference between disease groups (median 44 years[range 18-67] vs. 72[48-97];

p<0.001). It is possible that turnover of cells may be impeded in diseased conjunctiva due to epithelial cell damage or the presence of scar tissue.

Alterations in the CD4+ T cell population and CD4:CD8 ratio were demonstrated in a cohort of healthy volunteers with age (**Chapter 4**). These effects were circumvented by undertaking age-matched comparisons, indicating that changes were independent of natural ageing changes in the conjunctival epithelium. Whether naturally occurring elevations in CD4+ T cells contribute to an inflammatory phenotype and promote disease activity, and indeed damage, are undetermined.

The most compelling evidence for an underlying inflammatory process was seen by the significant increase in the percentage of $CD45^{INT}$CD11b+CD16+CD14- neutrophils in both the uninflamed and inflamed eyes of patients with MMP. In severe conjunctival inflammation however there is a 3-fold increase in neutrophils compared to controls (Bernauer et al., 1993a, Rice and Foster, 1990). Our data therefore confirm that neutrophils are present in the conjunctival epithelium, correlate with the grade of clinical inflammation but importantly can be demonstrated in a clinically Non-inflamed eye. The presence of neutrophils in the clinically 'white' eye was shown in a small study previously (in 5/6 conjunctival samples) (Rice and Foster, 1990), but the ability to demonstrate *ex vivo* has not previously been achieved.

Having previously established that progression rates were similar in clinically inflamed and uninflamed eyes (**Chapter 3**), it was important to determine the role of neutrophils might play in progressive cicatrisation. It was apparent that neutrophil numbers and percentage were higher among eyes at presentation that had evidence

of lower fornix shrinkage at 12 months follow up. Furthermore, this elevation in neutrophils was maintained over time, similar to the findings in the SJS-TEN cohort. This would indicate a persistent, chronic inflammatory process. When considering clinically uninflamed eyes, the number of neutrophils also determined the extent of lower fornix shrinkage and was more likely with an elevation of neutrophils. The presence of an elevation in neutrophils therefore suggests that chronic inflammatory responses in the conjunctiva may lead to an increase in scarring and potentially open an avenue for exploration of this cell population as a non-invasive biomarker of disease activity and/or damage. Changes in Tauber staging or secondary outcome measures could not be predicted by an elevation in neutrophils in this cohort which may relate to the duration of the study, or the difficulty in accurately quantifying symblephara numbers, even with an FDM. This warrants further investigation.

The importance of the complement-mediated component in the inflammatory process in OcMMP has previously been emphasised (Bernauer, 1997), with antibodies inducing the classical complement cascade via C3, leading to chronic recruitment of neutrophils to the conjunctival BMZ. Historically neutrophils were thought to be short lived, becoming primed and leaving the peripheral blood compartment via the endothelium and entering infected/inflamed tissue along a chemotactic gradient. By utilising lysosome bound peptides such as the azurophilic granules - elastase, cathepsin and proteinase 3 as well as toxic ROS e.g. superoxide (O_2^-) and Nitrous Oxide (NO) to destroy pathogens (Janeway, 2005, Doring, 1994). The short life-span of neutrophils is controlled in part by apoptosis of activated neutrophils, but alterations in apoptotic regulation can lead to a prolonged inflammatory response (Wright et al., 2010). This interaction with other inflammatory cells in an inflamed site

is a complex process but there is evidence for dysregulation resulting in a number of chronic inflammatory conditions e.g. rheumatoid arthritis (Witko-Sarsat et al., 2000).

Persistent elevation of neutrophils is known to induce localised tissue damage through the mechanisms outlined above including the destructive properties of the azurophilic granules such as elastases (Doring, 1994). Although azurophilic granules are normally confined to lysosomal compartments, they may extravasate in to tissue, inducing disruption that should ordinarily disrupt phagocytised bacteria (Witko-Sarsat et al., 2000, Nathan, 2006). Elastase release can also modulate innate responses and alter the epithelial glycocalyx in mucosal sites (Doring, 1994). It is unknown whether chronic elevation in neutrophils diminishes resistance to pathogens but a recent study revealed that there was an alteration in the normal micro flora with an increase in pathogenic micro-organism on the ocular surface of patients with MMP (Agarwal M *et al*. The Prevalence Of Bacterial Pathogens On The Lids And Conjunctiva In Ocular Cicatricial Pemphigoid IOVS 2011;52: E-Abstract 4250).

The possibility of a persistent localised destruction of tissue resulting in scarring has been recognised in other diseases. Mice lacking neutrophil elastase have been shown to be resistant to pulmonary fibrosis (Chua et al., 2007) and this finding may be important in the persistence of human scarring disease. Indeed, pulmonary fibrosis is characterised by a local infiltrate of neutrophils (Hara et al., 2009). Similar findings were found in the context of cryptogenic fibrosing alveolitis (CFA), with an elevation of neutrophil numbers and elastase seen in CFA compared to the clinically less severe fibrotic process evident in systemic sclerosis (Obayashi et al., 1997).

The evidence suggests that the recruitment of and persistence of conjunctival epithelial neutrophils is associated with progressive fibrosis, even in the absence of clinically identifiable disease activity. The presence in the very superficial layers may reflect what is occurring in the ‘business end’ of the scarring process, namely the underlying BMZ. This in turn could result in underlying stromal fibrosis. Further clarification and validation of this process is required to determine whether this neutrophil infiltrate contributes to the inflammatory process, disrupts the architecture of the epithelium e.g. reducing the CD8+ population and how the mechanisms of this progressive fibrosis takes place.

Chapter 6

General Discussion

6.1 Introduction

This thesis has provided an opportunity to measure clinical parameters in acquired ocular immunobullous disease, develop a non-invasive technique for phenotyping the resident healthy conjunctival epithelial leukocyte population and determine how this is altered in the context of age and diseases such as SJS-TEN and OcMMP. I have demonstrated that:

1. Early onset of OcMMP is associated with significant increases in the levels of clinically identifiable inflammation but less evidence of conjunctival scarring.
2. Progression rates between early onset or established disease are similar and confirmed that this can occur in the absence of clinically identifiable inflammation.
3. Clinical documentation rates may be sub-optimal, compounded by the absence of objective measurement of conjunctival scarring. This can be overcome by using a validated FDM and a CRF, with potential for use in other PCC.
4. OSIC combined with multi-colour flow cytometry offers a non-invasive means of characterising IEL and other leukocytes from the conjunctival epithelium
5. TCR$\alpha\beta$+CD3+CD56-CD8$\alpha\beta$+ lymphocytes are the dominant population in the healthy conjunctival epithelium. These are effector memory (CD45RA-CCR7-), CD103+ T cells with the ability to produce Granzyme B and IFN-γ. The ability to recognise CMV and EBV viral antigens correlate with peripheral blood.
6. The second most common cell population in the conjunctival epithelium are TCR$\alpha\beta$+CD3+CD56-CD4+ lymphocytes. These T cells also produce IFN-γ and a smaller population IL-17. Importantly the number of CD4+ cells increases with

age and while the IL-17 population is maintained, the IFN-γ producers increase. By contrast, the CD8+ population remains unaltered.

7. Both SJS-TEN and OcMMP are characterised by a significant elevation in $CD45^{INT}$CD11b+CD16+CD14- neutrophils. In OcMMP there is an altered CD4:CD8 ratio, resulting from a decrease in the dominant CD8+ population.
8. In OcMMP neutrophils correlate with clinically identifiable conjunctival inflammation, but this is still detectable in clinically Non-inflamed eyes.
9. In both SJS-TEN and OcMMP there is a persistent elevation in conjunctival epithelial neutrophils. In the case of OcMMP this is associated with evidence of progressive conjunctival scarring as measured by a FDM.
10. Even in a clinically uninflamed eye, an elevation of neutrophils is accompanied by evidence of lower fornix shrinkage.

The major changes in the conjunctival leukocytes are summarised in **Table 6.1**.

	Health	Ageing	SJS-TEN	PSS	Uninflamed OcMMP	Inflamed OcMMP
CD8+ T cells	80%	-	-	-	↓	↓
CD4+ T cells	10%	↑	-	↑	-	-
CD4:CD8 ratio	0.15	↑	↑	↑	↑	↑
Neutrophils	<1%	-	↑↑	-	↑	↑↑

Table 6.1 Summary of the major conjunctival epithelial cellular changes in health, ageing and in acquired ocular immunobullous disease. Key: Increases (↑), decreases (↓) or no change (-) where ↑/↓ ≈ twofold increase/decrease (T-cell/neutrophil % are of lymphocytes/leukocytes).

In this chapter, these results will be discussed in the context of their respective findings and how they may inform future studies.

6.2 Measuring clinical parameters in acquired ocular immunobullous disease

It was clear from undertaking a retrospective review of patients with OcMMP presenting to two large tertiary referral centres in the UK that there was a significant lag between the onset of symptoms and presentation to specialist centres for care (a median of 4 years). When patients were referred 'early' (<3 years) these individuals were more likely to have evidence of clinically identifiable inflammation but less fibrotic damage to the conjunctiva. Conversely, 'late' presenters had less manifest activity but greater evidence of cicatrisation. Crucially however, both groups needed more aggressive immunosuppression to control disease yet the rates of progression were no different. A further complexity was the conformation of the finding that clinically uninflamed eyes progressed in up to 42%.

Progression rates are difficult to assess in the clinical setting, not least because of the limitations of the Mondino and Foster systems (Mondino and Brown, 1981, Foster, 1986). In this thesis, I demonstrated sub-optimal documentation using the Foster scale and how the provision of a CRF can aid the ophthalmologist in recording changes when faced with a number of clinical parameters.

The production and validation of a FDM has also shown how more sensitive scales such as the Mondino system can be enhanced through objective measurement of fornix shrinkage. In addition, the FDM has demonstrated how Tauber's proposed system (Tauber et al., 1992) can be enumerated and introduced in to the clinical setting and how millimetric determination of fornix shrinkage rather than relying on an ordinal scale such as Mondino can be employed.

There is clearly additional work that can be undertaken to help improve the scoring in OcMMP. The work of Sotozono and colleagues has shown how a scoring system can be developed in PCC, with a 39-point scale utilised for chronic SJS-TEN (Sotozono et al., 2007). The scale considers the ocular surface sequentially, incorporating scales for the eyelids, conjunctiva, fornices, cornea and tear film. The corneal scales were adapted for our CRF but in order to standardise these scales and consider which others are acceptable, a consensus is required.

The first international consensus on MMP sought to address many areas of disagreement and confusion surrounding the disease. Indeed the mission statement was defined as follows: '*The purposes of the consensus statement are to define a single nomenclature for this groups of diseases, to establish a unified set of diagnostic criteria, to provide recommendations for medical treatment and to facilitate future clinical and laboratory investigation*' (Chan et al., 2002). The format of the consensus was to gather experts in their field (26/36 approached participated) based on a literature search undertaken by the principal author, Lawrence S. Chan. A consensus meeting was organised and 17 participants attended. Three rounds of revisions were undertaken to the consensus statement, based on the literature search undertaken. The first international consensus on MMP was the first attempt to standardise the nomenclature and definition of the disease as well as the other objectives outcomes in the statement. Three challenges have arisen from the consensus statement however, and the panel has not re-convened since.

The first challenge is the requirement for a confirmatory DIF positive biopsy. The difficulties surrounding this requirement have been discussed in Chapter 1 (**section**

1.7.2) but briefly, there is a cohort of MMP patients where the biopsy is 'negative', but have no other identifiable cause for PCC e.g. SJS-TEN to account for their identical clinical phenotype (Thorne et al., 2004, Bernauer et al., 1994, Saw, 2008). Consistent with the consensus statement a biopsy is certainly advocated, not least to exclude neoplastic causes of cicatrisation, but excluding the diagnosis of MMP based on a negative result could jeopardise patient care. These patients still require immunosuppressive intervention, as a patient with positive biopsy result would expect. Furthermore the consensus statement makes it clear that distinguishing OcMMP from Bullous Pemphigoid, Linear IgA disease and Epidermolysis Bullosa acquista should be made on clinical grounds as a biopsy positive result could be expected in all conditions (Chan et al., 2002).

The second challenge is that the mode of recording and grading the clinical features of MMP, crucially and especially OcMMP, were not addressed in the consensus statement. There has been no impetus therefore to address the difficulties outlined with current systems. Finally, and related to this point, the mechanism for reaching consensus was very different to the Delphi approach, utilised in dry eye and thyroid eye disease in the field of ophthalmology or the outcome measure in rheumatology (OMERACT) (Behrens et al., 2006, Douglas et al., 2009, Tugwell et al., 2007, Naredo et al., 2011). In order to determine a scoring system using the Delphi approach, it is suggested that questionnaires are employed following the establishment of an expert panel. It is important that patients are involved and the use of questionnaires avoids a situation where individuals can impose their views on participants. On publication the size and composition of the panel, the methodology and the results should be clearly stated (Sinha et al., 2011).

The Delphi approach has been utilised in numerous fields and adopted for clinical trials. By undertaking a systematic approach such as this there is also the ability to gather information form multiple experts without the risk of greater weight being favoured by senior or vocal experts (Sinha et al., 2011). This approach has allowed outcome measures in large-scale clinical trials to be agreed. It is crucial in the process of developing a disease scoring scale that good intra- and inter-observer agreement can be achieved so a process of validating the agreed scores is undertaken with patients to assess reproducibility among clinicians (Kempen et al., 2008, Bowman et al., 2007, Barry et al., 2008).

The implementation of such an approach for MMP would be welcome; in particular as there is a heavy reliance on subjective documentary systems. It is hoped that developing a method for producing an FDM in a mechanised or semi-automated fashion would allow cheap production for use in specialist clinics beyond our own. In order to aid the process of determining forniceal shrinkage, anthropological studies of upper fornix and inter-canthal distances could aid percentage shrinkage calculations akin to the lower fornix outlined by Schwab and colleagues (Schwab et al., 1992). Such studies are being conducted in both BMEC and MEH among White European and Asian populations.

6.3 The conjunctival epithelial leukocyte population in health and in acquired ocular immunobullous disease

Devising expanded multi-colour antibody panels for use with flow-cytometry has allowed a far greater understanding of IEL and other leukocyte populations in the conjunctiva. This has also facilitated the exploration of a non-invasive technique for collecting cells, previously restricted by tissue access and/or limitation of material.

The dominance of CD3+CD8+ T cells and the presence of CD45RO+ antigen experienced cells have been previously established in the conjunctiva. The novelty in the data generated in this thesis is the confirmation of a dominant Type a IEL TCR$\alpha\beta$CD3+CD56-CD8$\alpha\beta$+ lymphocyte population with a cytotoxic phenotype, which does not alter with age (**Chapter 4**). Cells were able to produce IFN-γ and Granzyme-B but did not have regulatory features (expression of IL-10 or FoxP3), seen in other mucosal CD8+ subsets such as the gut. The presence of CD103+ has also been associated with a regulatory phenotype (Uss et al., 2006) but this was not the case in this conjunctival IEL population and despite their cytotoxic phenotype, all expressed CD103.

Few Type b IEL were identified in health and alterations in disease were confined to the elevation in double negative (DN) cells seen in SJS-TEN and percentage changes within T cells in OcMMP, including and elevation of CD8$\alpha\alpha$+ populations. In chronic disease, this could represent a repair mechanism, in response to disruption of the ocular surface following acute disease. Type b IEL populations play a role in repair such as CD8$\alpha\alpha$+ and TCR$\gamma\delta$+ T cells. Depletion of TCR$\gamma\delta$+ T cells has been

shown to reduce epithelial barrier integrity in response to *Toxoplasma gondii* in mice (Dalton et al., 2006). An elevation in gamma-delta T cells has been observed in the context of OcMMP (Soukiasian et al., 1992), but no alterations in TCR$\alpha\beta$-(TCR$\gamma\delta$+) T cells were seen in the conjunctiva in this study however (Healthy 0.1% of T-cells vs. PSS 0.2% vs. OcMMP 0.2%;p=0.95, data not shown). Whether the Type b IEL play a minor role in human conjunctival epithelial immune regulation or have a more important function in the deeper stromal layers is not known.

The effector memory, cytotoxic status of the CD8+ population demonstrated in this thesis reflected the ability of these cells to recognise common immuno-dominant viral antigens in CMV and EBV (**Chapter 5**). Murine virus-specific mucosal IEL CD8+ memory cells differ from virus-specific CD8+ T cells isolated from the spleen, with a higher production of Granzyme B (Masopust et al., 2006). The role of CD8+ type a IEL in ocular surface viral regulation warrants further exploration, in particular their role in recognising viruses such as HSV. HSV is a major cause of ocular morbidity, causing an initial blepharo-conjunctivitis followed by persistent latent residence in the trigeminal nerve root ganglion, triggering recurrent episodic epithelial, stromal or endothelial reactivation. This results in potentially blinding complications including corneal scarring and neovascularisation. The prevalence has been reported as 150 cases per 100,000 and therapeutic intervention in the US was placed at $18 million per annum to treat recurrences (Liesegang et al., 1989, Lairson et al., 2003).

In mice infected by HSV via the skin, effector memory skin cells continue to reside within the epidermis, even following the transport of the virus to the trigeminal nerve ganglion and resolution of acute infection. Furthermore, grafting previously infected

skin on to naïve donors results in re-circulation of HSV specific CD103+ CD8+ T cells to the skin epidermis of the recipient but not splenic tissue, providing protection against HSV infection. After resolution of infection, these effector memory peripheral CD8+T cells persisted in the epithelium, even when they were not present in peripheral blood (Gebhardt et al., 2009, Gebhardt et al., 2011). The role of human conjunctival epithelial TCR$\alpha\beta$CD8$\alpha\beta$+ T cell in regulating HSV infection could be explored by HLA-typing patients with recurrent ocular viral infections such as HSV keratitis, and constructing HLA-typed tetramers bound to known viral antigens. This process is complex and lengthy however but could offer an interesting insight in to why such a dominant population of IEL is found in the conjunctival epithelium.

Questions remain about how CD8+ T cells are recruited and retained within the conjunctival epithelium and it may be that the epithelial cells themselves play a role in regulating the immune recruitment process. Certainly, the concept of the '*epimmunome – epithelial molecules that direct the actions of immune cells*' (Swamy et al., 2010) has garnered interest because of the possibility that rather than being simple barriers, epithelial cells respond to micro-organisms targeting mucosal epithelial surfaces and help establish and maintain immune responses via numerous pathways e.g. the TLR mediated regulatory pathway NF-κB, where inhibition of intestinal epithelial-cell-specific NF-kB results in colonic inflammation in mice (Nenci et al., 2007).

TGF-β, produced by the epithelium, is known to induce a switch from α4 to αE expression on CD8+ T cells, facilitating their recruitment to the epithelium from the underlying stroma. TGF-β derived from intestinal epithelial cells influences the

antigen- presenting cells, which migrate to the draining lymph nodes to prime local T cell responses (Sheridan and Lefrancois, 2011). OSIC may offer a novel avenue for exploring the epimmunome. Preliminary data has suggested that conjunctival epithelial cells can be isolated using the epithelial cell marker EpCam. These cells may then be subjected to microarrays to elucidate transcript profiles before formal validation by RT-PCR. This may provide another opportunity to understand conjunctival disease and to achieve this characterisation of human mucosal epithelial cells by using a readily accessible tissue and collection technique. In the presence of a reduced CD8$\alpha\beta$+ T cell population in both SJS-TEN and OcMMP, it is conceivable that up-regulation of TGF-β seen in OcMMP, may represent a way of replacing IEL lost during the scarring processes.

Disruption to the BMZ may also impair T cell trafficking as cicatrisation alters the architecture of the conjunctiva. The BMZ, which IEL would normally have to cross when trafficking to the epithelial surface (Chan et al., 2008), becomes disrupted in OcMMP and may therefore compromise CD8+ T cell recruitment. The reduction in CD8+ cells may therefore alter the natural equilibrium seen in human conjunctival epithelium by compromising immune regulatory mechanisms involved in ocular surface health such as targeting infected cells. This may be one of the reasons that alterations in the micro-flora were recently demonstrated in relation to OcMMP (Agarwal M *et al*. The Prevalence Of Bacterial Pathogens On The Lids And Conjunctiva In Ocular Cicatricial Pemphigoid IOVS 2011; 52: E-Abstract 4250). OSIC or even conjunctival swabs could be employed to recover cells and PCR-based technology may offer an insight in to the composition of the normal flora in health and how these changes occur in disease.

At a stromal level, elevation in CD4+ T cells has been recognised in relation to OcMMP during the initial inflammatory insult. Age-related elevations in CD4+ cells were demonstrated (**Chapter 4**) and with the careful age matching undertaken in this thesis, a change in CD4:CD8 ratio was observed (**Chapter 5**). In the gut lamina propria CD4+ cells are predominantly Th17 or T^{REG}s, and these are regulated by the presence of commensal bacteria (van Wijk and Cheroutre, 2009). Th17 cells can induce granulopoiesis and recruit neutrophils to inflammatory sites (Cua and Tato, 2010). Although not demonstrated directly in our patients with PCC, one would hypothesise that Th17 CD4+ cells are implicated in the process of recruiting neutrophils to the ocular surface and again alterations to the normal ocular surface flora my provoke such responses. Indeed, a small population of both CD4+ and CD8+ IL-17 producing cells were demonstrated in our healthy cohorts and our data suggests that IL-17 producing CD4+ cells are maintained with age (**Chapter 4**). In trachoma, an infective cause of cicatrisation induced by the pathogen Chlamydia trachomatis, this was recently associated with an elevation in IL-17 transcripts (Burton et al., 2011).

Whether locally produced anti-BMZ antibody contributes to the pathology in OcMMP has not been explored. At present there are no data regarding local anti-BMZ Ab production in affected tissue from secondary lymphoid tissue such as EALT or the lacrimal gland. Furthermore, there are no data to suggest whether local antibody targets local tissue more effectively e.g. tear anti-BMZ IgG directed against conjunctiva more than buccal mucosa. This may be of relevance as evidence of local inflammation may correlate with the presence of local antibody production and in turn fibrotic damage. Whether the inflammatory response is also influenced by

local antibody production e.g. by EALT, or whether tertiary lymphoid structures develop is unknown. Determining specific anti-BMZ tear and serum IgA levels could offer a means of exploring this question by calculating a Goldmann-Witmer co-efficient (van der Voet et al., 1989). Whether there is specific binding in this context is also unknown such as tear binding to conjunctiva or saliva to oral mucosa. This could be explored by immunofluorescent studies.

In OcMMP, the complement cascade is activated at the BMZ in response to autoantibodies. Presumably this involves the classical pathway via IgG binding to C1q (the first component of the complement cascade), resulting in the BMZ being coated in C3b. Although murine models have suggested that the complement cascade was not involved in subepithelial lesions in skin, closer scrutiny of the mouse conjunctiva has not been undertaken (Lazarova et al., 1996). The murine ocular surface may or may not have had evidence of blistering but in humans this again is not a prominent feature - scarring takes place without gross evidence of blister formation. It is therefore quite possible that autoimmune mediated recruitment of complement contributes to the inflammatory infiltrate by recruiting neutrophils. This may be compounded by recruitment of other cells to the inflammatory environment within the conjunctiva as in other mucosal sites. Indeed, CD4+ T cells have been shown to regulate neutrophil recruitment to the site of inflammation in a mouse colitis model (van Lierop et al., 2010). In addition, the alternative complement pathway is able to recruit phagocytic cells such as neutrophils, in the absence of antibodies.

Having established the elevation in $CD45^{INT}$CD11b+CD16+CD14- neutrophils in OcMMP, it should be possible to compare the presence of these cells collected and

recovered by OSIC with matched tissue sections. We are currently undertaking evaluation of matched OSIC and conjunctival biopsies to address this question. This will also provide histological evidence that these cells are neutrophils, as opposed to other granulocytes such as eosinophils and could be determined by cytospin and light microscopy (Saraiva-Romanholo et al., 2003).

Having a non-invasive test available in the clinic to detect ocular surface inflammation could provide a major improvement in monitoring disease activity, especially when considering patients who have an apparently uninflamed eye. Escalating therapeutic intervention is based on clinically identifiable inflammation (**Figure 1.12**) so selected immuno-modulatory targeting of patients at greater risk of progression could not only be more cost effective but hugely beneficial to patients.

Revalidation of $CD45^{INT}$CD11b+CD16+CD14- granulocytes by OSIC may help produce a simplified flow cytometric determination of neutrophils without having to use multi-colour panels including CD45, CD11b, CD14 and CD16. Alternatively OSIC or swabbing the conjunctiva may also facilitate a study of elevated azurophilic granules such as Proteinase 3. Again, PCR based assays could be employed to determine this. The advantage of course is that cells collected could be stored and transferred to a diagnostic lab if this was established as a biomarker.

Additional non-invasive means of characterising ocular surface inflammatory changes by in vivo confocal microscopy could also be explored. Alterations in dendritiform cells and other inflammatory profiles have been identified in trachoma (Hu et al., 2011a, Hu et al., 2011b). Although labelling of cells by monoclonal

antibodies is precluded in humans, correlating OSIC evidence of conjunctival neutrophils with a characteristic cell 'signature' by *in vivo* confocal microscopy could provide an additional clinic-based tool for determining disease activity.

How a persistent inflammatory infiltrate contributes to the fibrotic process has not been resolved. TGF-β has been heavily implicated in the fibrotic process in OcMMP and in many other fibrotic diseases (Bernauer et al., 1993b, Elder, 1997b, Goodwin and Jenkins, 2009). More recently pro-fibrotic IL-13 has been identified in the context of OcMMP (Saw et al., 2009b). Neutrophils may well be inducing fibrotic changes through local tissue damage and the marked reduction in goblet cells seen in both SJS-TEN and OcMMP may play a role in up-regulating IL-13, in particular as this is an important molecule in the control of goblet cell homeostasis (De Paiva et al., 2011).

In SJS-TEN, granulysin is a key mediator in the disease and recently, serum elevation has shown to correlate with systemic disease (Fujita et al., 2011). An obvious adaptation to this technique would be to collect tears (although this may be very challenging in patients with severe dry eye problems) or undertake OSIC to determine granulysin by ELISA or Western blotting. This may help predict local disease alterations and inform closer clinical monitoring and/or detection of cell changes to commensal organisms. This could help bridge the gap between the initial cell-mediated toxicity and elevation in innate cells such as neutrophils.

The contribution of persistently elevated neutrophils to the fibrotic as well as the inflammatory process may help to guide treatment. Anti TNF-α and anti CD20

therapies are used in MMP, but are usually reserved for refractory disease. Anti TNF-α therapy may play a role in modulating neutrophil function directly as this cytokine stimulates ROS while immune-complex induction of neutrophils could be abrogated by targeting antibody production by B cells with Rituximab (Wright et al., 2010). Future directions may also include the use of anti-complement therapy. C5 is a terminal component of the complement cascade which is cleaved into C5a and C5b convertases of the classical/lectin and alternative pathways (Woodruff et al., 2011). These in turn can recruit phagocytes such as neutrophils. Eculizumab is a humanised monoclonal antibody that inhibits C5, and has been demonstrated to prevent complement destruction of erythrocytes in paroxysmal nocturnal haemoglobinuria (Kelly et al., 2011). Although further investigation is clearly warranted, it illustrates how novel therapeutic intervention may be guided by the composition of the conjunctival inflammatory infiltrate.

6.4 Conclusions

In this thesis, I have tested the hypothesis that progressive fibrosis in OcMMP, occurring in the apparent absence of clinical inflammation, was driven by underlying inflammatory processes. The cohort of patients studied confirmed that an inflammatory infiltrate with neutrophils occurred in both SJS-TEN and OcMMP, and that this was significantly elevated in patients with clinical evidence of disease progression in OcMMP, *even* in Non-inflamed eyes. These findings have therefore confirmed my hypothesis and provide a platform for future studies in to the role of neutrophils in mediating or promoting fibrosis and developing OSIC as a means of biomarking these alterations.

Chapter 7

Appendices

7.1 Appendix 1: Clinical Record Form

Notes: 8

ML2876 14.12.10

Sandwell and West Birmingham Hospitals NHS
NHS Trust

Cornea and External Eye Diseases Clinic
New Patient Sheet

History Sheet - Ophthalmology

Date:

Reason for referral:
Source of referral:
Tertiary Internal GP BMEC A&E
Other

Surname			Reg no
Forename	Sex		Date of birth
Address			Cons
			Ward/Dept
			Hosp

Urinalysis
Blood
Protein
Sugar
Other

BMI:
Weight (kg):

Height (m):
BP:
BM:

Visual acuity (logMAR): Right Eye Left Eye

Unaided
Glasses/CL
Pin-hole

History: .....

- Dry Eyes ☐
- Foreign boby sensation ☐
- Using tear substitutes ☐
- Red eyes ☐
- Deterioration in vision ☐
- Duration of symptoms: ☐ .....

Diagnosis date: .....

CExEd New Patient CRF

Extra-ocular symptoms: Oesophageal / Nasopharyngeal/Tracheal / Oral / Skin / Genito-urinary / Colorectal / Other 2

Duration .....

.....

.....

Previous eye surgery:.

.....

.....

.....

Biopsy: Conjunctiva/ Oral/ Other/Not Done .....

Method (circle): Immunofluorescence / Histopathology / Not done

Findings:.....

.....

.....

Past Medical History:	**Present**	**Details**
Autoimmune disease:	☐	
Dermatological disease:	☐	
Cardiovascular Disease:	☐	
BP:	☐	
Diabetes Mellitus:	☐	
Renal Disease:	☐	
Liver Disease:	☐	
Anaemia:	☐	
Malignancy:	☐	

Other: .....

Family History: .....

Social History: Occupation: ..... Smoking Status:.....

Alcohol intake (units/week): ..... Home Circumstances: .....

Ethnic Group: ***Ask the patient which group describes their ethnic origin*** .....

Notes: 7

Impression: .....

.....

.....

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Photos:

Ocular (Upper and lower fornices mandatory) ☐ Other ☐

Oral (1 mandatory +/- lesional photo) ☐

Management plan: .....

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Drug Allergies:

Current Ocular Medication: .....

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Current Systemic Medication: .....

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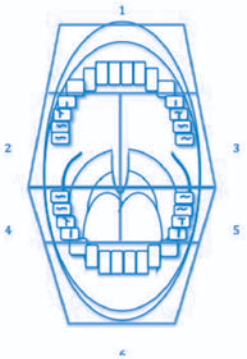
Oral Examination



Segment Key	Description
1	Upper anterior segment: Incisors to canines
2 & 3	Upper posterior segments: Canines to anterior pillars of fauces
4 & 5	Lower posterior segments: Canines to anterior pillars of fauces; Anterior 2/3rds of tongue (dorsal, lateral & ventral aspects to circumvallate papillae
6	Lower anterior segment: Incisors to canines

Assessment				Tic appropriate box
No Caries	Few caries	Excessive caries		
No Dentures	Partial dentures	Dentures: <1/2	Dentures: >1/2	

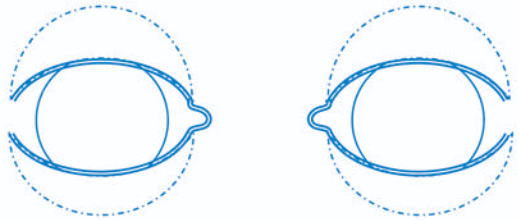
Score	Description	
0	Nil	
1	Minimal (<1 segment*)	
2	1-2 segments* involved or 1 ulcerated	
3	3-4 segments* involved or 2 ulcerated	
4	5-6 segments* ulcerated	
5	Severe generalised ulcerative desquamative gingivits	

Ocular Examination

Yes / No Proptosis Yes / No

Key:
PEDs: Green
Vessels: Red
Infiltrate: Yellow
Ulceration: Green
Oedema: Blue
Scarring: Black
Keratinisation:
Symblephara:
Subepithelial fibrosis:

Lids



AC

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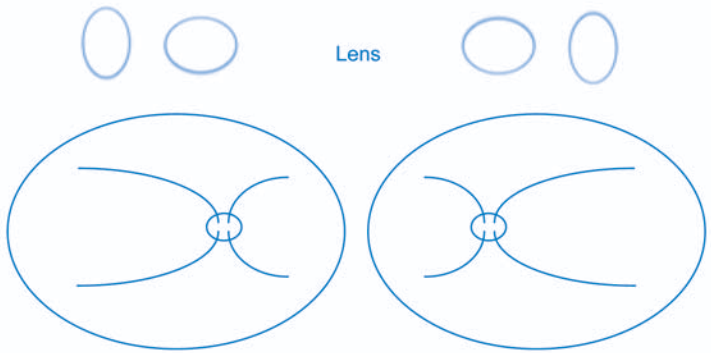
(Time IOP taken)

T

Lens



Examination Assessment Scores
(circle or score as indicated)

Tear Film

Normal None	Reduced Yes	Absent	.....	Break up time (s) Tear meniscus Filaments present	Normal None	Reduced Yes	Absent	
Normal	<10	<5	<2	Schirmer's test in mm	Normal	<10	<5	<2

Lids and Lashes

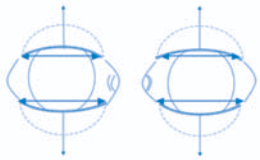
Normal	Anterior	Posterior	Metaplasia or lid notch	Lid Margin Disease	Normal	Anterior	Posterior	Metaplasia or lid notch
Normal	Lash metaplasia	Trichiasis	Entropion	Entropion	Normal	Lash metaplasia	Trichiasis	Entropion
Open	Small	Occluded	Plugged	Upper puncti	Open	Small	Occluded	Plugged
Open	Small	Occluded	Plugged	Lower puncti	Open	Small	Occluded	Plugged
Absent	Mild	Moderate	Severe	Lagophthalmos	Absent	Mild	Moderate	Severe

Conjunctiva/Limbitis

None (0)	Mild (1)	Mod (2)	Sev (3)	Inflammation (score each quadrant)	None (0)	Mild (1)	Mod (2)	Sev (3)
			No of bulbar quadrants of ulceration	Conjunctival ulceration				No of bulbar quadrants of ulceration
None (0)	Mild (i)	Mod (ii)	Sev (iii) or ischaemia	Limbitis (score each inner quadrant)	None (0)	Mild (i)	Mod (ii)	Sev (iii) or ischaemia
None	<3'	3-9'	>9'	Peripheral ulcerative keratitis (PUK)	None	<3'	3-9'	>9'
None	<50%	>50%	Perforation	PUK depth	None	<50%	>50%	Perforation
None	Mild	Moderate	Pseudo-membrane	Conjunctival mucous	None	Mild	Moderate	Pseudo-membrane
None	Mild	Severe		Keratinisation	None	Mild	Severe	

Upper Fornix Assessment

			.....	Horizontal obliteration by fibrosis (mm)	.....		
			.....	Central Depth (mm)	.....		
None	Involvement in mm		No.	Horizontal involvement by symblephara	None	Involvement in mm	No .....



Lower Fornix Assessment

			.....	Central Depth (mm)	.....			
			.....	Horizontal obliteration by fibrosis (mm)	.....			
None	Involvement in mm		No.	Horizontal involvement by symblephara	None	Involvement in mm		No. .....
None	Ankyloblepharon	Limitation	Frozen globe	Limitation in motility	None	Ankyloblepharon	Limitation	Frozen globe

Cornea

Normal	Diminished	Absent		Sensation	Normal	Diminished	Absent	
None	<1/3	1/3 to 2/3	>2/3	Punctate Keratopathy	None	<1/3	1/3 to 2/3	>2/3
None	<1/3	1/3 to 2/3	>2/3	Conjunctivalisation	None	<1/3	1/3 to 2/3	>2/3
None	<1/3	1/3 to 2/3	>2/3	Keratinisation	None	<1/3	1/3 to 2/3	>2/3
None	<3'	3-9'	>9'	Neovascularisation - total clock hours	None	<3'	3-9'	>9'
None	Periphery	Pupil margin	Central 4 mm	Neovascularisation - Encroachment to visual axis	None	Periphery	Pupil margin	Central 4 mm
None	<1/3	1/3 to 2/3	>2/3	Opacification /Scarring	None	<1/3	1/3 to 2/3	>2/3
None	<1/3	1/3 to 2/3	>2/3	Central corneal ulcer	None	<1/3	1/3 to 2/3	>2/3
None	<50%	>50%	Perforation	Central ulcer depth	None	<50%	>50%	Perforation

Sclera

None	Diffuse/ Nodular	Necrotising	Necrotising inflamed	Anterior scleritis	None	Diffuse/ Nodular	Necrotising	Necrotising inflamed
None	Present			Posterior scleritis	None	Present		

7.2 Appendix 2: Publications arising from the thesis

In Press/Online:

Williams GP, Denniston AKO, Oswal KS, Tomlins PJ, Barry RJ, Rauz S and Curnow SJ. 'The dominant human conjunctival epithelial CD8αβ+ T cell population is maintained with age but the number of CD4+ T cells increases'. *Age* 2011 doi: 10.1007/s11357-011-9316-3

Williams GP, Radford C, Nightingale P, Dart JKD and Rauz S. 'Referral and Disease Patterns of Ocular Mucous Membrane Pemphigoid Patients at Two Major United Kingdom Tertiary Referral Hospitals'. *Eye* 2011 Sep;25(9):1207-18.

Williams GP, Saw VP, Saeed T, Evans ST, Cottrell P, Curnow SJ, Nightingale P, Rauz S. 'Validation of a Fornix Depth Measurer - A Putative Tool for the Assessment of Progressive Cicatrising Conjunctivitis'. *British Journal of Ophthalmology* 2011 Jun;95(6):842-7.

Submitted/In preparation:

Williams GP, Tomlins PJ, Denniston AKO, Southworth HS, Sreekanthan S, Curnow SJ and Rauz S. 'Neutrophils – a potential biomarker for disease activity in ocular Stevens-Johnson Syndrome and Toxic Epidermal Necroylsis?'

Williams GP, Denniston AKO, Oswal KS, Tomlins PJ, Barry RJ, Rauz S and Curnow SJ. 'Resident human conjunctival epithelial leukocytes are predominantly effector memory TCRαβ+CD8αβ+ mucosal homing lymphocytes'.

Open

Eye (2011), 1–12

www.nature.com/eye

CLINICAL STUDY

Evaluation of early and late presentation of patients with ocular mucous membrane pemphigoid to two major tertiary referral hospitals in the United Kingdom

GP Williams[1], C Radford[2], P Nightingale[3], JKG Dart[2] and S Rauz[1]

Abstract

Purpose **Ocular mucous membrane pemphigoid (OcMMP) is a sight-threatening autoimmune disease in which referral to specialists units for further management is a common practise. This study aims to describe referral patterns, disease phenotype and management strategies in patients who present with either early or established disease to two large tertiary care hospitals in the United Kingdom.**

Patients and Methods **In all, 54 consecutive patients with a documented history of OcMMP were followed for 24 months. Two groups were defined: (i) early-onset disease (EOD: < 3years, $n = 26$, 51 eyes) and (ii) established disease (EstD: > 5years, $n = 24$, 48 eyes). Data were captured at first clinic visit, and at 12 and 24 months follow-up. Information regarding duration, activity and stage of disease, visual acuity (VA), therapeutic strategies and clinical outcome were analysed.**

Results **Patients with EOD were younger and had more severe conjunctival inflammation (76% of inflamed eyes) than the EstD group, who had poorer VA (26.7% = VA < 3/60, $P < 0.01$) and more advanced disease. Although 40% of patients were on existing immunosuppression, 48% required initiation or switch to more potent immunotherapy. In all, 28% (14) were referred back to the originating hospitals for continued care. Although inflammation had resolved in 78% (60/77) at 12 months, persistence of inflammation and progression did not differ between the two phenotypes. Importantly, 42% demonstrated disease progression in the absence of clinically detectable inflammation.**

Conclusions **These data highlight that irrespective of OcMMP phenotype, initiation or escalation of potent immunosuppression is required at tertiary hospitals. Moreover, the conjunctival scarring progresses even when the eye remains clinically quiescent. Early referral to tertiary centres is recommended to optimise immunosuppression and limit long-term ocular damage.**

Eye advance online publication, 29 July 2011; doi:10.1038/eye.2011.175

Keywords: cicatrising conjunctivitis; conjunctival scarring; progression; immunosuppression

Introduction

Mucous membrane pemphigoid (MMP) is a potentially fatal autoimmune disease[1] with a mortality usually secondary to aero-digestive tract stricture formation, quoted as 0.029 per 100 000 in the United States during 1992–2002.[2] Although the condition is associated with skin and mucous membrane involvement including the oral cavity, oesophagus, trachea and genitals,[3] ocular manifestations of MMP (OcMMP) are defined as 'high risk' and can be blinding.[3] Management strategies are aimed at early diagnosis together with the prevention of both life- and sight-threatening complications

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through the removal of factors that precipitate inflammation, careful immunomodulation and/or surgical intervention.

The course of ocular disease is variable, and determining disease activity and progression represents a major challenge.[4] Patients are often diagnosed at an advanced stage of disease or with an acutely inflamed eye,[5,6] which if left untreated, may result in an acceleration of disease progression that can be unresponsive to pharmacological manipulation.[7,8] In order to delay or arrest this process, early intervention with systemic immunosuppression is required,[3–5,9–12] usually by adopting either a validated stepladder approach based on the severity of disease activity,[10,13] or the primary use of oral cyclophosphamide,[11] both inducing long-lasting remission.

In the United Kingdom, streams of hospital referral are to tertiary care services specialising in the management of OcMMP, from ophthalmologists practicing in secondary care, directly from the primary care services such as general (family) practitioners or optometrists, and from dermatologists/oral medicine specialists referring patients for screening with a diagnosis of MMP at extra-ocular sites. Although the possibility of a referral bias of patients with a more severe phenotype is recognised,[11] there is limited information regarding the clinical features of patients with early or established disease who present to specialised services, whether these patients require continued tertiary care, or are discharged back to their referring unit.

Amongst our patient cohorts, we have noted that delaying referral of patients with OcMMP to our specialist hospitals seemed to augment a clinical phenotype that is refractory or only partially responsive to therapeutic intervention. The aim of this study, therefore, was to characterise referral patterns and disease phenotype (including activity, staging and progression) in patients with OcMMP who present either early or late according to duration of symptoms, to the two largest tertiary specialist hospitals in the United Kingdom, and the strategies employed to manage these patients.

Materials and Methods

Study population

A total of 54 consecutive patients with a documented history of OcMMP referred to dedicated ocular surface disease clinics over a 3-year period at the two largest specialist referral centres in the United Kingdom, Moorfields Eye Hospital (MEH, London, UK) and the Birmingham and Midland Eye Centre (BMEC, Birmingham, UK) were identified from electronic databases and followed for 24 months. Patients were stratified according to duration of symptoms, where symptoms were defined as redness, tearing, burning, decreased vision or foreign body sensation.[14] The frequency distribution of the duration of disease defined two groups straddling either side of the median (1460 days (4 years); Supplementary Figure 1). Group1 ($n=26$, 51 eyes) consisted of patients with <1095 days (<3 years) history and was termed the 'early-onset' disease (EOD) group, whereas group 2 ($n=24$, 48 eyes) comprised patients with >1825 days (>5 years) history and was termed the 'established' disease (EstD) group. Four patients had a duration of symptoms that fell on the median (4 years), and these patients were excluded from further analysis. The study was conducted following ethical approval and conformed to the tenets of the Declaration of Helsinki.

Diagnosis

Diagnosis of OcMMP was based on clinical findings characteristic for the disease, namely progressive conjunctival cicatrisation in the absence of other causes of conjunctival scarring. If patients did not had a previous positive tissue biopsy, a confirmatory perilesional conjunctival and/or oral mucosal biopsy for direct immunofluorescence was undertaken. A positive result was defined as linear deposition of immunoglobulin G, A or complement (C3) along the basement membrane zone.[3] If typical clinical characteristics were evident, a negative result did not exclude the diagnosis[10,14–16] because of the recognition of a subgroup of ocular patients who have ocular features consistent with OcMMP but have a negative biopsy.[15,17] In accordance with the first international consensus, a positive indirect immunofluorescence was not an essential requirement for diagnosis.[3]

Study design

Data were captured at presentation, immediately following first consultation, at 12 and 24 months for both the EOD and EstD groups. Visual acuity (VA) was classified as good (6/6–6/18), or in accordance with the WHO definitions of 'visual impairment' ($<$6/18–6/60), 'severe visual impairment' ($<$6/60) and 'blind' ($<$3/60). Disease activity was based upon the degree of conjunctival inflammation: absent, mild, moderate or severe (including inflammation in all four quadrants, limbitis and/or conjunctival ulceration).[18] Stage of disease and progression was determined by using the staging systems described by Mondino and Brown (I, 0–25%; II, 25–50%; III, 50–75%; IV, 75–100% loss of inferior fornix)[7] and Foster (I, subconjunctival scarring

and fibrosis; II, fornix foreshortening of any degree; III, presence of any degree of symblepharon; IV, end-stage cicatricial pemphigoid).[19] Progression was defined as an advance in either Mondino or Foster staging criteria. Immunosuppression strategies used a 'step–ladder' approach as previously described (Figure 1).[13] Information regarding surgical intervention was also recorded.

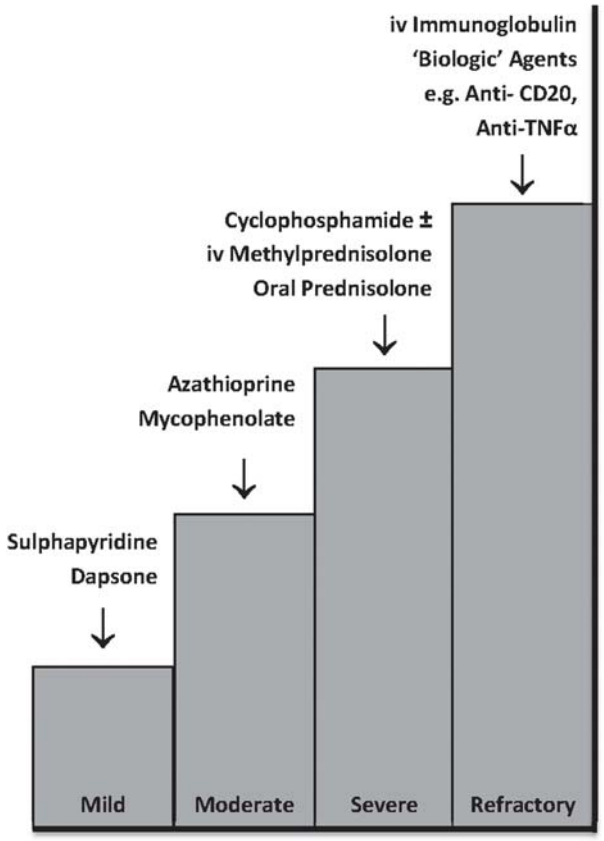

Figure 1 Immunosuppression strategies (based on Rauz *et al*[13]). A step–ladder approach to treatment with agents having the fewest side effects to those that have the greatest side effects is adopted according to disease activity (mild, moderate or severe), which is used to guide therapy. Dapsone (25–50 mg twice a day) or sulphapyridine (500 mg twice a day) can be used for mild inflammation; azathioprine (1–2.5 mg/kg/day) or mycophenolate mofetil (500–1000 mg twice a day if intolerant to azathioprine) may be added or substituted for persistent disease. Severe inflammatory disease is treated with cyclophosphamide (1–2 mg/kg/day) and adjuvant prednisolone (1 mg/kg/day with or without supplementary loading doses of 1 g intravenous methylprednisolone preceding oral therapy) for up to 3 months until the optimal effects of cyclophosphamide have taken effect. Patients with refractory disease are managed through intravenous immunoglobulin or 'biological' agents such as anti-CD 20 (rituximab) or anti-TNFα therapy.

Statistical analysis

Statistical analysis was by SPSS 16.0 for Macintosh and 14.0 for Windows (SPSS, Chicago, IL, USA; 2006), and Prism version 5.0 for Macintosh (GraphPad Software, CA, USA; 2008) using Fishers-exact test, McNemar's test and Kendall's τ-b for rank correlations. Continuous variables were analysed by non-parametric tests (Mann–Whitney *U*-test). Data were collected on all eyes and comparisons were undertaken between the worst affected eye for cross-sectional analysis of inflammation/fibrosis and the better-seeing eye for VA. When determining disease progression, comparisons were undertaken between patients (either one or both eyes). In order to determine whether changes seen at differing time points were significant rather than as a result of a change in the cohort (eg, because of the patients being discharged back to the referring hospital or missing data), longitudinal analysis of the same eye was undertaken. Owing to the referral of patients back to the originating physician, the sample sizes at the three time points differ and percentages rather than absolute counts are therefore reported.

Results

Demographic information and referral patterns

The geographical origin of our patient cohort is illustrated in Figure 2, where the furthest referral was from Newquay in Cornwall to MEH (238 miles). Associated patient demographics and subgroup stratification (EOD *vs* EstD) is detailed in Table 1. The EOD group was younger than the EstD group (62 (32–82) *vs* 69 (39–91) years (median, range; $P = 0.02$)). In all, 19 patients (37 eyes) from the EOD and 16 patients (32 eyes) from the EstD were followed for the full 24 months. Of the 15 patients, not reviewed at 24 months, 1 patient in the EstD group died before 12 months follow-up and 14 (28%) were referred back to the referring hospital. Of these, 11 had no clinically detectable inflammation at their last visit before discharge from the tertiary centre, 2 had mild inflammation, which continued to be monitored and treated at the local referring hospital, and 1 patient repeatedly failed to attend for follow-up despite recall. The remaining cohort (EOD 19; EstD 16) consisted of patients with more severe ocular disease.

Biopsies

A total of 87.2% (34/39) of patients, who underwent a biopsy, were direct immunofluorescence (DIF) positive. By contrast, indirect immunofluorescence studies were positive in only 34.8% (8/23) of tested individuals, all of whom were also DIF positive (Table 1). Although five

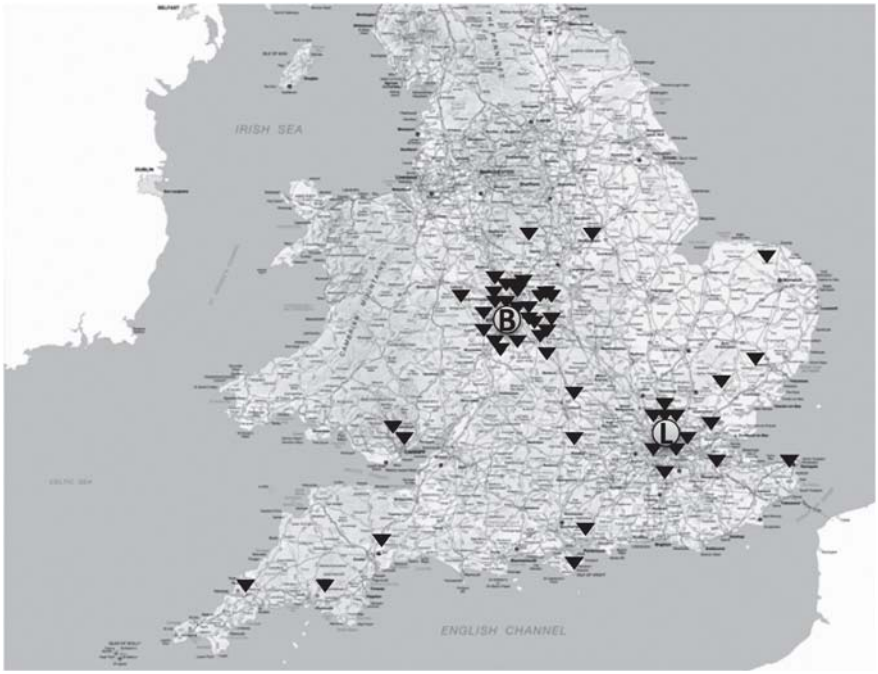

Figure 2 Map containing ordnance survey data (© Crown copyright and database right 2010) showing the combined geographical distribution of referrals (▼) to the two tertiary referral hospitals: Moorfields Eye Hospital, London, UK (circled, L) and the Birmingham and Midland Eye Centre, Birmingham, UK (circled, B). The furthest referral was for Newquay, Cornwall to Moorfields (238 miles).

(12.8%) patients were biopsy negative, these patients had clinical features consistent with OcMMP in the absence of other causes of progressive conjunctival scarring.
Ten patients in total did not undergo a conjunctival biopsy: seven patients were of advanced age (>80 years) with co-morbidities in which systemic immunosuppression was contraindicated; and the remaining three patients had end-stage disease (defined as Mondino/Foster stage 4) in which the sensitivity of a positive DIF conjunctival biopsy is low due to physical destruction of the basement membrane zone architecture.[15]

Extra-ocular features

Extra-ocular mucocutaneous involvement was present in 52% (26/50) of patients at presentation (62% (16/26) of the EOD group; 42% (10/24) of EstD group; $P=0.257$; Table 1). A total of 18% (9/50) patients had a history of skin involvement and this was more frequently reported in the EstD group (29.2% (7/24)) than the EOD group (7.7% (2/26)). Conversely, oral involvement was more common in the EOD group (57.7% (15/26)) compared with 20.8% (5/24) in the EstD group ($P=0.01$).

Visual acuity

After excluding other causes of reduced vision such as cataract, age-related macular degeneration, glaucoma and diabetic retinopathy ($n=14$, EOD 5; EstD 9) at presentation, 95% (20/21) of patients in the EOD group and 60% (19/15) in the EstD group had a Snellen VA of between 6/6 and 6/18 in the better-seeing eye. Only patients in the EstD group were severely visually impaired (<3/60, 26.7% (4/15)) and overall VA was significantly worse for the EstD group ($P<0.01$; Kendal τ-b; Table 1).

Inflammation

At presentation, 53% (50/94) of all eyes had clinical evidence of conjunctival inflammation where the majority (76% (38/50 eyes)) were in the EOD group ($P<0.001$) when comparing the worst affected eye (Figure 3a). Patients with moderate/severe inflammation were also more likely to have EOD. By 12 months follow-up (Figure 3a), inflammation had resolved in 78% (60/77) of all eyes (EOD = 83% (35/42) *vs* EstD = 71% (25/35), $P=0.917$) and there were no patients with residual severe conjunctival inflammation. These data were endorsed by McNemar's longitudinal analysis, showing a significant reduction in inflammation in the EOD ($P<0.001$) compared with the EstD group ($P=1.0$).

A recalcitrant group of patients with persistent inflammation not responsive or only partially responsive to treatment was identified in 29.9% (20/67) of eyes examined at 24 months. Interestingly, the persistence of inflammation was independent of group phenotype (EOD = 27% (10/37) *vs* EstD = 33.3% (10/30), $P=0.967$; Figure 3a).

Table 1 Patient demographics and characteristics

	All patients	*Early-onset disease*	*Established disease*	P-*value*
Total no. of patients	50	26	24	—
Total no. of eyes	99	51	48	—
Male: female (% female)	23:27 (54)	11:15 (58)	12:12 (50)	—
Median age (years; range)	67 (32–91)	62 (32–82)	69 (39–91)	$P=0.02$
Median duration of symptoms (years; range)	3 (0–41)	1.5 (0–3)	14 (5–41)	$P<0.0001$
Patient follow-up (eyes)				
12 months	43 (85)	23 (45)	20 (40)	—
24 months	35 (69)	19 (37)	16 (32)	—
No. of patients discharged back to referring hospital[a]				
Total number discharged	14	7	7	—
12 months follow-up	4	1	3	—
24 months follow-up	10	6	4	—
Biopsy[b]				
DIF positive	87.2% (34/39)	92% (23/25)	78.6% (11/14)	$P=0.33$
IIF positive[c]	34.8% (8/23)	42.9 % (6/14)	2.2% (2/9)	$P=0.4$
Extraocular mucocutaneous involvement				
All mucocutaneous tissues	52% (26/50)	62% (16/26)	42% (10/24)	$P=0.26$
Skin	18% (9/50)	7.7% (2/26)	29.2% (7/24)	—
Oral	40% (20/50)	57.7% (15/26)	20.8% (5/24)	$P=0.01$
Visual acuity[d]				
Normal: 6/6 to >6/18	80.6% (29/36)	95.2% (20/21)	60% (9/15)	$P=0.007$
Visual impairment: <6/18 to 6/60	8.3% (3/36)	4.8% (1/21)	13.3% (2/15)	
Severe visual impairment: 6/60 to 3/60	0% (0/36)	0% (0/21)	0% (0/15)	
Blind: <3/60	11.1% (4/36)	0% (0/21)	26.7% (4/15)	
Excluded due to other causes[d]	28% (14/50)	19% (5/26)	38% (9/24)	

Abbreviations: DIF, direct immunofluorescence; IIF, indirect immunofluorescence.
[a]Follow-up: one patient from the established disease group died before 12 months follow-up, and one from the established disease group failed to attend between 12 and 24 months, and was referred back to their local hospital for continuing care.
[b]Ten patients in total did not undergo a conjunctival biopsy (seven patients with advanced age (>80 years) and immunosuppression was systemically contraindicated and the remaining three patients had end-stage disease (defined as Mondino/Foster stage 4)). Data were missing for one individual and this patient was excluded from analysis.
[c]All patients who were IIF were also DIF positive. There were no patients who were IIF positive in the absence of positive DIF studies.
[d]Visual acutiy represents a comparison of visual acuity in the better-seeing eye, after exclusion of other causes of reduced vision such as cataract, glaucoma, age-related macular degeneration and diabetic retinopathy ($n=14$, early-onset disease 5; established disease 9).
The early-onset disease group consisted of a younger cohort of patients with increased frequency of oral mucous membrane pemphigoid. DIF and IIF refer to the proportion of patients who demonstrated the linear deposition of immunoglobulin G, A or complement (C3) along the basement membrane zone or had measurable titres of immunoglobulin in the serum, respectively. Comparisons were undertaken with Fishers-exact test, Kendall's τ-b for rank correlations and continuous variables were analysed by nonparametric tests (Mann–Whitney *U*-test).
Significant *P*-values are in bold text.

Stage of disease and progression

Eyes in the EstD group had more severe conjunctival fibrosis at presentation (Figures 3b and c) gauged by staging systems described by both Mondino (stage IV: EOD = 5% (2/40) *vs* EstD = 39.5% (17/45), $P<0.001$) and Foster (stage IV: EOD = 3.9% (2/51) *vs* EstD = 13% (16/46), $P<0.035$). At 12 months, the EstD group demonstrated significantly advanced stage of disease, irrespective of staging system used (Figures 3b and c), despite a total of 20.8% of all eyes having progressed according to both Mondino and Foster systems.

There was no significant difference when comparing progression (defined by worsening of clinical stage of disease in at least one eye) amongst patients in both the groups, neither between presentation and at 12 months (Mondino: EOD = 33.3% (4/12) *vs* EstD = 23.1% (3/13), $P=0.67$; Foster: EOD = 18.2% (4/22) *vs* EstD = 38.9% (7/18) $P=0.173$), nor during the subsequent 12 to 24 months follow-up period (Mondino: EOD = 53.8% (4/22) *vs* EstD = 16.7% (2/12), $P=0.10$; Foster: EOD = 23.5% (4/17) *vs* EstD = 28.6% (4/14) $P=1.0$; Fishers exact test).

Progression and the presence of conjunctival inflammation

Differences in the rates of progression (defined by an advance in Mondino or Foster staging) were stratified

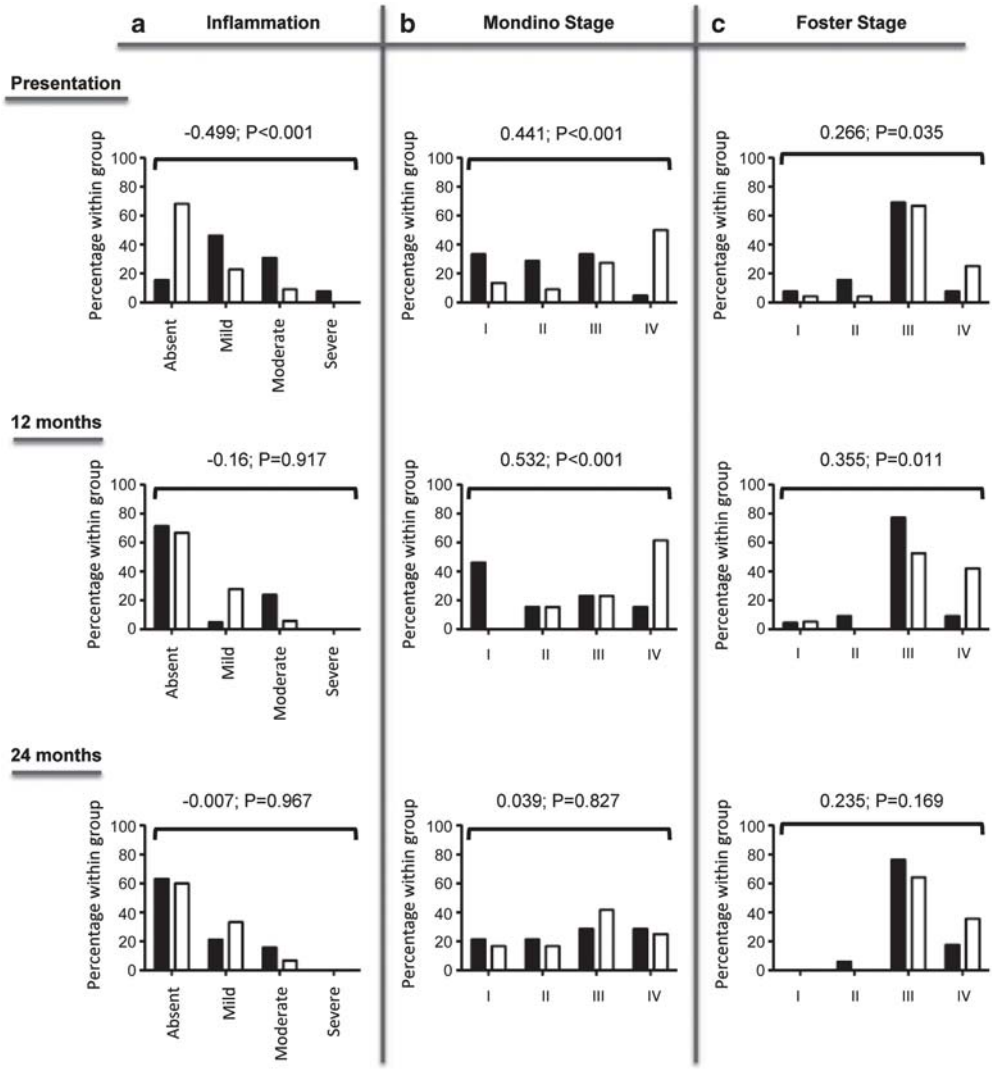

Figure 3 Cross-sectional analysis of clinically detected conjunctival inflammation (a) and ocular staging using Mondino (b) and Foster (c) systems in the worst eye at presentation, 12 months and 24 months follow-up in the EOD (■) and EstD groups (□). Differences in the extent of conjunctival inflammation and stage of disease were compared between the two groups by rank correlation using Kendal's τ-b. At 12 months follow-up, inflammation had resolved in the majority of eyes within both groups, and there were no patients with severe inflammation. By 24 months, 30% of the remaining patients at the tertiary centres had residual inflammation not responsive to treatment. Note that patients in the EstD had more advanced stage of disease compared with the EOD throughout the follow-up period, but there was no difference in the progression rate (worsening of clinical stage of disease) between the two groups. NB 14 patients had been referred back to their original hospital by 24 months and 1 had died. These patients have been excluded from the analysis thereby accounting for the apparent increase in the percentage of patients at stage 1 and decrease in the percentage of patients at stage 4 disease during the 12 and 24 months according to the Mondino staging system.

according to the presence or absence of clinically identifiable conjunctival inflammation in at least one eye. There was no significant difference observed between the first and second 12 months follow-up periods (data not shown). Moreover, despite the absence of clinically detectable inflammation, progression of disease occurred in 42% of patients according to the Mondino system (Figure 4a, left panel), and 16 and 38% of patients according to the Foster (Figure 4a, right panel) system for each of the 12 months follow-up periods, respectively.

Surgical intervention

At initial presentation to the specialist units, 32% (16/50) of patients had previously undergone eyelid or fornix reconstructive surgery by the referring hospital with the

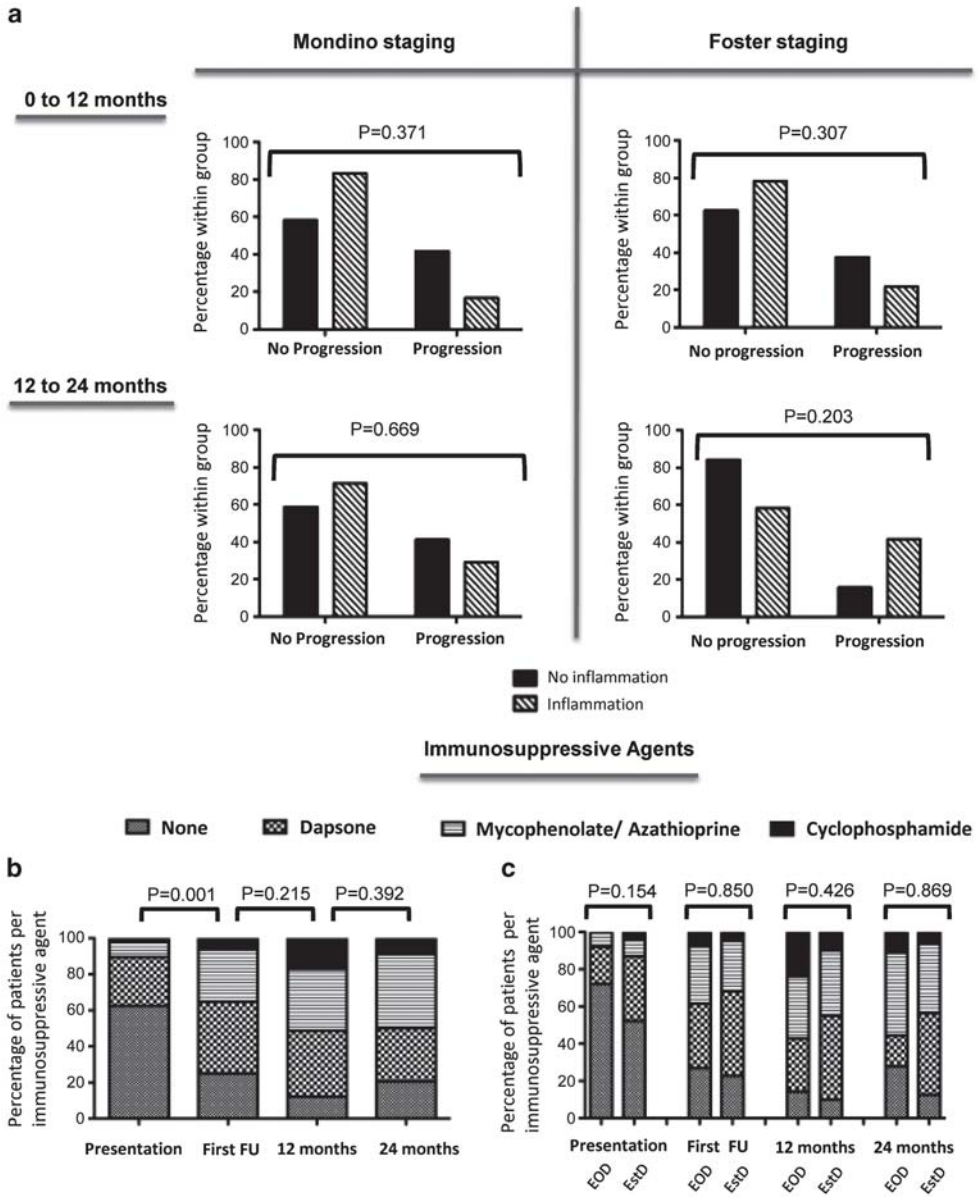

Figure 4 Progression rates, defined by worsening of either Mondino or Foster clinical staging of MMP, in the presence or absence of clinically detectable conjunctival inflammation are shown in the upper composite (a). Note there was no significant difference in progression between eyes with clinically detectable inflammation or those that were seemingly uninflamed (Fishers exact test). The percentage of patients requiring immunosuppression at presentation, following the first follow-up (FU) clinic visit, 12 months and 24 months follow-up time points are shown in the lower b and c. Immunosuppression strategies were ranked according to the hierarchy described by Rauz *et al*.[13] Overall, a significant initiation or escalation in 'strategic-step' was required at the first FU visit (b; McNemar's test), but this did not significantly differ when the early onset (EoD) and established disease (EstD) groups were compared (c; Kendal's τ-b). By 12 months follow-up, five patients stabilised on immunosuppression and were discharged back to their originating hospitals, and similarly a further 10 between the 12 and 24 months follow-up.

majority of cases being performed in the EstD group ($P<0.01$; Supplementary Table 1). Although both groups required oculoplastic surgical intervention at the tertiary hospitals, this did not differ between the two groups, nor during the first and second 12 months follow-up periods.

Immunosuppression strategies

In all, 40% (20/50) of all patients were on immunosuppression at the time of referral. After first consultation, 36% (18/50) required initiation and 12%

(6/50) a switch to a more potent immunomodulatory treatment representing a significant overall 'step-up' on the step ladder approach ($P=0.001$; Figure 4b). During the first 12 months follow-up period, a further 30% (13/43) of patients required 'step-up' treatment ($P=0.215$; Figure 4b) equating to a total of 88% (38/43) of patients requiring initiation or changes in immunosuppression at presentation or during the first year of follow-up. By 24 months, immunosuppression could be withdrawn in only one patient but no further escalation in therapy was required. In all, 28% (14) patients had stabilised and were discharged to the referring unit for immunosuppression monitoring (Table 1). There was no statistical difference between the requirements for immunosuppression for each of the patient groups at each of the time points (Figure 4c).

The most commonly used drug by the referring unit was Dapsone (26%, $n=13$) followed by either azathioprine or mycophenolate (8%, $n=4$) with only one patient on cyclophosphamide (2%, $n=1$). The majority of these patients were commenced on azathioprine or mycophenolate (10%, $n=5$) or switched to these drugs from dapsone (10%, $n=5$). Two further patients required oral cyclophosphamide to control inflammation. By 12 months, an additional seven patients had initiated cyclophosphamide therapy and this was either because of the presence of exuberant inflammation ($n=3$) not adequately responding to less potent agents (two requiring intravenous (i.v.) methylprednisolone) or there was a requirement for an increase in immunomodulation before ocular or eyelid reconstructive surgery. Resolution of inflammation occurred in two patients who were 'stepped down' to less potent agents. By 24 months, oral cyclophosphamide was withdrawn in three patients (because of completing the maximum safe duration of therapy of approximately 14 months, that is, a cumulative dose (oral or i.v.) of <20 g. The majority (40%) of patients were maintained on either mycophenolate or azathioprine. There was no statistical difference in the immunosuppressive agents used between the EOD and EstD groups. i.v. immunoglobulin or biological agents were not administered during the course of this study.

Adverse reactions to immunosuppression

Only 6 of the 38 patients that required immunosuppression suffered from adverse effects. Adverse events included one episode of anaemia following dapsone; two patients reported headaches after the use of azathioprine and one had induction of hepatic enzymes; three patients developed lymphopaenia while taking cyclophosphamide, including one patient who developed respiratory failure secondary to a combined cytomegalovirus and *Pneumocystis carinii* pneumonitis, which resolved following admission to the intensive care unit and treatment with i.v. ganciclovir and oral cotrimoxazole.

Discussion

OcMMP is a bilateral sight-threatening disorder characterised by progressive conjunctival cicatrisation associated with corneal vascularisation and scarring. The true incidence is not known although the outcome of a recent British Ophthalmological Surveillance Unit study suggests a minimum United Kingdom incidence of 0.7 per 1000 000 population with a regional variance exemplified by 1.1 per million in Greater London and 1.8 per million in the West Midlands[20] (Radford *et al*, unpublished data). MMP usually presents between 30 and 90 years of age, with a peak age of onset after 70 years.[3,7,19] Although disease progression is more aggressive in younger patients,[13] the disease is lifelong causing chronic discomfort, with 75% of patients requiring immunosuppression to control inflammation and limit disease progression.[8] The presence of extra-ocular manifestations of MMP in approximately half of our patients is consistent with other studies,[5,10] although higher rates have been reported.[17]

In this series, two disease phenotypes of OcMMP were statistically defined: (i) those with EOD who were characterised as having less advanced disease stage but significantly greater conjunctival inflammation, and (ii) those patients with EstD who had less clinically identifiable inflammation but more advanced stage of disease. Although 40% of the patient cohort were on existing systemic immunosuppression, the majority of patents required initiation or escalation in systemic immunosuppression following either their first clinic visit, or during the first year of follow-up in order to control inflammation, facilitate corrective eyelid surgery or prevent further progression in already advanced disease states. Despite these measures, 20.8% of eyes demonstrated disease progression during the first 12 months and another 20.8% between 12 and 24 months; and this progression was independent of the EOD or EstD clinical phenotypes. These results indicate that OcMMP may progress at any stage of disease,[7,8] and more importantly, progression rates amongst eyes that are clinically inflamed and those that are not do not differ. These data endorse previously reported literature,[4,10,21] and signify a molecular, fibrotic process independent of inflammation, which can be seen clinically.

Accurately identifying early disease and documenting progression presents difficulties. The staging systems currently used are reliant on subjective assessment of

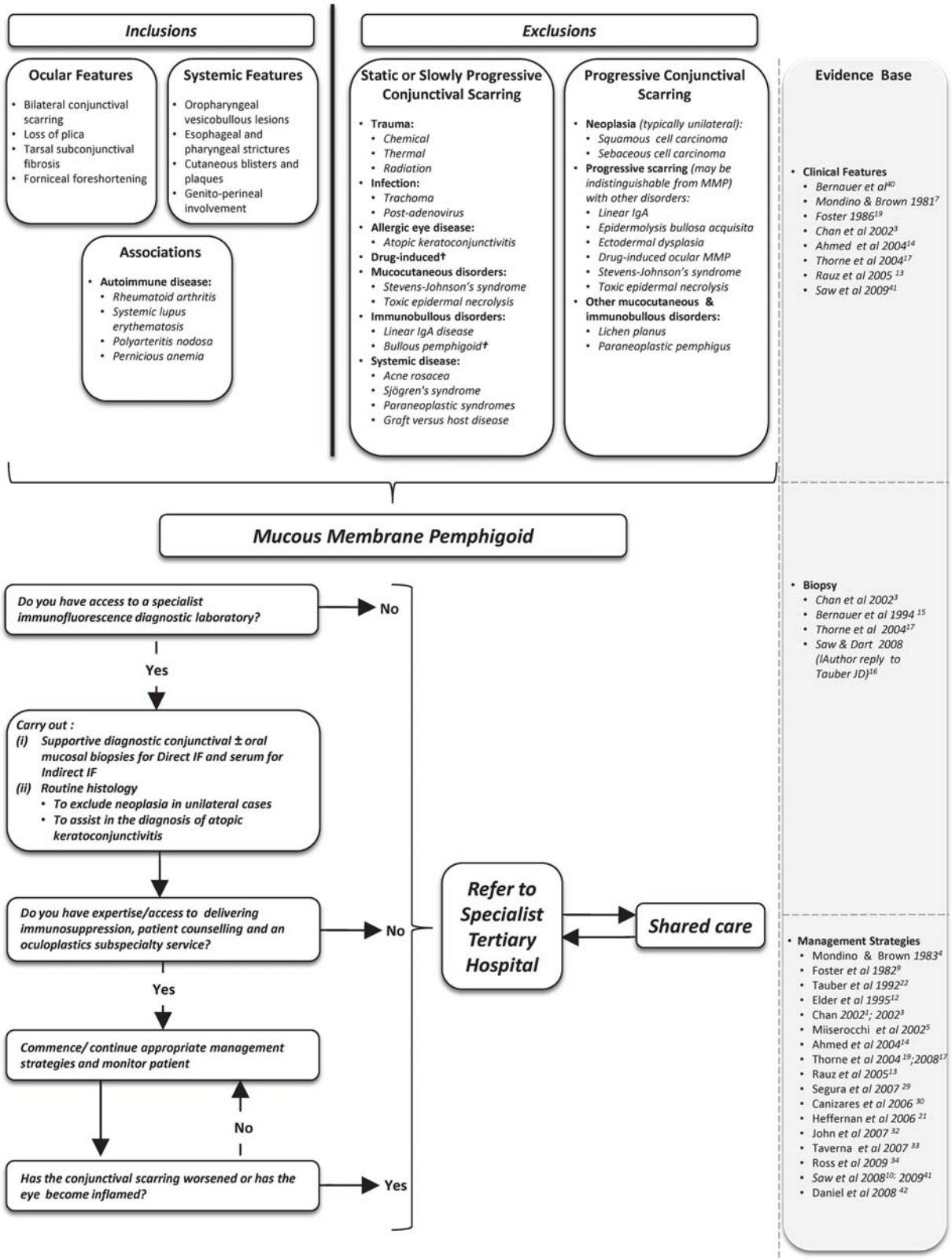

Figure 5 An algorithm highlighting clues to the diagnosis of OcMMP (ocular features, systemic involvement, autoimmune disease associations), together with differential diagnoses for conjunctival scarring subdivided into 'static or slowly progressive' or 'progressive' aetiologies is shown. A putative model for early referral to tertiary care hospitals is also suggested. †, A subset develop autoantibody-positive progressive conjunctival scarring similar to MMP; IF, immunofluorescence; MMP, mucous membrane pemphigoid.

conjunctival fibrosis and obliteration of the inferior fornix, with judgment of progression open to individual interpretation. Information regarding horizontal obliteration of the fornix by symblephara is not routinely documented, precluding use of the proposed and improved staging system described by Tauber.[22] In addition, there is no standardised method for measuring and documenting disease progression of the upper fornix, when the disease is clearly not confined to the inferior conjunctival surface. Subsequent to this study, we have designed and adopted the use of a validated bespoke Fornix depth measurer to routine clinical practise to enable quantification of upper and lower forniceal obliteration and to monitor disease progression.[23]

The difficulty lies not only in identifying early disease and determining which patients may progress, but also recognising when this is happening. 'Activity' and 'damage' indices have been validated and accepted for a number of autoimmune conditions including systemic lupus erythematosis and primary Sjögren's syndrome.[24–27] These indices facilitate not only comparison of clinical cohorts worldwide, but also inform clinical trials specifically those targeting therapeutic intervention. As such, we suggest that clearer strategies for discriminating MMP disease 'activity' and 'damage' are necessary to afford a uniform language and understanding when describing OcMMP phenotypes, before molecular targeting and evaluation of novel therapeutic approaches through randomised controlled trials can be considered.

These difficulties in determining activity and progression highlight the challenge in directing appropriate therapy. The issue of suboptimal therapeutic immuno-modulation of disease course has been widely described.[4,5,9–11,28] We highlight a recalcitrant group of patients with persistent mild or moderate inflammation in keeping with the findings of others.[10,11,13] Unfortunately, there is also a disease subset that either is completely refractory to conventional immunosuppression or relapses despite initial success.[11] A few isolated case reports indicate that 'biological' agents, such as rituximab (anti-CD20) or infliximab (anti-TNFα), may be beneficial in some of these patients, but as randomised trials are lacking, funding for such treatment in the United Kingdom prohibits regular use.[29–34] These data re-emphasise the fact that the pathogenesis of OcMMP is not resolved and strengthen the case for further study of clinically involved and seemingly uninvolved mucous membranes.[35–37]

The reasons for a delay in presentation in our EstD group are not clear. It is possible that the clinical features or the severity of the disease may not have been recognised until late, as early symptoms may have been insidious and non-specific.[9,38] A variable duration of disease and course have been described by others,[11] but the true definition of early disease in the context of OcMMP is not known. This may well lie under the 3 year duration of symptoms statistically defined in our cohort, and this is particularly relevant if disease activity or progression is initially either subtle or sub-clinical. Experience in other more common autoimmune diseases, such as rheumatoid arthritis, point to the clinician actively pursuing identification of early disease to enable early therapeutic intervention in order to limit tissue damage.[39] In the light of the potential for disease progression in both early or late onset OcMMP disease forms, irrespective of whether inflammation is clinically detected or not, it may be prudent for ophthalmologists to take precedence from the rheumatological concepts for capturing early disease, and adopt a similar approach.

Many of our patients travelled long distances to our centres and this may represent a barrier to early tertiary care of this rare disease, resulting in initiation of suboptimal immunomodulation and/or surgery. Stringent efforts to identify features characteristic of OcMMP are necessary in order to avoid missing an early diagnosis. Where local diagnosis or management is not possible, or where the identification of high risk features including severe refractory inflammation or evidence of progression is manifest, prompt referral of cases with OcMMP to specialised tertiary centres is essential for optimisation of immunosuppression aimed at limiting long-term tissue damage. Furthermore, this may include implementation of shared care pathways or stabilisation of disease before discharge back to local referring centres, as evident in many (28%) of our patients. We therefore propose a referral algorithm as a putative model to help educate these decisions and prompt early referral (Figure 5).

Although our study is limited by its retrospective nature and bias in our study population towards a more severe clinical phenotype, a high proportion (48%) of patients required initiation or a switch to more potent immunosuppression following referral to our tertiary centres. In addition, 28% of patients were eventually returned to their local referring unit for monitoring after stabilisation of disease. Most importantly, however, up to 42% of patients (irrespective of disease phenotype, early or late) continued to demonstrate progressive conjunctival scarring in the absence of clinically detectable inflammation. A greater understanding of disease pathology is required to facilitate earlier recognition of disease, improved activity and damage scores, and more accurate therapeutic targeting, specifically for patients recalcitrant to existing immunomodulatory therapy.

Summary

What was known before

- Mucous membrane pemphigoid is a potentially blinding autoimmune disease affecting multiple mucosal surfaces, where the presence of ocular involvement is defined as high-risk.
- There is evidence that young patients have a more destructive phenotype despite the average age of onset in the seventh decade.
- Progressive conjunctival scarring is thought to be more aggressive in the context of severe conjunctival inflammation that may or may not be responsive to immunotherapy.

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What this study adds

- There is a wide range of clinical characteristics that prompt referral to tertiary centres. Those with early-onset disease (defined according to the duration of symptoms at presentation) have more severe ocular surface inflammation than those with more established disease.
- Despite initiation or escalation to more potent immunosuppression at first visit, only 28% of patients are discharged back to the originating hospitals after stabilisation of disease. Almost 50% of the referred patients demonstrate progressive ocular surface scarring independent of disease duration, which continues to progress in a clinically quiescent eye.
- In the absence of a biomarker to facilitate monitoring of disease activity and/or progression, a putative algorithmic model for early detection of ocular mucous membrane pemphigoid is proposed, to enable prompt referral to specialist tertiary care clinics.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Validation of a fornix depth measurer: a putative tool for the assessment of progressive cicatrising conjunctivitis

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Notes

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Validation of a fornix depth measurer: a putative tool for the assessment of progressive cicatrising conjunctivitis

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ABSTRACT

Background/aims Documentation of conjunctival forniceal foreshortening in cases of progressive cicatrising conjunctivitis (PCC) is important in ascertaining disease stage and progression. Lower fornix shortening is often documented subjectively or semi-objectively, whereas upper forniceal obliteration is seldom quantified. Although tools such as fornix depth measurers (FDMs) have been described, their designs limit upper fornix measurement. The purpose of this study was to custom-design a FDM to evaluate the upper fornix and to assess variability in gauging fornix depth.

Methods A polymethylmethacrylate FDM was constructed using industry-standard jewellery computer software and machinery. Two observers undertook a prospective independent evaluation of central lower fornix depth in a heterogeneous cohort of patients with clinically normal and abnormal conjunctival fornices both subjectively and by using the FDM (in mm). Upper central fornix depth was also measured. Agreement was assessed using Bland—Altman plots.

Results Fifty-one eyes were evaluated. There was 100% intraobserver agreement to within 1 mm for each observer for lower fornix measurement. The mean difference in fornix depth loss using the FDM between observer 1 and 2 was 1.19%, with 95% confidence of agreement (±2SD) of −15% to +20%. In total, 86% (44/51) of measurements taken by the two observers agreed to within 10% of total lower fornix depth (ie, ±1 mm) versus only 63% (32/51) of the subjective measurements. Mean upper fornix difference was 0.57 mm, with 95% confidence of agreement of between −2 and + 3 mm.

Conclusions This custom-designed FDM is well tolerated by patients and shows low intraobserver and interobserver variability. This enables repeatable and reproducible measurement of upper and lower fornix depths, facilitating improved rates of detection and better monitoring of progression of conjunctival scarring.

INTRODUCTION

Sequential documentation of forniceal foreshortening in cases of progressive cicatrising conjunctivitis (PCC), such as ocular mucous membrane pemphigoid (OcMMP), is important in assessing stage and progression of disease.[1–3] The Foster staging relies on subjective evaluation of subepithelial fibrosis and extent of symblepharon formation,[3] and the system described by Mondino and Brown[2 4] describes grading of percentage shrinkage of the lower fornix. A modification encompassing both systems was described by Tauber *et al*,[1] who proposed that counting the number of, and percentage horizontal obliteration of the lower fornix by symblephara could potentially improve detection of disease progression.

We have previously shown that lower fornix shortening is documented either subjectively or semi-objectively by utilising a slit-light beam, the degree of upper forniceal obliteration is seldom quantified.[5] Furthermore, we have shown that, at the initial visit to tertiary referral centres, Foster's staging of disease is undertaken in 100% of patients' lower fornix, but only 78% of patients had quantification of forniceal shrinkage. This is probably related to the difficulty in assessing lower fornix depth accurately without the aid of a made-for-purpose tool.[6]

Measurement of the fornices using devices such as the fornix depth measurer (FDM) has previously been described, but their design restricts accurate upper fornix measurements.[7 8] Specifically, the depth and curvature of the upper fornix dictates that the ideal FDM must be sufficiently long and curved to enable comfortable and accurate assessment of it. We previously used in routine clinical practice an FDM based on that designed by Schwab *et al*,[7] but its design was suboptimal (figure 1). In 2004, a modification of the Schwab FDM was designed at Moorfields Eye Hospital for a clinical trial[9] and is currently being used in an epidemiological study. In 2007, we wanted to redesign and evaluate a bespoke comfortable prototype that could provide an accurate tool for improving forniceal sac documentation in the outpatient clinic setting, with potential for wider-scale commercial manufacture. In this study we describe this custom-designed FDM constructed specifically to facilitate evaluation of the depth of the upper conjunctival fornix, and we assess intraobserver and interobserver variability in gauging the extent of the upper and lower fornices by validating the FDM and comparing it with subjective assessment of fornix shrinkage.

MATERIALS AND METHODS

Design of a bespoke FDM

A polymethylmethacrylate FDM was designed using industry-standard jewellery computer software (CAD V5, 3Design, Brussieu, France, 2007). The virtual model was then exported as an. STL file into a program to set the cutting parameters for the milling machine (Modela Player V4; Roland DG, Shizuoka, Japan, 2002) (figure 2A). The virtual model

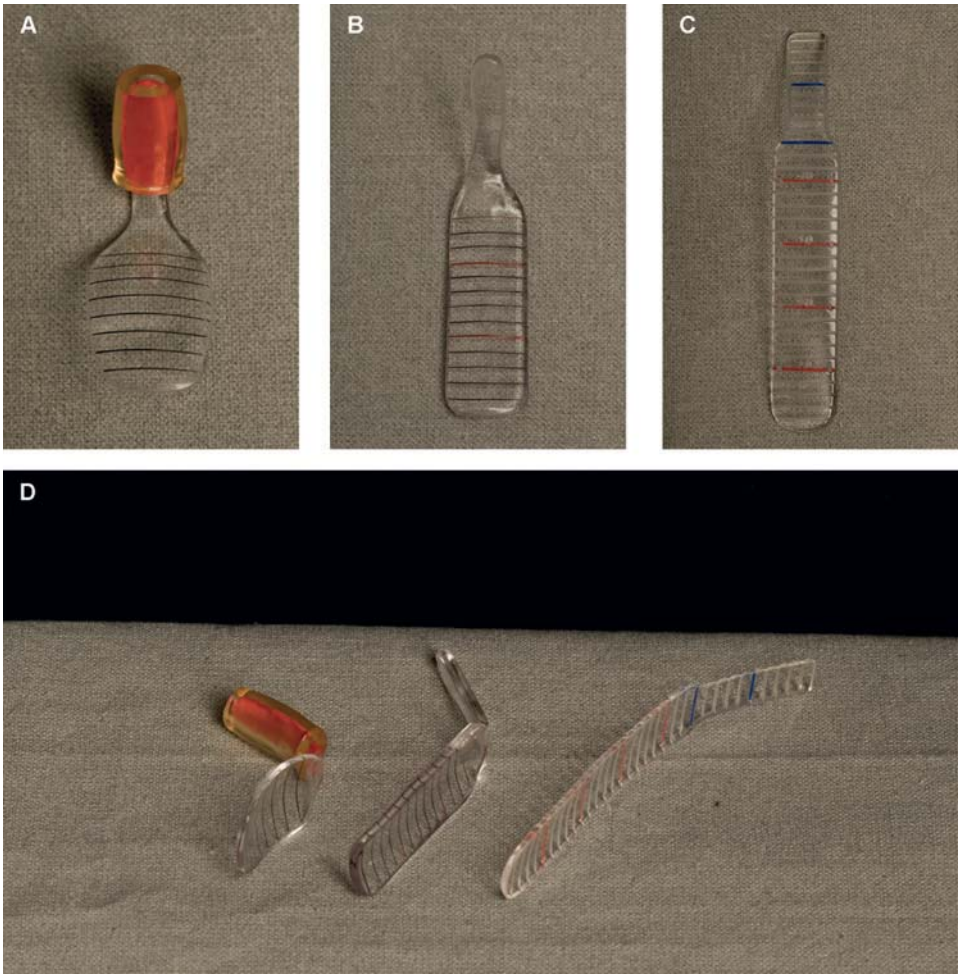

Figure 1 Colour photographs illustrating the evolution of the fornix depth measurer (FDM). (A) The original adaptation of an FDM described by Schwab *et al*[7] (produced by our local prosthetics department). (B) An FDM constructed at Moorfields Eye Hospital, which is an elongated polymethylmethacrylate modification of the Schwab FDM using a hand-made plaster cast (designed by VS, Scott Hau and David Carpenter, ocular prosthetist at Moorfields Eye Hospital, specifically to facilitate upper fornix depth measurement for use in a clinical trial). For comparison, (C) illustrates the computer-designed bespoke FDM prototype. This is an elongated, biconcave design with engraved markings to a precision of 2 μm/step, and increments expressed at 2 mm intervals on both the main body of the FDM and the narrower 'handle'. The markings on the handle facilitate upper fornix measurement and the ability to measure the fornix in the presence of symblephara (D). The accuracy and reproducibility of the computer-generated design and jewellery precision engraving provides potential for commercial manufacture.

was cut with a machine to a precision of 2 μm/step, and increments were expressed at 2 mm intervals at both ends of the FDM: the main 'body' and the 'handle'. This design feature enables measurement of the fornix in the context of multiple symblephara, which could potentially hinder the smooth passage of the FDM to the limits of the conjunctival sac. The FDM measured 25 mm × 5 mm and was moulded to a biconcave shape to ensure ease of insertion and comfort (figures 1C and 2B).

Patients

An evaluation of the bespoke FDM was undertaken through a prospective, masked, independent assessment of central lower fornix depth by two observers (GPW, TS), following the Tenets of the Declaration of Helsinki. This was undertaken as a service evaluation at the Birmingham and Midland Eye Centre (BMEC). The validation of the FDM was conducted on a heterogeneous group of patients comprising clinically normal and abnormal conjunctival fornices presenting to BMEC. This heterogeneity enabled a wide range of fornix depths to be tested with the bespoke FDM.

The cohort consisted of 51 eyes of 26 patients with a median age of 64 years (range 42–100), of whom 65% (17/26; 33/51 eyes) had an identifiable cause of cicatrising conjunctivitis (OcMMP, 10; dry eyes, five (including three with Sjögren's syndrome); Stevens–Johnson syndrome, two) and 35% (18/51) had no evidence of conjunctival scarring (age-related macular degeneration, four; uveitis, three; peripheral ulcerative sclerokeratitis, two).

After instillation of one drop of 0.4% oxybuprocaine hydrochloride, patients were asked to look in the opposite direction to the placement of the FDM (upper fornix, down-gaze; lower fornix, up-gaze) in order to protect the cornea, ensure consistency in readings, and circumvent variability in the eyelid position in primary gaze. The central conjunctival fornix was measured to the eyelid margin, defined as the posterior lip of the meibomian gland orifice (figure 1C). All FDM readings were taken in triplicate, with the first measurement taken used for interobserver comparison. A semi-objective estimation of lower fornix conjunctival shrinkage was also performed by gauging the central lower fornix depth, measured from the inferior fornix to the eyelid margin with the aid of a vertical 1 mm wide slit-lamp beam with illumination and observation axes in a coaxial position (NB a subjective assessment of the upper fornix is impossible). The FDM was sterilised by soaking the device in 0.05% sodium dichloroisocyanurate solution for 5 min between patients (as per the BMEC infection control policy for reusable tonometer heads). Patients were also asked about their tolerance to the FDM.

Calculations and statistical analyses

The percentage loss of lower fornix for both methods of measurement (subjective and objective) was calculated using the equation:

$$\{[\text{fornix depth (FD) age} - \text{FDM measurement}]/\text{FD age}\} \times 100 = \%\ \text{loss of fornix}$$

A correction factor for age was implemented, as the lower forniceal depth is known to progressively shorten with age.[7] The 'FD age' values were derived from published age-specific lower fornix depths in normal eyes detailed in table 1.[7] For example,

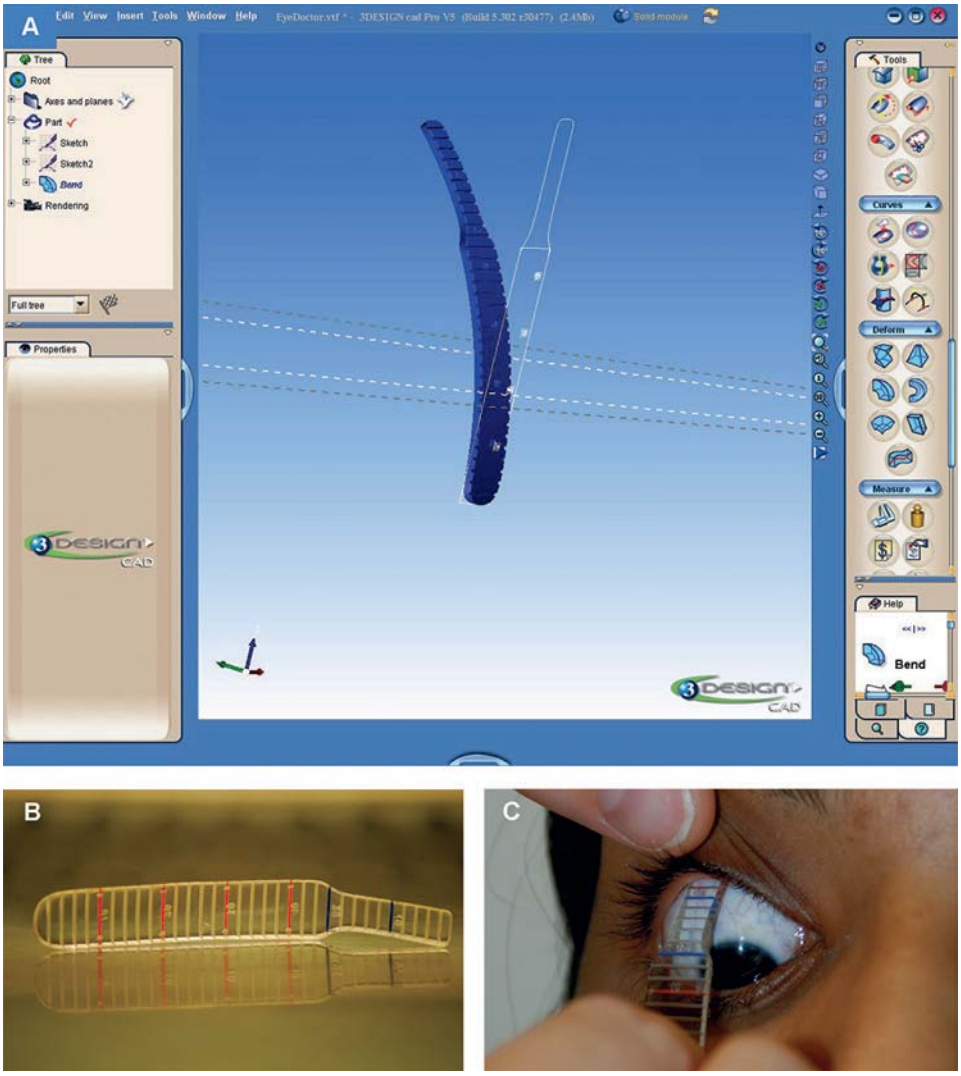

Figure 2 (A) A polymethylmethacrylate fornix depth measurer (FDM) was constructed using industry-standard jewellery software and machinery. A screenshot of the 3design prototype is shown in (A). The final prototype is illustrated in (B). Increments are expressed at 2 mm intervals to a precision of 2 μm/step, and the FDM was heat moulded to a biconcave shape for comfort. The FDM was applied after instillation of one drop of 0.4% oxybuprocaine hydrochloride (C). Patients were asked to look in the opposite direction to the placement of the FDM, and the central conjunctival fornix was measured to the eyelid margin, defined as the posterior lip of the meibomian gland orifice.

for a patient aged 80 years with a lower fornix measurement of 6 mm, the calculated percentage shrinkage is as follows:

$$\{[10.2-6]/10.2\times100\} = 41.2\%$$

It is important to note that it was not possible to calculate percentage loss of the upper fornix because the normal range of age-specific upper forniceal depths is currently unknown.

Intraobserver and interobserver agreement was assessed using Bland–Altman plots of differences in measurements versus mean measurements using Excel for Macintosh (Microsoft Office 2008).[10 11] The mean difference in observations and the 95% limits of agreement (the mean difference ±2 standard deviations)[10] were calculated using SPSS V16.0 for Macintosh. A continuity correction was applied to the 95% limits of agreement to take account of the fact that Bland–Altman plots assume that the variables measured are continuous.

A 10% threshold or 'tolerance' was chosen as an allowance for intraobserver variation. Agreement was also evaluated by determining the percentage of observations that agreed to within the 10% 'allowance' for both observers.

RESULTS

Lower fornix assessment

Intraobserver variation

Triplicate measurements of FDM readings of the same anatomical position by each observer (central lower fornix) showed exact agreement of 86% (42/49) and 89% (41/46) of measurements within observer 1 and observer 2, respectively. When allowing for 1 mm 'tolerance' (approximating to 10% of the normal lower fornix, see table 1), 100% of intraobserver observations fell within 1 mm for both observers.

Interobserver variation

Interobserver variation between the subjective and objective measurements of the central lower fornix by the two observers was also assessed. Assessment of the lower fornix shrinkage was expressed as a percentage for both subjective and objective estimations, the latter using the age correction factor described in the methods.[7]

Table 1 Age-specific normal values for the conjunctival lower fornix

Age	Mean depth of normal lower fornix (mm) ('100%')	10% of normal lower fornix (mm)
40–49	11.9	1.19
50–59	11.3	1.13
60–69	11.0	1.10
70–79	10.6	1.06
80+	10.2	1.02

Adapted from Schwab *et al*.[7]

The mean difference in calculated percentage fornix depth using measurements obtained from the FDM by observer 1 and 2 was 1.19%, and, with a continuity correction, the 95% limits of agreement (±2SD) were narrower for interobserver objective (FDM) measurements than for those obtained subjectively (−15% and + 20%) (figure 3). The interobserver agreement within the 10% allowance (ie, approximately ±1 mm) of total lower fornix depth was 86% (44/51) (figure 4).

In contrast, the interobserver mean difference in subjective estimation of percentage fornix depth was −1.86%, and, with a continuity correction, the 95% limits of agreements (±2 SD) were between −30% and +25% (figure 3). Only 63% (32/51) of the subjective measurements taken by the two observers agreed to within a 10% allowance of total lower fornix depth (figure 4).

These data highlight that the FDM afforded greater consistency in fornix depth measurement by each observer (intraobserver variation) and between observers (interobserver variation).

Upper fornix assessment

Intraobserver variation

Triplicate measurements (to assess intraobserver variation) of FDM readings of the same anatomical position of the central upper fornix by each observer showed minimal variation (ie, identical objective measurements) in 88% (45/51) and 70% (33/47) of measurements by observer 1 and observer 2, respectively. There are no data regarding normal upper fornix depth in the published literature, preventing calculations for age-based corrections and percentage fornix depth foreshortening and tolerance threshold.

Interobserver variation

Interobserver variation of the upper fornix showed a mean difference in fornix depth measurement using the FDM for observer 1 and 2 of 0.57 mm, with 95% limits of agreement (±2SD) of −2 and + 3 mm (figure 5). The absence of normal upper fornix values precludes evaluation of the 10% allowance; however, 84.3% (43/51) of upper fornix measurements were within 1SD of the mean difference of 0.57 mm (+2 to −1 mm of the mean with a continuity correction).

Patient comfort and tolerance

The FDM was well tolerated by patients, with only a few (three) experiencing mild discomfort during upper fornix measurement, despite repeated measurements. None of these patients reported prolonged discomfort or pain.

DISCUSSION

PCC comprises a group of disorders characterised by progressive scar formation in response to conjunctival inflammation affecting the stromal layers of the conjunctiva.[12] These include ocular immunobullous diseases such as OcMMP, Stevens–Johnson syndrome and toxic epidermal necrolysis, lichen planus, linear IgA disease, paraneoplastic pemphigus and epidermolysis bullosa. Other causes include graft-versus-host disease, Sjögren's syndrome, acne rosacea and those associated with topical therapy where progression is reported to be more insidious with less destructive clinical sequelae.

Because of the sight-threatening consequences of OcMMP, the ocular phenotype of MMP is regarded as 'high risk',[13] particularly as disease progression is more aggressive in younger

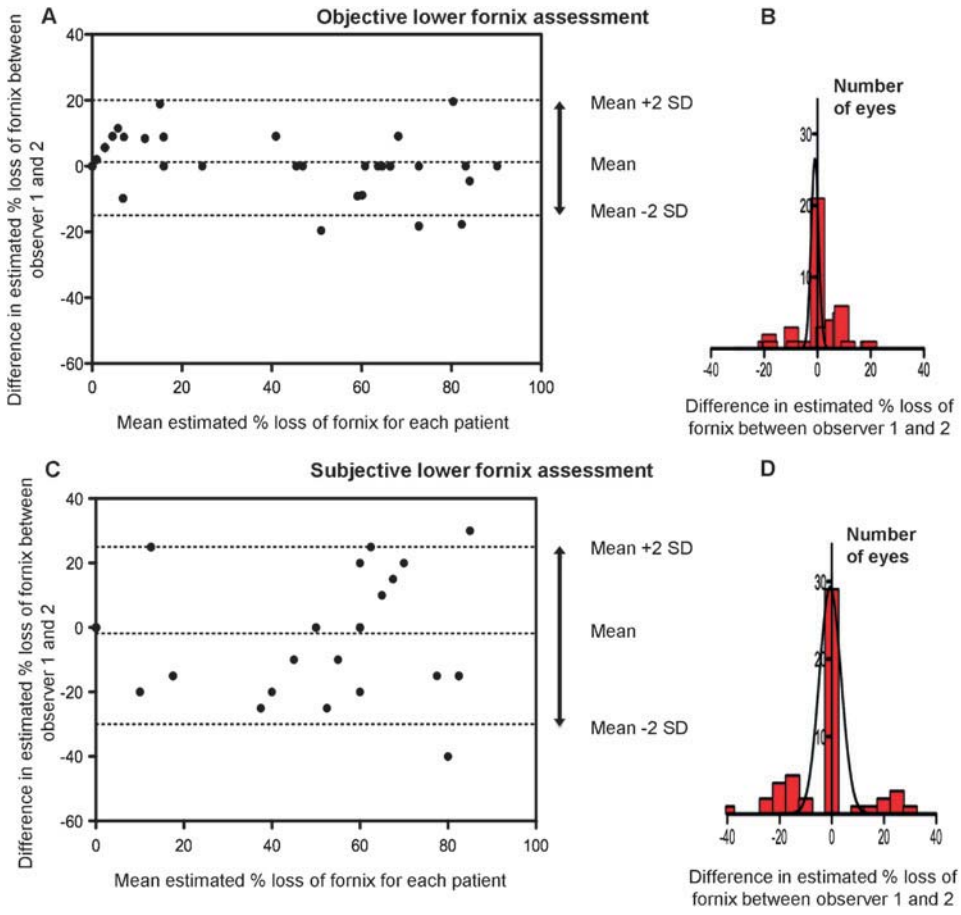

Figure 3 Bland–Altman plots showing interobserver variation in lower fornix assessment ((A) objective and (C) subjective). Some data points are identical and therefore overlay each other on the figures. The percentage difference in assessment between observer 1 and 2 is plotted against the mean percentage loss of fornix for each eye. If there was a completely normal fornix, this is represented as 0% loss of fornix on the x axis. Note the increase in the 95% limits of agreement (±2SD) for subjective assessment (arrowed), demonstrated also by the histograms (B,D).

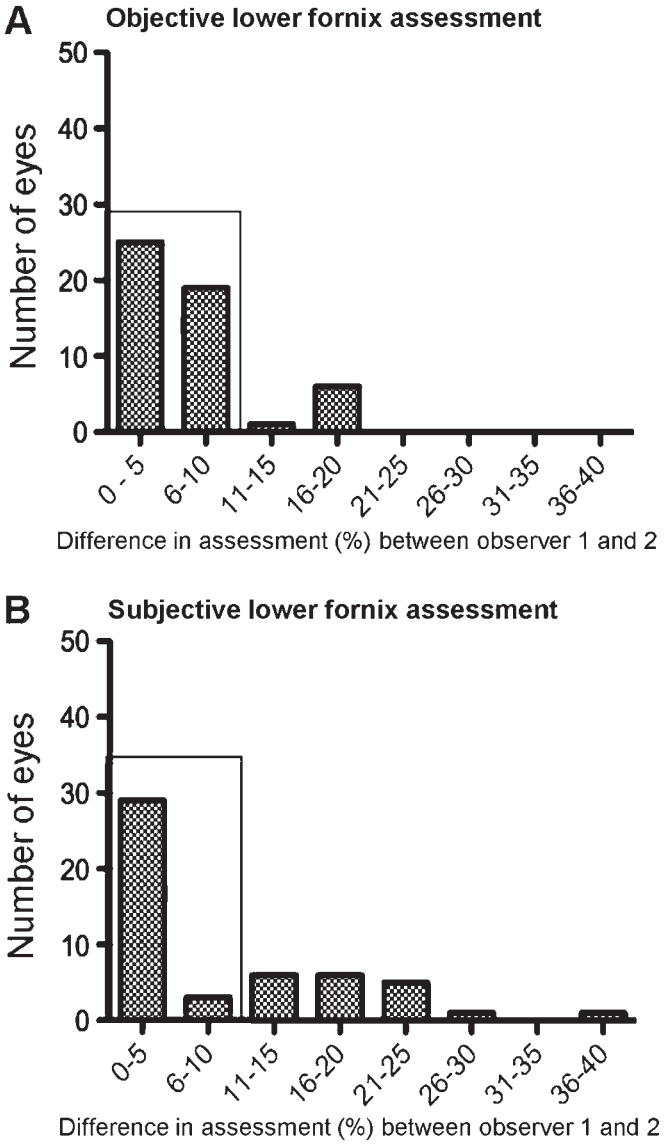

Figure 4 Difference in objective (A) and subjective (B) lower fornix assessment between observer 1 and 2. The number of individuals that agree to within a 10% 'allowance' are boxed and are higher for objective measurements (86% (44/51)) than for subjective measurements (63% (32/51)).

patients[14] and 50% of patients continue to have progression of cicatrising disease in the apparent absence of inflammation.[15]

Determining progression in PCC is a challenging aspect of patient management. When considering OcMMP, for example, the most common of the acquired immunobullous diseases that cause PCC,[12 16] progression may occur at any stage of disease,[2 17] which can be aggressive early in the disease course,[14] but, importantly, is often independent of clinically identifiable inflammation.[15] Determining disease progression in the cicatrising conjunctivitides therefore relies upon accurate documentation of disease, in particular conjunctival shrinkage of the fornices. Although the Mondino staging system is considered to be more sensitive than Foster's staging system[15] and is integrated into the system proposed by Tauber *et al*,[1] Mondino fornix depth measurement is reported to be undertaken in only 78% of new patients in tertiary referral centres compared with 100% documentation of the Foster's system.

Vigilant assessment, quantification and documentation of forniceal foreshortening is mandatory for enabling accurate patient follow-up, currently not achieved by subjective or semi-objective assessments of the fornix. These approaches are limited by inconsistency and poor reproducibility and reliability, but are overcome by the development and implementation of fornix depth measuring devices for the assessment and progression of diseases that cause conjunctival scarring.[7 8 18] Our aim was to design an FDM based upon the original concept of Schwab *et al*,[7] but with additional upper fornix depth capability. Specifically, our custom-made FDM was found to be comfortable and accurate. Designed and made using industry-standard computer software and machinery, this FDM prototype was modelled mathematically taking into account the curvature of the globe necessary for comfortable measurement of the upper fornix. We tested our bespoke FDM on patients with a range of fornix depths in the presence or absence of conjunctival fibrosis. Our findings showed low intraobserver and interobserver variability, enabling repeatable and reproducible measurements of both upper and lower fornix depths, highlighting its potential in facilitating both accurate and robust clinical documentation of disease stage. This FDM prototype is currently being optimised with further modifications to improve comfort and fit.

Crucially, the conjunctival cicatrising process is not confined to the lower fornix, and sight-threatening sequelae secondary to subtarsal fibrosis, upper lid entropion or lash trauma commonly ensue. These clinicopathological processes are not taken into

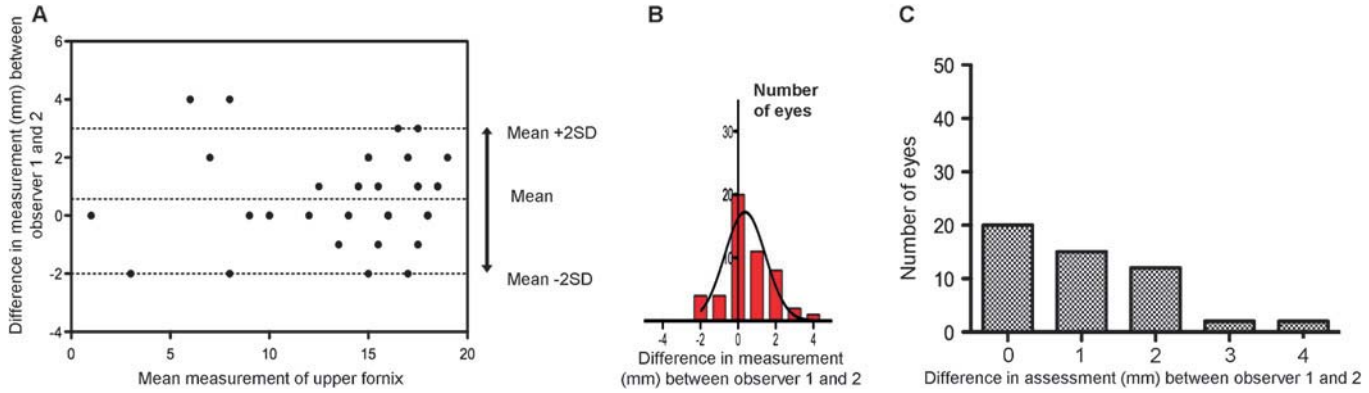

Figure 5 (A) Bland–Altman plot showing interobserver variation in upper fornix assessment. As there are no defined limits for the upper fornix, the calculations are in mm. The mm difference in assessment between observer 1 and 2 is plotted against mean mm measurement for each patient. The 95% limits of agreement are vertically arrowed and also represented by a histogram (B). (C) Difference in upper fornix assessment between observer 1 and 2; the 10% allowance cannot be calculated in the absence of normal upper fornix values, but 71% (36/51) of measurements were within 1 mm, and 92% (47/51) of observations were within 2 mm of each other.

consideration by ocular staging systems that calculate percentage obliteration of the lower fornix,[1 2 4] although are reflected in the staging system described by Foster where fornix shortening or symblephara of any degree throughout the conjunctival surface are thought to be important.[3] The decreased sensitivity in relying solely upon this system, or those that include direct measurement of the lower fornix alone, increase the risk of type 2 errors (false negatives) in determining disease progression, which are considerably rescinded by the use of fornix depth measuring devices for the upper fornix.

The larger depth of the upper fornix and difficulty of access explains the omission of detailed upper fornix pathology in currently recognised scoring systems.[1 2 4] Perhaps not surprisingly therefore, there appear to be few data in the ophthalmic literature regarding the anthropology and normal depth of the upper fornix. Kawakita and colleagues[8] have recently discussed the use of a non-curved 150×2 mm FDM in Japanese patients with Stevens–Johnson syndrome and healthy volunteers. They found that the mean superotemporal and superonasal upper fornix depths were 14.1±2.5 mm in normal people. Our findings show a median central upper fornix depth of 16 mm in a caucasian group of patients, even in the presence of recognised cases of cicatrising conjunctivitis. The variation in central upper fornix depth among healthy populations using anthropological ethnography together with differences in age remains unresolved. This has recently been highlighted in the context of other diseases affecting the size of the upper fornix such as the giant fornix syndrome described by Rose.[19] These data illustrate the need for population-based studies of normal age-based upper fornix depths to facilitate calculations of percentage shrinkage in conjunctival scarring diseases. Such studies are currently being undertaken at BMEC and Moorfields Eye Hospital.

In summary, our custom-designed FDM was well tolerated by patients in this study, with only three experiencing short-lived mild discomfort during assessment of the upper fornix. This FDM shows low intraobserver and interobserver variability, enabling repeatable and reproducible measurements of lower fornix depths. We believe that the custom design of an FDM using industry-standard jewellery software and machinery, curved to fit the globe, provides an accurate and comfortable means of assessing lower fornix depth. Furthermore, it offers the potential to measure upper fornix depth, currently not routinely carried out in clinical practice, thereby improving both the detection and monitoring of progressive conjunctival fibrosis in this group of devastating disorders.

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The dominant human conjunctival epithelial CD8αβ+ T cell population is maintained with age but the number of CD4+ T cells increases

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Abstract The conjunctiva is a highly specialized ocular mucosal surface that, like other mucosa, houses a number of leukocyte populations. These leukocytes have been implicated in age-related inflammatory diseases such as dry-eye, but their phenotypic characteristics remain largely undetermined. Existing literature provides rudimentary data from predominantly immunohistochemical analyses of tissue sections, prohibiting detailed and longitudinal examination of these cells in health and disease. Using recovered cells from ocular surface impression cytology and flow cytometry, we examined the frequency of leukocyte subsets in human conjunctival epithelium and how this alters with age. Of the total CD45+ leukocyte population within the conjunctival epithelium, 87% [32–99] (median) [range] comprised lymphocytes, with 69% [47–90] identified as CD3+CD56- T cells. In contrast to peripheral blood, the dominant conjunctival epithelial population was TCRαβ+CD8αβ+(80% [37–100]) with only 10% [0-56%] CD4+ cells. Whilst a significant increase in the CD4+ population was seen with age ($r=0.5$; $p<0.01$) the CD8+ population remained unchanged, resulting in an increase in the CD4:CD8 ratio ($r=0.5$;$p<0.01$). IFNγ expression was detectable in 18% [14–48] of conjunctival CD4+ T cells and this was significantly higher among older individuals (<35 years, 7[4–39] vs. >65 years, 43 [20–145]; $p<0.05$). The elevation of CD4+ cells highlights a potentially important age-related alteration in the conjunctival intra-epithelial leukocyte population, which may account for the vulnerability of the aging ocular surface to disease.

Keywords Aging · Tcell · Lymphocyte · Conjunctiva · Mucosa

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Introduction

The ocular surface consists of the cornea, corneal–scleral limbus and conjunctiva, closely interrelated with adnexal structures (lacrimal gland, eyelids and lashes) that together are vital for optical clarity, immune and mechanical protection. The conjunctiva is a highly specialized, delicate mucosa comprising of

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a bi-layered substantia propria underlying a non-keratinized, stratified squamous epithelium interspersed with goblet cells. It extends from the mucocutaneous junction of the eyelid margins, lining the posterior surface of the eyelids and reflects forward over the sclera to become continuous with the cornea at the corneal scleral limbus.

The avascular central cornea is largely devoid of immune cells (Knop and Knop 2007), whereas the vascularized conjunctiva contains numerous resident immune cells including intra-epithelial lymphocytes (IELs) (Allansmith et al. 1978; Hayday et al. 2001; Knop and Knop 2005; Knop and Knop 2007). In common with other mucosal sites, the presence of conjunctiva-associated lymphoid tissue provides a local immune microenvironment, which includes the production of immunoglobulins such as IgA that confers immuno-protection to the ocular surface.

Although an abundance of CD3+ cells (including CD8+ cells) has been identified in the human conjunctival mucosa (Hingorani et al. 1997), the subtypes of IELs have not been defined. Non-invasive means of sampling conjunctival leukocytes such as ocular surface impression cytology (OSIC) have been utilized to characterize ocular surface changes in diseases such as dry eye (Baudouin et al. 1997; Brignole et al. 2000; Baudouin et al. 2004), but this methodology has not been extended to afford a comprehensive examination of resident epithelial leukocytes in healthy conjunctiva.

Of interest, is the observation that the severity of ocular surface infections such as microbial or herpetic keratitis is clinically worse in the elderly (van der Meulen et al. 2008) and autoimmune diseases e.g. Mucous Membrane Pemphigoid (Foster 1986; Chan 2001) typically affect people in later life. Dry eye is a 'multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface' (2007). It is accompanied by increased osmolarity of the tear film and inflammation of the ocular surface (2007). Some studies have demonstrated an increased prevalence of dry eye problems with age (McCarty et al. 1998; Moss et al. 2000; Stern et al. 2010). Despite this, little is known about age-related changes in the leukocyte populations within the ocular surface (Gwynn et al. 1993). The aim of this study was therefore to utilize OSIC in combination with multi-color flow cytometry (Baudouin et al. 1992; Baudouin et al. 1997) to provide a detailed characterization of the frequency of leukocyte subsets in the healthy human conjunctival epithelium and whether these alter with age.

Materials and methods

Study subjects

Clinical data collection and patient sampling were undertaken following ethical approval in accordance with the Declaration of Helsinki. Healthy volunteers were defined as individuals with no history or current clinical evidence of ocular, systemic inflammatory or autoimmune disease (including dry eye) (Behrens et al. 2006; 2007), contact lens wear, previous ocular surgery, cataract surgery within 3 months or use of topical ophthalmic medication.

Two separate cohorts were evaluated:

Cohort 1 (Figs. 1, 2 and 3): OSIC of right and left eyes of 30 individuals (median age, 61 years [21–83]) together with matched peripheral blood were collected. Twenty out of 30 individuals were White (European) and ten out of 30 were Asian (defined according to the ethnic demographic categories employed in the UK census 2011). Fifteen were male and 15 were female.

Cohort 2 (Fig. 4): OSIC of right and left eyes were collected and pooled for each of ten healthy individuals (five young; median age, 24 [23–33] and five older; median age, 66 [65–83], $p=0.01$) in order to evaluate conjunctival T cell cytokine production. All were White (European) with five males and five females. Samples were pooled in this cohort in order to maximize the yield of cells for cytokine staining.

Conjunctival epithelial cell collection and recovery

Collection of conjunctival cells was undertaken with autoclaved synthetic membranes divided in two semicircles (measuring 13×6.5 mm^2 each) (Brignole-Baudouin et al. 2004). Supor 200 polyethersulfone filters (0.2 μm membranes) were applied following instillation of 0.4% Oxybuprocaine (as a topical anesthetic). Conjunctival OSIC was performed with

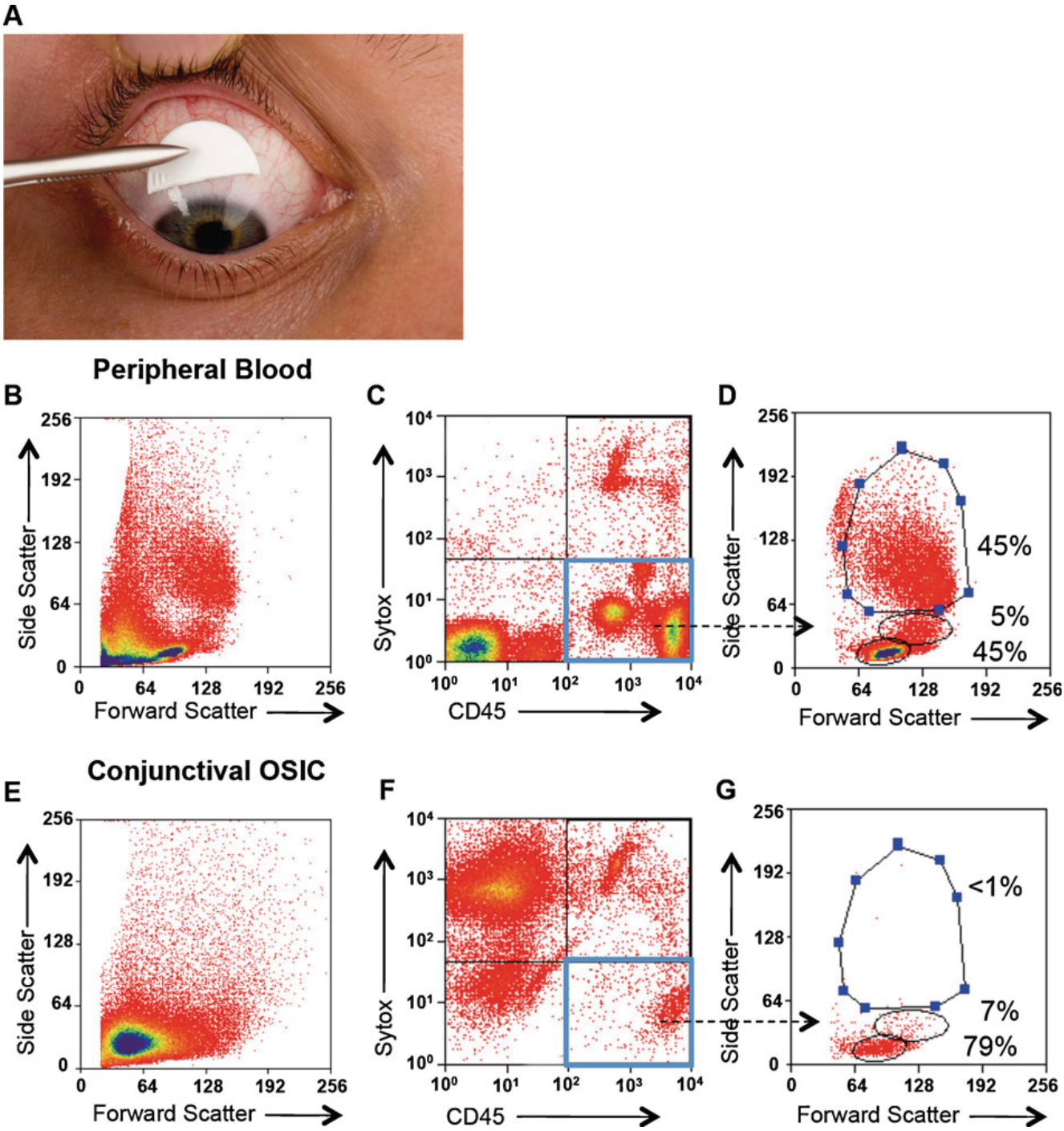


Fig. 1 Lymphocytes are the dominant conjunctival epithelial leukocyte population. Conjunctival OSIC of the superior unexposed bulbar conjunctiva is shown in **A**. Representative plots of a subject demonstrating the gating strategy used to identify conjunctival leukocytes. **B**, **E** The forward and side scatter profiles for peripheral blood and OSIC, respectively. Live leukocytes were identified by gating for CD45+ cells that were negative for the dead cell exclusion dye Sytox blue (*lower right box* in **C** and **F**) and back-gated to show the forward and side scatter profiles of the CD45+ live cells (**D** and **G**). Percentages are shown for the representative subject (n=30)

four semi-circle membranes per eye (equivalent to two full impressions) from the superior unexposed bulbar conjunctiva for 5–10 s using a sterile technique (Brignole et al. 2000; Brignole-Baudouin et al. 2004) and before the application of topical fluorescein drops for clinical examination (Fig. 1a).

Membranes were removed and placed in 1.5 ml of RPMI 1640 (Sigma-Aldrich, Dorset, UK) supplemented with 1% GPS (1.64 mM L-glutamine, 40 U/ml benzylpenicillin, 0.4 mg/ml streptomycin) (Sigma-Aldrich), 1% HEPES buffer (Sigma-Aldrich) and 10% heat-inactivated fetal calf serum (HIFCS–Biosera Ltd., Ringmer, UK) in a sterile 5-ml universal container and processed within 6 h after OSIC. In order to expedite cellular recovery, the cells were recovered by gentle agitation with a pipette tip for 1 min. Cell suspensions were transferred to a 1.5-ml Eppendorf tube and centrifuged (400×*g* for 5 min).

The majority of the supernatant was discarded, which was re-suspended in RPMI/10% HIFCS to a total volume of 200 μl (cohort 1) or 100 μl (cohort 2). One hundred microliters of cells were placed into each well of a 96-well plate for flow cytometric analysis.

Preparation of lysed peripheral blood

Peripheral blood was collected in EDTA tubes, centrifuged and re-suspended in 1:10 dilution of filter-sterilized red cell lysis buffer (8.29 g NH_4Cl, 1 g $KHCO_3$ and 37.2 mg of EDTA per liter of dH20). After 5 min at room temperature, the suspension was diluted with up to 15 ml of RPMI to block further lysis. Following centrifugation, the pellet was re-suspended in PBS at a concentration of 1×10^7 cells per milliliter and aliquoted at a volume of 100 μl in to individual wells.

Flow cytometry

Flow cytometry was undertaken with a Dako Cyan ADP high performance flow cytometer (Beckman Coulter, High Wycombe, UK). Multi-color cytometry compensation was performed using cells or compensation beads individually stained with each fluorochrome conjugated-antibody in order to circumvent spectral overlap by adjusting for false positives from other fluorochromes. An analysis was undertaken with Summit 4.3 for Windows (Dako, CO 2007). Non-parametric comparisons were undertaken with the Mann–Whitney *U* test, Wilcoxon signed rank test and correlations by Spearman's correlation using Prism version 5.0 for Macintosh (GraphPad Software, CA 2008).

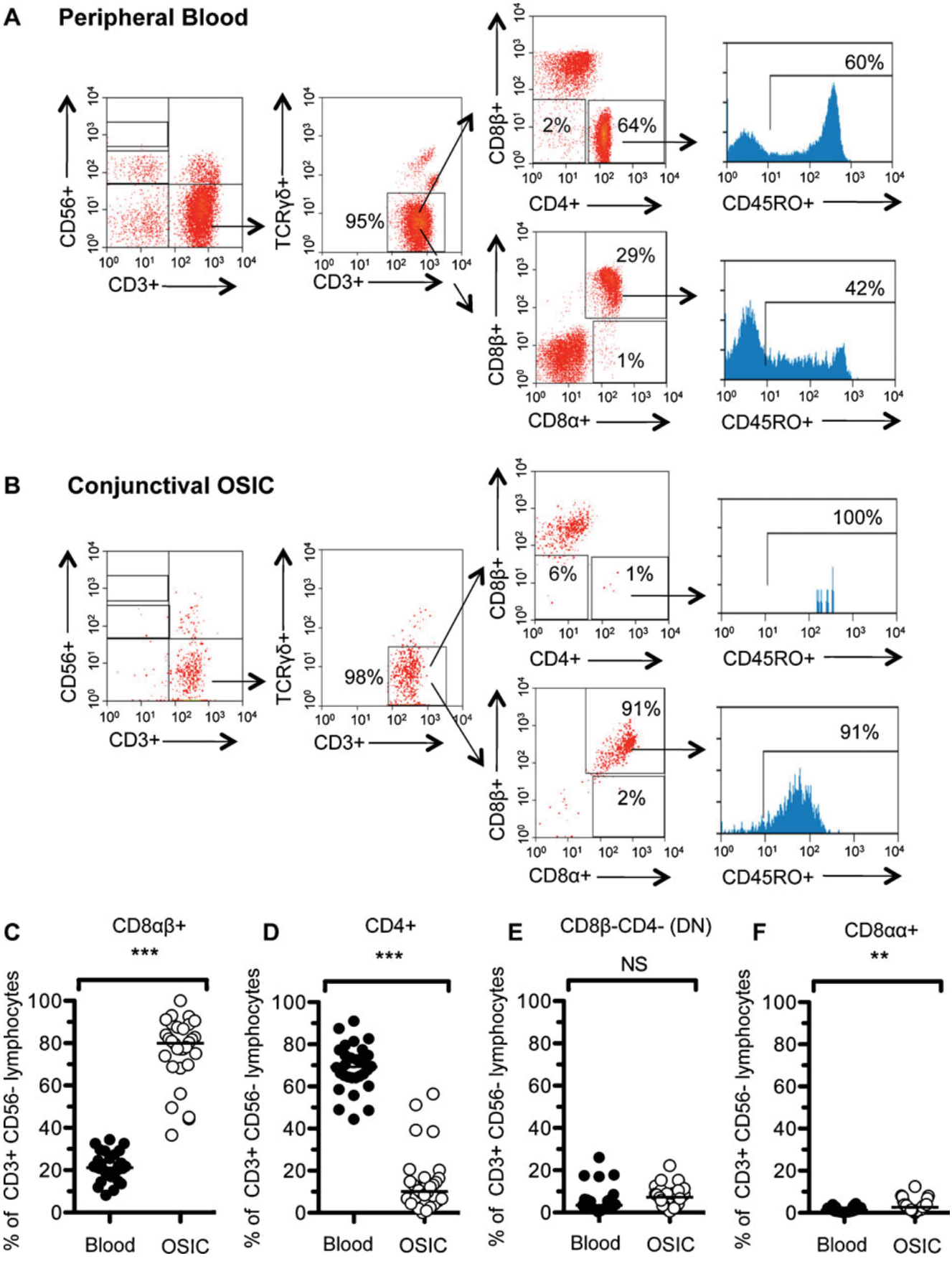
To characterize the cellular profile of the conjunctival ocular surface, nine color flow cytometry panels were developed. Commercially available antibodies to cell surface markers were employed in two panels; panel 1 mouse anti-human CD45RO (FITC), γδTCR (phycoerythrin), CD4 (PerCP Cy5.5), CD45 (allophycocyanin), CD3 (AlexaFluor 780) (Ebioscience, Hatfield, UK); CD8α (Pacific Orange) (Invitrogen, Paisley, UK); CD8β (PE Texas Red) (Beckman Coulter); CD56 (PE Cy7) (Biolegend, Cambridge, UK) and panel 2 mouse anti-human CD16 (FITC), CD45 (Allophycocyanin), CD14 (AlexaFluor 780) (Ebioscience); CD20 (Pacific Orange), CD19 (PE Texas Red) (Invitrogen); CD138 (PerCP Cy5.5) (BD, Oxford, UK) and CD11b (PE Cy7) (Biolegend). These were titrated to determine the optimal concentrations. Each panel was applied to cells recovered from conjunctival OSIC or peripheral blood.

One hundred microliters of cells were placed into 96-well plates (with a cell count per well ranging from 2×10^5–1×10^6 for PBMCs) or 20 μl of positive and negative compensation beads. Cells were centrifuged for 4 min at 1,200 rpm at 4°C, the supernatant removed and the 96-well plate gently vortexed. Cells were stained with surface marker antibodies (made up in 50 μl at appropriate dilutions) and incubated on ice in the dark for 20 min. One hundred microliters of PBS/0.5% BSA was added to each well prior to further centrifugation and removal of supernatant. Cells were re-suspended in 295 μl of FACS and 5 μl counting beads (1,002 beads per microliter) buffer prior to analysis. For dead cell exclusion, 30 μl Sytox blue (Invitrogen) was added at a concentration of 1/800 to the FACS tubes and incubated for 5 min prior to running on the flow cytometer.

Intracellular cytokine staining

For cytokine assays, conjunctival and lysed peripheral blood cells were stimulated with phorbol 12-

Fig. 2 TCRαβ+CD8αβ+T cells are the dominant population of lymphocytes in the conjunctival epithelium. Representative scatter profile of leukocyte populations derived from matched peripheral blood and conjunctival OSIC from a healthy subject. Data are shown for a representative subject, gated on CD45+ live cells for peripheral blood (**A**) and conjunctival OSIC (**B**). CD45RO staining is shown for CD3+CD56−TCRγδ−gated CD4+CD8β−(*top panel*) and CD8αβ+(*bottom panel*) cells. Percentages of CD3+CD56- lymphocytes are shown for the representative subject (n=30). Statistical comparisons between peripheral blood and conjunctival impression populations of CD8αβ+, CD4+, CD8αα+ and TCRαβ+CD4−CD8αβ− (*double negative*; DN) T cells (**C**–**F**) were undertaken by the Mann–Whitney *U* test (*NS* not significant [P>0.05], **P<0.01, ***P<0.001)


A
Peripheral Blood
CD56+
CD3+
95%
TCRγδ+
CD3+
2%
64%
CD8β+
CD4+
60%
CD45RO+
29%
1%
CD8β+
CD8α+
42%
CD45RO+
B
Conjunctival OSIC
CD56+
CD3+
98%
TCRγδ+
CD3+
6%
1%
CD8β+
CD4+
100%
CD45RO+
91%
2%
CD8β+
CD8α+
91%
CD45RO+
C
CD8αβ+
***
% of CD3+ CD56- lymphocytes
Blood
OSIC
D
CD4+
***
% of CD3+ CD56- lymphocytes
Blood
OSIC
E
CD8β-CD4- (DN)
NS
% of CD3+ CD56- lymphocytes
Blood
OSIC
F
CD8αα+
**
% of CD3+ CD56- lymphocytes
Blood
OSIC

mysristate 13-acetate (PMA) (Sigma-Aldrich) and ionomycin (Sigma-Aldrich). Briefly, cells were incubated in 200 μl containing PMA (250 ng/ml), ionomycin (250 ng/ml) and Brefeldin A (Sigma-Aldrich) 2 ug/ml for 3 h at 37°C, 5% CO_2.

A Live/Dead fixable yellow dye (Invitrogen) was used to discriminate dead cells. Cells were suspended in 100 μl of 1:1,000 Dye/DMSO for 30 min on ice in the dark.

An additional panel was utilized to determine cytokine expression by T cell subsets: mouse anti-human IFNγ (eFluor 450) (Ebioscience), IL-17 (FITC) (Ebioscience), CD4 (PerCP Cy5.5), CD45 (Allophycocyanin), CD3 (AlexaFluor 780) (Ebioscience), CD8β (PE Texas Red) (Beckman Coulter), CD56 (PE Cy7) (Biolegend) and rat anti-human IL-10 (Phycoerythrin) (Biolegend). For cytokine assays, surface marker antibodies in this panel were suspended in Fixation Medium A (Fix & Perm, Invitrogen) under the same conditions as described for "Flow cytometry." Intracellular antibodies were suspended in Permeabilization Medium B (Fix & Perm, Invitrogen) on ice in the dark for 20 min before centrifugation and re-suspension as described.

Results

Defining resident conjunctival leukocyte populations

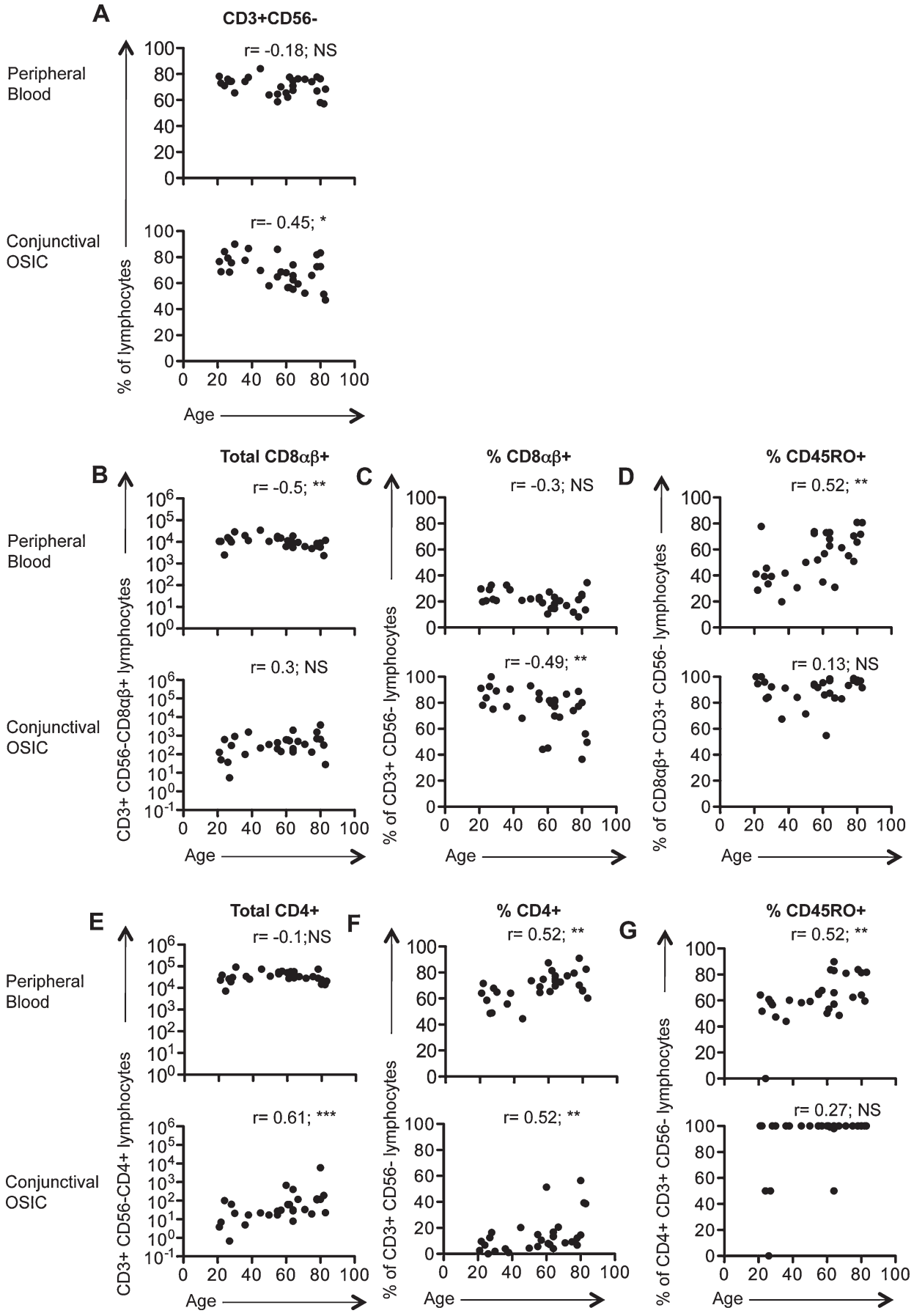
Conjunctival OSIC (Fig. 1a) and matched peripheral blood samples were taken from healthy subjects and recovered cells analyzed by flow cytometry. Whilst the light scatter profile for peripheral blood clearly delineated each leukocyte population (Fig. 1b), it was not possible to make this discrimination from OSIC (Fig. 1e). This was overcome by gating on CD45+ live cells, which permitted demonstration of lymphocytes as the dominant leukocyte population in the conjunctival epithelium (Fig. 1f, g). This approach also clarified the identity of the leukocyte populations found in peripheral blood (Fig. 1c, d).

There were no differences in the number of leukocytes between the right and left eyes (p=0.23; Wilcoxon signed rank test) and right and left eye leukocyte numbers highly correlated (r=0.72; p< 0.0001). Therefore, the mean of the right and left eyes for each subject was calculated (i.e. the right and left eyes per individual subject were considered to be experimental duplicates). CD45+ live leukocytes accounted for a median 834 [range, 6017,635] of total events. Of the cohort of 30 subjects (median age, 61 years [21–83 years]), the dominant conjunctival leukocyte population was lymphocytes (median, 89% [32–99%]) as defined by their forward and side scatter profiles; 9% [0–34] were monocytes and 1% [0–66] were neutrophils. This compared to 52% [18–75], 5% [3–28] and 42% [19–76], respectively in matched peripheral blood.

Fig. 3 Changes in T cell subsets and memory populations in peripheral blood and conjunctiva with age. Changes in the T cell populations in peripheral blood and conjunctival OSIC for CD3+ CD56− lymphocytes (**A**), as well as CD8αβ+ and CD4+ subsets **(B/C, E/F)** with their respective CD45RO frequencies (**D, G**). Statistical analysis was undertaken by Spearman's correlation (*NS* not significant [p>0.05], *P<0.05, **P<0.01) (n=30)

T and NK cell subsets were defined by the expression of CD3 and CD56 (Fig. 2). T cells (CD3+CD56−) dominated in both conjunctival OSIC (69% [47–90]) and in matched peripheral blood (74% [57–84]), and these were >98% TCRγδ-(TCRαβ+) in both (Fig. 2a, b). T cells were further characterized by the expression of the CD4, CD8α and CD8β cell surface co-receptors (Fig. 2a, b). Unlike in the peripheral blood where the dominant T cell population was CD4+ (Fig. 2d; 69% [45–91]), the dominant population from the conjunctival impression was CD8αβ+ (Fig. 2c, 80% [37–100]). The majority of CD4+ and CD8αβ+ T cells were CD45RO+ in the conjunctival epithelium (100% [0–100] and 94% [55–100], respectively), higher than the proportion of antigen experienced populations in blood (61% [0–90] and 56% [20–86], respectively). CD4−CD8αβ− (DN) T cells accounted for only 7.3% [0.7–22] and 3.5% [0.4–26] of conjunctival and peripheral blood T cells, respectively (Fig. 2e). Whilst CD8αα+ cells formed the minority of T cells, these were significantly higher in the conjunctiva than in peripheral blood (Fig. 2f; 2.6% [0–12.5] versus 1.4% [0.1–4.4] (p<0.001)).

NK cells represented 7% [0–20] of conjunctival epithelial lymphocytes compared with 9% [0–22] in the peripheral blood. There was also a greater proportion of NKT (CD3+CD56+) cells (conjunctiva 6% [0–17] versus peripheral blood 2% [0–6] (p> 0.05)) but fewer CD19+CD20+ B cells (3% [1–45] versus 9% [3–23], respectively, (p<0.0001)).


A
CD3+CD56-
Peripheral Blood
r= -0.18; NS
Conjunctival OSIC
r=- 0.45; *
% of lymphocytes
Age
B
Total CD8αβ+
Peripheral Blood
r= -0.5; **
Conjunctival OSIC
r= 0.3; NS
CD3+ CD56-CD8αβ+ lymphocytes
Age
C
% CD8αβ+
r= -0.3; NS
r= -0.49; **
% of CD3+ CD56- lymphocytes
Age
D
% CD45RO+
r= 0.52; **
r= 0.13; NS
% of CD8αβ+ CD3+ CD56- lymphocytes
Age
E
Total CD4+
Peripheral Blood
r= -0.1;NS
Conjunctival OSIC
r= 0.61; ***
CD3+ CD56-CD4+ lymphocytes
Age
F
% CD4+
r= 0.52; **
r= 0.52; **
% of CD3+ CD56- lymphocytes
Age
G
% CD45RO+
r= 0.52; **
r= 0.27; NS
% of CD4+ CD3+ CD56- lymphocytes
Age

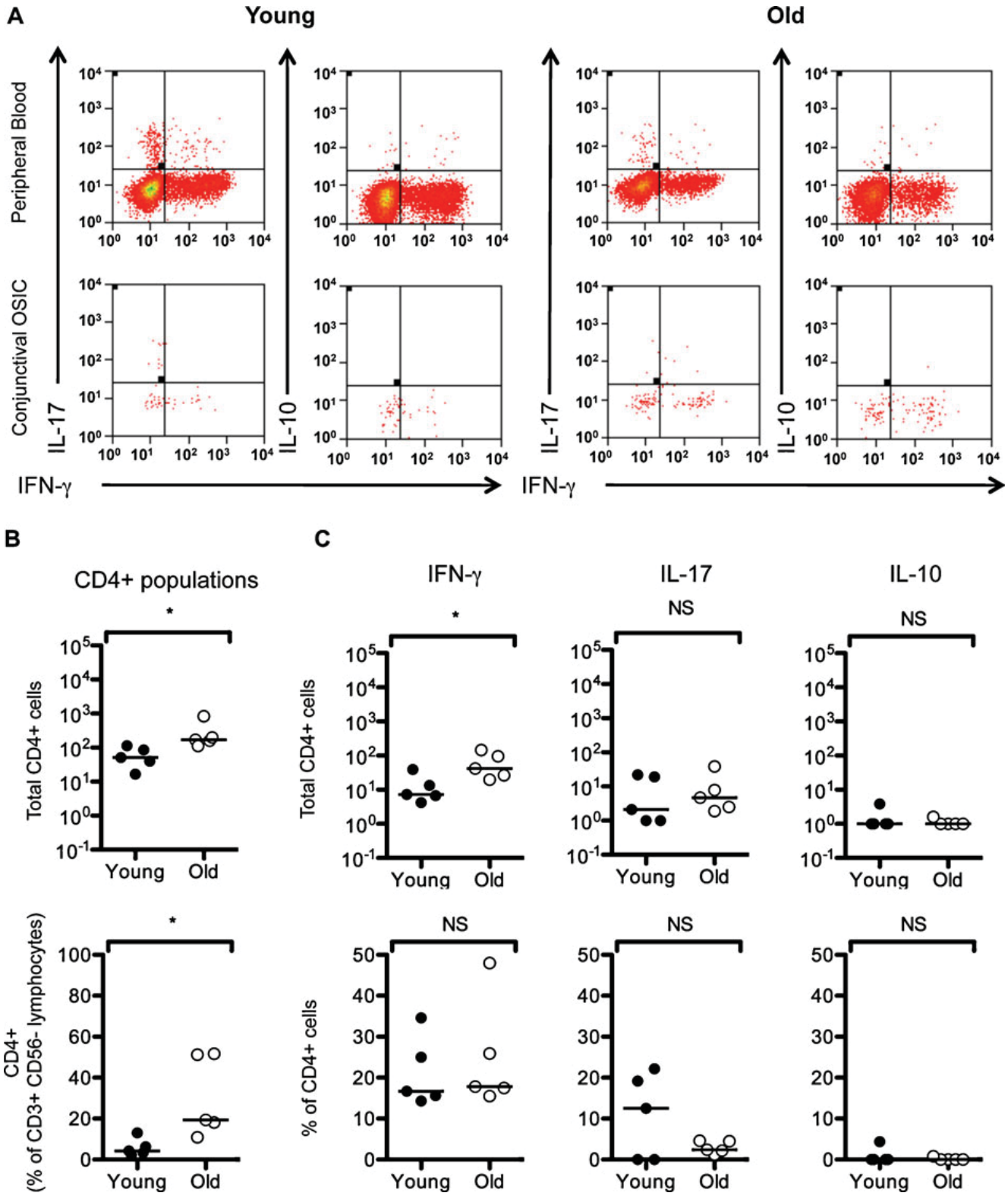


Fig. 4 TCRαβ+CD4+ T cells increase with age but do not alter their cytokine production. Expression of IFNγ, IL-17 and IL-10 in the T cell populations in stimulated peripheral blood (*PB*) and conjunctival OSIC for CD45+CD3+CD56−CD4+ live lymphocytes (**A**). Representative figures from a subjects aged <35 and >65 years are shown. The number and percentage of CD4+ T cells are shown for this cohort (**B**), as well as the number and percentages of cytokine-secreting cells (**C**). Statistical comparisons were undertaken by the Mann–Whitney *U* test (*NS* not significant [$p>0.05$], *$P<0.05$)

Age-related changes in leukocyte populations in the healthy human conjunctival epithelium

Changes in leukocyte populations with age were determined in this cohort (Table 1). Analysis of peripheral blood monocyte, neutrophil and lymphocyte frequencies showed that the only change was a decrease with age in lymphocytes calculated as a percentage of total leukocytes (Table 1). This was not observed in cells recovered from the conjunctival epithelium. By contrast, conjunctival cells showed an increase in the absolute numbers of lymphocytes and monocytes (but not neutrophils), resulting in a significant increase in the total number of leukocytes (Table 1).

Within the conjunctival epithelial lymphocyte population there was an age-related decrease in the proportion of T cells (Fig. 3a; Table 2), compensated for by an increase in the percentage of NK cells (Table 2). The dominant CD8αβ+cell population remained unchanged in the conjunctival epithelium with age but decreased in peripheral blood (Fig. 3b; Table 2). Conversely, the absolute number of CD4+ cells significantly increased in the conjunctiva but remained unchanged in peripheral blood (Fig. 3e; Table 2). This resulted in proportional changes in CD8αβ+and CD4+ lymphocytes (Fig. 3c and f; Table 2) with a consequent increase in the CD4/CD8 ratio with age (Table 2). In addition, the CD45RO+ memory population increased in the peripheral blood with age, a change that was not seen in conjunctival OSIC (Fig. 3d and g; Table 2), reflecting the high proportion of antigen experienced CD8+ (93%) and CD4+ cells (100%) present in the conjunctival epithelium from a young age.

The conjunctival CD4+ T cell populations of the cohort of five additional healthy younger (<35 years) and five older subjects (>65 years) were characterized for the expression of IFNγ, IL-17 and IL-10 following stimulation with PMA/ionomycin (Fig. 4a). The CD4+ population was significantly elevated in the older age group (19% [11–52) vs. those <35 years] (4% [2–13]; p=0.02) (Fig. 4b).

Eighteen percent [14–48] of conjunctival CD4+ T cells were capable of expressing IFNγ, 3.5% [0–22] IL-17 and 0% [0–4] IL-10. The absolute number of CD4+ T cells able to secrete IFNγ was significantly elevated with age (<35 years, 7[4–39] vs. >65 years, 43 [20–145]; p=0.03) while the percentage of IFNγ+ CD4+ remained unchanged (17% [14–35] vs. 18% [16–48], p=NS, respectively) (Fig. 4c). Changes in IL-17 and IL-10 producing CD4+ T cells were not observed with age (Fig. 4c).

Discussion

OSIC offers a non-invasive sampling technique which when combined with multicolor flow cytometric analysis of the recovered cells enables a comprehensive characterization of the conjunctival epithelial leukocytes superficial to the basement membrane zone. In this study, we have utilized this methodology to describe a detailed phenotyping of conjunctival

Table 1 Changes in leukocyte populations in peripheral blood and conjunctiva with age

Cell population	Age correlation		r value (p value[a])	
	Peripheral blood	Conjunctival OSIC	Peripheral blood	Conjunctival OSIC
Leukocytes numbers	No change	Increase	0.05 (NS)	0.43 (0.02)
Lymphocytes numbers	No change	Increase	−0.23 (0.03)	0.38 (0.04)
Lymphocytes (% of leukocytes)	Decrease	No change	−0.4 (0.03)	−0.08 (NS)
Monocyte numbers	No change	Increase	0.01 (NS)	0.45 (0.01)
Monocytes (% of leukocytes)	No change	No change	−0.01 (NS)	0.34 (NS)
Neutrophil numbers	No change	No change	0.20 (NS)	0.33 (NS)
Neutrophils (% of leukocytes)	No change	No change	0.34 (NS)	−0.09 (NS)

NS not significant

p>0.05, NS

[a] Spearman's correlation

Table 2 Changes in lymphocyte populations in peripheral blood and conjunctiva with age

Cell population	Age correlation		r value (p value[a])	
	Peripheral blood	Conjunctival OSIC	Peripheral blood	Conjunctival OSIC
T cells (% of lymphocytes)	No change	Decrease	−0.18 (NS)	−0.45 (0.01)
CD8αβ+ numbers	Decrease	No change	−0.5 (<0.01)	0.3 (NS)
CD8αβ+ (% of T cells)	No change	Decrease	0.31 (NS)	−0.49 (<0.01)
CD45RO+cells (% of CD8αβ+ cells)	Increase	No change	0.53 (<0.01)	0.13 (NS)
CD4+ numbers	No change	Increase	−0.1 (NS)	0.61 (<0.001)
CD4+ (% of T cells)	Increase	Increase	0.52 (<0.01)	0.52 (<0.01)
CD45RO+cells (% of CD4+ cells)	Increase	No change	0.53 (<0.01)	0.27 (NS)
CD4:CD8 ratio	Increase	Increase	0.41 (0.03)	0.49 (<0.01)
B cells (% of lymphocytes)	No change	No change	0.18 (NS)	−0.08 (NS)
NK cells (% lymphocytes)	No change	Increase	0.24 (NS)	0.4 (0.03)
NKT cells (% lymphocytes)	No change	No change	−0.05 (NS)	0.15 (NS)

NS not significant

$p>0.05$, NS

[a] Spearman's correlation

leukocytes in a cohort of healthy individuals and how these change with age. We have identified that the dominant conjunctival epithelial leukocyte population is CD3+CD56−TCRαβ+CD8αβ+ lymphocytes. Interestingly, although this population remained unchanged with age, there was an increase in the conjunctival epithelial CD4+ population resulting in an alteration in the CD4/CD8 ratio. We have demonstrated that 14–48% of conjunctival epithelial CD4+ cells were capable of producing IFNγ and 0–22% were capable of producing IL-17. This was maintained in older subjects and given the increase in absolute numbers of CD4+ cells with age, this resulted in a substantial increase in the number of pro-inflammatory conjunctival CD4+ T cells.

The role of conjunctival IELs is unresolved. In other mucosal tissues, two subtypes of IEL have been defined: type a (TCRαβ+CD8αβ+) 'conventional' and type b (TCRαβ+ CD8αα+, TCRγδ+ CD8αα+ and TCRγδ+ double negative (DN)) IELs, with differing roles in effector function and regulation (Hayday et al. 2001). Type b IELs are thought to represent an interface between the innate and adaptive immune response and are also implicated in the repair of damaged mucosa. Function is dependent on additional activation, and in their resting state a number of anti-proliferative genes are expressed e.g. Btg1 and 2. This has given rise to the concept of being 'activated but resting' (Hayday et al. 2001). Our study suggests that the dominant IEL population in the human conjunctiva is 'conventional' (type a) TCRαβ+CD8αβ+ with less than 1% TCRγδ+. Whether TCRγδ+ cells have a relatively minor role to play in conjunctival epithelial biology, or are confined to below the basement membrane zone within the substantia propria (which we were unable to sample using OSIC) remains unknown.

Tissue-based immunohistochemical analyses identified CD45RO+cells (75–100%) in the bulbar conjunctiva (Hingorani et al. 1997), but those studies were limited as precise T cell subsets were not quantified. Our data showed that antigen experience (defined by the expression of CD45RO) was evident in all CD4+ and almost al (median 94%) of CD8αβ+ conjunctival epithelial T cells, whereas a significant increase in the CD8αβ+ and CD4+ CD45RO+T cell population was observed in peripheral blood, in keeping with the findings of others (Saule et al. 2006; Utsuyama et al. 2009). The predominance of CD45RO+lymphocytes in the conjunctival epithelium is expected and consistent with the preferential recruitment of memory cells into mucosal tissues.

The major alteration in IELs was an increase in the number and percentage of CD4+ cells with age. This resulted in a reduction in the percentage of the dominant conjunctival CD8αβ+ population and an

increase in the CD4/CD8 ratio. By contrast, there was a decrease in the number of CD8αβ+T cells in blood, although this too resulted in an increase in the CD4/CD8 ratio, as previously reported by Utsuyama et al (Utsuyama et al. 2009).

As the proportion of T cells decreased in the conjunctival epithelium with increasing age, both the proportion and number of NK cells, increased. Although this observation has been previously described in peripheral blood (Borrego et al. 1999) (and there was an observed trend in our cohort) this is the first time those changes have been defined in the conjunctiva. Whether this represents an accumulation of NK cells in the ocular surface reflecting immune senescence, or a direct response to a specific change in ocular surface antigen exposure, remains unknown.

It is clear that the changes in IEL populations seen in our cohort, in particular the increased TCRαβ+ CD4+ T cell population, have implications for age matching when undertaking comparisons with disease populations, specifically in relation to infective or immune-mediated processes affecting the ocular surface. Dry-eye problems increase with age (Draper et al. 1999) including dry eye disease (McCarty et al. 1998; Moss et al. 2000). Although changes to the lacrimal acinar gland have been attributed to age-related ocular surface dryness (Draper et al. 1999), dry eye syndromes (including Sjögren's syndrome and non-Sjögren's syndrome-related dry eye) are thought to have an underlying inflammatory and autoimmune component.

Intriguingly, elevations of CD4+ T cells in both humans and in animal models of dry eye have been identified (De Paiva et al. 2010; Stern et al. 2010), but the contribution of elevated conjunctival intraepithelial CD4+ cells to a pro-inflammatory state is not known and may offer clues to dry eye vulnerability amongst older subjects. The absolute number of CD4+ T cells able to secrete interferon-γ was significantly elevated with age and IL-17 producers were maintained with age in this study. An elevation of IFNγ and IL-17 producing cells has been identified in the conjunctiva in murine models of dry eye (De Paiva et al. 2009). Furthermore, an increase in these cytokines is seen in tears of human subjects with dry eye disease (De Paiva et al. 2009). Whether age-associated accumulation of CD4+ cells predisposes to dry eye problems by an increased number of IFNγ and IL-17 secreting cells or whether an alteration in function occurs under dry eye conditions in humans, remains to be defined.

In murine models, there appears to be a defective suppressor function by T regulatory cells on Th17 cells (Chauhan et al. 2009). Few conjunctival CD4+ T cells were capable of producing IL-10 in this study, and no changes were observed with age. The expression of the transcription factor FoxP3 was seen in approximately 2% of CD3+ conjunctival T cells (data not shown). This suggests that in the healthy conjunctival epithelium, there is not a significant population of CD4+ T cells with an IL-10+ or FoxP3+ regulatory phenotype. An increase in the stromal CD4 + population has also been identified from histological conjunctival sections (Bernauer et al. 1993) taken from patients with Mucous Membrane Pemphigoid, a disease characterized histologically by antibody deposition in the basement membrane zone with subsequent conjunctival inflammation, scarring, corneal limbal epithelial stem cell failure and ocular surface keratinization (Foster 1986; Chan et al. 2002; Liesegang 2008), that typically affects older patient populations (although disease activity and progression is worse in younger patients) (Rauz et al. 2005).

The possibility of utilizing a non-invasive sampling technique such as OSIC to characterize changes in supra-basement membrane structures of the ocular mucosa in the context of infectious and non-infectious disease affords an attractive method for both cross-sectional and longitudinal research into human ocular surface inflammatory disease. The importance of aging on conjunctival leukocyte profiles in disease states is yet to be elucidated, but has implications in forming comparative healthy control cohorts, indicating that age-matching is essential.

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Chapter 8

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