Cross-talk between human T cells, mast cells and conjunctival epithelial cells

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I, Ifeoma Offiah confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed:

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“People are always blaming their circumstances for what they are. I don't believe in circumstances. The people who get on in this world are the people who get up and look for the circumstances they want, and, if they can't find them, make them”.

~G.B. Shaw, Mrs. Warren's Profession, 1893

Abstract

The ocular surface is continually exposed to the outside environment and is a common site of inflammation. Conjunctival epithelial cells are thought to play a role in innate responses at the ocular surface. The hypothesis of my study is that conjunctival epithelial cells also contribute to T cell and mast cell effector mechanisms in chronic allergic eye disease via secretion of cytokines. In this study we initially demonstrate that the conjunctiva expresses TLRs, and that the TLR3 ligand (poly I:C) activates conjunctival epithelial cells *in vitro* to secrete inflammatory mediators as part of the innate immune response. Conjunctival tissues were also shown to express the Th2 associated cytokine, IL-13 as well as TSLP – a cytokine thought to be involved in Th2 differentiation. Conjunctival tissues from chronic allergic eye disease subjects were found to have increased IL-13 and TSLP expression compared to normal controls. Using a human conjunctival epithelial cell line, cells could be induced to express increased levels of TSLP following exposure to poly I:C or pro-inflammatory cytokines. Th17 cells, identified by coexpression of CD4 and IL-17, were also detected in CAED tissues and a high level of expression of IL-17A was localised to the epithelium. However, although capable of secreting IL-25, IL-17A was not secreted by conjunctival epithelial cells, indicating that the IL-17 observed histologically may have been IL-17 binding to the surface of the epithelium. IL-17 receptor C (IL-17RC) expression was found to be increased in CAED tissues whilst IL-17RA was upregulated when conjunctival epithelial cells were stimulated with pro-inflammatory cytokines together with poly I:C. Blockade of IL-17RA and subsequent stimulation with IL-17 led to increased IL-8 and decreased TGF-β secretion. Although being implicated in the immunopathogenesis

of certain diseases, IL-17 and its other family members may potentially serve to play an immunoregulatory role in immunity at the ocular surface.

Abbreviations

AEC	3-amino-9-ethylcarbazole
AKC	Atopic keratoconjunctivitis
APC	Allophycocyanin
APC	Antigen presenting cells
BSA	Bovine serum albumin
CAED	Chronic allergic eye disease
CBMC	Cord blood mast cell
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	Cluster of differentiation
CFSE	Carboxyfluorescein diacetate succinimidyl ester
ChWK	Wong-Kilbourne derivative of Chang conjunctiva
DAB	3, 3'-diaminobenzidine
DC	Dendritic cell
DNA	Deoxyribonucleic acid

dsRNA	Double-stranded ribonucleic acid
EAE	Experimental allergic encephalitis
EAU	Experimental autoimmune uveitis
FCS	Foetal calf serum
FITC	Fluoroscein isothiocyanate
FSC	Forward scatter
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPC	Giant papillary conjunctivitis
HRP	Horseradish peroxidase
HLA	Human leukocyte antigen
ICAM	Intracellular adhesion molecule
iDC	Immature dendritic cell
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNKT	Invariant natural killer T cells

LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
MALT	Mucosa-associated lymphoid tissue
MAP	Mitogen-activated protein
MCP	Monocyte chemotactic protein
MFI	Mean fluorescent intensity
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
mRNA	messenger ribonucleic acid
Muc	Mucin
NK	Natural killer cells
NKT	Natural killer T cells
OCP	Ocular ciccatricial phemphigoid
PAMPs	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells

PBS	Phosphate buffered saline
PE	Phycoerythrin
PHA	Phytohemagglutinin
PI3K	Phosphatidylinositol 3 kinase
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear leukocytes
PRRs	Pattern recognition receptors
RA	Rheumatoid arthritis
ROR	Related orphan receptor
SAC	Seasonal allergic conjunctivitis
SCF	Stem cell factor
SD	Standard deviation
SEM	Standard error of the mean
SSC	Side scatter
STAT	Signal transducer and activator of transcription
TCR	T cell receptor

TGF	Transforming growth factor
Th	T helper cell
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Tregs	Regulatory T cells
TSLP	Thymic stromal lymphopoietin
VKC	Vernal keratoconjunctivitis

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1.1 The mucosal immune system

The mucous membranes which in addition to the conjunctiva cover other areas such as the respiratory, digestive and urogenital tract, have a highly specialised immune system which becomes activated in response to direct destruction or invasion of pathogens through the epithelial surface. The cells that form part of this local immune system are accumulated in or are in transit between mucosal organs and glands that form the mucosa-associated lymphoid tissues (MALT), a secondary lymphoid organ. The main functions served by the MALT are to protect the mucous membranes from colonisation and invasion by dangerous microbes, to prevent the uptake of non-degraded antigens and prevent the development of harmful immune responses to these antigens if they make their way through (Lugton, 1999). Within the MALT there are a number of cells that actively participate towards the immune response, some of which play a regulatory role. These cells include B lymphocytes, T helper (Th) and regulatory T cells (Tregs). There are also a number of accessory cells such as immature dendritic cells (iDCs) and macrophages involved in phagocytosis and presentation of antigen to T lymphocytes.

1.2 Innate immunity

The innate immune response has evolved as a first line of defence; its actions are immediate but non-specific and rapidly eliminate or diminish invading pathogens. Many different components make up the innate immune system; these can take the form of physical barriers to pathogen invasion such as the skin and mucous

membranes and also cellular and serum factors activated by secreted or cell surface products of the pathogen. These factors also induce an inflammatory response that leads to secretion of inflammatory cytokines and chemokines, which in turn leads to the recruitment of other cells regarded as part of the innate immunity.

1.2.1 Polymorphonuclear leukocytes

Some of the cells forming the innate immune system are termed polymorphonuclear leukocytes (PMNs) or granulocytes because of the granules stored within. There are three types, the most abundant being neutrophils which are characterised by their lobed nuclei and make up about 90% of circulating granulocytes and 65-75% of white blood cells in peripheral circulation. These are phagocytic cells that specialise in ingesting, killing and degrading phagocytosed microbes, and are the predominant cells in acute inflammatory infiltrates; once an inflammatory response is initiated, neutrophils are the first cells to be recruited to sites of infection or injury. The targets of this cell include bacteria, fungi, protozoa, viruses, virally infected cells and tumour cells (Smith, 1994). The other two forms of circulating granulocytes are eosinophils which account for 2-5% of white cells in the blood and basophils which make up less than 1%. Tissue mast cells are also included here as they are a close relative of basophils. These cells are important in the host defence to multicellular pathogens, in particular to helminths. Unlike neutrophils that phagocytose, the defensive function of these cells is due to their ability to discharge potent biological mediators into the cellular microenvironment, a process known as degranulation. Degranulation can be triggered by antigen-specific IgE molecules bound to basophils

and mast cells which express high affinity receptors for the Fc portion of IgE (FcεRI). Other cells considered part of the innate immune system are monocytes/macrophages, natural killer cells (NK cells) and dendritic cells (DCs), which can also be phagocytic but, unlike PMNs, they do not contain granules. Instead, they contain lysosomes within the cytoplasm that can secrete pre-formed proteins such as perforin and a family of serine proteases called granzymes (Chertov et al, 2000).

1.2.2 Mast cells

Mast cells are mainly found in the mucosal and connective tissues of the skin, lung and gut and, as previously described, have cytoplasmic granules containing heparin, histamine and proteases, which upon activation can be released into the tissues (Klaus *et al*, 1996). Although mast cells have a key role in immediate type I hypersensitivity responses, the early phase, it is also thought that mast cells take part in the late phase of an immune response or as part of the memory response. The binding of IgE to mast cell Fc receptors leads to their activation, but they can also be activated directly by pathogens or even microbial products via toll-like receptors (TLRs) or cytokines (Galli *et al*, 2005). Following activation, these cells do not only release preformed mediators, they can also secrete a variety of cytokines, chemokines and growth factors including interleukin-10 (IL-10) and TGF-β, which not only orchestrate the inflammatory response but underline a potential suppressive action of these cells (Galli *et al*, 2005). Mast cells are key cells in providing host resistance to parasites, as demonstrated in mice deficient in IgE, but they have also

been associated with the development of airway hyperresponsiveness in a mouse model of asthma (King *et al*, 1997; Kobayashi *et al*, 2000).

1.2.3 Epithelial cells

The cellular components of the innate immune system described earlier, are not the only innate cells capable of providing host defence to pathogens. Epithelial cells line the areas of the body such as the respiratory tract, intestines, skin and the ocular surface. These cells provide an interface between the internal and external environment and are the first point of contact to microorganisms, airborne allergens and environmental pollutants (Diamond *et al*, 2000).

The role of the epithelium in innate immunity is of great importance, considering that failure in their mechanism may result in microbial colonisation and subsequent infection. The mechanisms by which epithelial cells recognise pathogens is through pattern recognition receptors / molecules present in secretions such as mannan-binding lectin, or as transmembrane molecules like TLRs (see section 1.2.4.1) (Bals & Hiemstra, 2004). Upon recognition of a pathogen, epithelial cells respond by releasing antimicrobial peptides as well as cytokines and chemokines (refer to section 1.2.4). Secretion of these mediators allows the epithelium to chemoattract and activate innate and adaptive immune cells, immobilise and kill microorganisms and orchestrate the initiation of an adaptive immune response. In response to injury, epithelial cells can also induce wound healing and angiogenesis (Allan *et al*, 2008). In regards to the antimicrobial peptides, many groups have investigated the

expression and production of defensins and cathelicidin (LL-37) in the mucosa. In host defence, both types of peptides have two major functions; direct inhibition of pathogens and modulation of other innate and adaptive immune functions (Doss *et al*, 2010).

Within the epithelium, enterocytes and goblet cells produce mucin glycoproteins providing a barrier and thereby limiting the access of the microbiota to the apical side of the lumen (Rescigno, 2011). It has recently been shown that an increase in the production of the mucin Muc2, in mice infected with *Trichuris muris*, correlated with worm expulsion. However, mice deficient in Muc2 experienced delayed worm expulsion 20 days after infection, indicating the importance of mucins in innate defence and enteric infection (Hasnain *et al*, 2010). Such observations have further added to the opinion that epithelial cells not only provide a barrier function, but actively participate in the innate immune response.

1.2.4 Pattern recognition receptors

Although the innate immune system is regarded as relatively nonspecific, it still has the ability to discriminate between what is self and what is foreign, for example with defence to viruses. To carry out this role, the innate immune system relies on pattern recognition receptors (PRRs). These receptors can be secreted or expressed on cell surfaces and in intracellular compartments, in which their principal functions include opsonization, activation of complement and coagulation cascades, phagocytosis, activation of pro-inflammatory signalling pathways, and induction of apoptosis

(Janeway & Medzhitov, 2002). These receptors can be activated by interaction with specific molecular structural motifs on pathogens, called pathogen-associated molecular patterns [PAMPs] (Vroling *et al*, 2008). The best studied group of PRRs are the TLRs, so-called as they are the mammalian homolog of the Drosophila Toll receptor, responsible for this organism's activation of host-defence mechanisms in response to infection (Lemaitre *et al*, 1996).

1.2.4.1 TLRs

To date, 10 functional TLRs have been identified in humans and their expression has been found to have a very broad distribution. Innate immune cells such as PMNs, mast cells, NK cells and DCs express most classes of TLRs. This expression also extends to T and B cells as well as endothelial cells, epithelial cells, skin keratinocytes and fibroblasts (Table 1-1; Chang, 2010). The array of ligands that bind TLRs are diverse, and includes components of bacterial cell wall, bacterial genome DNA, viral, fungal and parasitic products and, as such, these receptors fall into two groups. The first group recognise PAMPs on extracellular pathogens; these TLRs are expressed on the cell surface and include TLR1, TLR2, TLR4, TLR5 and TLR6. Conversely, the second group of TLRs are found on the surfaces of endosomes (TLR3, TLR7, TLR8 and TLR9; Figure 1-1), in which they bind the nuclear acid of PAMPs from viruses and bacteria (Ishii & Akira, 2008).

1.2.4.2 TLR signalling

All TLRs belong to the type I transmembrane glycoprotein receptor family which are characterised by extracellular domains that contain varying numbers of leucine-rich repeat (LRR) motifs. Additionally, these receptors have a signal transmembrane α-helix portion and a conserved intracellular domain, homologous to the human interleukin-1 receptor (IL-1R) and IL-18R which is called the Toll/IL-1R (TIR) homology domain (Beutler *et al*, 2006). Upon recognition of their ligands, TLRs induce the expression of various host defence genes. As well as initiating the secretion of inflammatory cytokines and chemokines, TLR signalling can induce production of antimicrobial peptides, expression of costimulatory molecules and MHC molecules that are necessary in the host defence against invading pathogens.

Engagement of ligands to their respective TLR leads to the recruitment of various TIR domain containing adaptors, namely MyD88, TIRAP, TRIF and TRAM. Of the TLRs, TLR1-9, excluding that of TLR3 recruits MyD88 (the MyD88 dependent pathway). In some instances, namely involving TLR1, TLR2, TLR4 and TLR6, TIRAP is also recruited which serves as a link between the TIR domain and MyD88. In contrast to the majority of TLRs, TLR3 initiates the TRIF dependent pathway. TLR4 can also utilise this pathway via the TIR-containing adaptor TRAM (Figure 1-1). The activation of NFκB may occur by means of two pathways involving the N-terminal domain or C-terminal domain of TRIF. In each of these cases, the recruitment of these adaptors triggers a cascade in the signalling pathway, leading to the activation of the transcription factor NFκB as well as MAP kinases and

subsequent induction in the transcription of inflammatory cytokines, type I interferons and chemokines (Kumar *et al*, 2009).

1.2.4.3 TLRs and the immune response

TLRs play a crucial role in the initiation of innate and adaptive immune responses; as such it does not come as a surprise that they may also be involved in the pathogenesis of a number of chronic inflammatory diseases. To begin with, these receptors have demonstrated a protective role in the intestines in response to commensal bacteria as the ligation of their products to TLRs has been shown to result in the production of protective factors such as TGF-β and defensins (Ospelt & Gay, 2010). Inflammatory or autoimmune diseases could be the result of an inappropriate response which may be due to a particular genetic background or the quantity or quality of ligands. In type 1 diabetes mellitus, polymorphisms in TLR2 and TLR3 have been associated with the development of this disease (Park *et al*, 2004; Pirie *et al*, 2005). Furthermore in studies using pancreatic islet β cells isolated from rats and mice, activation of TLR3 on these cells by dsRNA resulted in their apoptosis, which is significant owing to the fact that the destruction of β cells is characteristic of type 1 diabetes (Dogusan *et al*, 2008). In other diseases such as rheumatoid arthritis (RA), the synovium of these patients have been assessed for their expression of TLRs where it was found that TLR2, TLR3, TLR4 and TLR7 are upregulated (Radstake *et al*, 2004; Roelofs *et al*, 2005; Pierer *et al*, 2004). Activation of TLRs can upregulate inflammatory mediators as shown by the increased

production of TNF-α and IL-6 following stimulation of RA derived DCs with ligands for TLR2 and TLR4 (Roelofs *et al*, 2005).

Table 1-1 The expression of TLR1-10 on a variety of cell types and the ligands which bind them (Chang, 2010)

TLRs	Major cell types	Recognised ligands
TLR1	Myeloid cells, T and B cells, NK cells, endothelial cells, epithelial cells, keratinocytes	Forms heterodimers with TLR2 for bacterial tri-acyl lipopeptide, OSP of *Borrelia* spp., and other ligands
TLR2	Myeloid cells, T cells, B cells, endothelial cells, epithelial cells, keratinocytes	Peptidoglycan from Gram-positive bacteria, *Mycoplasma* lipopeptide, LPS of leptospirosis and other spirochetes
TLR3	Myeloid cells, T cells, NK cells, endothelial cells, epithelial cells, keratinocytes, neurons	Single-stranded viral RNA (ssRNA) and double-strand-RNA (dsRNA)
TLR4	Myeloid cells, NK cells, mast cells, T cells, endothelial cells, epithelial cells, keratinocytes	BCG, LPS, lipoteichoic acid, respiratory syncytial virus, fibronectin, heparin sulfate, fusion protein
TLR5	Myeloid cells, T cells, NK cells, endothelial cells, epithelial cells, keratinocytes	Flagellin from Gram-positive or Gram-negative bacteria
TLR6	Myeloid cells, T cells, B cells, endothelial cells, epithelial cells, keratinocytes	Forms heterodimers with TLR2 for *Mycoplasma* di-acyl-lipopeptides, a peptidoglycan from Gram-positive bacteria and Zymosan from fungal cell wall
TLR7	Myeloid cells, NK cells, endothelial cell, T cells, B cells	Single-strand RNA (ssRNA) compounds, such as the imidazoquinolines
TLR8	Myeloid cells, NK cells, endothelial cell	Single-stranded RNA (ssRNA)
TLR9	Myeloid cells, T and B cells, NK cells, endothelial cells, epithelial cells, keratinocytes	Unmethylated CpG motifs found in microbial DNA
TLR10	Myeloid cells, T and B cells, endothelial cells, epithelial cells	Unknown, may interact with TLR2

Figure 1-1 Expression of TLRs and their signalling pathways. TLRs use the same adaptor as the IL-1R, TIR. TLR1/6, 4 and 5 are located on the surface of cells, whilst TLR3, 7, 8 and 9 are found in endosomes. With the exception of TLR3, all TLRs signal through a MyD88 pathway. TLR3 utilises the TRIF pathway, which can also be used by TLR4 (Taken from Casanova *et al*, 2011)

1.2.5 Chemokines and cytokines in inflammation

As part of the immune response, granulocytes as well as other cells are able to secrete cytokines and chemokines to aid in host defence, in addition to their other factors. Chemokines are a number of small (8-10kDa), inducible, pro-inflammatory proteins and can be divided between an α-chemokine subfamily and a β-chemokine subfamily. In humans, members of the α-chemokine subfamily include interleukin-8 (IL-8) and interferon-gamma-inducible protein-10 (IP-10; CXCL10), whereas eotaxin, monocyte chemotactic protein-1 (MCP-1; CCL2), MCP-2 (CCL8), MCP-3 (CCL7), MCP-4 (CCL13), macrophage inflammatory protein-1α (MIP-1α; CCL3), MIP-1β (CCL4) and regulated upon activation, normal T cell expressed and secreted (RANTES; CCL5) make up the β-chemokine subfamily (Ben-Baruch *et al*, 1995; Baggiolini *et al*, 1997). In inflammation, IL-8 has been identified as playing an important role in neutrophil recruitment and activation. Studies have shown that anti-IL-8 antibody treatment, in a model of LPS-induced inflammatory disease in rabbits, prevented neutrophil-dependent tissue damage as well as neutrophil infiltration (Harada *et al*, 1994). MCPs have been shown to attract monocytes, basophils and T lymphocytes, but not neutrophils. These chemokines are also closely related to eotaxin and MCP-2, 3 and 4 have been found to act on eosinophils (Baggiolini *et al*, 1997). It has also been observed that MCP-1 in addition to RANTES promotes mast cell activation (Conti & DiGioacchino, 2001).

Cytokines play a critical role in mediating and regulating immune effector function. They have multiple biologic activities and play a major role in host defence and in

the pathogenesis of various diseases. Some cytokines are pro-inflammatory and can act by worsening disease, whereas anti-inflammatory cytokines serve to reduce inflammation and promote healing (Dinarello, 2000). Cytokines such as IL-4, IL-5 and IL-13 are secreted by Th2 cells (discussed in section 1.3.2) These cells are potent activators of B lymphocytes; they also act as anti-inflammatory cytokines, IL-4 antagonises IFN-γ and the genes for pro-inflammatory cytokines such as IL-1, IL-6, IL-8, tumour necrosis factor-α (TNF-α) and MIP-1α can also be suppressed (Opal *et al*, 2000). Whilst some cytokines have proven to be anti-inflammatory, the most potent of these is IL-10 which inhibits the Th1 (see section 1.3.2) cytokines IL-2 and IFN-γ, although Th1 and Th2 cell clones have been reported to produce IL-10 (Opal *et al*, 1998; Del Prete *et al*, 1993). Another cytokine, TGF-β, also has both pro- and anti-inflammatory effects. It is an important regulator of cell proliferation, differentiation and formulation of the extracellular matrix. Although it suppresses the differentiation of T cells and B cells and limits the production of IL-2, IFN-γ and TNF, it is less potent than IL-10 and has little or no effect on IL-1 production (Letterio & Roberts, 1997). It is the balance between these pro-inflammatory and anti-inflammatory cytokines that is thought to determine the outcome of disease.

1.3 Adaptive immunity

The primary players involved in the adaptive immune system are antibodies, T lymphocytes, B lymphocytes and antigen presenting cells (APCs). The effector mechanisms involved are slower than those of the innate system and are based upon the amplification of soluble products acting systemically (humoral immunity), the requirement of direct cell-to-cell contact or the activity of cytokines and chemokines acting in the cellular microenvironment (cell-mediated immunity).

The adaptive immune system mounts a specific response through direct activation by antigens of lymphocytes bearing specific antigen receptors. There are four types of lymphocytes based on phenotypic and functional differences; B cells, T cells, NKT cells (discussed in more detail in section 1.4.8) and NK cells. Although the latter resemble lymphocytes in their morphology, they lack specific antigen receptors but are capable of killing virus-infected and tumour cells in a non-specific manner and therefore play a role in the innate immune system.

1.3.1 T cell differentiation

All lymphocytes differentiate from common lymphoid stem cells in the bone marrow along distinct pathways. For T cell development, precursor T cells must migrate to the thymus where they are phenotypically distinguished by the expression of CD3 and CD4 or CD8 co-receptors. In the thymus they also undergo further maturation and selection for expression of antigen receptors (Staal *et al*, 2001). B cells, on the other hand, continue their differentiation into antibody producing cells in the bone marrow.

1.3.1.1 T cell receptors

T cell receptors (TCRs) belong to the immunoglobulin superfamily and are composed of α and β or γ and δ chains. The potentially large numbers of antigen that organisms may encounter in their life span requires receptors with antigenic specificity. The αβ and γδ TCR pairs are encoded by genes formed by rearrangement of variable (V), diversity (D) and junctional (J) gene elements. The expression of CD4 or CD8 co-receptors is not common to all T cells. While the αβ T cells are $CD4^+$ or $CD8^+$, γδ T cells are negative for both of these (Ferrero *et al*, 2007).

1.3.1.2 Antigen presentation and T cell recognition

In order for T cells to fully mature they must undergo positive selection, whereby the TCR expressed on their surface must successfully and appropriately bind to self major histcompatibility complex (MHC) antigens (Petrie *et al*, 1995). These naïve T cells are programmed to re-circulate through secondary lymphoid tissues such as lymph nodes until they die or encounter their specific antigen (Butcher & Picker, 1996). These tissues collect antigen from epithelial surfaces, somatic tissues and blood and present it to these naïve cells in the context of an APC, leading to the formation of memory/effector cells. The main cell type responsible for antigen presentation is the DC, where in the absence of ongoing immune responses patrol through the blood, peripheral tissues, lymph and secondary lymphoid organs. Danger signals, such as the triggering of TLRs by pathogens, results in the maturation of DCs, transforming them into efficient APCs and T cell activators. In peripheral tissues, DCs take up self and non-self antigens which become internalised and

processed into proteolytic peptides. These peptides are loaded onto MHC class I and class II molecules and presented to T cells in secondary lymphoid organs (Guermonprez *et al*, 2002).

Peptide recognition and the events which follow are carried out in a localised environment between the TCR and APC called the immunological synapse, requiring the activities of TCR – associated molecules, including co-receptors CD3 and CD8 or CD4, and other costimulatory receptors (Rudolph et al, 2006). The introduction of antigenic peptide is essential for the differentiation of naïve T cells into effector T cell subsets. Although already activated these T cell subsets require restimulation in order to perform their function, whether that is via cytokines or cytotoxicity.

1.3.2 Th1 and Th2 cells

While in the thymus, T cells undergo differentiation into two distinct types of αβ T cells that exhibit different effector pathways, $CD8^+$ cytotoxic T cells and $CD4^+$ T helper (Th) cells. Using the study of mouse $CD4^+$ Th cell clones it was discovered that these cells could be further subdivided into two subsets; Th1 and Th2 based on their cytokine production (Mosmann *et al*, 1986). It has now been demonstrated that these two types of cells regulate different immune responses. In studies using mice it has been shown that Th1 cells produce large amounts of the pro-inflammatory cytokine IFN-γ and are thought to mediate cellular immunity while Th2 cells instruct humoral immune responses by producing IL-4, IL-5, IL-10 and IL-13 that regulate B cell proliferation and antibody class switching (Dong & Flavell, 2001). There are

also several molecules important during cell migration found on these cells that may also distinguish them such as CC chemokine receptor (CCR)5 and CXC chemokine receptor (CXCR)3 on Th1 and CCR3 and CCR4 on Th2 cells (Mackay, 2000).

In addition to the Th1 and Th2 subtypes, early mouse studies demonstrated another group of cells which have the capacity to produce all these cytokines and has been described as Th0 (Firestein *et al*, 1989), representing an intermediary in the differentiation of naïve T cells into Th1 or Th2. Studies investigating the factors influencing the differentiation pathway have revealed that in the presence of IL-4, the development of human T cells producing IL-4 and IL-5 (Th2) is favoured whereas the addition of IFN-γ and anti-IL-4 antibody in culture supported the differentiation of T cells able to produce predominantly IFN-γ (Th1) via IL-2 blockade (Maggi *et al*, 1992; Ghoreschi *et al*, 2011).

1.3.3 The role of Th1 and Th2 cells in inflammatory disorders

Researchers have applied the defined T helper subsets in order to characterise the mechanisms of certain disease processes. These effector T cells are key players in driving immune responses. Th1 cells are involved in the protection of the host from obligate intracellular pathogens, whilst Th2 cells are important in resistance to extracellular forms of pathogens. Dysregulation of T helper cell responses can lead to tissue damage and elicit unwanted chronic immune disorders such as allergy and autoimmunity. The Th2 cells are well known to be involved in allergic disease, examples of this are asthma and allergic rhinitis where peripheral blood mononuclear

cells (PBMCs) from atopic patients (individuals sensitized to environmental allergens) showed high levels of the Th2 cytokine IL-4 which was again correlated in their mRNA expression (Esnault *et al*, 1996). In asthma where there is chronic inflammation in the lungs, excess mucus secretion contributes to airway obstruction. The receptors IL-4R and IL-13R share a common receptor chain, IL-4Rα, it has been shown that even in the absence of IL-4 there is still production of mucus. Another cytokine, IL-13, may be the important Th2 cytokine involved in this, in addition to the requirement of IL-4Rα (Cohn *et al*, 1999).

In other chronic inflammatory diseases we see a different cytokine profile, that of the Th1 cells. These cells have been linked to conditions such as the inflammatory skin disease *Psoriasis vulgaris* where lesional and peripheral blood T cells have dominating Th1 cytokines (IFN-γ as well as high levels of TNF-α and IL-2) with very low levels of cells expressing IL-4 (Austin *et al*, 1999). The balance between Th1 and Th2 cells in disease has been hotly debated and now these views have been challenged by the emergence of a new subset of T helper cells and they may also play a role in the environment of an inflammatory response (Steinman, 2007).

1.4 Th17 effector cells

As previously described, much attention has been placed on the role of Th1 and Th2 cells in protecting against infection and the imbalance between the two subsets that accounts for different immune pathologies. Certain disease processes could not be explained as being mediated by Th1 or Th2 cells, especially when determining T-cell mediated tissue damage (Steinman, 2007). The question remained what other cells or cytokines could be contributing to this process? More recently there has been the emergence of another $CD4^+$ effector T cell subset termed Th17, so called for its ability to secrete IL-17 and other pro-inflammatory cytokines. This discovery evolved from studies focussing on two structurally related but functionally distinct cytokines, IL-23 and IL-12. IL-23 has been reported to be essential for the expansion of IL-17 producing T cells (Aggarwal *et* al, 2003; Langrish *et al*, 2005), while it is accepted that IL-12 is responsible for the differentiation of naïve $CD4^+$ T cells into IFN-γ producing cells (Th1) (Wu *et al*, 1993). These studies have now indicated that IL-23 is involved in playing a role in the pathogenesis of certain diseases such as experimental allergic encephalitis (EAE) (Cua *et al*, 2003). This same group later went on to show that IL-23 drives an IL-17 producing $CD4^+$ T cell population, that when transferred into recipient WT mice showed severe clinical signs of EAE compared to IL-12-driven Th1 cells (Langrish *et al*, 2005). With the identity of the IL-17 family of cytokines (IL-17A - IL-17F) it soon became evident that Th17 cells and its associated cytokines are thought to have an important function in inflammatory disease.

1.4.1 Th17 cell differentiation

Many of the reports investigating Th17 cells and IL-17 have utilised mouse models. It is now clear that the Th17 cell phenotype, origin and the role it plays in disease differs from that of humans. In mice it has been shown that, following the actions of transforming growth factor (TGF-β) and IL-6, naïve $CD4^+$ T cells differentiate into Th17 cells with IL-23 acting to stabilise the phenotype (Bettelli *et al*, 2006; Mangan *et al*, 2006; Veldhoen *et al*, 2006). Recently conflicting data have revealed that in humans differentiation into the Th17 cell subset requires IL-1β, IL-6 (Acosta-Rodriguez *et al*, 2007), IL-21 or IL-23 and TGF-β (Manel *et al*, 2008; Volpe *et al*, 2008). In their findings that IL-1β and IL-6, in the absence of TGF-β, is sufficient for Th17 differentiation, Acosta-Rodriguez et al also reported that the addition of TGF-β has an inhibitory affect on the number of IL-17 producing cells. There is also the suggestion that not only can Th17 differentiation occur without TGF-β (IL-1β plus IL-23 being effective) but this cytokine supports the development of Th17 cells by inhibiting IFN-γ producing cells (Th1) (Santarlasci *et al*, 2009).

Human Th17 cells have also been characterised by Acosta-Rodriguez and colleagues as cells that express the chemokine receptors, CCR6 and CCR4 and produce IL-17. These $CCR6^+$ $CCR4^+$ Th17 cells also expressed mRNA to the human ortholog of mouse RORγt (RORC variant 2), which is required for Th17 cell differentiation (Acosta-Rodriguez *et al*, 2007). CCR6 has been shown to be involved in the recruitment of dendritic cell populations as well as Th17 cells to inflamed sites via the ligand CCL20 (Dieu-Nosjean *et al*, 2000; Hirota *et al*, 2007).

1.4.2 Th17 cells in immunopathology

The pathogenic role of Th17 cells in humans has been widely studied to date. It has now been implicated in certain diseases although only established in a condition known as Hyper-immunoglobulin E syndrome (HIES) (Annunziato *et al*, 2008). HIES or Job's syndrome is a primary immune deficiency characterised by an abnormal susceptibility to infections (Ma *et al*, 2008). This study found that STAT3 mutations from $CD4^+$ T cells of HIES patients had a defect in Th17 differentiation and therefore are seen as the causative agent in HIES patients' inability to defend against certain pathogens (Ma *et al*, 2008). This further underscores the belief that Th17 cells are thought to play a role in host protection against extracellular pathogens.

The role of Th17 cells in human chronic inflammatory disease is not so definitively established. It has been demonstrated that IL-17 is present in biopsies from lesional psoriatic skin which for many years was considered a Th1 disease with IFN-γ playing a key role (Austin *et al*, 1999; van Beelen *et al*, 2007). As well as psoriasis, it has been shown that there is an infiltration of Th17 cells in tissues of Crohn's disease, rheumatoid arthritis and allergic asthma (Pène *et al*, 2008). This interplay between Th1 and Th17 cells in disease is again echoed in another report where researchers found that in experimental autoimmune uveitis (EAU) disease is inhibited when mice are treated with anti-IL-17 and in the absence of an IFN-γ response, Th17 effector cells still have the capability of inducing EAU. Th17 cells in this instance were thought to play a more dominant role, but when cells from a

uvetitogenic $CD4^+$ Th1 cell line were given to naïve syngeneic mice, EAU was induced (Luger *et al*, 2008). Although these discoveries are very insightful they do not produce a clear picture of the role of Th17 cells in inflammatory disorders. These cells produce several inflammatory cytokines but at the moment we cannot be sure which of these cytokines contribute to the inflammatory process in man and hence disease pathogenesis (Annunziato *et al*, 2008).

1.4.3 The IL-17 family of cytokines

Th17 cells are capable of producing various cytokines such as IL-22, IL-26 and TNF-α, one of their main effector cytokines is IL-17A, and its production in humans was first described in activated human peripheral T cells (Langrish *et al*, 2005; Yao *et al*, 1995). The IL-17 family of cytokines consists of six family members, IL-17A – IL-17F, with IL-17F being the other main cytokine secreted by human Th17 cells (Shen & Gaffen, 2008). Other cell types that are thought to produce IL-17 are NKT cells and γδ T cells (described in section 1.4.8); in mice it has been shown that γδ T cells are thought to be important in providing a protective response to *Mycobacterium tuberculosis* via their production of IL-17A (Lockhart *et al*, 2006). In humans it has been observed that a large proportion of IL-17 producing cells in the peripheral blood consists of γδ T cells, which become the vast majority of the $IL\text{-}17^+$ population in tuberculosis patients (Peng *et al*, 2008).

Another prominent member of the IL-17 family, which in the last few years has been the focus of much work, is IL-17E (IL-25). Unlike IL-17A and IL-17F, this cytokine has been found to be secreted by lung epithelial cells and has been implicated in

initiating Th2 type immunity by driving the expression of IL-4, IL-5 and IL-13 (Angkasekwinai *et al*, 2007; Fallon *et al*, 2006). A proposed model for the actions of these IL-17 family members during allergic inflammation has been described in Figure 1-2.

Figure 1-2 Diagram demonstrating the effector mechanisms of the IL-17 family of cytokines. The invasion of the epithelium by virus, fungi and allergens induces the infiltration or differentiation of IL-17 producing T cells. These cells release IL-17A, IL-17F and IL-22, inducing structural cells such as the epithelium to secrete large quantities of inflammatory cytokines and chemokines leading to the recruitment of other cells such as neutrophils to the site of injury. During chronic inflammation, mast cells, basophils and eosinophils infiltrate the area, which along with damaged structural cells secrete IL-25 and increase Th2 cytokine production from Th2 cells activated by antigen loaded DCs (Taken from Wang & Liu, 2008).

1.4.4 The family of IL-17 receptors

There are currently five members of the IL-17 receptor family (IL-17RA-RE) that display differing biological effects upon binding to their ligand, ranging from cytokine secretion and neutrophilia to mucus secretion (Wang & Liu, 2008). Within this family there is significant sequence divergence, although many of the genes encoding these receptors are linked, with clusters found on human chromosome 3. All the receptors contain a single transmembrane domain which range in size from 499-866 amino acids (Gaffen, 2009). These receptor subunits have conserved structural motifs which include an extracellular fibronectin III-like domain and a cytoplasmic tail encoding a SEF/IL-17 receptor (SEFIR) domain (Figure 1-3), which bears some homology with the TIR domains of TLRs (Onishi *et al*, 2010). Most of the IL-17 receptor family members exhibit broad distribution; earlier reports from mouse cells and cell lines have detected IL-17R mRNA in liver epithelial cells, fibroblasts and mast cells amongst others (Yao *et al*, 1995). It has now been shown that human fibroblasts, epithelial and endothelial cells coexpress both IL-17RA and IL-17RC, whilst T cells only express IL-17RA (Ishigame *et al*, 2009).

IL-17RA is a common signalling subunit used by multiple ligands (Figure 1-3). Studies on human IL-17R have demonstrated that, in order for IL-17A and IL-17F to elicit a response, IL-17RA (also known as IL-17R) and IL-17RC are necessary (Wang & Liu, 2008). More recently it has been shown that although IL-17A, IL-17F and a heterodimer of IL-17F/IL-17A all bind to IL-17RA albeit at differing binding kinetics, they bind to IL-17RC at similar rates. The results also showed that IL-17A

binds better to IL-17RA than IL-17RC with the opposite being true for IL-17F, while IL-17F/IL-17A binds with similar affinity to both receptors (Wright *et al*, 2008). Unlike TLRs which utilise the adaptors MyD88 and TRIF, IL-17 signals through the Act1 adaptor and U-box E3 ubiquitin ligase that contains both a SEFIR and TRAF domain (Gaffen, 2011). Stimulation of IL-17 receptors results in the activation of NFκB, MAP kinases and possibly PI3K. Other transcription factors such as IκBζ and C/EBP have also been reported to be activated (Gaffen, 2011).

To date the most studied of the receptors is IL-17RA and IL-17RC, although recent work has sought to identify the ligands and signalling components of the other receptors. IL-17RB is expressed by various endocrine tissues as well as the kidney, liver and Th2 cells. This receptor alone binds both IL-17B and IL-25; it can also pair with IL-17RA to form a functional IL-25R complex. Like the rest of the family, the cytoplasmic tail of IL-17RB contains a SEFIR domain and has been reported to bind Act1 in a SEFIR-dependent manner (Chang & Dong, 2011). It is difficult to speculate on the other family receptors as they have not been studied in depth. The literature at the moment has been unable to identify a ligand for IL-17RD. So far it seems to be the oldest member of the IL-17R family and can interact with IL-17RA, although the biological significance of this relationship is not clear. IL-17RE remains the least understood receptor (Gaffen, 2009).

Figure 1-3 IL-17 receptor family members. A diagram representing the five different receptor subunits and their ligands. The receptor for IL-17A, IL-17F and IL-17A/F heterodimer is composed of IL-17RA paired with IL-17RC. IL-17RB/IL-25R pairs with IL-17RA and its ligand is IL-17E/IL-25, on its own, IL-17RB/IL-25R binds IL-17B. IL-17RD and IL-17RE are more poorly understood (Taken from Gaffen, 2009).

1.4.5 Th9 cells

Since the emergence of Th17 cells, two further T helper cell subsets have come to light. The principal cytokine secreted by Th9 cells is IL-9, although it can also be secreted by other cell types such as mast cells, eosinophils and neutrophils (Soroosh & Doherty, 2009). Initially it was revealed that IL-9 is preferentially produced by Th2-like lymphocytes (Dugas et al, 1993). Later reports showed that activation of naïve $CD4^+$ T cells, in the presence of IL-4 and TGF-β significantly increased IL-9 and IL-10 production but not other Th2 cytokines, indicating a population distinct from Th2 cells (Veldhoen *et al*, 2008; Dardalhon *et al*, 2008). It is now thought that IL-9 may play a role in allergic airway disease. In mouse models, transgenic mice over-expressing IL-9 were found to have increased airway-hyperresponsiveness, eosinophilia and IgE levels after antigen challenge (McLane *et al*, 1998). In humans, bronchial biopsies from patients with atopic asthma have shown an elevated number of IL-9 mRNA-positive cells in the airway compared with normal controls (Ying *et al*, 2002). With the many sources of IL-9, it is difficult to draw conclusions as to the presence or role of Th9 cells in allergic disease.

1.4.6 Th22 cells

The defined Th22 cells were first thought to be a variant of Th17 cells, it has now been shown that IL-22 can be produced without production of IL-17 (Nograles *et al*, 2009). IL-22 belongs to the IL-10 cytokine family and has both pro-inflammatory and protective properties although its actions are not fully understood. The response to IL-22 normally comes from nonhematopoietic cells such as epithelial cells and

keratinocytes, as they express IL-22R. This triggers the production of antimicrobial peptides and expression of proteins involved in cellular differentiation and survival (Souwer et al, 2010). A pathogenic role for IL-22 has been proposed by studying IL-22 deficient mice, where a decreased incidence of disease was observed in an arthritis model (Geboes *et al*, 2009). In atopic dermatitis skin, an upregulated expression of IL-22 has also been reported (Nograles *et al*, 2009). More research is needed to further elucidate the function of IL-22 in allergic disease.

1.4.7 T regulatory cells

The host immune system also has specific cells that are involved in the regulation of immune responses. During the past decade, T regulatory (Treg) cells have emerged as key players in the development of immunological tolerance. Dysfunction of this cell can cause autoimmune disease, immunopathology and allergy (Sakaguchi *et al*, 2008). Tregs are naturally occurring and are characterised as $CD4^+$ $CD25^+$ cells (although in human studies, it has been argued that CD25 is not a definitive marker) expressing the transcription factor forkhead box P3 (FoxP3). They are capable of suppressing the activation and proliferation and effector functions of a wide range of immune cells such as, $CD4^+$ and $CD8^+$ cells as well as NK and NKT cells. Currently, the use of Treg cell-based therapy is the subject of much research for the application of immunological disease (Sakaguchi *et al*, 2010).

1.4.8 Unconventional lymphocytes

There are other lymphocytes that also contribute to the immune defence towards invading pathogens; these lymphocytes take the form of γδ T cells and NKT cells. In contrast to the more conventional αβ T cells previously discussed, a population of lymphocytes bearing a new TCR heterodimer, γδ, was discovered (Brenner *et al*, 1986). This lymphocyte subset acts in a way very distinct from αβ T cells, especially in its recognition of antigen. Although sharing certain cell surface proteins with αβ T cells, the interaction of γδ TCR with its ligand is more akin with antigen – antibody interactions, unlike the processing and presentation of antigen required for αβ T cells. The ability to recognise intact proteins directly is a characteristic of γδ T cells, enabling them to mediate their cellular immune functions faster than αβ T cell mediated immunity (Chien *et al*, 1996). The distribution of γδ T cells in humans is greater in the skin and mucosa, than in lymphoid organs or peripheral blood (Kaufmann, 1996). In response to antigenic stimulation, these cells can secrete cytokines and also take part in cytolytic activities. Studies in mice have found that they are capable of regulating the development of epithelial cells and αβ T cell responses (Komano *et al*, 1995; Kaufmann *et al*, 1993). More recent reports have observed the secretion of IFN-γ, IL-10 and high levels of TGF-β in human γδ T cells, and have subsequently been implicated as having potent regulatory potential (Kühl *et al*, 2009).

NKT cells are a population of lymphocytes that share some characteristics with NK cells. They are innate-like T lymphocytes that recognise glycoproteins in the context

of CD1d, the MHC class I-related glycoprotein (Van Kaer, 2007). There are two subtypes of NKT cells; type I NKT cells, also known as invariant NKT (iNKT) cells, have a highly restricted αβ TCR repertoire. In comparison, the TCR repertoire expressed by type II NKT cells is more diverse (Umetsu & DeKruyff, 2010; Van Kaer, 2007). In the healthy human liver, NKT cells make up 0.5% of $CD3^+$ cells and in the blood, 0.02% (Kenna *et al*, 2007). Most studies have focussed on iNKT cells, which upon activation have been found to produce high levels of IL-4, IFN-γ and TNF and can thereby activate DCs, NK cells B cells and conventional T cells (Umetsu & DeKruyff, 2010). In systemic lupus erythematosus, iNKT cells are also thought to play a pathogenic role (Major *et al*, 2006), later studies have now implicated these cells in allergic airway inflammation. In human asthma, increased numbers of iNKT cells have been observed, especially in patients with severe asthma. Some investigations have also reported contradictory findings in asthma patients (Iwamura & Nakayama, 2010). Further to this, some authors have suggested that these cells do not drive asthma pathogenesis but in fact modulate the asthmatic phenotype (Thomas *et al*, 2010).

1.5 Dysregulation of the immune response

In its aim to protect the body against disease, the immune system can sometimes cause harmful reactions in the host. Such reactions are known as hypersensitivity reactions, which can be divided into four types as originally conceived by Gell and Coombs (Gell & Coombs, 1963). Immediate hypersensitivity reactions (Type I) occur within minutes after contact with the antigen. A localised reaction usually ensues if the contact involves the epithelial tissues, such as that found in asthma or allergic rhinitis (hay fever). The reactions are commonly referred to as allergic and the antigens involved are allergens. This type of hypersensitivity is mediated by IgE which precedes the release of histamine and other mediators from mast cells and basophils.

Cytotoxic reactions (Type II hypersensitivity) are another form; in this case, IgG or IgM antibodies which bind to cell surface antigens leading to complement activation and destruction of local tissue cells. Antigens and antibodies may also form complexes that enter into the circulation and again can activate the complement system and cause local tissue damage. This type III hypersensitivity response is also known as immune – complex reactions.

The last group of hypersensitivity reactions are mediated by T cells rather than antibodies and is termed delayed hypersensitivity (Type IV). As opposed to immediate hypersensitivity, this reaction takes a day or more to develop. The responding T cells secrete chemokines that attract macrophages which take part in phagocytosis and subsequent tissue destruction (Rajan, 2003). Allergic disorders that

affect the ocular surface are generally thought to be classed as either type I or type IV hypersensitivity reactions – depending on the location and the degree of severity.

1.6 A role for the epithelium in adaptive immunity

As part of their innate function, epithelial cells produce a repertoire of cytokines and chemokines, which can be released into the local environment during mechanical injury, for example as a result of virus or bacterial invasion. Many studies have been conducted with the use of human colon and bronchial epithelial cells, to investigate their expression and production of cytokines and chemokines. These studies have demonstrated mRNA transcripts for IL-8 and TGF-β1, even in the absence of stimulation, as well as detectable levels of TNF-α, IL-1α, IL-1β, IL-10, GM-CSF and MCP-1, and of these, IL-8, MCP-1, TNF-α and GM-CSF can be upregulated in the presence of TNF-α stimulation or infection by certain bacterial strains (Jung et al, 1995). Within an asthma setting, bronchial epithelial cells from asthmatic patients have been shown to produce high levels of GM-CSF, IL-6 and IL-8 (Marini *et al*, 1992).

Within the last few years, the emergence of an epithelial derived cytokine called thymic stromal lymphopoietin (TSLP) has provided researchers with further evidence of a larger role for epithelial cells in the immune response. Thymic stromal lymphopoietin (TSLP) is an IL-7 – like, epithelial cell-derived cytokine that was first characterised in murine models of B cell development, where it was found to be an important factor in supporting the growth of B cells as well as exhibiting the ability

to induce the expansion of $B220^+$ pre-B cells (Sims *et al*, 2000). The functional receptor for this cytokine has already been characterised in the mouse. In human models this receptor complex is similar, consisting of two subunits – TSLPR and IL-7Rα – which have been found to be specific for TSLP and not IL-7, and which upon ligation lead to subsequent signal transducer and activator of transcription (STAT) 5 and STAT3 phosphorylation (Reche *et al*, 2001). Studies on the mRNA of this receptor have shown that its expression is restricted to DCs, monocytes, some T cell clones and more recently mast cells (Reche *et al*, 2001; Allakhverdi *et al*, 2007).

Studies using mouse models of allergic skin inflammation have shown that TSLP is vital for Th2 cytokine secretion by skin infiltrating effector T cells (He *et al*, 2008). The biological properties of TSLP differ between humans and mice and therefore the main focus here will be on the role of TSLP in modulating the human immune response. Analysis of mRNA has found that TSLP is not expressed by human endothelial cells or hematopoietic cell types such as B cells, T cells, NK cells or granulocytes, its expression is mainly by epithelial cells, stromal cells and mast cells (Soumelis *et al*, 2002).

Unlike murine TSLP, there have been no reports that human TSLP promotes the development of B and T cells although it has been shown to enhance the maturation of $CD11c^+$ DCs as well as slightly upregulating HLA-DR and CD86 expression and strongly inducing the co-stimulatory molecules CD40 and CD80 (Reche *et al*, 2001). In conjunction with each other, $CD11c^+$ DC in the presence of TSLP can provoke a very strong naïve T cell proliferation (Reche *et al*, 2001).

The microenvironment created by the presence of TSLP has also been studied and in mouse models it has been shown that this cytokine can drive Th2 development directly without any need for antigen presenting cells (APCs) (Omori & Ziegler, 2007). In addition, Ying et al observed that in diseases such as asthma there is a significantly increased expression of the mRNA of Th2 attracting chemokines TARC/CCL17 in the epithelium and submucosa which also correlated with the expression of TSLP in those areas. A role for TSLP in Th2 differentiation through the actions of TSLP stimulated DCs (TSLP-DCs) has been also been proposed. The mechanisms behind this polarising effect are thought to be due in part through OX40 ligand (ligand for the CD4 activation antigen OX40). Ito et al have demonstrated that TSLP-DCs expressing OX40L are responsible for the differentiation of $CD4^+$ T cells into Th2 cells (Ito *et al*, 2005). The actions of TSLP may elicit many effects as part of the epithelial response (Figure 1-4), although problems may arise – as seen with the secretion of other pro-inflammatory cytokines – if this response becomes exaggerated.

Figure 1-4 Diagrammatic representation of the effect of TSLP secretion in response to microbial invasion. Damage to the epithelium by pathogens leads to the secretion of TSLP by epithelial cells, this in turn upregulates the costimultory molecules OX40, CD40 and CD80 on DCs which are involved in T cell polarisation to the Th2 phenotype. The secretion of Th2 cytokines induces B cell IgE production leading to the activation of mast cells. IL-1 and TNF-α produced by the damaged epithelium also activates mast cells to secrete TSLP – thereby feeding back into the loop – and other cytokines, which along with IL-5 and GM-CSF produced by Th2 cells allow the recruitment of eosinophils (Taken from Holgate, 2007).

1.7 The ocular surface

The surface of the eye is the most exposed mucous membrane of the body (Buckley, 1998), coming into direct contact with the environment containing airborne debris or pathogens. It is therefore vital that there is some form of protection from these foreign particles. There are many layers that allow for this protection, of these the eyelid forms the outermost guard for the anterior of the eye. The exposed ocular globe is covered by the pre-ocular tear film which contains substances with bactericidal properties. Tearing also provides a flushing and cleansing action to remove foreign particles or noxious chemical substances (Holly, 1987). Beyond this a vascularised mucous membrane is revealed covering the anterior surface of the eyeball and the posterior surface of the upper and lower eye lids, known as the conjunctiva (Hoang-Xuan *et al*, 2001).

1.7.1 The conjunctiva

The conjunctiva is a membraneous junction between the lids and the globe of the eye; it ensures smooth movement of the eyelids over the globe (Figure 1-5A). It transitions from the skin of the lid margin where it lines the inner surface of the lids, is reflected beyond the tarsal plate and lies against the surface of the sclera, with which it fuses at the corneo-scleral junction therefore forming a conjunctival sac or fornix (Spooner, 1976). The tissue lining the eyelid is known as the palpebral conjunctiva which has a stratified non-keratinising squamous epithelium that is continuous with the keratinised squamous epithelium of the epidermis of the eyelid (Hoang-Xuan *et al*, 2001). The bulbar conjunctiva covers the sclera; it is thin and

translucent, allowing the sclera to show through. It loosely adheres to the underlying tissue within 3mm of the cornea. The fornix connects both these sections (Figure 1-5B).

The conjunctival glands are a number of small glands that participate in tear film secretion. The palpebral sebaceous glands contain meibomian glands, their secretions form the outermost lipid layer of the tear film, and Zeiss glands which release sebum into the eyelash follicle preventing the cilia from becoming dry and brittle (Remington, 1998). Within the fornix the ducts of the lacrimal gland and the accessory lacrimal glands can be found, the latter of which is separated into two groups according to the type of secretion (serous or mucous). The serous glands include the *glands of Krause* comprising of about 20 to 40 such glands in the superior fornix and 6 to 8 in the inferior and the *glands of Wolfring* or *Ciaccio* with no more than 6 being found in the upper lid and 2 located in the inferior edge of the lower tarsus. *Henle's glands* or *crypts* are part of the mucous glands; they arise in the palpebral conjunctiva between the tarsal plates and the fornices. They are composed of goblet cells which secrete mucus and form folds of mucous membrane lined by epithelium. The limbus houses *Manz's glands* found in many animal species but not present in humans (Spooner, 1976; Hoang-Xuan *et al*, 2001).

Figure 1-5 Anatomy of the eye. These diagrams represent a cross section of the human eye (**A**) and the location of the different accessory structures that make up the eye lid (**B**; Snell & Lemp, 1998).

1.7.2 The conjunctival epithelium

The epithelium of the conjunctiva is composed of between 2 and 10 layers of cells and, depending on its location, the size and shape of these cells are variable (Hoang-Xuan *et al*, 2001). Over much of the upper lid the epithelium is 2 to 3 cells thick whereas the lower lid is thicker with 3 to 4 cells (Remington, 1998). There are four distinct morphological components comprising the conjunctival epithelium. The eyelid has previously been described to be of a nonkeratinised and keratinised stratified squamous epithelium covering a connective-tissue stromal layer, the submucosa (Remington, 1998). Other areas such as the bulbar conjunctiva have a stratified epithelium with cylindrical superficial cells with other sites containing flattened superficial cells. In some cases, as the epithelium continues along it may change from a columnar to a squamous morphology.

Once again there are goblet cells, in this case scattered throughout the stratified columnar epithelium. Their number tends to decrease with age and greatly increases in some inflammatory conditions such as vernal conjunctivitis and contact lens-associated giant papillary conjunctivitis (Dartt, 2004). They are thought to be formed from the deepest layer of the conjunctiva, the cylindrical cells, where they pass towards the surface. They play an important role in moistening and protecting the conjunctiva and cornea via their production of mucins (Wolff, 1976).

1.7.3 Allergic conjunctivitis

Currently there are a few basic types of ocular allergy that constitute allergic conjunctivitis – which refers to a collection of hypersensitivity disorders – affecting about 20% of the population worldwide (Trocme & Sra, 2002). Allergic conjunctivitis can be divided into two main groups depending on the patients' signs and symptoms. The first group, also known as a milder disorder is more frequent and includes perennial (PAC) and seasonal allergic conjunctivitis (SAC), the latter of which is usually the ocular component of hay fever. The second group and the more severe form of allergic conjunctivitis have been given the term 'chronic allergic eye disease' (CAED) and it mainly describes two types, vernal keratoconjunctivitis (VKC) and atopic keratoconjunctivitis (AKC). Both forms are less frequent and the disease may be complicated by corneal involvement (Leonardi et al, 2008). The common clinical features of allergic conjunctivitis include redness, itching and tearing, there also tends to be eosinophil recruitment to the ocular surface (Stahl & Barney, 2004).

1.7.4 Seasonal allergic conjunctivitis

As discussed, one of the most prevalent forms of ocular allergy is SAC. In the UK it affects up to one-fifth of the adult population and represents the majority of all ocular allergy cases, with 98% of patients with ocular allergy having SAC or PAC. Although having similar characteristics, PAC differs from SAC as it is the rarer of the two and is caused by a constantly present allergen such as the house-dust mite as opposed to season-related pollens (Buckley, 1998). SAC is characterised by an IgE-

mediated response to environmental airborne allergens, such as grass and tree pollens. The binding of these allergens to specific IgE occurs on conjunctival mast cells – the main responding cell type in this disorder – and results in mast cell degranulation and their release of histamine, tryptase, prostaglandins and leukotrienes. Histamine in particular is the principal mediator thought to account for the itching, redness and swelling that are the hallmark signs of this disorder (Ono & Abelson, 2005).

Mast cells are particularly influential in the inflammatory response observed in SAC as the histamine and leukotrienes that they release are thought to contribute to the recruitment of neutrophils and eosinophils (Spada *et al*, 1986; Woodward *et al*, 1986). A correlation between these cells and adhesion molecule upregulation has been observed in atopic subjects challenged with allergen. This study demonstrated that 6 hours after allergen challenge, the expression of E-selectin and ICAM-1 was upregulated and there was infiltration of mast cells, neutrophils, eosinophils and macrophages compared to that of the control where infiltrating cells were not present (Bacon *et al*, 2000).

1.7.5 Vernal keratoconjunctivitis

This form of CAED is a disease that affects mostly young people, it is also more common in males and visual impairment may occur if the cornea is involved. Sufferers have a history of atopic disease in 49% of cases (Bonini *et al*, 2004) and, as such, some symptoms and cells (itching, redness and degranulated mast cells) are

similar to SAC, but there are major differences as VKC is a more chronic and severe form of ocular allergic disease. Clinical features of VKC include the presence of giant papillae on the upper tarsal conjunctiva or at the limbus and transient limbal or conjunctival yellow – white points or deposits known as Horner – Trantas dots which are clumps of eosinophils and epithelial cell debris (Kumar, 2009). The tissues from VKC may also undergo remodelling such as thickening of the conjunctival epithelium or epithelial loss which may be attributed to connective tissue deposition, oedema or inflammatory cell infiltration (Leonardi, 2002). The majority of VKC cases are seasonal and last from the beginning of spring until autumn, although there are perennial cases that persist throughout the year particularly in patients living in warm sub-tropical or desert climates (Leonardi, 2002). In the UK 75% of those affected have a history of atopic disease whilst 67% have a family history of atopy (Buckley, 1998).

Whilst the conjunctival epithelium and stroma are infiltrated with mast cells and neutrophils, T lymphocytes and eosinophils are the predominant cell type in VKC. T lymphocytes have been regarded as one of the principal mediators in the pathogenesis of this disorder. The phenotypic characteristics of VKC are marked by infiltration into the conjunctiva of $CD4^+$ $CD45RO^+$ T lymphocytes, with upregulated expression of HLA-DR in allergic tissues compared to normal controls. This expression was also observed on other cell types including macrophages, DCs and epithelial cells (Metz *et al*, 1996). Conjunctival specimens from VKC subjects have shown increased numbers of inflammatory cells that are positive for the chemokine receptor CXCR3, demonstrating a possible regulation of lymphocyte recruitment

within the conjunctiva of patients affected with this disorder (Abu-El Asrar *et al*, 2001). As well as the majority of T lymphocytes being $CD4^+$, an early report from Maggi and colleagues observed conjunctival tissue-derived T cell clones derived from VKC patients when stimulated were mainly producing IL-4, indicating that Th2 cells are the predominant cell type in this disease (Maggi *et al*, 1991). Later studies revealed increased mRNA expression of IL-3, IL-4 and IL-5 in the tissues of VKC patients and in the tears of these patients, there was an increased percentage of $CD4^+$ T lymphocytes expressing IL-4, which was thought to correlate with disease severity (Metz *et al*, 1997; Leonardi *et al*, 1999). Although being a very severe disease, VKC usually resolves at puberty but it can sometimes transform into AKC.

1.7.6 Atopic keratoconjunctivitis

Of all the ocular surface allergy–related disorders AKC is the most severe and chronic form. It is associated with atopic dermatitis and affects the conjunctiva and the lids of the eye (Bielory, 2000). The condition occurs in both children and adults although the onset is usually between 20 and 50 years of age (Stahl & Barney, 2004). The clinical signs include hyperaemia of the conjunctiva and papillae on the upper tarsal conjunctiva, in very severe cases conjunctival scarring with subepithelial fibrosis may occur and, like VKC, it can be sight threatening if there is corneal involvement (Bonini, 2004). Unlike that of VKC, the inflammatory cell infiltrate in AKC are predominated by $CD4^+$ T cells and neutrophils, with fewer eosinophils and mast cells than VKC. Where the two disorders differ is in their cytokine profile and hence the distribution of Th1 and Th2 cells. The same studies that detected large

numbers of IL-4 producing $CD4^+$ T cell clones in VKC also noted that there were a limited number of IFN-γ producing T cells, whilst in AKC the expression of IFN-γ localised to T cells was greater than in VKC, giant papillary conjunctivitis (GPC) and the controls (Maggi et al, 1991; Metz et al, 1997; Calder et al, 1999). These studies indicate that there is a shift towards a more Th1 cell population in this disorder.

The cytokines and other molecules upregulated as a result of foreign invasion are important in the inflammatory response especially for recruitment of cells to the site. It has already been shown that normal conjunctival epithelial cells secrete a range of cytokines such as IL-6, IL-8, TNF-α and RANTES that when upregulated, contribute to this process. During chronic inflammation such as that seen in CAED, conjunctival epithelial cells have also been shown to have greater expression of ICAM-1 and HLA-DR (Hingorani *et al,* 1998). Other cells infiltrating the conjunctiva contribute to changes in the cytokine environment during disease. Conjunctival T cells from subjects with CAED have increased levels of IL-3, IL-4 and IL-5 although the two different forms of CAED have differing cytokine profiles. In VKC conjunctival tissue sections, there is a Th2 like profile while in AKC it is more Th1 like (Metz *et al*, 1997). In VKC conjunctival-derived T cell lines, there is a mainly Th2 cytokine profile whereas in AKC conjunctival-derived T cell lines, there is also a Th1 profile (Calder *et al*, 1999). Within other tissues such as that of the lung, mRNA of an epithelial cell derived cytokine TSLP was reported to be increased in asthmatics (Ying *et al*, 2005). This cytokine has also been implicated in induction of Th2 attracting chemokines, thereby suggesting a role in asthma pathogenesis (Soumelis *et al*, 2002).

In diseases such as psoriasis and allergic asthma, infiltrating Th17 cells have been isolated in the tissues (Teunissen *et al*, 1998; Pène *et al*, 2008). These cells have also been shown to produce several inflammatory cytokines and therefore have been thought to contribute to chronic inflammatory diseases (Laan *et al*, 1999; Jones & Chan, 2002). Mast cells play an important role in allergy. They produce a range of

pro-inflammatory cytokines and chemokines – important in recruiting inflammatory cells – and can also cause direct tissue damage via secretion of molecules such as histamine and TNF-α. The hypothesis of my study is that conjunctival epithelial cells contribute to T cell effector mechanisms in CAED via the actions of their cytokines and chemokines on T cells and mast cells.

To investigate this hypothesis, I will aim to:

I. Identify novel epithelial derived cytokines as well as other cytokines known to be associated with tissue inflammation and their expression in conjunctival tissues during disease;
II. Investigate whether tissues from the conjunctiva express Th17 cells and if this is elevated during chronic eye disease.

I will examine the effect of co-culturing human T cells or mast cells with conjunctival epithelial cells, using an *in vitro* model of human conjunctival epithelial cells to establish the mechanism by which these cells and their cytokines and receptors are potentially capable of causing disease pathogenesis.

2.1 Culturing of human cells

2.1.1 *In vitro* culture of IOBA-NHC cells

A conjunctival epithelial cell line, IOBA-NHC (a kind gift from M. Calonge, Valladolid, Spain) was incubated at 37°C, 5% CO_2 in 5ml "IOBA" medium containing DMEM Nutrient Mixture F-12 HAM (Sigma-Aldrich, Dorset, UK) supplemented with 10% heat-inactivated foetal calf serum (FCS; Biosera, East Sussex, UK), Penicillin/Streptomycin (P/S) (100U/ml; Invitrogen, Paisley, UK), mouse endothelial growth factor (EGF) (2ng/ml; Sigma), bovine insulin (1µg/ml; Sigma-Aldrich), hydrocortisone [5µg/ml] and Cholera Toxin (Sigma-Aldrich; 0.1µg/ml) in a 25cm^2 flask (Diebold *et al*, 2003). At confluence the cells were detached using trypsin-EDTA (Invitrogen) and incubated at 37°C, 5% CO_2 for 3 minutes then centrifuged at 400rcf for 10 minutes and transferred to a 75cm^2 flask in 10ml IOBA medium and incubated at 37°C, 5% CO_2. Cells were split and fed when cell monolayers became confluent.

2.1.2 *In vitro* culture of ChWK cells

The conjunctival epithelial cell line, Wong-Kilbourne derivative of Chang conjunctiva-(ChWK; clone 1-5c-4 ECACC, Idmiston UK) was maintained in Medium- 199 (Sigma-Aldrich) supplemented with 10% FCS, P/S (100U/ml) and L-glutamine [2mM; Invitrogen] (Zhan *et al*, 2003). Upon reaching confluence, cells were detached using trypsin-EDTA (Invitrogen) and routinely split into new flasks.

2.1.3 *In vitro* culture of 16 HBE cells

The bronchial epithelial cell line, 16 HBE (a kind gift from P. Lackie, University of Southampton, UK) was cultured as described in 2.1.2.

2.1.4 *In vitro* culture of human cord blood mast cells (CBMC)

Mast cells were generated from the culture of human cord blood $CD34^+$ progenitors (Lonza Wokingham Ltd; Berkshire, UK). Cells (2 x 10^5 approx per vial) were cultured in 200µl StemSpan® medium (Stemcell Technologies, Grenoble, France) supplemented with IL-3 [1ng/ml], IL-6 [50ng/ml] and stem cell factor (SCF) [100ng/ml; all Peprotech, London, UK] and seeded onto a 96 well plate. Cells were routinely split then transferred firstly to a 24-well plate (Nunc) until sufficient numbers were then transferred to a $25cm^2$ flask and grown in 5-10 ml of culture medium. After the first 2 weeks of culture, IL-3 was removed from the culture medium and confluent cells were subsequently grown in $75cm^2$ flasks until 8 weeks. From 9 weeks onwards, 10% FCS was introduced into the culture medium to aid in the cells maturation. The cells were maintained at 1 x 10^6/ml (approx). At 11 weeks' culture, cells were counted and stained for their expression of c-Kit (SCF receptor) and FcεRI (IgE receptor), to determine phenotypically, if the cells had differentiated into mast cells.

2.2 Mast cell cytospins

To ascertain whether the mast cells contained the enzymes tryptase and chymase, 1 x 10^3 cells in 100µl of medium were placed into cytocentrifuge funnels with a slide attached and spun at 1000rpm for 3 minutes (Cytocentrifuge Cytospin 3; Fisher

Scientific, Leicestershire, UK). The slides were removed and allowed to dry before fixing in acetone for 1 minute, then stored for immunohistochemical staining.

2.3 Cytokine treatment of epithelial cells

Epithelial cells (described above) were detached as described in 2.1.1-2, centrifuged at 400rcf for 10 minutes, resuspended and plated out in a 24-well plate at $1x10^5$ cells/ml and left to incubate at 5% CO_2, 37^oC. When cells had reached pre-confluence (70-80%), culture supernatants were removed and replaced with fresh medium in the presence, absence or with a combination of human recombinant cytokines. Table 2-1 shows the cytokine concentrations that were used for titration experiments. In some combinations TNF-α, IL-4 or IL-13 were added at 100ng/ml as according to Kato *et al*, 2007. Epithelial cells were also treated with Phorbol 12-myristate 13-acetate (PMA) (1ng/ml), poly I:C [50μg/ml] (both Sigma-Aldrich), IL-1β [10ng/ml] (R&D Systems, Abingdon, UK) or a combination of TNF-α + IL-1β. Cells were incubated for 6, 24, 48 or 72 hours, after which time the supernatants were removed and centrifuged to remove cell debris. Cell-free supernatants were frozen in aliquots at -70^oC until ready for assay.

Table 2-1 Titration of recombinant cytokines for the treatment of epithelial cells

Cytokine	Concentration	Cytokine	Concentration
IFN-γ (PeproTech)	100U/ml	**TNF-α** (PeproTech)	100U/ml
	200U/ml		300U/ml
	500U/ml		500U/ml
	600U/ml		700U/ml
IL-4 (PeproTech)	25U/ml	**IL-13** (R&D Systems)	5U/ml
	50U/ml		10U/ml
	100U/ml		20U/ml
	150U/ml		30U/ml

2.4 Pre-treatment of epithelial cells for IL-17RA blocking experiments

Epithelial cells (2.1.1) were detached as previously described in 2.1.1, centrifuged at 400rcf for 10 minutes, resuspended and plated out in triplicate into a 24-well plate (Nunc) at $1x10^5$ cells/ml and incubated at 5% CO_2, 37^oC. When cells had reached pre-confluence (70-80%), supernatants were removed and replaced with fresh medium and cells were treated with either PMA [1ng/ml], TNF-α [25ng/ml] (Peprotech) in combination with IL-1β [10ng/ml] (R&D Systems), rIL-17A [5ng/ml] (R&D Systems), zymosan [20µg/ml] (InVivogen, Nottingham, UK), poly I:C [50µg/ml] (Sigma-Aldrich) or LPS [5µg/ml] (Sigma-Aldrich), the latter three indicating agonists for TLRs 2, 3 and 4, respectively. Some wells were also left unstimulated.

Cells were incubated for 6, 24 and 48 hours, after which time the supernatants were removed and centrifuged to remove cell debris. Cell-free supernatants were frozen in aliquots at -70^{o}C until ready for assay. Cells remaining in the wells were detached and stained for various markers and receptor expression.

2.5 IL-17RA receptor blocking

2.5.1 IL-17RA neutralisation titration

Before the neutralisation study was conducted, the neutralising IL-17RA antibody was first titrated onto the cells in order to see which concentration of the antibody had the ability to downregulate IL-17RA expression. Conjunctival epithelial cells that had been grown until pre-confluence had their medium removed and were washed twice with PBS before incubation with anti-IL-17RA at a concentration of 0.1, 0.5 and 1μg/ml for 2 hours. Some wells were left untreated. After this time, the cells were washed, trypsinized and transferred to micro-centrifuge tubes so that they can be stained for expression of IL-17RA and analysed by flow cytometry.

2.5.2 IL-17RA neutralisation

Epithelial cells were set up in parallel and treated as in 2.4. Cells were then incubated for 24 hours after which time cell-free supernatants were prepared and stored frozen until further use. After washing, the cells were given fresh medium. To one of the plates cells were treated with normal goat anti-IgG [0.5μg/ml] (R&D Systems), to another plate the cells were treated with goat anti-human IL-17RA antibody [0.5μg/ml] (R&D Systems) and incubated for 2 hours. To a third plate, wells were replaced with fresh medium alone and incubated for 24 hours. After 2 hours, medium

from the first two plates was removed and cells were washed twice. Fresh medium was added to all wells and cells were stimulated with rIL-17A [5ng/ml] (as above), including control cells that had not received any stimulation prior to treatment with IgG or anti-IL-17RA antibody. The cells were incubated for 24 hours with supernatants collected after this time and centrifuged to remove any cells and debris. Supernatants were frozen in aliquots and stored until use in an assay.

2.5.3 CFSE labelling of PBMCs

Frozen peripheral blood mononuclear cells (PBMCs) isolated from leukocyte depletions collected in cones (Blood transfusion service, UK) were thawed into RPMI containing 10% FCS and spun at 400rcf for 10 minutes. Cells were washed in RPMI and spun for a further 10 minutes then resuspended in 100µl RPMI containing 5µM carboxyfluorescein diacetate succinimidyl ester (CFSE) and incubated for 10 minutes at 37^{o}C. After this 1ml RPMI-10% FCS was added to stop CFSE ad spun at 4000rpm for 4 minutes, after which cells were resuspended in the same volume of medium and incubated for 30 minutes at room temperature. Cells were then spun at 400rcf and resuspended at a concentration of 1x10^{6}/ml in RPMI supplemented with 10% FCS (BIosera), non-essential amino acids, sodium pyruvate (2mM for both; Invitrogen), 2x10^{-5} mercaptoethanol (Sigma) and 50µg/ml gentamycin (Invitrogen)

2.5.4 Determining inhibition of lymphocyte proliferation

PBMCs labelled as in section 2.5.3 were plated out in a 96 well plate at $1x10^6$ cells/well in triplicate. Three wells were left unstimulated and three wells were stimulated with anti-CD3 (50ng/ml) and anti-CD28 (1µg/ml; BD Biosciences, Oxford, UK). The remaining wells were supplemented with supernatants collected in section 2.5.2 to make up a concentration of 5, 10 or 20% and also stimulated with anti-CD3/CD28. Cells were incubated for 5 days at 37^oC, 5% CO_2.

2.6 CBMC and epithelial cell interaction

2.6.1 CBMC sensitisation

In order to mimic the actions of allergens in stimulating mast cells, CBMCs must first be sensitised by treatment with IgE to upregulate their IgE receptors, before stimulation with anti-IgE, which acts by cross-linking two IgE molecules thus mimicking an allergen. The IgE used to sensitise the cells was dialysed before use to remove the sodium azide. To achieve this, 100µl IgE (Abd Serotec, Oxford, UK) was added to a dialysis unit (Dialysis unit, 3.5kDa; Thermo Scientific, Pierce; Leicestershire, UK), placed in a buoy and transferred to a beaker containing 2 litres PBS and left on a magnetic stirrer for at least 2 hours. As sodium azide is 65 daltons, IgE was retained within the dialysis tubing as the azide diffused out. CBMCs were plated out at $1x10^6$ cells/ml in 24 well plates and presensitised by adding IgE Ab [4µg/ml] for 24 hours.

2.6.2 CBMC, epithelial cell co-culture

Conjunctival epithelial cells plated out at $1x10^5$ cells/ml were grown until pre-confluence and stimulated for 4 hours with IL-17, TNF-α + IL-1β, poly I:C and poly I:C + TNF-α + IL-1β. In parallel, presensitised CBMCs that had been seeded onto 24 well plates at $1x10^6$ cells/ml were left untreated or stimulated with anti-IgE [25μg/ml] (Sigma-Aldrich), IL-17, TNF-α + IL-1β, PMA or poly I:C for 2 hours (at the concentrations indicated in section 2.4). CBMCs were harvested after this time and spun at 4000rpm for 4 minutes. The cell pellet was resuspended in 1ml mast cell culture medium and cultured with the appropriately stimulated epithelial cells. Supernatants were collected at 24 and 48 hours and stored at -70^{o}C. ELISAs were used to quantitate the production of chemokines.

2.7 Cytokine secretion by ELISA

Supernatants collected as described (2.3, 2.4, 2.5.2 and 2.6.2) were used to detect and quantify the level of various secreted proteins using commercially available ELISA kits. The ELISAs were performed according to the manufacturers' instructions. Briefly, a 96-well Immunolon microplate (Nunc, Leicestershire, UK) was coated with capture antibody (refer to Table 2.7) and incubated overnight at room temperature.

Medium was aspirated and wells washed 3 times with wash buffer [0.05% Tween® 20 (Sigma-Aldrich) in phosphate buffered saline (PBS; Sigma-Aldrich)] after which plates were blocked by the addition of Reagent Diluent [1% bovine serum albumin (BSA; Sigma-Aldrich) in PBS] and incubated at room temperature for a minimum of

1 hour. Wells were again washed 3 times after which samples or standards were added to each well and incubated for 2 hours at room temperature. Standards were prepared using recombinant human proteins of the targets of interest. A seven point standard curve was made using a 2-fold serial dilution in duplicate wells in Reagent Diluent, with the highest standard of 2000pg/ml for IL-8, IL-25, MMP3, MMP9 and CXCL10; 1000pg/ml for IL-17, CCL17 and CCL20 and 20,000pg/ml for IL-17F. After this time wells were aspirated, washed and the detection antibody (refer to Table 2-2) added for 2 hours at room temperature. Wells were again aspirated, washed and Streptavidin-horseradish peroxidase (HRP) was added to each well and incubated for 20 minutes at room temperature. After this time the aspiration/washing step was repeated and substrate solution was added to each well and incubated at room temperature for 20 minutes, after which time Stop Solution (2N H_2SO_4) was then added to each well. Plates were read at 450nm (405nm for IL-25) and the concentration of protein in the samples determined from a standard curve by taking the mean of each duplicate standard and plotting the absorbance against their known concentration. From the standard curve a best-fitting curve was generated allowing the concentrations of each sample under test to be calculated.

For the detection of sCD44 an ELISA kit (Bender MedSystems, Vienna, Austria) was used according to manufacturer's instructions. A plate pre-coated with the capture antibody was provided and the assay carried out as indicated above from the addition of the standards and samples.

Table 2-2 Proteins of interest targeted for detection by ELISA

Protein	Capture Antibody	Detection Antibody	Source
TSLP	0.8µg/ml	400ng/ml	R&D Systems
IL-8	4µg/ml	20ng/ml	R&D Systems
IL-17A	4µg/ml	150ng/ml	R&D Systems
IL-17F	0.8µg/ml	100ng/ml	R&D Systems
IL-25	1µg/ml	0.25µg/ml	PeproTech
MMP3	0.8µg/ml	200ng/ml	R&D Systems
MMP9	1µg/ml	200ng/ml	R&D Systems
sCD44	According to manufacturer	According to manufacturer	Bender MedSystems
CXCL10	2µg/ml	50ng/ml	R&D Systems
CCL17	2µg/ml	100ng/ml	R&D Systems
CCL20	2µg/ml	50ng/ml	R&D Systems

2.8 Detection of MMP secretion by antibody array

Another approach used to identify the presence of MMPs in culture supernatants was an antibody array (RayBiotech, Inc, Cambridgeshire, UK), that is capable of detecting a number of proteins simultaneously (Table 2-3), but only semi-quantitatively, hence providing a significant advantage over the more traditional method used for detecting MMPs (Zhou *et al*, 2005). This assay was carried out at room temperature according to manufacturer's instructions, using reagents that were

supplied. Membranes coated with antibodies were each placed in an 8 well plate and blocked with 2ml 1x Blocking Buffer for 30 minutes at room temperature. After this time, the blocking buffer was decanted and membranes were incubated with 1ml supernatant from control and stimulated wells at room temperature for 2 hours. Supernatants were decanted from the plate and each well was washed 3 times (5 minutes per wash) with 2ml of 1x Wash Buffer I on an orbital shaker. Membranes were again washed twice allowing 5 minutes per wash with 2ml of 1x Wash Buffer II with shaking. To the membranes, 1ml of diluted biotin-conjugated antibodies was added and incubated for 2 hours. The same washing steps were carried out before the addition of 2ml diluted HRP-conjugated streptavidin. Membranes were incubated at 4^{o}C overnight after which they were washed as before.

In order to detect the reaction, excess wash buffer was drained and each membrane was placed (protein side up) on a clean plastic sheet. Each membrane was then covered with a 500µl solution of detection buffer (made from a 1:1 mixture of two separate detection buffers) and incubated at room temperature for 2 minutes. Excess detection reagent was drained from the membranes and placed, protein side up, on another plastic sheet. A second sheet was placed on the membranes and any air bubbles gently smoothed away. The membrane array was exposed to x-ray film (Super RX Fuji Medical x-ray film) for up to 40 seconds and the signal detected using film developer. The signal intensities were quantified by densitometry (Kodak digital science 1D 2.0 software) and the positive control was used to normalise the results.

Table 2-3 Position of specific antibodies on the membrane, to the proteins of interest

	A	B	C	D	E	F	G	H
1	**POS**	**POS**	**NEG**	**NEG**	**MMP-1**	**MMP-2**	**MMP-3**	**MMP-8**
2	**POS**	**POS**	**NEG**	**NEG**	**MMP-1**	**MMP-2**	**MMP-3**	**MMP-8**
3	**MMP-9**	**MMP-10**	**MMP-13**	**TIMP-1**	**TIMP-2**	**TIMP-4**	**NEG**	**POS**
4	**MMP-9**	**MMP-10**	**MMP-13**	**TIMP-1**	**TIMP-2**	**TIMP-4**	**NEG**	**POS**

2.9 Detecting cytokine secretion by Multiplex cytokine bead arrays

Culture supernatants were simultaneously investigated for the presence of IL-8 as a positive control for epithelial cell responses, IL-10, IL-13 and TGF-β_1 by a multi-cytokine bead assay kit (Bender MedSystems), and carried out according to the manufacturers' instructions. TGF-β1 is secreted in a latent form, and therefore had to undergo acidification in order for the total amount of TGF-β1 to be detected in this assay. First, 20µl supernatant was diluted with 180µl assay buffer. To this 20µl 1N HCl (supplied in kit) was added to acidify the TGF-β1 and incubated at room temperature for 1 hour. After this time 20µl 1N NaOH (supplied in kit) was added to stop the reaction. Samples were then ready for assay.

Wells from a 96-well plate (multiscreen filtration plate; Millipore, Co Durham, UK) were pre-wet with 50µl assay buffer then aspirated using a vacuum manifold. Standards were prepared for each cytokine by setting up a 7 point serial dilution from 10000pg/ml to 14pg/ml and adding 25µl each to duplicate wells using the buffer as a blank. To the remaining wells 25µl of sample supernatants were added in duplicate

and 25µl bead mixture and 50µl biotin-conjugate mixture consisting of beads specific for TGF-β1 IL-8, IL-10 or IL-13 were added to all wells. Plates were covered and left to incubate on the orbital shaker at room temperature for 2 hours. After this time the wells were aspirated using vacuum filtration and washed twice with assay buffer. To each well 100µl assay buffer and 50µl streptavidin-PE solution was added and incubated for 1 hour at room temperature. After washing, any remaining liquid was removed by blotting on absorbent paper and 200µl assay buffer added to each well, mixed and then transferred to 5ml polystyrene tubes for analysis by flow cytometry.

The flow cytometer was set up using 2 sets of beads of differing sizes (4.4µm and 5.5µm), the bead populations being differentiated by varying intensities of an internal fluorescent dye, detected in the FL-3- channel. Streptavidin binding to the biotin conjugate was detected in the FL-2 channel therefore allowing the quantification of the analytes. Data was analysed using Flow Cytomix Pro 2.2 software (Bender MedSystems).

2.10 Immunofluorescence and Flow Cytometry

Cells in suspension were transferred to 1.5ml microfuge tubes for one or two parameter staining. The cell suspensions were first centrifuged in a microcentrifuge for 4 minutes at 4000rpm, the supernatant was removed and cells resuspended in PBS and washed again. Cells were then stained using directly conjugated antibodies (direct immunofluoresence) or with unconjugated antibodies (indirect immunofluorescence) with appropriate controls included as indicated in table 2-4).

To the pellet, antibodies were added at the appropriate volume or concentration and incubated at 4^{o}C for 30 minutes. After this time, cells were washed 3 times with PBS and finally resuspended in PBS and transferred to 5ml polystyrene tubes. For indirect immunofluorescence staining, a secondary antibody was added, anti-goat FITC (fluorescein isothiocyanate) [1µg/ml] (Southern Biotech, Cambridge, UK) and cells incubated for 30 minutes at 4^{o}C. The cells were washed and the pellet resuspended in PBS. Analysis was carried out by flow cytometry (FACScalibur; BD Biosciences), plotting forward scatter (FSC) vs. side scatter (SSC) to locate the live cell region then acquiring 10,000 events in the live region. The data was analysed using Winlist (Verity Software House Inc, Maine, USA). A region indicating the live cell population was drawn and from this a histogram was created looking at the cells in the FL1 channel for FITC or CFSE, FL2 channel for PE (Phycoerythrin) and FL4 channel for APC (Allophycocyanin). The mean fluorescence intensity or percentage expression was measured. To analyse proliferation, a gate was drawn around lymphocytes and lymphoblasts (proliferating cells) and the percent of proliferation was determined based on diminishing fluorescent intensity.

Table 2-4 List of antibodies used for immunofluorescence staining for flow cytometry

Primary Antibody	Raised In	Source	Volume / concentration
IgG control FITC	Mouse	BD Biosciences	5µl
IgG control PE	Mouse	BD Biosciences	5µl
IgG control APC	Mouse	BD Biosciences	5µl
CD54 (clone HA58) APC	Mouse	BD Biosciences	5µl
IL-17RD PE	Mouse	R&D Systems	5µl
c-Kit (clone 104D2) FITC	Mouse	Abcam	5µl
FcεRI (clone AER-37) PE		eBioscience	10µl
IL-17RA	Goat	R&D Systems	0.5µg/ml
IL-17RC	Goat	R&D Systems	0.5µg/ml

2.11 Donors

2.11.1 Biopsy specimens

All specimens used in this study were obtained from our collection of banked tissues in the laboratory. All procedures (2.11.1 and 2.11.2) were approved by the local Ethics Committee and informed consent was obtained from all subjects.

Conjunctival biopsies from 31 subjects attending clinics at Moorfields Eye Hospital were obtained, taken from one eye per donor. Biopsies were taken from the upper tarsal (AKC, VKC, normal controls) or bulbar conjunctiva (ocular ciccatricial phemphigoid [OCP], SAC). The control specimens (n=6; 69.0±19.9 years, three female) were from patients undergoing elective surgery. SAC subjects were biopsied

after 8 hours post conjunctival allergen challenge using grass pollen extract (n=6; 18-65 years, all male). CAED subjects all had active disease: VKC (n=6; 24.0±2.6 years, two male); AKC (n=6; 37.8±21.7 years, two male). Treatment in all cases had been stopped at least 4 weeks (for corticosteroid) or 7 days (sodium cromoglycate treatment) prior to biopsying. Of the OCP patients, those that were active (n=5; 70.2±16.0 years, 2 male) had acute inflammation whilst those that had chronic disease (n=5; 72.6±10.5 years, 1 male) did not have clinically apparent inflammation. None of the OCP patients were receiving anti-inflammatory therapy at the time of biopsying. The diagnosis of disease was made clinically and by direct immunofluorescence of the conjunctiva for OCP.

2.11.2 Ocular swab samples

A total of 58 subjects with either active or controlled disease were recruited, this included; AKC (n=7; 34.3±3.2 years, 5 male), OCP (n=35; 70.8±2 years, 19 male) and normal controls (n=16; 69.2±2.1 years, 8 males). The right conjunctiva was used in all cases and was anaesthetised with preservative-free proxymetacaine 0.5% eye drops (Minims™; Chauvin Pharmaceuticals, Kingston-Upon-Thames, UK). The upper eye lid was inverted and a swab (Dacron polyester-tipped swab; Hardwood Products Co LP, Maine, USA) was gently brushed along the tarsal conjunctiva and immediately placed in a tube containing 200µl RNA*later*® (Ambion Inc, Texas, USA). Samples were stored at 4^{o}C then transferred to a -20^{o}C freezer. All procedures (2.11.1 and 2.11.2) were approved by the local Ethics Committee and informed consent was obtained from all subjects.

2.12 Immunohistochemistry

Previously obtained conjunctival biopsy tissue sections (2µm thick, stored at -70^{o}C) as described (Hingorani *et al,* 1998) were obtained from our tissue bank, from subjects with AKC and VKC (CAED) as well as individuals with SAC and those without any inflammatory eye disorder (non-inflamed controls). Sections were allowed to thaw; each section or slide containing mast cell cytospins were ringed with a wax pen (Dako, Cambridge, UK) and blocked with peroxidase (3%) (Dako) for 10 minutes at room temperature. Slides were then washed 3 times for 5 minutes with PBS. Sections were blocked with 10% normal rabbit serum (Dako) at room temperature for 30 minutes. The slides were drained and negative control antibody (diluted to the same protein concentration as primary antibody) or appropriately titrated primary antibody of interest (Table 2-5) was added and incubated overnight at room temperature in a humidified chamber.

The slides were washed 3 times for 5 minutes in PBS and incubated at room temperature for 2 hours with a secondary biotinylated antibody (Table 2-6). After washing, streptavidin-horseradish peroxidase (1:200) was added for a further 2 hours followed by washing. A substrate was then applied and left for 25 minutes; using 3,3'-diaminobenzidine tetrahydrochloride (DAB), which stains brown. The slides were then washed under running tap water for 5 minutes. Mayer's haematoxylin (Dako) was applied to sections for 2-3 minutes then washed under tap water for 5 minutes; slides were drained then mounted with glycergel mounting medium (Dako) and covered with a coverslip. For sections that were double-stained, one of the molecules of interest was stained first and then the procedure repeated again for the

second molecule using a different substrate, 3-amino-9-ethylcarbazole (AEC), which gives a pink colour, before slides were mounted.

2.12.1 Quantification of immunohistochemical staining

In order to quantify the staining of the tissue sections, sections were scored as the total number of positively stained cells, or for expression intensity in the epithelium and stroma. The scoring system was graded on a scale of 0-3, with 0 meaning no staining, up to 3 meaning very intense staining.

Table 2-5 Primary antibodies and substrates used for detection in immunohistochemistry

Primary Antibody	Raised In	Source	Dilution	Substrate
IgG control	Mouse	Santa cruz	1:100	
IgG control	Rabbit	Dako	1:100	
IgG control	Goat	R&D Systems	1:100	
TSLP	Rabbit	Santa cruz	1:250	DAB
IL-13	Rabbit	Santa cruz	1:100	AEC
CD3	Mouse	Dako	1:10	DAB
IL-17	Rabbit	Santa cruz	1:100	DAB
CD4	Mouse	Dako	1:10	AEC
IL-17RA	Goat	R&D Systems	1:25	DAB
IL-17RC	Goat	R&D Systems	1:25	DAB
Chymase	Mouse	Abd Serotec	1:100	AEC
Tryptase	Mouse	Abd Serotec	1:100	AEC

Table 2-6 Secondary antibodies used for immunohistochemistry

Secondary Antibody	Raised In	Source	Dilution
IgG F(ab')2	Rabbit	Dako	1:200
IgG F(ab')2	Goat	Dako	1:200
IgG F(ab)	Donkey	Abcam	1:100

2.13 Quantification of mRNA from conjunctival epithelial cells

2.13.1 RNA extraction from conjunctival epithelial cells

IOBA cells were detached from flasks using trypsin, centrifuged at 400rcf for 10 minutes, resuspended in culture medium then seeded onto 24 well plates at a density of $1x10^5$ cells per well. Confluent cells were treated with TNF-α [25ng/ml] ± IL-1β [10ng/ml], IL-4 [20ng/ml] or IL-13 [20ng/ml], IL-17 [10ng/ml], zymosan [20µg/ml], poly I:C [50µg/ml] ± TNF-α + IL-1β and LPS [5µg/ml] for 1, 4 and 6 hours. Cells that had reached 70-80% confluence were treated for 24 hours.

For total RNA isolation, the procedure was carried out using RNeasy® Mini kit (Qiagen, Crawley, UK) according to the manufacturers' instructions. Epithelial cells were lysed using a buffer supplied in the kit and the lysate was then homogenised by centrifugation (QIAshredder spin column, Qiagen). The remaining procedure was carried out using RNeasy spin columns according to manufacturer's instructions.

RNA was eluted by the addition of 30µl RNase-free water to the spin column and centrifugation for 1 minute at 8000rcf into a 1.5ml collection tube. The concentration of RNA was measured by spectrophotometry (NanoDrop®; Thermo Scientific) and the purity analysed by taking the ratio of readings at 260nm and 280nm (A_{260}/A_{280}). RNA was either stored immediately at -70°C or reverse transcribed.

2.13.2 RNA extraction from conjunctival swabs

RNA was extracted using the same procedure detailed in section 2.12.1 with the exception of a slight modification. Swabs were thawed on ice then removed from their tube containing RNA stabiliser. Using cocktail sticks, the swabs were

transferred to a new tube containing the buffer supplied in the kit and vortexed. The lysate was then homogenised as previously described.

2.13.3 RNA transcription

Template RNA isolated as in 2.12.1and 2.12.2 was converted to cDNA by reverse transcription (QuantiTect® or Omniscript® reverse transription kit, Qiagen). All reactions were prepared on ice, the first step involved the elimination of genomic DNA using 0.5µg template RNA and a buffer supplied for the removal of genomic DNA. Samples were incubated at 42^{o}C for 2 minutes then placed on ice. A reverse-transcription master mix was made up using the reagents supplied, this included reverse transcriptase, a buffer and a primer mix containing Mg^{2+} and dNTPs. This master mix was added to each template RNA sample from the first step and incubated for 15 minutes at 42^{o}C. Reverse transcriptase in the reaction was inactivated by incubating at 95^{o}C for 3 minutes. Samples were stored at -20^{o}C until RT-PCR was carried out.

2.13.4 Real-time quantitative PCR of conjunctival epithelial cells

To quantify the relative amount of mRNA, 20x TaqMan gene expression assays were used which contain primer and probes (inventoried stock; refer to table 2.13). The assay includes the use of 2x TaqMan Gene Expression Master Mix which contains Ampli Taq Gold® DNA polymerase, Uracil-DNA Glycosylase and dTNPs with dUTP (Applied Biosystems, Warrington, UK). Each was mixed with cDNA and made up with RNase-free water to set up a 20µl reaction in a 96 well reaction plate (Applied Biosystems). Reactions were run in triplicate using a thermal cycler

(7900HT Fast Real-Time PCR System, Applied Biosystems) and analysed with SDS 2.2.2 software.

The following conditions were used for the cycling protocol; (1) Initial activation of DNA polymerase for 10 minutes at 95^{o}C; (2) denaturation for 15 seconds at 95^{o}C; (3) combined annealing and extension step for 1 minute at 60^{o}C with fluorescence data collection. Steps 2 to 3 were repeated for a total of 40 cycles. Cycle threshold (C_t) values were recorded and data was normalised by running each sample with Beta Actin as a housekeeping gene with the fold change in gene expression calculated using the $2^{-\Delta\Delta Ct}$ method:

$$\Delta C_t = C_t \text{ target gene} - C_t \text{ housekeeping gene}$$

$$\Delta\Delta C_t = \Delta Ct \text{ sample} - \Delta C_t \text{ unstimulated}$$

$$\text{Fold change} = 2^{-\Delta\Delta Ct}$$

2.13.5 Real-time quantitative PCR of conjunctival swabs

Quantification of mRNA from samples taken from conjunctival swabs was carried out by multiplex PCR. A four plex reaction mix was prepared with 2x QuantiTect Multiplex PCR kit (Qiagen), containing HotStarTaq DNA polymerase, QuantiTect Multiplex PCR buffer, dNTP mix including dUTP and 11nM $MgCl_2$. This was mixed in PCR tubes with cDNA, 10x forward and reverse primers (table 2-7) and sequence specific probes for all target genes. Reactions were set up with hypoxanthine phosphoribosyl transferase 1 (HPRT1) as the housekeeping gene with the following cycling conditions; (1) Initial activation of HotStatTaq for 15 minutes at 95^{o}C; (2) Denaturation for 60 seconds at 94^{o}C; (3) Annealing and extension step for 60

seconds at 60°C with 50 repeated cycles of steps 2 to 3 (Rotorgene 3000; Corbett). By taking the ratio of each target gene with HPRT1, mRNA was quantified using the $\Delta\Delta C_t$ method.

Table 2-7 Genes of interest and their target sequences

Gene	Assay ID / Part number / Target Sequence	Source
TSLP	Hs00263639_m1	Applied Biosystems
IL-8	Hs99999034_ml	Applied Biosystems
IL-13	Hs99999038_m1	Applied Biosystems
IL-25	Hs03044841_m1	Applied Biosystems
Actin	4352935E	Applied Biosystems
IL-17A	Forward - CCAAATTCTGAGGACAAGAACTTCC Reverse - CAAGGTGAGGTGGATCGGTTG	Sigma-Genosys Ltd.
TLR2	GGGTCATCATCAGCCTCTCC CCTGAGGGAATGGAGTTTAAAGATC	Sigma-Genosys Ltd.
TLR4	CAAATGCCCCTACTCAATCTCTC AAACTATCAAAATTATTTCTTAAAGTCAGC	Sigma-Genosys Ltd.
TLR9	GCAATGTCACCAGCCTTTCC GCAGTTCCACTTGAGGTTGAG	Sigma-Genosys Ltd.
IL-13	CCCTTGCCAGACATGTGGTG TCCCCAACGGTGACAAACAC	Sigma-Genosys Ltd.
HPRT1	TATGGACAGGACTGAACGTCTTG GATCTATCACTATTTCTATTCAGTGCTTTG	Sigma-Genosys Ltd.

TLR mediated immunity at the ocular surface mucosa

3.1 Introduction

The location of the eye presents the ocular surface with the means to interface with the external environment. The ocular mucosa is continually exposed to airborne particles such as dust, pollen and microbes. As well as functioning as a barrier to such contact, the mucosa also takes part in host defence by responding to invading pathogens. The conjunctival epithelium forms part of the ocular mucosa and is at the forefront of the innate immune response owing to its array of surface receptors, namely TLRs and production of cytokines and chemokines. As a result, TLRs of the epithelium are essential in recognising microbial structures and initiating an immune response. Problems may arise however when the immune response becomes exaggerated and uncontrolled, thereby exacerbating any ongoing inflammation.

It has been proposed that there is a correlation between TLRs and allergic disease (Bauer *et al*, 2007), and this association has also been highlighted in allergic conjunctivitis. One such study has demonstrated that subjects with VKC had increased TLR4 and decreased TLR2 compared to normal subjects (Bonini *et al*, 2005), whilst Cook et al observed increased TLR2 from the conjunctiva of AKC patients (Cook *et al*, 2006). As part of their role in the innate inflammatory response, agonists to TLRs1-4 have been shown to induce the upregulation of the proinflammatory cytokines, IL-6 and IL-8, in primary human conjunctival epithelial cells (Li *et al*, 2007; Chung *et al*, 2009). Moreover, upon activation of TLR2, 3 and 4

salivary epithelial cells upregulate their expression of CD54, CD40 and MHC class I molecules (Spachidou *et al*, 2007).

As part of their role in orchestrating the innate immune response, earlier studies have revealed that the conjunctival epithelium from chronic allergic tissues express ICAM-1 and HLA-DR to a greater extent than milder allergic eye disorders (Hingorani *et al*, 1998). Certain proinflammatory cytokines have also been detected in normal tissues, but more recently *in vitro* experiments have shown that upon stimulation, conjunctival epithelial cells upregulate the secretion of various cytokines and chemokines, such as IL-6, IL-8 and RANTES, that may influence the outcome of the inflammatory response (Hingorani *et al*, 1998; Enríquez-de-Salamanca *et al*, 2008).

The varying characteristics that distinguish the conjunctival epithelium have allowed researchers to speculate the contribution they make to not only innate immunity but the acquired immune response. By triggering an immune response through TLR signalling, the epithelium is poised to secrete products that will lead to the recruitment of inflammatory cells to areas of invasion. As part of the innate response, it would be important to establish TLR expression on conjunctival epithelial cells, which will be the focus of this chapter.

3.2 Expression of TLR2, TLR4 and TLR9 at the ocular surface

The expression of certain molecules on the conjunctiva allows the epithelium to respond, hence giving innate protection against invasion from foreign particulates. Unfortunately, with some chronic ocular inflammatory disorders there is a need to treat patients with immunosuppressive drugs such as steroids. Problems arise when patients become immunocomprimised and are therefore subject to secondary infections, which may be bacterial, fungal, viral or parasitic in origin. In such a case, there may be an increase in TLRs in response to this infection from subjects suffering from ocular inflammatory disorders. To address this we looked at the mRNA expression levels in freshly isolated conjunctival swab specimens of certain TLRs, the expression of which has been shown to be altered during disease.

There was basal expression of TLR2, TLR4 and TLR9 detected at low levels in normal conjunctival swabs. For both TLR2 and TLR4, expression in OCP and AKC conjunctival swabs appeared to be greater than that of normal controls. When compared with AKC, expression levels in OCP were also greater, though there was not a significant difference between the groups (Figure 3-1A and B). In contrast, the expression of TLR9 was slightly increased in AKC compared with normal and OCP samples, again this did not reach significance (Figure 3-1C). Taking into account the ratio between each gene and HPRT1, the level of expression of TLR2 and TLR4 between all three groups is greater than TLR9 expression. In this study it appears that there is no association between the level of TLR expression and ocular inflammation at the conjunctiva.

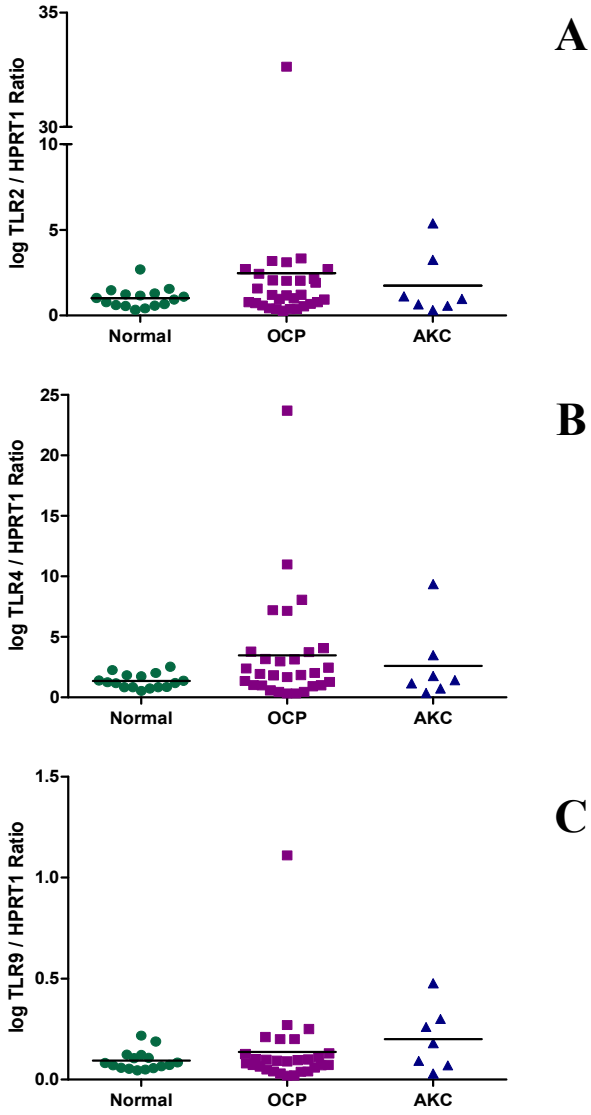

Figure 3-1 TLR mRNA expression remains unaltered in normal and inflamed conjunctiva. Conjunctival swabs taken from normal (n = 16), OCP (n = 35) and AKC (n = 7) subjects were used to isolate RNA. The expression of mRNA for TLR2 (**A**), TLR4 (**B**) and TLR9 (**C**) was assessed by real-time RT-PCR**.** Each symbol represents an individual and lines indicate the mean expression, statistical analysis was carried out using 2-tailed Mann Whitney U test.

3.3 Effect of microbial peptide exposure on conjunctival epithelial cells

To further investigate the role of TLRs in innate immunity at the ocular surface, it is important to determine whether the cells found at the conjunctiva have the ability to elicit an inflammatory response to microbial antigens. During inflammation, there is usually an influx of cells such as neutrophils, attracted to the site via the secretion of chemokines such as IL-8. Cell migration and adhesion to inflammatory sites are also directed by CD54 and CD44.

A conjunctival epithelial cell line was cultured in the presence of zymosan, poly I:C or LPS, ligands to TLR2, TLR3 or TLR4 respectively, and the response examined by looking at their secretion and expression of activation markers (sCD44, IL-8 and CD54) by ELISA and flow cytometry (Figure 3-2). The production of sCD44 was significantly increased in response to exposure to zymosan ($p<0.05$) and poly I:C ($p<0.01$) in comparison to untreated cells. Treatment with LPS did not result in a significant difference in sCD44 expression above that of unstimulated cells (Figure 3-2A). To further confirm whether the cells were indeed activated, IL-8 secretion was also quantitated. Unlike sCD44, exposure to poly I:C only resulted in a significant increase in IL-8 ($p<0.01$; Figure 3-2B). The expression of CD54 was very high in unstimulated cells and therefore the mean fluorescent intensity (MFI) was used to represent CD54 expression by epithelial cells (Figure 3-2C). Again, it was only upon treatment with poly I:C that significant expression of CD54 was observed ($p<0.01$) compared to unstimulated cells (Figure 3-2D). This suggests that the cells can be activated by at least two of the three TLR ligands used in this study.

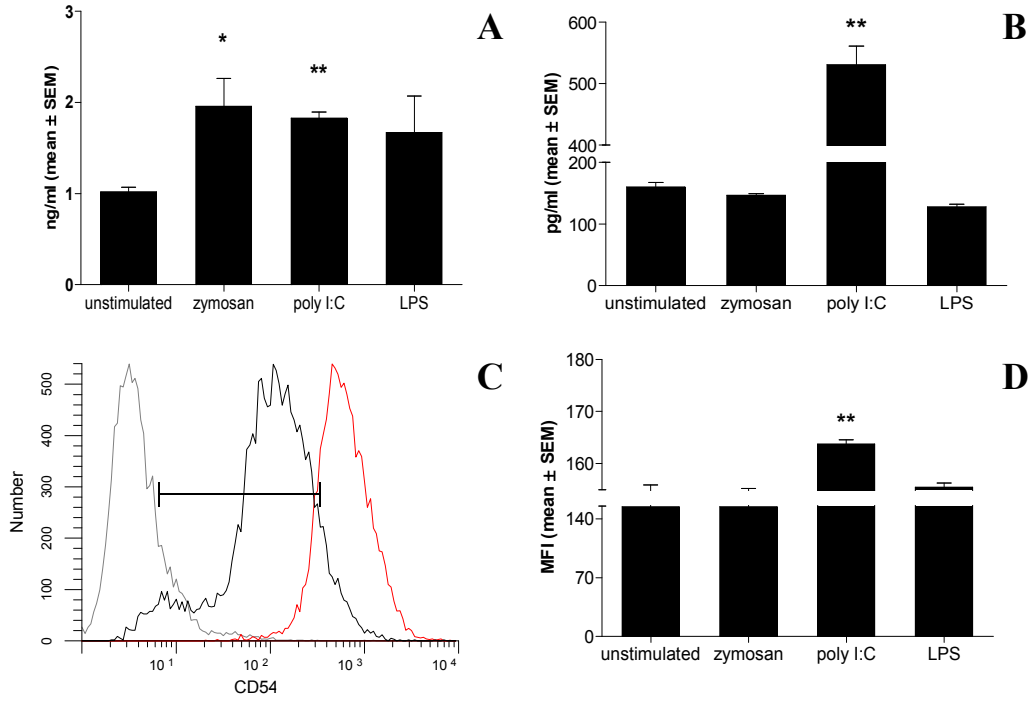

Figure 3-2 Conjunctival epithelial cells respond to certain fungal and viral ligands. IOBA-NHC epithelial cells were stimulated for 24 hours with zymosan, poly I:C and LPS after which, culture supernatants were collected for sCD44 (**A**) and IL-8 (**B**) production by ELISA. Cells were also harvested and stained for their expression of CD54 (**C**) and acquired by flow cytometry; where: - - - represents IgG control, — represents unstimulated cells and — represents poly I:C stimulated cells. Due to the high level of CD54 expression by unstimulated cells, the mean fluorescent intensity was used to show the level of CD54 expression (**D**). Results were analysed by taking a comparison between treated and untreated cells, *** *p*<0.05**; **** *p*<0.01**.

3.4 Effect of allergens on conjunctival epithelial cells

In addition to pathogens, allergens can induce inflammatory responses to some degree at the ocular surface during SAC but not chronic inflammation as seen in disorders such as AKC and OCP. In SAC there is usually infiltration of mast cells, eosinophils and neutrophils. We therefore hypothesised that conjunctival epithelial cells would have the ability to be stimulated by allergens found in the environment and initiate an inflammatory response. This was again monitored by their secretion of sCD44 and IL-8 and their expression of CD54.

Treatment with the allergen peptides led to a significantly increased secretion of sCD44 in cells exposed to *D.farinae* and *D.pteronyssinus* ($p<0.01$ for both) above that of untreated cells, the greatest effect was detected in cells treated with Timothy grass ($p<0.01$; Figure 3-3A). The level of IL-8 remained unaltered after 2 hours in the presence of the peptides, at 6 and 24 hours IL-8 secretion increased in relation to cells treated with the same peptide after 2 hours, this increase failed to reach significance when compared to unstimulated cells (Figure 3-3B). Again the expression of CD54 was already high in unstimulated cells and treatment with the 3 peptides failed to augment this expression (Figure 3-3C). This data implies that the conjunctival epithelial cell line used in this study may be partly activated by allergens; hence the increase in sCD44 but not IL-8 and CD54 compared to unstimulated cells.

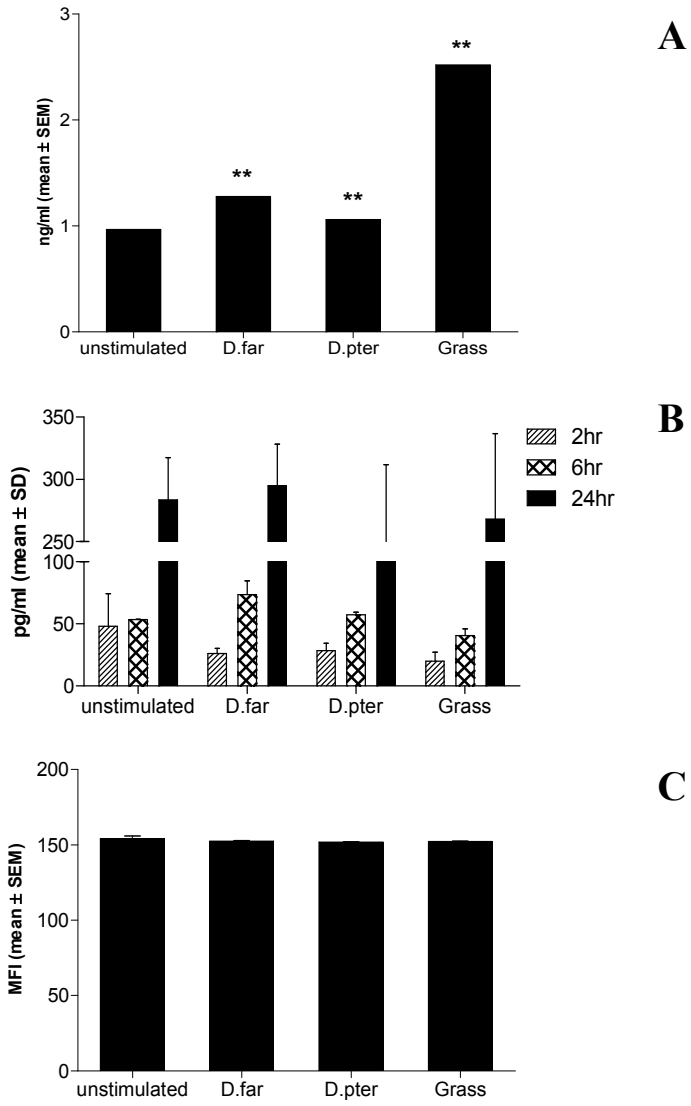

Figure 3-3 Conjunctival epithelial cells respond to allergens. IOBA-NHC epithelial cells were stimulated for 2, 6 and 24 hours with *D.farinae*, *D.pteronyssinus* and Timothy grass and culture supernatants were collected for sCD44 (**A**) and IL-8 (**B**) production by ELISA. Cells were also stained for their expression of CD54 (**C**) and acquired by flow cytometry. Statistical analysis was carried out using Student's T test, taking a comparison between treated and untreated cells, ** $p<0.01$.

3.5 Discussion

The ability of the epithelium to recognise microbial pathogens and produce cytokines that function to recruit other cells to the site of injury is crucial in immunity. This data confirms the expression of TLRs 2, 4 and 9 in the conjunctiva, which is necessary in recognising invading molecules. Epithelial cells of the conjunctiva also respond to microbial challenge, demonstrated here by the TLR3 ligand (poly I:C) induced increase in the secretion of IL-8 and upregulation of the adhesion molecule, CD54. In contrast, the allergen peptides failed to augment cytokine secretion and CD54 expression.

Earlier studies have indicated that allergic eye disease can augment the level of TLR expression, demonstrated by an increase in TLR4 expression and decrease in TLR2 expression in VKC subjects (Bonini *et al*, 2005). Our data was unable to correlate the expression of TLRs with disease, although we cannot draw the same conclusions as the Bonini study as the tissues used in this study were from AKC subjects. Subjects with VKC or AKC may be susceptible to different infections as they are mediated by different cell types. This may be of relevance for example during infection by intracellular pathogens. Such an infection can be diminished by IFN-γ present in Th1 mediated disorders such as AKC. Conversely, subjects with VKC that have dominant Th2 expression are afforded less immunity towards infection by intracellular parasites. This may explain the differential expression of TLRs observed by the authors. In contrast, another group has shown elevated levels of TLR2 from impression cytology AKC specimens, the authors also highlighted the finding that

IFN-γ and *Staphylococcus aureus* stimulated conjunctival epithelial cells as seen by upregulated TLR2 (Cook *et al*, 2006). It is therefore conceivable that IFN-γ present in the micro-environment of subjects with AKC could trigger enhanced TLR2 expression; unfortunately our findings were unable to prove this. We are unable to say whether these subjects exhibited secondary infections, and any steroids administered at the time may have had an effect on TLR expression. From the OCP and AKC tissue specimens that were investigated, it is clear that in each group there were a few subjects that had all three TLRs upregulated above controls. It is therefore possible that these subjects may have been suffering from an ongoing infection. TLR expression is not exclusive to the conjunctival epithelium, neutrophils, monocytes, dendritic cells and T cells have been shown to express a few if not all TLRs (O'Mahony *et al*, 2008; Bonini *et al*, 2005). Our method of obtaining samples was by the use of conjunctival swabs which will always result in a heterogeneous population of cells; as a result we are unable to differentiate any augmentation in TLR expression from epithelial cells only.

The expression of TLRs at the ocular surface has already been established, with TLRs1-10 present at the mRNA level of the conjunctival epithelium (Lambiase *et al*, 2011). Many reports have examined the role of *S. aureus* on the inflammatory response of human corneal or conjunctival epithelial cells. Notably, TLR2 becomes activated and cells respond by inducing pro-inflammatory cytokine production (IL-6, IL-8 and TNF-α) as well as CD54 (Li *et al*, 2008; Cook *et al*, 2005). The results from this chapter reveal a disparate profile in cells treated with the TLR2 ligand, zymosan. Other than upregulating sCD44 secretion, zymosan failed to alter IL-8 and CD54

responses; this was also true for LPS. One hypothesis which may explain the lack of response from zymosan and LPS may be due to two differing factors; the location of the TLRs and a possible deficiency in MD2 expression. It has been demonstrated that TLR2 and TLR4 are localised in the cytoplasm of human corneal epithelial cells and not on the surface, as such the cells were unresponsive to LPS as they failed to upregulate IL-6 and IL-8 (Ueta *et al*, 2005). Conversely the accessory molecule to TLR4 – MD2, which is essential in LPS recognition, has very minimal expression if at all in conjunctival epithelial cells. However, pre-treatment of these cells with IFN-γ augmented MD2 expression thus allowing the cells to respond to LPS (Talreja *et al*, 2005). In contrast to zymosan and LPS, the TLR3 ligand – poly I:C was a powerful stimulant on epithelial cells in our study. Poly I:C is a synthetic dsRNA analogue that mimics viruses, it has been implicated as an inducer of inflammatory cytokines, namely IL-6 and IL-8 (Ueta *et al*, 2005). More recently it has been described in human conjunctival epithelial cells as having the ability to upregulate nearly 200 transcripts of cytokines, chemokines and molecules of the epithelial cell surface, thereby indicating a role for TLR3 in regulating the allergic immune response (Ueta *et al*, 2010). As we have demonstrated that TLR ligands, especially poly I:C, are capable of activating conjunctival epithelial cells, they will be used throughout the remaining study to monitor the cells response.

Another important mediator in allergic disorders is that of allergens which normally induce a milder and more common form of allergic conjunctivitis such as that seen in SAC and PAC, where mast cells predominate. There are many sources of allergens in the environment, the most common of which in allergic conjunctivitis are thought to

be two species of house dust mite, *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* (Seto *et al*, 2009). Current literature has shown in both human bronchial and conjunctival epithelial cells that both allergens stimulate the induction of IL-8, IL-6 and GM-CSF cytokine release (King *et al*, 1998; Seto *et al*, 2009). In our study we had the addition of a further allergen – Timothy grass, but we were unable to reproduce such findings. As with the use of TLR2 and TLR4 ligands only sCD44 was modulated above that of unstimulated cells. Treatment with grass produced a pronounced sCD44 response but failed to provoke a similar effect in IL-8 secretion and CD54 expression, which may point towards divergent signalling pathways utilised by receptors to the allergens. The mechanism in which allergens can promote inflammation is usually through the production of proteases, in the case of house dust mites this could take the form of serine or cysteine proteases (Seto *et al*, 2009). Proteases activate protease-activated receptors (PARs), in which PAR1 and PAR2 have been demonstrated to be expressed in human conjunctival epithelial cells (Nickel *et al*, 2006). By stimulating the cells with PAR1 and PAR2 activators, which led to an increase in production of IL-6, the authors illustrated that both receptors are functionally active (Nickel *et al*, 2006). Different proteases activate different PARs, for example, thrombin activates PAR1 and trypsin PAR2 (Nickel *et al*, 2006). As our conjunctival epithelial cells were routinely passaged, it is possibly that before the addition of allergens to the culture, the receptors were already activated. These actions may also have resulted in p38 phosphorylation as described in studies involving PAR2 activation by trypsin on oral keratinocytes (Rohani *et al*, 2010). Their investigation also revealed that induction of

innate immune responses via phosphatidylinositol 3 kinase (PI3K) signalling, may be inhibitory to the actions of PAR1 and PAR2. Further interference with allergen treatment in our study may have activated this signalling pathway and subsequently dampened the cells response.

We have shown that the conjunctival epithelium does take part in innate immunity through the actions of receptors on their surface and by their production of inflammatory cytokines. By using a conjunctival epithelial cell line and culturing them with antigens that are commonly found in our surroundings, we have established a model that can be used to mimic the environment of the conjunctiva during allergic conjunctivitis. Further investigations will explore how the epithelium may be contributing to allergic disorders through their production of Th2 associated cytokines.

Cytokine micro-environment of conjunctival epithelial cells during chronic allergic eye disease

4.1 Introduction

Of the many forms of allergic conjunctivitis that have been documented, specific characteristics have been identified that can distinguish between the different types. The main features that typify allergy at the ocular surface usually arise from the inflammatory environment of the eye in the course of the conjunctivitis. During mild ocular allergy such as SAC, mast cells are the predominant cell type. In such a situation mast cells become activated leading to subsequent degranulation and release of histamine, prostaglandins and leukotrienes (Trocme & Sra, 2002). Although other cell types such as eosinophils and neutrophils have also been found to be present in conjunctival tissues during SAC, they are not considered to take part in driving this disorder (Anderson *et al*, 1997). As well as the different mediators that are released by mast cells, they also secrete the Th2 cytokine IL-13 during the late phase reaction (Trocme & Sra, 2002).

In ocular allergy such as SAC, there have not been any reports of the presence of T cells in this disorder in contrast to chronic disorders affecting the eye such as AKC and VKC which are dominated by T cells infilitrating the area. These subtypes of allergic conjunctivitis are characterised by increased mRNA expression and enhanced expression of IFN-γ in AKC tissues and IL-5 in VKC tissues (Metz *et al*, 1997; Calder *et al*, 1999). In addition to the T cell mediated effects seen in other chronic diseases in addition to CAED, an epithelial-derived cytokine has been

implicated in driving the allergic inflammatory response. TSLP is the cytokine in question and it has been reported that activated, naïve $CD4^+$ T cells are able to produce high amounts of IL-5, IL-13 and TNF-α when primed by TSLP-activated DCs and Langerhans cells (Soumelis *et al*, 2002). In contrast it has also been demonstrated that decreased levels of IL-10 and IFN-γ are seen under the same conditions (Ebner *et al*, 2007). Both of these observations suggest a role for TSLP in $CD4^+$ T cell differentiation into effector Th2 cells and subsequent Th2 mediated allergic disease. Further evidence to support the role of TSLP in the pathogenesis of allergic disease has shown an increase in the expression of this cytokine in the lesional skin of individuals with inflammatory disorders as well as in asthmatic airways (Soumelis *et al*, 2002; Ying *et al*, 2005).

With such compelling evidence it is tempting to speculate that, as well as the other cytokines conjunctival epithelial cells are known to produce, as detailed in chapter 3, they might also be capable of secreting T cell-associated cytokines. The ability to demonstrate this characteristic will enable the conjunctival epithelium to be directly involved in both the innate and adaptive arms of the immune response. This chapter will aim to identify whether this may indeed be true and if it has any bearing on the severity of allergic conjunctivitis.

4.2 IL-13 is localised to the conjunctival epithelium in CAED

Evidence to date has shown that the expression of IL-13 in CAED, either from mRNA expression, tear samples or T cells isolated from conjunctival biopsies, is found to be increased above normal control subjects (Montan *et al*, 2002; Leonardi *et al*, 2006; Calder *et al*, 1999). This has led to the belief that IL-13 may correlate with severity of disease in CAED. So far the expression of IL-13 has not been shown in conjunctival tissue specimens in both the mild and chronic ocular allergies. To study the expression of IL-13 at the conjunctival surface, biopsy sections were used to evaluate whether the expression of this cytokine is altered according to disease state.

4.2.1 IL-13 expression at the ocular surface

To investigate whether biopsies from CAED tissues express increased levels of IL-13 compared to normal controls, either expressed by conjunctival epithelial cells or by $CD3^+$ T cells, two colour immunohistochemical staining was used.

As a negative control, an AKC section was stained with a CD3 and IL-13 IgG control antibody, a minimal level of background staining was picked up along the epithelium (Figure 4-1A). Within SAC sections, goblet cells found in the epithelium demonstrated significant IL-13 staining (Figure 4-1B). In CAED sections (AKC) there was double staining of CD3 and IL-13 within the epithelium – localised to those areas where there is darker brown/black staining, indicating the combination of red and brown staining (Figure 4-1C). $IL\text{-}13^+$ staining was also observed to be scattered in the stroma (Figure 4-1C). In VKC tissues stained for IL-13 alone, the

epithelium was also observed to have intense IL-13 along the epithelium (Figure 4-1D&E).

The sections were graded to quantify the staining intensity of IL-13 staining between the different ocular disorders and the controls. Within the epithelium, IL-13 staining was intense in both CAED sections and GPC above that of the controls ($p<0.05$). There was also increased IL-13 staining in SAC sections which was predominantly found in the goblet cells within the epithelium (Figure 4-2A). Conversely, in the stroma IL-13 staining was not detectable in the controls, SAC or GPC groups, and the staining that was observed in VKC and AKC was weak and failed to reach significance above controls (Figure 4-2B).

The level of IL-13 expression at the ocular surface was further characterised by quantifying IL-13 mRNA in conjunctival swabs from normal, OCP and AKC subjects. As demonstrated with the results from the immunohistochemical staining, normal subjects had either very low expression of IL-13 mRNA or none at all. The same was also found within the OCP group, although one individual had higher levels of IL-13. There was a significantly greater expression of IL-13 in AKC compared with normals ($p<0.05$) and OCP ($p<0.01$) (Figure 4-3).

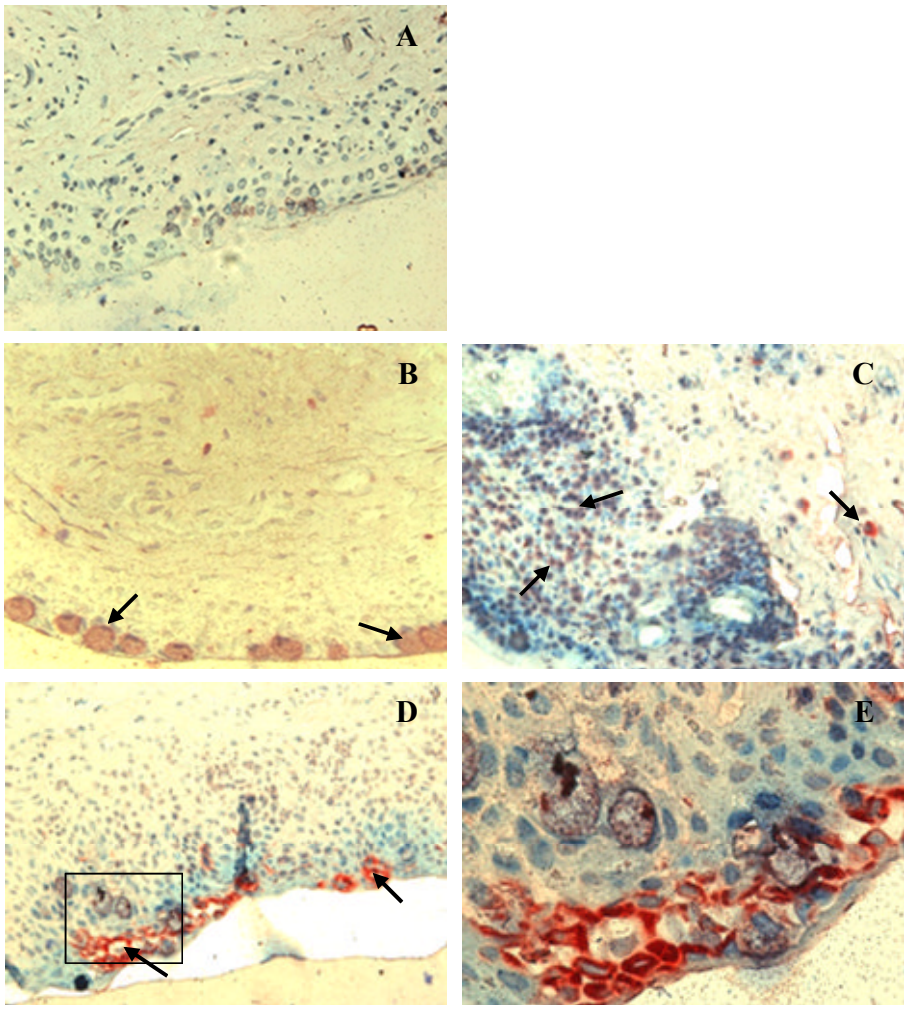

Figure 4-1 IL-13 is localised to the epithelium and stroma of conjunctival tissues. Conjunctival biopsies were stained for the expression of CD3 (brown) and/or IL-13 (red) by immunohistochemistry; (**A**) AKC; n = 6 [CD3 and IL-13 IgG control], (**B**) SAC; n = 3 [double stain, CD3 and IL-13], (**C**) AKC; n = 6 [double stain, CD3 and IL-13], (**D**) VKC; n = 6 [IL-13], (**E**) VKC; n = 6 [IL-13] magnified insert of D. Arrows indicate areas of positivity. Images were captured using Motic BA400; A- D, x400 magnification; E, x630 magnification.

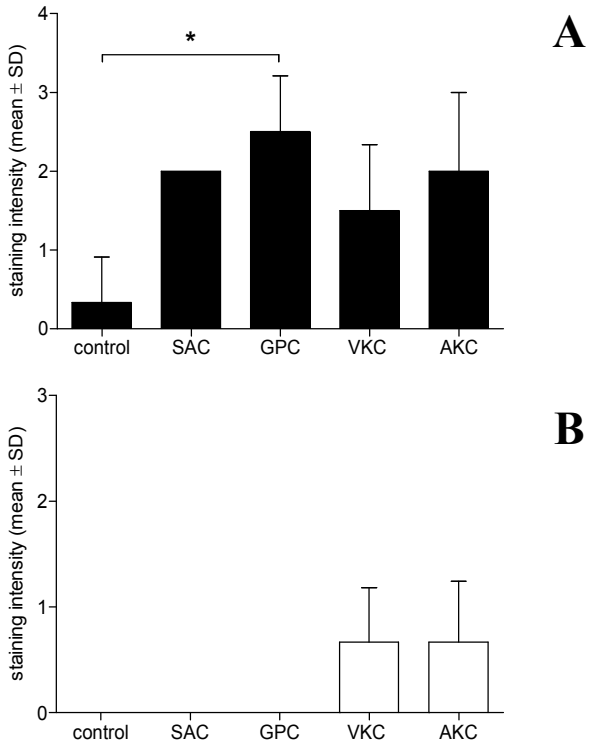

Figure 4-2 IL-13 expression at the conjunctiva. Conjunctival biopsies stained for IL-13 expression as indicated in figure 4-1 were graded based on their staining intensity in the epithelium (**A**) and stroma (**B**). Intensity of IL-13 staining from non-inflamed controls (n = 3), SAC (n = 3), GPC (n = 3), VKC (n = 6) and AKC (n = 3) were scored between 0-3 where 0 = no staining, 1 = weak, 2 = moderate, 3 = strong staining. Results are shown as mean ± SD. ***** $p<0.05$

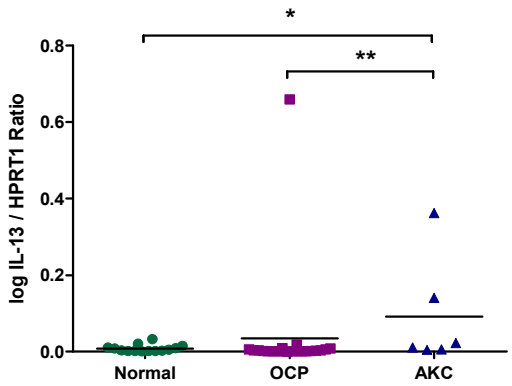

Figure 4-3 IL-13 mRNA is expressed at the ocular surface and increased in AKC. Conjunctival swabs taken from normal (n = 16), OCP (n = 35) and AKC (n = 7) subjects were used to isolate RNA. The expression of mRNA for IL-13 was assessed by real-time RT-PCR**.** Each symbol represents an individual and lines indicate the mean expression. ***** ***p* <0.05; ****** ***p* <0.01.**

4.2.2 IL-13 expression in conjunctival epithelial cells

So far, we have demonstrated that IL-13 is expressed at the conjunctival surface and is upregulated in CAED. Part of this expression was attributed to $CD3^+$ T cell expression of IL-13. In those sections where IL-13 staining alone was detected on the epithelium, its cellular source was unknown. One hypothesis is that the conjunctival epithelium secreted IL-13 in response to the cytokines that are associated with CAED pathogenesis. To investigate whether conjunctival epithelial cells secrete IL-13, the IOBA and ChWK cell lines were used and cells were stimulated for 24 and 48 hours in the presence of PMA, IFN-γ, IL-4, poly I:C, IL-1β, or TNF-α ± IL-1β. There were no detectable levels of IL-13 secreted by either conjunctival epithelial cell lines, suggesting that the staining observed is of IL-13 binding to the surface of the epithelium, and not secretion by conjunctival epithelial cells.

4.2.3 IL-13 mRNA expression in the conjunctiva

Although the conjunctival epithelial cells did not secrete IL-13, we wanted to confirm this finding by looking at IL-13 mRNA expression in the cell line. In this experiment only the IOBA cell line was used, the cells were treated with various stimuli for 1, 4, 6 and 24 hours before harvesting to isolate RNA and quantitate using real-time RT-PCR.

The expression of IL-13 mRNA could be found in all cells receiving treatment after 4, 6 and 12 hours, but there were no significant differences between the groups (data not shown). In contrast treatment of the cells for 1 hour with TNF-α, IL-4 or IL-13 led to significantly increased expression of IL-13 ($p<0.05$ in all). TNF-α synergised with IL-4 and IL-13 to significantly upregulate IL-13 expression ($p<0.01$; Figure 4-4A). As well as the pro-inflammatory and Th2 cytokines, all three TLR agonists - zymosan ($p<0.05$), poly I:C ($p<0.01$) and LPS ($p<0.05$) – induced significant increases in IL-13 expression. Of these, poly I:C appeared to have a greater effect above zymosan and LPS but this failed to reach significance (Figure 4-4B).

From this data we can speculate that IL-13 may be involved in CAED and although conjunctival epithelial cells do not secrete this cytokine, the ability to induce IL-13 mRNA expression in these cells leads us to assume that epithelial cells may be involved in supporting the IL-13 environment seen in CAED.

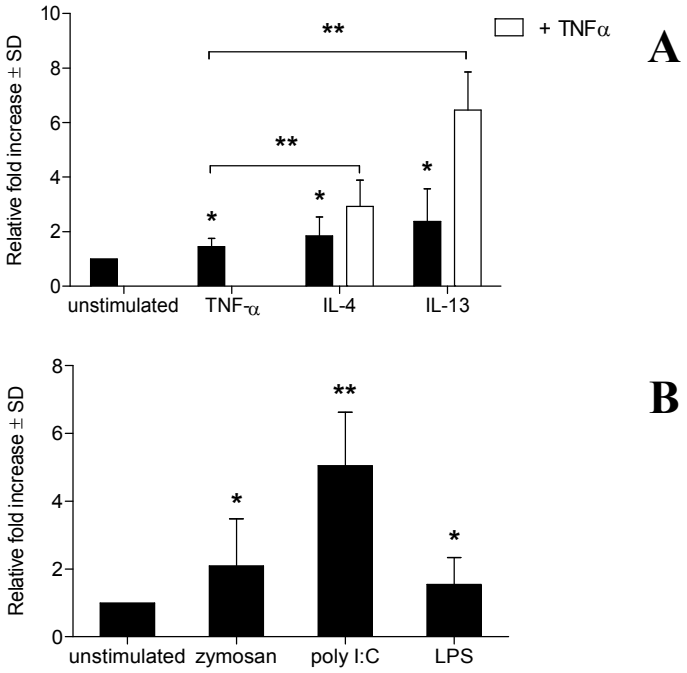

Figure 4-4 IL-13 mRNA expression can be induced in conjunctival epithelial cells. IOBA cells were either left untreated or treated with pro-inflammatory and Th2 cytokines (**A**) and TLR agonists (**B**) for 1 hour. Cells were harvested and RNA was isolated before conversion to cDNA by reverse transcription. IL-13 mRNA was analysed by real-time RT-PCR and the house keeping gene β-actin was run in parallel. *** *p*<0.05; ** *p*< 0.01**

4.3 TSLP is expressed throughout the conjunctiva in CAED

There are many mechanisms that may contribute to the pathogenesis of CAED; one hypothesis currently being explored involves the epithelial cell derived cytokine TSLP, which has been reported to be increased in inflamed tissues of the skin and lungs (Soumelis *et al*, 2002; Ying *et al*, 2005). As the epithelium of the conjunctival surface shares many properties with other mucosal sites, we speculated that like that of the lungs, the conjunctival tissues of CAED subjects will also show increased expression of TSLP. It has recently been reported that primary human conjunctival epithelial cells can be induced to upregulate TSLP transcripts and significantly increase TSLP protein production by poly I:C stimulation (Ueta *et al*, 2010).

4.3.1 TSLP expression at the ocular surface

To examine the expression of TSLP, GMA embedded conjunctival biopsy sections were stained by immunohistochemistry. The sections were also graded using a 4 point scale to demonstrate staining intensity.

Using an AKC section stained with a control antibody as our negative control, we detected some background staining (Figure 4-5A). The normal controls (Figure 4-5B) and SAC sections (Figure 4-5C) did not show any detectable TSLP expression. In comparison in CAED sections a very strong expression of TSLP was observed (Figure 4-5D and E). Within the AKC and VKC sections TSLP expression appears to be continuous in the epithelium and stroma in AKC sections (Figure 4-5D), whilst the staining found in VKC sections is very intense in the epithelium (Figure 4-5E) compared to AKC.

The staining results were graded based on TSLP staining intensity. TSLP expression within the epithelium was significantly increased in VKC than in AKC ($p<0.05$; Figure 4-6A). The distribution of TSLP expression in the stroma was less variable (Figure 4-6B); there were no significant differences between the two different forms of allergic eye disease. Also, the grading revealed that although epithelial TSLP was absent in SAC sections, within the stroma there was some expression evident (Figure 4-6B).

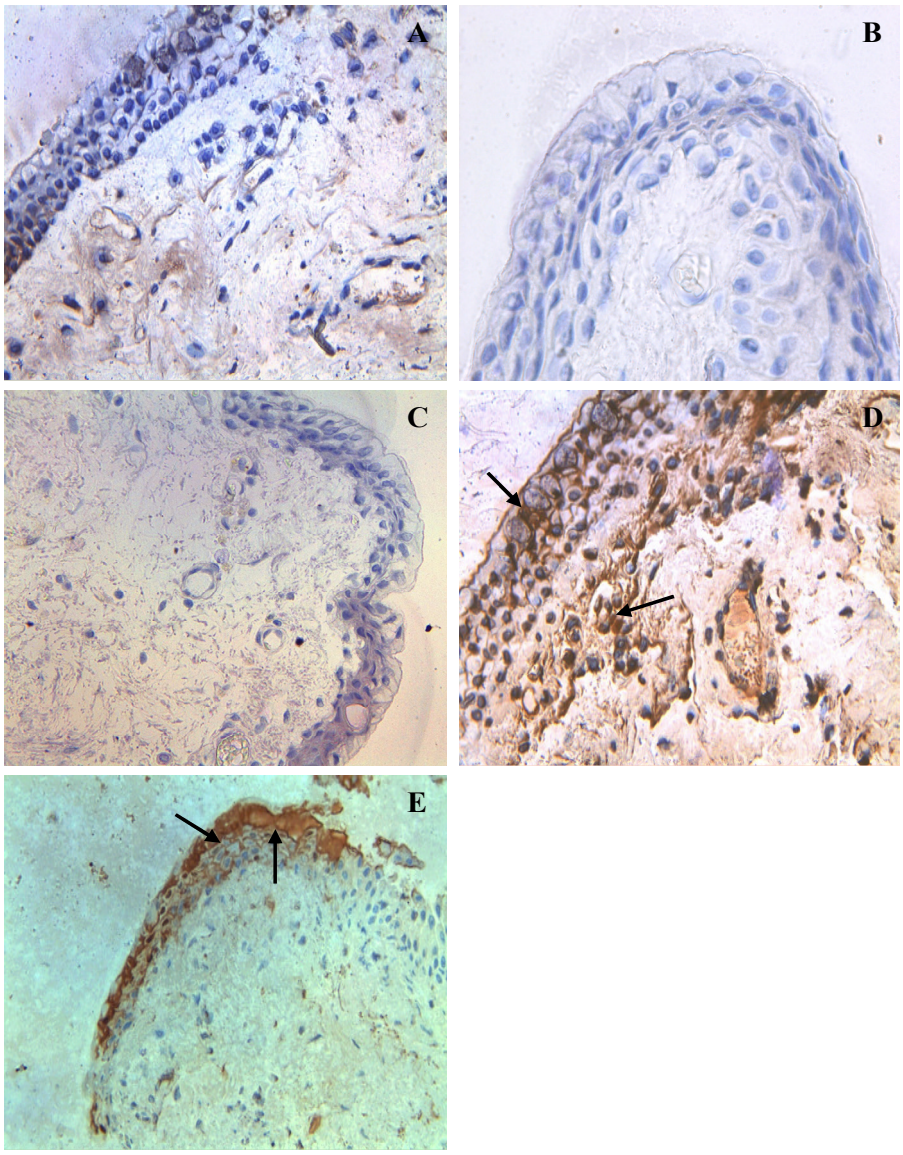

Figure 4-5 TSLP is localised to the conjunctival epithelium of CAED tissues. Conjunctival biopsies taken from (**A**) AKC (n = 5; IgG control), (**B**) non-inflamed control; n = 3, (**C**) SAC; n = 3, (**D**) AKC; n = 5 and (**E**) VKC; n =5, were evaluated for TSLP expression by immunohistochemical staining. Arrows pointing to brown staining indicate TSLP expression. Images were captured with Motic BA400; A and E, x200 magnification; B-D, x400 magnification.

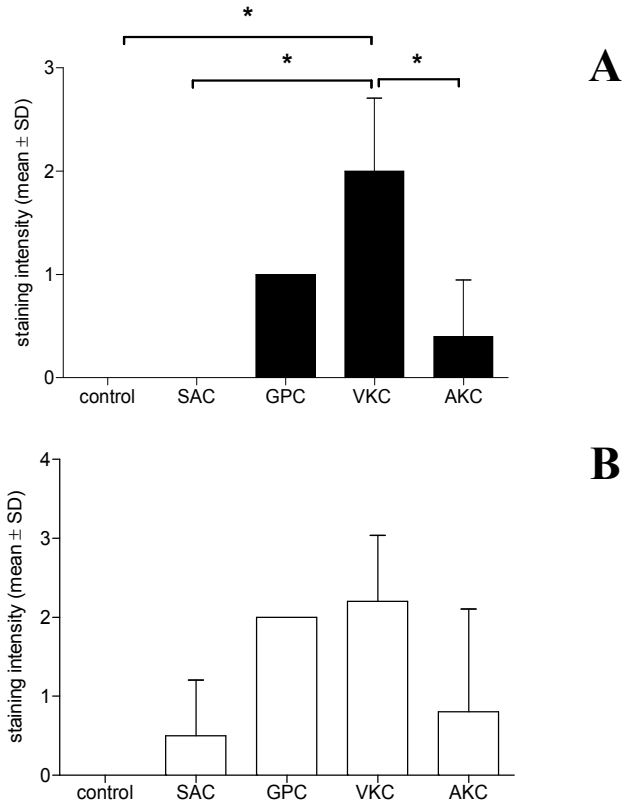

Figure 4-6 TSLP is upregulated in the conjunctival epithelium in VKC tissues. Conjunctival biopsies obtained from 18 subjects; non-inflamed controls (n = 3), SAC (n = 3), GPC (n = 3), VKC (n = 6) and AKC (n = 3), were stained for TSLP expression as described in figure 4-2. Biopsies from each subject were graded on the basis of TSLP staining intensity in the epithelium (**A**) and stroma (**B**) and scored between 0-3 where 0 = no staining, 1 = weak, 2 = moderate, 3 = strong staining. Results are shown as mean ± SD. *** *p*<0.05.**

4.3.2 TSLP protein production

Our findings demonstrated TSLP localisation in the epithelium of CAED tissues, this led us to examine whether TSLP is also secreted by conjunctival epithelial cells which has been reported to be expressed by bronchial epithelial cells (Kato *et al*, 2007). Based on their study, both IOBA and ChWK epithelial cells where treated with PMA, IFN-γ, IL-4 ± IL-1β, IL-13 ± IL-1β, , IL-1β, TNF-α ± IL-1β or poly I:C for 4, 24 and 48 hours. Supernatants were assayed for TSLP secretion by ELISA.

Both conjunctival cells lines had very minimal secretion of TSLP; the minimal detectable range for the ELISAs was 40pg/ml (Figure 4-7A & B). As Kato et al had shown TSLP secretion in bronchial epithelial cells, we used a bronchial cell line (16HBE) in parallel as a positive control. Like the conjunctival cells lines there was very low secretion of TSLP with the majority of treatments at all time points (data not shown), but treatment with poly I:C ($p<0.01$) and PMA ($p<0.01$) after 24 hours significantly upregulated TSLP secretion above untreated cells. Poly I:C also had the greatest effect in inducing TSLP secretion compared to PMA treated ($p<0.01$) (Figure 4-7C).

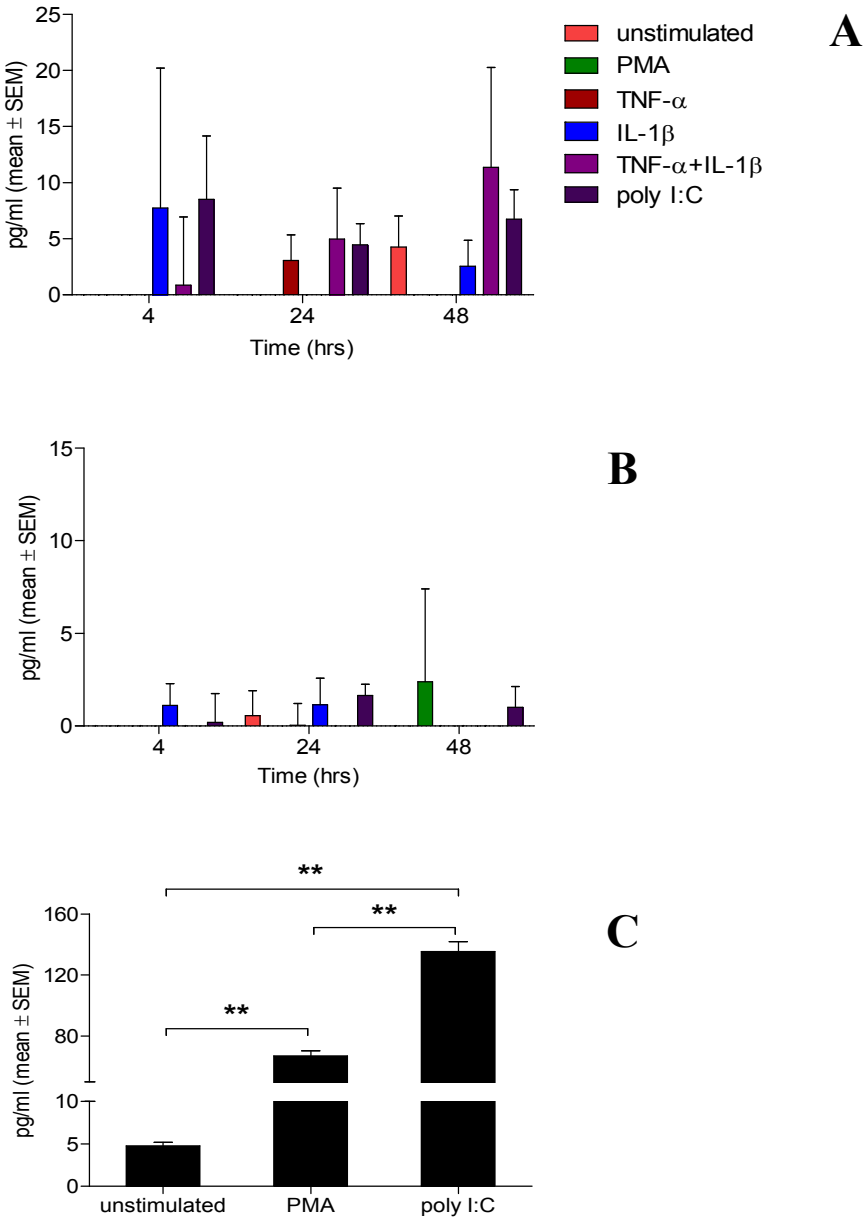

Figure 4-7 TSLP is secreted by human epithelial cells. Two human conjunctival epithelial cell lines; IOBA (**A**) and ChWK (**B**) and a bronchial epithelial cell line [16HBE] (**C**) were treated with various stimuli for 4, 24 and 48 hours. Supernatants were collected at each time point and assayed for TSLP secretion by ELISA. Results are taken from five independent experiments and are shown as mean ± SEM of the stimulations where TSLP was above the minimum detectable range. ****p* **<0.01**

4.3.3 TSLP mRNA is expressed in conjunctival epithelial cells

Unlike our finding and other reports citing TSLP secretion in bronchial epithelial cells, we were unable to detect secretion of this cytokine by conjunctival epithelial cells. As with IL-13 we sought to determine whether conjunctival epithelial cells express TSLP at the mRNA level. Of the different treatments that were used to address TSLP secretion in this study, we focussed on IL-4, IL-13 and their combination with TNF-α as well as TLR agonist, especially poly I:C which has been demonstrated to significantly upregulate TSLP mRNA (Kato *et al*, 2007). Cells were treated for 4 and 6 hours before harvesting and RNA isolation. Quantification was carried out by real-time RT-PCR.

At the 6 hour time point, both unstimulated and stimulated cells expressed TSLP mRNA, indicated by their cycle threshold values but there was no difference observed between the groups (data not shown). Within 4 hours of the cells being treated TNF-α stimulation significantly upregulated TSLP expression ($p<0.01$). Combination of TNF-α with IL-4 or IL-13 did not augment the level of expression. On their own, IL-4 and IL-13 also upregulated TSLP mRNA, IL-13 had the greatest effect increasing expression up to 150 fold (Figure 4-8A). All the TLR agonists induced significant upregulation of TSLP mRNA ($p<0.01$ in all), at least almost 200 fold compared with previous stimulations. Of the three TLR agonists, poly I:C treatment produced the greatest increase though it was not significantly higher (Figure 4-8B).

Taken together we can say that there is a strong association with TSLP and its role in CAED, which is especially apparent in VKC. Although the conjunctival epithelial

cells used did not secrete TSLP, we were able to show that in an inflammatory environment these cells are capable of inducing TSLP mRNA, which may in time be secreted in the right conditions.

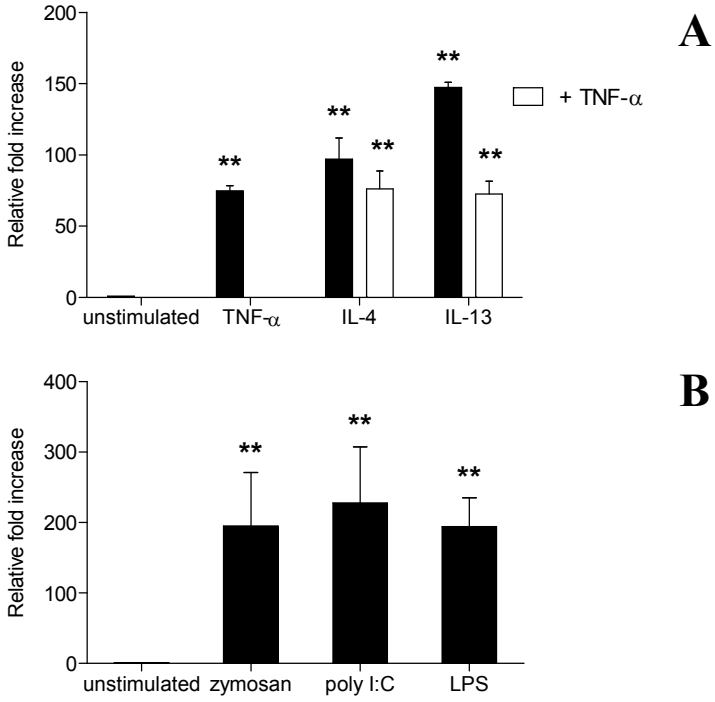

Figure 4-8 TSLP mRNA is expressed by conjunctival epithelial cells. The conjunctival epithelial cell line (IOBA) were treated with pro-inflammatory and Th2 cytokines (**A**) and TLR agonists (**B**), cells were harvested and RNA isolation was carried out. RNA was reverse transcribed and assayed for TSLP expression by real-time RT-PCR. The house keeping gene β-actin was assayed in parallel to account for any variations in the amount of cDNA in each sample. Results are shown as relative fold increase with unstimulated cells given a value of 1. ** $p<0.01$

4.4 Discussion

In the different forms of allergic conjunctivitis, the inflammatory environment of the conjunctiva is clearly influencing the development of the various disorders. As with current literature, we have been able to identify increased IL-13 expression at the ocular surface as well as the ability for conjunctival epithelial cells to express IL-13 mRNA when stimulated with inflammatory cytokines and TLR agonists. More interestingly, the proallergic Th2 cytokine, TSLP, is found to be increased in CAED. Most studies have shown that TSLP is mainly induced by the TLR3 agonist – poly I:C, this data also confirms that finding and shows that TSLP mRNA in conjunctival epithelial cells can be upregulated under Th2 inflammatory conditions as well as with TLR agonists.

Th2 derived cytokines such as IL-13 are important in the promotion of allergic inflammation. This cytokine has been found in significantly increased levels in tear specimens from subjects with SAC, VKC and AKC in comparison to controls (Leonardi *et al*, 2006). Our study has also demonstrated the expression of IL-13 in SAC and CAED conjunctival tissue sections, with CAED tissues showing very strong IL-13 staining and IL-13 positive goblet cells in SAC tissues. Although there was very intense staining of this cytokine, it may not represent actual secretion of IL-13 from epithelial cells but in fact binding of the cytokine to the epithelium. This idea is supported by the undetectable levels of IL-13 in the culture supernatants. IL-13 does appear to be expressed by, and not bound to $CD3^+$ T cells since $CD3^+$ T cells do not have receptors for IL-13. Previous reports support this finding as high levels

of IL-13 have been shown to be produced by conjunctival T cell lines derived from AKC and VKC subjects (Calder *et al*, 1999). The $CD3^+$ T cells in the biopsies may therefore be secreting IL-13 which is, in turn, binding to the epithelium. This further adds to the evidence that the chronic allergic inflammatory process is dependent on the expression of Th2 derived cytokines, perhaps secreted also by mast cells present at the site of inflammation.

There is no evidence of IL-13 secretion by epithelial cells but IL-13 is also present in goblet cells in VKC and AKC sections and it has been reported that IL-13 induces goblet cell hyperplasia of human airway epithelial cells *in vitro* (Atherton *et al*, 2003) and mucin production observed in rat models (Shim *et al*, 2001). This may prove true in VKC and AKC tissues as the source of IL-13 may be due in part to $CD3^+$ T cells; however it does not explain the expression of IL-13 seen in goblet cells of SAC conjunctival tissue, as there were no $CD3^+$ T cells present here. It may be possible that the increased numbers of goblet cells shown in SAC conjunctival tissue, compared to the controls, may have been as a result of IL-13 secreted by mast cells, which are increased in numbers in SAC (Anderson *et al*, 1997). This in turn may have led to the increased proliferation of goblet cells leading to mucus hypersecretion. The mechanism responsible for the presence of IL-13 in the goblet cells is unknown and further work will have to be carried out to elucidate the origin of IL-13 in these tissues.

It has already been reported that there is a role for TSLP in the development of inflammation (Al-Shami *et al*, 2005). It has also been linked to allergic diseases. This

theory has been supported by findings that the expression of TSLP is increased from cells within the bronchial epithelium and submucosa in asthmatics (Ying *et al*, 2005) and acute and chronic atopic dermatitis lesions (Soumelis *et al*, 2002) and TSLP expression here correlates with disease severity (Ying *et al*, 2005). These findings may therefore also be true for those with chronic forms of allergic eye disease (AKC and VKC), where TSLP was highly expressed in comparison to milder conditions such as SAC, therefore supporting the idea that TSLP is associated with the severity of disease, with epithelial cells being central to this pathogenesis.

The difference in TSLP expression demonstrated between VKC and AKC sections and the low level expression of this cytokine in the epithelium of SAC sections may be due in part to the functional characteristics of TSLP. Ying et al reported that TSLP is implicated in asthma pathogenesis, which may be acting through the Th2 cells whose cytokines are responsible for much of the disease pathogenesis (Ying *et al*, 2005). In allergic conditions such as that of SAC, Th2 cytokines are predominant (Fujishima *et al*, 1997) which is also seen in subjects with VKC (Metz *et al*, 1997). The reverse is true for AKC subjects, where there is a more Th1-skewed cytokine profile (Metz *et al*, 1997). Although T cells are not found in SAC, the actions of TSLP may be driving cells capable of secreting Th2 type cytokines, such as eosinophils or mast cells. These differences in cytokine profiles may account for the differences in TSLP expression seen between VKC and AKC, where production of TSLP leads to a more Th2 permissive environment like that seen in VKC.

It has been postulated that upon stimulation of the epithelium, in this case airway epithelial cells, these cells can be induced to secrete TSLP. In these studies the combination of TNF-α and IL-4 or IL-13 proved to be the most effective stimulation for the epithelial cells (Kato *et* al, 2007) as well as airway tissues from patients with asthma (Huang *et al*, 1995). TNF-α and IL-1β as well as double stranded RNA such as poly I:C (Allakhverdi *et al*, 2007) were also shown to be effective stimuli. *In vivo* we have shown that TSLP is expressed in conjunctival tissues from CAED patients. It was therefore important to go back and look at *in vitro* models. Our data was only able to demonstrate TSLP secretion in the bronchial epithelial cell line (16HBE) and only following PMA and poly I:C stimulation. While evidence for TSLP mRNA expression does not prove that it is secreted at the protein level by the cells, it does give us an indication of how the cytokine milieu may alter the characteristics of conjunctival epithelial cells. We were able to show that TSLP mRNA expression can be upregulated in line with recent data that has shown expression of TSLP mRNA in CAED tissues as well as increased TSLP mRNA in a primary human conjunctival epithelial cell line stimulated with poly I:C (Matsuda *et al*, 2010). More interestingly, one study has defined a role for TSLP and its dependency on IL-13 to induce cell proliferation and wound closure in asthmatic bronchial epithelial cells (Semlali *et al*, 2010).

The investigation into IL-13 and TSLP at the ocular surface has allowed us to add to the growing body of evidence that both cytokines are involved in allergic conjunctivitis. By showing IL-13 treatment of conjunctival epithelial cells enhances TSLP mRNA expression we have demonstrated what may be a causal link between

IL-13 and TSLP expression. At this point it is not enough to say that Th1 and Th2 cells and cytokines only contribute to CAED pathogenesis. Other T helper subtypes have been implicated and are thought to play a role in chronic disease, which will be the focus of the next study.

Th17 cells infiltrate the conjunctiva, epithelial cells of which respond to IL-17

5.1 Introduction

The pathogenesis of Th17 cells in human inflammatory disorders has been the subject of much debate. As well as Th1 and Th2 cells, Th17 cells have been found to be present in inflamed tissues of patients suffering from psoriasis, arthritis, Crohn's disease and severe asthma (Pène *et al*, 2008). It is thought that the IL-17 produced by these cells may contribute to the pro-inflammatory environment observed in inflammatory disorders. As well as a pro-inflammatory role, it has been described that Th17 cells are important in host defence towards bacterial and fungal pathogens. This protective function has been reported to work through differing actions such as neutrophil recruitment via IL-8 induction by IL-17, as well as the production of chemokines thereby permitting the recruitment of protective IFN-γ producing $CD4^+$ T cells (Wu et al, 2007; Khader *et al*, 2007). It has also been shown that knockout mice lacking IL-17A and IL-17F have decreased expression of the antimicrobial peptide important in host defence, β-defensin (Ishigame *et al*, 2009). Although known as the signature cytokine for Th17 cells, IL-17A as well as IL-17F can be produced by a number of other cell types such as γδ T cells, NKT cells, NK cells, neutrophils and eosinophils (Korn *et al*, 2009).

The pathogenesis of CAED has been described as involving $CD4^+$ T lymphocytes, namely that of mainly Th1 in AKC and Th2 in VKC, as shown by their cytokine profile during disease (Metz *et al*, 1997; Calder *et al*, 1999). Both disorders have

other cell types present in inflammatory areas of the conjunctival tissues such as mast cells which have been found in AKC and increased in the conjunctival epithelium of VKC (Foster *et al*, 1991; Abu el-Asrar et al, 1989). Mast cells are characterised by their ability to produce an array of mediators such as histamine and cytokines, including IL-4, IL-5, IL-8 and IL-13, giving these cells the ability to initiate allergic inflammation (Church & Levi-Schaffer, 1997; Anderson *et al*, 2001). More recently it has been observed that in the synovium of joints affected by RA, the majority of mast cells found were IL-17A$^+$ and stimulation of mast cells *in vitro* induced IL-17A production (Hueber *et al*, 2011).

With such interplay of cells at the conjunctiva in CAED it is impossible to say which cells and their cytokines are taking part in the pathogenesis of the disease, especially with the emergence of Th17 cells. The aim of this chapter is to establish whether Th17 cells are present in conjunctival tissues and if their levels vary during CAED and to determine the effects of IL-17 at the conjunctival surface.

5.2 Th17 cells infiltrate the conjunctival epithelium in CAED

In the past it was widely thought that the pathogenesis of certain diseases was through the actions of Th1 or Th2, for example RA was described as Th1 mediated. With the discovery of Th17 cells many studies have been trying to ascertain whether this disease is Th1 mediated or Th17 mediated (Yamada *et al*, 2008). As Th17 cells have been found in various inflamed sites (Pène *et al*, 2008), we sought to determine whether these cells are also present in the tissues of CAED, which has been reported to be either Th1 or Th2 mediated.

5.2.1 Th17 cells are localised to the conjunctiva in CAED

To examine whether there is a correlation between chronic diseases affecting the eye and Th17 cells, conjunctival biposy sections were stained for co-expression of CD4 T cells and IL-17 in normal controls, SAC, OCP, VKC and AKC. The normal control sections were negative for both $CD4^+$ T cells and IL-17 (Figure 5-1A); this was also observed in SAC tissues, which were negative in all sections (Figure 5-1B) whilst in OCP tissues few $CD4^+$ T cells and IL-17^+ cells were present (Figure 5-1C). In contrast, increased numbers of $CD4^+$ T cells appeared to be infiltrating areas of VKC tissues (Figure 5-1D) and in the same section, $CD4^+$ IL-17^+ T cells were observed localising to the conjunctival epithelium (Figure 5-1E). Similarly in AKC, $CD4^+$ IL-17^+ T cells were found within areas of the epithelium as well as in the stroma (Figure 5-1F).

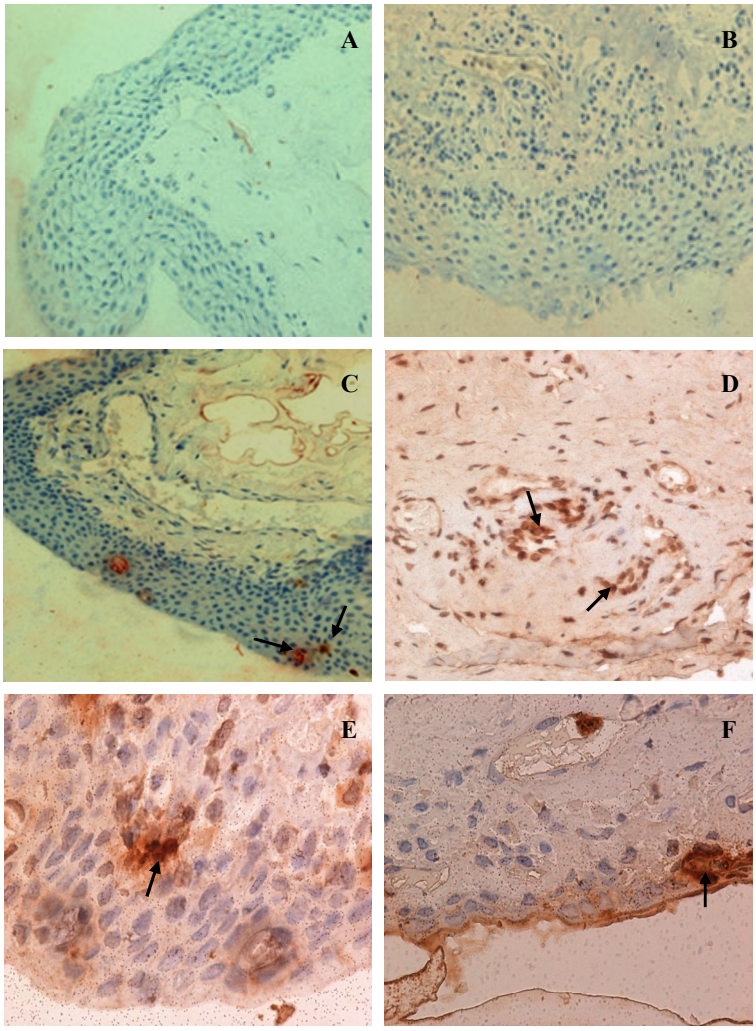

Figure 5-1 Th17 cells localise to the conjunctival epithelium in CAED tissues. Conjunctival biopsies were immunohistochemically stained for the expression of Th17 cells, characterised by $CD4^+$ $IL\text{-}17^+$ cells. Sections were double stained for CD4 (red) and IL-17 (brown), combined the cells have a very dark colouration; (**A**) normal control; n = 6 [double stain, CD4 and IL-17], (**B**) SAC; n = 6 [double stain, CD4 and IL-17], (**C**) OCP; n = 5 [double stain, CD4 and IL-17], (**D**) VKC; n = 6 [1L-17] (**E**) VKC; n = 6 [double stain, CD4 and IL-17] , (**F**) AKC; n = 6 [double stain, CD4 and IL-17]. Arrows indicate positive stained cells. Images were captured using Motic BA400; A - D, x200 magnification; and E - F, x630 magnification.

5.2.2 Th17 attracting chemokines are preferentially secreted by conjunctival epithelial cells

As we have detected an infiltration of Th17 cells in the conjunctival tissues in CAED, it was important to ascertain whether the conjunctival epithelium can orchestrate T cell infiltration into the area, especially that of Th17 cells. Certain pro-inflammatory cytokines that have been observed in the conjunctiva of subjects with CAED were used to stimulate conjunctival epithelial cells as well as co-culturing with pre-activated mast cells. Their supernatants were collected and assayed for secretion of Th1, Th2 and Th17 attracting chemokines by ELISA.

After 24 hours there was minimal secretion of the Th1 attracting chemokine CXCL10 in poly I:C – and TNF-α + IL-1β – treated epithelial cells whilst the combination of these treatments significantly augmented the production of this chemokine (Figure 5-2). In contrast, after 48 hours treatment CXCL10 secretion was detected in unstimulated cells and significantly upregulated with all treatments. There did not appear to be any difference between epithelial cells treated with TNF-α + IL-1β alone or in combination with poly I:C, except in epithelial cells cultured with non-activated CBMCs (Figure 5-2A) or in poly I:C treated CBMCs (Figure 5-2F). However, the addition of CBMCs to epithelial cells did not augment this response as the levels of CXCL10 were similar between the different epithelial treatments that had been cultured with untreated or treated CBMCs.

In the same way we also wanted to determine whether the epithelial cells secreted the Th2 attracting chemokine – CCL17, but unlike CXCL10 the level of CCL17 was undetectable.

Another chemokine studied was CCL20, the Th17 attracting chemokine. Secretion of this chemokine was observed after 24 hours even with unstimulated epithelial cells and it was significantly upregulated after IL-17 and poly I:C treatment ($p<0.01$). Upon treatment with TNF-α + IL-1β and its combination with poly I:C, the response was more pronounced, combination of these treatments failed to have a synergistic effect (Figure 5-3). After 48 hours there was only a slight diminution of CCL20 secretion in the majority of cells. There also appeared to be a synergistic effect between poly I:C + TNF-α + IL-1β treated cells cultured with poly I:C treated CBMCs ($p<0.05$; Figure 5-3F).

The localisation of Th17 cells in the conjunctiva of CAED tissues has led us to the idea that this cell type may play a role in disease. Although the conjunctival epithelium secretes Th1 attracting chemokines, we have shown that CCL20 is produced within the first 24 hours and is sustained over 48 hours. This could indicate that Th17 cells may be the first T cell subset to infiltrate diseased areas, followed by Th1 then Th2 cells, depending on the inflammatory environment of the particular disease. This data, although being insightful, is unable to establish whether Th17 cells are pathogenic in CAED.

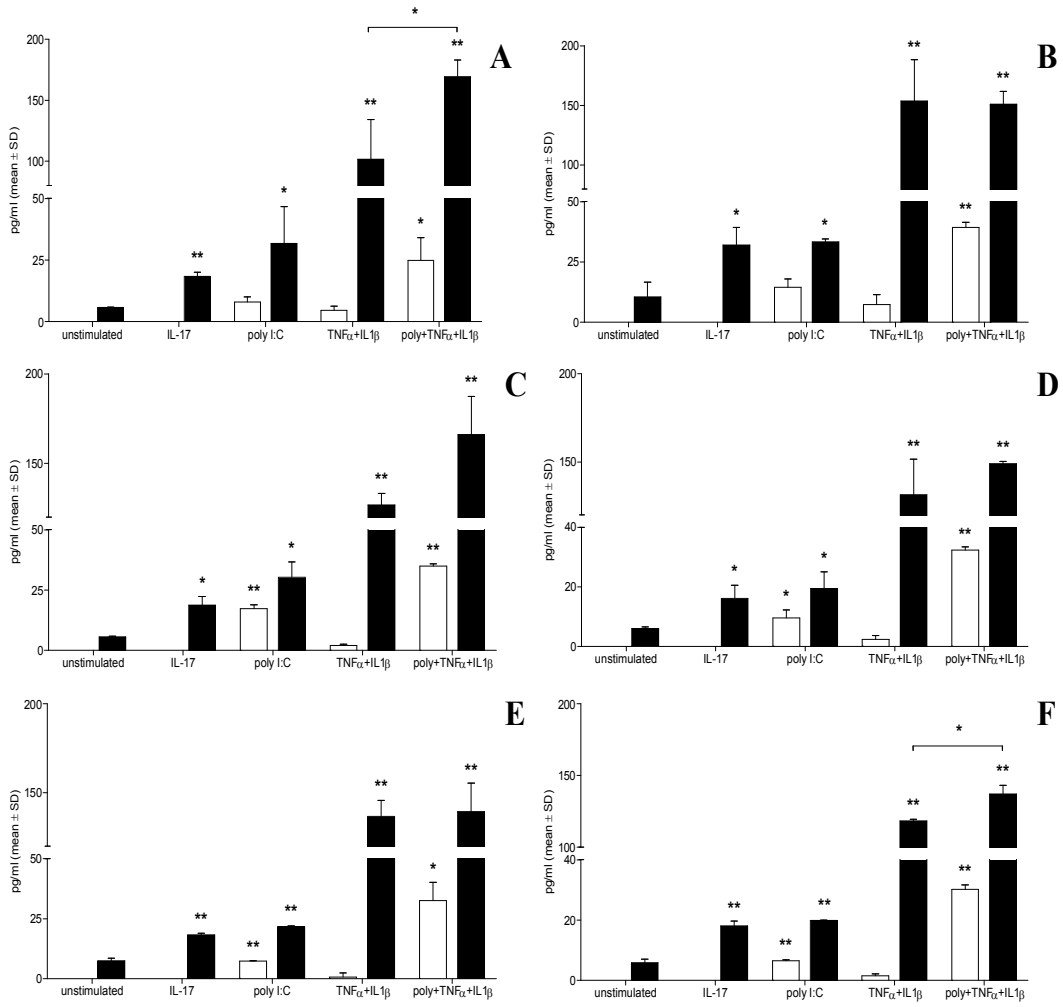

Figure 5-2 The Th1 attracting chemokine – CXCL10 is secreted greatly in the later stages of stimulation. IOBA cells were either left untreated or stimulated with IL-17, poly I:C, TNF-α + IL-1β for 24 hours before being cultured with CBMCs that had been unstimulated (**A**) or treated with (**B**) IgE, (**C**) IL-17, (**D**) TNF-α+IL-1β, (**E**) PMA and (**F**) poly I:C. Supernatants were collected after 24 (open bars) and 48 hours (black bars) and assayed for CXCL10 by ELISA. Results are shown as mean ± SD of three separate experiments; ***** $p < 0.05$, ****** $p < 0.01$.

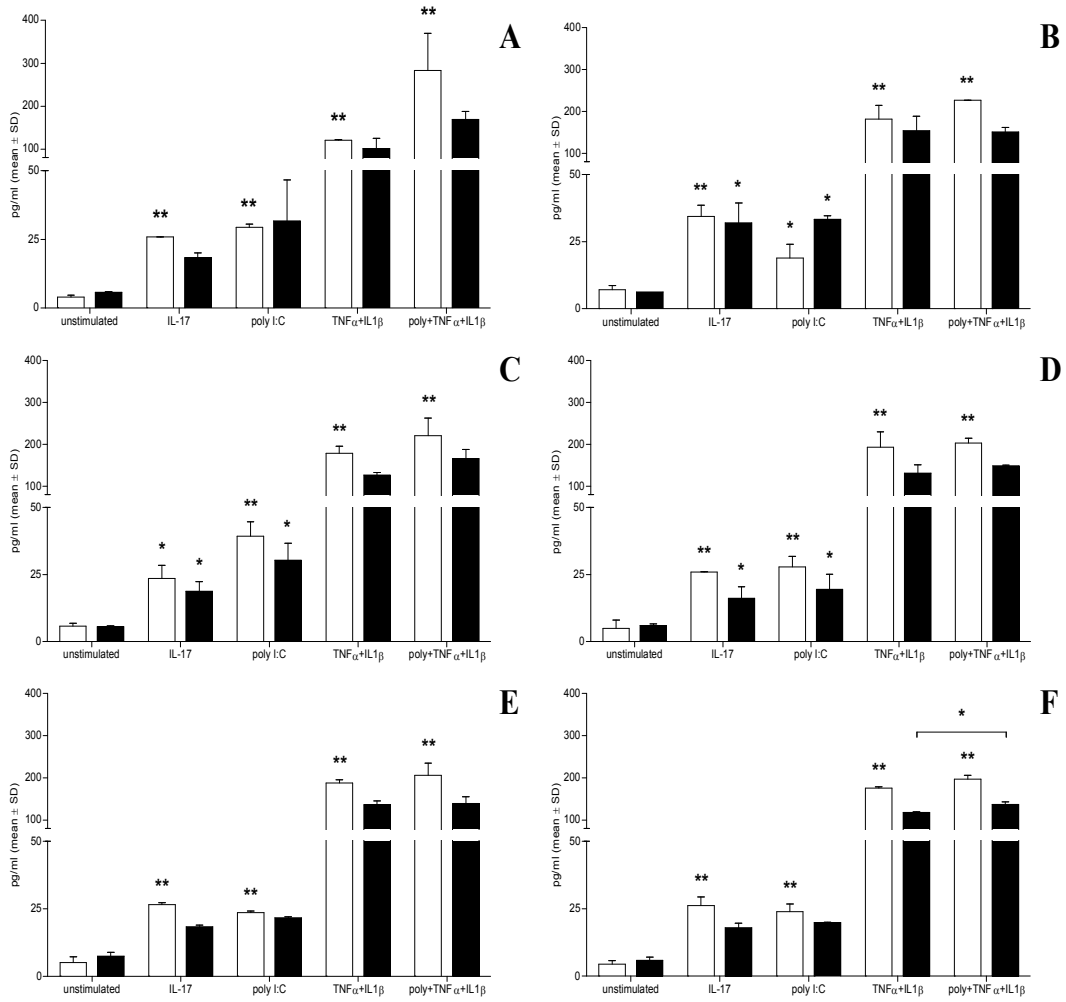

Figure 5-3 The Th17 attracting chemokine – CCL20 is preferentially secreted in the early phase of stimulation and is sustained over 48 hours. IOBA cells were either left untreated or stimulated with IL-17, poly I:C, TNF-α + IL-1β for 24 hours before being cultured with CBMCs that had been (**A**) unstimulated or treated with (**B**) IgE, (**C**) IL-17, (**D**) TNF-α+IL-1β, (**E**) PMA and (**F**) poly I:C. Supernatants were collected after 24 (open bars) and 48 hours (black bars) and assayed for CCL20 by ELISA. Results are shown as mean ± SD of three separate experiments; ***** $p < 0.05$, ****** $p < 0.01$.

5.3 IL-17 can be found at sites of conjunctival tissues

The actions of IL-17 have been given two differing roles, one of mediating pathogenesis the other of protecting the host from infection. As Th17 cells have been found to be localised in CAED tissues, it is important to determine whether the ocular surface has the ability to produce IL-17 and other Th17 related cytokines, therefore shedding a light on any further cytokines that may be participating in the inflammatory environment seen in disease.

5.3.1 IL-17 is expressed in the conjunctiva and increased in CAED

In addition to its co-localisation to $CD4^+$ T cells we also examined IL-17 expression on non-T cells in CAED tissues. Compared to OCP tissues (Figure 5-4A) we found increased intensity of IL-17 staining on the epithelium and stroma in CAED (Figure 5-4 B), the level of which was not found to be restricted to any particular area but widespread throughout the tissue. In conjunction we quantified the level of IL-17 staining intensity in all the tissues. Within the epithelium we could not identify any IL-17 expression in the normal controls and only minimal expression in SAC and OCP tissues, which was significantly upregulated in CAED tissues ($p<0.05$). In the stroma we detected expression of IL-17 in OCP which was increased significantly in CAED tissues ($p<0.05$), this expression did not differ between the epithelium and stroma (Figure 5-4C).

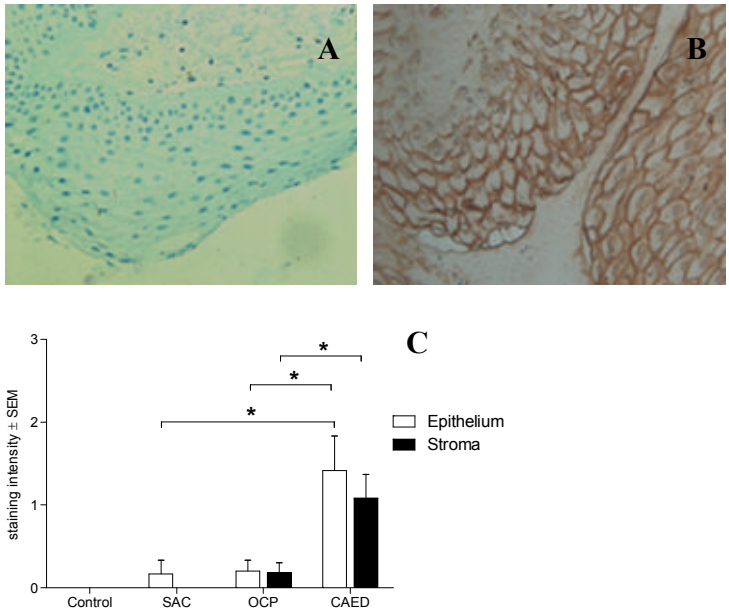

Figure 5-4 IL-17 is found at the conjunctival epithelium of CAED tissues. Conjunctival biopsy sections stained as in figure 5-1 were also evaluated for their expression of IL-17. Positive IL-17 staining is depicted by brown staining; (**A**) OCP, (**B**) AKC. Intensity of IL-17 staining (**C**) was quantified by grading sections from normal controls (n = 6), SAC (n = 6), OCP (n = 10), CAED (VKC – n = 4; AKC – n = 5). Sections were scored between 0-3 where 0 = no staining, 1 = weak, 2 = moderate, 3 = strong staining. Results are shown as mean ± SEM. ***** ***p* <0.05**

5.3.2 IL-17 mRNA is expressed at the conjunctival surface

To further investigate the expression of IL-17 at the conjunctiva, mRNA from conjunctival swabs was isolated and assayed to quantify their expression of IL-17 mRNA. The results indicate the expression of IL-17 in controls as well as in OCP and AKC specimens. Although not significant, there was increased expression of IL-17 in AKC above that of normal controls and OCP specimens. In using this method we are unable to establish which cell type is responsible for the IL-17 expressed here,

in such a case it would be interesting to determine whether conjunctival epithelial cells are capable of producing this cytokine.

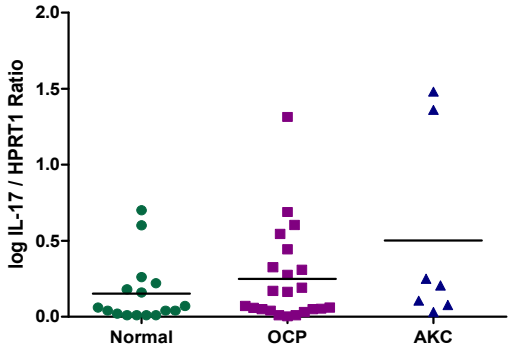

Figure 5-5 IL-17 mRNA is expressed at the conjunctival surface. RNA was isolated from conjunctival swabs taken from normal (n = 16), OCP (n = 35) and AKC (n = 7) subjects. The expression of IL-17 mRNA was evaluated by real-time RT-PCR**.** Each symbol represents an individual and lines indicate the mean expression.

5.4 Conjunctival epithelial cells produce IL-17 related cytokines

So far this study has shown that the chemokine involved in Th17 infiltration is produced in high levels by conjunctival epithelial exposed to pro-inflammatory cytokines and a TLR agonist, which may explain the Th17 cells observed at conjunctival sites. Just as the conjunctiva in CAED has demonstrated high IL-17 staining intensity in both the stroma and epithelium, we wanted to confirm whether the IL-17 seen in the tissues was produced from epithelial cells. To examine this we stimulated IOBA cells and assayed their supernatants for the presence of cytokines from the IL-17 family.

Contrary to the IL-17 staining observed in the tissue sections (Figure 5-5B); conjunctival epithelial cells were unable to produce the major Th17 cell cytokines IL-17A and IL-17F, nor did they produce IL-22 or IL-23. Nevertheless, in the presence of poly I:C, the cells were induced to secrete significantly higher levels of IL-25 (IL-17E; $p<0.01$) compared to the relatively lower levels observed in unstimulated cells and cells cultured with other treatments (Figure 5-6).

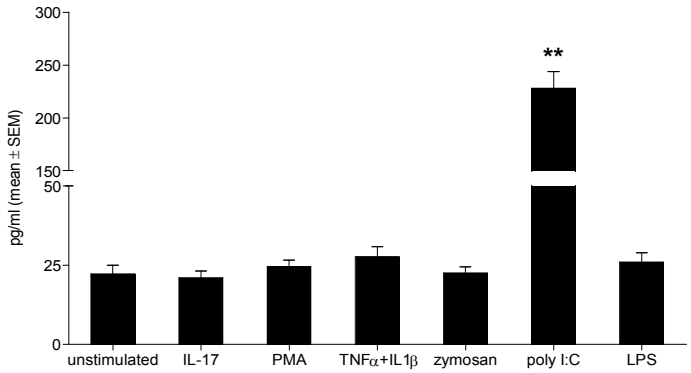

Figure 5-6 IL-25 (IL-17E) is secreted by conjunctival epithelial cells stimulated by a TLR3 ligand. IOBA cells were stimulated with a panel of treatments for 24 hours after which supernantants were collected and used to assay IL-25 production by ELISA. Experiments were repeated four times and results are shown as mean ± SEM of the four experiments; ** ***p*** **<0.01**.

5.5 IL-17 stimulates conjunctival epithelial cells to produce pro-inflammatory cytokines.

It is thought that IL-17 mediates its pathogenic activity via its ability to induce proinflammatory cytokines as well as chemokines such as TNF-α, IL-1β, IL-6 and IL-8 (Onishi & Gaffen, 2010). These cytokines can then work by exacerbating any ongoing inflammation whilst chemokines aid in the recruitment of other cells, thereby increasing further sources of cytokines. To understand whether IL-17 contributes to the pathogenesis of ocular disease, it is necessary to establish if this cytokine has any effect on conjunctival epithelial cells.

5.5.1 IL-17 induces the secretion of pro-inflammatory mediators

To confirm whether conjunctival epithelial cells can be activated by IL-17, the cells were treated with IL-17 for 24 hours after which supernatants were assayed for secretion of IL-8 and sCD44, and CD54 expression in detached cells. In parallel, stimulated cells were detached in order to isolate RNA and look for mRNA expression of IL-8, IL-13 and TSLP. In contrast to untreated cells, IL-17 stimulation caused a significant upregulation of both IL-8 and sCD44 (Figure 5-7A; $p<0.01$). This was further confirmed at the mRNA level with significant expression of IL-8 (Figure 5-7B). IL-13 mRNA expression in the cells failed to be altered by IL-17 treatment but the level of TSLP was markedly increased (Figure 5-7B; $p<0.01$). In contrast to their ability to upregulate IL-8 and TSLP, IL-17 stimulation did not result in augmentation of CD54 (data not shown).

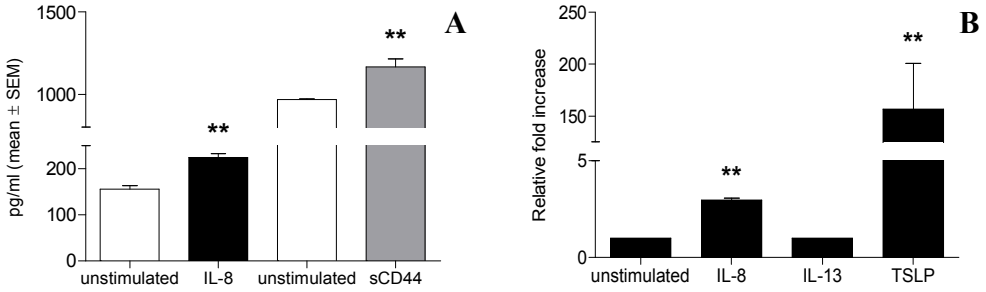

Figure 5-7 IL-17 stimulation induces the production and mRNA expression of inflammatory markers. IOBA-NHC cells were stimulated for 24 hours with IL-17A after which supernatants were collected and assayed for IL-8 and sCD44 production (**A**). Results are shown relative to unstimulated cells. Cells were also stimulated with IL-17A for 4, 6 or 24 hours upon which cells were detached and RNA isolated. Using RT-PCR, the level of mRNA for IL-8, IL-13 and TSLP were analysed (**B**). Results are shown as mean ± SEM of four experiments for the production of cytokines and three experiments for mRNA expression; ** ***p*** **<0.01**.

5.5.2 IL-17 promotes the upregulation of MMPs in the epithelium

MMPs have been associated with different disorders and it is thought that they contribute to inflammation and tissue remodelling. In mice, desiccating stress has been reported to increase MMP9 as well as Th17 associated genes (De Paiva *et al*, 2009). To investigate whether pro-inflammatory stimuli, especially IL-17 and TLR ligands, can induce conjunctival epithelial cells to produce MMPs, cell-free supernatants from treated cells were assayed for MMP9 production with the use of a multicytokine bead array. This method was unable to detect MMP9; therefore the use of a MMP antibody array which is more sensitive in detecting MMP expression was adopted. In this instance we detected significant changes in MMP2 and MMP9 expression. The intensity of MMP2 (Figure 5-8A) as well as MMP9 expression

(Figure 5-8B) in IL-17 stimulated cells was found to be significantly increased compared to unstimulated cells and cells treated with the remaining stimuli ($p<0.05$). The intensity of MMP9 expression was also increased in TNF-α + IL-1β treated cells but this failed to reach significance. We also detected the expression of MMP1 and MMP3 which was not altered upon stimulation (data not shown).

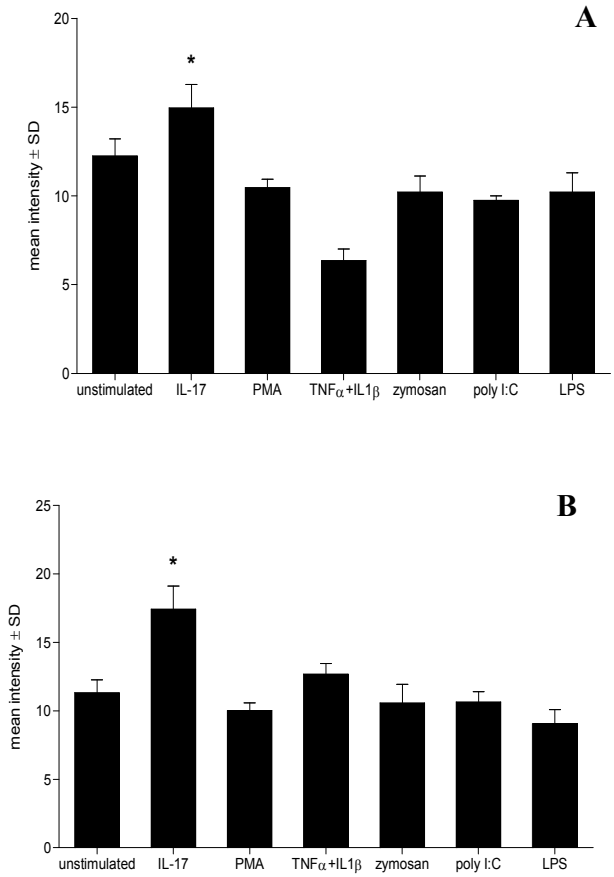

Figure 5-8 IL-17 upregulates the expression MMPs. IOBA cells were stimulated with various treatments, after 24 hours supernatants were collected for use in an antibody protein array to determine MMP2 (**A**) and MMP9 (**B**) expression. Graph represents results from mean ± SD of three separate experiments; *** *p* <0.05**.

5.6 Discussion

Th17 cells have been implicated in various chronic inflammatory conditions in addition to playing a part in host defence. In this present chapter we investigated the expression of a T helper cell subset in tissues of chronically inflamed conjunctiva and the effect of its primary cytokine – IL-17 on epithelial cell responses. So far we have found evidence of Th17 cells localising to the conjunctiva of chronically inflamed tissues and indications that Th17 cells may be the first T cell subset to arrive at inflamed sites, based on the secretion of CCL20 by conjunctival epithelial cells as early as 24 hours post treatment. As well as IL-17 mRNA expression found in conjunctival swabs, inflamed tissues, in particular in CAED, exhibited increased levels of IL-17 staining. Upon further investigation, IL-17 was capable of stimulating conjunctival epithelial cells to induce increased pro-inflammatory markers in addition to upregulating MMPs.

Much like this study only one group have reported the presence of Th17 cells in the conjunctiva of OCP patients, where OCP specimens were found to have increased staining of Th17 cells above that of healthy subjects (Lambiase *et al*, 2009). Although Th17 cells were present in our biopsy specimens, it is not clear whether these cells have been recruited and mediate inflammation as part of the disease pathogenesis or if these cells are responding as part of the defence mechanism against microbial infection. As just described, in order for $CD4^+$ T cells to take part in the immune response, they must first be recruited to the specific site, normally initiated by chemoattractants secreted by other cells in that area, for Th17 cells the

chemoattractant of interest is CCL20. While there has not been much attention focusing on secretion of specific chemoattractants by ocular epithelial cells, one study has described the expression of CCL20 mRNA in primary human conjunctival epithelial cells, which can be significantly upregulated more than 10-fold after poly I:C stimulation (Ueta *et al*, 2010). Furthermore, the authors also noted a significant increase in CCL20 protein secretion (Ueta *et al*, 2010). The authors also reported the same findings with CXCL10, which adds to our data showing both chemokines are secreted by conjunctival epithelial cells. When we the address the expression of CCL20 in context with disease it has been shown that 24 hours after the addition of allergen there is an increase in epidermal CCL20 mRNA in atopic dermatitis patients (Gros *et al*, 2009). In contrast, the same study showed that CCL17 – a Th2 chemokine is found to be increased at a later time point – after 72 hours allergen exposure (Gros *et al*, 2009). Another role for CCL20 has also been proposed, as an antimicrobial peptide. Reports have shown that CCL20 peptide is effective in killing *Pseudomonas aeruginosa* (Huang *et al*, 2007), therefore pointing towards a model of host defence by this conjunctival epithelial cell line.

It is interesting that we were unable to detect CCL17 in our cultures during this study; some evidence has suggested that this chemokine can be induced in bronchial epithelial cells. The authors here found that stimulation with TNF-α + IL-4 induced CCL17 secretion up to 72 hours in one of their cell lines (Sekiya *et al*, 2000). Stimulation of a different bronchial epithelial cell line with the allergen *Der P* combined with IL-4 and TGF-β, also significantly increased CCL17 production (Heijink *et al*, 2006). Contrary to this another study demonstrated that CCL17

protein is not detected in primary human keratinocytes upon stimulation with various cytokines, whereas a keratinocyte cell line they used in parallel produced this chemokine without stimulation (Tsuda *et al*, 2002). Since mast cells are important in allergic inflammation and they are capable of producing various cytokines such as TNF-α and IL-4, we began to consider what role, if any, mast cells may play whilst in contact with the conjunctival epithelium. Although culturing mast cells with conjunctival epithelial cells appeared not to have an effect on the secretion of the various chemokines, one study has examined the effect of histamine on Th1 and Th2 chemokines by keratinocytes. The investigators reported that TNF-α + IFN-γ induce both CXCL10 and CCL17 and that through the actions of one of the histamine receptors (HR1) found on keratinocytes, histamine suppressed CCL17 and enhanced CXCL10 production (Fujimoto *et al*, 2011). This may not fully explain why we did not detect CCL17 in our cultures but as the conjuctiva is known to express histamine receptors (Bielory & Ghafoor, 2005); it may be an important factor in any future studies.

Further to the presence of Th17 cells in the biopsies, we also found whole areas of conjunctival tissue from CAED tissue, exhibiting very strong staining of IL-17 in comparison to healthy controls. In a study investigating IL-17 in dry eye disease of mice, $CD4^+$ $IL\text{-}17^+$ cells and IL-17 mRNA levels were also found at increased levels to control (Fukushima *et al*, 2008; Chauhan *et al*, 2009). We therefore looked for the production of Th17 associated cytokines in an attempt to ascertain whether the conjunctival epithelium secretes IL-17 observed in these sections. Although there was very intense staining of this cytokine, conjunctival epithelial cells failed to

produce any IL-17A, IL-17F, IL-22 or IL-23. However, they were able to produce IL-25 which, in mice is thought to provide a regulatory role to Th17 by inhibiting Th17 responses and inducing Th2 responses (Kleinschek *et al*, 2007). As we only stained for T cell markers, it is impossible to ascertain what other cell type may have expressed the IL-17. There are a number of candidates that may be responsible namely gamma delta (γδ) T cells. To date there are no studies focussing on γδ T cells in the conjunctiva but recent literature has implicated these cells as being critical in allergic conjunctivitis. By using a model of allergic conjunctivitis using γδ T cell deficient mice, the authors observed a decrease in the clinical signs of allergic conjunctivitis as well as reduced eosinophil infiltration; this was also associated with decreased production of Th2 associated cytokines (Reyes *et al*, 2011). If γδ T cells are required for greater expression of allergic conjunctivitis, it would be interesting to know whether there would be an increase in IL-17 production by these cells, which may explain the level of IL-17 staining in CAED sections.

Several reports have demonstrated the stimulatory ability of IL-17 on different cell types. Although this cytokine can upregulate the IL-8 responses by cells, alone it is not capable of augmenting CD54 expression in human keratinocytes and bronchial epithelial cells (Albanesi *et al*, 1999; Kawaguchi *et al*, 2001), which we too have now found in conjunctival epithelial cells. This however does not detract from the growing evidence of a supposed central role for IL-17 in certain pathologies. In disorders such as rheumatoid arthritis (RA), IL-17 is thought to be involved in matrix and cartilage degradation. Studies investigating this have revealed the presence of elevated IL-17 in the serum of RA patients compared to healthy controls, as well as

the observation that IL-17 was able to upregulate various MMPs (Moran *et al*, 2009). At the ocular surface, it is thought that IL-17 has the same effect; in an experimental model of dry eye, neutralisation of IL-17 resulted in decreased expression of MMP3 and MMP9, possibly leading to dysfunction of the corneal epithelial barrier (De Paiva *et al*, 2009). Although we also found increased MMP expression in IL-17 stimulated conjunctival epithelial cells, we can only speculate as to the effect this may have in CAED pathology.

As a whole, the data from this study suggests that Th17 cells and its cytokine – IL-17 may play a part in CAED, whether this is a supportive or a pathogenic role remains unclear. Our results have shown IL-17 expression in CAED and we now know that it has the ability to stimulate conjunctival epithelial cells – but to what end? To answer these questions the next chapter will aim to uncover the function of IL-17 on conjunctival epithelial cells by looking at its family of receptors and how this may have an impact on chronic inflammation.

IL-17 receptor subsets in tissues and cells

6.1 Introduction

As the previous chapter has discussed, IL-17 is able to drive inflammatory responses at the mucosal surface; conversely, this cytokine also plays a major role in host protection. The main Th17 cytokines, IL-17A and IL-17F, both require IL-17RA and IL-17RC for signalling, which have been found to be coexpressed by fibroblasts, epithelial and endothelial cells (Toy *et al*, 2006; Ishigame *et al*, 2009). Studies have shown that IL-17 receptor signalling is essential when addressing the protective response of IL-17. Freitas et al noted that mice deficient in IL-17RA that had been subjected to polymicrobial sepsis had a significantly reduced survival rate compared to wild type mice, the authors also observed a significant decrease in neutrophil migration in the IL-17RA deficient mice (Freitas *et al*, 2009). Whilst IL-17 receptor signalling can be beneficial to the host, its actions have been proposed as a major contributor to the pathogenesis of certain diseases. In a mouse model of arthritis, blockade or neutralisation of IL-17 resulted in suppression of joint swelling and inflammation as well as prevention of bone erosion which occurs as a result of joint inflammation (Koenders *et al*, 2005)

If we follow this line of reasoning it is possible that if IL-17 receptor signalling - and therefore IL-17 - has such an influential role in disease pathogenesis, the affected tissues may also have increased IL-17 receptor expression leading to a greater inflammatory response. We therefore hypothesised that these receptors may be upregulated in CAED, which would support our observations that there is a

significant level of IL-17 staining increased in these inflamed conjunctival tissues. This chapter will explore this theory in more detail as well as what effects blocking this receptor might have.

6.2 IL-17 receptor expression is regulated by pro-inflammatory cytokines and TLR ligands

The cytokine milieu often found in inflammatory disease can have devastating effects to the local tissues especially if left unchecked, where it could exacerbate disease. Studies have shown that under the influence of pro-inflammatory cytokines, conjunctival epthielial cells can be induced to upregulate HLA-DR, ICAM-1 and two neuroreceptors (Calonge et al, 2005). As with these receptors, the inflammatory environment characterised by CAED may also drive IL-17 receptor upregulation. The following studies will focus on certain molecules that could possibly provoke such actions.

6.2.1 IL-17 receptor expression is upregulated in CAED

We have already observed the intensity of IL-17 staining to be increased in CAED tissues (Chapter 5). To determine whether this correlates with a concomitant expression of receptors for IL-17 on conjunctival tissues, sections were stained for IL-17RA and IL-17RC. Whilst there is very low expression of IL-17RA at the epithelial layer in controls (Figure 6-1A), in AKC, IL-17RA expression was detected along the surface of a section of the subepithelium (Figure 6-1B). In contrast, the epithelial surface of normal controls had greater expression of IL-17RC than IL-17RA (Figure 6-1C); this expression was further enhanced in AKC – affected tissues (Figure 6-1D).

When we graded the intensity of IL-17 receptor expression, we discovered that whilst both receptors were shown to be expressed at some degree in normal controls,

SAC and CAED sections, the level of IL-17RC staining was more than that of IL-17RA when comparing SAC with CAED (Figure 6-1E). Although IL-17RA was found to be increased in CAED tissues over SAC, it failed to reach significance. The staining intensity of IL-17RC, on the other hand, was moderate within normal controls and SAC, whereas in CAED, this expression intensity was significantly increased in comparison to the two other groups (Figure 6-1E; $p<0.05$ for both).

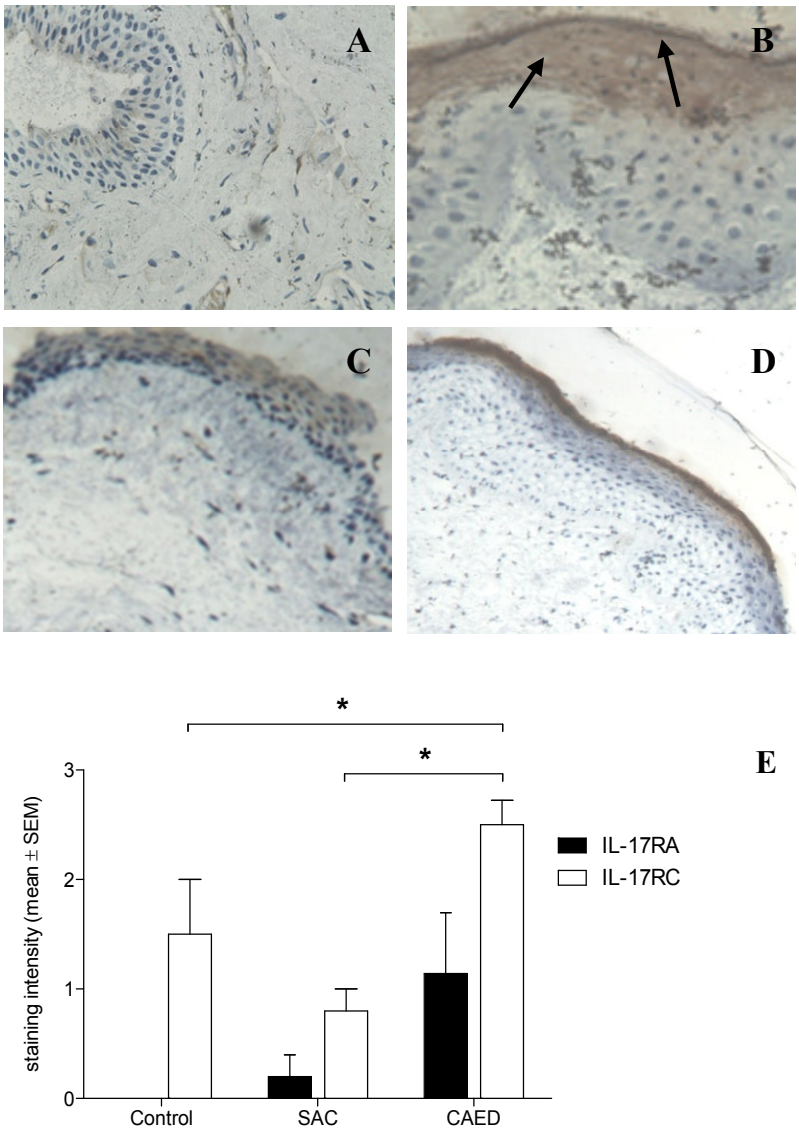

Figure 6-1 IL-17 receptor expression at the conjunctiva. Conjunctival biopsies were stained for the expression of IL-17RA and IL-17RC by immunohistochemistry; (**A**) non-inflamed control [IL-17RA], (**B**) AKC [IL-17RC], (**C**) non-inflamed control [IL-17RC], (**D**) AKC [IL-17RC]. Images were captured using Motic BA400; A - C, x400 magnification; D, x200 magnification. Biopsy staining was also graded to quantify the level of intensity of both receptors (**E**) from non-inflamed controls (n = 3), SAC (n = 5) and CAED (VKC, n = 5; AKC, n = 4). Staining was also scored between 0-3 where 0 = no staining, 1 = weak, 2 = moderate, 3 = strong staining. Results are shown as mean ± SD; *** *p*<0.05**.

6.2.2 IL-17RA is upregulated in conjunctival epithelial cells

In earlier chapters we described the response of conjunctival epithelial cells to certain stimuli, which allowed us to examine whether, under the same stimulation conditions, these cells can be induced to regulate the expression of IL-17RA. Unstimulated cells already had a high level of receptor expression, at around 50%, when we treated the cells with the three separate allergens they failed to augment IL-17RA expression. Alone, TNF-α + IL-1β significantly upregulated receptor expression ($p<0.01$), and when combined with the different allergens there was increased expression of IL-17RA but it did not reach significance in any of the combinations (Figure 6-2A). Upon challenge with IL-17A, there was decreased expression of IL-17RA, owing to receptor internalisation ($p<0.05$; Figure 6-2B). This expression was significantly increased when epithelial cells were exposed to PMA and poly I:C ($p<0.01$ and $p<0.05$ respectively; Figure 6-2B). Although treatment with zymosan and LPS had no initial affect on IL-17RA expression, when combined with TNF-α + IL-1β, there was a significant additive effect when compared to TNF-α + IL-1β alone ($p<0.05$ and $p<0.01$ respectively). Treatment with poly I:C and TNF-α + IL-1β also resulted in a synergistic effect above that of TNF-α + IL-1β alone ($p<0.01$; Figure 6-2B).

We also investigated the expression of IL-17RC and the relatively unknown IL-17RD. Both of these receptors were abundantly expressed by conjunctival epithelial cells, but unlike IL-17RA where treatment with TNF-α + IL-1β upregulated IL-17RA (Figure 6-2B &C), stimulation of the cells did not result in augmentation in the

levels of IL-17RC and IL-17RD expression, as illustrated in Figure 6-2D & E. The histograms show that whilst there is a shift in IL-17RA expression, the same treatment had no effect above untreated cells (Figure 6-2D & E). As with the biopsies, the intensity of expression of both IL-17RC and IL-17RD at the conjunctival epithelium was greater than that of IL-17RA (Figure 6-2D & E). We can therefore suggest that the conjunctival epithelium expresses at least three of the IL-17 receptor subtypes, but only speculate as to their relative expression levels in disease. Of these three receptors, IL-17RA appeared to be the most susceptible to modulation, possibly due to their low baseline expression. Further examination into their role in epithelial cell immune responses will be investigated.

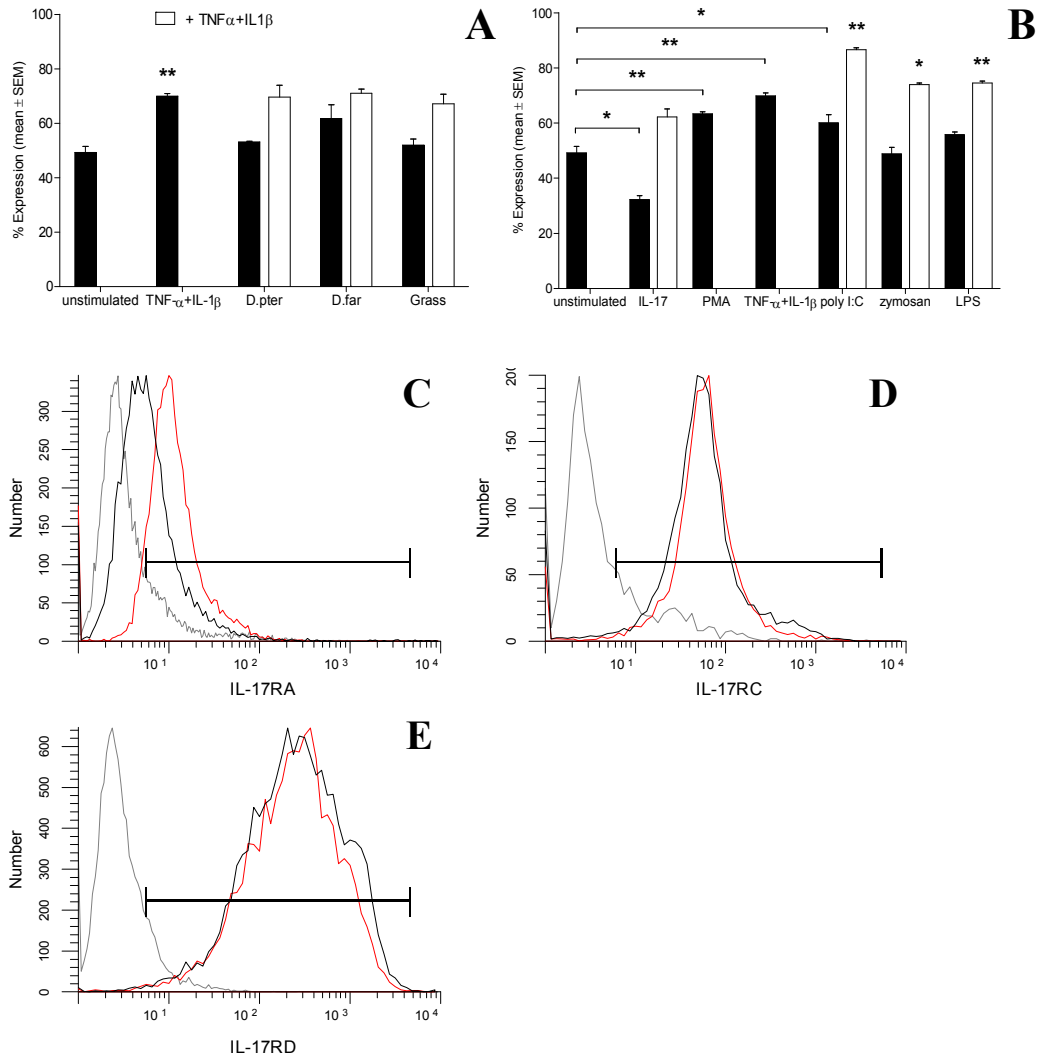

Figure 6-2 Expression of IL-17 receptor subtypes by the conjunctival epithelium. Conjunctival epithelial cells were treated with various allergens, cytokines or TLR ligands either alone or in combination with TNF-α+IL-1β. Cells were detached and stained for IL-17RA, expression was quantitiated by flow cytometry (**A - C**). Epithelial cells were also stained for (**D**) IL-17RC and (**E**) IL-17RD. The intensity of receptor expression was compared between histograms C – D, where: - - - represents IgG control, — represents unstimulated cells and — represents TNF-α+IL-1β treated cells. Results are shown as mean ± SEM of four independent experiments; ***** $p<0.05$, ****** $p<0.01$.

6.3 IL-17 receptors mediate conjunctival epithelial cell responses

So far, many reports have discussed a role for IL-17 and IL-17 receptor signalling in disease pathogenesis. A few groups have found that certain growth factors and cytokines such as growth regulated oncogene-alpha (GRO-α), IL-1β and TNF-α, induced by IL-17, are significantly decreased after receptor blockade (McAllister *et al*, 2005; Koenders *et al*, 2005). As some of these cytokines are pro-inflammatory, we investigated whether the presence of these cytokines, such as that seen at inflamed sites may also upregulate IL-17 receptor expression.

6.3.1 IL-17 downregulates conjunctival epithelial cell responses

So far we have reported on the effect of IL-17 on the conjunctival epithelium. To investigate the contribution of IL-17 receptor expression in epithelial cell responses to IL-17 and whether the expression of certain surface molecules and chemokines can be altered, epithelial cells were firstly treated with a panel of stimuli to upregulate IL-17RA, receptors were then blocked using an anti-IL-17RA antibody or treated with an isotype-matched control antibody and then cells were re-stimulated with IL-17A. To determine the concentration of anti-IL-17RA needed to saturate IL-17RA receptors, the antibody was titrated. As we have observed previously, unstimulated cells expressed 25-30% IL-17RA and incubation with three different concentrations of a control antibody resulted in a similar level of expression (Figure 6-3A). However, after incubation with 0.1μg/ml anti-IL-17RA, IL-17RA expression was diminished by about 40% ($p<0.05$) and by about 70% with 0.5-1μg/ml of anti-

IL-17RA (p<0.01 for both; Figure 6-3A). For the remaining experiments 0.5µg/ml of anti-IL-17RA was used.

In comparison between control antibody treated and anti-IL-17RA antibody treated cells, there was a significant increase in the level of CD54 expression in those cells pre-treated with PMA and TNF-α + IL-1β (which have already been shown to significantly upregulate CD54) prior to incubation with the control antibody and further stimulation with IL-17A (p<0.05 for both; Figure 6-3B). Interestingly, pre-treatment with poly I:C and subsequent stimulation with IL-17A significantly reduced CD54 expression (p<0.05; Figure 6-3B). Furthermore, with respect to IL-17RA expression, IL-17A ligation after treatment with the control antibody resulted in a significant decrease in IL-17RA expression as before, whilst pre-treatment with TNF-α + IL-1β and poly I:C resulted in significantly increased receptor expression when compared to unstimulated cells (p<0.01 for all; Figure 6-3C). When the cells were treated with anti-IL-17RA, prior stimulation with PMA, TNF-α + IL-1β and poly I:C significantly increased IL-17RA expression (p<0.05; p<0.01; p<0.01 respectively; Figure 6-3C) in comparison to unstimulated cells. In this instance, pre-treatment with IL-17A did not augment receptor expression. When comparing the two separate treatments, blockade of IL-17RA significantly decreased receptor expression following poly I:C pre-treatment in comparison to control antibody treated cells (p<0.01; Figure 6-3C).

To address whether the blockade of IL-17RA affects other epithelial cell responses, IL-8 levels were also measured. Pre-treatment of the cells before receptor blocking

demonstrated a similar epithelial cell response as that reported in chapter 3, with PMA, TNF-α + IL-1β and poly I:C being the main stimulants responsible for increased production of IL-8 (data not shown). When the cells were treated with control antibody, the levels of IL-8 were significantly increased in PMA, and poly I:C pre-treated cells only ($p<0.05$; Figure 6-3D). The remaining stimulations failed to augment IL-8 production above unstimulated cells (Figure 6-3D). These results indicate our baseline data. A significantly increased production of IL-8 was the result of cells being pre-activated with IL-17, PMA and poly I:C in the presence of anti-IL-17RA antibody compared to control antibody ($p<0.01$, 0.05 respectively; Figure 6-3D). This suggests that IL-17RA ligation mediates a down-regulation of IL-8.

IL-8 plays a vital role in the immune response namely because it has the ability to recruit neutrophils to sites of infection, overproduction of which may exacerbate inflammation. Our findings that IL-17 may in fact decrease the production of IL-8 led us to examine whether levels of another immunoregulatory cytokine TGF-β may also be modulated. Pre-treatment with the various stimuli, before the addition of anti-IL-17RA antibody, appeared to decrease the production of TGF-β1 compared to control antibody under the same conditions, although this failed to reach significance (Figure 6-3E). Surprisingly, unstimulated cells treated with anti-IL-17RA antibody, significantly down-regulated TGF-β1 secretion in comparison to unstimulated cells exposed to control antibody (Figure 6-3E). From this data, epithelial cell responses appear to deviate from their expected response following IL-17A ligation, this divergence may provide IL-17A with the capacity to carry out a regulatory role.

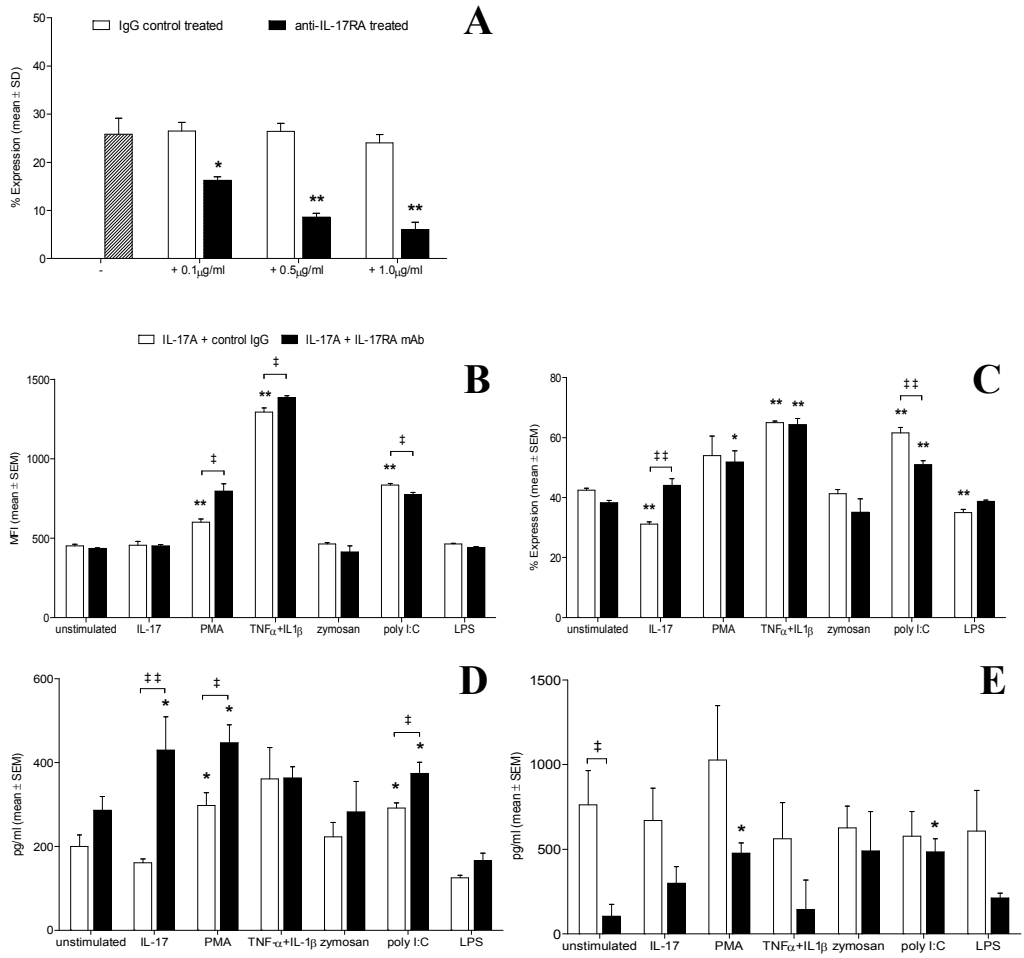

Figure 6-3 The conjunctival epithelial cell response to IL-17 receptor ligation and blockade. The anti-IL-17RA mAb used for these experiments were titrated using three different concentrations (0.1 – 1.0μg/ml) along with and IgG control. Conjunctival epithelial cells were left untreated or treated separately with the control antibody of anti-IL-17RA mAb for 2 hours. Detached cells were stained for IL-17RA expression and analysed by flow cytometry (**A**). For the remaining experiments, 0.5μg/ml of both control antibody and anti-IL-17RA mAb were used. Epithelial cells were pre-treated for 24 hours with the panel of stimuli then incubated for 2 hours with control antibody or anti-IL-17RA mAb, after which all cells were stimulated with IL-17A for 24 hours. Detached cells were stained for CD54 (**B**) and IL-17RA (**C**), whilst cell-free supernatants were assayed for IL-8 (**D**) and TGF-β1 (**E**; medium alone contained 1656.72pg/ml ± 11.22 which was subtracted from TGF-β cell production). Results are shown as mean ± SEM from three independent experiments; open bars represent control treated cells and solid bars represent anti-IL-17RA treated cells * denotes comparison between unstimulated and stimulated cells under the same conditions, ‡ denotes comparison between opposing conditions; ***/‡ $p<0.05$; **/‡‡ $p<0.01$**.

6.3.2 A role for IL-17 in lymphocyte proliferation

At this stage, two lines of evidence had been investigated that pointed towards a possible regulatory role for IL-17, which included the question as to the differential levels of TGF-β1 between unstimulated cells and cells that received stimulation prior to anti-IL-17RA treatment. To address whether conjunctival epithelial cells, after IL-17A ligation (control antibody treated cells) are proficient in inhibiting lymphocyte proliferation, through the actions of TGF-β1, we titrated a range of concentrations of supernatants from the treatments that reflected strong epithelial cell responses and cultured them with anti-CD3/28 stimulated CFSE-labelled PBMCs. In parallel, unstimulated and anti-CD3/28 stimulated CFSE-labelled PBMCs were cultured alone as controls and proliferation measured by flow cytometry.

To measure the percentage of proliferation, a gate was drawn around the area where proliferating lymphocytes, based on cell size and granularity were found. A marker was drawn to indicate undivided cells from unstimulated cultures, shown by the lack of CFSE dilution (Figure 6-4A); percentage division of stimulated cells was shown by an increase in CFSE dilution (Figure 6-4B).

As expected there was minimal lymphocyte proliferation in the unstimulated lymphocyte population in contrast to stimulated cells where there was up to 50% proliferation. The addition of 5% culture supernatant from all cell treatments, did appear to inhibit lymphocyte proliferation, although there was only a significant decrease with supernatants from unstimulated cells treated with control antibody and PMA – anti-IL-17RA treated cells ($p<0.05$; Figure 6-5A). In the presence of

unstimulated – anti-IL-17RA supernatants, the level of proliferation was significantly reduced compared to control antibody ($p<0.05$; Figure 6-5A). Lymphocytes cultured with 10% culture supernatants from unstimulated and PMA pre-treated cells, prior to the addition of control antibody or anti-IL-17RA resulted in reduced lymphocyte proliferation ($p<0.05$; Figure 6-5B). Between the two antibody treatments, lymphocyte proliferation was significantly inhibited when cultured with supernatants from unstimulated cells that had received anti-IL-17RA, compared to control antibody ($p<0.01$; Figure 6-5B). Supernatants from TNF-α + IL-1β treated cells appeared to decrease proliferation, but this did not reach significance. Interestingly, lymphocytes in the presence of supernatants from poly I:C – control antibody treated cells were observed to have reduced levels of proliferation ($p<0.05$), more so than anti-IL-17RA when comparing between the two ($p<0.05$; Figure 6-5B). Although there was a significant decrease, in some instances, in the proliferation of lymphocytes cultured with 5 and 10% culture supernatant, it remained that with all the treatments the 20% concentration had the strongest effect, inhibiting lymphocyte proliferation by about 50% (Figure 6-5C).

This data could not confirm whether IL-17 plays a regulatory role, possibly due to the experimental design. However, the conjunctival epithelium has been reported to express TGF-β mRNA (De Paiva *et al*, 2009) and therefore may still be able to dampen lymphocyte actions through constitutive production of TGF-β.

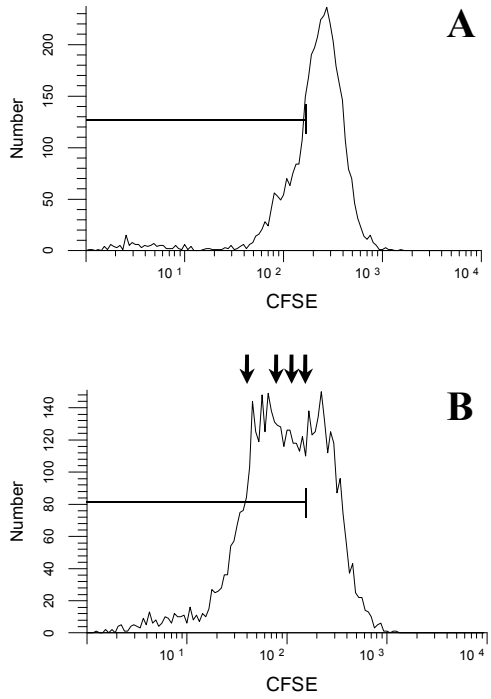

Figure 6-4 CFSE dilution profile. Unstimulated and stimulated PBMCs labelled with CFSE were cultured alone or in the presence of epithelial cell supernatants for 5 days. After this time cells were harvested and analysed by flow cytometry. A marker was positioned to set the area of cell proliferation as shown by unstimulated PBMCs (**A**) indicating the baseline levels of undivided cells. As dividing cells lose their intensity of CFSE after each division, indicated by arrows (CD3/28 stimulated PBMCs; **B**), the marker was used to record CFSE dilution and hence the percentage of proliferation. Proliferation experiments were repeated three times and the means taken for each condition.

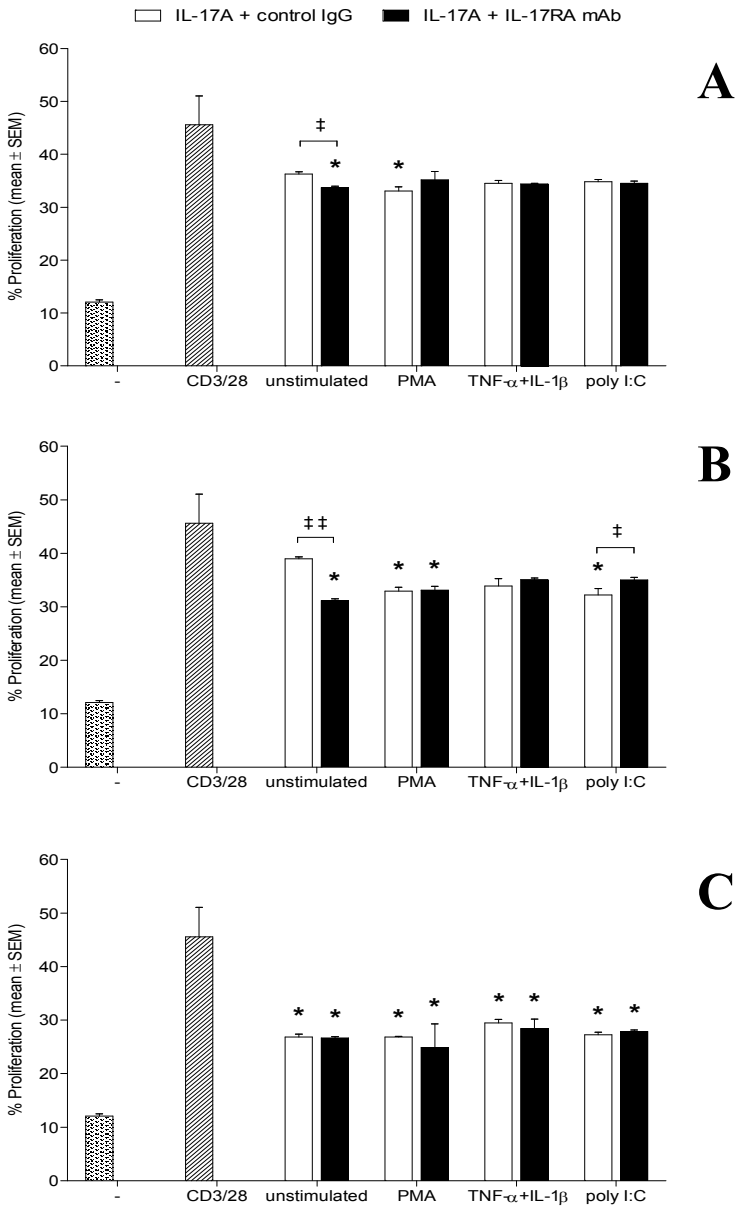

Figure 6-5 Inhibition of human lymphocytes by conjunctival epithelial cell products. The effect of the epithelial cell response on human lymphocyte proliferation was measured by culturing lymphocytes stained with CFSE for 5 days with supernantants collected from the IL-17RA blockade experiments**.** Lymphocytes were also stimulated with CD3/28 as well as an unstimualted control and analysed by flow cytometry. Analysis was conducted by gating on the lymphoblast region and looking at CFSE dilution in that population – indicating proliferating cells. Percent proliferation in comparison with CD3/28 stimulated lymphocytes was taken for lymphocytes cultured with 5% (**A**), 10% (**B**) and 20% (**C**) epithelial cell supernatants. Results are shown as mean ± SEM; * denotes comparison to CD3/28 stimulated cells, ‡ denotes comparison between opposing conditions; ***/‡ *p*<0.05; ‡‡ *p*<0.01**.

6.4 Discussion

It has already been established that IL-17 receptors can be found on many cell types (Yao *et al*, 1997); our study has taken this further and shown that both IL-17RA and IL-17RC are expressed on conjunctival tissues. This expression of IL-17RC in this study was shown to be upregulated in chronic disorders. Further analysis provided evidence that IL-17RA can be upregulated by pro-inflammatory cytokines and TLR agonists, such as that seen in the inflammatory environment of chronic allergic disorders. More interestingly we discovered that IL-17 ligation reduces the level of IL-8 production by epithelial cells under specific conditions.

The evidence from the literature tells us that IL-17 receptor signalling drives the production of pro-inflammatory cytokines; it was therefore not surprising that our data suggests that TLR ligands as well as inflammatory cytokines - which can be detected at the inflamed ocular surface – were shown to upregulate IL-17RA in our model. Hence, IL-17R expression may be part of an important defence mechanism against ocular infection, which may be essential to subjects that risk the development of secondary infections during anti-inflammatory therapy. Conversely, the expression of IL-17RC and IL-17RD was not affected by epithelial cell stimulation; this may be due in part to the very high levels of expression of both these receptors on unstimualted cells. Unlike IL-17RA and IL-17RC, IL-17RD is poorly understood and therefore we are currently unable to draw any conclusions regarding its expression on the cells or its role in epithelial cell responses.

In studies investigating IL-17R signalling, mice deficient in IL-17R displayed significantly reduced expression of IL-1α, IL-1β and IL-6 (Koenders *et al*, 2005) and a decrease in neutrophil migration (Conti *et al*, 2009; Freitas *et al*, 2009). Our hypothesis in this study was that IL-17RA blockade downregulates conjunctival epithelial cell responses. We therefore investigated the contribution of IL-17RA in epithelial responses by, firstly, stimulating conjunctival epithelial cells for 24 hours to upregulate the receptor, blocking IL-17RA function and then determining epithelial cell responses to IL-17A. Our initial observations demonstrated that IL-17A ligation had no effect on CD54 expression as previously shown by our earlier studies. During receptor blockade, the effect of IL-17A was reversed and the expression of IL-17RA was comparable to the level found on unstimulated cells. In relation to the effect of anti-IL-17RA treatment on receptor expression, treatment of cells with control IgG lead to down-regulation of IL-17RA, probably due to receptor internalisation. Unexpectedly we detected enhanced IL-17RA expression in response to IL-17A in those cells pre-treated with TNF-α + IL-1β, which was unaffected by anti-IL-17RA, presumably due to the overriding effect with such a potent stimulation. The anti-IL-17RA antibody did significantly reduce IL-17RA expression in epithelial cells if they were pretreated with the poly I:C.

In contrast to our current understanding of a pro-inflammatory role for IL-17 in upregulating IL-8, IL-17RA neutralisation on conjunctival epithelial cells increased the levels of IL-8 when compared to controls, but only in those cells prestimulated with PMA, IL-17A or poly I:C. This unexpected finding suggested that IL-17RA plays a role in modulating IL-8 production. The mechanism for these actions remains

unclear but a recent study focussing on the interaction of IL-17 with its receptors revealed that IL-17A bound to both IL-17RA and IL-17RC with high affinity. In conjunction, the affinity for IL-17RC was greatly increased after IL-17A was bound by one IL-17RA molecule (Ely *et al*, 2009). Since we know that our epithelial cells also express IL-17RC, it might explain the enhanced levels of IL-8 in response to IL-17RA blocking, though this does not explain why a similar, if not increased production of IL-8 was not seen in cells treated with control IgG.

In the last chapter we briefly discussed another member of the IL-17 family, IL-25, which is thought to be involved in the initiation of Th2 immunity through the induction of IL-5 and IL-13 (Fort *et al*, 2007). IL-25 has, however, been demonstrated to be immunoregulatory for IL-8 responses (Xu *et al*, 2010), and for pro-inflammatory cytokine production (Owyang *et al*, 2006; Kleinschek *et al*, 2007). A recent study has shown that, in order for IL-25 to be activated, it must first be captured by IL-17RB before binding to IL-17RA (Ely *et al*, 2009; Rickel *et al*, 2008). We found in our previous study that poly I:C treatment enhanced conjunctival epithelial cell secretion of IL-25. The resulting IL-25 could have bound to IL-17RA which would explain why an increase in IL-8 production was seen in those poly I:C treated cells receiving anti-IL-17RA antibody since IL-25-mediated regulation of IL-8 production would have been bypassed. Clearly our findings highlight IL-17RA as being important for downregulating IL-8 production by PMA-, IL-17A- or poly I:C-stimulated epithelial cells in response to IL-17A. Other groups have reported a possible role for IL-17 in regulating immune responses. As well as initiating allergic asthma, IL-17 was able to reduce pulmonary eosinophil recruitment and bronchial

hyperreactivity in a mouse model (Schnyder-Candrian *et al*, 2006). In addition, Voo et al observed $CD4^+$ $FOXP3^+$ cells that express the Th17 selective receptor, CCR6, and produce IL-17 (Voo *et al*, 2009). These studies demonstrate the opposing roles IL-17 may play in the pathogenesis of disease and controlling the immune response.

TGF-β1 secretion by unstimulated conjunctival epithelial cells was significantly downregulated in response to IL-17 in the presence of anti-IL-17RA antibody, suggesting an important role for IL-17RA ligation in TGF-β1 production by epithelial cells. Whilst TGF-β is not necessary for Th17 differentiation in humans, in the context of our study, it may serve to play its role as an important anti-inflammatory cytokine. It is interesting that this effect was only observed in unstimulated cells, whereas the IL-8 modulation was only found on stimulated cells, suggesting these two IL-17RA-dependent responses occur under different culture conditions. The signalling events may provide an explanation for the opposing effects following IL-17A ligation to IL-17RA on IL-8 and TGF-β observed in this study. Currently, IL-17RA signal transduction is poorly understood (Gaffen, 2009). Studies have identified two possible signalling pathways that are involved in IL-17A-IL-17R ligation. One of the pathways that have been described works through ACT1/TRAF6/TAK1-dependent NF-κB activation (Huang *et al*, 2007). Normal IL-17RA signalling requires the binding of ACT1 to TNFR-associated factor 6 (TRAF6) leading to its related downstream molecule TGF-β-activated kinase 1 (TAK1) and subsequent NF-κB activation. In our study, IL-17RA blockade resulted in a decrease in TGF-β which may have resulted in a lack of TAK1 phosphorylation. Similarly, TRAF6 is also required for myeloid differentiation primary-response protein 88

(MyD88) (Huang *et al*, 2007), one of the adaptor proteins involved in TLR signalling. Pre-activation of the cells may have upregulated certain signalling molecules and skewed responses towards the MyD88 pathway, which could have resulted in IL-8 upregulation. Although we offer many suggestions as to the mechanisms involved, the supernatants from the control treated cells were unable to suppress lymphocyte proliferation below that of anti-IL-17RA treatment. This could be due to the high levels of TGF-β that these conjunctival epithelial cells express constitutively, therefore abrogating any effect that IL-17 ligation may have had. Any future studies will aim to investigate these IL-17R-mediated effects in more detail, in particular the contribution of IL-17RC and the signalling molecules involved.

This study has highlighted a potential immunoregulatory role for IL-17 at the level of its receptors. We have shown increased expression of IL-17RA localised to the conjunctival epithelium, and our model has demonstrated that IL-17RA ligation leads to a decrease in IL-8 and an increase in TGF-β1 secretion. Taken together, it is clear that the conjunctival epithelium possess an array of features that allow it to play an active role in inflammation but more importantly it also displays anti-inflammatory characteristics.

Summary & conclusion

The epithelium can mediate inflammation through its ability to secrete various cytokines and chemokines, thereby allowing the recruitment and activation of other cell types as well as enhancing T helper cell responses (Swamy *et al*, 2010). With the increasing literature directed towards inflammation, the epithelium is fast becoming a focal point in allergy. The key observations of this study have highlighted increased expression of the Th2 associated cytokines, IL-13 and TSLP as well as IL-17 in chronic inflammation such as that seen in CAED. More interestingly we discovered that under the influence of its receptor, IL-17 may downregulate inflammation under certain circumstances. These results are the first of its kind to be demonstrated in CAED and human conjunctival epithelial cells.

The increasing prevalence of allergy has in the past been attempted to be explained in the context of T helper cell subsets, this concept has been referred to as the 'hygiene hypothesis' and it describes the events that are thought to contribute to atopy. This idea was first proposed by the observations made by Strachan on hay fever and the number of older children in the household, the author suggests that if there are older siblings in the family, younger siblings have a decreased prevalence of hay fever (Strachan, 1989). One theory for this phenomenon is that early childhood infection via unhygienic contact with older siblings or acquired prenatally by a mother infected by contact with her older children, may prevent the onset of allegic diseases. The increased expression of atopic disease may therefore be as a result of higher standards of personal hygiene thereby reducing the occurrence of

cross infection (Strachan, 1989). In relation to T helper subsets, it has been argued that bacterial and viral infections during early life direct the immune system towards a Th1 phenotype and the production of IFN-γ, inhibiting Th2 responses. Further viral or bacterial infection and therefore Th1 activation may prevent the proliferation of Th2 cells and thus the development of allegic disease (von Mutius, 2001). In keeping with this theory, a more recent study has demonstrated that mouse models of allergic airway disease, when infected by *Helicobacter pylori*, exhibit reduced airway hyperresponsiveness as well as the lessening in inflammation and goblet cell metaplasia which are characteristic of asthma. Within the BALF of these mice, IL-5 secretion and pulmonary infiltration of Th2 and Th17 cells was diminished. These observations were found to be more strongly induced in infected neonates than adult infected mice (Arnold *et al*, 2011). From both studies it can be thought that an increase in allergy may ensue due to a reduction in microbial burden, leading to diminshed Th1 activation and uncontrolled Th2 responses.

We have previously discussed the findings of recent years which have implicated another T helper cell subset – Th17 in the pathogenesis of certain diseases; our findings have also shown these cells to be present in CAED. As Th1 and Th2 cells have been the focus of the hygiene hypothesis, it is likely that recent discoveries concerning Th17 cells would require this idea to be revised. The question can be asked as to where Th17 cells fit in regarding this hypothesis. Although differing in their development and phenotypical characteristics, reports have suggested that there are some shared similarities between Th1 and Th17 cells, mainly in their expression of surface molecules (Nakae *et al*, 2007). This similarity may be important as some

diseases that were once classified as being Th1 driven have been redefined and show that Th17 cells may influence the pathology such as that seen in EAE. Adoptive transfer experiments in these mice concluded that IL-17 producing T cells were more efficient at inducing EAE than T cells producing IFN-γ alone (Langrish *et al*, 2005). The ability of IL-23 to induce IL-17 expressing T cells can also be expanded to include IFN-γ production and proliferation of T cells (Oppmann *et al*, 2000). More interestingly, recent data has now shown that there are some cells which have the ability to produce both IL-17 and IFN-γ, and Th17 cells can be converted to IFN-γ producing cells by IL-12 and IL-23, highlighting the plasticity of Th17 cells and the parallels they share with Th1 cells (Lee *et al*, 2009). If we go back and put this in context with the hygiene hypothesis, the proposed favouring of Th1 activation may also include that of Th17 cells, perhaps even more so as they are involved in host defence and their cytokine IL-17 is also produced by innate cells.

The role of Th17 cells and IL-17 in the immune response may be of great importance when considering inflammatory disease, particularly in the lungs as demonstrated by new studies. Secondary lymphoid organs create an environment that facilitates the activation of T cells and B cells, allowing immune reactions to occur more efficiently (Cupedo, 2011). Recently one group has defined a role for IL-17 in the development of lymphoid tissues in the lung. These tissues that form in the lung are generated ectopically and are known as inducible bronchus-associated lymphoid tissue (iBALT) which occur after infection or inflammation and is more apparent in chronic disease (Randall, 2010). Their development in LPS treated mouse neonate models has now been associated with T cell - IL-17 signalling; furthermore the chemokines

involved in conventional lymphoid organ formation, CXCL13 and CXCL19 are thought to be induced by IL-17 (Rangel-Moreno *et al*, 2011). The formation of lymphoid tissues due to exposure to microbes would therefore be important in influencing the immune response.

It is unclear as to whether the hygiene hypothesis can be used at this juncture to explain the increased frequency of allergy. Exposure to bacteria and viruses in neonates or in early childhood and the resulting production of Th1 and Th17 cells cannot justify a causal link to exaggerated Th2 responses. The actions of TSLP in the induction of proallergic Th2 responses have already been proposed. Tanaka and colleagues further show that the response to TSLP and poly I:C by dendritic cells promotes the differentiation of Th17 cells and reduces IL-17 and IFN-γ double producing cells (Tanaka *et al*, 2008). Moreover, in lung tissues of a mouse model, IL-17 was reported to be induced by allergen and was capable of reducing eosinophil recruitment and Th2 cytokine production (Schnyder-Candrian *et al*, 2006). This evidence casts more doubt onto the hygiene hypothesis as it fails to take into account the regulatory role T helper cytokines play between the different subtypes and suggests that there may be more of a balancing act between these cells and the outcome of allergic disease.

As well as TSLP, other epithelial cell derived cytokines have now become the focus of attention, such as IL-33. This new member of the IL-1 super family is also produced by other cell types and can activate Th2 cells and mast cells leading to the secretion of proinflammatory and Th2 cytokines (Schmitz *et al*, 2005). IL-33 has

been implicated in allergic asthma with studies showing increased immunoreactivity of epithelial IL-33 in patients with severe asthma (Préfontaine *et al*, 2010). This cytokine also has the ability to stimulate innate cells such as basophils, to secrete Th2 cytokines and can work without the aid of T cells (Kondo *et al*, 2008). More recently studies involving mouse models of airway hyper-reactivity (AHR) have revealed that its induction can occur via IL-33 activation of an innate lymphoid cell type termed 'natural helper cells' (Chang *et al*, 2011). The authors demonstrate that AHR development by H3N1 infection can induce IL-33 production which in turn activates natural helper cells to produce IL-13 – more so than other cell types (Chang *et al*, 2011). This may have implications in CAED as one study has reported a possible role for IL-33 in chronic allergic conjunctivitis (Matsuda *et al*, 2009) and our study has shown non T cell expression of IL-13 in the conjunctival tissues, albeit the expression of IL-13 could be because of its binding to other cells.

As new reports reveal the presence of certain cell types and cytokines in inflammation and disease, it has become increasingly important to determine effective therapies to overcome an over-active immune system. This may involve targeting receptors on the cells that would block their activation or antibodies that neutralise specific cytokines and chemokines. As this study has demonstrated the presence of TSLP and IL-17 in CAED, they could be important targets in disease control or prevention. To date a few groups have focussed their investigations on blocking the TSLP receptor (TSLPR). One such group demonstrated an increase in TSLP in asthmatic mice, following blockade with a TSLPR neutralising antibody led to a decrease in eosinophils and lymphocytes as well as a reduction in IL-4 and IL-5.

Whilst there was a decrease in Th2 cytokines, IFN-γ and IL-10 were increased (Shi *et al*, 2008). At the same time a study was published on the use of imidazoquinoline compounds – imiquimod and its derivative R848 (ligands to TLR7 and TLR8) – to suppress TSLP-DC mediated inflammatory Th2 cell responses. Treatment of human DCs with TSLP and R848 significantly reduced levels of IL-4, IL-5, IL-13 and TNF-α whilst increasing IL-10 and IFN-γ (Torii *et al*, 2008).

There are also endogenous mechanisms that have been proposed to serve a role in inhibiting TLR-dependent inflammatory responses, one such example is SHP (small heterodimer partner); an orphan member of the nuclear receptor superfamily, that contributes to transcriptional regulation. A recent study has shown that LPS-stimulated macrophages from SHP knockout mice have greater TNF, IL-1β and IL-6 protein and mRNA expression than $SHP^{+/+}$ mice (Yuk *et al*, 2011). This same study demonstrated that the mechanisms involved were due to the inhibition of TLR signalling pathways – both MyD88 dependent and independent (TRIF pathway) – downstream of various ligands (Yuk *et al*, 2011). In context with inflammatory diseases, this may be a potential therapeutic target. Certain agents, MSP (macrophage stimulating protein), metformin and AICAR (5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside) have already been found to induce the expression of SHP in hepatocytes (Chanda *et al*, 2009; Kim *et al*, 2008). MSP in particular has shown that it can down-regulate TLR4 inflammatory responses in $SHP^{+/+}$ mice (Yuk *et al*, 2011).

Like TSLP, the increasing literature on Th17 pathogenesis has led researchers to investigate therapies aimed towards reducing IL-17 in order to ameliorate inflammatory disease. So far certain drugs, namely Methotrexate and/or Etanercept have been used to redress the balance of Th17 cells in RA patients (Li *et al*, 2011; Lina *et al*, 2011). In other disease settings it may be useful to target the cytokines that drive Th17 expansion such as IL-23. Previous work has described the protection of mice from psoriasis after treatment with an anti-IL-12 mAb which reacts against the IL-12 (p35/p40 (p70)) heterodimer, and a fully human anti-IL-12/23 antibody called ABT-874 has been reported to reduce the psoriatic area by 75% (Hong *et al*, 1999; Kimball *et al*, 2008). As IL-23 consists of a p19/p40 chain it could be a target for the amelioration of disease. The inhibition of IL-17 signalling through its receptor may also be an effective method. As IL-17RC has a higher affinity for IL-17A, an antibody directed towards this receptor could neutralise both IL-17A and IL-17F. Investigators may also target IL-17A, but as this cytokine appears to have many functions, blocking its total production may not be wise. As innate cells can also produce IL-17, researchers should possibly concentrate on targeting the production of IL-17 by Th17 cells therefore allowing innate IL-17 production to carry out its role in host defence. Furthermore, it may also be possible to exploit the plasticity of Th17 cells in order to convert them to regulatory T cells, thereby suppressing inflammation. The most recent studies have shown that human myeloid derived suppressor cells ($CD14^{+}HLA\text{-}DR^{-/low}$) when cultured with Th17 cells decreased the frequency of IL-17 secreting $CD4^{+}$ cells while increasing $FoxP3^{+}$ $IL\text{-}17^{-}CD4^{+}$ cells (Hoechst *et al*, 2011). Moreover, human Th17 cells generated from tumour-

infiltrating lymphocytes that had been expanded in the presence of OKT3 and allogeneic PBMCs, decreased in number in contrast to increased percentages of IFN-γ producing and $FOXP3^+$ cells (Ye *et al*, 2011). The authors suggest that this may be due to repeated TCR stimulation. More interestingly, not only do these cells have potent suppressive ability but they do not convert back to Th17 cells even under Th17 polarizing conditions (Ye *et al*, 2011).

Through our own work and other studies to date, new theories have come to light, but yet many questions remain unanswered regarding the complexity of immune cell interactions and the role of the cytokine microenvironment in the immune response. When we consider the range of receptors expressed and the ability to produce cytokines that can both initiate and regulate inflammation, we can say that the epithelium is central to immunity with characteristics that bridge both the innate and adaptive arms of the immune response.

Abu El-Asrar, A.M., S. Struyf, A.A. Al-Mosallam, L. Missotten, J. Van Damme and K. Geboes 2001. Expression of chemokine receptors in vernal keratoconjunctivitis. *Br J Ophthalmol* 85:*1357*.

Abu el-Asrar, A.M., O.o.J. Van den, K. Geboes, L. Missotten, M.H. Emarah and V. Desmet 1989. Immunopathological study of vernal keratoconjunctivitis. *Graefes Arch Clin Exp Ophthalmol* 227:*374*.

Acosta-Rodriguez, E.V., G. Napolitani, A. Lanzavecchia and F. Sallusto 2007. Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol* 8:*942*.

Aggarwal, S., N. Ghilardi, M.H. Xie, F.J. de Sauvage and A.L. Gurney 2003. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *J Biol Chem* 278:*1910*.

Al-Shami, A., R. Spolski, J. Kelly, A. Keane-Myers and W.J. Leonard 2005. A role for TSLP in the development of inflammation in an asthma model. *J Exp Med* 202:*829*.

Albanesi, C., A. Cavani and G. Girolomoni 1999. IL-17 is produced by nickel-specific T lymphocytes and regulates ICAM-1 expression and chemokine production in human keratinocytes: synergistic or antagonist effects with IFN-gamma and TNF-alpha. *J Immunol* 162:*494*.

Allakhverdi, Z., M.R. Comeau, H.K. Jessup, B.R. Yoon, A. Brewer, S. Chartier, N. Paquette, S.F. Ziegler, M. Sarfati and G. Delespesse 2007. Thymic stromal lymphopoietin is released by human epithelial cells in response to microbes, trauma, or inflammation and potently activates mast cells. *J Exp Med* 204:*253*.

Allan, G.J., J. Beattie. and D.J. Flint 2008. Epithelial injury induces an innate repair mechanism linked to cellular senescence and fibrosis involving IGF-binding protein-5. *J Endocrinol* 199:*155*.

Anderson, D.F., J.D. MacLeod, S.M. Baddeley, A.S. Bacon, J.I. McGill, S.T. Holgate and W.R. Roche 1997. Seasonal allergic conjunctivitis is accompanied by

increased mast cell numbers in the absence of leucocyte infiltration. *Clin Exp Allergy* 27:*1060*.

Anderson, D.F., S. Zhang, P. Bradding, J.I. McGill, S.T. Holgate and W.R. Roche 2001. The relative contribution of mast cell subsets to conjunctival TH2-like cytokines. *Invest Ophthalmol Vis Sci* 42:*995*.

Angkasekwinai, P., H. Park, Y.H. Wang, Y.H. Wang, S.H. Chang, D.B. Corry, Y.J. Liu, Z. Zhu and C. Dong 2007. Interleukin 25 promotes the initiation of proallergic type 2 responses. *J Exp Med* 204:*1509*.

Annunziato, F., L. Cosmi, F. Liotta, E. Maggi and S. Romagnani 2008. The phenotype of human Th17 cells and their precursors, the cytokines that mediate their differentiation and the role of Th17 cells in inflammation. *Int Immunol* 20:*1361*.

Arnold, I.C., N. Dehzad, S. Reuter, H. Martin, B. Becher, C. Taube and A. Muller 2011. Helicobacter pylori infection prevents allergic asthma in mouse models through the induction of regulatory T cells. *J Clin Invest* 121:*3088*.

Atherton, H.C., G. Jones and H. Danahay 2003. IL-13-induced changes in the goblet cell density of human bronchial epithelial cell cultures: MAP kinase and phosphatidylinositol 3-kinase regulation. *Am J Physiol Lung Cell Mol Physiol* 285:*L730*.

Austin, L.M., M. Ozawa, T. Kikuchi, I.B. Walters and J.G. Krueger 1999. The majority of epidermal T cells in Psoriasis vulgaris lesions can produce type 1 cytokines, interferon-gamma, interleukin-2, and tumor necrosis factor-alpha, defining TC1 (cytotoxic T lymphocyte) and TH1 effector populations: a type 1 differentiation bias is also measured in circulating blood T cells in psoriatic patients. *J Invest Dermatol* 113:*752*.

Bacon, A.S., P. Ahluwalia, A.M. Irani, L.B. Schwartz, S.T. Holgate, M.K. Church and J.I. McGill 2000. Tear and conjunctival changes during the allergen-induced early- and late-phase responses. *J Allergy Clin Immunol* 106:*948*.

Baggiolini, M., B. Dewald and B. Moser 1997. Human chemokines: an update. *Annu Rev Immunol* 15:*675*.

Bals, R. and P.S. Hiemstra 2004. Innate immunity in the lung: how epithelial cells fight against respiratory pathogens. *Eur Respir J* 23:*327*.

Bauer, S., D. Hangel and P. Yu 2007. Immunobiology of toll-like receptors in allergic disease. *Immunobiology* 212:*521*.

Ben-Baruch, A., D.F. Michiel and J.J. Oppenheim 1995. Signals and receptors involved in recruitment of inflammatory cells. *J Biol Chem* 270:*11703*.

Bettelli, E., Y. Carrier, W. Gao, T. Korn, T.B. Strom, M. Oukka, H.L. Weiner and V.K. Kuchroo 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441:*235*.

Beutler, B., Z. Jiang, P. Georgel, K. Crozat, B. Croker, S. Rutschmann, X. Du and K. Hoebe 2006. Genetic analysis of host resistance: Toll-like receptor signaling and immunity at large. *Annu Rev Immunol* 24:*353*.

Bielory, L. 2000. Allergic and immunologic disorders of the eye. Part II: ocular allergy. *J Allergy Clin Immunol* 106:*1019*.

Bielory, L. and S. Ghafoor 2005. Histamine receptors and the conjunctiva. *Curr Opin Allergy Clin Immunol* 5:*437*.

Bonini, S. 2004. Atopic keratoconjunctivitis. *Allergy* 59 Suppl 78:*71*.

Bonini, S., M. Coassin, S. Aronni and A. Lambiase 2004. Vernal keratoconjunctivitis. *Eye (Lond)* 18:*345*.

Bonini, S., A. Micera, A. Iovieno, A. Lambiase and S. Bonini 2005. Expression of Toll-like receptors in healthy and allergic conjunctiva. *Ophthalmology* 112:*1528; discussion 154*.

Brenner, M.B., J. McLean, D.P. Dialynas, J.L. Strominger, J.A. Smith, F.L. Owen, J.G. Seidman, S. Ip, F. Rosen and M.S. Krangel 1986. Identification of a putative second T-cell receptor. *Nature* 322:*145*.

Buckley, R. J. 1998. Allergic eye disease – a clinical challenge. *Clin Exp.Allergy* 28 Suppl 6:39.

Butcher, E.C. and L.J. Picker 1996. Lymphocyte homing and homeostasis. *Science* 272:*60*.

Calder, V.L., G. Jolly, M. Hingorani, P. Adamson, A. Leonardi, A.G. Secchi, R.J. Buckley and S. Lightman 1999. Cytokine production and mRNA expression by conjunctival T-cell lines in chronic allergic eye disease. *Clin Exp Allergy* 29:*1214*.

Calonge, M., A.E. De Salamanca, K.F. Siemasko, Y. Diebold, J. Gao, M. Juarez-Campo and M.E. Stern 2005. Variation in the expression of inflammatory markers and neuroreceptors in human conjunctival epithelial cells. *Ocul Surf* 3:*S145*.

Casanova, J.L., L. Abel and L. Quintana-Murci 2011. Human TLRs and IL-1Rs in host defense: natural insights from evolutionary, epidemiological, and clinical genetics. *Annu Rev Immunol* 29:*447*.

Chanda, D., T. Li, K.H. Song, Y.H. Kim, J. Sim, C.H. Lee, J.Y. Chiang and H.S. Choi 2009. Hepatocyte growth factor family negatively regulates hepatic gluconeogenesis via induction of orphan nuclear receptor small heterodimer partner in primary hepatocytes. *J Biol Chem* 284:*28510*.

Chang, S.H. and C. Dong 2011. Signaling of interleukin-17 family cytokines in immunity and inflammation. *Cell Signal* 23:*1069*.

Chang, Y.J., H.Y. Kim, L.A. Albacker, N. Baumgarth, A.N. McKenzie, D.E. Smith, R.H. Dekruyff and D.T. Umetsu 2011. Innate lymphoid cells mediate influenza-induced airway hyper-reactivity independently of adaptive immunity. *Nat Immunol* 12:*631*.

Chang, Z.L. 2010. Important aspects of Toll-like receptors, ligands and their signaling pathways. *Inflamm Res* 59:*791*.

Chauhan, S.K., J. El Annan, T. Ecoiffier, S. Goyal, Q. Zhang, D.R. Saban and R. Dana 2009. Autoimmunity in dry eye is due to resistance of Th17 to Treg suppression. *J Immunol* 182:*1247*.

Chertov, O., D. Yang, O.M. Howard and J.J. Oppenheim 2000. Leukocyte granule proteins mobilize innate host defenses and adaptive immune responses. *Immunol Rev* 177:*68*.

Chien, Y.H., R. Jores and M.P. Crowley 1996. Recognition by gamma/delta T cells. *Annu Rev Immunol* 14:*511*.

Chung, S.H., M.N. Kweon, H.K. Lee, S.I. Choi, J.Y. Yang and E.K. Kim 2009. Toll-like receptor 4 initiates an innate immune response to lipopolysaccharide in human conjunctival epithelial cells. *Exp Eye Res* 88:*49*.

Church, M.K. and F. Levi-Schaffer 1997. The human mast cell. *J Allergy Clin Immunol* 99:*155*.

Cohn, L., R.J. Homer, H. MacLeod, M. Mohrs, F. Brombacher and K. Bottomly 1999. Th2-induced airway mucus production is dependent on IL-4Ralpha, but not on eosinophils. *J Immunol* 162:*6178*.

Conti, H.R., F. Shen, N. Nayyar, E. Stocum, J.N. Sun, M.J. Lindemann, A.W. Ho, J.H. Hai, J.J. Yu, J.W. Jung, S.G. Filler, P. Masso-Welch, M. Edgerton and S.L. Gaffen 2009. Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis. *J Exp Med* 206:*299*.

Conti, P. and M. DiGioacchino 2001. MCP-1 and RANTES are mediators of acute and chronic inflammation. *Allergy Asthma Proc* 22:*133*.

Cook, E.B., J.L. Stahl, S. Esnault, N.P. Barney and F.M. Graziano 2005. Toll-like receptor 2 expression on human conjunctival epithelial cells: a pathway for Staphylococcus aureus involvement in chronic ocular proinflammatory responses. *Ann Allergy Asthma Immunol* 94:*486*.

Cua, D.J., J. Sherlock, Y. Chen, C.A. Murphy, B. Joyce, B. Seymour, L. Lucian, W. To, S. Kwan, T. Churakova, S. Zurawski, M. Wiekowski, S.A. Lira, D. Gorman, R.A. Kastelein and J.D. Sedgwick 2003. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* 421:*744*.

Cupedo, T. 2011. An unexpected role for IL-17 in lymphoid organogenesis. *Nat Immunol* 12:*590*.

Dardalhon, V., A. Awasthi, H. Kwon, G. Galileos, W. Gao, R.A. Sobel, M. Mitsdoerffer, T.B. Strom, W. Elyaman, I.C. Ho, S. Khoury, M. Oukka and V.K. Kuchroo 2008. IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(-) effector T cells. *Nat Immunol* 9:*1347*.

Dartt, D.A. 2004. Control of mucin production by ocular surface epithelial cells. *Exp Eye Res* 78:*173*.

Del Prete, G., M. De Carli, F. Almerigogna, M.G. Giudizi, R. Biagiotti and S. Romagnani 1993. Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production. *J Immunol* 150:*353*.

De Paiva, C.S., S. Chotikavanich, S.B. Pangelinan, JD 3rd. Pitcher, B. Fang, X. Zheng, P. Ma, W.J. Farley, K.F. Siemasko, J.Y. Niederkorn, M.E. Stern, D.Q. Li and S.C. Pflugfelder 2009. IL-17 disrupts corneal barrier following desiccating stress. *Mucosal Immunol* 2:*243*.

Diamond, G., D. Legarda and L.K. Ryan 2000. The innate immune response of the respiratory epithelium. *Immunol Rev* 173:*27*.

Dieu-Nosjean, M.C., C. Massacrier, B. Homey, B. Vanbervliet, J.J. Pin, A. Vicari, S. Lebecque, C. Dezutter-Dambuyant, D. Schmitt, A. Zlotnik and C. Caux 2000. Macrophage inflammatory protein 3alpha is expressed at inflamed epithelial surfaces and is the most potent chemokine known in attracting Langerhans cell precursors. *J Exp Med* 192:*705*.

Dinarello, C.A. 2000. Proinflammatory cytokines. *Chest* 118:*503*.

Dogusan, Z., M. Garcia, D. Flamez, L. Alexopoulou, M. Goldman, C. Gysemans, C. Mathieu, C. Libert, D.L. Eizirik and J. Rasschaert 2008. Double-stranded RNA induces pancreatic beta-cell apoptosis by activation of the toll-like receptor 3 and interferon regulatory factor 3 pathways. *Diabetes* 57:*1236*.

Dong, C. and R.A. Flavell 2001. Th1 and Th2 cells. *Curr Opin Hematol* 8:*47*.

Doss, M., M.R. White, T. Tecle and K.L. Hartshorn 2010. Human defensins and LL-37 in mucosal immunity. *J Leukoc Biol* 87:*79*.

Dugas, B., J.C. Renauld, J. Pene, J.Y. Bonnefoy, C. Peti-Frere, P. Braquet, J. Bousquet, J. Van Snick and J.M. Mencia-Huerta 1993. Interleukin-9 potentiates the interleukin-4-induced immunoglobulin (IgG, IgM and IgE) production by normal human B lymphocytes. *Eur J Immunol* 23:*1687*.

Ebner, S., V.A. Nguyen, M. Forstner, Y.H. Wang, D. Wolfram, Y.J. Liu and N. Romani 2007. Thymic stromal lymphopoietin converts human epidermal Langerhans cells into antigen-presenting cells that induce proallergic T cells. *J Allergy Clin Immunol* 119:*982*.

Ely, L.K., S. Fischer and K.C. Garcia 2009. Structural basis of receptor sharing by interleukin 17 cytokines. *Nat Immunol* 10:*1245*.

Enriquez-de-Salamanca, A., V. Calder, J. Gao, G. Galatowicz, C. Garcia-Vazquez, I. Fernandez, M.E. Stern, Y. Diebold and M. Calonge 2008. Cytokine responses by conjunctival epithelial cells: an in vitro model of ocular inflammation. *Cytokine* 44:*160*.

Esnault, S., N. Benbernou, F. Lavaud, H.C. Shin, G. Potron and M. Guenounou 1996. Differential spontaneous expression of mRNA for IL-4, IL-10, IL-13, IL-2 and interferon-gamma (IFN-gamma) in peripheral blood mononuclear cells (PBMC) from atopic patients. *Clin Exp Immunol* 103:*111*.

Fallon, P.G., S.J. Ballantyne, N.E. Mangan, J.L. Barlow, A. Dasvarma, D.R. Hewett, A. McIlgorm, H.E. Jolin and A.N. McKenzie 2006. Identification of an interleukin (IL)-25-dependent cell population that provides IL-4, IL-5, and IL-13 at the onset of helminth expulsion. *J Exp Med* 203:*1105*.

Fort, M.M., J. Cheung, D. Yen, J. Li, S.M. Zurawski, S. Lo, S. Menon, T. Clifford, B. Hunte, R. Lesley, T. Muchamuel, S.D. Hurst, G. Zurawski, M.W. Leach, D.M. Gorman and D.M. Rennick 2001. IL-25 induces IL-4, IL-5, and IL-13 and Th2-

associated pathologies in vivo. *Immunity* 15:*985*.

Foster, C.S., B.A. Rice and J.E. Dutt 1991. Immunopathology of atopic keratoconjunctivitis. *Ophthalmology* 98:*1190*.

Freitas, A., J.C. Alves-Filho, T. Victoni, T. Secher, H.P. Lemos, F. Sonego, F.Q. Cunha and B. Ryffel 2009. IL-17 receptor signaling is required to control polymicrobial sepsis. *J Immunol* 182:*7846*.

Fujimoto, S., M. Komine, M. Karakawa, H. Uratsuji, S. Kagami, Y. Tada, H. Saeki, M. Ohtsuki and K. Tamaki 2011. Histamine differentially regulates the production of Th1 and Th2 chemokines by keratinocytes through histamine H1 receptor. *Cytokine* 54:*191*.

Fujishima, H., I. Saito, T. Takeuchi, N. Shinozaki and K. Tsubota 1997. Characterization of cytokine mRNA transcripts in conjunctival cells in patients with allergic conjunctivitis. *Invest Ophthalmol Vis Sci* 38:*1350*.

Fukushima, A., T. Sumi, W. Ishida, J. Yamada, Y. Iwakura and H. Ueno 2008. Endogenous IL-17 does not play a significant role in the development of experimental murine allergic conjunctivitis. *Int Arch Allergy Immunol* 147:*206*.

Gaffen, S.L. 2009. Structure and signalling in the IL-17 receptor family. *Nat Rev Immunol* 9:*556*.

Gaffen, S.L. 2011. Recent advances in the IL-17 cytokine family. *Curr Opin Immunol* 23:*613*.

Galli, S.J., S. Nakae and M. Tsai 2005. Mast cells in the development of adaptive immune responses. *Nat Immunol* 6:*135*.

Geboes, L., L. Dumoutier, H. Kelchtermans, E. Schurgers, T. Mitera, J.C. Renauld and P. Matthys 2009. Proinflammatory role of the Th17 cytokine interleukin-22 in collagen-induced arthritis in C57BL/6 mice. *Arthritis Rheum* 60:*390*.

Gell, P.G.H, Coombs R.R.A, eds. *Clinical Aspects of Immunology*. 1st ed. Oxford, England: Blackwell; 1963

Ghoreschi, K., A. Laurence, X.P. Yang, K. Hirahara and J.J. O'Shea 2011. T helper

17 cell heterogeneity and pathogenicity in autoimmune disease. *Trends Immunol* 32:*395*.

Gros, E., C. Bussmann, T. Bieber, I. Forster and N. Novak 2009. Expression of chemokines and chemokine receptors in lesional and nonlesional upper skin of patients with atopic dermatitis. *J Allergy Clin Immunol* 124:*753*.

Guermonprez, P., J. Valladeau, L. Zitvogel, C. Thery and S. Amigorena 2002. Antigen presentation and T cell stimulation by dendritic cells. *Annu Rev Immunol* 20:*621*.

Harada, A., N. Sekido, T. Akahoshi, T. Wada, N. Mukaida and K. Matsushima 1994. Essential involvement of interleukin-8 (IL-8) in acute inflammation. *J Leukoc Biol* 56:*559*.

Hasnain, S.Z., D.J. Thornton and R.K. Grencis 2011. Changes in the mucosal barrier during acute and chronic Trichuris muris infection. *Parasite Immunol* 33:*45*.

He, R., M.K. Oyoshi, L. Garibyan, L. Kumar, S.F. Ziegler and R.S. Geha 2008. TSLP acts on infiltrating effector T cells to drive allergic skin inflammation. *Proc Natl Acad Sci U S A* 105:*11875*.

Heijink, I.H., P. Marcel Kies, A.J. van Oosterhout, D.S. Postma, H.F. Kauffman and E. Vellenga 2007. Der p, IL-4, and TGF-beta cooperatively induce EGFR-dependent TARC expression in airway epithelium. *Am J Respir Cell Mol Biol* 36:*351*.

Hingorani, M., V.L. Calder, R.J. Buckley and S.L. Lightman 1998. The role of conjunctival epithelial cells in chronic ocular allergic disease. *Exp Eye Res* 67:*491*.

Hirota, K., H. Yoshitomi, M. Hashimoto, S. Maeda, S. Teradaira, N. Sugimoto, T. Yamaguchi, T. Nomura, H. Ito, T. Nakamura, N. Sakaguchi and S. Sakaguchi 2007. Preferential recruitment of CCR6-expressing Th17 cells to inflamed joints via CCL20 in rheumatoid arthritis and its animal model. *J Exp Med* 204:*2803*.

Hoang-Xuan, T., C. Baudouin and C. Creuzot-Garcher. *Inflammatory Diseases of the Conjunctiva*. Thieme Medical Publishers. 2001

Hoechst, B., J. Gamrekelashvili, M.P. Manns, T.F. Greten and F. Korangy 2011. Plasticity of human Th17 cells and iTregs is orchestrated by different subsets of myeloid cells. *Blood* 117:*6532*.

Holgate, S.T. 2007. The epithelium takes centre stage in asthma and atopic dermatitis. *Trends Immunol* 28:*248*.

Hong, K., A. Chu, B.R. Ludviksson, E.L. Berg and R.O. Ehrhardt 1999. IL-12, independently of IFN-gamma, plays a crucial role in the pathogenesis of a murine psoriasis-like skin disorder. *J Immunol* 162:*7480*.

Huang, F., C.Y. Kao, S. Wachi, P. Thai, J. Ryu and R. Wu 2007. Requirement for both JAK-mediated PI3K signaling and ACT1/TRAF6/TAK1-dependent NF-kappaB activation by IL-17A in enhancing cytokine expression in human airway epithelial cells. *J Immunol* 179:*6504*.

Huang, L.C., D. Jean, R.J. Proske, R.Y. Reins and A.M. McDermott 2007. Ocular surface expression and in vitro activity of antimicrobial peptides. *Curr Eye Res* 32:*595*.

Huang, S.K., H.Q. Xiao, J. Kleine-Tebbe, G. Paciotti, D.G. Marsh, L.M. Lichtenstein and M.C. Liu 1995. IL-13 expression at the sites of allergen challenge in patients with asthma. *J Immunol* 155:*2688*.

Ishigame, H., S. Kakuta, T. Nagai, M. Kadoki, A. Nambu, Y. Komiyama, N. Fujikado, Y. Tanahashi, A. Akitsu, H. Kotaki, K. Sudo, S. Nakae, C. Sasakawa and Y. Iwakura 2009. Differential roles of interleukin-17A and -17F in host defense against mucoepithelial bacterial infection and allergic responses. *Immunity* 30:*108*.

Ishii, K.J. and S. Akira 2008. Potential link between the immune system and metabolism of nucleic acids. *Curr Opin Immunol* 20:*524*.

Ito, T., Y.H. Wang, O. Duramad, T. Hori, G.J. Delespesse, N. Watanabe, F.X. Qin, Z. Yao, W. Cao and Y.J. Liu 2005. TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. *J Exp Med* 202:*1213*.

Iwamura, C. and T. Nakayama 2010. Role of NKT cells in allergic asthma. *Curr Opin Immunol* 22:*807*.

Janeway, C.A., Jr. and R. Medzhitov 2002. Innate immune recognition. *Annu Rev Immunol* 20:*197*.

Jones, C.E. and K. Chan 2002. Interleukin-17 stimulates the expression of interleukin-8, growth-related oncogene-alpha, and granulocyte-colony-stimulating factor by human airway epithelial cells. *Am J Respir Cell Mol Biol* 26:*748*.

Jung, H.C., L. Eckmann, S.K. Yang, A. Panja, J. Fierer, E. Morzycka-Wroblewska and M.F. Kagnoff 1995. A distinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion. *J Clin Invest* 95:*55*.

Kato, A., S. Favoreto, Jr., P.C. Avila and R.P. Schleimer 2007. TLR3- and Th2 cytokine-dependent production of thymic stromal lymphopoietin in human airway epithelial cells. *J Immunol* 179:*1080*.

Kaufmann, S.H. 1996. gamma/delta and other unconventional T lymphocytes: what do they see and what do they do? *Proc Natl Acad Sci U S A* 93:*2272*.

Kaufmann, S.H., C. Blum and S. Yamamoto 1993. Crosstalk between alpha/beta T cells and gamma/delta T cells in vivo: activation of alpha/beta T-cell responses after gamma/delta T-cell modulation with the monoclonal antibody GL3. *Proc Natl Acad Sci U S A* 90:*9620*.

Kawaguchi, M., F. Kokubu, H. Kuga, S. Matsukura, H. Hoshino, K. Ieki, T. Imai, M. Adachi and S.K. Huang 2001. Modulation of bronchial epithelial cells by IL-17. *J Allergy Clin Immunol* 108:*804*.

Kenna, T., M. O'Brien, A.E. Hogan, M.A. Exley, S.A. Porcelli, J.E. Hegarty, C. O'Farrelly and D.G. Doherty 2007. CD1 expression and CD1-restricted T cell activity in normal and tumour-bearing human liver. *Cancer Immunol Immunother* 56:*563*.

Khader, S.A., G.K. Bell, J.E. Pearl, J.J. Fountain, J. Rangel-Moreno, G.E. Cilley, F. Shen, S.M. Eaton, S.L. Gaffen, S.L. Swain, R.M. Locksley, L. Haynes, T.D. Randall

and A.M. Cooper 2007. IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during Mycobacterium tuberculosis challenge. *Nat Immunol* 8:*369*.

Kim, Y.D., K.G. Park, Y.S. Lee, Y.Y. Park, D.K. Kim, B. Nedumaran, W.G. Jang, W.J. Cho, J. Ha, I.K. Lee, C.H. Lee and H.S. Choi 2008. Metformin inhibits hepatic gluconeogenesis through AMP-activated protein kinase-dependent regulation of the orphan nuclear receptor SHP. *Diabetes* 57:*306*.

Kimball, A.B., K.B. Gordon, R.G. Langley, A. Menter, E.K. Perdok, R.J. Valdes and ABT-874 Study Investigators 2008. Safety and efficacy of ABT-874, a fully human interleukin 12/23 monoclonal antibody, in the treatment of moderate to severe chronic plaque psoriasis: results of a randomized, placebo-controlled, phase 2 trial. *Arch Dermatol* 144:*200*.

King, C.L., J. Xianli, I. Malhotra, S. Liu, A.A. Mahmoud and H.C. Oettgen 1997. Mice with a targeted deletion of the IgE gene have increased worm burdens and reduced granulomatous inflammation following primary infection with Schistosoma mansoni. *J Immunol* 158:*294*.

King, C., S. Brennan, P.J. Thompson and G.A. Stewart 1998. Dust mite proteolytic allergens induce cytokine release from cultured airway epithelium. *J Immunol* 161:*3645*.

Kleinschek, M.A., A.M. Owyang, B. Joyce-Shaikh, C.L. Langrish, Y. Chen, D.M. Gorman, W.M. Blumenschein, T. McClanahan, F. Brombacher, S.D. Hurst, R.A. Kastelein and D.J. Cua 2007. IL-25 regulates Th17 function in autoimmune inflammation. *J Exp Med* 204:*161*.

Kobayashi, T., T. Miura, T. Haba, M. Sato, I. Serizawa, H. Nagai and K. Ishizaka 2000. An essential role of mast cells in the development of airway hyperresponsiveness in a murine asthma model. *J Immunol* 164:*3855*.

Koenders, M.I., J.K. Kolls, B. Oppers-Walgreen, L. van den Bersselaar, L.A. Joosten, J.R. Schurr, P. Schwarzenberger, W.B. van den Berg and E. Lubberts 2005.

Interleukin-17 receptor deficiency results in impaired synovial expression of interleukin-1 and matrix metalloproteinases 3, 9, and 13 and prevents cartilage destruction during chronic reactivated streptococcal cell wall-induced arthritis. *Arthritis Rheum* 52:*3239*.

Koenders, M.I., E. Lubberts, B. Oppers-Walgreen, L. van den Bersselaar, M.M. Helsen, F.E. Di Padova, A.M. Boots, H. Gram, L.A. Joosten and W.B. van den Berg 2005. Blocking of interleukin-17 during reactivation of experimental arthritis prevents joint inflammation and bone erosion by decreasing RANKL and interleukin-1. *Am J Pathol* 167:*141*.

Komano, H., Y. Fujiura, M. Kawaguchi, S. Matsumoto, Y. Hashimoto, S. Obana, P. Mombaerts, S. Tonegawa, H. Yamamoto, S. Itohara et al 1995. Homeostatic regulation of intestinal epithelia by intraepithelial gamma delta T cells. *Proc Natl Acad Sci U S A* 92:*6147*.

Kondo, Y., T. Yoshimoto, K. Yasuda, S. Futatsugi-Yumikura, M. Morimoto, N. Hayashi, T. Hoshino, J. Fujimoto and K. Nakanishi 2008. Administration of IL-33 induces airway hyperresponsiveness and goblet cell hyperplasia in the lungs in the absence of adaptive immune system. *Int Immunol* 20:*791*.

Korn, T., E. Bettelli, M. Oukka and V.K. Kuchroo 2009. IL-17 and Th17 Cells. *Annu Rev Immunol* 27:*485*.

Kuhl, A.A., N.N. Pawlowski, K. Grollich, M. Blessenohl, J. Westermann, M. Zeitz, C. Loddenkemper and J.C. Hoffmann 2009. Human peripheral gammadelta T cells possess regulatory potential. *Immunology* 128:*580*.

Kumar, H., T. Kawai and S. Akira 2009. Toll-like receptors and innate immunity. *Biochem Biophys Res Commun* 388:*621*.

Kumar, S. 2009. Vernal keratoconjunctivitis: a major review. *Acta Ophthalmol* 87:*133*.

Laan, M., Z.H. Cui, H. Hoshino, J. Lotvall, M. Sjostrand, D.C. Gruenert, B.E. Skoogh and A. Linden 1999. Neutrophil recruitment by human IL-17 via C-X-C

chemokine release in the airways. *J Immunol* 162:*2347*.

Lambiase, A., A. Micera, F. Mantelli, C. Moretti, A. Di Zazzo, E. Perrella, S. Bonini and S. Bonini 2009. T-helper 17 lymphocytes in ocular cicatricial pemphigoid. *Mol Vis* 15:*1449*.

Lambiase, A., A. Micera, M. Sacchetti, F. Mantelli and S. Bonini 2011. Toll-like receptors in ocular surface diseases: overview and new findings. *Clin Sci (Lond)* 120:*441*.

Langrish, C.L., Y. Chen, W.M. Blumenschein, J. Mattson, B. Basham, J.D. Sedgwick, T. McClanahan, R.A. Kastelein and D.J. Cua 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med* 201:*233*.

Lee, Y.K., H. Turner, C.L. Maynard, J.R. Oliver, D. Chen, C.O. Elson and C.T. Weaver 2009. Late developmental plasticity in the T helper 17 lineage. *Immunity* 30:*92*.

Lemaitre, B., E. Nicolas, L. Michaut, J.M. Reichhart and J.A. Hoffmann 1996. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell* 86:*973*.

Leonardi, A. 2002. Vernal keratoconjunctivitis: pathogenesis and treatment. *Prog Retin Eye Res* 21:*319*.

Leonardi, A., S.J. Curnow, H. Zhan and V.L. Calder 2006. Multiple cytokines in human tear specimens in seasonal and chronic allergic eye disease and in conjunctival fibroblast cultures. *Clin Exp Allergy* 36:*777*.

Leonardi, A., G. DeFranchis, F. Zancanaro, G. Crivellari, M. De Paoli, M. Plebani and A.G. Secchi 1999. Identification of local Th2 and Th0 lymphocytes in vernal conjunctivitis by cytokine flow cytometry. *Invest Ophthalmol Vis Sci* 40:*3036*.

Leonardi, A., L. Motterle and M. Bortolotti 2008. Allergy and the eye. *Clin Exp Immunol* 153 Suppl 1:*17*.

Letterio, J.J. and A.B. Roberts 1997. TGF-beta: a critical modulator of immune cell

function. *Clin Immunol Immunopathol* 84:*244*.

Li, J., J. Shen and R.W. Beuerman 2007. Expression of toll-like receptors in human limbal and conjunctival epithelial cells. *Mol Vis* 13:*813*.

Li, Y., L. Jiang, S. Zhang, L. Yin, L. Ma, D. He and J. Shen 2011. Methotrexate attenuates the Th17/IL-17 levels in peripheral blood mononuclear cells from healthy individuals and RA patients. *Rheumatol Int*

Lina, C., W. Conghua, L. Nan and Z. Ping 2011. Combined Treatment of Etanercept and MTX Reverses Th1/Th2, Th17/Treg Imbalance in Patients with Rheumatoid Arthritis. *J Clin Immunol* 31:*596*.

Lockhart, E., A.M. Green and J.L. Flynn 2006. IL-17 production is dominated by gammadelta T cells rather than CD4 T cells during Mycobacterium tuberculosis infection. *J Immunol* 177:*4662*.

Luger, D., P.B. Silver, J. Tang, D. Cua, Z. Chen, Y. Iwakura, E.P. Bowman, N.M. Sgambellone, C.C. Chan and R.R. Caspi 2008. Either a Th17 or a Th1 effector response can drive autoimmunity: conditions of disease induction affect dominant effector category. *J Exp Med* 205:*799*.

Lugton, I. 1999. Mucosa-associated lymphoid tissues as sites for uptake, carriage and excretion of tubercle bacilli and other pathogenic mycobacteria. *Immunol Cell Biol* 77:*364*.

Ma, C.S., G.Y. Chew, N. Simpson, A. Priyadarshi, M. Wong, B. Grimbacher, D.A. Fulcher, S.G. Tangye and M.C. Cook 2008. Deficiency of Th17 cells in hyper IgE syndrome due to mutations in STAT3. *J Exp Med* 205:*1551*.

Mackay, C.R. 2000. Follicular homing T helper (Th) cells and the Th1/Th2 paradigm. *J Exp Med* 192:*F31*.

Maggi, E., P. Biswas, G. Del Prete, P. Parronchi, D. Macchia, C. Simonelli, L. Emmi, M. De Carli, A. Tiri, M. Ricci et al 1991. Accumulation of Th-2-like helper T cells in the conjunctiva of patients with vernal conjunctivitis. *J Immunol* 146:*1169*.

Maggi, E., P. Parronchi, R. Manetti, C. Simonelli, M.P. Piccinni, F.S. Rugiu, M. De

Carli, M. Ricci and S. Romagnani 1992. Reciprocal regulatory effects of IFN-gamma and IL-4 on the in vitro development of human Th1 and Th2 clones. *J Immunol* 148:*2142*.

Major, A.S., R.R. Singh, S. Joyce and L. Van Kaer 2006. The role of invariant natural killer T cells in lupus and atherogenesis. *Immunol Res* 34:*49*.

Manel, N., D. Unutmaz and D.R. Littman 2008. The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgammat. *Nat Immunol* 9:*641*.

Mangan, P.R., L.E. Harrington, D.B. O'Quinn, W.S. Helms, D.C. Bullard, C.O. Elson, R.D. Hatton, S.M. Wahl, T.R. Schoeb and C.T. Weaver 2006. Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature* 441:*231*.

Marini, M., E. Vittori, J. Hollemborg and S. Mattoli 1992. Expression of the potent inflammatory cytokines, granulocyte-macrophage-colony-stimulating factor and interleukin-6 and interleukin-8, in bronchial epithelial cells of patients with asthma. *J Allergy Clin Immunol* 89:*1001*.

Matsuda, A., N. Ebihara, N. Yokoi, S. Kawasaki, H. Tanioka, T. Inatomi, M.a.R. de Waal, J. Hamuro, S. Kinoshita and A. Murakami 2010. Functional role of thymic stromal lymphopoietin in chronic allergic keratoconjunctivitis. *Invest Ophthalmol Vis Sci* 51:*151*.

Matsuda, A., Y. Okayama, N. Terai, N. Yokoi, N. Ebihara, H. Tanioka, S. Kawasaki, T. Inatomi, N. Katoh, E. Ueda, J. Hamuro, A. Murakami and S. Kinoshita 2009. The role of interleukin-33 in chronic allergic conjunctivitis. *Invest Ophthalmol Vis Sci* 50:*4646*.

McAllister, F., A. Henry, J.L. Kreindler, P.J. Dubin, L. Ulrich, C. Steele, J.D. Finder, J.M. Pilewski, B.M. Carreno, S.J. Goldman, J. Pirhonen and J.K. Kolls 2005. Role of IL-17A, IL-17F, and the IL-17 receptor in regulating growth-related oncogene-alpha and granulocyte colony-stimulating factor in bronchial epithelium: implications for airway inflammation in cystic fibrosis. *J Immunol* 175:*404*.

McLane, M.P., A. Haczku, R.i.M. van de, C. Weiss, V. Ferrante, D. MacDonald, J.C. Renauld, N.C. Nicolaides, K.J. Holroyd and R.C. Levitt 1998. Interleukin-9 promotes allergen-induced eosinophilic inflammation and airway hyperresponsiveness in transgenic mice. *Am J Respir Cell Mol Biol* 19:*713*.

Metz, D.P., A.S. Bacon, S. Holgate and S.L. Lightman 1996. Phenotypic characterization of T cells infiltrating the conjunctiva in chronic allergic eye disease. *J Allergy Clin Immunol* 98:*686*.

Metz, D.P., M. Hingorani, V.L. Calder, R.J. Buckley and S.L. Lightman 1997. T-cell cytokines in chronic allergic eye disease. *J Allergy Clin Immunol* 100:*817*.

Montan, P.G., A. Scheynius.and P.l.I. van der 2002. Similar T helper Th2-like cytokine mRNA expression in vernal keratoconjunctivitis regardless of atopic constitution. *Allergy* 57:*436*.

Moran, E.M., R. Mullan, J. McCormick, M. Connolly, O. Sullivan, O. Fitzgerald, B. Bresnihan, D.J. Veale and U. Fearon 2009. Human rheumatoid arthritis tissue production of IL-17A drives matrix and cartilage degradation: synergy with tumour necrosis factor-alpha, Oncostatin M and response to biologic therapies. *Arthritis Res Ther 11:R113.*

Mosmann, T.R., H. Cherwinski, M.W. Bond, M.A. Giedlin and R.L. Coffman 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 136:*2348*.

Nakae, S., Y. Iwakura, H. Suto and S.J. Galli 2007. Phenotypic differences between Th1 and Th17 cells and negative regulation of Th1 cell differentiation by IL-17. *J Leukoc Biol* 81:*1258*.

Nickel, T.J., M.H. Kabir, J. Talreja, D.J. Stechschulte and K.N. Dileepan 2006. Constitutive expression of functionally active protease-activated receptors 1 and 2 in human conjunctival epithelial cells. *Mediators Inflamm* 2006:*61359*.

Nograles, K.E., L.C. Zaba, A. Shemer, J. Fuentes-Duculan, I. Cardinale, T. Kikuchi, M. Ramon, R. Bergman, J.G. Krueger and E. Guttman-Yassky 2009. IL-22-

producing "T22" T cells account for upregulated IL-22 in atopic dermatitis despite reduced IL-17-producing TH17 T cells. *J Allergy Clin Immunol* 123:*1244*.

O'Mahony, D.S., U. Pham, R. Iyer, T.R. Hawn and W.C. Liles 2008. Differential constitutive and cytokine-modulated expression of human Toll-like receptors in primary neutrophils, monocytes, and macrophages. *Int J Med Sci* 5:*1*.

Omori, M. and S. Ziegler 2007. Induction of IL-4 expression in CD4(+) T cells by thymic stromal lymphopoietin. *J Immunol* 178:*1396*.

Onishi, R.M. and S.L. Gaffen 2010. Interleukin-17 and its target genes: mechanisms of interleukin-17 function in disease. *Immunology* 129:*311*.

Onishi, R.M., S.J. Park, W. Hanel, A.W. Ho, A. Maitra and S.L. Gaffen 2010. SEF/IL-17R (SEFIR) is not enough: an extended SEFIR domain is required for IL-17RA-mediated signal transduction. *J Biol Chem* 285:*32751*.

Ono, S.J. and M.B. Abelson 2005. Allergic conjunctivitis: update on pathophysiology and prospects for future treatment. *J Allergy Clin Immunol* 115:*118*.

Opal, S.M. and V.A. DePalo 2000. Anti-inflammatory cytokines. *Chest* 117:*1162*.

Opal, S.M., J.C. Wherry and P. Grint 1998. Interleukin-10: potential benefits and possible risks in clinical infectious diseases. *Clin Infect Dis* 27:*1497*.

Oppmann, B., R. Lesley, B. Blom, J.C. Timans, Y. Xu, B. Hunte, F. Vega, N. Yu, J. Wang, K. Singh, F. Zonin, E. Vaisberg, T. Churakova, M. Liu, D. Gorman, J. Wagner, S. Zurawski, Y. Liu, J.S. Abrams, K.W. Moore, D. Rennick et al 2000. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* 13:*715*.

Ospelt, C. and S. Gay 2010. TLRs and chronic inflammation. *Int J Biochem Cell Biol* 42:*495*.

Owyang, A.M., C. Zaph, E.H. Wilson, K.J. Guild, T. McClanahan, H.R. Miller, D.J. Cua, M. Goldschmidt, C.A. Hunter, R.A. Kastelein and D. Artis 2006. Interleukin 25 regulates type 2 cytokine-dependent immunity and limits chronic inflammation in the gastrointestinal tract. *J Exp Med* 203:*843*.

Park, Y., S. Park, E. Yoo, D. Kim and H. Shin 2004. Association of the polymorphism for Toll-like receptor 2 with type 1 diabetes susceptibility. *Ann N Y Acad Sci* 1037:*170*.

Pene, J., S. Chevalier, L. Preisser, E. Venereau, M.H. Guilleux, S. Ghannam, J.P. Moles, Y. Danger, E. Ravon, S. Lesaux, H. Yssel and H. Gascan 2008. Chronically inflamed human tissues are infiltrated by highly differentiated Th17 lymphocytes. *J Immunol* 180:*7423*.

Peng, M.Y., Z.H. Wang, C.Y. Yao, L.N. Jiang, Q.L. Jin, J. Wang and B.Q. Li 2008. Interleukin 17-producing gamma delta T cells increased in patients with active pulmonary tuberculosis. *Cell Mol Immunol* 5:*203*.

Petrie, H.T., F. Livak, D. Burtrum and S. Mazel 1995. T cell receptor gene recombination patterns and mechanisms: cell death, rescue, and T cell production. *J Exp Med* 182:*121*.

Pierer, M., J. Rethage, R. Seibl, R. Lauener, F. Brentano, U. Wagner, H. Hantzschel, B.A. Michel, R.E. Gay, S. Gay and D. Kyburz 2004. Chemokine secretion of rheumatoid arthritis synovial fibroblasts stimulated by Toll-like receptor 2 ligands. *J Immunol* 172:*1256*.

Pirie, F.J., R. Pegoraro, A.A. Motala, S. Rauff, L. Rom, T. Govender and T.M. Esterhuizen 2005. Toll-like receptor 3 gene polymorphisms in South African Blacks with type 1 diabetes. *Tissue Antigens* 66:*125*.

Prefontaine, D., J. Nadigel, F. Chouiali, S. Audusseau, A. Semlali, J. Chakir, J.G. Martin and Q. Hamid 2010. Increased IL-33 expression by epithelial cells in bronchial asthma. *J Allergy Clin Immunol* 125:*752*.

Radstake, T.R., M.F. Roelofs, Y.M. Jenniskens, B. Oppers-Walgreen, P.L. van Riel, P. Barrera, L.A. Joosten and W.B. van den Berg 2004. Expression of toll-like receptors 2 and 4 in rheumatoid synovial tissue and regulation by proinflammatory cytokines interleukin-12 and interleukin-18 via interferon-gamma. *Arthritis Rheum* 50:*3856*.

Rajan, T.V. 2003. The Gell-Coombs classification of hypersensitivity reactions: a re-interpretation. *Trends Immunol* 24:*376*.

Randall, T.D. 2010. Bronchus-associated lymphoid tissue (BALT) structure and function. *Adv Immunol* 107:*187*.

Rangel-Moreno, J., D.M. Carragher, M. de la Luz Garcia-Hernandez, J.Y. Hwang, K. Kusser, L. Hartson, J.K.Kolls, S.A. Khader and T.D. Randall 2011. The development of inducible bronchus-associated lymphoid tissue depends on IL-17. *Nat Immunol* 12:*639*.

Reche, P.A., V. Soumelis, D.M. Gorman, T. Clifford, M.r. Liu, M. Travis, S.M. Zurawski, J. Johnston, Y.J. Liu, H. Spits, R. de Waal Malefyt, R.A. Kastelein and J.F. Bazan 2001. Human thymic stromal lymphopoietin preferentially stimulates myeloid cells. *J Immunol* 167:*336*.

Remington, L. *Clinical Anatomy of the Visual System*. Butterworth-Heinemann. 1998

Reyes, N.J., E. Mayhew, P.W. Chen and J.Y. Niederkorn 2011. Gammadelta T cells are required for maximal expression of allergic conjunctivitis. *Invest Ophthalmol Vis Sci* 52:*2211*.

Rickel, E.A., L.A. Siegel, B.R. Yoon, J.B. Rottman, D.G. Kugler, D.A. Swart, P.M. Anders, J.E. Tocker, M.R. Comeau and A.L. Budelsky 2008. Identification of functional roles for both IL-17RB and IL-17RA in mediating IL-25-induced activities. *J Immunol* 181:*4299*.

Roelofs, M.F., L.A. Joosten, S. Abdollahi-Roodsaz, A.W. van Lieshout, T. Sprong, F.H. van den Hoogen, W.B. van den Berg and T.R. Radstake 2005. The expression of toll-like receptors 3 and 7 in rheumatoid arthritis synovium is increased and costimulation of toll-like receptors 3, 4, and 7/8 results in synergistic cytokine production by dendritic cells. *Arthritis Rheum* 52:*2313*.

Rohani, M.G., D.H. DiJulio, J.Y. An, B.M. Hacker, B.A. Dale and W.O. Chung 2010. PAR1- and PAR2-induced innate immune markers are negatively regulated by PI3K/Akt signaling pathway in oral keratinocytes. *BMC Immunol* 11:*53*.

Rudolph, M.G., R.L. Stanfield and I.A. Wilson 2006. How TCRs bind MHCs, peptides, and coreceptors. *Annu Rev Immunol* 24:*419*.

Sakaguchi, S., M. Miyara, C.M. Costantino and D.A. Hafler 2010. FOXP3+ regulatory T cells in the human immune system. *Nat Rev Immunol* 10:*490*.

Sakaguchi, S., T. Yamaguchi, T. Nomura.and M. Ono 2008. Regulatory T cells and immune tolerance. *Cell* 133:*775*.

Santarlasci, V., L. Maggi, M. Capone, F. Frosali, V. Querci, R. De Palma, F. Liotta, L. Cosmi, E. Maggi, S. Romagnani and F. Annunziato 2009. TGF-beta indirectly favors the development of human Th17 cells by inhibiting Th1 cells. *Eur J Immunol* 39:*207*.

Schmitz, J., A. Owyang, E. Oldham, Y. Song, E. Murphy, T.K. McClanahan, G. Zurawski, M. Moshrefi, J. Qin, X. Li, D.M. Gorman, J.F. Bazan and R.A. Kastelein 2005. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* 23:*479*.

Schnyder-Candrian, S., D. Togbe, I. Couillin, I. Mercier, F. Brombacher, V. Quesniaux, F. Fossiez, B. Ryffel and B. Schnyder 2006. Interleukin-17 is a negative regulator of established allergic asthma. *J Exp Med* 203:*2715*.

Sekiya, T., M. Miyamasu, M. Imanishi, H. Yamada, T. Nakajima, M. Yamaguchi, T. Fujisawa, R. Pawankar, Y. Sano, K. Ohta, A. Ishii, Y. Morita, K. Yamamoto, K. Matsushima, O. Yoshie and K. Hirai 2000. Inducible expression of a Th2-type CC chemokine thymus- and activation-regulated chemokine by human bronchial epithelial cells. *J Immunol* 165:*2205*.

Semlali, A., E. Jacques, L. Koussih, A.S. Gounni and J. Chakir 2010. Thymic stromal lymphopoietin-induced human asthmatic airway epithelial cell proliferation through an IL-13-dependent pathway. *J Allergy Clin Immunol* 125:*844*.

Seto, T., T. Takai, N. Ebihara, H. Matsuoka, X.L. Wang, A. Ishii, H. Ogawa, A. Murakami and K. Okumura 2009. SLPI prevents cytokine release in mite protease-exposed conjunctival epithelial cells. *Biochem Biophys Res Commun* 379:*681*.

Shen, F. and S.L. Gaffen 2008. Structure-function relationships in the IL-17 receptor: implications for signal transduction and therapy. *Cytokine* 41:*92*.

Shi, L., S.W. Leu, F. Xu, X. Zhou, H. Yin, L. Cai and L. Zhang 2008. Local blockade of TSLP receptor alleviated allergic disease by regulating airway dendritic cells. *Clin Immunol* 129:*202*.

Shim, J.J., K. Dabbagh, I.F. Ueki, T. Dao-Pick, P.R. Burgel, K. Takeyama, D.C. Tam and J.A. Nadel 2001. IL-13 induces mucin production by stimulating epidermal growth factor receptors and by activating neutrophils. *Am J Physiol Lung Cell Mol Physiol* 280:*L134*.

Sims, J.E., D.E. Williams, P.J. Morrissey, K. Garka, D. Foxworthe, V. Price, S.L. Friend, A. Farr, M.A. Bedell, N.A. Jenkins, N.G. Copeland, K. Grabstein and R.J. Paxton 2000. Molecular cloning and biological characterization of a novel murine lymphoid growth factor. *J Exp Med* 192:*671*.

Smith, J.A. 1994. Neutrophils, host defense, and inflammation: a double-edged sword. *J Leukoc Biol* 56:*672*.

Soroosh, P. and T.A. Doherty 2009. Th9 and allergic disease. *Immunology* 127:*450*.

Soumelis, V., P.A. Reche, H. Kanzler, W. Yuan, G. Edward, B. Homey, M. Gilliet, S. Ho, S. Antonenko, A. Lauerma, K. Smith, D. Gorman, S. Zurawski, J. Abrams, S. Menon, T. McClanahan, Rd.R. de Waal-Malefyt, F. Bazan, R. Kastelein and Y.J. Liu 2002. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat Immunol* 3:*673*.

Souwer, Y., K. Szegedi, M.L. Kapsenberg and E.C. de Jong 2010. IL-17 and IL-22 in atopic allergic disease. *Curr Opin Immunol* 22:*821*.

Spachidou, M.P., E. Bourazopoulou, C.I. Maratheftis, E.K. Kapsogeorgou, H.M. Moutsopoulos, A.G. Tzioufas and M.N. Manoussakis 2007. Expression of functional Toll-like receptors by salivary gland epithelial cells: increased mRNA expression in cells derived from patients with primary Sjogren's syndrome. *Clin Exp Immunol* 147:*497*.

Spada, C.S., D.F. Woodward, S.B. Hawley and A.L. Nieves 1986. Leukotrienes cause eosinophil emigration into conjunctival tissue. *Prostaglandins* 31:*795*.

Staal, F.J., F. Weerkamp, A.W. Langerak, R.W. Hendriks and H.C. Clevers 2001. Transcriptional control of t lymphocyte differentiation. *Stem Cells* 19:*165*.

Spooner, J. D. *Ocular Anatomy*. Butterworth-Heinemann Ltd. 1976

Stahl, J.L. and N.P. Barney 2004. Ocular allergic disease. *Curr Opin Allergy Clin Immunol* 4:*455*.

Steinman, L. 2007. A brief history of T(H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage. *Nat Med* 13:*139*.

Strachan, D.P. 1989. Hay fever, hygiene, and household size. *BMJ* 299:*1259*.

Swamy, M., C. Jamora, W. Havran and A. Hayday 2010. Epithelial decision makers: in search of the 'epimmunome'. *Nat Immunol* 11:*656*.

Talreja, J., K. Dileepan, S. Puri, M.H. Kabir, D.M. Segal, D.J. Stechschulte and K.N. Dileepan 2005. Human conjunctival epithelial cells lack lipopolysaccharide responsiveness due to deficient expression of MD2 but respond after interferon-gamma priming or soluble MD2 supplementation. *Inflammation* 29:*170*.

Tanaka, J., N. Watanabe, M. Kido, K. Saga, T. Akamatsu, A. Nishio and T. Chiba 2009. Human TSLP and TLR3 ligands promote differentiation of Th17 cells with a central memory phenotype under Th2-polarizing conditions. *Clin Exp Allergy* 39:*89*.

Teunissen, M.B., C.W. Koomen, M.a.R. de Waal, E.A. Wierenga and J.D. Bos 1998. Interleukin-17 and interferon-gamma synergize in the enhancement of proinflammatory cytokine production by human keratinocytes. *J Invest Dermatol* 111:*645*.

Thomas, S.Y., Y.H. Chyung and A.D. Luster 2010. Natural killer T cells are not the predominant T cell in asthma and likely modulate, not cause, asthma. *J Allergy Clin Immunol* 125:*980*.

Torii, Y., T. Ito, R. Amakawa, H. Sugimoto, H. Amuro, T. Tanijiri, Y. Katashiba, M.

Ogata, T. Yokoi and S. Fukuhara 2008. Imidazoquinoline acts as immune adjuvant for functional alteration of thymic stromal lymphopoietin-mediated allergic T cell response. *J Immunol* 181:*5340*.

Toy, D., D. Kugler, M. Wolfson, T. Vanden Bos, J. Gurgel, J. Derry, J. Tocker and J. Peschon 2006. Cutting edge: interleukin 17 signals through a heteromeric receptor complex. *J Immunol* 177:*36*.

Trocme, S.D. and K.K. Sra 2002. Spectrum of ocular allergy. *Curr Opin Allergy Clin Immunol* 2:*423*.

Tsuda, T., M. Tohyama, K. Yamasaki, Y. Shirakata, Y. Yahata, S. Tokumaru, K. Sayama and K. Hashimoto 2003. Lack of evidence for TARC/CCL17 production by normal human keratinocytes in vitro. *J Dermatol Sci* 31:*37*.

Ueta, M., J. Hamuro, H. Kiyono and S. Kinoshita 2005. Triggering of TLR3 by polyI:C in human corneal epithelial cells to induce inflammatory cytokines. *Biochem Biophys Res Commun* 331:*285*.

Ueta, M., K. Mizushima, N. Yokoi, Y. Naito and S. Kinoshita 2010. Gene-expression analysis of polyI:C-stimulated primary human conjunctival epithelial cells. *Br J Ophthalmol* 94:*1528*.

Ueta, M., T. Nochi, M.H. Jang, E.J. Park, O. Igarashi, A. Hino, S. Kawasaki, T. Shikina, T. Hiroi, S. Kinoshita and H. Kiyono 2004. Intracellularly expressed TLR2s and TLR4s contribution to an immunosilent environment at the ocular mucosal epithelium. *J Immunol* 173:*3337*.

Umetsu, D.T. and R.H. Dekruyff 2010. The regulatory role of natural killer T cells in the airways. *Int J Biochem Cell Biol* 42:*529*.

van Beelen, A.J., M.B. Teunissen, M.L. Kapsenberg and E.C. de Jong 2007. Interleukin-17 in inflammatory skin disorders. *Curr Opin Allergy Clin Immunol* 7:*374*.

van Kaer, L. 2007. NKT cells: T lymphocytes with innate effector functions. *Curr Opin Immunol* 19:*354*.

Veldhoen, M., R.J. Hocking, C.J. Atkins, R.M. Locksley and B. Stockinger 2006. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 24:*179*.

Veldhoen, M., C. Uyttenhove, J. van Snick, H. Helmby, A. Westendorf, J. Buer, B. Martin, C. Wilhelm and B. Stockinger 2008. Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat Immunol* 9:*1341*.

Volpe, E., N. Servant, R. Zollinger, S.I. Bogiatzi, P. Hupe, E. Barillot and V. Soumelis 2008. A critical function for transforming growth factor-beta, interleukin 23 and proinflammatory cytokines in driving and modulating human T(H)-17 responses. *Nat Immunol* 9:*650*.

von Mutius, E. 2001. Infection: friend or foe in the development of atopy and asthma? The epidemiological evidence. *Eur Respir J* 18:*872*.

Voo, K.S., Y.H. Wang, F.R. Santori, C. Boggiano, Y.H. Wang, K. Arima, L. Bover, S. Hanabuchi, J. Khalili, E. Marinova, B. Zheng, D.R. Littman and Y.J. Liu 2009. Identification of IL-17-producing FOXP3+ regulatory T cells in humans. *Proc Natl Acad Sci U S A* 106:*4793*.

Vroling, A.B., W.J. Fokkens and C.M. van Drunen 2008. How epithelial cells detect danger: aiding the immune response. *Allergy* 63:*1110*.

Wang, Y.H. and Y.J. Liu 2008. The IL-17 cytokine family and their role in allergic inflammation. *Curr Opin Immunol* 20:*697*.

Wolff, E. *Anatomy of the Eye and Orbit*. Saunders. 1976

Woodward, D.F., C.S. Spada, S.B. Hawley and A.L. Nieves 1986. Conjunctival eosinophil infiltration evoked by histamine and immediate hypersensitivity. Modification by H1- and H2-receptor blockade. *Invest Ophthalmol Vis Sci* 27:*1495*.

Wright, J.F., F. Bennett, B. Li, J. Brooks, D.P. Luxenberg, M.J. Whitters, K.N. Tomkinson, L.J. Fitz, N.M. Wolfman, M. Collins, K. Dunussi-Joannopoulos, M. Chatterjee-Kishore and B.M. Carreno 2008. The human IL-17F/IL-17A

heterodimeric cytokine signals through the IL-17RA/IL-17RC receptor complex. *J Immunol* 181:*2799*.

Wu, C.Y., C. Demeure, M. Kiniwa, M. Gately and G. Delespesse 1993. IL-12 induces the production of IFN-gamma by neonatal human CD4 T cells. *J Immnol* 151:*1938*.

Wu, Q., R.J. Martin, J.G. Rino, R. Breed, R.M. Torres and H.W. Chu 2007. IL-23-dependent IL-17 production is essential in neutrophil recruitment and activity in mouse lung defense against respiratory Mycoplasma pneumoniae infection. *Microbes Infect* 9:*78*.

Xu, G., L. Zhang, D.Y. Wang, R. Xu, Z. Liu, D.M. Han, X.D. Wang, K.J. Zuo and H.B. Li 2010. Opposing roles of IL-17A and IL-25 in the regulation of TSLP production in human nasal epithelial cells. *Allergy* 65:*581*.

Yamada, H., Y. Nakashima, K. Okazaki, T. Mawatari, J.I. Fukushi, N. Kaibara, A. Hori, Y. Iwamoto and Y. Yoshikai 2008. Th1 but not Th17 cells predominate in the joints of patients with rheumatoid arthritis. *Ann Rheum Dis* 67:*1299*.

Yao, Z., W.C. Fanslow, M.F. Seldin, A.M. Rousseau, S.L. Painter, M.R. Comeau, J.I. Cohen and M.K. Spriggs 1995. Herpesvirus Saimiri encodes a new cytokine, IL-17, which binds to a novel cytokine receptor. *Immunity* 3:*811*.

Yao, Z., S.L. Painter, W.C. Fanslow, D. Ulrich, B.M. Macduff, M.K. Spriggs and R.J. Armitage 1995. Human IL-17: a novel cytokine derived from T cells. *J Immunol* 155:*5483*.

Ye, J., X. Su, E.C. Hsueh, Y. Zhang, J.M. Koenig, D.F. Hoft and G. Peng 2011. Human tumor-infiltrating Th17 cells have the capacity to differentiate into IFN-gamma+ and FOXP3+ T cells with potent suppressive function. *Eur J Immunol* 41:*936*.

Ying, S., Q. Meng, A.B. Kay and D.S. Robinson 2002. Elevated expression of interleukin-9 mRNA in the bronchial mucosa of atopic asthmatics and allergen-induced cutaneous late-phase reaction: relationships to eosinophils, mast cells and T

lymphocytes. *Clin Exp Allergy* 32:*866*.

Ying, S., B. O'Connor, J. Ratoff, Q. Meng, K. Mallett, D. Cousins, D. Robinson, G. Zhang, J. Zhao, T.H. Lee and C. Corrigan 2005. Thymic stromal lymphopoietin expression is increased in asthmatic airways and correlates with expression of Th2-attracting chemokines and disease severity. *J Immunol* 174:*8183*.

Yuk, J.M., D.M. Shin, H.M. Lee, J.J. Kim, S.W. Kim, H.S. Jin, C.S. Yang, K.A. Park, D. Chanda, D.K. Kim, S.M. Huang, S.K. Lee, C.H. Lee, J.M. Kim, C.H. Song, S.Y. Lee, G.M. Hur, D.D. Moore, H.S. Choi and E.K. Jo 2011. The orphan nuclear receptor SHP acts as a negative regulator in inflammatory signaling triggered by Toll-like receptors. *Nat Immunol* 12:*742*.

Zhou, Q., T. Desta, M. Fenton, D.T. Graves and S. Amar 2005. Cytokine profiling of macrophages exposed to Porphyromonas gingivalis, its lipopolysaccharide, or its FimA protein. *Infect Immun* 73:*935*.

Publications

Saw VP, Schmidt E, **Offiah I**, Galatowicz G, Zillikens D, Dart JK, Calder VL, Daniels JT. Profibrotic phenotype of conjunctival fibroblasts from mucous membrane pemphigoid. **Am J Pathol**. 2011 Jan;178(1):187-97.

Saw VP, **Offiah I**, Dart RJ, Galatowicz G, Dart JK, Daniels JT, Calder VL. Conjunctival interleukin-13 expression in mucous membrane pemphigoid and functional effects of interleukin-13 on conjunctival fibroblasts in vitro. Am J Pathol. 2009 Dec;175(6):2406-15.

Offiah I, Calder VL. Immune mechanisms in allergic eye diseases: what is new? Curr Opin Allergy Clin Immunol. 2009 Oct;9(5):477-81.

Abstracts

Offiah I, Seow EL, Galatowicz G, Leonardi A, Calder VL. IL-17 receptor expression by human conjunctival epithelial cells: modulation by TLR agonists. 7th EAACI-GA^{2}LEN Immunology Winter School, 2009, Davos.

Offiah I, Seow EL, Mellors R, Galatowicz G, Calder VL. Factors contributing to the modulation of IL-17R expression on human conjunctival epithelial cells. Allergy & Asthma: Bridging Innate and Adaptive Immunity – Abcam meeting, 2009, Bruges.

Offiah I, Mellors R, Galatowicz G, Calder VL. An immunoregulatory role for IL-17 and IL-17 receptors in conjunctival epithelial cells. World Immune Regulation Meeting – IV, 2010, Davos.

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"Look at a stone cutter hammering away at his rock, perhaps a hundred times without as much as a crack showing in it. Yet at the hundred-and-first blow it will split in two, and I know it was not the last blow that did it, but all that had gone before". ~Jacob A. Riis