The role of mast cells, T cells and their cytokines in Allergic Conjunctivitis

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Declaration

I, Amirah Mohd Zaki 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.

May 2019
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Last but not least, this PhD is dedicated to my late father, who had also completed his PhD and successfully made his way up in the academic field. I’m
sure you will be proud of me if you know that I have followed your footsteps. You have always been my idol and I always pray for you every day.
Abstract

The pathology and mechanisms underlying allergic conjunctivitis are still poorly understood. Even though most studies focusing on the mechanisms of allergic diseases, only few have focused on the mechanisms of allergic conjunctivitis. Basic and clinical research have been done in order to understand the cells and mediators that involved in the immunologic events of ocular allergy. Immunologic mediators, including cytokines are important in mediating intracellular communication networks during allergy, and they play important roles in the contribution of disease pathology.

IL-9 is a well-established growth factor, mediating mast cell and T cell production of proinflammatory cytokines. IL-9 has recently been associated in the development of allergic diseases including allergic asthma and allergic rhinitis, however, its contribution in allergic conjunctivitis is still unknown. IL-9 is one of the initially thought Th2 cytokine, but recent studies showed that there are many different cell types that also secretes IL-9, including mast cells and Th9 cells. (more details)

The data obtained from this thesis is the first to demonstrate the upregulation of IL-9 expression by mast cells in the conjunctiva of experimental allergic conjunctivitis mice and allergen challenged allergic conjunctivitis donors. Further in vitro studies using has revealed that IL-9R blockade resulting in the downregulation of mast cell secretion products including histamine and Th2 cytokines, IL-4, IL-5 and IL-13.
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<th>Description</th>
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<tbody>
<tr>
<td>AC</td>
<td>allergic conjunctivitis</td>
</tr>
<tr>
<td>ABC</td>
<td>avidin-biotin complexes</td>
</tr>
<tr>
<td>ACQ-6</td>
<td>Asthma Control Questionnaire 6</td>
</tr>
<tr>
<td>AD</td>
<td>atopic dermatitis</td>
</tr>
<tr>
<td>AHR</td>
<td>allergic hyperresponsiveness</td>
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<tr>
<td>AIT</td>
<td>allergen immunotherapy</td>
</tr>
<tr>
<td>AKC</td>
<td>Atopic Keratoconjunctivitis</td>
</tr>
<tr>
<td>alum</td>
<td>aluminium hydroxide</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting conjunctivitis</td>
</tr>
<tr>
<td>BCL</td>
<td>B cell lymphoma</td>
</tr>
<tr>
<td>BFA</td>
<td>brefeldin A</td>
</tr>
<tr>
<td>BMMC</td>
<td>bone marrow mouse mast cells</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>CTMC</td>
<td>connective tissue mast cells</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>dLNs</td>
<td>draining lymph nodes</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DNP</td>
<td>Anti-dinitrophenyl</td>
</tr>
<tr>
<td>EAC</td>
<td>experimental allergic conjunctivitis</td>
</tr>
<tr>
<td>EAU</td>
<td>experimental autoimmune uveoretinitis</td>
</tr>
<tr>
<td>EAE</td>
<td>encephalomyelitis</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>EPR</td>
<td>early phase response</td>
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<tr>
<td>ETOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>eTreg</td>
<td>effector regulatory T cells</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FcεRI</td>
<td>high affinity IgE receptor</td>
</tr>
<tr>
<td>FGF-2</td>
<td>fibroblast growth factor 2</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<td>FoxP3</td>
<td>forkhead box P3</td>
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<tr>
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<td>forward scatter</td>
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<td>GATA-3</td>
<td>GATA binding protein-3</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>stimulating factor</td>
</tr>
<tr>
<td>GMA</td>
<td>glycol methacrylate</td>
</tr>
<tr>
<td>GPC</td>
<td>giant papillary conjunctivitis</td>
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<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
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<tr>
<td>HBE</td>
<td>human bronchial epithelial</td>
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</table>
SLE  systemic lupus erythematosus
SRW  short ragweed pollen
SSC  side scatter
STAT signal transducer and activator of transcription
TCR  T cell receptors
Tfh  T follicular helper cells
TGF-β transforming growth factor beta
Th   T helper
TJ   tight junction
TLR  toll like receptors
TMB  3,3′,5,5′-tetramethylbenzidine
TNF-α tumour necrosis factor-α
Treg  T regulatory cells
TSLP thymic stromal lymphopoietin
UC   ulcerative colitis
VEGF vascular endothelial growth factor
VKC  Vernal Keratoconjunctivitis
ZO   Zonula Occludens
βc   β cytokine
°C   degrees centigrade
Chapter 1.
Introduction and hypothesis
In this chapter, I will describe various allergic diseases and their subtypes including allergic conjunctivitis (AC), what is currently known about the immune cells and immune pathways involved in AC and the relevance and significance of current treatment for AC.

1.1. Allergic Diseases

1.1.1. Asthma

Asthma is a chronic inflammatory disease of the airways which involves bronchial hyper-reactivity (BHR), mucus overproduction, airway wall remodelling and airway narrowing. It is a very common disease that affects up to 300 million people worldwide and results in substantial morbidity and is a huge burden to healthcare expenditure.

There are two forms of asthma which have been identified in the clinic; allergic and non-allergic (intrinsic asthma). Asthma, which coincides mostly with allergic sensitization, affects children and about 15% of adults. Allergic asthma is caused by commonly inhaled or ingested allergens such as house dust mite (HDM), grass and tree pollen, animal dander and fungal spores. In children, asthma initially develops through sensitization, and is often accompanied by eczema in the first year of life and, in later years, these children can develop allergic rhinitis and allergic asthma. The progression of these symptoms has been recognised as the ‘atopic march’. Based on population-based birth cohort studies, children with multiple allergies are likely to develop asthma (1,2) and approximately half of children with eczema will develop asthma later in life (3). Intrinsic asthma often develops later in life and has neither any allergen involvement nor any obvious adaptive immune system markers such as Th2 cells. This form of asthma is normally more difficult to treat and requires long-term systemic steroids.

Therapy for asthma includes managing symptoms usually by a combination of short or long-acting β agonists with inhaled corticosteroids and some patients with chronic asthma require systemic corticosteroids. Leukotriene antagonists
(such as montelukast) as an add-on therapy have been shown to have a positive effect in patients with mild to moderate asthma (4). Omalizumab, a monoclonal antibody directed against IgE, has also been used to treat patients with severe asthma (5).

Some patients, however, are more difficult to treat and are resistant to corticosteroids. With current advances and the availability of genome-wide expression studies in asthma, clinicians realised that there are different asthma phenotypes, each having a different pathophysiology. The differences in asthma phenotypes are now being recognised as asthma endotypes, which differ in terms of genetic susceptibility, environmental risk factor, age of asthma onset and molecular basis of the disease (6–8).

Besides improving and modifying currently available treatments, through current understanding of asthma, newly developed biologic agents targeting specific pathways are now in trials. Biologic compounds directed against transcription factors and small molecule inhibitors for chemokines and cytokines, include blocking chemokine receptors on eosinophils (CCR3), and Th2 cells (CCR4, CCR8 and CXCR4), and cytokines IL-4, IL-5, IL-13 and IL-17, are potentially promising for asthma treatment (9) of which, anti-IgE and anti-IL-5 have been licensed for asthma treatments (10). Specific immunotherapy (SIT) to inhibit allergic responses has been shown to be successful in treating individuals with allergic rhinitis where the allergens were identified and can be effective in reducing symptom scores and medication used in asthma patients (11). SIT decreases the development of sensitization to new allergens and decreases the risk of asthma developing in rhinitis patients (12,13).

1.1.2. Allergic Rhinitis

Allergic rhinitis (AR) is a common condition, which is an IgE-mediated inflammatory response in the nose caused by the interactions of specific airborne allergens binding to IgE antibodies on the surface of nasal mast cells. It affects nearly 400 million people around the world, a large proportion of which are still under-diagnosed and under-treated (2). In the United States, although
10-30% of the adult population and 40% of children are affected by AR, the health and economic impact of AR are hugely underestimated (14). Only recently AR has been recognised as a serious issue of epidemic proportion involving enormous medical costs. Typical symptoms of AR include rhinorrhea, sneezing, nasal itching, and nasal congestion.

A significant percentage of rhinitis patients, about one third, suffer from non-allergic rhinitis, (15). These patients have rhinitis symptoms, but not as a result of IgE-mediated events. Compared to non-allergic rhinitis, AR typically presents at younger age in which symptoms can be seasonal, episodic or all year round (perennial), depending on the type of allergen to which they are sensitized. Since seasons do not exist in many areas of the world, this classification does not represent a true AR classification. The Allergic Rhinitis and its Impact on Asthma (ARIA) guidelines for classifications have led to the definition of AR based on the severity of the symptoms ranging from intermittent or persistent and mild to moderate or severe (2,16,17). AR is often associated with asthma and other allergic diseases which can contribute to asthma exacerbations, making asthma more difficult to control and requiring hospitalizations and physician visits (18).

Successful treatments involve allergen avoidance and medication with oral and intranasal antihistamines, nasal and systemic corticosteroids, intranasal cromolyn sodium and more recently anti-leukotriene, which has recently been approved in the United States for the treatment of AR. Non-pharmacologic measures for AR treatment includes SIT, which has been demonstrated to be extremely beneficial to selected AR patients. Immunotherapy could represent a cure for AR. Subcutaneous immunotherapy has been shown to be effective in AR, with significantly long lasting symptom reduction, reducing the use of other medications and preventing new sensitisations and the onset of asthma (19–22). Sublingual immunotherapy is a safer alternative than subcutaneous immunotherapy with side effects restricted to the upper airways and gastrointestinal tract with rare episodes of anaphylaxis (20).
1.1.3. Food Allergy

Food allergy (FA) is a pathological reaction of the immune system in response to the ingestion of a food protein antigen. In food allergic individuals who are sensitised to a specific food protein, exposure to even trace amounts of food allergens can trigger clinical symptoms such as gastrointestinal disorders, urticaria and airway inflammation. These symptoms can range from mild to severe which can lead to life-threatening anaphylaxis. FA is common globally especially in younger children. It is estimated that about 6-8% of children suffer from FA (23–25). Sensitivity to some allergens (cow’s milk, egg, wheat) are outgrown, but allergy to peanut, tree nut and seafood normally persists into adulthood (26,27). FA is highly prevalent in atopic individuals; approximately 35% of children with severe persistent eczema also suffer from FA (28).

Food allergies can be classified into three different groups; those which are IgE-mediated, those which are both IgE-dependent and IgE-independent (mixed), and those which are not IgE-mediated (26). IgE-mediated FA is normally associated with the risk of severe, and possibly fatal, anaphylactic responses. Individuals with IgE-mediated FA are normally sensitized to food allergen (those who have had initial exposure to food allergen and caused initial immune reaction) and IgE-mediated immune cells triggered by subsequent exposure to specific food allergen. Oral allergy syndrome (OAS) is a variant of FA in which individuals with rhinitis have specific IgE towards fruits and vegetables as a result of cross-reactivity between pollen and plant-derived food (29). OAS results in less severe symptoms like oral pruritus, mucosa angioedema and/or abdominal pain but these symptoms will not lead to anaphylaxis. Mixed food allergy is characterized by both IgE-dependent and IgE-independent pathways. IgE-independent pathways cause delayed food-allergy-associated atopic dermatitis which happens around 6-48 hours post exposure, due to Th2 cells (30,31). Eosinophilic gastrointestinal disorders, such as eosinophilic esophagitis, are caused by eosinophil infiltration of the tissues after exposure to milk allergen (32) but can also be due to IgE-independent pathways. The difference between IgE-mediated FA and mixed FA with non-IgE mediated FA
is that the symptoms of non-IgE mediated FA normally affect the gastrointestinal tract, rather than the skin and respiratory tract.

Besides food allergen avoidance and treating anaphylactic responses with adrenalin as the standard of care, there is currently no definitive treatment for FA. Antihistamines are used to treat FA symptoms like itching, pruritus and oedema. Currently, immunotherapy against specific food allergen that can be delivered sublingually, orally or through the skin has been seen to have some potential as the treatment for FA (33–35). A monoclonal antibody against IgE has been tested in combination with oral immunotherapy in order to reduce allergic responses (36,37). Other examples that use monoclonal antibody to target upstream mediators in allergic pathways, such as IL-5, have also been used successfully in clinical trials as a treatment for eosinophilic esophagitis (38,39). An anti-IL-33 antibody is also currently being evaluated in late phase 2 clinical trial for treating food allergy (40).

### 1.1.4. Atopic Dermatitis

Atopic dermatitis (AD), also known as atopic eczema, is the most common chronic inflammatory skin disease which affects up to 7% of adults and over 20% of children (41–43). AD was thought to be the first manifestation of atopy and the first step in the atopic march, leading to asthma and allergic rhinitis. The disease manifests from the first year of life, but it can start at any age. In individuals with AD, the earliest observed clinical signs are rough and dry skin and the eczematous lesions only start later on and can continue for long periods or become relapsing-remitting in nature with repeated flare-ups (44). A birth cohort study suggested that the prognosis of AD depends on the severity, the presence of early onset of atopic sensitisation and a family history of AD, which are the risk factors for a long disease course (45).

Rather than an IgE-mediated disease, AD has been associated with activation of T cell subsets. Several different subtypes have been described and these are
based on IgE levels (intrinsic and extrinsic AD) and FLG (fillagrin gene) mutation. Extrinsic AD, also known as allergic AD, is associated with high total serum IgE levels towards environmental and food allergens and this is the most common form of disease with high prevalence (46,47). Intrinsic AD, or non-allergic AD, normally exhibit normal serum IgE levels and an absence of specific IgE. This type of disease normally affects about 10-45% of the population, predominantly females (47,48). Fillagrin, an important skin barrier protein has shown to be important in individuals with AD. FLG gene mutations that lead to FLG deficiency have been associated with a higher risk and a more persistent and severe form of the disease (49,50).

At present, only the symptoms of AD can be managed to achieve long-term disease control. Continuous repairing of the epidermal barrier with emollients, allergen avoidance and application of topical corticosteroids or calcineurin inhibitors have been used for managing the symptoms. Short term phototherapy treatment can be considered if the disease cannot be controlled by topical application of corticosteroids or calcineurin inhibitors (51). Systemic immunosuppressive therapies can be considered in cases where both topical measures and photo treatment fail. Targeting acute phase inflammation or Th2-driven immunity has been the aim for developing new therapeutic strategies for AD. Dupilumab, an antibody targeting IL-4 receptor alpha chain has shown to have marked and rapid improvement in the disease symptoms (52). Other newly developed monoclonal antibodies are already in trials and could potentially offer new targeted therapy for AD.

1.1.5. Ocular Allergy

Allergic inflammatory symptoms affecting the eye are less well studied as compared to other allergic conditions since often, information on allergic conjunctivitis (AC) is included within studies on rhinoconjunctivitis. AC is a global disorder that affects 19.1% to 20% of the general population worldwide. According to the Allergic Rhinitis and its Impact on Asthma (ARIA) initiative, AC is also a comorbidity of allergic rhinitis and asthma (53). AC is commonly under-diagnosed among patients with asthma and rhinitis even though almost 50% of
seasonal allergic rhinitis patients have eye symptoms (SAC; hay fever) and sometimes it also co-exists with atopic dermatitis and food allergy. Recent studies have highlighted the significance of ocular symptoms showing that they are as severe or sometimes even more severe than nasal symptoms in 70% of hay fever sufferers (54). This thesis will further investigate the immunological mechanisms which are involved in ocular allergy. Using in vitro and in vivo models of AC, we will study the immunopathogenic mechanisms involving T cell and mast cell-derived cytokines, which are commonly associated with the immunopathogenesis of allergy.

1.1.5.1. Classification and Symptoms

Ocular allergies can be classified in different categories, depending on the severity of the symptoms. The mild forms of AC are seasonal (SAC; hayfever) and perennial (PAC), while the more severe and chronic forms of AC include vernal keratoconjunctivitis (VKC) and atopic keratoconjunctivitis (AKC). VKC and AKC have quite different clinical and pathophysiological features as compared to SAC and PAC. Giant papillary conjunctivitis (GPC) is another category of ocular allergy, most commonly seen in contact lens wearers.

Seasonal allergic conjunctivitis (SAC) (where inflammation occurs seasonally) and perennial allergic conjunctivitis (PAC) (where inflammation occurs all year round) are immediate (type 1) hypersensitivity reactions believed to be caused by allergen-induced IgE-mediated mast cell activation. In the United States, SAC and PAC make up about 95% of ocular allergy cases (55). Different reports on rates of AC incidence all over the world is due to AC often being classified as rhinoconjunctivitis rather than AC (56). Most cases of SAC and PAC have been shown to have specific IgE antibodies to allergen (57). Signs and symptoms of both SAC and PAC are the same; they differ on the specific allergens to which the patients are allergic. Common AC symptoms include watery, itchy, red, sore, swollen and stinging eyes and they can cause a significant reduction in the quality of life. SAC is more common than PAC, whilst PAC is caused by allergens that are present all year round (animal dander and dust mites) resulting in more prolonged symptoms than in SAC. Hence PAC is considered to be clinically mild but chronic in its symptoms. Although SAC and
PAC are generally not sight-threatening, discomfort from ocular symptoms can reduce productivity levels for example from daily activities including reading, computer use, and restricted outside activity. As a result, the loss of productivity leads to costs estimated at €350 per patient per annum in Spain (58).

Vernal keratoconjunctivitis (VKC) and atopic keratoconjunctivitis (AKC) are clinically more severe forms of AC. VKC is a rare chronic allergic inflammatory disease of the ocular surface which occurs in less than 1% of the population (59,60). It is more common in young males, especially those living in hot and dry climates (like the Mediterranean and West African countries) and, in more than 95% of VKC children, the symptoms resolve upon puberty and only a minority go on to develop adult AKC. In the Asia-Pacific region, the age of onset is before the age of 10 years (61,62). Based on an epidemiological report in Sweden, VKC is more prevalent amongst the immigrant children of African and Asian origin, which suggests that VKC has both genetic and environmental impacts on the incidence of the disease (63). VKC never occurs in adults but it is not a rare disease amongst children aged 15 years and under (64, 65). Two thirds of VKC patients are atopic and VKC is often associated with a previous history of other allergic diseases (eczema, asthma and rhinitis). The inflammation persists throughout the year but there will often be seasonal worsening due to re-exposure to allergens.

Symptoms of VKC worsen in the allergy season but symptoms can also be all year round. Typical signs include the presence of giant papillae on the upper tarsal conjunctiva, production of sticky mucus, Trantas Dots at the limbus (clumps of eosinophils with dead epithelial cells), corneal ulceration and scarring. Giant papillae can be easily seen by eye on the tarsal conjunctiva (65). Trantas dots can be a measure of disease activity as they only appear in active VKC (66). Vernal plaque ulcers can occur which are deposited on the surface of the corneal stroma where there is no epithelium. Severe itching, photophobia, tearing and redness are normally present in VKC patients.
AKC is also a chronic inflammatory disease of the conjunctiva and cornea but, unlike VKC, it also involves the eyelids with eczematous appearance. It is always associated with another severe allergic disease, either eczema (where 15-40% of eczema patients also have an ocular involvement) or asthma. It usually affects adults between 20 and 50 years of age (67). Eczematous lesions of the eyelids are normally red, elevated and very itchy. Conjunctival giant papillae might or might not be present and conjunctival scarring is frequent in patients with AKC. Unlike VKC, in AKC, patients tend to develop atopic cataracts and sometimes, AKC patients require cataract surgery at a young age although it is very uncommon (68).

In giant papillary conjunctivitis (GPC), the inflammation is usually related to the wearing of contact lenses or ocular prostheses or due to sutures protruding on the ocular surface. GPC affects 5-10% of soft contact lens wearers. GPC is characterized by papillary hypertrophy of the superior conjunctiva and its appearance is similar to VKC, but GPC is stimulated by an inert substance rather than allergen. It can occur in patients at any age, in atopic or non-atopic individuals. GPC is not associated with atopy and resolves upon cessation of contact lens wear or removal of the protruding suture.

1.1.5.2. Pathophysiology

The pathogenesis of AC is predominantly due to allergen interacting with IgE bound to sensitised mast cells. During early phase responses, mast cells release their mediators (such as histamine, prostaglandins and leukotrienes) upon degranulation, as a result of allergen binding to the IgE FceRI receptor on its surface (69). This then causes itching, vasodilatation and increased vascular permeability. These symptoms normally occur during the first hour after allergen exposure. Mast cell degranulation also leads to the activation of vascular endothelial cells, which in turn express chemokines (such as RANTES and monocyte chemoattractant protein (MCP)) and adhesion molecules (such as intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM)) (70). These factors initiate the beginning of late phase responses by recruiting more inflammatory cells to the conjunctival mucosa (the central role of
conjunctival mast cells in the pathogenesis of ocular allergy). Late phase responses occur 6-24 hours after allergen exposure and are dependent on an initial interaction of mast cells with antigen during the early phase response (71). This involves the recruitment of eosinophils and a few T lymphocytes, which release pro-inflammatory Th2 type cytokines (IL-4, IL-5, IL-13).

Immune mechanisms of both AKC and VKC are more complex than the milder forms of the disease as they are not triggered by common allergens, rather they possess more complex immune mechanisms involving T cells (72). There are common pathways between VKC and AKC. Both VKC and AKC involve both mast cell degranulation and Th2 type cytokines. Whilst VKC involves mainly Th2 lymphocytes, immune mechanisms in AKC involve both Th1 and Th2 lymphocytes and cytokines. This will be discussed below in further detail.

VKC is a combination of type I and delayed (type IV) hypersensitivity (73). VKC is a result of a complex pathogenesis that involves mainly CD4+ T lymphocytes (Th2) with overexpression of mast cells, eosinophils, neutrophils and fibroblasts (74). The formation of giant papillae is the result of IL-4 and IL-13 involvement by inducing the production of extra-cellular matrix and the proliferation of conjunctival fibroblasts (74,75). High levels of IgE and mast cell mediators are found in VKC patients (76). Since it involves the cornea, VKC can be sight threatening but usually leaves no permanent alterations in visual acuity except in 6% of patients.

The pathophysiologic mechanisms of AKC involve both mast cell degranulation and also immune mechanisms, involving both Th1 and Th2 type cytokines (77). This is similar to AD, which is not exclusively mediated by Th2 lymphocytes and where involvement of both Th1 and Th2 lymphocyte-derived cytokines leads to a severe conjunctival reaction. T cell polarization in AD and in AKC is biphasic. During the acute phase, there is a predominantly Th2 cell response whilst during the chronic phase, Th1 cells are more predominant (78). Briefly in AD, during acute phase, FcεRI engagement with antigen presenting cells in the skin contributes to the initiation of inflammation by resident Langerhans cells with Th2 cytokine predominance. Thereafter, inflammatory dendritic epidermal cells
(IDEC) are recruited into the epidermis and produce high amounts of proinflammatory cytokines that prime naïve CD4+ T cells into Th1 cells producing IFN-γ (79,80). Acute AD skin lesions are characterised by the presence of high levels of Th2 cells and eosinophils with increased levels of cytokine IL4, IL-5, and IL-13, and little IFN-γ expression. However, as AD becomes more chronic, lesions exhibit increased levels of IFN-γ, IL-12, and GMCSF, suggesting the presence of Th1 cells to maintain chronic inflammation (81). However, the mechanism of chronic AC and the switch between Th2 to Th1 predominant pathways in AKC are still yet to be explored. The switch from Th2 to Th1 is thought to involve microbial products from bacterial colonisation in the skin that activates Toll-like receptor 2 (TLR2) (82). Unlike AD, in AKC, the mechanism behind the shift between Th2/Th1 like pattern is still unknown but high levels of IL-4 and IL-13 were found in the tears of AKC and VKC patients whereas IFN-γ levels were increased in the tears of patients with corneal damage, suggesting that in chronic AC, IFN-γ is important in maintaining chronic inflammation in AKC (83,84).

Some of the important complications in chronic AC include a wide range of corneal clinical manifestations. In the pathogenesis of punctate keratopathy, the tear film becomes unstable due to the changes in the mucin layer of the tear film and changes to corneal epithelium permeability. The dots of punctate keratopathy may combine together to form syncytial opacity, which often leads to a whitish or greyish plaque beneath the epithelium. These plaques may interfere with vision and lead to scarring of the cornea. Punctate keratitis may evolve to confluent ulceration and shield ulcer as a result of epithelial toxicity caused by mediators released by activated eosinophils (85). The vernal plaque ulcer is a deposition on the surface of the cornea stroma where there is no epithelium. Patients with shield ulcers with visible plaque formation had delayed re-epithelization which can cause bacterial keratitis as the cornea is not protected by epithelium (Cameron JA Ophthalmology 1995). Mechanical eye rubbing, although not the primary cause, can contribute to the worsening of keratoconus, which usually causes blurry vision. Trantas dots consist of necrotic eosinophils, neutrophils and epithelial cells and represent an almost pure collection of eosinophils. The formation of giant papillae is due to
overgrowth of conjunctival connective tissue and abundance of collagen fibres. Excessive deposition of extracellular matrix and the formation of giant papillae are due to imbalance of the expression of matrix metalloproteinases (MMPs) and tissue inhibitors of MMP (TIMP) (86). Conjunctival resident cells, including epithelium and fibroblasts, play an important role in the formation of giant papillae. Th2 cytokines, growth factors including vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and transforming growth factor β1 (TGF-β1) were demonstrated to be increased in VKC and which promote fibroblast growth and procollagen production and may be involved in tissue growth and remodelling (87–89). Fibroblast and keratocytes may also promote inflammation by producing chemokines including G-CSF, eotaxin 1 and MCP-1 or by expressing adhesion molecules like ICAM-1 in response to Th2 cytokines which could facilitate eosinophil infiltration (90,91).

**1.1.5.3. Diagnosis and treatment**

In both SAC and PAC, the diagnosis is usually confirmed by a history of atopic conditions (from personal and family), clinical observation and positive skin prick test to suspected allergens. The skin prick test is not always reliable in assisting with the diagnosis whereas the conjunctival provocation test, in which very small amounts of allergen are administered to the tear film, can be more useful in leading to AC diagnosis (92). In PAC, the presence of eosinophils in the conjunctival swab and/or an increase of total IgE in serum and tears can also confirm the diagnosis (93).

The first line of treatment for both SAC and PAC is allergen avoidance. In most mild cases of AC, oral and topical antihistamines (e.g. mast cells stabilisers like cromolyn sodium, or olopatadine which is an H1 receptor blocker and mast cell stabiliser) (94) can be helpful in reducing symptoms. Antihistamines, which reversibly block histamine binding to its receptor, provide quick relief from redness and itch but do not prevent action from other proinflammatory mediators secreted by activated mast cells such as prostaglandin and leukotrienes (95). Mast cell stabilisers are also frequently used to treat SAC and PAC. Cromolyn sodium inhibits mast cell degranulation (96), and is generally
safe with minimal side effects. Currently, antihistamines combined with mast cell stabilisers are the most commonly prescribed group of agents as these provide better symptom relief and are well tolerated with milder side effects (94). Specific immunotherapy can also provide systemic relief in SAC and PAC but this does not work in clinically severe disease (VKC and AKC).

In severe chronic AC diagnosis can be done using similar criteria described above for clinically less severe AC (SAC and PAC), which include measuring eosinophil and IgE levels in tear samples, although these tests are not usually required in clinical practice as the diagnosis is usually made clinically based on typical symptoms and signs. High levels of IgE and mast cell mediators are present in the tear fluids of VKC patients (76,97).

To date, one of the main options to treat severe AC (VKC and AKC) is the administration of topical (eye drops) or systemic (oral) corticosteroids, in addition to anti-histamine and mast cell stabilising treatments previously discussed. Corticosteroids exert rapid anti-inflammatory effects via their interactions with glucocorticoid receptors, which modulate protein synthesis of cytokines through the blocking of nuclear factor-κB (NFκB). Topical or subtarsal steroids may be required to reduce or inhibit the production of inflammatory mediators. Systemic steroids which are more potent, are very rarely required for more severe cases, and can cause significant side effects in the eye, when given long term, including an increase in intraocular pressure (IOP), cataracts, glaucoma and susceptibility to infections.

Calcineurin inhibitors such as cyclosporine A (CsA) or tacrolimus can be used as a valid alternative to steroids to treat severe VKC and AKC due to their ability to block CD4\(^+\) T-lymphocyte proliferation, IL-2 production and histamine release by mast cells through the inhibition of calcineurin (a phosphate that plays an important role in FcεRI-mediated exocytosis of pre-formed mediators from mast cells). CsA inhibits histamine release from mast cells and basophils through an IL-5 reduction, which may also affect eosinophil recruitment and cause tissue destruction on the ocular surface (98). Frequent side effects of topically applied
CsA include burning and irritation of the eye. Randomised, double-blind, placebo-controlled trials concluded that the use of topical CsA is safe and effective in improving symptoms in both VKC and AKC (99,100). More than half of patients who were using topical steroids at the beginning of a trial were able to be tapered off steroid drops after treatment with topical CsA (101)(101). Systemic cyclosporin or tacrolimus may also be required for recalcitrant atopic dermatitis associated with AKC. Topical tacrolimus is more potent than cyclosporin (77) and is very useful for eyelid eczema (102). In resistant AD cases, systemic treatment with mycophenolate (103), methotrexate or dupilumab (an anti-IL-4 immunotherapy) (104) may also be required.

As a consequence of the limited availability of therapeutic options and the significant side effects from current treatments, additional strategies for the treatment of chronic AC are needed for improvement of clinical outcomes and better management for patients.

1.2. Basic Immunology of Allergic Responses

1.2.1. Allergic sensitisation

Dendritic cells (DC), acting as professional antigen presenting cells (APC), will first encounter and take up antigen, then migrate back to the local lymph nodes in order to induce T cell tolerance or sensitization. Antigen taken up by DC undergoes proteolytic cleavage into smaller fragments before being presented on the surface of DC via major histocompatibility complex (MHC) class II molecules. In order to induce tolerance under normal circumstances, CD103+ DC are thought to induce Foxp3+ regulatory T cells (Treg) which then suppress the generation of Th2 cytokines and IgE production (105,106).

In order to elicit allergic responses, an atopic individual must first be exposed to a specific allergen, which then leads to sensitization. During allergic sensitization, presentation of allergen by DC to naïve T cells induces their differentiation into Th2 T cells in the presence of IL-4, which then undergo clonal expansion. IL-4 and IL-13 secreted by Th2 cells induce immunoglobulin
(Ig) class switching from IgG to IgE and clonal expansion of naïve B-cell populations after allergen-specific binding on B cell receptor, which allows IgE production by B cells. IgE sensitises both mast cells and basophils by firstly upregulating IgE receptors (IgER) on the surface and then IgE will bind to the high affinity receptors (FcεRI) on the surface of the mast cells/basophils. Besides FcεRI, IgE also binds to the low-affinity receptor, FcεRII (also known as CD23) on the surface of B cells. Subsequent presentation of allergen-derived peptides to specific CD4+T cells, mast cells and basophils, will drive both the early and late phases of allergic responses.

1.2.2. Allergic responses

During an allergic response, allergens from the environment penetrate through the epithelium into the subepithelial and stromal layer of tissues. This foreign allergen will bind to the surface of DC for antigen presentation. DC express high affinity FcεRI receptors (trimeric isoform) for binding allergen which is then shuttled into lysosomal compartments for loading and presentation on MHC class II molecules (107). Besides DC, other myeloid cells can function as APC, including macrophages, monocytes and granulocytes. Native allergen protein is first degraded by the APC and is then loaded onto MHC molecules for antigen presentation to T cells. In allergic individuals (atopics), allergen uptake by DC is facilitated by interaction of the allergen with IgE attached to FcεRI.

Immune responses are divided into two components, the innate (non-specific) and adaptive (specific) immune responses. During the first exposure to microorganisms or allergen that managed to penetrate the epithelial surface, cells such as macrophages, DC, neutrophils and mast cells are the first line of innate immune cells that will respond. Their responses are rapid and non-specific. Both macrophages, DC and neutrophils express Toll-like receptors (TLRs) that recognise bacterial cell wall (TLR1, 2, 4, 5 and 6) and nucleic acids (TLR3, 7, 8 and 9),) enabling recognition and removal of bacteria by engulfing and destroying the invading micro-organisms.
The innate immune response is crucial in contributing to the activation of the adaptive immune response. Innate immune responses can occur alone or can be followed by with the slower adaptive immune responses. After the first encounter during the innate immune response, antigen-bearing cells will move to the lymphoid tissue, whilst some mediators released by immune cells during the innate response recruit more immune cells to the site of inflammation, hence amplifying the immune response. At the lymphoid tissues, specialized long-lived phagocytic cells like the DC interact with antigen-specific lymphocytes, which then migrate to the site of inflammation and activate the adaptive immune response. Activated DC and mast cells are said to be important in linking the innate and adaptive responses since these cells secrete cytokines that influence both innate and adaptive immune responses.

1.3. Immune cells in allergic responses

1.3.1. Mast cells

Mast cells (MCs) were first described by Paul Ehrlich in 1878 as cells that stained uniquely with large granules. Mast cells are potent effector cells of the innate immune system that are haematopoietic in origin. Mast cell precursors migrate to tissues where they mature and differentiate depending on the local cytokine milieu to become tissue resident cells in the host environment. The maturation of mast cells is dependent on the presence of stem cell factor (SCF) and its c-kit receptor (108). Besides maturation of mast cells, SCF also promotes mast cell adhesion and survival (109).

In the eye, mast cells are mostly found in the conjunctival region. MCs can be distinguished by their protease contents and their location in the tissue. In mice, MCs can be distinguished based on their anatomical sites; connective tissue mast cells (CTMC; located around venules, intestinal submucosa and skin) contain both tryptase and chymase while mucosal mast cells (MMC; located in the epithelial cell layer of mucosal tissues in lung and intestines) contain only tryptase. In humans, mast cell subtypes depend on their protease content; MC_{TC} mast cells contain both tryptase and chymase, (resemble murine CTMC)
and MC$_T$ mast cells only contain tryptase (resemble murine MMC) in their granules (110).

It is clear that mast cells exist as subtypes, and migrate depending on their environment and this change can occur in both directions. By performing immunohistochemistry staining of normal and allergic human conjunctivae, Baddely et al. reported that tissue-resident mast cells in the healthy conjunctiva contain both MC$_{TC}$ and MC$_T$ phenotypes (111). During AC, the distribution of mast cell protease expression was altered and an increase in MC$_T$ was observed in the allergic conjunctivae (111). A similar study was performed by staining human conjunctival tissue sections of normal and allergic conjunctivae, where it was observed that the proportion of MC$_T$ was increased in the substantia propria of VKC individuals as compared to normal control individuals (76).

Mast cells can be activated in response to many different stimuli including activation through toll like receptors (TLRs) in response to pathogens (112,113), activation through aryl hydrocarbon receptor (114), the CD40 ligand (115), the OX40 ligand (116) or by immune complexes of IgG (117). However, during allergic inflammation, mast cells are activated through allergen cross-linking with surface IgE receptors (Fc$\varepsilon$RI), which are present on the surface of the allergen-sensitised MC. Another study has demonstrated that, during allergic responses, mast cells can also be activated upon IgG binding to surface Fc$\gamma$RI and Fc$\gamma$RIII receptors (118). Mast cells can respond differently depending on the strength of the Fc$\varepsilon$RI signals. (119). Either low or high affinity stimuli, different receptor cluster size, mobility and distribution, can all activate signalling molecules which are required to release different secretory products. For example, adaptor protein LAT-2 is enhanced in high affinity signals resulting in MCP-1 production.

In allergic inflammation, mast cells are important during the sensitisation process, and to mediate both early and late phase allergic reactions. Once activated, mast cells secrete preformed mediators from their granules within minutes. One of the secreted mediators, histamine, then binds to its receptors
(H1R and H2R), causing the immediate symptoms seen in SAC and PAC such as redness of the eye, itching, lacrimation and chemosis. Besides SAC and PAC, histamine and tryptase levels are also increased in tears of VKC and AKC patients (120,121). Mast cell granules also contain some preformed cytokines, chemokines and growth factors including TNF-\(\alpha\), IL-6, IL-8, IL-10, vascular endothelial growth factor (VEGF) and SCF. Beside histamine and cytokines, mast cells also secrete proteases including tryptase and chymase and other de novo bioactive compounds such as lipid mediators (leukotriene C4, prostaglandin D2 and platelet activating factor). Tryptase is highly expressed in mast cells, but not in basophils.

After 6-12 hours of activation, mast cells also synthesise a wide range of cytokines, which are important in the late phase or for initiating a more chronic response. Among the cytokines, IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, IL-13, IL-16, TNF-\(\alpha\) and granulocyte-macrophage colony stimulating factor (GM-CSF) can be secreted by human mast cells (122). Depending on the inflammatory signals, mast cells are able to replenish their granules upon stimulation. These cytokines are responsible for cellular infiltration and eventually lead to tissue damage, which can be seen during chronic allergic responses.

After stimulation, mast cells function as the promoters and effectors of adaptive immunity. They also shape the inflammatory milieu, which is crucial for adaptive immunity. Secreted mast cell products are responsible for recruiting inflammatory cells.

1.3.2. T cells

During development, T cell precursors leave the bone marrow and migrate to the thymus via the blood stream where they differentiate and begin to express T cell receptors (TCR), CD4 and CD8 molecules and develop into naïve T cells. Committed T cells subsequently seed the secondary lymphoid organs where they encounter antigens and differentiate into distinct T cell subsets. T cells are differentiated based on their expression of CD4 and CD8. However, double-positive CD4\(^+\)/CD8\(^+\) cells have been described in the blood and peripheral
lymphoid tissues where this T cell subset is regulated by a very strict transcriptional program (123). Cytotoxic CD8+T cells are mainly involved in killing cancer cells, virus-infected cells and dying cells by expressing death signals and secreting lytic enzymes (granzymes). CD4+T cells, originally termed T helper (Th) cells, are responsible during the later stage of inflammation where they mainly secrete cytokines and chemokines, the key players in upregulating/modulating immune responses.

CD4+T cells are the major contributors in the immunopathology of autoimmune diseases and allergic inflammation (124–128), hence, in this section I will describe the role of CD4+ T cells in more detail. During cell-mediated immunity of adaptive immune responses, CD4+T-cells are polarised to distinct T cell subsets according to the cytokines present in the microenvironment. Naïve CD4+ T-cells can differentiate into Th1, Th2, Th9, Th17, Th22, T regulatory cells (Treg), and T follicular helper cells (Tfh)(Figure 1).
Figure 1: Different T cell subsets. Naïve CD4$^+$ T cells polarise to different T-cell subsets; Th1, Th2, Th9, Th17, Th22, iTreg. Tfh, secreting different cytokine profiles. These T-cell subsets promote different types of inflammatory responses depending on their respective cytokine profiles and specific transcription factors. Adapted from (128).
1.3.2.1. Th1 cells

Th1 cells, which are mostly defined by their secretion of IFN-γ, are normally involved in cell-mediated inflammatory responses and delayed-type hypersensitivity and are important in immunity against intracellular pathogens. Th1 cells are also defined by the master regulator transcription factor, Tbet, which is important in promoting Th1 differentiation. T-bet and GATA3 are co-expressed in Th1 cells and Kanhere and colleagues found that in Th1 cells, GATA 3 is distributed away from Th2 genes and binds to T-bet binding sites and T-bet can directly activate its own expression (129). The Th1 signature cytokine IFN-γ has long been known to be involved in the immunopathology of several autoimmune diseases including multiple sclerosis (MS) and rheumatoid arthritis (130). High levels of IFN-γ in MS serum, plus extensive studies using adoptive transfer of CD4+ T cell subsets in experimental mouse models suggested that Th1 cells are immunopathogenic (131,132). IFN-γ can also be anti-inflammatory as it is antagonistic for IL-4, by dampening the differentiation of other pathogenic T cell subsets and downregulating lymphocyte trafficking into the draining lymph nodes (133). Although IFN-γ is considered to be the protective response in allergic disorders, many studies have shown that allergic inflammation is often due to mixed responses involving both Th1 and Th2 cells (134,135).

1.3.2.2. Th2 cells

Th2 cells secrete IL-4, IL-5, IL-9 and IL-13, which are the key cytokines in host defence against multi-cellular parasites and in the development and immunopathogenesis of allergic diseases. IFN-γ, secreted by Th1 cells, acts as a negative regulator for Th2 differentiation, hence both Th1 and Th2 T cell subsets can be clearly defined based on their cytokine profiles and transcription factors. IL-4, a pleiotropic cytokine which is secreted by activated Th2 cells, T follicular helper cells, mast cells, innate lymphoid cells and basophils, is important as the survival factor for lymphocytes besides promoting B cell differentiation and IgG and IgE class switching (136–140). GATA-3 is the master regulator of Th2 differentiation although it was found that GATA-3 is also important in the differentiation of innate lymphoid cells (141). The GATA-3
transcription factor binds to multiple proximal and distal sites to IL4, IL5 and IL13 in order to activate the Th2 cytokine locus (129,142–144). IL-4 activates downstream signalling transducer and activator of transcription (STAT)-6 which, in turn, promotes the expression of GATA binding protein 2 (GATA-2) to further activate Th2 cytokine secretion. Besides IL-4, Th2 cells also secrete large amounts of IL-5 and IL-13. IL-5 is known to stimulate antibody secretion from activated B cells and enhances proliferation and differentiation of eosinophils. IL-13 is known to inhibit the proliferation of other proinflammatory cytokines secreted from monocytes, B cell co-stimulator for B cell maturation, and promotes mucus production (145). Both IL-5 and IL-13 are regarded as important in driving the pathology of allergic asthma.

1.3.2.3. Th9 cells

Th9 cells develop from naïve CD4+ T cells in the presence of TGF-β and IL-4 (146–148). In the presence of IL-4, TGF-β reprograms Th2 cells to differentiate into Th9 cells, which produce high levels of IL-9 and IL-10 (148). The Th9 cell subset, characterized by its production of IL-9, was recently described as a distinct T cell subset, whereas IL-9 was initially identified as a Th2 cytokine. Similar to other T cell lineages, Th9 cells are regulated by specific transcription factors: STAT6, GATA-3, IRF4 and PU.1. However these transcription factors are not specific for Th9 cells as their expression overlaps with other T cell lineages, including Th2 and Th17 cells (146–150). Different cytokines and transcription factors that regulate and promote Th9 cell development as a separate T cell lineage will be discussed later in this chapter. Although IL-9 was first described as a growth factor for T cells and mast cells, it is increasingly being described now as playing important roles in the pathogenicity of various allergic and autoimmune diseases including SLE (127,151–153). Other functions of Th9 cells will be discussed later in this chapter.

1.3.2.4. Th17 cells

Besides Th1, Th2 and Th9 cells, Th17 cells are another CD4+ T cell subset which plays an important role in inflammation. This CD4+ T-cell subset is
characterised by its secretion of IL-6, IL-8, IL-9, IL-17A, IL-17E, IL-22 and IL-26 under the control of the master transcription factor RORγt. Th17 cells are known to be involved in autoimmune tissue injury and host defence against extracellular bacteria and pathogens in the mucosa. Naïve CD4⁺T cells differentiate into Th17 cells in the presence of combinations of IL-23, TGF-β, IL-6 and IL-21. Much of the pathogenic roles for Th17 cells have been attributed to their secretion of IL-17, which is classically known to enhance neutrophil differentiation of CD34⁺ hematopoietic progenitors in a coculture system with fibroblasts, to activate innate immune cells and to enhance B cell functions (154).

1.3.2.5. Th22 cells

The Th22 cell subset, secreting predominantly IL-22, is considered to be a sibling of Th17 cells but having distinct gene expression and functions. IL-22, a member of the IL-10 cytokine family, is produced by immune cells including CD8⁺ and CD4⁺T cell subsets (Th17 and Th22), natural killer (NK) cells and gamma/delta (γδ) T cells. The functional effects of IL-22 are confined to non-hematopoietic cells especially to epithelial cells of the skin, liver, lung and intestine (155–157). Increased populations of CD8⁺ T cells expressing IL-22 have been observed in the skin of patients with the more severe form of atopic dermatitis (158). In patients with rheumatoid arthritis, IL-22 has been shown to be important for promoting the differentiation of synovial fibroblasts (159).

1.3.2.6. Other T cell subsets

Other T cell subsets include follicular helper T-cells (Tfh), which secrete IL-21, provide helper function to B cells to synthesize antibodies and differentiate into plasma and memory B cells in the germinal centre. Similar to other T cell lineages, Tfh are associated with a canonical transcription factor. High expression levels of Bcl-6 were identified in Tfh (160,161) and are necessary for Tfh survivability and development (162,163). Tfh help B cells to regulate antibody secretion that precisely target specific pathogens for elimination, at the same time limiting autoimmunity and excessive inflammation.
Another T cell subset, regulatory T cells (Treg), downregulates CD4+ T cell proliferation, and suppresses potentially deleterious activities of Th cells by maintaining peripheral tolerance. Tregs are the important basic regulators that are designed to maintain immune tolerance. Unlike other Th cell subsets which respond and proliferate upon T cell receptor (TCR) engagement, Treg cells respond by suppressing proliferation of other T cell subsets in response to TCR stimulation (164). Treg cells are important to dampen the overwhelming responses by other Th subsets and are considered to be the master modulator in promoting anti-inflammatory responses. Treg cells have a distinct phenotype expressing the transcription factor Foxp3. Two major populations of Tregs have been identified; thymically-derived naturally-occurring Tregs (nTregs) and peripherally-induced Tregs (iTregs).

nTregs are characterized by CD4+CD25+ surface receptor expression, strongly express the Foxp3 transcription factor and secrete both IL-10 and TGF-β. Foxp3 expression is maintained irrespective of extracellular conditions or Foxp3 signals (165) The main task of nTreg is to dampen inflammatory responses mediated by various lymphocytes, especially helper T cell subsets (166–168). There are four different suppression mechanisms used by Treg cells; suppression by inhibitory cytokines IL-10, IL-35 and TGF-β, suppression by cytolyis, including granzyme A- and B-dependent and perforin-dependent killing mechanisms, suppression by metabolic disruption and suppression by targeting DC function and maturation (169).

iTregs, on the other hand, have different suppressive properties than nTregs, which are cell contact-independent and are mediated mainly by cytokines IL-10 and TGF-β. iTregs also bear similar phenotypes with nTregs such as the expression of CD25, CTLA-4 and Foxp3 (165,170). However, Foxp3 expression in iTregs is unstable. In a study done by Floess and colleagues, they demonstrated that TGF-β-induced Foxp3 expression is lost after removal of TGF-β or TCR stimulation in vitro suggesting that the stability of Foxp3 expression depends on extracellular conditions (165). These cells developed from CD4+ T cells and consist of two different types: Tr1 and Th3 cells, which
have different capabilities in secreting cytokines. Tr1 are defined by their ability to be induced by IL-10 and secrete large amounts of IL-10 and lower amounts of TGF-\(\beta\), whereas Th3 cells secrete mostly TGF-\(\beta\). Tr1 cells’ immunosuppressive activity is mostly mediated by IL-10 (171). The Tr1 cell plays an important role in regulating immune responses against commensal organisms and promotes tolerance in the gut (172). Th3, on the other hand, plays an important role in tolerance induced by oral antigen delivery (173).

1.3.3. Basophils

Basophils are a rare population of granulocytes, which represents less than 1% of peripheral blood leukocytes. After the discovery of mast cells, Paul Ehrlich in 1879 described basophils as a population of leukocytes which contains cytoplasmic granules that stain with basophilic dyes.

Basophils are developmentally similar and share characteristics with tissue resident mast cells. Like mast cells, basophils express the high affinity receptors for IgE, FcεRI, on their surface (174) that cross-link upon engagement with allergen, resulting in release of a number of mediators including histamine, but not prostaglandin 2 (PGD2) or mast cell tryptase (175). Basophils also rapidly secrete IL-4, IL-13 and express CD40L and CCR3 (176–178). Basophils can be distinguished phenotypically from mast cells by a lack of, or only weak expression of cell surface expression of c-kit (SCF receptor). Exogenous IL-3 is a prerequisite requirement for basophil development and maturation whereas mast cells require both IL-3 and SCF as the main factors for growth and development (179,180). Unlike mast cells, which become fully mature in peripheral tissues, basophils are believed to complete their maturation in the bone marrow before being released into the circulation.

Basophils are known to be key regulators of allergic inflammation and Th2 cell-mediated disease due to their ability to secrete high levels of IL-4 and IL-13 (181). After allergen challenge, basophils together with eosinophils and Th2 lymphocytes were recruited to the site of inflammation including to the skin, lung and nose after allergen challenge (182).
1.3.4. Eosinophils

Eosinophils are bone marrow-derived leukocytes and can be found in both peripheral blood and/or in tissues. They represent 1% to 5% of the whole leukocyte population. Eosinophils can easily be identified because of their strong affinity to the acidic dye eosin which led to their discovery by Paul Ehrlich in 1879. In the bone marrow, IL-3, GM-CSF and IL-5 drive eosinophil differentiation. IL-5 is crucial in the final maturation stage in the bone marrow as well as eosinophil release into the circulation (183).

Eosinophils are an important source of cytotoxic proteins that can mediate pathology of a disease and can modulate immune responses and allergic diseases. Granules in mature human eosinophils contain cationic proteins which include major basic proteins (MBPs), eosinophil cationic protein, eosinophil derived neurotoxin and eosinophil peroxidase (184). Besides cationic proteins, eosinophil granules also contains preformed cytokines, chemokines, enzymes and lipid mediators such as IL-2, -3, -4, -5, -10, -12 and -13, IFN-γ, GM-CSF, RANTES, eotaxin-1 and CXCL5 which are capable of activating T cells, mast cells, and epithelial cells (185–187). For example, eosinophils express Th2 cytokines (IL-4, IL-5, IL-9 and IL-13), Th1 cytokines (IL-12 and IFN-γ), proinflammatory cytokines (TNFα, IL-1β, IL-6 and IL-8) and regulatory cytokines (TGFβ and IL-10) (188,189). Besides secreting mediators, eosinophils also can act as an antigen presenting cells since they express MHC class II and costimulatory molecules that directly modify T cell activities (190). To my knowledge they do not, however, have the capacity to take up antigen for processing, so are considered ‘non-classical APC’.

Eosinophil-rich inflammation has long been associated with parasitic inflammation and allergy. Comparing allergic asthma pathophysiology at the cellular level, increased numbers of infiltrating eosinophils at the airway mucosa are normally associated with severe asthma (191,192). Hence, higher levels of eosinophils in peripheral blood or within specific tissues can be an indicator of a pathologic process.
1.3.5. Neutrophils

One subpopulation of polymorphonuclear cells (PMNs), neutrophils, are important effector cells in immune defence and in IgG-mediated humoral immune responses due to their expression of surface receptors including Fc, IL-2R, IgE-binding proteins and Mac-2/epsilon binding protein (BP) that binds to inflammatory mediators including IgE, IL-4 and IL-13 (193–195). Besides functioning as phagocytic cells, PMNs also secrete inflammatory mediators including TNF-α, TGF-β and IL-1β in response to cell activation. They have also been demonstrated to secrete IL-17 (196,197).

Despite being commonly associated with increased inflammation, the role of neutrophils in allergy in general and in the pathogenesis of bronchial asthma remains unclear (198,199). However, an increased number of neutrophils has been found in the bronchoalveolar lavage of severe asthma, especially after allergen challenge (191,200).

1.3.6. Innate lymphoid cells

In addition to mast cells, eosinophils, neutrophils and T cells which are known to be involved in the pathogenesis of allergic inflammation, innate cells have also been described which are lineage-negative, not expressing surface markers of lineage-positive cells, but secrete large amounts of cytokines (201–203). It was found that innate lymphoid cells (ILCs) consist of three different subsets, namely ILC1, ILC2 and ILC3. ILC2 is a subtype that is of interest with regards to allergy since this cell secretes large amounts of classical ‘Th2 cytokines’ including, IL-4, IL-5, IL-9 and IL-13. It is thought that ILC2 secrete cytokines during the early stage of allergic responses, preceding T cell-mediated inflammation. Whilst most studies on ILC2 were performed in mice (137,201,204), other groups have also reported an upregulation of ILCs in the lungs of patients with chronic obstructive pulmonary disease (COPD) and in the gastrointestinal tract of patients with eosinophilic oesophagitis as compared to
control subjects (205,206). However, their exact contribution to inflammation in relation to T cells and other immune cells is still being investigated.

This brief description of different immune cell types has led us to a greater understanding of the role for secreted cytokines in modulating different cell types during inflammation, a very important aspect in the regulation of immune responses. In this thesis, the role of cytokines, especially IL-9, in the development and pathogenesis of allergic conjunctivitis will be investigated and discussed in further detail.

1.4. The cytokine response in allergy

Since classical Th2 cytokines (IL-4, IL-5, IL-9, IL-13) are mostly involved in the development and pathology of allergic diseases, further roles for these cytokines will be discussed in more detail in this section.

These cytokines are derived from mast cells, basophils, eosinophils, B cells and T cells during acute and chronic allergic inflammation. During acute allergic conjunctival (AC) responses, mast cells release pro-inflammatory pre-formed mediators such as IL-4, IL-13 plus other cytokines, chemokines, tryptase, chymase, prostaglandins and leukotrienes, all leading to symptoms seen in SAC. The release of these mediators also promotes the recruitment of other pro-inflammatory cells, which drive the inflammation to become more chronic. In PAC, there is an increase in neutrophils and T cells. VKC is marked by an increase in eosinophil and T cell infiltration, releasing IL-5 (for upregulating eosinophils migration and activation), IL-4 (for driving B-cell activation and IgE release) and IL-13 (for promoting mucus secretion and tissue remodelling). This inflammatory cascade results in persistent and more pronounced symptoms as seen in chronic ocular allergy (83). However, different cytokine profiles can be seen in AKC such as an increase in T cell-derived IFN-γ in conjunctival tissues (207) and in CD4+ T cells derived from AKC tear samples, which suggests that AKC may also be driven, in part, by Th1 cells (208).
In general, Th2 cytokines have been extensively studied in allergic diseases. Besides being secreted by different immune cells including CD4^+ T helper cells, eosinophils, basophils and ILC2 cells, IL-4 is known to be the first cytokine to be secreted by mast cells upon stimulation via IgE receptor (209). An earlier study conducted by Bradding and colleagues used mast cells purified from the skin and lung and demonstrated that human mast cells contain and release IL-4 upon IgE stimulation (210). A few years later, a study done by Wilson and colleagues confirmed that IL-4 was stored within mast cell granules and could be one of the earliest cytokines released by mast cells upon activation (209).

The distribution of IL-4 receptor (IL-4R) expression is extremely broad, suggesting that IL-4 has pleiotropic effects that can potentially affect many different cell types (211). In the presence of IL-13, IL-4 controls IgE class switching in B cells (212) and IL-4 is known to control Th2 phenotype differentiation in the presence of IL-2 (213). With its various roles being documented, especially the involvement of IL-4 in allergic diseases, IL-4 has also been shown to be upregulated in allergic conjunctivitis patients’ tear samples. It is also postulated that IL-4 is involved in stimulating conjunctival fibroblasts in chronic ocular allergy (214).

Despite sharing a common receptor subunit and sharing many functional properties with IL-4, IL-13 has been shown to have distinct functions, especially in the development of airway hyperresponsiveness and mucus production (215). In hematopoietic cells like monocytes and macrophages, IL-13 enhances the expression of integrins, which are important in cell adhesion (216) and inhibits the secretion of pro-inflammatory mediators including IL-12 and TNF-α (217). IL-13 is mostly functionally important on non-hematopoietic cells like endothelial cells, smooth muscle cells, fibroblasts, and epithelial cells. IL-13 induces vascular adhesion molecule 1 on endothelial cells, which is important for eosinophil recruitment (218), upregulates goblet cell metaplasia and decreases beat frequency of ciliated epithelial cells (219,220). This summarises the importance of IL-13 in the contribution to the pathogenesis of allergic asthma.
Besides IL-4 and IL-13, IL-5 is another classical Th2 cytokine, which is also secreted by mast cells. IL-5 acts on target cells by binding to specific IL-5 receptors (IL-5R), which contains IL-5Rα (IL-5 specific) and the common signal transducing β cytokine (βc) chain (221). Besides eosinophils, IL-5R is also expressed on human basophils and B cells (222–224). Since IL-5 is important in eosinophil differentiation, migration, activation and survival, IL-5 has been recognised as the most specific cytokine for the development of the eosinophil lineage (225,226). Besides eosinophils, IL-5 also promotes B cell growth and induces terminal differentiation of activated B cells into antibody-forming plasma cells (227,228). Horikawa and colleagues showed that IL-5 exerts its effects on B cell maturation by regulating several gene clusters including immunoglobulin-related genes and genes involved in B cell maturation including B lymphocyte-induced maturation protein 1 (Blimp-1) (229). However, the effects of IL-5 on B cells were only observed in mice whereas IL-5 exerted an effect on both mouse and human eosinophils. Human B cells also express IL-5Rα and respond to IL-5 for B cell maturation.

1.4.1 The role of Th2 cytokines: IL-9

The role of IL-9 in ocular allergy, however, is less clear although IL-4, IL-5 and IL-13 are all upregulated in tears and tissues of patients with chronic allergic conjunctivitis (214). This study will be investigating the contribution of IL-9 in ocular allergy in more detail.

Il9 has been shown as a candidate gene for asthma (230). In mice, the Il9 gene is located on chromosome 13, whereas in humans, the Il9 gene is located in a syntenic region on chromosome 5 (231). When comparing human and mouse gene which encodes for Il4, Il5 and Il13, the region for the interleukin gene cluster in human is on chromosome 5q31-q33, and this is syntenic to the mouse interleukin gene family, which is located on the long arm of chromosome 11, 13 and 18 (232). Since in humans, the Il9 gene is located within close proximity of the other Th2 cytokine genes (Il4, Il5 and Il13) on human chromosome 5q31 (233), the role for IL-9 in AC responses will likely be influenced by these other Th2 cytokines. Most of the studies on IL-9 have looked at its contribution in
asthma but to date no one has studied the contribution of IL-9 in ocular allergy, which could also potentially have important roles in AC. Since IL-9 is the cytokine of interest in my thesis, more details on IL-9 will be provided below.

1.4.1.1 Sources of IL-9

IL-9, a 28-30 kDa monomeric glycosylated polypeptide, is a pleiotropic cytokine which was identified and described more than two decades ago (234). Historically, IL-9 was considered to be part of the Th2 cytokine family, but more recently our understanding of IL-9 has changed. IL-9 is expressed and secreted by many other cell types (235), including natural killer cells (NK cells) (236,237), mast cells (238), Th17 (239,240) and Treg cells (241,242), as illustrated in Figure 2. Th9 cells predominantly secrete IL-9 and not other T helper cell lineage-specific cytokines (147,148). Recently, Wilhem et al. showed that ILC can also produce IL-9 in the presence of IL-2 and IL-33 (243).

1.4.1.1.1 Th2 cells

A protein of 144 amino acids, IL-9 was initially described as a Th2 cytokine based on the production of IL-9 from T cells isolated from Leishmania major-infected, Th2-prone Balb/c mice (244). They have also shown that the expansion of Th2 cell populations was correlated with IL-9, hence supporting the idea of IL-9 as a Th2 cytokine. However, a new CD4+ T cell subset that secretes mainly IL-9 was subsequently described as Th9. It is unclear whether the correlation of IL-9 secretion with levels of Th2 cells is due to its direct production by Th2 cells or whether IL-9 secretion results indirectly from the IL-4 derived from Th2 cells that supports the development of Th9 cells. To date, Chang et al. managed to report low but detectable levels of IL-4 and IL-9 co-expression during in vitro differentiation of Th2 cells (245). Another study from Temann and colleagues reported that Th2 cytokine transcripts of IL-5, IL-13 and IL-9 could be detected in the lung tissues of IL-9 transgenic mice, suggesting that IL-9 is also a Th2 cytokine (246). However, the evidence linking IL-9 and Th2 cells is still not clear and it is known that IL-9 is not a Th2-specific cytokine.
As compared to other Th2 cytokines, IL-9 mRNA expression is delayed (246). This is true in CD4+ T cells from PBMC of healthy donors which showed delayed induction of IL-9 mRNA in which its expression only peaked at 24 hours post activation (247). IL-9 expression by T cells may depend on IL-4 and IL-10 which are secreted at earlier time points than IL-9 following cell activation (248). Since there is a possibility that IL-9 expression might be a consequence of other cytokines produced earlier, it is also worth investigating the effects of other Th2 cytokines on IL-9 expression.

Figure 2: Sources of IL-9. Different immune cells which produce IL-9
Th9 cells were initially characterised as the specific CD4\(^+\) T cell subset that secretes IL-9 (148). Th9 T cells are distinct from other T helper subsets because they secrete IL-9, but not signature cytokines of other Th1 (IFN-γ), Th2 (IL-4, IL-5 and IL-13) and Th17 (IL-17) subsets. Th9 cell differentiation is induced and regulated downstream of the T cells receptors (TCR) via different co-stimulatory and cytokine receptor signalling pathways.

The development of Th9 cells is regulated by the transcription factors PU.1, Interferon-Regulatory Factor 4 (IRF-4) and B cell, activating transcription factor-like, (BAT-F) (147,245,249). Besides Th9 cells, IRF4 also regulates Th2 and Th17 cytokine production (149,250). In Th9 cells, IRF4 directly binds to the \(il9\) promoter and increases \(il9\) transcription, suggesting that IRF4 has an important role in directly inducing the transcription of \(il9\) gene and blocking the expression of Th1 associated transcription factors (147,249). PU.1, which is induced by TGF-β together with antigen receptor stimulation, is crucial for Th9 differentiation and its capacity to produce IL-9 by binding to the \(il9\) promoter (245,251) even though a very recent study from Koh and colleagues have demonstrated that another ETS family transcription factor, Etv5, also shows similar function promoting the development of Th9 cells (252). BAT-F is required for the development of Th17, Th2 cells and more recently, Th9 cells (249,253). BAT-F deficiency resulted in the reduction of nearly all of the Th9 cell-associated genes suggesting that transcription factor BAT-F is required for the expression of IL-9 and other Th9 associated genes and it also cooperates with IRF4 in activating \(il9\) locus (249).

In separate \textit{in vitro} murine studies, both Veldhoen \textit{et al.} and Dardalhon \textit{et al.} showed that Th9 cell differentiation is dependent upon exposure to TGF-β and IL-4 (146,148). This is also true in human T cells where CD4\(^+\) memory T cells can become IL-9-producing cells in the presence of IL-4 and TGF-β (254). IL-4, which is the major signalling component of the IL-4 receptor (IL-4R), activates STAT6 (146,148) and IRF4, whereas TGF-β activates SMAD which activates
PU.1 and suppresses T-bet (T-box expressed in T cells) and GATA3 (GATA-binding protein 3) (255). A combination of these two events eventually drives IL-9 secretion from the Th9 cells. Low levels of GATA3 are expressed in Th9 cells, although GATA3 expression is higher in Th2 cells (251), and GATA3 is still required for IL-9 production (146).

The addition of IL-25 and IL-33 to CD4^+ T cells in the presence of IL-4 and TGF-β can enhance the production of IL-9 by Th9 cells by activating nuclear factor kappa B (NF-κB) which, in turn, induces STAT-1/IRF-1 expression (256). Interestingly, a study of murine CD4^+ T cells suggested that IL-9-producing T cells can also be generated in culture in the presence of IL-1β and TGF-β, replacing the need for IL-4 (257).

IL-2 is also needed for Th9 development since IL-2 activates STAT5 phosphorylation in T cells. Deletion of STAT5 in cultured Th9 cells resulted in a dramatic decrease in IL-9 production (258,259). Thymic stromal lymphopoietin (TSLP) may work together with IL-2 to promote Th9 cell differentiation (260). Recently, TL1A was shown to promote Th9 cell differentiation in combination with Th9-polarizing cytokines (IL-4 and TGF-β; (153). TL1A is a member of the TNF family of cytokines that binds to death receptor signal 3 (DR3); DR3 is a member of the TNF receptor family (TNFR), mostly expressed on lymphocytes. Binding of TL1A to DR3 enhances IL-2 production and STAT5 activation, which is required for IL-9 secretion. TL1A enhances IL-9 production independently of PU.1 and STAT6. TL1A co-stimulates T-cells to produce a wide variety of other cytokines (261) and can also affect T cell polarisation. In contrast, STAT3 functions as a negative regulator of IL-9 production. Activation of STAT5 is attenuated when STAT3 is activated, hence decreasing IL-9 production (262). Another promoter that was also found to be required for Th9 differentiation is Itk. Itk is a member of the Tec family of cystolic tyrosine kinases, which is also found to be an important component for TCR-mediated signalling and was demonstrated to be important in promoting STAT5/Irf4 binding for Th9 differentiation (263) (Figure 3).
Besides IL-9, activated Th9 cells also produce low levels of IFN-γ, IL-17 and IL-22 (264). However, Th9 cells also secrete high levels of IL-10, comparable to the levels of IL-9 (148,264). Although both IL-9 and IL-10 secretion increased markedly following stimulation, their kinetics of cytokine production is different. IL-9 secretion seems to peak on day 3 of stimulation and decline thereafter, but IL-10 secretion increases gradually until day 10 (264).

The pathogenicity of Th9 cells depends on the levels of IL-9 (264). Th9 cells that express highest levels of IL-9 induced inflammation whereas Th9 cells which express low levels of IL-9 did not induce inflammation. Depending on the type of inflammation, Th9 cells could be more or less pathogenic than Th1 or Th17 cells (264,265).

Unlike Th1 cells, Th17 and Treg lineage cells exhibit high levels of plasticity when exposed to polarising cytokines of specific lineage (266,267). Th9 cells on the other hands exhibit various degrees of plasticity, and are able to switch towards different phenotypes depending on the cytokine environments. Following Th9 cell exposure to various culture conditions used to polarise cells towards Th1, Th2 or Th17 lineages, Th9 cells showed moderate switching towards Th1 and Th17 cells but massive switching towards a Th2 population in which IL-4 intracellular expression levels increased significantly (264). TGF-β is responsible for ‘reprogramming’ a Th2 pathway towards a Th9 phenotype (148). Stimulation of the Th2 population in the presence of TGF-β causes a significant loss in the expression level of Th2 transcription factor (GATA3) and an increase in IL-9 levels in vitro. These studies show the close relationship between Th2 and Th9 cells although Th9 cells are distinct from Th2 cells. It has been proposed that there is plasticity between the two populations, depending on the environment (268).
Figure 3: Signal transduction pathways that promotes Th9 differentiation. The polarisation of IL-9 secreting cells requires IL-2/STAT5, IL-4/STAT6 and TGF-β derived signalling. A number of other signalling pathways including Activin A, TSLP, IL-1β, IL-33 and IL-25 derived signalling have also been identified that promote il9 transcription. Adapted from (269).
1.4.1.1.3 Th17 cells

Besides Th2 and Th9 cells, there have been reports that Th17 cells, which are identified either by their production of IL-17 or expression of the Th17-associated transcription factor retinoic acid receptor related orphan receptor γt (RORγt), can also produce IL-9. Based on an in vivo study using experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis, Nowak et al. reported that IL-9 blockade in this model downregulated Th17 responses (240). They also found that cells cultured either with TGF-β and IL-6 (to differentiate into Th17) or with TGF-β and IL-4 (to differentiate into Th9 cells) secreted similar amounts of IL-9. Besides being produced by Th17 cells, IL-9 also synergizes with TGF-β, replacing IL-6 to differentiate naïve CD4 cells into Th17 cells (239). Co-production of IL-17A and IL-9 by CD4+ T cells can be further enhanced by IL-1β or IL-21 (270). Although all of the studies mentioned above were performed in mice, this has also been demonstrated in human cells where human Th9 cells are a distinct T cell subset which lacked coproduction of Th1, Th2 and Th17 signature cytokines (271,272).

1.4.1.1.4 T regulatory cells

IL-9 is important in the differentiation and survival of Treg cells (239) but these cells themselves can also produce IL-9. FoxP3-expressing populations of mouse nTreg cells and iTreg cells in vivo secrete high amounts of IL-9 (242). Co-expression of FoxP3 and IL-9 was also found in isolated Treg cells in vitro (240,273). The level of Treg cells in both wild type and IL-9−/− mice were identical but Treg cells from IL-9−/− mice were less suppressive as compared to wild type mice (239).

1.4.1.1.5 Eosinophils

Besides mediating differentiation, maturation and the survival of eosinophils, IL-9 was also shown to be synthesized and secreted by eosinophils (274,275). Immunostaining of the nasal mucosal tissues from patients with allergic rhinitis showed that eosinophils contributed as the second main source of IL-9 after
mast cells in these tissues although T cells were found to be another source of IL-9 in the allergic nasal mucosa (276). Louahed et al. studied the role of IL-9 on eosinophils using IL-9 transgenic mice (277), highlighting that IL-5 and IL-9 had synergistic effects in sustaining eosinophilia.

1.4.1.1.6. Mast cells

Mast cells are mainly studied as the target of IL-9 due to their expression of IL-9 receptors. However, mast cells have also been reported to produce IL-9. Following IgE crosslinking of the FcεR1 on mast cells, degranulation leads to an immediate release of preformed mediators. During this degranulation process, mast cells also secrete IL-9. However it was also found that the secreted products histamine and IL-1β can further upregulate IL-9 production by mast cells (278,279). IL-9 secretion by mast cells can promote the survival and expansion of mast cells during immune responses.

1.4.1.1.7. Innate lymphoid cells (ILCs)

Lineage negative cells (Lin-) that display lymphoid tissue inducer (LTi) cell-like properties such as IL-7 receptor expression, but lack of CD4 and RORγt expression and have different cytokine expression profiles are called the innate lymphoid cells (ILCs). ILC2 cells are an ILC subset which is characterised by the secretion of high levels of IL-5, IL-6 and IL-13 (203,280). By using IL-9 fate reporter mice with a papain-induced lung inflammation model, Wilhelm et al. have shown that these ILC2 cells also secrete high levels of IL-9, more than from IL-9-producing T cells (281). The IRF4-driven IL-9 production by activated ILC2, induced by IL-33 and TSLP at the lung epithelium, is responsible for autocrine IL-9 secretion (282).

1.4.1.1.8. NK T cells

Natural killer T (NKT) cells, a distinct type of T cells, are characterised by the expression of CD-1b, a major histocompatibility complex (MHC) class 1 like
protein expressed by antigen presenting cells as the surface marker (283). NK T cells express both T cell receptors (TCR) and NK receptors. Once activated, invariant NKT (iNKT) cells rapidly secrete large amounts of both Th1 and Th2 cytokines including IL-4, IFN-γ, GMCSF, and IL-10 (284). NKT cells are found to be important in secreting IL-13 which regulates immune responses in asthma and oxazolone-induced colitis (285,286). NKT cells also secrete IL-9 (236,287). In the presence of IL-4 and TGF-β, iNKT (type 1 NKT cells) cells demonstrated enhanced IL-9 production upon TCR stimulation (288). Thus IL-9-producing iNKT cells were found to be protective against DSS-induced colitis by suppressing IFN-γ and IL-17A production, whilst this secreted IL-9 exacerbated allergic inflammation in the airways upon intranasal immunisation with house dust mite (289).

1.4.1.2. Functions of IL-9

1.4.1.2.1. IL-9R

The functions of IL-9 are mediated by the specific IL-9 receptor (IL-9R), which belongs to the hematopoietin superfamily (290). The IL-9R comprises a ligand-specific alpha chain (IL-9Rα) and a common gamma chain that is shared with IL-2, IL-4, IL-7 and IL-15 receptors (291). IL-9Rα can be produced in two forms: soluble and membrane-bound forms. IL-9R activation results in signal transduction via STAT1, STAT3 and STAT5 activation (292). Besides STAT activation, it was found that IL-9-mediated responses are also dependent on the Janus kinase-transducer pathway (293). Activation of STAT is required for IL-9-induced proliferation and inhibition of T cell apoptosis (292,294). IL-9R signalling also activates MAP kinase and insulin receptor substrate-P13 kinase pathway (293,295). Expression of IL-9R in distinct membrane areas could potentially have a crucial role in the regulation of its function. According to Nizsalóczki et al. (296), the IL-9Rα chain is co-localised within the molecular proximity of IL-2Rα (common gamma c chain), MHC I and MHC II in human T lymphoma cell lines.
The IL-9R is found on the surface of many different cell types, both in hematopoietic and non-hematopoietic cells. Briefly, IL-9R activation on T cells and mast cells promoted their survival and proliferation (297,298) and enhanced the mRNA expression of several other cytokines in activated mouse mast cells: IL-1β, IL-5, IL-6, IL-13 and TGF-β (299). Of these, IL-5 and IL-13 had direct effects on promoting eosinophil and mucus production in the lung. IL-9 also upregulated IgE production by B cells (300), inducing chemokine expression in epithelial cells (301) and exerting anti-apoptotic effects on neurons (302). Since IL-9 induced chemokine expression in epithelial cells, it is also possible that it can exert a similar effect on conjunctival epithelial cells.
Figure 4: The IL-9 receptor. IL-9 receptor subunits and its activation pathways. Adapted from (303)
1.4.1.2.2. IL-9 specific functions on cells and tissues.

As suggested by the distribution of IL-9R expression, IL-9 is a pleiotropic cytokine that exerts diverse biological effects on many different cell types. Besides being described as a mast cell and T cell growth factor, IL-9 may affect different immune cells and also tissue resident cells to promote the development of inflammation (243,279,303). IL-9 can function as both a positive and a negative regulator of immune responses. In response to helminth infections, IL-9 was demonstrated to function as an immune regulator, dampening the inflammation caused by helminth parasites whereas, during allergic inflammation, IL-9 was found to contribute to the development of allergic diseases including allergic asthma (304,305). During parasitic infection, IL-9 promoted pathogen clearance from the gastrointestinal tract (306).
Figure 5: IL-9 functions. Roles of IL-9 secreted by different cell types
1.4.1.2.2.1. CD4+ T cells

The function of IL-9 on T cells is to promote T cell growth but this is limited only to CD4+ T cells, not CD8+ T cells, and IL-9 is unable to support long term T cell growth (234,307). The ability to support the growth of cultured CD4+ T cells was reported to be independent of IL-2 and IL-4 (234). In an in vivo study, one group has shown that IL-9 promoted Th2 cytokine production but the direct or indirect effects of IL-9 on Th2 cells are still unclear (246,308). IL-9 also acts in an autocrine manner to further activate Th9 cells. The expression of IL-9Ra is important for the anti-apoptotic signals (309).

IL-9 has also been shown to influence Th17 and Treg cell development and function. IL-9 suppressed the immunomodulatory function of Treg cells but facilitated the expansion of Th17 cells in vitro and in vivo, highlighting its role in immunopathogenic and protective immune responses (239). IL-9 is a cytokine that affects the differentiation of Th17 and Treg cells. It is known that TGF-β alone induced Foxp3+ Treg cells but, in the presence of IL-6 or IL-21, induced Th17 cells. IL-9, in the presence of TGF-β, differentiated CD4+ T cells into Th17 cells. Besides being known to produce IL-17, Th17 cells also secrete IL-9. IL-9Ra deficiency was shown to attenuate disease severity of the mouse model (experimental autoimmune encephalomyelitis) which correlated with the downregulation of Th17 cells in the central nervous system (240). IL-9, secreted from Treg, can also promote the maintenance of a tolerant environment during skin transplantation (310). IL-9 also modulated virus-initiated inflammation in respiratory syncytial virus infection (311).

1.4.1.2.2.2. Mast cells

Cross-linking of allergen-specific IgE bound to FcεRI on the surface of mast cells leads to immediate activation, degranulation and release of proinflammatory mediators including IL-9. Besides being known to secrete IL-9, mast cells also express IL-9R on their surface and IL-9 was found to affect mast cell functions by supporting the growth of BMMC in vitro (234), inducing IL-6
secretion by BMMC, hence, increasing the survival and proliferation of these cells (312). Moreover, IL-9 in the presence of IL-1β and SCF, enhanced BMMC secretion of other Th2 cytokines and their long term survival, respectively (238,279,298).

Based on these studies on IL-9 functions on mast cells, in allergy IL-9 was found to promote mast cell expansion and IL-13 production which, in turn, promoted mucus secretion in the lung (313). Increased numbers of mast cells were observed in the airway epithelium of IL-9 transgenic mice in which the expression of murine IL-9 cDNA was under the control of the rat Clara protein CC10 promoter, which was selective for lung tissue, suggesting that accumulation, recruitment, enhanced survival and proliferation, differentiation and the pathogenicity of mast cells were due to IL-9 (314).

1.4.1.2.2.3. B cells

IL-9 promotes the development and function of B cells but did not affect the survival of these cells (315). A study reported an increase in peritoneal CD11b+ B cells in in vivo IL-9 transgenic mice (303). Increased accumulation of B cells has been detected in these IL-9 transgenic mice (314). IL-4-mediated IgE and IgG production by human B cells was enhanced by B cells (300). Increased IgE levels produced by B cells were observed in human tonsillar B cells as a synergistic effect of IL-9 in IL-4-induced IgE production (316). IL-9Rα and CD27 expression were also found to be greater on germinal centre B cells than on other B cells.

1.4.1.2.2.4. Eosinophils

Increased airway eosinophilia is the predominant feature of allergic asthma. An increased level of eosinophilia was observed in IL-9 transgenic mice as well as wild type mice which had been administered with recombinant IL-9 intratracheally (314,317). High levels of IL-9Rα mRNA and protein were observed in
peripheral blood eosinophils and in eosinophils from broncho-alveolar lavage of asthmatic patients (274,275). They have also found that IL-9 may potentiate eosinophil function by increasing their survival, differentiation and maturation via upregulation of IL-5Rα cell surface expression.

IL-9 is also thought to potentiate eosinophilia through inhibition of apoptosis and to promote eosinophil differentiation and maturation via IL-5-driven eosinophil maturation (277). Together with IL-5, IL-9 appears to have regulatory a role in the development of eosinophilia. IL-9 demonstrated a synergistic effect in combination with IL-5, resulting in a significant increase in eosinophil number and maturation both in vitro and in vivo in peritoneal lavage cells in mice.

1.4.1.2.2.5. Neutrophils

A study using PMNs from asthmatic patients showed high levels of both IL-9Rα mRNA and protein as compared with normal subjects (318). Based on immunohistochemistry on purified PMNs, they also found that IL-9Rα was expressed in the cytoplasm of PMNs from asthmatic donors whilst this was not detected in PMNs from normal controls. A functional study of PMNs from the same asthmatic donors suggested that IL-9 had a direct biological effect on IL-9R expression by upregulating IL-8 release from human PMNs, demonstrated by adding exogenous IL-9 or a neutralising anti-IL-9 antibody to human peripheral blood PMNs.

1.4.1.2.2.6. Antigen Presenting cells

There were also indications that antigen-presenting cells (APC) were one of the targets for IL-9. IL-9 promoted TGF-β production in lipopolysaccharide-induced activation of mixed monocytes and macrophages (319,320) and limited IL-12 production by APC, suggesting that IL-9 can suppress the capability of APC to promote a Th1 type immune responses which may contribute to the development of tuberculosis (321).
1.4.1.2.2.7. Innate lymphoid cell 2 (ILC2)

Recently, the ILC2 was found to secrete significant amounts of Th2 cytokines including IL-5, IL-13 and also IL-9. Wilhelm and colleague have reported that IL-9 secretion by ILC2 cells is transient and IL-9 has a positive feedback effect on upregulating IL-5 and IL-13 secretion from ILC2 (281). Furthermore, IL-9 supported the production of IL-5 and IL-13 from ILC2 cells through IL-2 signalling, which is required for IL-9 expression in ILC2 (281). IL-9 secreted by ILC2 cells could potentially be important in the development of allergic lung disease via the production of IL-5 and IL-13.

A few years after this finding, using the same reporter mice and IL-9R knockouts (i9r−/−), the production of IL-5 and IL-13 from ILC2 was found to be dependent on IL-9R signalling, which was crucial for the survival of ILC2s, but not Th2 cells (322). It was also found that besides being the main source of IL-9, in contrast to Th2 cells, ILC2 also expressed high levels of IL-9R (322). The survival of activated ILC2 was dependent on IL-9R signalling. IL-9R signalling upregulated the anti-apoptotic protein BCL-3. IL-9 functions in an autocrine manner to amplify ILC2 function by promoting tissue repair after heminth-induced lung inflammation. IL-9-induced ILC2 activation also promoted rapid IL-5 and IL-13 production by ILC2, which is required for optimal epithelial responses in the airways (282).

1.4.1.2.2.8. Airway epithelial cells

The airway epithelium produces a variety of cytokines and chemokines to promote activation and proliferation of inflammatory cells during immune responses. The IL-9Rα component of the IL-9R was found to be expressed within human airway epithelium of the bronchus (323,324). The effects of IL-9 on the airway epithelial cell functions have been investigated by studies in vitro and in vivo. Dong and colleagues found that IL-9 increased the level of expression of eotaxin (CCL11), macrophage inflammatory protein 1α (MIP-1 α; CCL3) and monocyte chemotactic proteins 1, 3, and 5 (MCP-1, -3, -5; CCL2, CCL7, CCL12, respectively) (301). Even in the absence of IL-4 (an inducer of
chemokine expression on epithelial cells), IL-9 upregulated eotaxin expression on cultured primary epithelial cells.

Increased mucin secretion is one of the characteristic features of allergic asthma. In a transgenic mouse model, expression of IL-9 in the lung resulted in changes in the gene expression of airway epithelial cells, including goblet cell metaplasia (314). Many effects of IL-9 on lung epithelial cells have been found to be due to the indirect effects of IL-13 on the epithelium. Steenwinckel et al. managed to show that mucus production in the lung was only upregulated when both il9r and il13 genes were expressed simultaneously (313). This suggests that IL-9 promotes mucin secretion through the induction of IL-13 production. After stimulation with IL-9, the expression level of mucin genes muc2 and muc5ac were increased (324).

### 1.5. IL-9 and allergic diseases

IL-9 has been associated with allergic disease. However, to date, there are no studies investigating the role of IL-9 in ocular allergy. Most studies have investigated the role of IL-9 in the development of allergic airway disease (314,325). Initially, IL-9 protein expression was shown to be strongly associated with asthma-like phenotypes. In murine studies, it was found that IL-9 contributed to the disease by promoting mast cell expansion and mucus production, leading to airway hyperresponsiveness (313,326). In mice, Th9 cells also independently induced bronchial hyperresponsiveness (BHR) mediated by eosinophils (327). IL-9 was also reported to promote mast cell expansion, growth and activation in chronic asthma (328). Kearley et al. also found that reduction in mast cell number upon IL-9 inhibition correlated with the reduction in Th2 cytokines (IL-5 and IL-13) and pro-fibrotic mediators associated with airway remodelling in the lung. On the other hand, McMillan et al. showed that IL-9 is not crucial for the development of allergic eosinophilia and airway hyperreactivity in a murine model (329,330).
One genetic study concluded that the IL-9 receptor region that is specific to IL-9 is more often homozygous in asthma patients and may have a role in the pathogenesis of asthma (331). Due to differences in the outcome of studies which investigate associations of human IL-9 gene with high total IgE levels and asthma (332,333), Kauppi and colleagues investigated the association of IL-9R gene instead of IL-9 gene with regard to asthma and IgE level. IL-9R was identified to be at the pseudoautosomal region of the Xq and Yq chromosomes, which are expressed by both X and Y chromosomes, and are found to have positive linkage with asthma (331).

The functional role of Th9 cells in asthma pathogenesis was recently reported. Jones et al. showed that circulating Th9 cells are detectable in human atopic peripheral blood but their numbers were increased in allergic patients compared to non-allergic donors (334) and that Th9 cells play an important role in asthma development both in humans and in mice. They have demonstrated that by transferring Th9 cells to naïve mice before inducing allergic inflammation, heightened allergic inflammation in vivo was observed and this was thought to be due to infiltration and activation of mast cells into the airways and lung. Activin A, a member of the TGF-β superfamily, replicated the function of TGF-β in driving the in vitro generation of Th9 cells in the presence of IL-4 (334). Furthermore, a humanised anti-IL-9 mAb (MEDI-528), which may be of clinical benefit in asthmatic patients, has been developed and its safety profile and efficacy are currently under investigation (335). These investigations clearly suggest that the functional roles of IL-9 in asthma pathogenesis may be clinically relevant.

Besides asthma, IL-9 also plays a significant role in allergic rhinitis, food allergy and eczema. During the pollen season, IL-9 levels in the nasal mucosa of allergic rhinitis patients were upregulated and these levels correlated with the increased number of infiltrating eosinophils (276). Following grass pollen immunotherapy, both IL-9 and nasal mast cell expression were reduced, suggesting that the IL-9 pathway can represent a therapeutic target.
Studies in a murine model of experimental intestinal anaphylaxis demonstrated that systemic anaphylaxis was attenuated in IL-9 knockout mice (306,336). This is also supported by evidence from studies in patients with peanut allergy, where IL-9 responses can potentially be a promising biomarker to differentiate peanut-sensitive from peanut-tolerant individuals (337). Interestingly, another study also reported that, based on the IL9 gene profile in peanut allergic children, they were able to differentiate peanut allergic children from peanut sensitised children based on expression profiles of IL5 and IL13. This finding suggests that there might be a potential role in characterising children who react to peanut allergens with peanut-sensitised and non-atopic children (Brough et al., 2014). Another subset of CD4+ T cells, which are IL-9+ IL-10+, has been described (146), and provides the link between the immediate allergic response with the late phase reaction in the intestine (339).

A recent finding in atopic dermatitis patients revealed that IL-9 expression was significantly increased in lesional skin areas but not in serum (340). They also found that IL-9 interacted with mast cells to release VEGF and this contributed to the initiation of the pathogenesis of atopic dermatitis via angiogenesis.

Although IL-9 has been implicated in the pathogenesis of allergic diseases, the contribution of this cytokine to the inflammatory processes occurring during the various clinical subtypes of ocular allergy has not yet been explored. This study will examine the contribution of this cytokine in the development and pathogenesis of allergic conjunctivitis.
1.4. *In vivo* models of Allergic Conjunctivitis

Animal models are important for investigating the potential inflammatory mechanisms involved during AC, for identifying molecular targets and for testing novel compounds for therapeutic interventions. There are a few models available to study experimental ocular allergy. The guinea pig model of AC was the first to be established as an AC model to test the effect of drugs. Both rat and murine models were subsequently developed but mice are the preferred species to study AC due to the availability of gene-knockout models (341).

In order to elicit AC responses in mice, allergen is administered topically following systemic sensitisation (342). Common allergens such as pollens (birch, ragweed or house dust mite) and cat dander have been used. Sometimes, ovalbumin (OVA) can also elicit AC responses in mice. Following sensitisation, this normally resulted in the early phase response (EPR), which is thought to be mediated through an allergen-specific interaction with IgE receptors on mast cells which cause degranulation and the release of a variety of mediators including chemokines and platelet activating factor (PAF). These EPR-associated mediators can sustain the allergic reaction until the late phase response (LPR) develops. EPR responses are normally recorded within the first 30 minutes after conjunctival challenge, evaluating the scores of itch, chemosis, conjunctival redness, lid oedema and tearing.

To induce a more severe form of AC, mice were challenged with a multi-hit topical allergen challenge following systemic sensitisation. Repeated challenge with allergen mimics the physiology of more severe forms of AC. Persistent exposure towards allergen causes a more severe reaction in late phase response (LPR) after 6-12 hours of allergen exposure. LPRs in this model are more pronounced with infiltrating eosinophils and increased expression of Th2 cytokines, namely IL-4, IL-5 and IL-13 as a result of the release of mediators by mast cells degranulating during the EPR. It has been found that IFN-γ also contributes to the development of murine AC during both EPR and LPR (343). Studies in a mouse model of AC suggested that allergen-specific IgE antibody is not required to develop LPR once AC is initiated and that LPR can be
induced by adoptively transferring T cells from allergen-sensitised donors to naïve mice prior to short ragweed pollen (SRW) topical challenge (344,345)

A study using daily allergen challenge mouse models have provided important evidence that IFN-γ is crucial for optimum induction of conjunctivitis (343). This corresponds well with the increased expression of IFN-γ in AKC and VKC patient conjunctival tissues (207) and significant levels of IFN-γ detected in tear specimens from SAC, VKC and AKC donors (83). Fukushima and co-authors highlighted the importance of co-stimulatory molecules which influence Th2 responses in LPR and also the presence of Th2 cytokines and IL-17 in EAC (346). All of these in vivo murine AC models have been used as important tools to further study and understand the mechanism of AC.

Although much effort has been made to understand the molecular mechanisms involved in AC, these models have only been useful to investigate the cellular involvement in the clinically milder forms of AC. There are very few T cells found in the conjunctival tissues in these models, making it impossible to study the contribution of T cell-derived cytokines in clinically more severe forms of chronic allergic eye disease. Therefore, the relevance of these models to clinically more severe forms of AC in man is less obvious.

Schlereth et al. developed a clinically more severe model of murine AC by injecting OVA-loaded dendritic cells into the subconjunctival (SCJ) space and then immediately challenging the mice topically with OVA via eyedrops (347). They repeatedly challenged mice for up to 10 days following SCJ injection, which resulted in successful induction of severe AC model with the presence of T cells and dendritic cells.

Using a clinically severe mouse model, this thesis will be investigating the contribution of Th2 cytokines, in particular IL-9, towards the immunopathogenesis of severe AC.
1.6. Conjunctival epithelial cells

Mucosal surfaces are the largest surface area of contact with the external environment and provide the main gateway for both allergens and pathogens to access the body. A great amount of research has been invested to understand the molecular components which are the first line of defence at the mucosal surfaces and how the components are altered during disease. The ocular surface consists of the cornea and conjunctivae that are constantly being exposed to the environment. The components of the mucosal surface of cornea and conjunctival epithelium prevent damage to the ocular surface but can be disrupted under several pathological conditions. The basis of the ocular surface epithelial barrier will be discussed in more detail below.

1.6.1. Structure

The conjunctiva consists of two layers: the epithelium and substantia propria. The epithelium is continuous with the skin junction on the lid and the corneal epithelium at the limbus. The stratified epithelium normally consists of a few layers of epithelial cells, goblet cells, melanocytes, Langerhans cells and inflammatory cells intercalated within them. Desmosomes and tight junctions connect these epithelial cells and provide a seal to the barrier. The substantia propria is located subjacent to the epithelium, providing space for immune cells including mast cells, lymphocytes, plasma cells and neutrophils of which will increase in number during allergic inflammation.

The ocular surface epithelium consists of different layers, which are important in providing a barrier against the external environment (Figure 6). The tear film is important in lubricating the eye as well as continuously flushing out noxious agents from of the ocular surface. The tear film consists of 2 layers: the outer layer is a lipid-based layer; the middle layer is an aqueous layer. The lipid layer is mainly present to protect the tear film from evaporation of the natural tears whereas the aqueous layer is mainly important for lubricating the eye and washing away particles. It contains antimicrobial peptides such as lysozymes,
lipocalin and lactoferrin, and, together with immune cells within the conjunctival tissues, protects the epithelial barrier.

The conjunctival epithelium consists of two major cell types, stratified squamous cells and goblet cells. Stratified squamous cells form another layer that also provides an additional layer of protection at the ocular surface epithelium. This layer forms the heavily O-glycosylated transmembrane mucins MUC1, MUC4 and MUC16. Suppression of MUC16 on the corneal epithelial cells leads to loss of surface protection (348).
Figure 6: The structure of the normal conjunctival epithelium. The normal conjunctival epithelium consists of tight junctions that seal the barrier from external pollutants and allergens. Adapted from (120).
1.6.1.1. Goblet cells

Goblet cells are another major cell type found in the conjunctivae. These cells occur singly or in clusters and are surrounded by stratified squamous cells. In comparison to stratified squamous epithelial cells, goblet cells secrete different types of mucins, including MUC5ac, which is a large, high molecular weight molecule, and a gel-forming mucin. MUC5ac is secreted into the tear film, forming a mucous layer that covers the glycocalyx and the epithelium underneath. It functions by trapping ocular allergens and removing them from the surface of the eye, which is important in ocular surface disorders including allergy, dry eye and infection where goblet cells are severely depleted during disease (349).

MUC2 is another soluble gel-forming mucin produced by goblet cells. MUC2 transcripts have been detected by RT-PCR on the ocular surface of normal conjunctiva but their abundance is lower than that of MUC5ac (350). A few studies have described the changes in MUC2 expression in patients with ocular allergy and dry eye disease where the expression levels were significantly lower in dry eye disease compared to normal conjunctiva (351). The expression of MUC2 in the conjunctival epithelium was reported to be increased in AKC tissues (352). IL-13 levels were found to be elevated in the tears of AKC patients (214). MUC2 mRNA expression and protein expression were both found to be increased in the presence of IL-13 in primary conjunctival epithelial cell cultures (353).

1.6.1.2. Tight junctions

Intracellular junctional complexes exist at different depths on the stratified epithelium. There are four different types of junctions. These include tight junctions (zonula occludens), desmosomes (macula adherens), adherens junctions (zonula adherens), and gap junctions. Besides cellular components, conjunctival barrier functions and permeability are also determined by tight junctional complexes, which consist of predominantly proteins - occludins, zonula occludins and claudins. These tight junctions appear as a series of
connections between adjacent cells, serving as a barrier to the diffusion of molecules by sealing the intercellular space, thereby preventing the free passage of molecules between adjacent cells (paracellular pathway).

Occludin is a 65-kDa tetraspan intergral transmembrane protein associated with the cytoplasmic face of the junction (354). Occludin contributes to the stability of tight junctions and maintains optimal barrier functions. This protein can be regulated by phosphorylation at multiple sites. For example, phosphorylation of occludin at a highly conserved motif containing tyrosine may disrupt its binding to ZO-1 and ZO-2 which then leads to separation of occludin from the membrane tight junctions, causing barrier dysfunction (355). It has been reported that in low humidity conditions, the c-jun N-terminal kinase 2 (JNK2) can trigger occludin dysfunction together with the involvement of other proinflammatory cytokines including IL-1α and IL-1β (356). Vitamin D, on the other hand, has been shown to be involved in the remodelling of the cornea after injury via upregulation of occludin expression after stimulation with vitamin D metabolites 25(OH)D3 and its active metabolite 1,25(OH)2D3 (357).

Another component of the tight junction (TJ), the Zonula Occluden (ZO) proteins (ZO-1, ZO-2 and ZO-3) are cystolic scaffolding proteins that regulate the assembly of multiple proteins including occludin at the intercellular TJ. These proteins also provide a link between integral membrane proteins on the plasma membrane (like occludins) and the cell nucleus through a filamentous cytoskeleton of the cytoplasm (358). ZO proteins can also be a component of adherens junctions (359). Lipopolysaccharides (LPS), normally associated with gram negative bacteria, were found to be detrimental to epithelial tight junctions by disrupting ZO-1 and ZO-2 proteins (360).

Proinflammatory cytokines, such as TNF-α, were found to be able to activate the NFκB pathway and disrupt both tight junctional proteins, occludin and ZO-1 (361). As shown in cultured human corneal epithelial cells, another proinflammatory cytokine IL-1β can also disrupt the subcellular localisation of these TJ proteins (362).
1.7. Hypothesis and aims of this thesis.

Previous studies with lung epithelium specimens and bronchoalveolar lavage fluids from individuals with asthma revealed that a high level of IL-9 correlates with disease severity (325,363). Our group has been studying the contribution of cytokines in allergic conjunctivitis. To date, Th2 cytokines including IL-4, IL-5 and IL-13 were found to be upregulated in the conjunctival tissue sections of VKC & AKC patients (207,364,365). In addition, we observed an upregulation of MUC2 mRNA after the exposure of a human conjunctival epithelial cell line (JOBA-NHC) to rhIL-9 (Porter et al, unpublished data). It has therefore been proposed that IL-9 might play a proinflammatory role in allergic conjunctivitis.

Based on these data, the hypothesis of this study is that conjunctival IL-9 is upregulated during allergic conjunctivitis and contributes to the immunopathogenesis of the disease.

To address this hypothesis, the specific aims of this thesis were as follows:

1. To investigate the presence of IL-9 during allergic conjunctivitis in both human and mouse conjunctival tissues.

2. To investigate the role of IL-9 secreted by mast cells and its effect(s) on mast cell responses.

3. To investigate the impact of IL-9 on conjunctival epithelial cells.

The novel findings from this thesis contribute to our understanding of the role of IL-9 during allergic conjunctivitis, especially the role of IL-9 secreted by mast cells. Understanding the IL-9 mechanisms in AC could potentially lead to IL-9 being used as a biomarker for the disease and to the discovery of a potential therapeutic target for the treatment of allergic conjunctivitis.
Chapter 2

Materials and Methods
2.1. Mouse models of experimental allergic conjunctivitis

2.1.1. Mice

For the purpose of this study, eight to twelve-week-old female C57BL/6 mice (Charles River, UK) were used for the severe model of allergic conjunctivitis. Mice were housed in the UCL Institute of Ophthalmology animal facility.

2.1.2. Induction of murine model of allergic conjunctivitis

Mouse studies were carried out in accordance with the Animals (Scientific Procedures) Act 1986 and housed in accordance with Home Office Regulations. Treatment of animals conformed to the ARVO statement for the use of animals in ophthalmic and vision research.

2.1.2.1. C57BL/6 mice induced by ovalbumin (OVA).

The severe allergic conjunctivitis model was induced using the protocol described (347). Mice were immunized once by intraperitoneal injection with a 100µl of suspension containing 1mg of aluminium hydroxide (Alum Imject, Sigma-Aldrich) diluted in phosphate buffer saline (PBS), 300ng of pertussis toxin (Sigma-Aldrich), and 100µg of ovalbumin (Sigma-Aldrich). Mice were left for 21 days before the first OVA topical challenge. This time is required for the generation of systemic T cell responses. On day 22, mice were challenged topically once daily for 7 days with 5µl of OVA eye drops per eye, containing 50mg/ml of OVA diluted in sterile PBS. Mice were harvested for whole eye, conjunctiva and draining lymph nodes on day 28, after 7 days of topical challenge. The mouse model of EAC that was used in this thesis is illustrated in Figure 7 below:
OVA sensitised and challenged C57BL/6 mice (347):

Immunize i.p. with OVA+ Alum + Pertussis

Daily OVA topical challenge

Day 0

Day 22 23 24 25 26 27 28

harvest

Samples collected:
1. Whole eye: H&E, Toluidine blue and immunofluorescence staining.
2. Conjunctiva: Cells explanted from conjunctiva, re-stimulated with PMA/ionomycin and analysed by FACS.
3. Draining lymph nodes: Preparation of single cell suspension, re-stimulated with PMA/ionomycin and analysed by FACS.

Figure 7: Diagram of allergic conjunctivitis (AC) mouse model with OVA sensitised and challenged in C57BL/6 mice.
In each experiment, there were 5 mice in PBS-challenged groups (control) and 10 mice in the OVA challenged groups. A clinical scoring scheme previously described (366) was used to evaluate chemosis, lid edema, tearing and conjunctival vasodilation (redness). Each parameter was graded on a scale ranging from 0 (absent) to 3+ (maximal) by two independent masked observers. Mice were sacrificed one-hour post OVA challenge and whole eyes were enucleated from each mouse. Each eye was kept in 4% glutaraldehyde for 2 hours then fixed in formalin before being embedded in paraffin wax. Eyes were processed and prepared for staining.

2.1.2.2. Mouse conjunctival mast cell isolation

After 20 minutes following the 7th topical challenge, mice were euthanized, and conjunctivae were surgically removed and placed on ice cold PBS. Single cell suspensions were prepared for conjunctival explants. Conjunctivae were minced into small fragments and left in 24-well culture plates containing just enough supplemented RPMI-1640 (Sigma-Aldrich) medium to cover the bottom of the well. The culture medium was supplemented with 10% heat inactivated fetal calf serum (Sigma-Aldrich), 1mM sodium pyruvate, 100 µg/ml penicillin streptomycin, 1x non-essential amino acid (Thermo Fischer), and 10 ng/ml SCF (PeproTech, London, UK). Cultures were left in a 5% CO\textsubscript{2} humidified incubator at 37°C for 3 days to allow mast cells to migrate out from the tissues.

2.1.2.3. Mouse draining lymph nodes isolation

After mice were euthanized, draining lymph nodes were placed on ice cold PBS. Single cell suspensions were prepared for intracellular cytokine staining where cells were stimulated with 500ng/ml of ionomycin and 50ng/ml of phorbol 2-myristate 13-acetate (PMA) in the presence of brefeldin A (BFA) for 3 hours prior to staining with fluorochrome conjugated antibodies.
2.2. Intracellular cytokine staining

Cell culture plates containing the cells were spun at 200g for 5 minutes, the medium aspirated and the cells resuspended by vortexing. For detection of cell surface CD4 expression, cells were incubated for 30 minutes at 4°C with anti-CD4 mAb (BD Biosciences). To wash, plates with cells were spun at 200xg for 5 minutes and vortexed. 100µl/well of Cytoperm (BD Biosciences) was added and incubated at 4°C for 20 minutes. 100µl/well diluted Perm/Wash (BD Biosciences) was then added and plates were spun and vortexed again to stop permeabilisation. Directly conjugated antibodies were added to the pellet. After 30 minutes, 100µl/well of Perm/Wash was added and plates were spun and vortexed as above. 200µl per well PBS was added and cells were run on a LSRFortessa-x20 using FACSDiva, with compensation applied where necessary (BD Biosciences). This data was analysed using FlowJo with 30,000 events in a live gate acquired, as determined by forward and side scatter plots (FSC v SSC).

Similar staining techniques were also used in in vitro stimulated single cell suspensions from mouse draining lymph nodes (dLNs), and conjunctival explants were fixed in 1x Cell Fix (BD Bioscience, UK), as described above (see 2.11). All antibodies used for staining single cell suspensions from mouse dLNs and conjunctival explants are listed in Table 1. Staining was detected on a flow cytometer LSR Fortessa-x20 and analysed by FlowJo software (FlowJo LLC, Ca, USA).

For conjunctiva and lymph node gating, a gate was drawn around the cells/lymphocyte region respectively. Within this gate, another region was drawn around single cells to remove doublets, followed by a live cell gate. Gating strategy will be explained further in chapter 3. Results are shown in percentages or in some samples, the number of cells were calculated using FACS counting beads which was added to each sample of 200µl. Compensation was set using BD Multicolor CompBeads (BD Biosciences) during acquisition.
Table 1: List of anti-mouse antibodies used for intracellular cytokine staining

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Conjugates</th>
<th>Volume/concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-kit,</td>
<td>eBioscience</td>
<td>PE</td>
<td>1µl/test</td>
</tr>
<tr>
<td>FCεR1</td>
<td>eBioscience</td>
<td>PE</td>
<td>1µl/test</td>
</tr>
<tr>
<td>IL-9</td>
<td>BioLegend</td>
<td>BV 421</td>
<td>1µl/test</td>
</tr>
<tr>
<td>IL-4</td>
<td>BD Bioscience</td>
<td>AF 488</td>
<td>1µl/test</td>
</tr>
<tr>
<td>CD4⁺</td>
<td>BioLegend</td>
<td>BV 395</td>
<td>1µl/test</td>
</tr>
<tr>
<td>PU.1</td>
<td>BioLegend</td>
<td>APC</td>
<td>1µl/test</td>
</tr>
<tr>
<td>GATA-3</td>
<td>BD Bioscience</td>
<td>AF 700</td>
<td>1µl/test</td>
</tr>
</tbody>
</table>

2.3. Immunostaining

2.3.1. Immunohistochemistry on human tissue sections with DAB staining.

Human conjunctival tissue sections were obtained from the archived UCL Institute of Ophthalmology Tissue Bank (Calder Lab). These were conjunctival punch biopsies, which had been obtained from out-of-season challenged allergic conjunctivitis (AC) donors at 8 hours post allergen challenge (n=8 from 3 males; age 31-57 years). Normal, non-inflamed conjunctival tissues were previously collected from anonymised donors (n=8, from all males; age: 16-65 years). Prior ethical approval had been obtained to use these tissues for ongoing immune studies. Tissues were embedded in glycol methacrylate (GMA) using a standard protocol, prior to cutting 3µm thick sections for immunostaining.

Sequential tissue sections were ringed with a wax pen (DAKO, UK) and fixed in acetone for 10 minutes at 4°C. Endogenous peroxidase was blocked using peroxidase blocking solution (Dako UK) for 15 minutes and then washed twice with PBS (5 minutes each). To prevent non-specific binding to Fc, a blocking buffer (1.5% goat serum in 10% bovine serum albumin BSA) was added for 30 minutes at room temperature. Sections were incubated with purified rabbit polyclonal anti-human IL-9 antibody and IL-9R mAb (Abcam, Cambridge, UK)
diluted 1:300 and anti-human tryptase (DAKO; clone AA1) diluted 1:100 overnight in a humidified chamber at room temperature. For negative controls, primary antibody was replaced with PBS.

After washing twice with PBS, biotinylated rabbit and mouse anti-rat Fab fragment IgG (DAKO UK Ltd.; 1:200 dilution) was added for 2 hours at room temperature. After washing, avidin-biotin complexes (ABC)-alkaline phosphatase (V) was added for 2 hours at room temperature. Unbound ABC was removed by washing twice with PBS and Ab binding was visualized using substrate 3', 3'-diaminobenzidine tetrahydrochloride (DAB; Vector Labs), incubating for 3 to 4 minutes depending on the intensity of staining. After rinsing with PBS and running tap water for 3 minutes, slides were counterstained with haematoxylin (DAKO UK Ltd) for 1 minute and mounted with fluorescence mounting medium (Vector Labs). Positively stained cells were visualized using light microscopy at x200 and x400 magnification.

2.3.2. Double immunofluorescence staining of human sections on GMA embedded sections.

In order to investigate the colocalisation of IL-9 or IL9R on mast cells, tissue sections from human biopsies were double stained for both IL-9 (or IL-9R) and tryptase. The tissue area was ringed with a wax pen (Dako UK Ltd) and fixed in acetone for 10 minutes at 4˚C. The slides were then washed twice in PBS and non-specific binding sites were blocked by adding 5% goat serum (in 1% PBSA, 0.1% triton). Excess serum was tipped off and slides were incubated with purified rabbit anti-human IL-9 or IL-9R mAb (Abcam; diluted 1:100) and anti-human tryptase (Dako UK Ltd; clone AA1; diluted 1:100) in 1% PBSA (BSA diluted in PBS) overnight in a humidified chamber at room temperature. After overnight incubation, slides were washed twice in PBS before incubation with Alexa Fluor® 488 Goat Anti-Rabbit IgG and Alexa Fluor® 546 Goat Anti-Mouse IgG (Life Technologies), diluted 1:250 for 2 hours at room temperature. Slides were then washed twice in PBS (5 minutes each wash) and stained with DAPI (Vector Labs) for 5 minutes. The slides were washed twice again with PBS and mounted in fluorescence mounting medium. Slides were stored in the dark at
4°C and visualized using a Zeiss 710 laser scanning confocal microscope with the 40x objective.

To quantify the staining of the tissue sections, positively stained individual cells were counted from a total of 3 fields (at x400 magnification) per section.

2.3.3. Double immunofluorescence (immunocytochemistry) staining of human epithelial cells.

In order to investigate the expression of IL-9 on human conjunctival epithelial cells, the IOBA-NHC cell line cells (Diebold et al., 2003; a kind gift from Prof. M. Calonge, University of Valladolid, Spain) were grown in Lab Tek® chamber slides (Thermo Scientific) until 80% confluent. Cells were fixed with 4% paraformaldehyde (PFA) in TBS for 10 to 15 minutes before being washed twice with TBS. Slides were incubated with 1% Triton X-100 in TBS for 3-5 minutes at room temperature and washed again twice with TBS before incubation with blocking reagent or 10% BSA for 30 minutes to block non-specific Fc binding. Excess blocking reagent was tipped off and diluted primary antibodies (Table 2) in 1% BSA were added to the slides and incubated for 1 hour at room temperature. Slides were washed 3 times for 2 minutes each with TBS-Tween 20 (0.1% v/v). Secondary antibodies (diluted 1:300 in 1% BSA) were added for 1 hour at room temperature and washed again 3 times for 2 minutes each with 0.1% TBS- Tween 20. Slides were mounted with Vectashield-DAPI and stored in the dark at 4°C. Cells and sections were viewed on a Zeiss 710 confocal microscope using 40x water immersion objective (Zeiss, UK).
Table 2: List of conjugated primary and secondary antibodies used for immunofluorescence staining on human conjunctival sections and IOBA epithelial cells.

<table>
<thead>
<tr>
<th>Anti-human Antibodies</th>
<th>Host Species</th>
<th>Conjugate</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-9</td>
<td>Rabbit</td>
<td>-</td>
<td>1 in 100</td>
<td>Abcam</td>
</tr>
<tr>
<td>IL-9R</td>
<td>Rabbit</td>
<td>-</td>
<td>1 in 300</td>
<td>Abcam</td>
</tr>
<tr>
<td>tryptase AA1</td>
<td>Mouse</td>
<td>-</td>
<td>1 in 100</td>
<td>DAKO</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Goat</td>
<td>-</td>
<td>1 in 300</td>
<td>Abcam</td>
</tr>
<tr>
<td>Occludin</td>
<td>Rabbit</td>
<td>-</td>
<td>1 in 100</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>Secondary</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit anti-goat</td>
<td></td>
<td>FITC 488</td>
<td>1 in 200</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>Donkey anti-rabbit</td>
<td></td>
<td>PE 555</td>
<td>1 in 200</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>Goat anti-rat</td>
<td></td>
<td>APC 633</td>
<td>1 in 200</td>
<td>Life Technologies</td>
</tr>
</tbody>
</table>

2.4. Generation of BMMC

BALB/c mice were chosen as they are a Th2-dominant strain and are commonly used in studies of allergic responses.

Naïve BALB/c mice (4-6 weeks old) were sacrificed by cervical dislocation. Intact tibias were dissected and stored in 70% ethanol on ice. Under sterile conditions, bone marrow cells were carefully harvested by repeated flushing with IMDM.

The cell culture was established at a density of $1 \times 10^6$ cells/ml, in Iscove’s Modified Dulbecco’s Medium (IMDM) (Sigma Aldrich, Dorset, UK), supplemented with 10% heat-inactivated foetal calf serum (FCS; Biosera, East Sussex, UK), 4mM L-glutamine, 1mM sodium pyruvate, 100 U/ml penicillin, 100μg/ml of streptomycin, 0.1mM non-essential amino acid (NEAA), and 50μM 2-mercaptoethanol (Life technologies, UK), 5ng/ml recombinant murine IL-3 and 10ng/ml stem cell factor (SCF; PeproTech, London, UK)(as shown in
Cells were incubated at 37°C in a 5% CO₂ humidified incubator.

Non-adherent cells were transferred to fresh 75mm² culture flasks every 2-3 days for a total of at least 3 weeks to remove any contaminating macrophages and fibroblasts. After 4-5 weeks of culture, cells were assessed for phenotypic expression of both FcεRI and c-Kit by flow cytometry. Cells were used for further assays when the population consisted of >95% FcεRI and c-Kit positive cells (BMMCs).

Figure 8: Bone marrow mouse mast cells extraction
2.5. In vitro BMMC cytokine assays

2.5.1. In vitro BMMC stimulation

BMMCs were counted and plated at 10^6/ml in culture medium without IL-3 and SCF. Prior to IgE stimulation, cells were sensitised overnight with monoclonal anti-DNP (2µg/ml; clone SPE-7; Sigma) and washed twice by spinning down the cells at 200g for 5 minutes, removing the supernatants and resuspending with fresh medium to eliminate the unbound IgE. Cells were stimulated with either phorbol 2-myristate 13-acetate (PMA) and ionomycin, 0.5µg/ml ionomycin (Sigma, Dorset, UK) alone or with 50µg/ml DNP-BSA (Biosearch Technologies). Supernatants were collected at different time points (24, 48, 72, 96 and 120 hours) and stored at -20°C for further analysis.

2.5.2. Pre-treatment of BMMCs for IL-9 and IL-9R neutralising experiments.

In some experiments, cells were treated with anti-mouse IL-9R mAb (R&D Systems) or isotype-matched antibodies (R&D Systems), 30 minutes prior to stimulation. Table 3 lists the concentrations of antibodies that were used for titration experiments.

Table 3: Antibody titration in BMMC stimulated cells

<table>
<thead>
<tr>
<th>Anti-Mouse Antibodies</th>
<th>Host Species</th>
<th>Ig Subclass</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-IL-9 (R&amp;D) clone ab203386</td>
<td>Goat</td>
<td>IgG</td>
<td>5µg/ml 9µg/ml 20µg/ml</td>
</tr>
<tr>
<td>anti-IL-9R (R&amp;D) clone ab61196</td>
<td>Rat</td>
<td>IgG2a</td>
<td>5µg/ml 10µg/ml 20µg/ml</td>
</tr>
<tr>
<td>Rat IgG2a (R&amp;D)</td>
<td>Rat</td>
<td></td>
<td>9µg/ml</td>
</tr>
<tr>
<td>Goat IgG (R&amp;D)</td>
<td>Goat</td>
<td></td>
<td>10µg/ml</td>
</tr>
</tbody>
</table>
2.5.3. IL-9 rescue experiment in BMMCs.

In order to confirm that the effects of blocking IL-9R with neutralizing IL-9R antibodies in BMMC were in fact due to an IL-9-specific inhibition, rescue experiments were done where anti-IL-9R mAb was added to BMMC culture 10 minutes prior to ionomycin stimulation and recombinant mouse IL-9 (mIL-9; R&D Systems) was added 30 minutes following the addition of IL-9 and IL-9R neutralizing antibodies and ionomycin stimulation (2.3.2. Pre-treatment of BMMCs for IL-9 and IL-9R neutralizing experiments). Different concentrations of mIL-9 were added to the culture (0.01, 0.05, 0.1 ng/ml) in order to titrate the optimal mIL9 concentration to reverse the effect of the neutralizing antibodies. Briefly, cells were plated at a concentration of 1x10^6/ml in 96 well plates. Neutralizing antibodies were added to appropriate wells after 30 minutes stimulating cells with ionomycin and anti-IgE. Plates were left at 37°C, 5% CO2 for 30 minutes before mIL9 was added at different concentration. Cells were incubated and supernatants were collected at 0, 24, 48 and 72 hours for cytokine analysis by multiplex bead arrays. Cell-free supernatants were stored in aliquots at -20°C.

2.5.4. IL-9 and IL-9R gene expression inhibition in BMMC with the addition of small interfering RNA (siRNA) IL-9 and IL-9R.

Besides blocking IL-9 and IL-9R functions with neutralizing antibodies, it is also possible to investigate the effect of IL-9 and IL-9R gene knock down. For this, pre-designed small interfering RNA (siRNA) of IL-9 and IL9R (Thermo Scientific) was used. These siRNA have specific sequences that are known to target the genes for IL-9 and IL-9R in mouse (Table 4). A scrambled gene that targets the non-targeting siRNA was used as the negative control since it has minimal targeting of known genes to human, mouse and rat cells. Ionomycin-stimulated cells and unstimulated cells were used as the positive and negative controls for the experiment.

Briefly, 3μl of IL-9 and IL-9R siRNA and scrambled control (final concentration of 1μg) was diluted in 50μl Optimem serum reduced medium (Life technologies
UK) for each reaction. Lipofectamine 2000 Reagent (Life Technologies) was used as the transfection reagent. 2 μl of Lipofectamine was diluted in 50 μl Optimem for each reaction/ well. The diluted siRNA and diluted Lipofectamine were mixed and left for 20 minutes at room temperature in order for the gene to bind to the transfection reagent. Cells were plated in 24 well plates at 1x10^6/ml and 500 μl per well in supplemented medium without Penicillin and Streptomycin as antibiotics could interfere with the transfection process. 100 μl of the mixed Lipofectamine and siRNA were then added to each well and incubated at 37˚C, 5% CO₂ for 24 hours for transfection to take place. IL-9 and IL-9 knockdown efficacy was tested by real time PCR.

Table 4: Small interfering RNA (siRNA) transfection.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Assay ID</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-9</td>
<td>62595</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>IL-9R</td>
<td>62596</td>
<td>Thermo Scientific</td>
</tr>
</tbody>
</table>

2.6. Measurement of histamine release (ELISA)

Histamine levels in the supernatants of BMMC cultures were measured by ELISA (IBL International, Hamburg). The method was carried out according to the manufacturers’ instructions. Briefly, cell culture standards, control and samples were acylated in a 96 well plate for 30 minutes. The reaction was stopped by diluting with assay buffer. A six points cell culture standard curve was provided with the highest standard of 219ng/ml and the minimum standard of 0.67ng/ml. Diluted acylated standard, control and samples were transferred to a 96-well microtiter plate pre-coated with anti-histamine antibody, and enzyme conjugate and histamine antiserum were added. Plates were incubated for 3 hours on an orbital shaker at room temperature. After washing, TMB (3,3',5,5'-tetramethylbenzidine) substrate solution was added and incubated for 20 minutes at room temperature on an orbital shaker. To stop the enzymatic reaction, TMB stop solution was added to each well. The optical density at 405 nm, with a reference wavelength of 600nm, was read with a microplate reader.
(Titertek™, Vienna, Austria). The concentrations of histamine in the samples were determined from a best-fitting standard curve (absorbance against known concentration) generated by the plate reader. All assays were conducted in triplicate.

2.7. Measurement of cytokine release (Multiplex bead arrays)

Cell-free supernatants from unstimulated and stimulated BMMC cultures were simultaneously assayed for IL-4, IL-5, IL-9, IL-10 and IL-13 using customised 5-plex bead immunoarrays (Bio-Rad, Hertfordshire) according to manufacturers’ instructions. Single vial standards were reconstituted in culture medium. Eight point serial dilution standards including blanks were prepared (Table 5) 96-well filter plates were pre-wet with 100µl of assay buffer and liquid then removed using a vacuum manifold (wash). Previously prepared 1x beads were then added to the assay plate. 50µl of standards, blank and samples were added to the plates in duplicate. Plates were covered and incubated in the dark at room temperature on orbital shaker for 30 minutes. After the incubation time, plates were washed 3 times with wash buffer. 25µl/well of detection antibody was added, the plate covered, and incubated in the dark on an orbital shaker for 30 minutes. Plates were washed 3 times and 50µl/well of freshly prepared streptavidin-PE were added. After 10 minutes in the dark at room temperature on an orbital shaker, plates were washed 3 times with wash buffer and beads were resuspended in 125µl assay buffer for analysis. Cytokine measurements were acquired using a Luminex-100 plate reader and analysed from a standard curve for each cytokine using Luminex software. Cytokine levels were expressed in pg/ml.
Table 5: Bio-Plex Pro™ (Bio-Rad) Mouse Cytokine Standard 5-Plex

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Highest standard (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4</td>
<td>125 315</td>
</tr>
<tr>
<td>IL-5</td>
<td>48 602</td>
</tr>
<tr>
<td>IL-9</td>
<td>33 358</td>
</tr>
<tr>
<td>IL-10</td>
<td>23 425</td>
</tr>
<tr>
<td>IL-13</td>
<td>48 663</td>
</tr>
</tbody>
</table>

2.8. ELISA for determining IL-9 pAb specificity

Enzyme-linked immunosorbent assay (ELISA) was used to determine the IL-9 pAb (Abcam, Ab111915) specificity for immunostaining on human conjunctival tissue sections. Recombinant human (rh) IL-9 (R&D 209-IL/CF) in bicarbonate buffer (Sigma; C304) was coated onto an ELISA plate (NUN96ft) at 4µg/ml and incubated overnight at room temperature. After incubation, plate was washed three times for 5 minutes with 0.1% Triton x100 or PBS with 300µl per well. Plate was then blocked with 3% BSA or PBS (at 300µl per well) for 1 hour at room temperature. Rabbit polyclonal Anti-IL9 (Abcam, Ab111915) was added at 6 different dilutions (1:125, 1:250, 1:500, 1:1000, 1:2000, 1:4000) plus a blank control and incubated for 1.5 hour at room temperature. IL-9 pAb was also prepared in carbonate/bicarbonate buffer. Plate was then washed 6 times for 5 minutes each 0.1% Triton x100 or PBS at 300µl per well. Goat anti-Rabbit HRP (Dako, P0448, 1:2000) was added and incubated for 1.5hr at room temperature. Plate was then washed 6 times for 5 minutes each 0.1% Triton x100 or PBS at 300µl per well. Then, ELISA Substrate Reagent kit (R&D Systems, DY999) was added to each well. Substrate A (stabilised hydrogen peroxide) and substrate B (stabilised tetramethylbenzidine) were mixed in equal portions just before use and 100µl per well of the mixture was added and left to develop in the dark for 20 minutes. 2N sulphuric acid was then added at 50µl per well to stop the reaction. The plate was read in a plate reader (TECAN, software; Safire, XFLUOR4) with the absorbance at 450nm (reference wavelength 540nm).

In another experiment, rhIL9-coated wells were incubated with neutralising human IL-9 (R&D,AB-209-NA), or neutralising mouse IL-9 (R&D, AB-209-MA).
Ab-409-NA) at 4 different dilutions (1:250, 1:500, 1:1000 and 1:2000) at the same time as IL-9 pAb at 1:1000 (based on previous experiment). Plate was incubated for 1.5 hour at room temperature and then subsequent steps were followed for ELISA plate as described above.

2.9. Human epithelial cell culture

2.9.1. In vitro culture of IOBA-NHC cells

IOBA-NHC is a conjunctival epithelial cell line spontaneously immortalised from a normal human conjunctival explant (a kind gift from M. Calonge, Valladolid, Spain). The cells were cultured in DMEM Nutrient mixture F-12 HAM medium (Sigma-Aldrich, Dorset, UK), supplemented with 10% heat inactivated foetal calf serum (FCS; Biosera, East Sussex, UK), 100U/ml Penicillin/Streptomycin (Life Technologies, UK), 2ng/ml of mouse endothelial growth factor (EGF) (Sigma-Aldrich), 1μg/ml bovine insulin (Sigma-Aldrich), 5μg/ml hydrocortisone and 0.1μg/ml Cholera Toxin (Sigma-Aldrich) at 37°C, in a 5% CO₂ humidified incubator. At confluence, cells were detached using trypsin-EDTA (Life Technologies) and were incubated at 37˚C, 5% CO₂ for 5 minutes. Once detached, cells were centrifuged for 5 minutes at 1200 rpm and resuspended in fresh medium in a 75cm² flask and cultured as above. Cultures were split and fed when the monolayer became confluent.

2.9.2. In vitro culture of 16-HBE cells

16-HBE cell line is a human bronchial epithelial cell line (a kind gift from P. Lackie, University of Southampton, UK). The cells were cultured in DMEM Nutrient mixture F-12 HAM medium (Sigma-Aldrich), supplemented with 10% heat inactivated foetal calf serum (FCS; Biosera) and 100U/ml Penicillin/Streptomycin. Cells were split and fed as described in 2.4.
2.10. Epithelial cell cytokine treatments

Epithelial cells were detached from the culture flask as described above. Cells were centrifuged for 5 minutes at 1200 rpm, suspended at 1x10^5 cells/ml, plated in 24-well plates and incubated overnight. The medium was replaced with serum free medium in the presence or absence of recombinant human cytokines (all from R&D Systems) as described in Table 6. Cells were incubated as above and harvested at 2, 6 and 24 hours post cytokine treatment where RNA were removed and kept frozen in -20°C until ready for qPCR.

Table 6: Recombinant human cytokines for human epithelial cell cytokine treatments.

<table>
<thead>
<tr>
<th>Human Recombinant Cytokine</th>
<th>Final Concentration (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>20</td>
</tr>
<tr>
<td>IL-9</td>
<td>10</td>
</tr>
<tr>
<td>IL-13</td>
<td>40</td>
</tr>
<tr>
<td>IL-17A</td>
<td>0.2</td>
</tr>
</tbody>
</table>

2.11. RT-PCR

2.11.1. RNA isolation

For total RNA isolation, the procedure was carried out using RNeasy Mini kit (Qiagen, Crawley, UK), according to the manufacturers' instructions. For human epithelial cells, 300µl of RLT-lysis buffer (supplied in the kit) was added to the cells, using a vortex, and kept frozen at -70°C until ready for RNA isolation. For suspension cells like BMMC, cells were centrifuged for 5 minutes at 1200rpm and washed once with PBS to remove FCS. BMMC were then washed again and resuspended in 300 µl of RLT-lysis buffer. To lyse mouse conjunctival tissues, Trizol was used instead of adding lysis buffer. All lysates were kept frozen at -70°C until ready for RNA isolation.
Cell lysates were homogenized by centrifugation using Qiashredder spin columns (Qiagen). The remaining procedure was carried out using RNeasy spin columns according to manufacturers’ instructions. 20 µl of RNase free water was added to the column to elute the RNA in 1.5ml RNase-free Eppendorf tubes (supplied in the kit). The concentration of RNA was measured by spectrophotometry (Nanodrop) and its purity was analysed by measuring the ratio of the readings at 260nm and 280nm \( (A_{260}/A_{280}) \), with readings greater than 1.9 indicating a high level of purity. Extracted RNA specimens were then stored at -80°C or used immediately for making cDNA.

2.11.2. Reverse Transcription (RT)

RNA was reverse transcribed into cDNA copies using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). All samples were prepared on ice. Firstly master mix, which comprises dNTP mix, Multiscribe® Reverse Transcriptase, RT buffer, RNase inhibitor, RT Primers and Rnase free water (supplied in the kit), was prepared according to Error! Reference source not found.7 below:
Table 7: Reagents added to a master mix per reaction.

<table>
<thead>
<tr>
<th>Component</th>
<th>Per 7.5µl reaction/reaction(µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dNTPs (100mM)</td>
<td>0.075</td>
</tr>
<tr>
<td>Multiscribe® Reverse Transcriptase</td>
<td>0.500</td>
</tr>
<tr>
<td>RT Buffer</td>
<td>0.750</td>
</tr>
<tr>
<td>Rnase Inhibitor</td>
<td>0.094</td>
</tr>
<tr>
<td>RT Primers Or Random Hexamers</td>
<td>1.5</td>
</tr>
<tr>
<td>RNase free water</td>
<td>2.081</td>
</tr>
</tbody>
</table>

3.5µl/well of reverse transcription buffer was aliquoted into wells of a 96-well reaction plate in triplicate followed by 2.5 µl aliquots of RNA added into respective wells, giving a total volume of 6 µl per well. Plates were capped and spun for 10 seconds before being incubated for 10 minutes at 25˚C in a thermal cycler, followed by 30 minutes at 48˚C and 2 minutes at 95˚C (to inactivate the reverse transcriptase in the reaction), before an indefinite holding stage at 4˚C. Samples were stored at -20˚C until qPCR was carried out.

2.11.3. qPCR

In order to quantify the relative amount of mRNA, TaqMan gene expression assays were used (Applied Biosystems, Warrington, UK) which contain primers
and probes, all from Applied Biosystems (refer to Table 8). 2X Taqman Gene Expression Master mix containing Ampli Taq Gold NA polymerase, Uracil-DNA Glycosylase and dNTPs with dUTP (Applied Biosystems) was diluted in RNase-free water (Table 9) to set up a 10 µl reaction in a 96-well reaction plate.

9.33 µl of TaqMan Master Mix was added to each well followed by 0.67 µl of the cDNA product to corresponding wells in the reaction plate. Each reaction was set up with β actin as the housekeeping gene with the following cycle condition: a) 50 °C for 2 minutes; b) Initial activation of DNA polymerase at 95 °C for 10 minutes; c) denaturation for 15 seconds at 95 °C; d) annealing and extension step at 95 °C for 60 seconds in 40 cycles using the 7500 HT Real-Time PCR system (Applied Biosystems). An annealing temperature of 95 °C was used in all primers. Results were analysed with SDS 2.2.2. software.

Table 8: Genes of interest and their target sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Assay ID/Part number/ Target sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human/Mouse Beta-actin</td>
<td>Hs01060665_g1</td>
</tr>
<tr>
<td>Human MUC2</td>
<td>Hs03005103_g1</td>
</tr>
<tr>
<td>Human MUC5ac</td>
<td>Hs00873651_mH</td>
</tr>
<tr>
<td>Mouse IL-9</td>
<td>Mm00434305_m1</td>
</tr>
<tr>
<td>Mouse IL-9R</td>
<td>Mm00434306_m1</td>
</tr>
</tbody>
</table>
Table 9: Conditions for qPCR

<table>
<thead>
<tr>
<th>Component</th>
<th>Per 10 µl reaction (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqMan 2x Universal PCR Master Mix, No AmpErase UNG</td>
<td>5.000</td>
</tr>
<tr>
<td>RNase-free Water</td>
<td>3.335</td>
</tr>
<tr>
<td>AOD OR 18S or Housekeeping gene</td>
<td>1</td>
</tr>
</tbody>
</table>

2.11.4. Analysis of Real Time qPCR

RNA was normalized to expression levels of β-actin. Relative RNA expression was calculated using the change-in-threshold method of $-\Delta\Delta CT$:

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

where

$\Delta$= change;

$\Delta Ct = Ct$ target gene - $Ct$ housekeeping gene;

$\Delta\Delta Ct = \Delta Ct$ sample - $\Delta Ct$ unstimulated

2.12. Statistical analysis of results

Data were analysed using Prism 6 (GraphPad Software Inc, LA, Jolla, Calif). Data were shown as means ± SEMs and SDs. For human data, analyses used Mann-Whitney U-tests for comparison of means. Student’s T-tests were used to evaluate statistical significance in all mouse experiments. A P value of <0.05 was considered statistically significant.
CHAPTER 3

Mouse model of Allergic Conjunctivitis
3.1. INTRODUCTION

Animal models are important for investigating the potential inflammatory mechanisms involved during allergic conjunctivitis (AC), for identifying molecular targets and for testing novel compounds for therapeutic interventions. Since studies done in human samples can give limited information due to limited availability of human samples, the stage of disease at the time of sampling, and the effects of treatment on the specimens, having access to allergic conjunctivitis animal model enable us to assess the effects of anti-allergic and newly-developed compounds and also to study in more detail the cellular and molecular mechanisms in ocular allergy.

There are different strains of mice that have been used for establishing models of AC which includes SWR/J mice, AJ mice, BALB/c mice and C57BL/6 mice. This study used the C57Bl/6 mice since C57Bl/6 mouse immune responses are more biased towards Th1 responses, which can cause a clinically more severe allergic reaction (368).

Mouse models of AC have been used to study the effects of topical application of inhibitors (369,370). Besides testing new pharmacological compounds, these mice have also been extensively used to study the mechanisms underlying AC. A study done by Li and colleagues has found that AC mediated by short ragweed pollen in BALB/c mice involves a TLR-4-dependent, TSLP/OX40 ligand binding leading to activation of the OX40 signalling pathway, which triggers the secretion of IL-4, IL-5 and IL-13 in the cornea, conjunctiva and cervical lymph nodes (371).

Although much effort has been made to understand the molecular mechanisms involved in AC, most AC models have only been useful to investigate the cellular involvement in the clinically milder forms of AC. There are very few T cells found in the conjunctival tissues in these models, making it impossible to study the contribution of T cell-derived cytokines in clinically more severe forms.
of chronic allergic eye disease. Therefore, the relevance of most AC models to clinically more severe forms of AC in man is less obvious.

This study uses an adaptation of an AC model previously described by Schlereth et al (347). Schlereth and colleagues developed a clinically more severe model of murine AC by injecting OVA-loaded dendritic cells into the subconjunctival (SCJ) space and then immediately challenging the mice topically with OVA via eyedrops. The mice were repeatedly challenged for up to 10 days following SCJ injection. This model was based on a similar approach previously developed for an allergic asthma model (372). Combining the approach from Kuipers et al., with the understanding of the interaction of antigen-specific T cells as playing a key role in experimental immune-mediated blepharoconjunctivitis (373), Schlereth et al successfully induced a severe model of AC. Schlereth et al investigated expression of CCR7, as the marker for activated dendritic cells, and demonstrated that antigen-specific T cell-dendritic cell interactions are crucial for inducing AC in this model. The presence of antigen-specific T cells and dendritic cells in this model were evidenced by the similar levels of Th2 cytokines (IL-4 and IL-13) in AC-induced mice receiving CCR7−/− dendritic cells as compared to AC induced mice that did not receive SCJ injection of wild type dendritic cells. This suggests the importance of activated dendritic cells in eliciting chronic AC responses in C57BL/6mice.

This model was adapted, by omitting the antigen-loaded DC and chosen for this study due to the outcome of repeated challenge with allergen that mimics the physiology of more severe forms of AC with increasing eosinophils infiltration and expression of Th2 cytokines. Following sensitisation and repeated OVA challenge, early phase responses which are normally recorded within 30 minutes after conjunctival challenge can be evaluated by scoring of itch, whilst late phase responses were also recorded after 4-24 hr (conjunctival redness, lid oedema and tearing (374)).

This severe form of AC requires relatively high doses of topical antigen during the repeated challenge at the ocular surface, making it physiologically less relevant to chronic AC in man. However, this model is more similar to chronic
AC affecting man with respect to its CD4+ T cell involvement, making it possible to study the role of Th9 cells in the chronic AC model.

To date, most studies investigating the role of IL-9 have been done using mouse models of allergic airway disease. Sehra et al have used both BALB/c and C57BL/6 mice to study the functions of Th9 cells in promoting mast cell responses during both acute and chronic allergic lung inflammation (127). Due to the availability of genetically modified mice, mouse models are normally preferred for studying the mechanisms underlying certain diseases. Liao et al used Il2−/− knockout mice to study in detail the downstream mechanisms of il9 promoter expression and Th9 differentiation which is modulated by B cell lymphoma 6 (BCL6) regulation (258). Another study using IL-9 knockout mice with oxazalone-induced colitis suggested the pathogenicity of Th9 subset as an important role in driving ulcerative colitis (375). Together, using mouse models, these studies have highlighted the current understanding for the role of IL-9 in various diseases.

Using the eye as the model of inflammation in transgenic mice expressing anti-hen egg lysozyme (HEL) TCR-specific T-cells, Tan et al., showed that after activation with HEL and antigen presenting cells (APC) in the presence of IL-4 and TGF-β, IL-9 expressing Th9 cells were immunopathogenic in mice receiving Th9 cells via their lens (264). Tan et al., studied in greater detail the kinetics of IL-9 secretion relative to IL-10 secretion by Th9 cells in whole eyes. In 2015, the same group had also discovered that the TNF family cytokine TL1A promoted IL-9 producing T cells (Th9) by signalling through DR3 receptor, which resulted in a more severe form of ocular inflammation (153). However, to date, no published study has used a mouse model of AC to study the contribution of IL-9 and Th9 cells in ocular allergy. This chapter describes our study to detect the presence of IL-9 in AC and investigating which immune cells secrete IL-9, using the mouse model of the severe form of AC as developed by Schlereth et al (347). We hypothesise that IL-9 is contributing to the severity of the disease in this mouse model.
To address this hypothesis, the specific aims were as follows:

1. To determine the expression of IL-9 in the conjunctiva and draining lymph nodes of mice with AC;
2. To define the cellular source of IL-9 during disease in this model.
3.2. RESULTS

3.2.1. OVA induced a clinically severe form of AC.

In order to investigate the expression of IL-9, its cellular sources during disease and the functions of IL-9 in vivo, experiments were performed using a mouse model of AC. The C57BL/6 mice were sensitised and subsequently topically challenged with OVA (376). Generally, OVA-challenged mice exhibited a clinically severe form of AC. Data shown in this chapter is a representative from three independent experiments that were performed at different times, unless stated otherwise. In each experiment, mice were divided into two different groups; 1) antigen-immunised mice topically challenged with OVA as EAC and 2) antigen-immunised mice topically challenged with PBS (control); with each group consisting of 5 or more mice per group (n ≥5).

EAC was induced in C57BL/6 mice, which were immunised with OVA together with alum and pertussis toxin and left for 21 days. After that time, the mice were challenged once daily with topical OVA for 7 days. In OVA-challenged mice, inflammatory responses were observed that mimic allergic conjunctivitis symptoms in man. Inflammatory responses in this model were recorded and scored according to a scoring system previously described (374); scoring the following symptoms; lid oedema, redness (hyperemia), itching and tearing. Mice were scored by two independent masked observers (Dr. Virginia Calder and Dr. Malihe Eskandarpour). EAC mice resulted in a more severe inflammation with higher total clinical score as compared to control mice (Figure 9). This experiment was recently repeated and data shown in Figures 13 to 19 were provided by Dr. Malihe Eskandarpour.
Figure 9: Clinical scoring

A. Clinical features of mouse eye in both control and EAC group

B. Clinical scoring of control and EAC mice. Daily scores were based on lid swelling, tearing, redness and conjunctival oedema, ranges from 0-3 for each symptom. Mice were scored by two independent masked observers and plotted as mean±SEM.
In order to analyse the histological changes in the eye during disease, 7-days post challenge, mice were sacrificed and whole eyes were enucleated, processed and embedded in paraffin. 5 μm thick sections were prepared and stained for haematoxylin and eosin (H&E) to identify nucleated cells in the tissue, allowing a good observation on the overall structure of the tissues and cell distribution throughout the tissue. As compared to controls, more cells were observed at the forniceal area of the conjunctival sections from EAC mice (Figure 10). There was a high number of cells at the forniceal area of the conjunctivae in EAC mice, suggesting that the conjunctivae of OVA-challenged mice were severely inflamed, which was supported with the inflammatory scoring data. In Figure 10, EAC slides were presented at different exposure than control slides due to the microscope automatic exposure setting that was initially set when obtaining the photos. These photos were obtained on different days.

In addition to H&E staining, tissue sections were stained with toluidine blue to determine the distribution of mast cells within the conjunctiva. The thiazine metachromatic dye in toluidine blue will stain acidic tissue components including mast cell granules. As observed in Figure 11, in control tissues, mast cells were mostly dispersed within the stromal region of the conjunctiva, however most of these positively stained mast cells were observed within the subepithelial layer of the conjunctiva of EAC tissues. Positively stained cells were counted within three separate fields (x400) and total numbers of positively stained cells were plotted in graphs (Figure 12). Significant increases in the levels of mast cells were observed in EAC tissues as compared to the control group (p<0.05).
Figure 10: H&E staining of conjunctival sections from control and EAC mice, 7 day post challenge. Conjunctival fornix (B and D). Fewer cells visible at the forniceal conjunctiva in control challenged sections (A and B) as compared to more infiltrating cells that are visible (in dark purple) in EAC challenged conjunctiva (C and D). Images were taken with x200 (A and C) and x400 magnification (B and D). Scale bar: 20µm.
Figure 11: Toluidine blue staining for mast cells on control and EAC tissue sections. Positive mast cells were stained deep blue as compared to other cells, A and C (x200); B and D (x400). Scale bar: 20µm.
3.2.2. Cell characterisation in the murine conjunctiva

In order to analyse different cell types that are present in the mouse conjunctivae, most studies have used enzyme digestion of the fibrous layers of conjunctival tissues to allow single cells to be released into the medium for further analysis by flow cytometry. The aim of this study is to investigate the presence of different cell types during AC, including mast cells and CD4+T cells. However, mast cells and activated T cells have low viability and potentially may not survive a strong enzymatic conjunctival digestion process. Another way to isolate the single-cell suspension is by conjunctival explant, a method that is widely used to isolate and expand conjunctival epithelial and goblet cells in culture (377). Based on the methods used for conjunctival explants, this experiment was modified and optimised, tailoring to the aim of the experiment.

Figure 12: Toluidine blue positive cells in control and EAC mice. Positive cells were counted from a total of 3 fields per sections for each mouse, and from 10 mice per group. Data represented from one of two independent experiments and were plotted as mean±SEM. * p< 0.05 by Student’s t-test.
Briefly, small pieces of conjunctival tissues were left in supplemented medium with the addition of mast cell and T cell growth factors to support viability and create a concentration gradient that will encourage both mast cells and T cells to migrate out from the tissue into the medium. In our system, leaving these tissues in supplemented medium for 3 days was sufficient to obtain enough cells whilst maintaining >90% cell viability (based on trypan blue exclusion assay) for further cell staining. A similar method was also used by Miyazaki et al, where conjunctival tissue explants were left in supplemented medium for two days in order to isolate mast cells from the tissue (378).

3.2.2.1 Gating strategy

For repeat experiments and data presented in this chapter, cells were stained for flow cytometry and acquired on a BD LSRFortessa-x20 using FACSDiva, with compensation applied where necessary. The data was analysed using FlowJo v10.

Cells were first gated for all cells or lymphocytes. Subsequently, live cells were gated on an area which was negative to zombie NIR™ which binds to dead cells. In order to remove the doublets, live cells that appear at a perfect diagonal line (at 45 degrees, passing the zeros) when viewed in FSCH and FSCA were gated as single cells. Further gating was performed from a live single cell gate.

In some samples, the numbers of cells were calculated using FACS counting beads which were added to each sample of 200µl.
3.2.2.2 Analysis of murine conjunctiva.

In order to investigate the presence of mast cells in EAC conjunctiva, conjunctival cells were stained for mast cell markers, c-kit and FceRI. Initial gates were applied as seen in figure 13 where all cells were selected, followed by live cells and the single cell gate. Further gates were drawn for c-kit and FceRI respectively (Figure 14). In order to draw gates, a fluorescence minus one (FMO) control is needed. The FMO control includes cells stained with all the fluorophores minus one, and this is normally used to set the cut-off point between positive and negative gates. FMO is especially important for intercellular staining since there is no clearly defined population that can be seen during staining. However, no FMO controls were acquired in this experiment due to initial experiment set up which did not include FMO controls and subsequent gates were not set using the FMOs. The percentages of mast cells (c-kit and FceRI positive) increased significantly in EAC mice in comparison to control mice. This data also correlated with the levels of mast cells in the conjunctiva of these mice stained with Toluidine blue (Figure 12), where the levels of mast cells were significantly increased in EAC mice as compared to control. It is not possible to draw gates for CD4+ and ckit+ cells in one dot plot since markers for mast cells and T cells in the conjunctiva were stained in different tubes.

Conjunctival cells were also stained for intracellular expression of IL-9. A representative FACS dot plot for c-kit+/IL-9+ cells from single cell gate is shown in Figure 15A. Significant increases in the levels of c-kit+/IL-9+ cell expression were observed in EAC as compared to controls (Figure 15B) suggesting that IL-9 expression by mast cells might reflect a role for IL-9 in the ocular allergy in OVA challenged mice (P<0.005). Not all IL-9 positive cells are c-kit positive (mast cells) cells suggesting there could be IL-9-expressing cells within the conjunctival tissues which are not mast cells. To address this possibility, IL-9 expression by conjunctival CD4+T cells was investigated. Similar experiments have been repeated by Dr. Malihe Eskandarpour after this data has been produced and through personal communication, she has confirmed similar results to the ones presented in this chapter.
Figure 13: Representative dot plot analysis for initial gating for cells from the conjunctivae of control and EAC mice. A. Initial gates around cells, followed by B. live cells and C., single cells.
Figure 14: Summary of total percentages of FcεRI and c-kit positive cells in the conjunctivae based on FACS plots, in 200μl volumes of cells. A. Representative FcεRI gating in both control (n=5) and EAC (n=5) mice with total percentage of FcεRI positive cells shown in the far right graph. B. Representative c-kit positive gating in both control and EAC mice with total percentage of c-kit positive cells shown in the far right graph. C. Representative double c-kit+ FcεRI+ dot plot from EAC conjunctiva. Data were plotted as mean±SEM from a representative from 3 experiments. * p<0.05. Student’s t test was performed on this data.
Figure 15: Summary of IL-9 staining in c-kit positive cells. A. Representative of c-kit+/IL-9+ from single cell gate in control and EAC mice. B. Summary of total number of IL-9 positive, c-kit positive cells. Data were plotted as mean ± SEM from a representative from 3 separate experiments. * p< 0.005 by Student’s t-test.

In addition to conjunctival mast cell infiltration, the presence of CD4+ T cells in the conjunctivae of allergic mice was also investigated. Based on the gating
strategy shown in Figure 16, the percentages and number of CD4+ T cells increased significantly in EAC mice as compared to PBS challenged control group (Figure 16A) suggesting that CD4+ T cells are involved in mediating the disease in EAC mice, ***p<0.0005.

In order to investigate expression within these CD4+ T cells, conjunctival cells were also intracellularly stained for IL-9 and PU.1 (Figure 16B and C) and GATA3 (Figure 17). The levels of CD4+/IL9+ and CD4+/PU.1+ cells significantly increased in challenged mice *p<0.05 (Figure 16B and C). Representative of quadrant gates drawn from CD4+ cells looking at IL-9+/PU.1+ and IL-9+/GATA3+ are also shown in Figure 17C and D. This data suggests that Th9 and Th2 cells are involved in mediating the disease in EAC mice.
Figure 16: Representative dot plot analysis for CD4+T cells, CD4+ /IL-9+ cells and total PU.1+ cells on single cell suspension from the conjunctiva of PBS control and OVA challenged EAC mice. A. CD4+ gates and total percentages and total numbers of CD4+ cells as shown in graphs, B. CD4+/IL-9+ gates and total percentages of IL-9+ in CD4+ cells, C. PU.1 gates and total percentages of PU.1 positive cells in live cells. Data were plotted as mean±SEM from a representative from 3 independent experiments, *p<0.05, **p<0.005, ***p<0.0005. Student's t test was performed on this data.
Figure 17: Representative dot plot analysis for CD4$^+$ cell in conjunctivae of control and EAC mice. Representative quadrant gates from single cell population for A. CD4$^+$ vs IL-9$^+$ cells, B. CD4$^+$ vs IL-9$^-$ cells. C. and D. show gates from CD4$^+$ population. Representative of C. IL-9$^+$ vs PU.1$^+$ quadrant gates and D. IL-9$^+$ vs GATA3$^+$ quadrant gates.
3.2.3. Cell characterisation in the murine draining lymph nodes.

In order to investigate the different types of CD4⁺T cells that are present during disease, a single cell suspension from the draining lymph nodes from each mouse was prepared for intracellular cytokine staining. Similar to the conjunctiva, since this study aimed to investigate the different sources of IL-9 during AC, cells were stained for different T cell subsets, Th2 and Th9. Different CD4⁺T cell subsets were distinguished based on their specific transcription factors and cytokine profiles. The Th2 cell lineage was identified by GATA3/IL-4, whilst the Th9 cell lineage was identified by expression of PU.1 transcription factors and can be identified by PU.1/IL-9 positive cells.

Based on size and granularity, cells were initially gated for lymphocytes (Figure 18). Within the lymphocyte gate, the live cell gate was drawn around the APC Cy7 negative population before further selecting the single cell population when viewed as FSCH and FSCA. All further gates were drawn from the hierarchical single cell population based on clearly defines.

There were similar percentages of CD4⁺T cells in draining lymph nodes of EAC mice as compared to control mice (Figure 19A). Within the CD4⁺ population, there was no significant difference in intracellular expression of IL-4+/GATA3+ cells of EAC mice and control mice (Figure 19C). However, levels of CD4+/IL-9+/PU.1+ cells were significantly increased in EAC mice as compared to control (p<0.005). This data suggests that IL-9 expressed by Th9 cells, but not IL-9 expressed by Th2 cells, might correlate with disease in this model.

This experiment shows that in EAC mice, both IL-9 secreted by mast cells and Th9 cells could potentially drive the disease within the conjunctiva whilst IL-9 secreted by Th9 cells, but not Th2 cells, could be pathogenic by exacerbating the disease by migrating from the lymph nodes to the conjunctiva.
Figure 18: Representation of FACS dot plot which shows the initial gates for draining lymph nodes in control and EAC mice. A., Initial gates were selected for lymphocytes and subsequently for live cells in B. Further gates for single cells (C) were selected from live cells gates.
Figure 19: Representation of flow cytometric analysis for Th2 and Th9 positive cells on the draining lymph nodes of control and EAC mice. Following single cell gates, A, Dot plot of CD4+ gates and summary of total percentages of CD4+ cells shown in graph. B, Dot plot of CD4+/IL9+/PU.1+ gates and summary of total percentages of IL9+/PU.1+ in CD4+ cells shown in graph. C, Dot plot of CD4+/IL4+GATA3+ gates and summary of total percentages of IL-4+/GATA3+ in CD4+ cells shown in graph Data were plotted as mean±SEM from a representative from 3 independent experiments. ** p< 0.005. Student’s t test was performed on this data.
3.3. DISCUSSION

Since, at the time of this study, there was no evidence to suggest that IL-9 is upregulated in AC, we first used a mouse model to study the presence and the contribution of IL-9 in EAC. Animal models have been extensively used to study mechanisms of disease, some studies have highlighted the importance of different signalling molecules, chemokines, inflammatory cells and mediators in AC, but very few have focused on the role of cytokines, especially the role of IL-9, in AC mouse models.

Firstly, there is one main technical issue which should be addressed when presenting results in this chapter. As previously mentioned, there are no FMO controls to set the gates during analysis. FMO controls are essential especially in setting gates for intracellular cytokine staining where cytokines can be partially expressed in cells with no obvious positive population to be seen. All gates in this chapter were set not based on FMO controls since no FMO controls were included during experiment setup.

The initial aim of this chapter was to investigate the presence of IL-9 in the conjunctival tissues of the mouse models. During innate immune responses, mast cells are known to be one of the first cells that respond to allergen on the surface of conjunctival tissues. The initial mast cell responses then result in further recruitment of inflammatory cells, including CD4+ T cells that are responsible for adaptive immune responses and prolonging allergic reactions. Since both mast cells and CD4+ T cells can express IL-9, this experiment investigated the presence of IL-9 in mast cells and in CD4+ T cells within the conjunctivae of AC mouse models.

To date, this is the first study to investigate mast cells secreting IL-9 in the conjunctiva in a mouse model of AC. Based on this study, mast cells expressing IL-9 were significantly upregulated in EAC mice compared to controls. Chen and colleagues showed that IL-9 producing mucosal mast cells (MMC) promoted IgE-mediated food allergy both in mice and humans (379). This data is also supported by other studies. IL9/IL-9R signalling is shown to be
important in mediating IgE-dependent oral and systemic anaphylaxis in mouse models (336,380).

Since CD4+ T cells regulate late phase responses during AC (381), a significant increase was observed in the levels of CD4+ T cells in the conjunctivae of OVA-challenged mice. The data also shows that IL-9 secreted by Th2 and Th9 cells was significantly upregulated in the conjunctivae of EAC mice as compared to control mice. In a recent study, Tan and colleague showed that Th2 and Th9 cells that were adoptively transferred to recipient mice migrated to the conjunctiva and initiated allergy-like inflammatory responses (382) which support data presented in this chapter that Th2 and Th9 can drive allergic responses in the eye. As most studies have investigated the role of CD4+ T cells during disease by investigating the lymph nodes, subsequent experiments were performed by staining CD4+ T cells in the draining lymph nodes.

In order to identify the CD4+ T cell region in the conjunctivae, besides CD4, extra surface markers including CD3 and CD45 can be useful in selecting CD45 lymphocyte populations followed by CD4- and CD3- positive gates. An initial region of CD45+ cells to gate all lymphocytes can be then followed by CD4+ and CD3+ gates. However, in some cell samples, I have managed to stain for CD3 and CD45 and, by backgating the plots, cells selected for CD4 expression are also positive for both CD3 and CD45, suggesting that selected CD4+ cells are lymphocytes (data not shown). Besides Th2 and Th9 cells, it will also be useful to be able to distinguish other cell types that secrete IL-9 in both conjunctivae and draining lymph nodes including ILCs, given that ILC2 cells are known to secrete high levels of cytokines including IL-9 and the role of ILC2 and IL-9 during AC has not yet been explored. In order to test this aim, more antibodies need to be included in this antibody panel.

Th2 cells are known to be involved in allergic hypersensitivity, including ocular allergy. During allergic hypersensitivity, the Th2 cytokine IL-4 plays many different roles including regulating B cell IgE production (383). In our experiments, we investigated the presence of Th2 cells and the possibility of Th2 cells as a source for IL-9 in AC. The data suggests that when using Th2
cell transcription factor GATA3 as a marker of Th2 cells, there is a significant increase in the levels of Th2 cells in the conjunctivae of EAC mice but no difference in the level of Th2 positive cells in the draining lymph nodes of EAC as compared to control mice. Schlereth et al have also reported similar findings on Th2 cytokine secretion using this same OVA model, by measuring the level of Th2 cytokines (IL-4, IL-5 and IL-13) in the supernatants of the recall stimulated lymph nodes from these mice (347). Since Th2 T cells are upregulated in the conjunctiva, but not in the draining lymph nodes of EAC mice, it suggests that Th2 cells play a role in driving AC.

Besides Th2 cells, Th9 cells were initially thought to be the main source of IL-9. However, a recent study found that innate lymphoid cells-2 (ILC2) secrete most Th2 cytokines, including IL-9 (127). IRF4 and PU.1 transcription factors are important in driving CD4+T cells to differentiate into Th9 cells. IRF4 is important during Th9 cell differentiation in which IRF4 levels were upregulated after T cell stimulation (384) and T cells cannot differentiate into Th9 cells in IRF4-deficient T cells (147). Another study characterised Th9 cells as CD4+/PU.1+/IL-9+ since the PU.1 transcription factor controls the transcription of Il9 gene and Th9 differentiation (251). In this study, Th9 cells were significantly upregulated in both conjunctivae and draining lymph nodes of EAC mice as compared to control mice, suggesting that Th9 cells play important roles in the pathogenesis of the disease. Th9 cells have been shown to be upregulated in several diseases including allergic asthma, food allergy, inflammatory bowel disease, and multiple sclerosis (239,304,375,385,386).

In addition, this study has also shown that IL-9 secreted from mast cells in the conjunctivae can also be contributing to the disease in mice. Since mast cells, producing IL-9 in the conjunctivae also contribute to the pathogenesis of AC, further in vitro experiments on IL-9 functions in mast cells will be presented in the next chapter.
CHAPTER 4

Mast cells as IL-9 producers and responders in vitro.
4.1. INTRODUCTION

IL-9 is a pleiotropic cytokine that exerts heterogeneous effects on different cell types (as outlined in Chapter 1). For mast cells, IL-9 promotes their proliferation, activation, protease expression and also upregulates IgE receptor expression on the surface of mast cells. Apart from its effects on T cells and mast cells, IL-9 also exerts effects on epithelial cells; decreased mucus secretion in the lungs has been observed in mice with allergic hyperresponsiveness treated with neutralising anti-IL-9 antibody (387). The effects of IL-9 on lung epithelium (for example mucus production) could also occur due to the indirect effects of IL-13 (388). In addition, IL-9 enhances IL-4-mediated IgE and IgG production from human B cells (388). Despite many groups researching the roles of IL-9, the effect of IL-9 on other cytokine networks has not yet been thoroughly investigated.

As mentioned previously in chapter 1, IL-9R is a heterodimeric protein composed of specific IL-9Rα chain and γ chain, the common subunit of which is shared with different cytokine receptors including IL-7. IL-9R binding activates signal transducer and activator of transcription-1 (STAT-1), STAT-3 and STAT-5 (292,389). IL-9R was found to be expressed on the surface of mast cells and polymorphonuclear cells (390,391).

Although IL-9 is not thought to be important in mast cell accumulation in bronchial tissues, IL-9 is important for promoting mast cell growth and functions (238). Wiener and colleagues found that IL-9 secretion by mast cells was upregulated in the presence of IL-9, SCF and IL-1β, suggesting that IL-9 secreted by mast cells works in a positive feedback loop (279). Another study has found that IL-9 can also act as a growth enhancer together with SCF in human mast cells (238).

Having detected IL-9 expression in mouse conjunctival tissues (chapter 3), it was important to identify the contribution of mast cell-derived IL-9 to mast cell responses. Hence, in this chapter, bone marrow-derived mouse mast cells (BMMC) were used to further investigate the role of IL-9 on mast cell
responses. The reasons for using mouse mast cells are several: they can be expanded in sufficient numbers for experimental studies; they share similar characteristics to human mast cells, as both of these cells express both FcεRI and c-kit on their surface; they are characterised by their FcεRI surface receptor expression. Other cells that also express FcεRI include basophils. However, only mast cells express both FcεRI and c-kit. These mouse mast cells also require both IL-3 and SCF (392) to proliferate and differentiate into mature mast cells. Previous studies used the RBL-2H3 cell strain to mimic real mast cell stimulation in vitro (393,394). However, this is a rat basophilic leukaemia cell line, which does not exhibit the full characteristics of mast cells. Other mouse mast cell lines including CTFL-15 and MCP 5/L also express high affinity IgE receptor alpha chain but they have relatively immature mast cell phenotypes (395). Hence, using primary BMMC is thought to be the best in vitro model to study mature mast cell functions, as these cells are relatively easy to proliferate in abundance.

In this chapter, three different types of stimulation were initially used to stimulate BMMC; PMA and ionomycin stimulation, ionomycin stimulation alone and a physiological stimulant, anti-IgE. These stimulations work in different ways. PMA is a small organic compound which diffuses through the cell membrane to the cytoplasm and directly activates Protein Kinase C (PKC), causing degranulation. Cell degranulation via PMA stimulation omits the need for cell surface receptor stimulation. Ionomycin, a calcium ionophore, on the other hand triggers calcium release to promote exocytosis of granules, which is needed for nuclear factor for T cell (NFAT) signalling during degranulation. Ionomycin can be used as an addition to PMA stimulation in order to achieve maximal degranulation or it can be used on its own as a stimulant. Ca^{2+} responses coupled with PKC activation are essential for mast cell degranulation (396,397).

Anti-IgE antibody stimulation is a more physiologically relevant stimulant as it involves IgE ligand binding to specific FcεRI surface receptor on mast cells. IgE crosslinking activates a series of biochemical events which is regulated by NFAT (398) that leads to the release of preformed mediators from granules and the generation of newly synthesized mediators. FcεRI is a tetrameric formed by
the complex αβγ2 chains of which is activated by the activation of non-receptor protein tyrosine kinases. Once aggregated, a series of phosphorylation of kinases downstream of FcεRI leads to release of Ca2+ from endoplasmic reticulum (ER) resulting in a decrease of free Ca2+ from the ER. When ER Ca2+ stores are depleted, more Ca2+ influxes across the plasma membrane result in activation of calcineurin which in turn dephosphorylates NFAT, which is responsible for regulating the transcription of several cytokine genes. FcεRI stimulation also activates phosphorylation and degradation of inhibitor IκB, which allows the release of NFκB via nuclear translocation in order to activate genes which are responsible for the synthesis of several cytokines.

Several previously mentioned studies have highlighted the role of IL-9 in mast cell functions (279,328,399). Mast cells clearly play important roles in contributing towards innate and adaptive immunity in several acute and chronic diseases including allergy, due to their ability to secrete many different proinflammatory cytokines including IL-9. However, these studies have not focussed on the effect of IL-9 on other mast cell functions such as histamine secretion and cytokine secretion; especially, proinflammatory Th2 cytokines (IL-4, IL-5 and IL-13) which are commonly associated with allergic diseases including ocular allergy. Since in the previous chapter (Chapter 3), it was found that IL-9-expressing conjunctival mast cells are upregulated in the EAC mouse model as compared to PBS-challenged controls, we wished to further investigate the effect(s) of IL-9 on mast cells in vitro, using BMMC. The hypothesis of this chapter is that IL-9 upregulates Th2 cytokine (IL-4, IL-5 and IL-13) and histamine secretion by mast cells in vitro.
To address this hypothesis, the specific aims were as follows:

1. To establish cultures of differentiated, purified mouse bone marrow-derived mast cells *in vitro*;
2. To utilise these cells to determine the effects of IL-9 and IL-9R on mast cell expression of FcεRI.
3. To determine the effects of IL-9 and IL-9R on mast cell degranulation;
4. To determine the effects of IL-9 and IL-9R on mast cell secretion of cytokines.
4.2. RESULTS

Given that levels of IL-9 expressing mast cell levels were significantly increased in the conjunctival tissues of allergic conjunctivitis mice relative to controls, the aim of this chapter was to investigate the effects of IL-9 on mast cell functions in vitro. In this chapter, BMMC were used for all in vitro experiments.

4.2.1. Phenotypic characterisation of BMMC

After 4-5 weeks in culture, BMMC were stained for c-kit (stem cell factor receptor) to characterise the cells. Cells were considered to be mature mast cells only when they were >95% c-kit positive (Figure 20). The maturation status of the mast cells was also characterised by its FcεRI expression (>95% FcεRI expression). Once the mast cells are mature they are then ready to be used for experiments.
Figure 20: Phenotypic characterization of BMMC after 4-5 weeks in culture. A. Forward and side scatter (FSC, SSC) of single population of live cells, containing the mast cells (R1)(A). FACS plot of c-kit and FcεRI staining showing double positive c-kit+/FcεRI+ mast cells in the upper right-hand quadrant (R6) (B).
4.2.2. BMMC viability

BMMC were also stained with propidium iodide (PI) in order to determine the percentage of dead cells (staining positively for PI) in the cell culture flask. Approximately 99% of cells were shown to be live cells (Figure 21) and this is also similar when the cells were counted under the microscope with trypan blue exclusion staining, which will stain dead cells blue (data not shown). BMMC viability was checked weekly when in culture and each time prior to conducting experiments.

Figure 21: BMMC Viability staining with PI. Cells were stained with propidium iodate (PI) in order to determine the percentage of live cells before experiments were performed. Representative FACS plot and gating strategy for dead cells in BMMC culture. Cells stained with PI can be viewed in a. Dead cells (blue dots) were observed in FCS vs SSC scatter plot (b).
4.2.3. Functional BMMC characterisation

Once the cultured cells were phenotypically confirmed to be fully differentiated mast cells, these cells were then compared for their ability to release histamine following ionomycin stimulation or FcεRI crosslinking relative to unstained controls (Figure 22). Cells stimulated with ionomycin secreted significantly increased levels of histamine (P<0.005), whilst anti-IgE stimulation also resulted in increased levels of histamine although the changes did not reach significance. Anti-IgE stimulation did not significantly increase levels of histamine relative to controls, which could be due to the IgE stimulation not properly working in this setting. These cells were then used for further experiments.

Figure 22: Histamine secretion (mean ± SEM) at 24 hours post ionomycin or anti-IgE stimulation. Cells were used for further in vitro experiments once their ability to secrete histamine had been confirmed by histamine ELISA. Data reflect one of four experiments (n=4)
4.2.4. EFFECTS OF IL-9 ON BMMC FUNCTION

4.2.4.1. Effects of functional blocking of IL-9 on histamine secretion

Since histamine is one of the earliest mediators released by mast cells upon stimulation and histamine is largely stored preformed in mast cell granules, the effect of IL-9 on histamine secretion was investigated. IL-9 had previously been shown to increase histamine release by BMMC in response to ionomycin and IL-9 stimulation (279). In that study, Wiener and colleagues used histamine-free mast cells from transgenic mice to further study the relationship between IL-9 and histamine. They concluded that IL-9 production by mast cells might act in a positive feedback loop and that a lack of histamine can disturb this loop. Another group has also confirmed that IL-9 increased histamine release from mast cells in mice treated with IL-9 (399). Based on this evidence, the effects on histamine secretion upon blocking IL-9 or IL-9R in response to PMA and ionomycin, ionomycin alone or FcεRI crosslinking were investigated in this chapter.

4.2.4.2. Anti-IL-9 and anti-IL-9R antibody optimisations

Throughout this chapter, three different types of mast cell stimulation will be used; PMA+ionomycin, ionomycin alone and anti IgE stimulation. PMA activates protein kinase C and in the presence of ionomycin (which In this calcium ionophore), it becomes a strong cell stimulus for mast cell degranulation. Anti-IgE stimulation is a more physiological stimulus, requires IgE crosslinking of cell surface IgE receptors to cause downstream activation and signalling which activate mast cells and only causes partial mast cell degranulation.

In order to identify the optimal working concentration for mouse anti-IL-9 mAb in vitro, antibody was titrated at different concentrations; 8, 9, 10µg/ml (according to the manufacturers’ instructions). In this preliminary experiment, histamine secretion was measured in response to PMA+ionomycin stimulation in the presence of different concentrations of anti-IL-9 mAb. Histamine secretion was
downregulated in cells treated with all concentrations of anti-IL-9 mAb (Figure 23) but the lowest level of histamine was detected in cells treated with 9µg/ml of anti-IL-9 mAb. Hence, further subsequent experiments were done using this concentration of anti-IL-9 mAb.

Figure 23: Histamine secretion in BMMC supernatants following PMA+ionomycin stimulation treated with different concentrations of anti-IL-9 mAb for 120 hours (5 days). Data shown as mean ± SD from triplicate wells from a single preliminary experiment.

Since PMA combined with ionomycin is a very potent stimulus, causing mast cells to release very high levels of histamine and cytokines, subsequent experiments were performed by stimulating cells with either ionomycin alone or with FcεRI cross-linking. Based on previous experimentation using PMA+ionomycin stimulation, histamine was maximally secreted at 72 hours post-stimulation, and the larger difference on histamine levels was observed at 48 hours post stimulation; hence, the following experiments measuring histamine release were repeated up to 48 hours post-stimulation.
In order to find the optimal working concentration of anti-IL-9R mAb concentration, BMMC were treated with different concentrations of anti-IL-9R (5, 10, 20µg/ml) prior to stimulation with ionomycin and anti-IgE, relative to an IgG control. Histamine secretion was downregulated in cells treated with all concentrations of anti-IL-9R mAb, with 5µg/ml and 10µg/ml doses inducing the most profound effects on histamine release (Figure 24).

The most striking effect on histamine secretion was observed at 48 hours (Figure 24) where histamine levels were downregulated in cells treated with neutralising antibody as compared to untreated (stimulated) cells with isotype-matched control antibodies. Histamine secretion decreased when IL-9 function was neutralised (in PMA with ionomycin-stimulated cells, figure 23) and a similar effect was observed when blocking IL-9R (in ionomycin-stimulated cells, figure 24). Since the maximum inhibitory effects on histamine release were seen with adding 10µg/ml of anti-IL-9R mAb to mast cell cultures (Figure 24a and b), this concentration was used in all subsequent experiments. Figure 24c shows that adding anti-IL-9R antibody significantly downregulated histamine levels at 48 hours post ionomycin and anti-IgE stimulations, p<0.05; p<0.005.
Figure 24: Histamine secretion upon a. ionomycin and b. anti-IgE stimulation in BMMC supernatants treated with anti-IL-9 (9µg/ml) and different concentrations of anti-IL-9R. c. Summary of histamine secretion treated with 10µg/ml of anti-IL-9R at 24 and 48 hours post stimulation. Values were plotted as mean ± SD of triplicate wells from one experiment in a and b whilst values in c were plotted as mean ± SEM from a total of 3 independent experiments, *p<0.5, **p<0.005. Student’s t test was performed on this data.
4.2.4.3. Anti IL-9R antibody downregulates surface FcεRI receptor expression in BMMC.

Once both anti-IL-9 and anti-IL-9R mAbs were optimised in the BMMC assays, we investigated the effects of these antibodies on mast cell functions.

Firstly, the effect of IL-9 on FcεRI expression in BMMC was investigated. A previous study by Lora et al. demonstrated the differential effects of Th2 cytokines (including IL-9) on FcεRI expression on human mast cells (400). Hence, further experiments aimed to investigate the effect of IL-9 alone on FcεRI expression after stimulating the mouse BMMC via FcεRI cross-linking (by adding anti-IgE mAb). BMMC were subsequently stained for surface FcεRI and the percentages of FcεRI-positive cells were obtained by flow cytometric analysis. Median fluorescence intensity (MFI) of FcεRI positive cells were calculated and then normalised relative to stimulated cells without treatment at each time point (Figure 25b).

MFI of FcεRI expression on the BMMC cell surface from flow cytometric histogram plots were analysed at 24, 48 and 72 hours post stimulation (Figure 26). The level of FcεRI expression was initially upregulated after 24 hours of anti-IL-9R mAb treatment as compared to stimulated cells with no treatment. However, after 48 and 72 hours post anti-IgE stimulation, the level of FcεRI expression on the surface of BMMC showed downregulated trend as compared to anti-IgE stimulated control cells even though these levels did not reach significance.
Figure 25: Gating strategy for FCεRI positive cells. a. Representative FACS dot plot of single population in BMMC viewed in FSC vs SSC. b. Representative of gating on FCεRI positive cells (FL4-H).
4.2.5. Effect of functionally blocking IL-9 on cytokine secretion

Mast cells are known to secrete a range of cytokines upon stimulation. Since previous experiments have shown that exogenously added IL-9 can upregulate histamine secretion by mast cells, the effect of IL-9 on other cytokines produced by BMMC was investigated.

4.2.5.1. Cytokine secretion upon BMMC stimulation.

In order to establish a cytokine secretion profile for BMMC, a panel of cytokines (IL-4, IL-5, IL-9, IL-10 and IL-13) was chosen for multiplex bead arrays to detect simultaneous secretion in BMMC culture supernatants, comparing mast cells

Figure 26: Relative FCζRI MFI by anti-IgE-stimulated cells upon treating with either IL-9 or IL-9R (a). antibodies over 72 hours post stimulation. Data was normalised relative to stimulated cells with no treatment (a-IgE) at each time point. Normalised values were calculated as $2^{x-y}$ with $x= y-z$; $y= \%$ of stimulated cells without treatment; $z= \%$ of stimulated cells with treatments. Normalised values were representative from three independent experiments.
either unstimulated (in the presence of isotype-matched control antibodies) or stimulated with PMA+ionomycin, ionomycin or anti-IgE (DNP-BSA) (Figure 27).

IL-9 was secreted in response to all modes of stimulation. Since PMA and anti-IgE stimuli (which cross-links FcεRI surface receptors) induced an immediate release of secretory granules from mast cells as compared to ionomycin stimulation, which triggers calcium release needed for NFAT signalling, the time courses of IL-9 secretion in response to ionomycin alone, PMA+ionomycin and anti-IgE stimulated cells are different.

In response to PMA+ionomycin or ionomycin stimulation alone, BMMC secreted IL-4, IL-5, IL-9, IL-10 and IL-13. However, following anti-IgE stimulation, much lower levels of these cytokines were detected. Ionomycin (a Ca^{2+} ionophore) stimulation resulted in exocytosis of granules in an all-or-none process whereas cell surface stimulation via FcεRI crosslinking induced only a partial degree of degranulation (401), explaining the reduced levels of cytokines secreted by mast cells in response to IgE crosslinking. Even though anti IgE stimulation can be very limited in the amount of secreted mediators, it is more physiologically relevant since this type of stimulation can also be observed in response to allergen exposure in man. Nevertheless, significant increases in cytokine secretion were detected in response to each mode of BMMC stimulation, relative to unstimulated BMMC.
Figure 27: Cytokine secretion (IL-4, IL-5, IL-9, IL-10 and IL-13) from supernatants of BMMC stimulated with PMA+ionomycin, ionomycin alone and anti-IgE over 120 hours (5 days) post-stimulation with pma+ionomycin and over 72 hours post stimulation with ionomycin or anti-IgE. For PMA+ionomycin stimulation, data were plotted in means ± SD of triplicate wells from one representative experiment. For ionomycin and anti-IgE stimulation (b and c), data were plotted in means ± SEM from a total of 3 independent experiments, *p<0.05, **p<0.005. Student's t test was performed at each individual time point.
The effects of different stimuli were also observed in more detail at 24 hours post stimulation, as shown in Figure 28. IL-4, IL-5 and IL-13 were significantly upregulated 24 hours post stimulation by ionomycin; p<0.05, p<0.005 (Figure 28b). Although cytokines were secreted at lower levels in anti-IgE stimulated cells, all cytokines IL-4, IL-5, IL-9, IL-10 and IL-13 were significantly upregulated 24 hours post stimulation with anti-IgE (Figure 28c).
Figure 28: Cytokine secretion in BMMC 24 hours post stimulation with a. PMA and ionomycin, b. ionomycin and c. anti-IgE crosslinking. Data were plotted in means ± SD from triplicate wells from a preliminary experiment in a. For ionomycin and anti-IgE stimulation (b and c), data were plotted in means ± SEM from a total of 3 independent experiments, *p<0.05, **p<0.005. Student's t test was performed on this data.
4.2.5.2. Blocking IL-9 and IL-9R with neutralising antibodies affects cytokines secreted from BMMC

After confirming that IL-9 is secreted by BMMC upon degranulation in response to a range of different stimuli, this in vitro model was used to further investigate the effect of IL-9 on cytokine secretion by BMMC. Upon neutralising IL-9 function using a neutralising mouse anti-IL-9 mAb (described in Chapter 2 of this thesis) in PMA+ionomycin treated cells in comparison with cells treated with an isotype-matched control Ab, both IL-4 and IL-13 secretion increased markedly in those wells in which IL-9 function had been neutralised (Figure 29). This preliminary experiment was performed only once, and further experiments were undertaken using ionomycin and anti-IgE stimulation.
Figure 29: Time course of cytokine secretion in PMA+ionomycin stimulated cells treated with neutralizing anti-IL-9 antibody over 120 hours (5 days). Values were plotted as mean ± SD of triplicate wells from one experiment.
The levels of cytokines secreted in response to PMA and ionomycin stimulation were very high, and often they exceeded the values within the standard curve of the multiplex bead array hence, the values were read after extrapolating the standard curve and some values exceeded those extrapolated from the standard curve. Since the levels of cytokines were too high to be assayed by multiplex bead arrays in PMA and ionomycin stimulated cells, the levels of cytokines were assayed in cells treated with only ionomycin in order to determine the effects of neutralising the functions of both anti-IL-9 and anti-IL-9R in BMMC cultures (Figure 30). The most obvious effect was seen in anti-IL-9R mAb-treated mast cells. Upon blocking IL-9R function, all cytokines (IL-4, IL-5, IL-9, IL-10 and IL-13) were markedly reduced at 48 and 72 hours after which time these levels increased again. It appears that blocking IL-9R function with anti-IL-9R antibody resulted in reducing cytokine production by BMMC. However, this effect did not last beyond 72 hours’ stimulation, possibly due to degradation of the anti-IL-9R antibody (which was added 30 minutes prior to stimulation) or internalisation of the anti-IL-9R antibody after 3 days in culture.
**Figure 30:** Cytokines secretion in ionomycin stimulated cells treated with anti-IL-9 or anti-IL-9R antibody. Cells treated with anti-IL-9 (green) downregulated the level of IL-4, IL-5, IL-9, IL-10 and IL-13 at 48 and 72 hours post ionomycin stimulation. Cytokine levels increased again at 96 hours post stimulation suggesting that the effects of anti-IL-9R antibody diminished after 72 hours post stimulation. Values were plotted as means of duplicate wells from a single preliminary experiment.
Subsequent experiments were repeated up to 72 hours post stimulation when the maximum differences in cytokine levels were observed. Blocking IL-9R significantly decreased levels of IL-4, IL-5, IL-10 and IL-13 secreted at 48 and 72 hours (p<0.05, p<0.005) (Figure 31).

However, in anti-IgE stimulated mast cell cultures, neutralising both IL-9 and IL-9R did not affect other cytokines secreted, although IL-9 levels in cells treated with anti-IL-9R were significantly reduced (*p<0.05) (Figure 32). The levels of cytokines were very low, suggesting that the IgE stimulation was not potent enough to be used to investigate the effects of IL-9 and IL-9R neutralising antibodies on cytokine secretion in BMMCs. It is also possible that the levels of cytokines were very low due to IgE stimulation not working optimally.
Figure 31: a. Cytokine expression profile of ionomycin stimulated BMMC over 72 hours post stimulation with ionomycin. Downregulation of cytokine secretion was observed in cells treated with anti-IL-9R antibody, but not in cells treated with anti-IL-9 antibody. Mean ± SEM from 3 independent experiments (n=3) is shown b. Summary of all cytokine levels at 72 hours on a log scale (pg/ml). *p<0.05, **p<0.005. Student’s t test was performed at each time point.
**Figure 32:** a. Cytokine expression profile of anti-IgE stimulated BMMC over 72 hours post stimulation with anti-IgE. Downregulation of IL-4 secretion was observed in cells treated with anti-IL-9R antibody, but all other cytokines were unaffected by treatment with anti-IL-9R or anti-IL-9 antibodies. Mean ± SEM from 3 independent experiments (n=3) is shown. Summary of all cytokine levels at 72 hours (pg/ml). *p<0.05. Student’s t test was performed at each time point.
4.2.5.3. Reversing the inhibitory effects of anti-IL-9 and anti-IL-9R neutralizing antibodies in vitro with IL-9.

In order to confirm that the effects of blocking IL-9R with mouse neutralizing IL-9R antibodies in BMMC were in fact due to an IL-9-specific inhibition, rescue experiments were done where anti-IL-9R mAb was added to BMMC culture 10 minutes prior to ionomycin stimulation. These cells were then treated with mouse recombinant IL-9 (mrIL-9), 30 minutes after antibodies and ionomycin stimulation were performed.

4.2.5.3.1. Histamine secretion

Histamine levels were measured in BMMC supernatants from this reconstitution (rescue) experiment. Previous experiments showed that adding anti-IL-9R antibody downregulated histamine secretion. The mIL-9 was first titrated at different concentrations in order to find the optimum concentration that was able to reverse the decrease of histamine levels to normal levels. As expected, by adding mIL-9 to ionomycin stimulated cells, histamine levels increased markedly (blue bar) as compared to stimulated cells (Figure 33). At 48 hours post ionomycin stimulation, cells treated with mIL-9 at different concentrations (0.01, 0.05 and 0.1ng/ml) secreted histamine at similar concentrations as the cells that were stimulated with only ionomycin. The ability of histamine secretion in anti-IL-9R antibody-treated BMMC to return to baseline levels (ionomycin stimulated cells) after rescuing with mrIL-9 suggests that the downregulation of histamine secretion was a specific effect of IL-9 due to the effects of the anti-IL-9R neutralizing antibody treatment.
4.2.5.3.2. Cytokine secretion.

BMMC supernatants from the rescue experiments were also assayed for cytokine secretion. Cells rescued with rIL-9 which had previously been treated with anti-IL-9R mAb, secreted similar levels of IL-4, IL-5, IL-10 and IL-13 as control treated cells after 72 hours post stimulation with ionomycin (Figure 34). However, IL-9, IL-10 and IL-13 levels were significantly upregulated at 48 and 72 hours post stimulation in cells treated with anti-IL-9 mAb as compared to stimulated cells as control.

This experiment was able to demonstrate that cytokine secretion in rescued cells treated with anti-IL-9R antibody returned to the baseline level (cytokine secretion in response to ionomycin stimulation) and this could support previous results described in Figure 31 and 32 of this chapter which showed that blocking IL-9R downregulate mast cell cytokine secretion.

Figure 33: Histamine secretion after rescuing with mIL-9. Histamine level in the supernatants returned back to the baseline level after rescuing BMMC with different concentrations of mIL-9. Data shows values in mean ± SD represented from 2 independent experiments.
Figure 34: Cytokine secretion level in BMMC supernatants after rescuing with mIL-9 over 72 hours post ionomycin stimulation. As compared to ionomycin stimulated cells, cytokine levels in cells treated with anti-IL-9 and anti-IL-9R antibodies remain lower and higher respectively and failed to return to baseline (ionomycin stimulated cells) after rescuing with mIL-9. All data are shown as mean±SEM from 2 independent experiments.
4.2.5.4. The effects on histamine and cytokine secretion by blocking il9 and il9r gene expression in mast cells.

In addition to inhibitory antibodies, another approach to demonstrate that IL-9 functions via IL-9R on the mast cell surface would be to block IL-9 and its receptor at the gene level. This could be achieved by using silencing RNA gene specific to IL-9 or IL-9R. This section will discuss in more detail the results from silencing RNA gene experiments to validate previous data on the effects of blocking IL-9R on mast cell functions.

4.2.5.4.1. Optimising the gene transfection.

Most gene transfection studies have used cell lines transfected with the gene of interest (402,403) and very few studies have used primary cells, in particular BMMC. A study by Zhang et al used BMMC to investigate the effect of silencing mouse phosphatase gene (404). Based on the protocol from that study, the first thing I did was to optimise the duration of transfection. The aim was to achieve a maximum silencing of the gene and to determine the level of silenced gene expression by means of quantitative PCR.

After 24 hours post siIL-9 gene transfection (Figure 35), relative to the scrambled control, the level of il9r was reduced by 30% in siIL-9 transfected cells whilst in cells transfected with siIL-9R, il9r expression decreased by 50%. When the transfected cells were cultured for longer times, after 48 hours post transfection with both siIL-9 and siIL-9R, il9r gene expression in BMMC was reduced by more than 60%, suggesting that the transfection with both silencer genes supressed il9r expression in mast cells after 48 hours post transfection. Hence, for subsequent experiments, cells were used only after 48 hours’ transfection.

When il9 was knocked down, il9r expression was also reduced. This is most likely due to positive feedback loop regulation where the presence of IL-9 is required to enhance IL-9R expression, whilst Figure 35 shows that the absence of il9 also downregulates il9r expression. This is also similar to what was
previously observed where IL-9R blocking also downregulated IL-9 protein secretion in BMMC (Figure 30 and 31). In this experiment, I also measured the levels of IL-9 mRNA after stimulating IL-9- and IL-9R- transfected BMMC with ionomycin. However, the level of IL-9 mRNA expression was undetectable in all cells (control, siRNA IL-9 and siRNA IL-9R) except in ionomycin stimulated cells alone (data not shown). This is probably due to il9 only being upregulated to detectable levels in non-treated ionomycin stimulated cells.

Besides measuring the levels of il9r gene, another way to measure transfection efficiency is by measuring IL-9R protein in transfected cells. However, due to limited numbers of cells and the main aim of this experiment being to show the function of mast cells after il9r gene suppression, only the levels of IL-9 protein were measured in this experiment.

Figure 35: Relative il9r expression in BMMC after transfection with silencer genes, il9 and il9r over 24 and 48 hours by qPCR. il9r was more than 60% downregulated only after 48 hours post-transfection. Il9r expression was measured relative to scrambled control. Data shows mean ± SEM as a representation from 3 independent experiments, *P<0.05; **P<0.005. Student’s t test was performed on this data.
4.2.5.4.2. Histamine secretion

Transfected cells were stimulated with ionomycin and histamine levels in the BMMC supernatants were measured by ELISA. Following 24 hours’ stimulation of cells transfected 48 hours previously, cells transfected with siIL-9R secreted the lowest levels of histamine as compared to cells transfected with siIL9 or scrambled controls (Figure 36). This result suggests that blocking IL-9R at the gene level could have a downregulatory effect on histamine secretion in ionomycin-stimulated BMMC, although the levels did not reach significance.

Figure 36: Histamine secretion in transfected BMMC 24 hours after stimulation with ionomycin. Cells transfected with IL-9R (siIL-9R) resulted in downregulated histamine secretion after ionomycin stimulation. Values are plotted as mean±SEM from 3 independent experiments.
4.2.5.4.2. Cytokine secretion

BMMC supernatants of ionomycin-stimulated cells were also assayed for cytokine production. A time course of secretion of Th2 cytokines IL-4, IL-9 and IL-13 is shown (Figure 37). IL-5 was not detected in this assay. As can be seen, the decreased levels of cytokines secreted were only observed 72 hours’ post ionomycin stimulation. The levels of IL-9 and IL-13 were significantly decreased in mast cells transfected with siIL-9R as compared to control cells and cells transfected with siIL-9 (Figure 37). However, the levels of IL-4 were significantly decreased in cells transfected with IL-9, and not IL-9R, as compared to scrambled controls (p<0.05). This suggests that silencing il9r and il9 downregulates the proinflammatory cytokines (IL-4, IL-9 and IL-13) secreted by mast cells. Blocking IL-9R is more effective in downregulating the secretion of proinflammatory cytokines since it also downregulated IL-9 secretion, where IL-9 was working in a positive feedback loop. This also suggests that IL-9 binds specifically to IL-9R and by blocking IL-9R, a downstream decrease in IL-9 secretion was observed followed by the downregulation of other Th2 proinflammatory cytokines (IL-4, IL-13) produced by mast cells (Figure 37).
Figure 37: Cytokine secretion over 72 hours post stimulation with ionomycin. Relative to the scrambled control, cells transfected with *il9r* demonstrated decreased levels of pro-inflammatory cytokines, especially IL-9 and IL-13. Data were plotted as a representative with mean±SEM from 3 independent experiments. P<0.05, p<0.01. Student’s t test was performed on this data at each time point.
4.3. Discussion

In this study, BMMCs were used as an *in vitro* model of mast cells to further evaluate the effect of IL-9 secreted by mast cells following stimulation. BMMCs are a commonly used mast cell population for *in vitro* studies although they have a distinct lineage from connective tissue mast cells (MCTc) which normally reside in the conjunctival tissues (405). However, during allergic inflammation, both types of mast cells (MCT and MCTc) are found in the inflamed conjunctiva of active vernal conjunctivitis sections, and it would be expected that MCT increase in allergic disease (406). BMMCs, grown in the presence of SCF and IL-3, most closely correspond with the characteristics of human mucosal type mast cells (MCT), hence, this *in vitro* model is relevant when studying ocular mast cells in allergic conjunctivitis. Human cord blood derived mast cells is another *in vitro* model that could be more relevant in studying mast cell function. However, since I had previously demonstrated IL-9 expression in mouse, using mouse mast cells to determine IL-9 function *in vitro*, BMMC was considered a more relevant source of cells than human mast cells hence, BMMC was the preferred *in vitro* model for these experiments.

There were a few technical issues that I would like to address in regard to conducting experiments in this chapter. Firstly, a wider range of antibody (anti-IL-9 and anti-IL-9R) concentrations should be used during antibody titration experiments. This would enable a correct dose response curve to be drawn by determining the optimum concentration of antibodies to be used for subsequent experiments. Although IL-9R was suppressed and resulted in downregulation of secretion of histamines and cytokines, it is also possible that a suboptimal concentration of anti-IL-9 antibody (too low) was used, which resulted in no difference being observed in the levels of histamine and cytokines after treating ionomycin-stimulated cells with anti-IL-9 antibody. Also, the fact that I was able to measure IL-9 protein in the supernatants despite anti-IL-9 having been added to the cells suggests that the wrong concentration of anti-IL-9 antibody was used in these experiments. Anti-IL-9 antibody binds to soluble IL-9 whilst anti-IL-9R antibody binds to IL-9R on the surface of mast cells. When antibodies are
not titrated properly, there will be some free circulating IL-9 and unbound IL-9R which are able to elicit downstream IL-9/IL-9R signalling upon activation, hence can give rise to false positive results on mast cell functions. However, in this study, when mast cells were treated with anti-IL-9R antibody, no IL-9 was secreted by mast cells suggesting this was not the case and that IL-9R blocking did not give false positive results. Another possible reason as to why IL-9 suppression by anti-IL-9 antibody did not work efficiently might be due to the antibody itself which was unable to bind to IL-9 within the cytoplasm of mast cells which can also cause IL-9/IL-9R activation.

Secondly, in the siRNA experiments, there could be alternative approaches for showing transfection efficiency despite monitoring the level of *il9r* expression after IL-9 and IL-9R knockdown. It is possible to measure IL-9R protein level in these cells, as another way to show the efficiency of IL-9 and IL-9R knockdown. However, since this experiment was focusing more on the functional effects on mast cells after gene knockdown, I only measured the levels of IL-9 protein in the supernatants of these cells, not IL-9R protein.

Mast cells express various types of receptors on their surface including chemokines, interleukins (IL), amines (histamines), Toll-Like Receptors, and Fc receptors. Since the aim of this study was to investigate the effects of mast cell degranulation in cytokine release, BMMCs were stimulated through FcεRI crosslinking, to mimic an allergen-specific stimulation via IgE receptor (FcεRI), which is a more physiological stimulant that mimics an allergic reaction. However, as stimulation via FcεRI is not a very potent stimulus as compared to calcium ionophore (ionomycin), it resulted in a lower concentration of secreted mast cell products such as histamine and cytokines (407). It is also possible that in these experiments, the anti-IgE stimulation was not properly optimised and resulted in technical error. Based on these possibilities with anti-IgE stimulation, I continued using ionomycin instead of FcεRI crosslinking for stimulating BMMC. It was also previously shown that FcεRI crosslinking and treatment with calcium ionophore on murine mast cell lines increased secretion of classical Th2 cytokines (408). Since ionomycin stimulation of BMMC resulted in higher levels of degranulation products as compared to stimulation via FcεRI,
it was easier to observe a larger difference in histamine and cytokine secretion in ionomycin-stimulated mast cells. Addition of PMA and ionomycin for activation is a very strong stimulus, which resulted in an even higher level of histamine and cytokine secretion. Similarly, this was observed by Plaut et al. who found that addition of PMA to ionomycin stimulation lowered the threshold ionomycin concentrations required for $[^3]$Hthymidine uptake (as a marker of cell division) by mast cells (407). In this study, Plaut and colleagues used murine mast cell lines that were derived from foetal liver. These cell lines are similar to BMMC, requiring IL-3 for growth. Hence, based on the previous study by Plaut et al., ionomycin stimulation was considered the best mode for BMMC stimulation for this current study, given that stimulation by ionomycin produced levels that were detectable by multiplex bead arrays.

In this chapter, we chose primary BMMC as the *in vitro* model to study the functions of mast cells, as these cells have many shared characteristics of human mast cells. These cells have been used extensively by other groups to study mast cell functions. More experiments can be done using these cells, as they can divide in culture in large quantities. Previous studies investigating mast cell functions have used transformed mast cell lines including rat basophilic leukaemia cells (RBL), human mast cell lines (HMC-1, LAD-1 and LAD-2) which are readily available in large quantities and easier to grow in culture. However, transformation of cell lines alters the normal functions of the cells in which KIT in HMC-1 is permanently phosphorylated, hence becoming independent of SCF. Another type of *in vitro* mast cell model would be primary cultures of mast cells derived from tissues. This was not feasible for culturing conjunctival mast cells due to the low amounts of tissue available. In mice, resident peritoneal-cell derived mast cells (PCMC) have been shown to have the characteristics of mature serosal-type mouse mast cells with retained morphological, phenotypic and functional characteristics (409). However, as compared to BMMC, PCMC secreted very low amounts of chemokines and cytokines, making it unsuitable to study the effects on cytokine secretion by mast cells.

The data included in this chapter has suggested that IL-9 downregulates BMMC histamine secretion. More than a decade ago, a study done by Temann *et al.*
reported that mast cells are significantly reduced in the lung tissues of IL-9-deficient mice that were stained with toluidine blue although the histamine levels observed in lung lavage fluid, as determined by ELISA, did not increase significantly in IL-9 expressing mice (314). A few years later, Wiener et al. found that IL-9 enhanced the ability of ionomycin-stimulated mast cells to secrete histamine (279). Through in vivo studies, they found an indirect effect on the reduction in IL-9 expression in histamine-deficient mast cells. Another study reported the effect of IL-9 to increase histamine release in a mast cell line which then led to an exacerbation of neonatal exocytotoxic damage in a mouse model of cerebral palsy (410) which was also shown previously (400).

The functional effect of blocking IL-9 and its receptor (IL-9R) on surface expression of the IgE receptor (FcεRI) on the BMMCs was investigated. Blocking IL-9R in vitro resulted in the downregulation of FcεRI surface expression. In 1993, IL-9 was first shown to promote IgE production via IL-4-induced human B cells (300) and later this was supported by others (316). Our findings are consistent with another study which demonstrated that mRNA expression of FcεRI was upregulated in the presence of IL-9 in a few mast cell lines including primary mast cell cultures, BMMC (411). Since FcεRI is also expressed on the surface of other immune cells including neutrophils, an interesting study has suggested that IL-9, not IL-4, has a direct effect on neutrophils by increasing FcεRI expression in neutrophils isolated from allergic asthmatics (412).

Since the il9 gene is located in the exon in close proximity to other Th2 cytokine genes (il5 and il13), it was postulated that IL-9 secretion could also be linked to the secretion of other Th2 cytokines. Previously, it has been shown that IL-9 can have co-stimulatory functions with ionomycin and antigen-specific IgE to stimulate secretion of several Th2 cytokines (IL-4, IL-5, IL-9, IL-13) by BMMC (279). Similarly, the in vitro study of IL-9 neutralisation described herein was to investigate the functional role of IL-9 on mast cell cytokine secretion but my results suggested otherwise. In my system, blocking IL-9 function in vitro alone did not have a significant effect on cytokines secreted by mast cells. This was probably due to the nature of the antibody which only binds to secreted IL-9, but
not the IL-9 that might still be stored within the cytoplasmic granules of mast cells. Upon secretion of this IL-9 protein, it can further bind to the IL-9R, thus activating further downstream signalling for Th2 cytokine secretion.

Hence, it was thought that since IL-9 binds specifically to its receptor (IL-9R), blocking the IL-9R could also be a more effective approach for downregulating Th2 cytokine secretion by BMMC. In this chapter, for the first time, I have been able to demonstrate that blocking IL-9R is effective in downregulating mast cell cytokine secretion. This novel finding suggests that by blocking IL-9R, soluble IL-9 will be prevented from binding to its receptor, thus blocking further downstream signalling for cytokine secretion.

Results in this chapter also suggested that blocking *il9* and *il9r* at the gene level resulted in similar effects to those seen when neutralising antibodies were used. In most transfection experiments, gene suppression can only be achieved 48 hours' post transfection with small interfering RNA, which is the same time frame required for gene transfection to occur in this study. 48 hours was required for gene suppression to occur probably due to the recently plated cells that need time to recover within the first 24 hours prior for the transfection to occur. The effect of *il9r* blocking can only be observed at 72 hours post ionomycin stimulation, 24 hours later than the effect observed in mast cells treated with blocking IL-9R with neutralising antibody. This was probably due to the effect of gene silencing that might take longer time to exert a regulatory effect as compared to when just adding neutralising antibodies that have immediate regulatory effects.

Since IL-9 secretion was also downregulated upon IL-9R blocking (in both conditions with anti-IL-9R antibody and siil9), this study demonstrated that secreted IL-9 binds specifically to mast cell IL-9R in an autocrine manner to amplify inflammatory responses. This positive feedback loop of IL-9 secretion suggests that IL-9 and other Th2 cytokines could be secreted via the activation of MAPK signalling downstream of IL-9R activation. IL-9 positively regulates cytokine production from mast cells. Based on this study, it is postulated that blocking the function of IL-9R on the surface of mast cells can modulate
cytokine secretion from mast cells (Figure 38). In this chapter, we have demonstrated that IL-9, which is secreted by mast cells, could have functional stimulatory effects on mast cell degranulation, including histamine and cytokine secretion.

Figure 38: Schematic diagram of proposed mechanism of IL-9 on mast cells via IL-9R which then leads to the secretion of other mast cell mediators including cytokines that causes the symptoms observed during allergic conjunctivitis.
Chapter 5

IL-9 Expression in Human Conjunctival tissues
5.1. Introduction

Initially, in chapter 3, it was shown that IL-9 was upregulated in the conjunctivae of EAC mice and, besides CD4\(^+\) T cells, mast cells were reported to be one of the cell types which secrete IL-9 (as discussed in Chapter 1). In chapter 4, further in vitro experiments were performed using BMMC as a mast cell in vitro model in order to understand the role of IL-9 on mast cell functions. In this chapter, I investigate IL-9 expression levels in human conjunctival tissues.

It has been well established that IL-9 supports allergic inflammation, especially in promoting mucus production and mastocytosis in the lung of an allergic airway disease mouse model (251). IL-9 has been detected in the lungs of asthmatic patients and its overexpression results in an asthma-like phenotype of inflammation and bronchial hyperresponsiveness (314,413).

Besides studies on the role of IL-9 in asthma patients, many other studies have also investigated the contribution of IL-9 in allergic diseases including allergic rhinitis, food allergy and allergic skin disease. Recently, it was concluded that IL-9 plays a role in regulating allergen-specific Th1 responses in allergic contact dermatitis (414), while Ma and colleagues found that IL-9 and Th9 cells were upregulated in atopic dermatitis patients (415).

It is understood that IL-9 plays an important role in driving allergic diseases and studies are now focused on understanding the mechanisms by which IL-9 drives allergic responses. However, it is still not known whether IL-9 plays a role in the pathogenesis of allergic conjunctivitis. Based on findings of upregulation of IL-9 in allergic diseases, we hypothesise that IL-9 is present and upregulated in patients with allergic conjunctivitis.
To address this hypothesis, the specific aims were as follows:

1. To compare the expression of IL-9 in donor conjunctival biopsies from allergen-challenged, out-of-season AC individuals with normal, unchallenged controls, using immunohistochemistry staining.
2. To investigate whether IL-9 in these biopsies is expressed by mast cells using confocal microscopy to visualise immunofluorescence stained tissue sections.
3. To analyse the proportion of IL-9- and IL-9R-expressing cells that are mast cells (tryptase positive) with those IL-9 and IL-9R-expressing cells that are tryptase negative.
5.2. Results

5.2.1. Determining polyclonal Ab (pAb) specificity for human IL-9 by ELISA.

At the time of undertaking this study, the only anti-human IL-9 Ab available for tissues staining was a polyclonal Ab. To ensure that the polyclonal Ab (pAb) recognising human IL-9 used for experiments in this chapter will bind specifically to IL-9, antibody validation experiments were done by Dr. Malihe Eskandarpour. Firstly, an ELISA plate was coated with rh IL-9 and subsequently incubated with different concentrations of rabbit polyclonal anti-human IL-9 Ab (Abcam Ab111915). Figure 39A shows that OD value increased with increasing concentrations of IL-9 Ab, suggesting that IL-9 Ab at increasing concentrations binds to rhIL-9, which resulted in increased absorbance values. A 1:1000 dilution factor for IL-9 Ab was chosen for subsequent experiments since, at this concentration, IL-9 Ab produced an absorbance value which was halfway between the baseline and the maximum value.

Subsequent experiments were done to confirm that the anti-IL-9 Ab binds specifically to rh IL-9, even in the presence of neutralising human IL-9 Ab. In a plate which was pre-coated with rh IL-9, the anti-IL-9 Ab competed with neutralising human IL-9 Ab and neutralising mouse IL-9 antibody respectively. Two neutralising antibodies against mouse and human were used in this experiment in order to confirm that this antibody did not cross-react between different species. Figure 39B shows that at increasing neutralising human IL-9 Ab concentrations, absorbance values decreased. This shows that neutralising human IL-9 Ab can bind to rhIL-9 at increasing concentrations and competes with anti-IL-9 Ab binding to rhIL-9 on the plate, resulting in reduced absorbance values. On the other hand, similar absorbance values were observed in plates with rhIL-9 which was subsequently incubated with IL-9 Ab, in the presence of increasing concentrations of neutralising mouse IL-9 Ab. This data shows that unlike neutralising human IL-9 Ab, neutralising mouse Ab binds less specifically to IL-9 Ab, which allows IL-9 Ab to bind to rhIL-9 on the plate and resulted in similar absorbance values across increasing concentration of neutralising mouse IL-9 Ab (Figure 39C). This experiment shows that the IL-9 pAb used for
human conjunctival tissue immunostaining is specific for IL-9, although it does not demonstrate whether the pAb binds to other proteins.

Figure 39: Absorbance values from IL-9 pAb validation experiments. Data shows absorbance values from plates incubated with increasing concentration of: A. IL-9 pAB; B. Neutralising human IL-9 Ab; C. Neutralising mouse IL-9 antibody. Data are presented as mean±SD from triplicate wells.
5.2.2. IL-9 and IL-9R expression in human conjunctival tissue sections.

In order to investigate the baseline expression of IL-9 in normal, uninflamed tissues, non-AC control human conjunctival biopsy sections were stained with polyclonal anti-IL-9 antibody. The same anti-IL-9 antibody, which was used for staining these conjunctival biopsies shown in this chapter was previously validated by ELISA (as explained in 5.2.1).

Sequential biopsy sections were singly stained for tryptase (as a marker for mast cells) as the positive control for the staining, and for IL-9, to investigate IL-9 colocalization with mast cells. For negative controls, primary antibody was replaced with water. A few attempts were made to repeat the staining however, due to the quality of the tissues and limited tissue availability, staining on these conjunctival biopsies was unable to be repeated.

5.2.2.1. Immunohistochemistry- Healthy tissues

Both IL-9 and tryptase were expressed in normal uninflamed conjunctivae and these positive cells were localised predominantly within the vessels (Figure 40). These sections were obtained from non-allergic, non-inflamed donors.
Figure 40: Expression of IL-9 protein (immunohistochemistry) in sequential sections of normal, unchallenged tissues. a. Unstained tissue section incubated without primary antibody as negative control. b. IL-9 positive cells are seen within the vessels (x200 magnification). c. IL-9 positive cells at higher magnification (x400). d. Tryptase positive cells (marker for mast cells) within vessels (x200). e. Tryptase positive cells at (x400). Scale bar: 20µm.
Since a low level of IL-9 expression was detected at baseline in normal, uninflamed tissues, this was compared with IL-9 expression in allergen-challenged SAC tissues. Allergen-challenged SAC tissues were stained for IL-9 and tryptase. These tissue sections were obtained from a previous clinical trial which investigated the role of a chemokine inhibitor in donors with SAC. In this trial, there were two groups; the control group comprising challenged SAC donors receiving placebo, and the treatment group comprising challenged SAC donors who received the treatment. Therefore, I was unable to obtain sections from a non-allergen challenged SAC group as the study did not include non-allergen challenged SAC donors.

In allergen-challenged AC tissues, both IL-9 and tryptase positive cells were found to be localised within the subepithelial and stromal layers of the tissues (Figure 41). This suggests that in normal tissues, IL-9 positive staining that colocalised to areas containing mast cells was contained within the vessels and that, during allergen challenge (as seen in the challenged AC biopsies), IL-9-expressing mast cells migrate to stromal and sub epithelial layers. Since IL-9 positive cells and tryptase expressing cells were seen within the same areas of the sequential sections, it is postulated that mast cells express IL-9 in conjunctival tissues of both normal and challenged AC donors. Cells stained positively for IL-9 were then counted in a masked fashion where two independent, masked observers were blinded by using numbers allocated to each slide without knowing the details of the slides. A total of three random fields per section were recorded. IL-9 expression was significantly upregulated in challenged AC tissues (n= 8) as compared to normal tissues (p<0.01; n=8) (Figure 42).
Figure 41: Expression of IL-9 protein in sequential sections of challenged AC (Allergic Conjunctivitis) tissues. AC eyes were challenged with mixed grass pollen extract. SAC section stained only with secondary antibody as control. IL-9 positive cells are seen within sub-epithelial and stromal layers of the tissue (b, d, and f.). Tryptase positive cells (c, e, and g) are also seen colocalised with IL-9 positive cells in the sub-epithelial layers (x200 and x400 magnification). Scale bar: 20μm.
Figure 42: Total IL-9 and tryptase expressing cells. IL-9 and tryptase expressing cells were counted and data normalized from 3 separate fields. Data were shown as means±SEM of 8 donors per group. AC = allergen-challenged. *P<0.001
5.2.2.3. Immunofluorescence staining- Healthy and allergen challenged tissues

Besides IL-9, sections were also stained for IL-9 receptor (IL-9R) as an indication of which cells could respond to IL-9. Sections were stained by three-colour immunofluorescence staining for IL-9 or IL-9R, tryptase and DAPI (fluorescent dye that bind strongly to DNA to detect nuclei on tissue sections) on the same slide and visualised by confocal microscopy (Zeiss 710). Three-colour staining was done in order to determine the proportion of IL-9 and IL-9R expressing mast cells. Double-positive cells (yellow), suggesting the colocalisation of IL-9, or IL9R, and tryptase, were counted in at least 3 fields.

Similar to the previous immunohistochemical single colour staining results, with normal unchallenged tissue sections, IL-9 and IL-9R expressing mast cells (yellow) were seen within the vessels, whereas few mast cells (stained in red) were seen in the tissue stroma (Figure 43). However, in allergen challenged tissues, more cells stained positively for IL-9 and IL-9R (Figure 44). Double positive cells (stained yellow) for both IL-9/IL-9R and tryptase are seen within the stromal areas of the tissues. There were also some IL-9 and IL-9R positively stained cells that did not colocalise with tryptase-positive mast cells.

IL-9 and IL-9R expressing cells were counted within 3 fields per slide and were expressed in total number of cells. Figures 45a and b depict the absolute number of IL-9 and IL-9R positive cells that colocalised with mast cells, whilst Figure 45c and d shows absolute numbers of IL-9 and IL-9R positive cells that did not colocalise with mast cells. Based on figures 45a and b, mast cells in both normal and challenged tissues expressed both IL-9 and IL-9R and there were significantly more mast cells expressing IL-9 and IL-9R in challenged biopsies as compared to control (p<0.05). When compared to control conjunctivae, there are significantly more IL-9 and IL-9R expressing cells that did not colocalise with mast cells (single green colour staining) in challenged biopsies (Figure 45c and d) (p<0.005, p<0.0005; n=8), suggesting that IL-9 is also secreted by other immune cells (for example eosinophils, CD4+T cells and ILC) during allergic responses. However, due to limitations of time and
availability of tissue biopsies, and given that the aim of this study was to investigate IL-9 and IL-9R expression on mast cells, no further staining was performed to investigate the other cell types which also expressed IL-9 and IL-9R in these biopsies.
Figure 43: IL-9 and IL-9R expression in normal unchallenged tissues. a and b. IL-9 positive cells (green) are seen within the vessels whereas a few mast cell (red) are seen within the stromal area of the tissues, c and d. IL-9R and mast cell double-positive cells (yellow) are seen within the vessels. e. negative control. Scale bar: 20μm. Arrows indicate double positive cells which are IL-9/IL-9R positive mast cell positive.
Figure 44: IL-9 and IL-9R expression in challenged tissues. a and b. IL-9 positive cells and c and d. IL-9R positive cells which colocalise with mast cells (yellow) are seen within the stromal areas of tissues. A few IL-9 positive cells (green) which are not mast cells are also seen within this area. X400 magnification. e. negative control. Scale bar: 20μm. Arrows indicate double positive cells which are IL-9/IL-9R positive mast cell positive.
Figure 45: Absolute numbers of IL-9 and IL-9R expressing cells. IL-9 and IL-9R expressing cells were counted from 3 separate fields. Data were shown as means +/- SEM of 8 donor specimens per group. Total number of IL-9 a. and IL-9R b. positive and tryptase positive cells (double positive) in normal and challenged biopsies. Total number of c. IL-9 and d. IL-9R positive cells but tryptase negative in both normal and challenged AC tissues. *p<0.05, **p<0.005, ***p<0.0005.
5.3. DISCUSSION

IL-9 has long been associated with allergic diseases, especially in asthma, where this has been supported by a recent genome-wide association study which has identified that severe asthmatic patients with IL-9 polymorphisms are more likely to interact with house dust mite and can lead to exacerbations of severe asthma (416). Besides asthma, increased IL-9 levels have also been associated with other allergic diseases including food allergy, allergic rhinitis, and eczema. However, there are still no reports on the role of IL-9 in allergic conjunctivitis. Data shown in previous chapters supported the concept that IL-9 is upregulated in a mouse model of allergic conjunctivitis and that mast cells were a source of IL-9 in these tissues. Further in vitro studies shown in chapter 4 were carried out in order to understand the role of IL-9 on mast cell function. The next aim was to investigate IL-9 levels in allergic conjunctivitis in human conjunctival tissues. Since the level of IL-9 is known to be upregulated in allergic asthma and given that the conjunctiva is also a mucosal surface, we postulated that IL-9 might also be involved in allergic conjunctivitis. First, we needed to investigate the expression of IL-9 in the conjunctiva of AC donors with and without allergen challenge. However, due to the study design that initially took place, only allergen-challenged out-of-season SAC samples were obtained, but not conjunctiva of AC donors without allergen challenge. Hence, in this study, instead of using conjunctiva of AC donors without allergen challenge, I used normal conjunctivae without SAC as control sections.

Despite several studies which have investigated the role of cytokines in eye diseases, including the roles of pro-inflammatory cytokines in allergic conjunctivitis (347,417), there have been very few studies focussing on the role of IL-9 in eye diseases, in particular AC. In an in vivo study on experimental autoimmune uveoretinitis (EAU), it was found that IL-9 was upregulated in the splenocytes and draining lymph nodes of EAU mice (418). Recently, it was also shown that IL-9 produced by Th9 cells drive inflammation in the eye (264,382).
There are a few technical issues which I would like to address in this chapter. Firstly, this experiment did not include unchallenged SAC samples due to the design of the initial study, which did not include unchallenged SAC. It would have been ideal to obtain unchallenged AC samples due to the fact that baseline IL-9 and IL-9R expression might also be upregulated in these samples, although since all patients recruited to that trial were “out-of-season”, it is unlikely.

In this chapter, a polyclonal IL-9 antibody was used for immunohistochemical staining instead of a monoclonal antibody, as that was unavailable at the time. A monoclonal antibody would have been preferable to use for immunostaining since it would bind more specifically to the designated IL-9 epitopes on tissue sections. Polyclonal antibodies are a heterogenous mixture of antibody which recognizes different epitopes from an antigen. Different antibodies are produced against these different epitopes and then harvested from the serum of the animal directly. Monoclonal antibodies on the other hand are produced from single clone of B cell which recognizes and bind to single epitope on the antigen. For immunostaining purposes, polyclonal antibody is not suitable since it binds to many different epitopes of specific antigen and could cause false positive binding. In this chapter, an attempt to show IL-9 pAb specificity by ELISA was not sufficient since this experiment only showed that IL-9 pAb could bind specifically to rhIL-9, but did not take into account the other possibility that other immunoglobulin which present in the antibody could bind non-specifically on other epitopes expressed in the conjunctival tissues. Hence, a better way to demonstrate IL-9 pAb specificity is by doing Western blot, which was not done in this study due to technical limitations.

Based on immunostaining of human conjunctival tissue sections, it was found here that IL-9 expression was upregulated in challenged AC tissues as compared to normal biopsies. Tryptase positive staining was used to identify mast cells in AC. It is already well known that mast cells are upregulated in the lamina propria of SAC conjunctiva (419). To date, no studies have investigated the presence of IL-9 in the conjunctiva. However, Metz et al. previously
discovered the presence of Th2 proinflammatory cytokines namely IL-4, IL-5 and IL-13 in the conjunctiva of VKC patients using *in situ* hybridization (207). This is the first time that IL-9 has been shown to be present in the conjunctiva of both normal and challenged SAC tissues and our findings demonstrated that IL-9 expression levels were upregulated in challenged SAC tissues.

Since increased numbers of tryptase-positive and IL-9-positive cells in the same area of the tissues was observed by light microscopy, these IL-9 positive cells were thought to be mast cells using serial sections, although this could not be confirmed using light microscopy. Double immunofluorescence staining confirmed that IL-9 expression colocalised with tryptase expression in some cells, suggesting that the mast cell can potentially secrete IL-9 in the conjunctiva during allergen challenge.

Double immunofluorescence staining also revealed that IL-9 and IL-9 receptor (IL-9R) positive cells also colocalised with tryptase positive cells, suggesting that mast cells in the conjunctiva express IL-9 and IL-9R and these levels increased significantly in challenged biopsies as compared to control biopsies. This study shows that in challenged biopsies, there are more IL-9/IL-9R positive mast cells which might contribute to the symptoms seen in AC.

Although no studies have investigated the expression of IL-9 and IL-9R in conjunctivae, Lin and colleagues have investigated the expression of IL-9 and IL-9R protein in the nasal tissue sections of control subjects and patients with chronic rhinosinusitis, where they found that IL-9 and IL-9R were both present in control tissues but these levels were upregulated in patients with chronic rhinosinusitis. In Lin’s study, IL-9 and IL-9R positive cells were seen as positively stained mucosal epithelial and submucosal inflammatory cells (420). This IL-9 and IL-9R mucosal study confirmed the presence of IL-9 and IL-9R positive cells in the nasal mucosa in normal control tissues similar to our study of the conjunctiva. Apart from the nasal mucosa, IL-9 and IL-9R are also highly expressed in the mucosa of patients with ulcerative colitis (421).
Based on double immunofluorescence staining, my study has found that IL-9R is expressed on the surface of mast cells in conjunctival biopsies. This result is also in line with a study done by Kearly et al., where they found that IL-9R is present on the surface of mast cells in the lung of mice with chronic allergic asthma (328).

Immunostaining of IL-9 and IL-9R in the conjunctiva of both normal and allergen-challenged SAC biopsies revealed that some of the IL-9+/IL-9R+ cells were also positive for tryptase, suggesting that the mast cell secretes IL-9 and expresses IL-9R. However, in allergen-challenged conjunctival biopsies, a considerable proportion of IL-9 and IL-9R positive cells did not colocalise with tryptase (tryptase negative). This suggests that other cells secrete IL-9 and express IL-9R during allergen challenge. IL-9R is present on the surface of T lymphocytes, eosinophils, mast cells and also epithelial cells. Since in SAC there are very few T cells present (72), they are unlikely to be a source of IL-9 in these tissues. Eosinophils could be the IL-9-expressing, tryptase-negative cells since this cell type was also found to be upregulated in the conjunctiva during AC (422) and they have also been reported to secrete high amounts of IL-9 during inflammation (423). Other cell types that also secrete IL-9 and express IL-9R during allergy include CD4+T cells and ILCs, although there is no direct evidence of the contribution of these cells and IL-9 secretion during AC.

Another way to confirm the colocalisation of IL-9 and mast cells is through in situ hybridisation staining, looking at the expression of IL-9 mRNA and mast cells, as previously shown for nasal biopsies of allergic rhinitis patients (276). However, I did not perform that technique in my studies. Given more time, and sample availability, it would have been an opportunity to be able to explore other types of cells including eosinophils, CD4+ T cells and ILCs, which also secrete IL-9 in the conjunctiva during AC.

In summary, despite a polyclonal anti-IL-9 Ab being used for staining tissue sections we could still postulate that IL-9 was present in both healthy controls and challenged biopsy sections and the level was upregulated on cells outside the blood vessels, in the stromal tissues, in allergen-challenged biopsies. These
IL-9-positive cells colocalised with mast cells but, in the allergen-challenged biopsies, other cells were also a source of IL-9. Besides conjunctival tissues, IL-9 was also found to be increased in the cell-free tear fluids of VKC patients relative to healthy control tears, and this level increased with disease severity (424). This data may suggest that IL-9 could play an important contribution in chronic allergic conjunctivitis and further studies with VKC/AKC tissues will be performed to investigate IL-9 expression in more clinically severe forms of AC.
CHAPTER 6

The Role of IL-9 on Conjunctival Epithelial Cells
6.1. INTRODUCTION

IL-9 is a pleiotropic cytokine that exerts a wide spectrum of functions on hematopoietic and non-hematopoietic cells. Apart from its role on the survival and proliferation of T cells and mast cells (297,425), IL-9 is also important in modulating B cell function by upregulating IL-4-mediated IgE and IgG production from human B cells (300). IL-9 specifically binds to IL-9Rα to deliver its signals into target cells. The IL-9R was reported to be expressed on the surface of eosinophils, as shown in a mouse model of allergic airway inflammation, where blocking the function of IL-5 in mice overexpressing IL-9 in the lungs resulted in reduced eosinophil recruitment (387), suggesting that eosinophil functions were affected by IL-9 indirectly via IL-5 secretion. More recently, IL-9 was shown to promote Th2 cytokine expression and to regulate the functions of innate lymphoid cells (ILCs) (281). Besides its influences on immune cells, IL-9 also directly affects tissue resident cells including epithelial cells. A few studies have investigated the role of IL-9 on lung epithelial cells in allergic airway disease and studies in this thesis have demonstrated that IL-9 is expressed on epithelial cells in human conjunctival tissues and in mouse models of allergic conjunctivitis. Based on these findings, I was interested in determining the functional effects of IL-9 on conjunctival epithelial cell function. The aim of this chapter was to investigate the effects of IL-9 added exogenously to human conjunctival epithelial cells, using a well-known in vitro human conjunctival spontaneously transformed epithelial cell line (IOBA-NHC).

Normally, both stratified squamous and stratified columnar epithelial cells play important roles by forming a barrier, which is the first line of defence against external microorganisms and allergens. However, in the lung, epithelial cells play another important role in the regulation of immune cells. During allergic airway inflammation, epithelial cells secrete TSLP and IL-33 to enhance immune cell recruitment, promoting Th2 and Th9 cells and, in studies involving functional blocking of IL-9 in mice with allergic airway inflammation, this downregulates TSLP-induced lung inflammation (260).
Conjunctival epithelial cells contain goblet cells that secrete mucus in response to a range of stimuli. Increased mucus secretion occurs due to the expansion in the numbers of goblet cells in the epithelium. IL-9 is known to cause an increase in secretion of mucus as well as goblet cell expansion (goblet cell hyperplasia), in the lung epithelium. Various studies have shown that in the lung and colon of IL-9 transgenic mice, mucus production and mucus-associated genes including Muc2, Muc5ac and TFF2 (mucus associated peptide that is responsible for mucus viscosity) mediated by IL-13 was upregulated (246,314,388,426). Although mucus upregulation by IL-9 was mediated by IL-13, IL-9 did not synergise with IL-13 and IL-9 alone did not induce goblet cell hyperplasia, as observed in paediatric bronchial epithelial cells (427). Absence of IL-9 in mice did not affect the level of mucus secretion, suggesting that IL-13 is probably able to compensate for the effects of IL-9 on lung epithelium (329).

Encoded by MUC genes, mucins are synthesised by both goblet cells and submucosal glands. There are 9 different derivations of MUC genes. Both MUC2 and MUC5ac (gel forming mucin) are found to be present in human conjunctival tissues (350) and are expressed in the epithelium (428).

In this chapter, different cytokines were used to compare their effects on the levels of MUC mRNA expressions in the conjunctival epithelial cells in vitro. The panel of cytokines consisted of IL-1β, IL-9, IL-13 and IL-17α. IL-1β, IL-13 and IL-17α, which are known to upregulate mucins in the epithelium and can be used as controls to compare MUC mRNA in cells treated with IL-9. These cytokines have also been shown to be present in the tear fluids of AC patients (64,424,429), hence these cytokines were considered relevant for this study. Since both MUC2 and MUC5ac genes are consistently associated with goblet cells, these two MUC genes were used as markers for mucin in this chapter. However, only MUC2, and not MUC5ac, gene expression had previously been reported in IOBA-NHC cells (367).

The ocular surface is constantly exposed to a myriad of pathogens and allergens from the environment. In order to maintain ocular surface protection, molecular components that seal the space in between adjacent ocular surface
epithelial cells like TJ and a second layer of protection (the transcellular barrier) is provided by membrane-associated mucins, which are available at the ocular surface. During allergy and inflammation, cells, cytokines, pathogens and allergens are known to disrupt these barriers, enabling pathogens and allergens to penetrate through the ocular surface and cause further immune-mediated tissue responses.

Besides upregulation of mucin production, IL-9 also regulates epithelial barrier function. Since the epithelial tight junction is important in ensuring the integrity of the epithelium and preventing allergen penetration, studies have investigated the role of IL-9 in maintaining epithelial tight junctions. Mutations of the filaggrin gene that contribute to barrier function especially in the skin were found to be associated with a predisposition to atopic dermatitis (AD) (430). The junctional adhesion superfamily of tight junctions including zonula occludens (ZO) 1-3, occludin and claudins 1-5, was also shown to be important in regulating the lung and nasal mucosa epithelium integrity during allergic asthma and allergic rhinitis (431,432). While one study using a mouse model of induced intestinal anaphylaxis demonstrated that IL-9 had an indirect effect in altering intestinal permeability via mast cell activity (433), another study found that IL-9 exerted a direct effect on maintaining intestinal barrier integrity in an oxazolone-induced colitis model in mice (375).

All of the aforementioned studies demonstrated functional effects of IL-9 on the mucosal epithelium. Despite studies aiming to understand the mechanisms of AC at the epithelium, to my knowledge no study has directly investigated the functional properties of IL-9 on the conjunctival epithelium. Here, the aim of this chapter was to investigate the functions of IL-9 on conjunctival epithelial cells. All of our experiments used the fully differentiated and spontaneously transformed human conjunctival epithelial cell line (IOBA-NHC) (367) as the in vitro model with which to study the effect of IL-9 on the conjunctiva. This cell line has previously been used to study immunomodulatory functions within the conjunctival epithelium (434,435). The IOBA-NHC cell line has the characteristics of primary human conjunctival epithelial cells (367), that is characterized by the presence of neutral mucin-like glycoprotein which stained
positively with periodic acid Schiff (PAS). These cells also express EFGR, ICAM-1 and HLA-DR, secrete mucus and form a barrier with resistance, hence making it a good \textit{in vitro} model for the purpose of this study. Many of the functions of IL-9 previously reported include upregulating mucin secretion on epithelial cells. In preliminary studies, our group had observed an upregulation of MUC2 mRNA following exposure of the IOBA-NHC cells to hrIL-9 (Calder \textit{et al}, unpublished preliminary data).

My hypothesis was that IL-9 upregulates mucus production and supports barrier integrity of conjunctival epithelium. This chapter will describe the effects of IL-9 on human conjunctival epithelial cells.
To address this hypothesis, the specific aims were as follows:

1. To investigate the effects of exogenously added IL-9 and other cytokines in MUC gene expression on conjunctival epithelial cells and to compare with the effects of these cytokines on human bronchial epithelial cells.

2. To investigate IL-9R expression on human conjunctival epithelium of AC tissue sections and the IOBA-NHC cell line.

3. To investigate the effects of IL-9 and other cytokines on conjunctival epithelial cell tight junctions.
6.2. Results

6.2.1. Upregulation of MUC mRNA in conjunctival epithelial cells in response to pro-inflammatory cytokines.

Mucin is secreted by goblet cells in the epithelium in response to inflammation and its secretion is important to dampen down inflammatory responses. There are different mucin subtypes (MUC) and the ones that are commonly found in the conjunctiva are MUC1, 2, 4, 5ac and 16. However, since MUC2 was known to be expressed in IOBA-NHC cells (367), we first assayed the effects of IL-9 as well as other cytokines on the level of MUC2 mRNA expression using 80% confluent IOBA-NHC cells plated in 6-well plates with triplicate wells per condition. The cytokines (IL-1β, IL-9, IL-13 and IL-17α) were first titrated in order to observe the optimal concentration for upregulating MUC2 expression in IOBA-NHC cells with an exposure time of 24 hours (based on unpublished work previously done in the lab). (Figure 46) illustrates the experimental design, including the different concentrations of cytokines that were assayed and fold change calculated for MUC2 mRNA expression at different cytokine concentrations relative to untreated controls cells. Data were analysed statistically using the student’s t test. MUC2 mRNA expression was upregulated in a dose-dependent manner in response to treatment with IL-1β, IL-9 or IL-13. However, only a very low concentration of IL-17A (0.2ng/ml) was required to upregulate MUC2 gene expression (p<0.005). In subsequent experiments, 20, 10, 40 and 0.2 ng/ml of IL-1β, IL-9, IL-13 and IL-17α respectively, were added to IOBA cells.
Figure 46: Fold change of MUC2 mRNA expression upon exposure to IL-1β, IL-9, IL-13 and IL-17α stimulation at different concentration relative to unstimulated cells. Data were plotted as mean±SEM from three independent experiments, *p<0.05, **p<0.005, and analysed by student’s T test.
Having demonstrated which concentrations of cytokines were able to upregulate MUC2 expression, the next study involved determining the time course of MUC2 upregulation. In this study, each cytokine was added to 80% confluent wells of IOBA cells and left for 2, 6 and 24 hours in culture. In this experiment, the mRNA expression levels in both MUC2 and MUC5ac were assayed. Relative to untreated cells, MUC2 mRNA expression was not upregulated at 2 hours’ post cytokine exposure in IOBA-NHC cells treated with all cytokines (Figure 47) However, MUC2 mRNA expression was significantly downregulated at 6 hours post IL-13 exposure (P<0.05). The effects of all cytokines on MUC2 mRNA expression by IOBA cells were detected at 24 hours’ treatment. At this timepoint, all cytokines in the panel induced an increase in MUC2 mRNA expression, especially in those cells exposed to IL-9, where MUC2 mRNA expression was significantly increased by more than 2-fold relative to untreated cells (P<0.05).
Figure 47: Fold change of MUC2 mRNA expression level (relative to unstimulated cells) in IOBA-NHC cells normalized to β-actin upon exposure to different cytokines at 2, 6 and 24 hours post stimulation. 20, 10, 40, and 0.2 ng/ml of IL-1β, IL-9, IL-13 and IL-17α respectively, were added to IOBA cells. Data were plotted as mean±SEM from four independent experiments, *p<0.05 and analysed by student’s T test.
I also investigated the expression of MUC5ac mRNA expression in this model. The cytokines induced different levels of MUC5ac mRNA expression as compared to MUC2 gene expression (Figure 48). Most cytokines did not affect the levels of MUC5ac gene expressions at 2 hours’ post stimulation. However, MUC5ac mRNA expression in IOBA-NHC cells was significantly downregulated after 6 hours of treatment to IL-9 or IL-13, (p<0.005, p<0.0005, respectively). After 24 hours post cytokine exposure, only cells exposed to IL-1β and IL-17A had increased levels of MUC5ac mRNA expression although the levels did not reach significance.

To compare results obtained with the IOBA-NHC conjunctival cell line, I also investigated the effect of these cytokines on a well-established lung epithelial cell line (HBE-16). To investigate the contribution of IL-9 and other cytokines (IL-1β, IL-3 and IL-17A) on MUC2 mRNA expression on human lung epithelial cells, these cytokines were added to the HBE-16 cell line in culture and left for 2 and 6 hours. IL-9 had no effect on MUC2 expression even at 6 hours post stimulation. In contrast, at 2 hours’ exposure MUC2 expression upregulated in cells treated with IL-13 and IL-17A (Figure 49). IL-13 also consistently upregulated MUC2 expression level even at 6 hours post IL-13 stimulation.
Figure 48: Fold change of MUC5ac mRNA expression level (relative to unstimulated cells) in IOBA-NHC cells normalized to β-actin upon exposure to different cytokines at 2, 6 and 24 hours post stimulation. 20, 10, 40 and 0.2 ng/ml of IL-1β, IL-9, IL-13 and IL-17α respectively, were added to IOBA cells.

Data were plotted as mean±SEM from 4 independent experiments, **p<0.005, ***p<0.0005 and analysed by student’s T test.
Figure 49: Fold change of MUC2 mRNA expression level (relative to unstimulated cells) in HBE-16 cells normalized to β-actin upon exposure to different cytokines at 2 and 6 hours post stimulation. 20, 10, 40, and 0.2 ng/ml of IL-1β, IL-9, IL-13 and IL-17α respectively, were added to IOBA cells Data were plotted as mean±SD from a single experiment and analysed by student’s T test.

6.2.2. IL-9R expression on the surface of a human conjunctival epithelial cell line.

IL-9R is expressed on different cell types including T helper cells, mast cells and on the surface of epithelium (as previously discussed in chapter 4). So far, it was already reported that IL-9R is expressed on the surface of human lung epithelium (363,436) but the presence of IL-9R on the surface of conjunctival epithelium was still unknown. In order to investigate the presence of IL-9R on the conjunctival epithelium, IOBA-NHC cells were stained with anti-IL-9R antibody. As seen in (Figure 50) IL-9R (green) is highly expressed on the surface of IOBA-NHC cells, whilst MUC2 expression (red) was localised to the cytoplasm.
Figure 50: Immunofluorescent staining of IOBA-NHC cell line with IL-9R (green, pink arrows), MUC2 (red) in a. 200x magnification and b. at 400x magnification c. negative control staining x200 without primary antibodies.
6.2.3. Effects of cytokines on conjunctival epithelial cell tight junctions.

Besides upregulation of mucins, we also investigated the effects of IL-9 on the epithelial cell tight junctions of IOBA-NHC cell monolayers. Tight junctions (TJ) that maintain the intracellular junctional complexes of an epithelial cell layer are composed of transmembrane proteins occludin, claudins and junctional adhesion molecules (refer to Chapter 1, section I.6.1.2.). Integral TJ protein, occludin and TJ plaque proteins which express PZD domains, zonal occludin 1 (ZO-1) that serve as the links between integral TJ proteins and actin cytoskeleton, functioning as adapters for the recruitment of cytosolic molecules implicated in cell signalling, are the components of the TJ complex.

In order to investigate the effects of cytokines on conjunctival epithelium TJ, IOBA-NHC cells were stained with anti-occludin and anti-ZO-1 antibodies after 24 hours treatment with cytokines, based on a previous time course study done in the lab. For each slide, cells were visualised using ZEISS 710 Confocal microscopy. From the immunofluorescence staining, all cells stained positively for both ZO-1 and occludin. Differences in overall changes observed were based on differences in intensity and localisation of the staining rather than the positivity of the staining (Figures 50 to 53). Hence, these images were analysed based on the intensity of expression by the cells and the values were measured as the number of pixels. These images were analysed using Fiji Image J software as this software can detect the moderate effect that the cytokine has on cells. Images were analysed as follows:
1) Single colour images were dragged into the Fiji window and displayed as below.

![Image of Fiji window with two images: No treatment and +IL-1β 40ng/ml]

2) For each image, click IMAGE tab followed by ADJUST tab and then select COLOR THRESHOLD.
3) Adjust the colour by moving the saturation and brightness tab. Click original button at the bottom of the screen in order to view the original image.

4) Choose SELECT, then go to ANALYSE tab and click MEASURE.
5) Values are shown as pixels of the area from the whole image. Each image was analysed using the same method.

No treatment

+IL-1β 40ng/ml
Pixel values obtained from analysis of the confocal images were translated into the intensity of TJ in IOBA-NHC cells. The intensity of expression by treated cells was calculated as the percentage of intensity relative to cells without treatment. The intensity of ZO-1 (Figure 51) and occludin (Figure 52) on IOBA-NHC cells that were treated with different concentrations of IL-9, TNF-α, IL-1β, IL-4, IL-13 and IL-17A was recorded. TNF-α was selected as the positive control, since it was already demonstrated that TNF-α downregulates epithelial barrier functions (437). TNF-α was titrated and 40ng/ml was found to be the optimal concentration to use for subsequent experiments. Although adding higher concentrations of TNF-α further downregulated both ZO-1 and occludin, there was also a decrease in cell viability at these higher concentrations.

TNF-α was added in each experiment as the positive control. IL-1β downregulated ZO-1 (Figure 51b) and the greatest decrease of occludin (Figure 52b) was observed after treating the cells with 40ng/ml of IL-1β. Treating cells with IL-4 (Figure 51c and 52c), IL-13 (Figure 51e and 52e) and IL-17A (Figure 60f and 61f) also downregulated TJ intensity, where increasing concentrations of cytokines downregulated the intensity of both ZO-1 and occludin. Similar effects were observed when IOBA-NHC cells were treated with IL-9. IL-9 (Figure 51d and 52d) decreased molecules associated with barrier functions (as measured by decreases in ZO-1 and/or occludin expression) where the intensity of expression of the TJ-associated molecules were downregulated to that detected in untreated cells. This experiment was repeated three times by treating IOBA-NHC cells with cytokines at their optimal concentration; IL-9: 30ng/ml, TNF-α: 40ng/ml, IL-4: 40ng/ml, IL13: 40ng/ml and IL-17A: 80ng/ml. Figure 62 summarises ZO-1 and occludin staining (pixel intensity). The levels of ZO-1 pixel intensity were significantly downregulated as compared to unstimulated cell after treating cells with TNF-α, IL-9 and IL-4, p<0.01. However, only TNF-α caused a significant downregulation in occludin intensity, p<0.01. This way of measuring tight junction intensity using pixels could be one way to detect a general overview of TJ intensity on confocal photos, but is not suitable for quantitative measurements, given that the purpose of immunostaining is to analyse in detail the quality and distribution of cells during staining, rather than measuring photos using quantitative measures. In order to
obtain more quantitative data in the future, this experiment could be repeated and analysed by qPCR and western blot.

Figure 51: ZO-1 intensity (pixels) from confocal images (n=1) of IOBA-NHC cells after treatments with different concentrations of a. TNF-α, b. IL-1β, c.IL-4, d. IL-9, e. IL-13 and f. IL-17A. Data shown were from a single experiment.
Figure 52: Occludin intensity (pixels) from confocal images of IOBA-NHC cells after treatment with different concentrations of a. TNF-α, b. IL-1β, c. IL-4, d. IL-9, e. IL-13 and f. IL-17A. Data shown were from a single experiment.
Figure 53: Summary of ZO-1 and occludin intensity (pixels) from confocal images of IOBA-NHC cells after treatments with TNF-α, IL-1β, IL-4, IL-9, IL-13 and IL-17. Data shows mean ± SEM from 3 independent experiments. *p<0.05, **p<0.01 by Student’s t test.
Interestingly, based on immunofluorescence staining photomicrographs (Figure 54), in some cells, the distribution of ZO-1 and occludin revealed a remarkable difference in the localization pattern as compared to unstimulated cells. In unstimulated cells, both ZO-1 and occludin showed a diffuse staining and were always expressed in the plasma membrane in cell-to-cell contact points. Cells treated with TNFα had marked changes in the distribution and intensity of both ZO-1 and occludin expression, as predicted from the literature. Both ZO-1 and occludin disappear from the adjacent conjunctival cells and the distribution becomes more localized within the cytoplasm. In cells treated with all other cytokines, the ZO-1 staining showed a more predominant cystolic localization with disappearance of cell-to-cell contact points. More irregular distribution of the staining was also observed. IL-9 treatment did not affect occludin distribution where occludin was observed localized within the plasma membrane and had a diffuse staining pattern where cell-to-cell contact points were still visible. A similar effect was observed with IL-4 treatment. Although the intensity of the protein expression was similar to unstimulated cells, some occludin was observed localized within the cytoplasm in cells stimulated with IL-17.
Figure 54: ZO-1 and occludin intensity (pixels) from confocal images (representative from 3 independent experiments) of IOBA-NHC cells after treatments with TNF-α, IL-1β, IL-4, IL-9, IL-13 and IL-17.
6.3. DISCUSSION

In this chapter, I have detected some effects of IL-9 on human conjunctival epithelial cells using the IOBA-NHC cell line as the in vitro model. The design of the study was to compare a range of relevant cytokines with IL-9, and to use TNF-α as a positive control. Generally, data in this chapter did not focus on the effect of IL-9 in particular as these are preliminary works comparing the effect of IL-9 and different cytokines on epithelial cells. Given more time, further experiments focusing on IL-9 in combination with different cytokines could conclude the effects of IL-9 on epithelial cells.

There are a few technical issues I would like to address in regard to the experiments performed in this chapter. Firstly, besides using IOBA-NHC cell line, I also tried to investigate the expression levels of MUC2 and MUC5ac on primary human corneal epithelial cells (HCEC). However, after several attempts, I failed to grow these cells to the required amount to perform experiments, perhaps due to these cells being more delicate to handle and not being as robust as the transformed IOBA-NHC cell line. Also, as I did not have access to fresh human conjunctival biopsies at the time I was performing the MUC expression studies, I was unable to compare MUC2 staining in the cell line with conjunctival tissue sections.

Given more time, it would have been preferable to repeat TJ barrier staining with different TJ proteins including Claudin 1 and Claudin 2 in light of a study done by Gerlach and colleagues which reported that the protein Claudin 1 showed lower expression levels in IL-9 knockout mice (370). They managed to show this analysis by performing qPCR and western blot to obtain quantitative data and immunostaining tissue sections for qualitative analysis- some experiments which I would like to do to bring this study forward.

Immunostaining of ZO-1 and occludin was not optimised as the staining was not localised within the cell membrane. This study could have been improved if the staining was optimised properly with correct concentration of antibodies and
techniques plus optimised microscopy technique. Hence, the effects of cytokines on tight junction could not be concluded concretely until the immunostaining has been optimised.

In response to inflammatory cytokines, both MUC2 and MUC5ac mRNA expression was upregulated in IOBA cells. Similarly MUC5ac expression in HBE cells was found to be upregulated in cells treated by IL-1β and IL-17 via NFκβ activation (438). This finding was also supported by Enss et al, who found that IL-1β upregulated both MUC2 and MUC5ac expression in an intestinal human colon cell line (439). IL-13 was reported to have a direct stimulatory effect on conjunctival goblet cell proliferation but did not affect their mucin secretion (440). In contrast, Tukler Henriksson et al demonstrated that IL-13 stimulated mucin gene expression in cultured conjunctival goblet cells obtained from mouse conjunctival explants (353). Addition of IL-13 also induced the increase of MUC2 mRNA expression in a human colonic cell line (441).

IL-9 was also found to upregulate MUC2 gene expression. In the colon of IL-9 transgenic mice, MUC2 expression was upregulated (426). Similarly, both MUC2 and MUC5AC expression was elevated in airway epithelial cells of IL-9 transgenic mice (324). Study by Temann and colleague found that IL-9 promotes IL-13-mediated effects on mucus production in mice but IL-9 alone was not essential for mucus production in the lung (442). However, Louahed and colleagues reported that IL-9 alone can induce expression of both MUC2 and MUC5AC both in human primary lung culture and in IL-9 transgenic mice suggesting that IL-9 has important role in mucus production (324). This controversy in findings is likely due to the different cell lines and mouse models used, and further studies are required in this area.

The conjunctival surface area is formed by an extensive network of epithelial cells that form an intact barrier to prevent inflammatory diseases. In allergic diseases, allergens, irritants and pollutants to varying degrees can weaken the integrity of the epithelial barrier, allowing allergens and pollutants to cross the barrier, facilitating easy entry of allergens to cause inflammatory responses by immune cells. TJ complexes form a seal between the epithelial cells, and
impairment in TJ complexes contributes to barrier dysfunction, as observed in atopic dermatitis patients (443), ulcerative colitis (444), asthmatic patients (445). By immunofluorescence staining of asthmatic tissue biopsies, it was revealed that both ZO-1 and occludin in the TJs of asthmatic epithelium was poorly developed as compared to normal epithelium (431).

TJ permeability can be affected by many different stimuli including cytokines, chemokines and growth factors. During allergy, several cytokines including IL-4, IL-5 and IL-9 are known to be involved in mediating the response, and these cytokines are known to affect the permeability of epithelial TJ’s. In this chapter, these classical allergy-associated cytokines (IL-4, IL-9 and IL-13) alongside other cytokines (TNFα, IL-1β and IL-17A) were added to IOBA-NHC cells and the intensity of ZO-1 and occludin was visualised by immunofluorescence staining and the level of intensity was analysed by Image J.

Based on in vitro studies with different cell lines, TNFα has been shown to directly impair TJ functions by downregulating occludin and e-cadherin, hence, lowering the complexity of TJ (446). Others have found that TNFα induces a drop in transepithelial resistance (TER) and that effect was dose-dependent (447). TJ junction disruption by TNFα treatment was associated with transmembrane protein internalisation (448). This is consistent with our results where the intensity and distribution of ZO-1 and occludin in IOBA cells after TNFα treatment was downregulated in a dose-dependent manner and associated with a change in pattern of staining. In all experiments, TNFα was used as the positive control for barrier dysfunction.

IL-1β, which is released by immune cells such as macrophages, was reported to cause an increase in intestinal epithelial TJ permeability at physiologically relevant concentrations (1-10ng/ml) (449). In this experiment, IL-4 and IL-13 also decreased TJ integrity by downregulating ZO-1 and occludin. IL-4 and IL-13 treatments decreased barrier functions in Calu-3 airway epithelial cells, as assessed by electrophysiological and [14C]mannitol flux measurements (450). Further analysis of TJ-associated proteins ZO-1 and occludin revealed that IL-4 and IL-13 treatment caused a significant decrease in ZO-1 protein expression.

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and slowly decreased occludin expression as compared to control cells, hence diminishing the capacity of Calu-3 cells to maintain barrier function and repair wounds (450). Another study has also confirmed that TNFα and IL-13 induced structural and functional disruption of human corneal epithelial barrier (451)

Following IL-9 treatment, the levels of ZO-1 were significantly downregulated as compared to untreated cells, suggesting that IL-9 also affects TJ permeability. However, the levels of occludin were only slightly downregulated after IL-9 treatment. This is the first time that IL-9 has been found to selectively downregulate ZO-1 expression in human conjunctival epithelial cells, although the data is preliminary and requires further investigation. Another study has explored the relationship between IL-9 and tight junction integrity using a model of IL-9-deficient experimentally-induced colitis in mice. In that study, the levels of pore-forming factor claudin-2, sealing factor claudin-3 and TJ protein occludin in IL-9-deficient colitis mice were supressed as compared to wild type mice, suggesting that IL-9 controlled barrier functions of the intestine (375). Parker and colleagues have found that IL-9 alone or in combination with IL-13 has a significant role in reducing ciliated cell numbers and transepithelial electrical resistance due to degradation of tight junction in normal epithelium, suggesting the role of IL-9 in inhibiting mucociliary clearance in asthmatic lung via degradation of tight junction (427).

In this current study, IL-17 was found to have no effect on the TJ proteins ZO-1 and occludin. Another study found that IL-17A promoted, instead of downregulated the development of TJ barriers in intestinal epithelial cells (452,453). According to Kinugase et al, the enhancing effect by IL-17 of epithelial TJ barrier may be mediated by ERK activation.

In conclusion, this chapter describes preliminary experiments on the general effects of cytokines including IL-9 on MUC expressions and barrier functions. Further experiments as previously discussed in this chapter could be done in the future in order to solidify these preliminary conclusions which will also focussing on IL-9 functions on epithelial cells.
Chapter 7

GENERAL DISCUSSION AND FUTURE WORK
In summary, this thesis has focussed on the role of cytokines secreted by mast cells and T cells, in particular IL-9, in allergic conjunctivitis. Firstly, Chapter 3 described experiments that demonstrated that IL-9 intracellular expression was upregulated in conjunctival mast cells of EAC mice, as compared to control mice. There were also increased levels of CD4^+IL-9^+ T cells, CD4^+PU.1^+ T cells and CD4^+IL-4^+GATA2^+ T cells in the conjunctiva of EAC mice compared to control mice. Significant increases in CD4^+PU.1^+IL-9^+ T cells were observed in the draining lymph nodes of EAC mice as compared to naïve mice, suggesting that IL-9 is upregulated within the Th9 T cell population. Since we know that IL-9 was expressed by mast cells in the conjunctiva in EAC mice, further in vitro experiments were done to investigate the role of IL-9 secreted by mast cells on mast cell functions. As demonstrated in chapter 4 blocking IL-9R function, either by applying an IL-9R blocking antibody or by silencing IL-9R function at the gene level, downregulated histamine and cytokine secretion by mast cells. Interestingly, neutralising IL-9 functions did not significantly affect mast cell function. In addition to investigating the effects of IL-9 secreted by mast cells in vitro, the next aim was to investigate the presence of IL-9 in human conjunctival tissue sections. In chapter 5, IL-9 expression was found to be upregulated in the conjunctival tissue sections from allergen-challenged SAC donors as compared to normals. These IL-9-expressing cells were also colocalised with a mast cell marker, suggesting that mast cells were the source of IL-9 during AC, although some IL-9 positive cells were clearly not mast cells. Lastly, Chapter 6 describes the effect of IL-9 on conjunctival epithelial cell responses. Based on the preliminary in vitro studies using the human conjunctival epithelial cell line, IOBA-NHC cells, data in Chapter 6 suggests that IL-9 upregulated Muc2 and Muc5ac mRNA expression and, to a lesser extent than other cytokines, IL-9 also downregulated ZO-1, a TJ protein on the epithelial cell surface. However, upon IL-9 treatment, occludin expression was slightly upregulated as compared to the non-treated cells.

Based on the hypothesis of this study, which is that conjunctival IL-9 is upregulated during allergic conjunctivitis and contributes to the immunopathogenesis of the disease, overall, this thesis managed to demonstrate an upregulation of IL-9 during allergic conjunctivitis and IL-9...
secreted by mast cells and T cells correlated with the pathogenesis of the disease, which also affected the conjunctival epithelium.

As has previously been discussed in each results chapter, there are several limitations in this study. In chapter 3, there were no FMOs as the control to set the FACS gates hence, gates drawn in the FACS plots might not be accurate. However, based on the results, there are clear differences in the levels of IL-9 from CD4\(^+\) T cells and mast cells in the conjunctiva and CD4\(^+\) T cells in the lymph nodes. In chapter 4, IL-9 and IL-9R blocking antibodies were not adequately titrated hence this might have resulted in an insufficient block of IL-9 function. However, IL-9 levels were dampened down in mast cells upon IL-9R antibody blocking suggesting that IL-9 secreted by mast cells affects mast cell functions. Immunostaining on human conjunctival biopsies in chapter 5 might not be convincing due to the polyclonal IL-9 antibody used for staining. Based on IL-9R staining (of which antibody used was monoclonal antibody), significant increases in the levels of IL-9R were observed in allergen-challenged biopsies and these IL-9R positive cells were identified as mast cells. Lastly, chapter 6 was primarily preliminary data and solid conclusions cannot be drawn from this chapter. In order to be able to conclude IL-9 functions on conjunctival epithelial cells, further functional experiments like Western blotting or blocking IL-9R function on human conjunctival epithelial cells to observe an effect on mucus production and tight junction formation will enable further understanding of the effects of IL-9 on epithelial cells.

This study has raised more research questions and there are several areas which could be done in the future in order to bring the research forward. Since current data in this thesis managed to show that blocking IL-9R in mast cells in vitro affects mast cell functions, further studies using anti-IL-9R blocking antibody in mouse models of AC will be one way to confirm in vivo the mast cell in vitro data. In order to fully determine the functional properties of IL-9/IL-9R in vivo, AC could also be induced in IL-9R knockout mice (322). The present study demonstrated that IL-9 expression was upregulated in the conjunctivae and draining lymph nodes of mice with AC. Further in vivo studies with the application of anti-IL-9R blocking antibody and IL-9R knockout mice would be
ideal in order to confirm the functionality of IL-9R activation during AC. The present study has also demonstrated that there were significant levels of CD4⁺ T cells expressing IL-9 in the conjunctivae of EAC mice. It will be very interesting to further explore the role of IL-9 and CD4⁺ T cells in allergic conjunctivitis. In order to translate these *in vitro* and *in vivo* findings into human disease, further immunostaining of IL-9 on CD4⁺ T cells and other cell types, and analysis of tear samples could confirm the findings in human disease. Once the role of IL-9 in allergic conjunctivitis has been establish, further studies could also investigate possible therapeutic intervention using IL-9 blockade in allergic conjunctivitis.

Cytokines are small protein molecules that are important in cell signalling and are secreted by pro-inflammatory cells during inflammation. Targeting cytokines in order to dampen immune response can be a good therapeutic target since certain cytokines are upregulated during inflammation. Anti-cytokine treatment has been used in treating autoimmune diseases like rheumatoid arthritis and allergic diseases including allergic asthma. However, to date, anti-cytokine treatment has not yet been used as the choice of treatments for severe AC.

A number of different anti-cytokine therapies have already been used in human trials for clinical targets. Targeting key cytokines that drive type 2 inflammation in allergic diseases have been the areas that interest clinicians and researchers in these past years. To date, efforts have been put into researching key Th2 type cytokines, IL-4, IL-5 and IL-13 as prime targets to supress allergic inflammation and have shown to be promising and achieved therapeutic benefits across diseases.

IL-4 receptor (IL-4Rα) can be found on most haematopoietic and non-haematopoietic cells. Type 1 IL-4R, which consists of heterodimeric chains of IL-4Rα and common γc chain can be found exclusively on haematopoietic cells whereas type 2 receptor which consists of IL-4Rα and IL-13Rα1 chain can be found mostly on non-haematopoietic cells including epithelial cells and fibroblasts. IL-4R can have distinct and overlapping functions with IL-13 partly due to the shared receptor moiety in which IL-13 receptor functions through
activation of specific IL-13α1 receptor. Having understood IL-4R structure in more details, several molecules targeting IL-4 and IL-13 were developed, with the idea that once these key cytokines are blocked, further inflammation will be dampened.

In a preclinical study, the attenuation of allergic airway disease in IL-4−/− mice resulted in the reduction of eosinophil recruitment into the airways and increased epithelial hypertrophy and allergic hyperresponsiveness (AHR) (454). Another study on IL-4 in mouse model of allergic airway disease demonstrated beneficial effects of suppressing IL-4 activities, resulted in suppression of eosinophil infiltration into the airways and inhibit IL-5 release from T cells (455).

Besides IL-4, there have been significant experimental studies to support IL-13 as a potential target in asthma. IL-13−/− mice induced for allergic asthma showed attenuation of mucus hypersecretion despite the persistence AHR (456). Neutralisation of IL-5 in these mice inhibits AHR suggesting that tissue eosinophilia accumulation is linked to the mechanism underlying AHR only in the absence of IL-13. This study shows the pro-inflammatory role of IL-13 when challenged. Administration of humanized anti-IL-13 suppressed AHR and eosinophil influx in bronchoalveolar lavage from models of allergic airway disease (AAD) (457,458). These studies indicate a good potential for anti-IL-13 to be used as therapy for allergic asthma.

After gathering information from mouse studies, two IL-4 inhibitors have been developed. Altrakincept, administered by a nebuliser, is a recombinant soluble IL-4Rα which competitively binds to IL-4 has shown to have positive outcome on asthma symptoms (459). Another IL-4 inhibitor is paslizumab, which is a humanized IL-4 specific antibody. Several IL-13 inhibitors, including anruknizumab, IMA-026 and Lebrikizumab, a humanized monoclonal antibody which bind to soluble IL-13 with high affinity, have shown to be promising in improving asthma symptoms during preclinical and early clinical study. However, all of these inhibitors failed to show efficacy and inconsistent results during larger Phase II and Phase III clinical study (460–463). Besides asthma, lebrikizumab is also currently being evaluated in atopic dermatitis and has
entered phase II clinical study. There are several reasons why a single cytokine target may not be effective in allergic disease. Among the reasons are the fact that many of these diseases utilise a range of inflammatory cytokines, and that the disease may be heterogeneous.

Although several attempts failed in targeting cytokines IL-4 and IL-13 individually, blocking dual cytokines by targeting the shared IL-4Rα is a novel approach to modulate type 2 inflammation. A few inhibitors targeting both IL4/IL-13 have shown some promising data. Pitrakinra, a mutated recombinant human interleukin 4 which competitively inhibits IL-4Rα receptor complex to interfere with the actions of both IL-4 and IL-13 significantly improved FEV1 upon allergen challenge and reduced spontaneous asthma attacks (464). Aeroderm, a modified version of pitrakinra with added polyethylene glycol (PEG), has also been tested in atopic dermatitis. Even though Aeroderm shows improvement in clinical symptoms, results from clinical studies failed to show statistical significance on disease end points. Dupilumab on the other hand has very recently received FDA approval for use as injection for the treatment of uncontrolled, moderate-severe atopic dermatitis. Previous clinical studies showed marked and rapid improvement in all evaluated measures for atopic dermatitis (52). In asthma, dupilumab has demonstrated several improvements by significantly downregulated several biomarkers related to type 2 diseases and also downregulates asthma exacerbation and FEV1 in Phase IIb of clinical studies (465,466).

Besides studies on IL-4 and IL-13, studies on mice showed that pre-treatment of a single dose of anti-IL-5 neutralizing antibodies (TRFK-5) was able to inhibit eosinophil influx into the airways of the challenged mice (467). Treating mice with allergic asthma has been shown to effectively suppress eosinophil recruitment and accumulation of inflammatory cells in the airways but failed to inhibit airway hyperresponsiveness (AHR) (468). Several IL-5 inhibitors have been developed such as IL-5-specific monoclonal antibodies, mepolizumab and reslizumab and IL-5 receptor α (IL-5R α)-specific monoclonal antibody, benralizumab, primarily targeting eosinophils during allergic airway disease. Administration of mepolizumab during a clinical trial in moderate asthma has
been shown to have similar effects as observed in the models of asthma in mice (469). It is safe to use and has been shown to reduce the number of eosinophils in the airways and blood (470), but did not improve asthma symptoms (471). A similar study on reslizumab has reported a decrease in the number of eosinophils in patients with severe eosinophilic asthma but modest improvement in airway symptoms (472). However, more recent clinical studies demonstrated that mepolizumab significantly reduced exacerbation in subset of asthma with high eosinophil count (473,474). Renewed interest in mepolizumab and reslizumab has led to FDA approval for both of these inhibitors for the treatment of severe asthma. Benralizumab is currently in Phase III clinical trial and has already demonstrated an ability to reduce the use of oral steroids in asthma patients and significantly decrease the frequency of asthma exacerbations in two trials (475,476).

Recently, many researchers have been focusing on IL-9 functions in allergy. Based on different evidence of IL-9 in the pathogenesis of allergic diseases especially in asthma, MEDI-528 (MedImmune), an antibody to IL-9, has been developed and studied in an early phase clinical trial for patients with asthma. Although it was shown to be safe for mild, moderate to severe asthma (335,477), a large phase 2b study with 329 subjects with moderate to severe persistent asthma failed to demonstrate improvement in Asthma Control Questionnaire 6 (ACQ-6) score, asthma exacerbation rates or FEV1 values (478).

Despite convincing preclinical studies providing strong evidence that IL-9 expression was upregulated in the airways of patients with asthma and that IL-9 functions to regulate mucus production, airway responsiveness and subepithelial fibrosis (479), the application of anti-IL-9 therapy in asthmatic subjects failed to achieve a clinically important effect on related quality of life and lung function (478). However, a previous study with a smaller sample size had observed a potential improvement in asthma exacerbation rates in subjects with mild asthma where fewer subjects experienced ≥ 1 asthma exacerbation in group treated with MEDI-528 as compared to placebo control group (335).
IL-9 is believed to play important roles in the trafficking and functions of mast cells. IL-9 is responsible for the development and recruitment of mast cells from the bone marrow to the lung in allergic asthma (236,480). Hence, it is also possible that therapeutic blocking of IL-9 would not expect to have a direct immediate clinical effect in asthma but could possibly affect mast cell functions at a later stage, and will have effects on other immune cells during disease and this will take a longer time to take effect. This explains why, in the clinical trials mentioned above, it did not result in short term improvements in asthma symptoms.

It is apparent that the heterogeneous nature of asthma makes it difficult to treat and is challenging for developing a single targeted therapy with monoclonal antibodies. Targeting mono cytokine in a non-selected asthma population might be hard to observe significant beneficial improvements in this cohort. Identification of single cytokine could be a great help in determining the disease marker, leading to a more accurate diagnosis and treatment as observed by Corren and colleagues, where subjects were grouped according to baseline type 2 helper T-cell (Th2) status which then led to a greater improvement on lung function after treatment with Lebrikizumab (anti-IL-13 mAb) (481). Unfortunately, a marker of the IL-9 pathway has not yet been identified although recent studies on IL-9 pathways in allergic diseases (276,340,363) are leading in this direction, making IL-9 a promising disease marker.

Targeting IL-9 could be an effective treatment for allergic conjunctivitis, since my data suggests that IL-9 is important for upregulating the Th2 cytokine response by mast cells. AC is classified as either mild, moderate or severe forms of disease and typically is more localized to the eye area. Based on a preliminary study by Bonini et al (unpublished observations), in cell-free tear specimens of VKC patients treated with cyclosporine, lower IL-9 expression levels were observed in comparison with untreated, active VKC, suggesting that the level of IL-9 increased in the active form of the disease.

Other studies on monoclonal anti-cytokine targets have also focused on targeting cytokine receptor functions. One mouse study revealed the
attenuation of allergic airway disease in mice in IL-4R$^{-/-}$ mice and by co-administration of a murine IL-4R antagonist (215,482). These are due to the inhibition of both IL-4 and IL-13 functions as compared to studies done in IL-4$^{-/-}$ mice (483). A soluble anti-IL-4Rα Ab seems to have beneficial effects in preserving FEV1 and asthma symptoms score of participants with moderate asthma compared to placebo (459).

Since current anti-IL-9 antibody has shown inconsistent results in treating asthma patients, it might be worth considering using an anti-IL-9R antibody as an alternative treatment. The data presented in this thesis are the first to show that blocking IL-9R affects mast cell function, suggesting that targeting IL-9R rather than the cytokine itself might be more effective in regulating mast cell functions. It is possible that IL-9R-directed blocking could potentially be a good therapeutic target for AC treatment. A study used IL-9R knockout mice to show that IL-9, as an autocrine amplifier, mediated the survival of ILC2 via activation of IL-9R (322).

In conclusion, based on the work in this thesis, IL-9 that is secreted by different cell types could be a proinflammatory cytokine that contributes to AC. The discovery of the increased expression of IL-9 during AC makes targeting the IL-9/IL-9R axis an attractive new target for anti-inflammatory therapy in allergic diseases and further studies are needed to confirm my findings in this thesis. Since the role of IL-9 has been extensively studied in asthma, it might also play important roles in AC. Inhibition of IL-9R activation could be promising in mast cell-mediated diseases such as allergic asthma as demonstrated by Kearly et al. where they demonstrated that IL-9R was mainly expressed by mast cells in human asthmatic lung tissue (328). Additionally, they have also demonstrated that mice treated with anti-IL-9 antibody showed protection from airway remodeling, characterized by reduction in the number of active mast cells, plus, decreased expression of profibrotic factors; TGFβ, vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF-2) in the lung.
Chapter 8

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