Characterisation of the immune cells and cytokines involved in a model of atopic dermatitis

by

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Abstract

Atopic dermatitis (AD) is a highly debilitating disease with significant health impacts worldwide. It is a chronic and relapsing inflammatory skin disease which often poses a life-long burden for the affected individuals. AD has been a difficult disease to treat as it manifests with a wide spectrum of clinical phenotypes and the current clinical management strategies are non-specific. Therefore, it is imperative to identify specific immunological pathways that could be targeted to treat this disease. Previous studies have documented that AD disease progression is precipitated by a combination of skin barrier dysfunction, itch and immune dysregulation that are responsible for AD progression. However, the precise role of effector cells and cytokines have not been fully elucidated. To address this, I established a clinically relevant model of AD, using the vitamin D analogue, MC903. This MC903 model closely resembles the AD phenotype in patients, including inflammatory parameters, barrier dysfunction, itch, and histopathological characteristics, providing a novel platform to evaluate targets for the treatment and prevention of AD. Furthermore, this model exposed the cells and cytokines that are critically associated with disease severity, including eosinophils, mast cells, TSLP, IL-4 and IL-9, but not CD4⁺ T cells. The instrumental role of these effector cells and cytokines was established by their stepwise depletion or blockade. Indeed, functional eosinophil depletion via the use of inducible eosinophil (iPHIL) mice significantly ameliorated AD pathology, most notably itch. Similar results were obtained after blockade of the IL-4/IL-13 axis by genetic deletion of STAT6. The clinically more relevant use of soluble inhibitors targeting IL-9 and CRT_h2 (in a prophylactic and therapeutic setting, respectively), both resulted in a

substantial reduction in AD phenotype. In summary, this body of work led to the identification of key disease-initiating and effector cells and molecules that represent attractive targets for the treatment of AD.

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processing for flow that is described in this thesis. I was responsible for the data collection and analysis.

With these disclosures, I declare the content of this document is my own work.

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Abbreviations

α (prefix)	anti
AA	arachidonic acid
AD	atopic dermatitis
APC	antigen presenting cell
CAE	chloroacetate esterase
COX	cyclooxygenase
CRTh2	chemoattractant receptor on Th2 cell
CRTh2ant	chemoattractant receptor on Th2 cell antagonist
DC	dendritic cells
dDC	dermal dendritic cell
DETC	dendritic epidermal T cells
EASI	Eczema Area and Severity Index
EC	epicutaneous sensitization
ELISA	Enzyme linked immunoabsorbant assay
EPO/EPX	eosinophils peroxidase
EtOH	ethanol
FACS	Flourescence-activated cell sorting
FoxP3	Forkhead box P3
FDA	Food and Drug Administration
FLG	filaggrin
γ c	common gamma chain
GM-CSF	granulocyte-macrophage colony stimulating factor

GPCR	G protein coupled receptor
H & E	haematoxylin and eosin
HDM	house dust mite
i.g.	intragastric
i.n.	intranasal
i.p.	intraperitoneal
ICS	intracellular cytokine staining
Ig	immunoglobulin
IHC	immunohistochemistry
IL-	interleukin
ILCs	innate lymphoid cells
iPHIL	inducible eosinophil
K5	keratin 5
KO	knock-out
LC	Langerhans cell
-mAB	monoclonal antibody
MBP	major basic protein
MC	mast cell
MMCP	mouse mast cell protease
NK	natural killer
PCR	Polymerase chain reaction
PMA	Phorbol 12 myristate 13 acetate
OVA	ovalbumin
Ox	oxazolone

PG	prostaglandin
PGDS	prostaglandin d synthase
PFA	paraformaldehyde
r (suffix)	recombinant
R (suffix)	receptor
RAR	retinoic acid receptor
RXR	retinoid x receptor
SCORAD	scoring atopic dermatitis
STAT	signal transducer and activator of transcription
TEWL	transepidermal water loss
Th	T helper
TMA	Trimethyl anhydride
TNCB	2,4,6-trinitrochlorobenzene
TNF	tumour necrosis factor
TSLP	thymic stromal lymphopoietin
Treg	T regulatory
VCAM	Vascular cell adhesion molecule-1
VDR	1,25-dihydroxyvitmain D3 receptor
WMS	whole mount staining
WT	wild type

1 Introduction

1.1 The skin is a barrier

As our understanding of skin immune-biology is evolving we begin to appreciate the role of the skin, a highly-specialized organ, as the interface between the host and the environment. An outer epidermal non-vascular layer harbours a network of keratinocytes, and Langerhans cells. There has been an additional epidermal population of cells recently characterized in murine skin named the dendritic epidermal T cells (DETCs) [1]. However, these DETCs have not been found in human skin tissue. The inner dermal layer being highly vascularized, contains most of the immune cell populations, such as macrophages, mast cells, eosinophils, dermal dendritic cells (DCs), and innate lymphoid cells (ILCs) [2]. Additionally, the dermis contains a network of fibroblasts, collagen and elastin fibres; hair follicles, sebaceous and sweat glands and nerve endings [3]. Skin resident cells respond to physical, pathogenic and/or environmental insults to provide protection to the host. A disruption to this barrier as well as the inability to respond to invading pathogens and/or stimuli results in allergic disease. It is only through gaining mechanistic knowledge regarding the epithelial-immune crosstalk that occurs during atopic dermatitis (AD) [4], that we can value the true role of the skin barrier. This body of work is focused on AD, commonly known as eczema, and is an attempt to characterise the immune cells and cytokines associated with AD.

1.2AD & its prevalence

Atopic dermatitis (AD) is a chronic and relapsing inflammatory disease [5], characterized by pruritic lesions or itch [6]. AD was described by the Japanese Dermatological Association as an itching disease with recurrent remissions, exacerbations and most patients have some atopic conditions [7]. This highly debilitating condition has a major emotional and financial impact on patients and their families thereby causing significant effects on their quality of life [8]. The prevalence of AD is estimated between 15%-30% in children and 2%-10% in adults and thus is becoming a substantial health concern worldwide [9]. In New Zealand, at least 15.8% of children between the ages of 3-5 years are diagnosed with AD [10]. Several pioneering studies into the development of AD have dramatically improved our current understanding of the disease. These investigations have illustrated that genetic predisposition [11], an underlying barrier dysfunction [12, 13], environmental factors [14], as well as an altered immune function, all contribute to the chronic inflammation [15] and contact sensitization [16]. In most countries, there are more households adopting an urban lifestyle, there is an increased use of soaps that remove the natural oils of the skin [17], along with implementation of western diets (high fat and sugar, low fibre diet) [18]. These environmental changes could all influence the development of AD [14, 18]. Furthermore, recent studies have highlighted the impact of skin microbiota on AD [19, 20]. During AD flares, colonization of *Staphylococcus aureus* was observed in skin lesions [19, 21]. The role of Immunoglobulin (Ig) E in AD is still a matter of debate, as it remains unknown whether IgE plays a central part in the pathogenesis of AD or whether its presence is

a bystander effect [22]. Most AD patients exhibit high levels of serum IgE (extrinsic AD), however, there are a subgroup of AD patients with normal levels of IgE. The latter cohort of AD patients is referred to as “intrinsic AD” or “non-atopic” AD patients [2, 23].

Therefore, to date, two hypotheses have been formulated to explain the pathogenesis of AD:

an immune-regulatory dysfunction which results in a predominant T helper type (Th) 2- and Ig E-mediated sensitization [24] and

an intrinsic defect in skin barrier function (e.g. a mutation in the filaggrin gene) which allows allergens and pathogens to penetrate the skin [25, 26].

1.3 The AD phenotype

AD is characterized by a wide spectrum of clinical phenotypes. Illustrated below are some of the manifestations that either assist in the diagnosis of AD or contribute to disease severity.

1.3.1 Pruritus/Itch

Clinical presentation of AD includes, xerosis or dry skin and intense pruritus or itching. Itch results in scratching which further disrupts the barrier, making the skin more susceptible to infection [6]. It is due to this intense scratching that leads to cytokine release such as thymic stromal lymphopoietin (TSLP) [27, 28] and IL-31 [29, 30], many

different cell types being activated and release of mediators which induces further pruritus thereby allowing for entry of allergens into the skin [31]. Interestingly, a recent study has focused on the impact of IL-4R α , through the signaling of IL-4 and IL-13 on sensory neurons to initiate chronic itch in both an inflammatory and non-inflammatory setting [32]. Following intense itching, mast cells have been reported to become activated, degranulate and release histamine [33]. In addition, a murine model of allergic skin inflammation has documented itching and innervation to be associated with eosinophils in the skin [34]. Furthermore, it has been revealed that keratinocytes release TSLP which stimulates sensory neurons thus triggering further itching [27, 28]. Moreover, the release of interleukin (IL)-31 by T cells and keratinocytes has been reported to induce itch [35]. The reduction of itch in AD patients would be a critical goal of therapeutic intervention.

1.3.2 Skin barrier dysfunction

The skin barrier controls transcutaneous movement of water, and prevents irritants, microbes as well as allergens from entering the skin. Barrier integrity of the skin is assessed by measuring the trans-epidermal water loss (TEWL) [36]. It has been observed that disruption of the skin barrier leads to a defect in the filaggrin (FLG) gene, a decrease in ceramide levels in the skin and/or an over-activation of epidermal proteases. FLG, itself has been reported to be important in the maintenance of skin integrity [12] as up to 50% of AD patients present with mutations in FLG. Studies involving the use of the flaky tail mice, deficient in FLG, have been conducted to determine its role [37-39]. Thus far, at least 20 mutations of the FLG gene have been described [13]. Furthermore, while a deficiency in filaggrin does provoke skin barrier

dysfunction it does not always lead to AD. This is seen in patients with ichthyosis vulgaris where the same FLG mutation occurs but not the inflammation as seen in AD [40]. Accompanying the loss of filaggrin, is often a substantial reduction of ceramide levels; a lipid of the stratum corneum; has been documented in AD patients and this results in epidermal water loss [41]. The increased activity of these proteases induces an increase in the pH of the skin, thereby further disrupting the skin barrier. A compromised barrier surface occurs bi-directionally, as it allows for epidermal water loss and allergen penetration. This high pH results in the breakdown of the enzymes, and proteases such as kallikrein, and lipids that are responsible for holding together the various structural layers of the skin [17, 42]. Desmosomes are integral proteins which bind the epidermal cells to the cytoskeleton [43]. In addition, this compromised barrier is often accompanied by the entry of microbes and in AD patients there is colonisation of the skin by *Staphylococcus Aureus* [21]. Many individuals who present with a dysfunctional barrier in the skin are often predisposed to developing other allergic diseases such as asthma, allergic rhinitis and/or food allergy [44-47]. This phenomenon is known as the allergic march and this breach in the skin barrier is often a trigger for the allergic march [48, 49].

1.3.3 Histopathology and clinical hallmarks of AD

This interplay between skin barrier dysfunction, pruritus and the immunological response is responsible for AD progression [50]. Furthermore, microscopic diagnostic criteria are assessed and evaluated in the epidermis and the dermis. The hallmarks

of chronic AD, particularly in the epidermis, are acanthosis, spongiosis and hyperkeratosis [11, 24, 51]. They describe the thickening of the epidermal layer, the presence of intercellular edema and the presence of nuclei in the stratum corneum, respectively. The stratum corneum is the uppermost layer of the epidermis [52]. The complication with AD diagnosis is often the histopathology appears similar to psoriasis. However, psoriasis is a completely different disease with a T helper type (Th) 17 profile as opposed to AD which is more of a Th2 profile [53-55]. Spongiosis is a hallmark unique to AD and not in psoriasis. Hence, good diagnostic evaluation is critical [24]. The spongiotic defect is mainly due to the disruption of the epidermal architecture in the tissue and this becomes more pronounced as the inflammation persists [56]. Over time, with more chronic AD lesions, inflammation is followed by epithelial cells hyper-proliferating [57] and this causes a further dysfunction in the barrier integrity [58]. At this stage, large amounts of fibrous connective tissue such as collagen and fibroblasts accumulate around the inflamed tissue [59, 60] and this leads to the leathery (lichenified) appearance of the skin [9, 61]. This phase of skin remodelling and fibrosis is associated with chronic itching [62] via the activation of neuropeptides in the skin [63] (as seen illustrated in figure 1.3 [64]).

A

Acanthosis

B

Hyperkeratosis

C

Spongiosis

Figure 1.1. Histopathology of AD patient lesions

Haematoxylin and Eosin staining depicting A) Acanthosis which is the epidermal thickening as indicated by the arrows. B) Hyperkeratosis which is the presence of the nuclei (as indicated by arrows) in the upper most layer of the epidermis (i.e. the stratum corneum) C) Spongiosis which is the intercellular edema (white spaces as indicated by arrows) seen in the epidermis (figure adapted from Shimizu's textbook of dermatology by Hiroshi Shimizu 2nd edition chapter 2.)

1.4 The phases of AD & its manifestations

Clinical presentation of AD occurs during three periods of life: infantile, childhood and adolescent-through to adulthood. The infantile phase manifests from birth to 2 years of age and these children experience intense pruritus. Erythematous (redness) papules typically begin on the cheeks, forehead and scalp and then extend to the trunk and extremities [65]. The childhood phase occurs from 2 years to puberty, these lesions exhibit more lichenified (leathery) papules and occur on the hands, feet, wrists and ankles. Additionally, these individuals experience severe pruritus which results in infections and sleep disturbance. The adult phase begins at puberty and frequently continues to adulthood. Affected areas extend from the face, neck, upper arms and back, to the hands, feet, fingers and toes. These skin eruptions are characterized by dry scaling erythematous papules, large lichenified papules, crusty areas on the skin and infections [47, 66].

Individuals that develop AD in early life are at a higher risk for developing other allergic diseases (e.g. food allergy, asthma and allergic rhinitis) later on. This progression is termed the allergic march [18, 45, 47]. AD is hypothesized to be the initial trigger that drives the allergic march [45-47]. The cellular and molecular mechanisms involved remain enigmatic [24]. Furthermore, some infants who develop mild-moderate AD during infancy experience AD manifestations throughout their lifespan [67].

1.5AD treatment and management

The current management strategies for AD entail proper skin care, the daily use of emollients, and in some cases anti - inflammatory treatment. AD patients are advised to avoid allergens and irritants that could possibly penetrate the skin. Therapeutic interventions are based on the mild-severe manifestation of AD, which is determined based on a classification of AD severity either by the SCORAD (scoring AD) [68] or the EASI (eczema area and severity index) system [69]. More recently, clinical studies have merged the two classification systems for clinical phenotyping of AD patients [66]. Historically anti - histamines have been used to treat AD, in particular to provide relief of pruritus. However, there is still insufficient evidence regarding both the efficacy and mechanism of action of these drugs in the treatment of AD [70]. Therefore, using anti - histamines as a treatment for AD is still being debated [71, 72]. Below is a brief outline into the various treatment interventions as AD lesions present from mild – severe and where the field of dermatology proposes the treatment options are headed.

1.5.1 First-line (basic) treatment

This primary treatment for mild AD lesions entails the use of moisturizers or emollients which aims to reduce the patients skin dryness. The type of emollient selected is based on the AD lesion. A more active emollient will be prescribed to restore lipids and contribute to repair of the skin barrier [73]. Daily lubrication of the skin reduces pruritus, erythema, hydrates the skin and alleviates inflammation.

1.5.2 Second-line (mild) treatment

This includes treatment of AD with either topical steroids, calcineurin inhibitors, and antimicrobial interventions.

Topical corticosteroids alleviate the cutaneous symptoms [68]. These are used mostly in the form of creams or lotions and aims to dampen down the skin immune response, as well as reduce skin colonization by *Staphylococcus aureus*. Corticosteroids however, are only recommended to be used as intermittent therapy, to be used in conjunction with emollients and used with extreme caution in children. Unfortunately, this treatment is accompanied with many side effects namely; skin atrophy, other bacterial or fungal infections and reduced efficacy after prolonged use [74].

The calcineurin inhibitors, such as tacrolimus and pimecrolimus, contain immunosuppressants which are used to treat AD in both children and adults. Its precise mechanism of action is to inhibit the activity of the calcineurin enzyme thereby preventing T cell activation. In addition, these drugs can decrease the production of pro-inflammatory cytokines, tumour necrosis factor and granulocyte-macrophage colony stimulating factor, necessary for the maturation of dendritic cells. Pimecrolimus is a cream used to treat patients with mild to moderate AD skin lesions [75, 76] while Tacrolimus ointment is prescribed for more moderate to severe AD [77]. As a short-term measure eradication of the bacterial colony does reduce the severity of AD, however long-term use will result in drug resistance [78]. The skin harbours many different bacterial colonies and many of which are necessary to ensure a healthy function of the skin barrier, during AD dysbiosis occurs where 90% of the patients' skin is colonized by *Staphylococcal* strains. Topical corticosteroids that are used to treat AD exert their action by inhibiting phospholipase A2 in the skin

[79]. This results in a reduction of lipid mediators such as prostaglandins and leukotrienes in the skin [80]. In addition, it has been documented that these corticosteroids inhibit cytokine activity and modulate mast cells influx [81]. As such, long-term use of these corticosteroid creams create a immunosuppressive environment and makes the individual susceptible to infections.

1.5.3 Third line of therapy-systemic immunotherapy

In more severe and some chronic cases of AD systemic immunotherapy is used. Immunosuppressive drugs such as Cyclosporine A has been successful in reducing inflammation, pruritus, size of lesions and improve quality of sleep [82]. This drug exerts its effects by blocking the apoptosis of immature T cells however this potentially perturbs self-tolerance mechanisms in the body [83]. In addition, systemic long-term administration of recombinant interferon- γ (rIFN- γ) has shown safety and efficacy in the treatment of severe AD. This aims to reduce disease pathology by decreasing eosinophil infiltration and normalizing the CD4+ and CD8+ T cell ratio [84].

1.5.4 Future therapeutic interventions for AD

Targeted immunotherapy has been the only causal treatment of AD [74]. As more evidence regarding the crosstalk between epithelial cells and immune cells emerge,

there have been considerable efforts to find specific targets to treat AD. A reflection of this has been the recent success of the human monoclonal antibody; Dupilumab. Commercially known as Dupixent®, it was approved by the U.S.A Food and Drug administration (FDA) to treat moderate-to-severe AD as of 29th March 2017. This monoclonal antibody targets the subunit of the IL-4 receptor resulting in the inability of IL-14/IL-13 signalling. However, not all AD patients responded to Dupilumab and it caused side effects such as nasopharyngitis, conjunctivitis, and inflammation of the cornea [85, 86]. It is unclear why Dupilumab did not effectively treat all patients and there have been some studies looking into the different endotypes of AD patients [66, 87, 88]. More studies are needed to fully understand this disease and the various ways it presents. Therefore, it is imperative to identify specific immunological pathways that may be targeted for the treatment and prevention of AD.

The evidence described herein was either from AD patient studies and/or AD murine models, with emphasis on the MC903 model of skin inflammation.

Figure 1.2 Immune – epithelial cell crosstalk

This occurs between the epidermis (LCs and epithelial cells) and the dermis (immune cells) of the skin in response to a disrupted skin barrier either due to an allergen, pathogen or infection. Keratinocytes express Toll-like receptors (TLRs) either on their surface (TLR1,2,4,5,6) or within their endosome (TLR 7 & 9). It is via these TLRs that keratinocytes sense insult thereby initiating an appropriate immune response. In addition, receptors for various cytokines and chemokines allow initiation of a pro-inflammatory response, such as IL-1 β , IL-6 and IL-18 which leads to the activation of LCs. Tissue resident dermal DCs are also involved in the inflammatory response and influenced by the skin environment (adapted from Nestle et al 2009 [4]).

1.6 The epidermal network and its role on AD

1.6.1 Keratinocytes

The main constituent in the epidermis are the keratinocytes and these cells play a vital role in cutaneous immune responses. This epithelial network senses noxious stimuli and signals from the environment which is then transmitted to the immune cells in the skin. Keratinocytes are highly specialized at sensing the environment as they express several pattern recognition receptors including toll like receptors (TLRs) 1,2,4,5 and 6, cytokine receptors such as TSLP, IL-6, IL-1, IL-31, IL-4Ra and IL-13 [4 Tuzun, 2007 #1097]. In addition, they express receptors for a number of chemokines and hormones such as CXCR2, CCR3 and vitamin D (Figure 1.2). The stress induced on the keratinocytes causes them to proliferate and produce cytokines, in particular TSLP, IL-33 and IL-25 [89-91]. Sensory innervation also occurs in the epidermis due to the presence of neuropeptide receptors for example substance P receptor NK1 is highly expressed on keratinocytes and involved in the induction of itch. These epithelial derived cytokines have been detected in increased amounts in AD skin lesions and have been reported to modulate keratinocyte function and skin barrier integrity [64, 92, 93]. This is the beginning of the inflammatory cascade that ensues in AD.

1.6.2 Langerhans cells (LC)

Epidermal immune cells, LCs, present in both mouse and human skin, also interact closely with the keratinocytes. Langerhans cells are anchored in the keratinocyte layer via an adhesion molecule E-Cadherin [94]. Upon receiving signals from the keratinocytes, the LCs and dermal dendritic cells (DC) acquire antigen, and transport the antigen to the draining lymph node. The antigen is then presented to a naive T cell which results in the initiation of either an adaptive or an innate immune response [95]. CD1a and CD207⁺ are used to detect LCs in humans while in mice LCs are CD207⁺ and these cells account for 3-5% of the epidermal cells in both mice and humans [96].

1.7 The dermal immune network and its role in AD

It is in the dermis that we can appreciate the involvement of both the adaptive and innate arms of the immune system [4]. Dysregulation of the immune system is an important factor in AD and many studies have reported an infiltration of immune cells into the inflamed tissue namely; DCs [97], mast cells [98], eosinophils [99], ILC2s [90, 91], basophils [100] as well as T cells and monocytes [101]. There is also a release of various cytokines which contribute to the pathogenesis of AD. In addition, AD patients are associated with increased IgE serum levels and a type two cytokine profile in the

skin such as TSLP, IL-4, and IL-13 [61]. The role of IL-4/IL-13 signalling is further illustrated by the success of Dupilumab, a monoclonal antibody targeting IL-4 receptor alpha (R α) significantly reduced disease severity [102, 103]. Figure 1.3 attempts to illustrate some of the critical cytokines and cells involved in the progression human AD in both an acute and chronic setting. The manner in which the immune cells orchestrate an inflammatory response in patients with AD is key to understanding AD pathogenesis.

1.7.1 Immune Cells

1.7.1.1 T cells

T cells are well known for their contribution to the adaptive arm of the immune response. For several years, AD has been reported to be a Th2 mediated disease, and many AD patients display high levels of serum IgE and a Th2 cytokine milieu. Both memory and effector T cells express cutaneous lymphocyte associated antigen (CLA) receptor which is crucial for skin homing. Interestingly, elevated levels of CD4⁺ T cells, expressing CLA have been documented in patients with AD [104]. The Th2 skewed immune response in AD has been attributed to the increased levels of TSLP produced by the keratinocytes in skin lesions [105]. TSLP is well known for its ability to promote a potent Th2 response [106] which include increased production of cytokines such as IL-4 [107], IL-13 [108], IL-5, IL-9 [109] and IL-31 [30, 110, 111], thereby promoting AD pathology.

In addition to Th2 cells, there has been an association of Th17 immunity and AD [112] which has cause a shift in the paradigm of T cell mediation and AD. Previously, a

Th17 immune response was typically associated with skin diseases such as psoriasis [113]. Recent studies have portrayed Th17 infiltration in AD skin lesions and, in addition to IL-17 being increased [114], there was enhanced production of IL-6 and IL-8 from keratinocytes [112]. Conversely, others have disputed the involvement of Th17 cells in AD [115, 116] and this is still highly debated. Notably, success has been obtained using antibodies against the p40 subunit of IL-12/ IL-23 which effectively blocked the IL-23 Th17 pathway in AD patients [117]. This warrants more investigation into the role of Th17 in AD.

Furthermore, there have been reports of increased numbers of Tregs in peripheral blood of AD patients [118] and in skin lesions [119]. Under homeostatic conditions, Tregs are present both in the skin of mice and humans, and are identified by their expression of forkhead box protein 3 (Foxp3) [120]. However, little is known regarding the molecular mechanisms of Tregs in the skin. Collectively, these studies from AD models and patient samples reflect the complexity of the T cell repertoire in AD. We can no longer view AD as a purely Th2 driven disease, thereby making AD an extremely difficult condition to treat.

Intriguingly, the MC903 model is independent of both T & B cells [89]. Skin inflammation continues to develop when RAG1KO mice were treated with MC903. However, there is an infiltration of CD4⁺ and CD3⁺ T cells [89] as well as potent Th2 cytokine [106] profile following MC903 treatment of C57Bl/6 mice. This model is interesting as it proposes that there is an AD phenotype that occurs independent of T cells but with a dependency on ILC2s [1, 91].

1.7.1.2 Dendritic cells (DCs)

DCs are involved in the early events of AD, functioning as sentinels of the skin in the epidermis [4]. The epidermal layer harbours a unique DC subset called Langerhans cells (LCs) in both mouse and human skin, which has been previously discussed. In the dermis of mice, there are several DC subsets known collectively as dermal DCs (dDCs). The dDCs comprise of three phenotypically distinct populations; CD103⁺ CD326⁺ CD11b⁻ (CD103⁺) [121], CD103⁻ CD326⁻ CD11b⁺ (CD11b⁺) [122] and CD103⁻ CD326⁻ CD11b⁻ [123] (triple negative) dDC. In human skin, equivalent dDC subsets have been identified are the CD141^{hi} (CD103 mouse), and the CD1C⁺ and CD14⁺ (CD11b⁺ mouse), with the exception of the triple negative population [95, 124]. Leyva-Castillo et al have elegantly shown once the DCs detect antigen in the skin, the activated DCs migrate to the skin draining lymph node. At the site of the lymph node (LN), DCs prime the naïve CD4⁺ T cells and this results in both cytokine release (e.g. IL-3 and IL-4 from the CD4⁺ T cells) and recruitment of innate immune cells to the LN such as basophils to produce IL-4 and induce further T cell priming [125]. In AD, this occurs under the influence of TSLP (discussed below); a cytokine often referred to as the master regulator; responsible for driving initiation of a Th2 response. TSLP, which is activated with mechanical barrier injury, induces DCs to trigger a Th2 response. This involves the production of type two cytokines such as IL-4 and IL-13 [126]. An elegant study comparing lesions from both AD and psoriasis patients documented elevated mRNA expression levels of TSLPR, CCL17 and CCL18 from AD lesions which was not present in psoriasis lesions. Additionally, this study proposed that the TSLPR was highly expressed on the DCs which also released the Th2 polarizing chemokines CCL17 and CCL18 in AD [97].

1.7.1.3 Innate lymphoid cells (ILCs)

ILCs are a newly identified population of immune cells involved in skin immunity [1, 127]. These are hematopoietic cells that express CD45 and Thy1 (CD90) but lack the expression of cell surface molecules that are associated with other immune cell lineages. This cell subset is classified into three distinct populations i.e. group one ILCs (ILC1), group two ILCs (ILC2) and group three ILCs (ILC3); based on the expression of developmental markers as well as cytokine profile. ILC1s are T-bet dependent and produce IFN γ and TNF, while ILC2s express GATA-3 and produce IL-4, IL-5 and IL-13. ILC3s are a population dependent on ROR γ t and produce IL-17A and/or IL-22.

Kim et al provided the first evidence of ILC2s being constitutively present in both mouse and human skin [90]. Recent studies, have demonstrated a significant accumulation of ILC2s in human AD skin lesions and murine AD models [90, 91, 128] that possess a higher expression of cytokine receptors, IL-25R (IL-17B), IL-33R (ST2) and TSLPR. In steady-state human keratinocytes express adhesion molecule E-cadherin which inhibits the activation of ILCs. Conversely, in AD skin there is a significant reduction of E-cadherin, skin barrier dysfunction and an increase in the keratinocytes producing IL-25, IL-33 and/or TSLP which could potentially activate ILC2s. Roediger et al elegantly showed through intra-vital multiphoton microscopy, the ability of ILCs to patrol the skin microenvironment and interact with mast cells. Interestingly, inducing spontaneous skin inflammation in Rag1KO mice resulted in accumulation of eosinophils, and mast cells and the ILC2s in the skin showed an activated phenotype [1].

To induce AD in mice, a vitamin D analogue, MC903 was topically applied to mouse ears. This resulted in AD like inflammation and an increase in ILC2s in the ear pinna and ear draining lymph nodes (DLN) of mice. MC903-treated Rag1KO mice induced AD like inflammation, suggesting that this MC903 model was independent of T and B cells [89]. The role of ILC2s in the MC903 model was further observed by depleting ILC2s by using anti - CD90.2 antibodies in Rag1 KO mice. This resulted in a significant amelioration of AD-like inflammation in Rag1 KO mice [90].

1.7.1.4 Basophils

Basophils are rare, short-lived, blood-borne cells, expressing FcεR1α and CD49b. AD has been associated with basophil recruitment and activation [129]. It has been reported that TSLP is responsible for eliciting basophils [130] and ILCs [90] in AD murine models. Basophils have been detected in skin lesion of AD but the cell density was sparse in comparison with lesions from other skin diseases namely, prurigo and urticaria [129]. In the context of chronic AD, basophils express higher amounts of IL-4 *in vivo* and promote the recruitment of eosinophils [131, 132]. More recently, basophils and ILCs have been revealed to be in close proximity to each other in skin lesions of both AD patients and AD-like murine skin [127]. In a murine model of IgE dependent skin inflammation, it was demonstrated that activated basophils secreted IL-4, which resulted in the induction of vascular cell adhesion protein (VCAM)-1, thereby recruiting eosinophils to the site of inflammation. This activity of eosinophil recruitment and VCAM-1 expression was reduced in basophil deficient mice. The study suggested that basophils were 'arrested' along the endothelium where they might encounter the antigen and initiate an appropriate response [100]. To date, the

precise role basophils play in AD remains unclear, however, there is sufficient evidence to suggest basophils may act in concert with ILCs to regulate skin inflammation [100, 127, 129, 131, 132]. It has been demonstrated that depleting basophils in an acute MC903 setting, resulted in abrogation of IL-4 from CD4⁺ T cells. However, in a longer 10 day MC903 model depleting the basophils resulted in significantly lower levels of IL-4, i.e. induction of IL-4 was not completely abolished [125].

1.7.1.5 Mast cells (MCs)

Mast cells are unique in that they are long-lived, tissue resident cells and are found at barrier surfaces like the skin and mucosa. MCs, similar to basophils, express FcεR1α, and MCs are often identified by its expression of CD117. MCs were initially identified in the mesentery of the frog, and the discovery of histamine [133] and IgE [134] provided initial insights into the role MCs play in allergic reactions. MCs are derived from hematopoietic progenitor cells in the bone marrow and migrate to the tissue where they mature. In rodents; two different types of mast cells have been classified, the mucosal MCs and the connective tissue MCs [135]. These MC subtypes are usually distinguished by their phenotypic and functional differences. Mucosal MCs preferentially express mouse MC protease (MMCP)-1 and 2 whereas connective tissue MCs express MMCP-4, -5, -6 and carboxypeptidase [136]. In the context of humans, MCs are classified by their content of serine proteases as tryptase - only MC (MCT), chymase – only MC (MCC) or both tryptase and chymase positive MC (MCTC). The MCs between the two species are comparable as MCTs share characteristics with murine mucosal MCs and the MCTC in humans share

characteristics with murine connective tissue MCs [137]. It is however, important to note that tissue distribution is not as specifically demarcated as in mice. Most human tissue have a mixed population of MC types [138]. In terms of the skin, MCs are distributed throughout the dermis and found in close proximity to nerve, lymphatic vessels and blood vessels [139]. This anatomy allows MCs to modulate vascular functions, acquisition of circulating IgE regulate for inflammatory changes and efficiently secrete mediators in response to inflammation of infection [140, 141]. Situated in a prime location; MCs serve as sentinels of the skin, thus enabling them to detect and initiate responses against invading allergen and or microbes [142]. The mouse skin harbours connective tissue mast cells whereas mouse mucosal sites like the gut contain both connective tissue and mucosal mast cells [143].

There is evidence in the literature, that human AD skin lesions [98] and AD mouse models contain increased mast cell numbers [1, 89] however, the precise role mast cells play in AD pathogenesis remains unclear. Healthy human skin, harbours 90% of MCT phenotype [144]. Many studies indicate there is an increase in the MCT phenotype in the skin of AD patients [145]. In an inflammatory setting, FcεR1 aggregates in the tissue, thereby leading to the degranulation of MC mediators such as histamine [146], prostaglandin (PG) D2 [147] as well as cytokines such as IL-31 and IL-33 [144]. All these preformed mediators are likely to be responsible for the symptoms experienced during allergy. Histamine has been shown to disrupt skin barrier dysfunction [148] by downregulating tight junctions [149] in the skin and suppression of filaggrin in human keratinocytes [146].

Prostaglandin D2 (PG)D2 is a chemoattractant for eosinophils and is released from activated mast cells in response to allergic inflammation [150]. MC derived PGD2 is

triggered by the presence of an allergen and this released PGD₂ has been detected in the lungs of asthma patients [151] as well as in skin biopsies from AD patients [150]. PGD₂ exerts its activity via two receptors DP1 [152] and CRTh2 [153]. CRTh2 is expressed on basophils and eosinophils [151] and it has been demonstrated that the PGD₂-CRTh2 system plays a crucial role in allergic skin inflammation [154]. It is essential to bear in mind that CRTh2 cellular interactions are quite different between humans and mice. In humans, CRTh2 is preferentially expressed in Th2 cells rather than Th1 whereas in mice CRTh2 is not biased toward Th2 cells [154].

IL-31 has been described as a pruritogenic cytokine, appreciating its involvement with itch in AD patients [30] and AD mouse models [155]. Interestingly, Niyonsaba et al showed another source for IL-31 in the human skin were mast cells [156].

Allakhverdi et al, demonstrated that in AD skin MCs were activated in a TSLP dependent manner [157]. Roediger et al exhibited, with the use of intravital microscopy, the close proximity of mast cells and dermal ILC2s in murine skin [1].

There is also some evidence to suggest that MCs are involved in the skin with tissue repair and neo-genesis via the formation of new vessels and inflammatory cells [158]. At this stage, further investigations need to be undertaken to fully elucidate the contribution of MCs to AD disease severity.

1.7.1.6 Eosinophils

This cell is unique in morphology, and contains cationic proteins. Paul Ehrlich first described this bi-lobar nucleated cell by the use of an acid dye called eosin [159]. Hence this cell was called eosinophil, named after the dye that was used to detect it. Eosinophils are derived in the bone marrow, under the influence of IL-3, IL-5 and

granulocyte-macrophage-colony-stimulating-factor (GM-CSF), from haematopoietic stem cells. During haematopoiesis, common myeloid progenitors give rise to eosinophil progenitors which express CD34⁺ and IL-5R⁺. It has been shown, that IL-5 signalling is most crucial for eosinophilia to develop [160]. In healthy non-allergic individuals, eosinophils represent at least 1-2% of the leukocytes in the blood [161], and these granulocytes are confined specifically to the digestive tract [162].

The recruitment of activated eosinophils from the blood to the tissue occurs under different inflammatory conditions [163]. This recruitment subsequently leads to the release of various cytokines, chemokines, lipid mediators and cytotoxic granule proteins. Once eosinophils mature they contain highly charged basic proteins namely, major basic protein (MBP), eosinophilic cationic protein (ECP), eosinophil-derived neurotoxin (EDN) and eosinophil peroxidase (EPO) [164]. It has been revealed that in eosinophil associated diseases, activated eosinophils undergo degranulation in the tissue, thereby releasing these mediators. This process of degranulation and deposition of granules contribute to disease pathogenesis. MBP and EPO are toxic to several cell types and lead to tissue damage and organ dysfunction.

It was initially thought that eosinophils were solely terminally differentiated pro-inflammatory effector cells, however, recent reports have highlighted eosinophil involvement in the initiation, the propagation and even the resolution of both the innate and adaptive arms of the immune system [165]. Both tissue eosinophilia and increased blood eosinophil levels correlates with disease severity in a number of different diseases; including asthma [166, 167], inflammatory bowel disease (IBD) [168], eosinophilic oesophagitis (EOE) [169] and even some leukaemias and carcinomas [170]. It has been documented in these disease contexts, that

accumulated eosinophils in the tissue, subsequently secrete either cationic proteins (e.g. MBP), cytokines (e.g. IL-5), lipids, chemokines, and/or oxygen radicals, which resulted in direct tissue damage or induction of other innate cells and/or cytokines [171].

This innate cell has often been associated with a classical Th2 immune response in parasitic infection and allergic disease, however, studies have indicated that it may play a part in some Th1 conditions [172]. A substantial body of evidence, in both mouse models and human clinical studies, has established a role for eosinophils in the pathogenesis of asthma [167, 173]. This includes a role for eosinophils in airway hyperplasia, elevated mucus production and remodelling [174, 175].

The development of an eosinophil peroxidase antibody (EPX), has been a significant contribution to the field. This monoclonal antibody has provided an efficient strategy to quantify eosinophil infiltration as well as degranulation in tissue biopsies [169]. Thereby, using eosinophils as a diagnostic tool in eosinophilic driven allergic diseases for e.g. in EOE patients [169] and in patients with bladder carcinoma [170].

Under homeostasis, the skin does not contain eosinophils, however it has been observed that AD skin biopsies contain large depositions of eosinophil granule proteins in the dermis. Moreover, Yawalkar et al showed the increase in eotaxin, a chemoattractant for eosinophils, and its receptor chemokine receptor type three (CCR3) to be upregulated in AD lesions [176]. PGD₂ has been highlighted as another chemoattractant for eosinophils in the context of allergic diseases such as asthma [177]. Lee et al illustrated that eosinophils may play an essential role in skin innervation, remodelling and itching. These findings were conducted in a trimethyl anhydride (TMA) induced murine model of allergic skin inflammation [34]. Many

treatment strategies have been explored to either prevent recruitment of eosinophils into the affected area; or to inhibit the cytokines and/or mediators that eosinophils release; or to prevent the survival of eosinophils altogether i.e. with the use of anti - IL-5 treatment. Further investigations need to be undertaken to determine the precise roles eosinophils play in each disease to determine the best therapeutic strategy.

1.7.2 Cytokines

1.7.2.1 Thymic stromal lymphopoietin (TSLP)

TSLP is a member of the four-helix bundle cytokine, and a distant paralog of IL-7. It is expressed by epithelial cells in the skin, lung and intestine. TSLP is able to support the growth and differentiation of B cells and T cell proliferation [125, 178]. The TSLP receptor (TSLPR) is a heterodimer consisting of IL-7Ra chain and a common γ receptor-like chain [179]. Many studies have implicated TSLP as a master regulator of Th2 inflammation in allergic disease [178]. Its regulation has been shown to be multifactorial. Mechanical injury, often a compromised barrier [89, 126], the presence pro-inflammatory cytokines [105, 106] and other proteases such as papain have all been documented to induce TSLP production from the epithelial cells [180]. It has been observed that TSLP expression was increased in cells that had reduced expression of cell to cell adhesion molecules like Caveolin-1 and E-cadherin in patients with asthma [181].

In both acute and chronic AD lesions, TSLP is highly expressed by keratinocytes and influences both the migration and activation of LCs [182, 183]. Increased serum levels of TSLP in children with AD was detected [184]. The literature, with regards to TSLP levels in the serum in adults with AD, appears conflicted. Nakamura et al and Miyagaki et al have reported that adults with AD do not have increased serum levels of TSLP [185, 186] while a smaller study by Alysandratos et al have reported an increase in serum TSLP levels [187]. Furthermore, pro-inflammatory cytokines; such as IL-1 β , TNF, IL-4 and IL-13; were detected at high levels in AD patients and these cytokines have also been reported to synergize and induce more TSLP expression by

keratinocytes [188]. In addition, TSLP acts on various cells types including DCs, T cells [125], mast cells [125, 157], basophils [130] and eosinophils, all in an attempt, to create a type two inflammatory response.

Human TSLP polymorphisms have been linked to increased risk for AD, asthma and allergic rhinitis [189, 190]. Interestingly, recent studies have shown TSLP to directly activate sensory neurons and induce itch, [27] thereby contributing to the pathogenesis of AD. Studies in patients with Netherton syndrome have illustrated a direct link between TSLP and the atopic march. Netherton syndrome is a genetic skin disease with severe atopic manifestations, caused by a mutation in SPINK5 [191]. These individuals have recurrent AD, high IgE titres, asthma and multiple food allergies. The keratinocytes and airway epithelial cells of these patients have high expression levels of TSLP [191, 192].

Intradermal TSLP injections, in a mouse model, were used to induce inflammation and this inflammation was reported to be dependent on eosinophils [193]. Zhang et al demonstrated that TSLP produced by keratinocytes, in an MC903 model, represented a key factor in the progression from AD to asthma [194]. In addition, Han et al demonstrated TSLP is crucial to the allergic march, using a TSLP-OVA mouse model [195]. Hence, TSLP is suggested as an initiator of an inflammatory cascade. In transgenic mouse models, overexpression of TSLP leads to systemic inflammation [196] and when the overexpression of TSLP is specific to airway epithelial cells or keratinocytes, it resulted in an asthma and AD like phenotype respectively [197-199]. Interestingly, similar to the MC903 models, TSLP transgenic mice also develop a phenotype that is independent of T and B cells [89, 199].

Collectively, these murine findings suggest TSLP produced by the keratinocytes promote allergen sensitization in the skin and thereby triggers the allergic march.

1.7.2.2 Interleukin 25 (IL-25/IL-17E)

IL-25 is a distinct member of the IL-17 family [200]. IL-25 shares a receptor IL-17 receptor (IL-17R) B with IL-17 B, although IL-25 is capable of binding to this receptor with a much higher affinity [201, 202]. Both IL-25 and IL-33 (discussed below 1.7.2.3) are stored in the nucleus but IL-25 differs in the fact that it is released from extra-nuclear cell components. Studies have revealed that IL-25 is produced by a number of different cells namely; Th2 cells [200], dendritic cells [203] mast cells [204], eosinophils [205] and epithelial cells [206]. It exerts its activity on T cells, innate immune cells and epithelial cells to drive type two cytokine production such as IL-4, IL-5 and IL-13 [200]. IL-25 mRNA levels have been shown to be higher in lungs of asthmatic patients and the skin of AD patients [203, 207]. Moreover, this cytokine has been described as important in type two directed immune responses in murine models [208]. Wang et al exhibited that TSLP, through the activation of DCs, was able to induce a Th2 response and this response was enhanced by IL-25 [207]. Hvid et al demonstrated that dermal DCs, which were increased in an AD murine model, play a role in the pathogenesis of disease through the production of IL-25 [203]. Furthermore, it has been documented that ILC2s activate IL-25, thereby reducing Th2 cytokine responses in the presence of E-cadherin. This demonstrated that cell to cell adhesion, provided by E-cadherin influenced the regulation of IL-25 [91]. Another study revealed that an increase in IL-25 inhibited the expression of filaggrin on both a gene and protein level in healthy human keratinocytes. This proposes that a

compromised skin barrier, as seen with a reduction in filaggrin may promote IL-25 activity, which will in turn affect filaggrin in a positive feed forward loop [209]. More investigations into the precise cellular mechanism of the regulation of IL-25 activity is required but at this stage all the above-mentioned studies suggest that the tissue microenvironment is essential.

1.7.2.3 Interleukin 33 (IL-33)

IL-33 is a member of IL-1 family and exerts its activity by binding to a heterodimer formed by its primary receptor ST2 and co-receptor, IL-1 receptor accessory protein (IL-1RAcP) [210]. It is constitutively expressed in epithelial barrier tissues and lymphoid organs. The endogenous IL-33 protein remains localized in the nucleus of producing cells in both human and mouse tissue [211]. IL-33 is often described as an alarmin, rapidly released from producing cells upon cellular damage or cellular stress. It is thought that due to the fact that IL-33 is constitutively present in normal tissue, it is ready to be released upon cell injury and 'alarm' other immune cells. However, this is only the case in murine tissue as human keratinocytes display low or absent expression of IL-33 at steady-state. Human tissue does upregulate IL-33 within 24 hours of cellular injury [212].

In response to cellular damage, IL-33 is released from the cells and binds to ST2 on Th2 cells as well as various types of innate immune cells including mast cells, basophils and eosinophils [213] or ILC2s [91]. Membrane bound expression of ST2 is highest on mast cells [214], Th2 cells and eosinophils [215] which are all critical in AD. Recent studies indicate that IL-33 induces ILC2s to produce large amounts of IL-5 and IL-13, and play vital roles in type 2 immunity, allergic inflammation and eosinophil

homeostasis [216-218]. It has been proposed that the regulation of IL-33 may occur on many levels including; genetic encoding, the presence of its decoy receptor soluble ST2 and factors that influence the biological activity and degrading of this cytokine itself. In addition, the synergistic interaction between another epithelial cytokine IL-25 has been shown to increase IL-33 expression in a house dust mite model of airway inflammation [219] and an MC903 model in the skin [91].

Furthermore, human polymorphisms of the ST2 gene has been associated with a high risk for developing AD [220]. Skin-derived ILC2s express IL-33 receptor ST2, which is upregulated during activation, and this population is also enriched in the epidermis of AD patients [218].

Transgenic mice expressing IL-33 specifically from the keratinocytes, via the keratin 14 promoter, were generated by Iami and colleagues and these mice developed spontaneous dermatitis and presented an AD-like phenotype. In addition, IL-33 was shown to be a potent stimulator for skin ILC2s, and in the absence of IL-33 signalling there was a reduction in skin inflammation [91]. Cumulatively, these studies all illustrate the important role for IL-33-ST2 interaction in the pathogenesis of AD [91, 218, 220].

1.7.2.4 Interleukin 9 (IL-9)

This pleiotropic cytokine was initially characterized as a T cell and mast cell growth factor however once its structure was more carefully studied, it became apparent that it was structurally quite different to other T cell growth factors [221]. IL-9 has been often associated with Th2 associated immunity. The first evidence that made the association between IL-9 and Th2 was in a murine model of *Leishmania major*. In this

study, the CD4⁺ T cells produced increased amounts of IL-9 in infected mice and by treating these mice with an anti-IL-4 mAb, mice became cleared of their infection and suppressed IL-9 production [222]. Furthermore, many reports have suggested a specialized subset of T cells, the T helper type 9 (Th9) cells are dedicated to the production of IL-9, in which the gene *il9* is regulated by many different cytokines and transcription factors [223]. Th9 cells have been shown to express IL-9 but not the key signature cytokines of other Th cell subsets. The characterisation of these Th9 cells have caused the direct association of Th2 cells and IL-9 to be somewhat unclear. To date, it has yet to be determined if there is a direct link between Th2 immunity and IL-9 or whether Th2 cytokines e.g. IL-4 promotes the production of IL-9 by Th-9 cells. Interestingly, there have also been reports that IL-9 may mediate a Th17 inflammatory response [224]. In addition, type 1 IFNs are able to promote IL-9 production which is dependent on IL-21 expression.

Two transcription factors were reported to bind directly to the *il9* gene and these were essential for the development of IL-9 secreting cells [225, 226]. These transcription factors are PU.1 and IFN-regulating factor 4 (IRF4). PU.1 has been shown to be a critical factor when diverting cells from a Th2 into an IL-9 secreting lineage [223]. IRF4 expression was correlated with mouse and human Th9 differentiation and is induced by either IL-4, IL-2 or TGF- β , all in an attempt to promote IL-9 production [226]. IL-9 receptor (IL-9R) has two subunits; the alpha chain (IL-9 α) and the common γ chain receptor, which is also shared by other cytokines including IL-2, IL-4 and IL-7. IL-9 and its receptor exerts its activation via STAT1, STAT3 and STAT5. IL-9R has the highest expression on Th2, Th17 [224] and mast cells [227].

Increased IL-9 expression has been associated with airway inflammation [228, 229] and food allergy [230]. In allergic responses in the lung, IL-9 has been demonstrated to contribute to diseases by inducing IL-13, thereby more mucus is released by promoting mast cell expansion [231]. There is increased expression of IL-9 and IL-9R expression in the lungs of asthmatic patients while it is undetected in the healthy controls [232]. In these different contexts of allergy, the source of IL-9 has been either mast cells [233], eosinophils [234], basophils [235], T cells [225], and ILC2s [229]. In AD patients both IL-9 and its receptor genes are overexpressed [236]. Interestingly, there has been a study revealing a correlation between IL-9 and IL-9R polymorphisms and AD [237]. Ma et al conducted comparative studies between AD and psoriasis patients. This study exhibited both higher serum and tissue mRNA levels of *il9* in AD patients compared to the psoriasis patients [109]. Murine food allergy models have revealed IL-9 contributing to mastocytosis, thereby increasing intestinal permeability [238, 239].

Concomitantly, these investigations suggest a role for IL-9 in AD, but this has yet to be fully explored.

1.7.2.5 Prostaglandins (PGs)

PGs are a family of lipid derived mediators and are produced by metabolism of arachidonic acid (AA), cyclooxygenase (COX) and prostaglandin synthase enzymes (PGDS). PGs are ubiquitously produced, and these inflammatory mediators act via G protein coupled receptor (GPCRs) [240, 241]. PGD₂ is one particular PG that has been most implicated to be associated with inflammatory and atopic diseases [240].

PGD2 has been typically associated with mast cell degranulation in acute conditions of human AD (Figure 1.3). Hence, this body of work will focus on PGD2.

1.7.2.5.1 Prostaglandin D2 (PGD2)

PGD2 exerts its activity through activation of two GPCRs namely DP1 and chemoattractant receptor homologous molecule expressed on Th2 cells (CRTh2). Both receptors have been shown to bind to PGD2 with similar high affinity. CRTh2 (also known as DP2) has been more recently identified and it surprisingly is more related to chemoattractant molecules than to the other prostaglandin receptors [242]. In peripheral tissues, activated mast cells have been reported to be the richest source of PGD2 [243, 244], however, later studies revealed Th2 cells [245], DCs [246] and epithelial cells [247] are all capable of producing this mediator.

The expression of CRTh2 has been shown on Th2 lymphocytes, eosinophils, mast cell, basophils [242] and ILC2s [248]. Notably, PGD2 caused activation of ILC2s [248] as well as eosinophils [177].

Reports have revealed that the presence of an allergen in the airways triggered the release of PGD2 into the bronchiolar lavage fluid of asthmatic patients [151]. Evidence of PGD2 has been detected in the nasal mucosa of allergic rhinitis individuals [249] and in the skin of AD patients [250]. Interestingly, when PGD2 was injected into human skin, erythema and leukocyte infiltration resulted [251]. Conversely, through the development of small molecule CRTh2 antagonists, investigations were conducted into the role of CRTh2 in allergic disease. [252, 253]. Uller et al highlighted the efficacy of a CRTh2 antagonist by reducing disease in an experimental model of

eosinophilic airway inflammation [253]. Stebbins and colleagues used CRTh2 antagonism to inhibit symptoms of allergic rhinitis [252].

In the context of the AD, the understanding of PGD2-CRTh2 axis is still in its infancy. Recently, CRTh2 deficient mice were utilized to illustrate the importance of this axis in chronic allergic skin inflammatory model [154]. It has been postulated that blocking the PGD2-CRTh2 axis is a suitable strategy in AD. Furthermore, Sarashina et al developed a transgenic mouse strain overexpressing PGD synthase and their investigations tried to attribute the DP1-CRTh2 axis as an anti-inflammatory pathway and PGD2-CRTh2 as a pro-inflammatory pathway in the skin [254].

Futures studies will still need to be conducted to fully elucidate the PGD-CRTh2 axis in the skin.

1.7.2.6 Interleukin 4/Interleukin 13 axis (IL-4/IL-13)

1.7.2.6.1 IL-13

IL-13, also cloned from T cells of a mouse [255], shares 25% sequence homology with IL-4 [256]. Both IL-4 and IL-13 signal through IL-4R α and STAT6 [257]. IL-13 signals through a heterodimeric receptor composed of IL-4R α and IL-13R α 1 [258]. Furthermore, IL-13 binds to IL-13R α 2, which at this stage is hypothesised to be a decoy receptor. IL-13 has been shown to be critical in allergic inflammatory pathways, in particular asthma [259]. The sources of IL-13 that have been described include Th2 cells [260], ILC2s [261], mast cells [262], eosinophils [263] and basophils [264]. Unlike IL-4, there have been fewer studies, evaluating the role of IL-13 in models of AD. The classical regulation of IL-13 activity is often due to Th2 differentiation occurring and causes binding to the IL-4 receptors thereby inducing

phosphorylation of STAT6. STAT6 dimerizes and translocates to the nucleus to induce GATA3 expression [110]. Interestingly, IL-13R α 2 has been described to be upregulated in the skin of AD patients [265], and mice lacking this IL-13R α 2 show an increase of AD phenotype [266]. David et al illustrated this decoy receptor to be under the regulation of Th2 cytokines produced by keratinocytes [267].

1.7.2.6.2 IL-4

Initially known as an IgG1 induction factor, IL-4 was first cloned from a mouse T cell [268]. IL-4 exerts its activity by binding two types of IL-4 receptors; namely IL-4R type I and IL-4R type II [110]. This cytokine is critical for Th2 cell differentiation [269] and causes IgE class switching to occur in B cells. The sources for IL-4 include Th2 cells, basophils, eosinophils, mast cells, NKT cells and gamma delta T cells. Many studies have appreciated the role of IL-4 in regulating allergic conditions and a protective response against helminths [110, 111, 258].

Murine studies in IL-4 and IL-4R α knockout mice have shown defects in Th2 cell differentiation, fewer infiltrating eosinophils and T cells as well as significantly reduced levels of IgG1 and IgE serum levels [270]. Chan et al developed transgenic mice with the overexpression of IL-4 in the epidermis, and these mice developed skin inflammation and pruritus [107]. Conversely, in another study mice developed spontaneous AD in the absence of STAT6, which suggested that in this model the IL-4 in the skin was independent of STAT6 signalling [271].

There has been a correlation between elevated levels of serum IL-4 levels and a predisposition for developing AD [272]. Furthermore, the role of IL-4 in regulating skin homeostasis has been explored and this adds to our understanding of IL-4 in the skin

[273]. Human epidermal keratinocytes when treated with IL-4 showed an increase of eotaxin 3 expression [274]. Eotaxin, as mentioned earlier, is an important chemokine for the recruitment of eosinophils. The inhibition of a Th2 inflammatory response by targeting both IL-4 and IL-13 is being explored [275]. Recent FDA approval of Dupilumab has been the most promising treatment option [86, 102, 103] for the treatment of AD. This antibody specifically targets IL-4R α , thereby impairing the signalling of both IL-4 and IL-13.

Figure 1.3 Current paradigm of the pathogenesis of human AD

The current mechanism of the pathogenesis in human AD. A) Representation of an acute manifestation: allergens penetrate the skin barrier and this damage leads to TSLP release, inflammation, pruritus and a subsequent Th2 dominated milieu of cells and cytokines such as mast cells, eosinophils, basophils, ILC2s, IL-4, IL-5, IL-13, IL-31 and PGD2. B) Representation of a chronic manifestation of AD which comprises of a mixed immune response. IL-12 and IL-18 secreted by DCs support the activation of IFN γ , thereby inducing keratinocyte apoptosis. Activation of Th22 cells results in tissue remodelling and lichenification of the skin. (adapted from Peng and Novak 2015 [64]).

1.8AD current knowledge

1.8.1 Murine models of AD

Much of the current knowledge and understanding of allergic skin inflammation has arisen from murine models. A few models have been described below and are categorized into the following; 1) epicutaneous sensitizations with allergen 2) hapten-induced, 3) transgenic mice models, 4) mutant mouse strains, [199, 276] and 5) topical applications to induce skin inflammation. The transgenic strains either overexpress or lack the certain molecules that are thought to be important in the disease progression of AD. The mutant mouse strains develop spontaneous AD.

1.8.1.1 Epicutaneous sensitization (EC) with allergen

1.8.1.1.1 Ovalbumin (OVA)

This murine model of AD involved repeated tape stripping of the back of the skin, followed by topical application of OVA protein. The method of tape stripping is an artificial way to mimic the itching that occurs in patients with AD and to mechanically breach the skin barrier [277]. Ova-sensitised mouse models have been reported in five strains of mice and requires shaving of the hair on the back of the mouse. It is an attempt to mimic the mechanical injury to the skin in patients with AD following continuous scratching. This model of AD resulted in epidermal and dermal thickening, increase in CD4⁺ T cells, eosinophils, IL-4, IL-5 and IL-13 [277]. Mast cells and natural killer (NK) T cells were not necessary for the development of skin inflammation in this EC OVA model [278, 279].

1.8.1.1.2 House dust mite (HDM)

In a similar manner to the EC OVA model, an EC model using recombinant mite allergen Der p8 has been established in BALB/c mice. This model has revealed epidermal hyperplasia, spongiosis, an infiltration of both CD4+ and CD8+ T cells. Interestingly, nerve fibres were seen by immunohistochemistry (IHC), to be in close proximity to mast cells [280]. The use of the HDM model is clinically relevant as there has been considerable evidence that HDM is associated with human AD [281].

1.8.1.2 Hapten – induced AD model

Multiple challenges with different haptens such as oxazolone (Ox) or 2,4,6-trinitrochlorobenzene (TNCB) to the skin of shaved mice induces a Th2 inflammatory response of allergic skin inflammation [282, 283]. This model demonstrated infiltration of mast cells, eosinophils, elevated IL-4 in the dermis and high IgE levels. A decreased expression of skin proteins such as filaggrin, loricrin, and involucrin were seen. These are important proteins that maintain the skin barrier integrity [283].

1.8.1.3 AD transgenic models

1.8.1.3.1 IL-4 transgenic mice

These transgenic mice overexpress IL-4 specifically in the skin and develop spontaneous pruritus as well as dermatitis lesion from 4 months of age. This model presents with either an acute phenotype (early lesions) or a chronic phenotype. The acute lesions contain T cell infiltrate in the epidermis and dermis and elevated IgE and IgG1 levels. The chronic lesions contain T cell infiltrate in the dermis only and have mild spongiosis, acanthosis and dermal eosinophils [107].

1.8.1.3.2 TSLP transgenic mice

In patients with AD, TSLP is produced by keratinocytes where it has been associated with both the activation as well as the migration of DCs in the dermis. To exploit this feature of AD, mice that specifically express TSLP by keratinocytes were generated [182]. Similarly, the keratin (K)5-TSLP mice were established to study the inflammatory mechanism of AD. These mice overexpress tetracycline inducible TSLP in the skin, which is under the direct control of the K5 promoter. These mice exhibit a Th2 cell profile, and higher expression of IL-4, IL-5 and tumour necrosis factor (TNF) [197]. When these mice were crossed with mice that lack T cells, the TSLP transgenics continued to develop AD-like lesions with an accumulation of eosinophils and mast cells in the dermis. This implies skin inflammation in this model is T-cell independent.

1.8.1.3.3 Retinoid X receptor knockout (KO) mice

Retinoid X receptor (RXR) is a part of the super family of nuclear receptors which play an important role in cell development and immune functions. RXR has three isotypes: α , β , and γ and it is able to form heterodimers with other nuclear receptors such as retinoic acid receptor (RAR) and 1,25-dihydroxyvitamin D3 receptor (VDR) [284]. Li et al generated mice that lacked RXR α and RXR β in the epidermal keratinocytes, which are directly under the control of keratin 14 promoter. These RXRKO mice displayed the clinical features of xerosis, reddening of the skin and pruritus of AD as well as an infiltration of CD4⁺ T cells, mast cells and eosinophils in the dermis. In addition, this RXRKO AD model was documented to exhibit a Th2 cytokine profile. Intriguingly, this model demonstrated that TSLP, produced by the keratinocytes, is the initiator of the skin inflammatory pathway [199].

1.8.1.4 Spontaneous AD development in NC/Nga mice

The NC/Nga mice originated in Japan was an inbred strain that has been reported to develop spontaneous AD-like lesions, when these mice were in conventional surroundings. These mice developed AD-like lesions from 8 weeks of age with it reaching its peak around 17 weeks. However, when these mice were kept in specific pathogen free conditions, this phenotype was lost [51, 285]. It has been documented that these mice display infiltration of CD4⁺ T cells, high levels of IL-4 and IL-5, degranulation of mast cells and eosinophils which all correlated to the AD progression. Surprisingly, NC mice that were crossed to signal transducer and activator of transcription (STAT)6KO mice was still able to elicit AD-like lesions to the same extent as NC control mice. Contrary to what has previously been hypothesized [286, 287] STAT6 signalling did not play a role in this model of atopic disease [271]. Unfortunately, the spontaneous AD development in this model produced variable results across different lab groups, and this is possibly due to the different mouse facility environments. The more sterile the environment the less the AD phenotype developed [285, 288].

1.8.1.5 Topical application to induce skin inflammation

1.8.1.5.1 TMA application

A contact toxicant, Trimethyl anhydride (TMA), has been topically applied to BALB/c mice to induce skin inflammation [289]. This TMA model was also utilized to explore the mechanistic role between eosinophils and neurosensory responses in the skin, with a particular focus on itching [34]. In this study, mice treated with TMA exhibited

an increase in tissue eosinophilia and degranulation. In addition, it was documented by using TMA-treated eosinophil deficient mice that both inflammation and itch were dependent on eosinophils. Furthermore, Lee et al, showed a decrease in substance P (a neuropeptide on sensory nerves) [290] in the TMA treated IPHIL mice, suggesting an association between nerve innervation and eosinophils [34].

1.8.1.5.2 MC903 application

Elegant investigations have demonstrated that topical application of the vitamin D3 analogue MC903 (also known as Calcipotriol) was sufficient to induce allergic skin inflammation similar to AD [1, 89-91, 125]. This characteristic allergic skin inflammation includes epidermal thickening, dermal hyperplasia as well as an increased number of inflammatory cells in the skin, most notably mast cells, eosinophils and Th2 cells [89]. To date, many cell types and cytokines have been implicated to be involved with the MC903 AD model. However, there have been murine strain differences reported, model differences in terms of the concentration and duration of application and transient involvement of cytokines [89-91]. Many mouse models of AD depict very high prolonged levels of circulating (systemic) TSLP and this is not consistently seen in patients with AD [195, 291]. The kinetics of TSLP accumulation in AD is still a feature of the disease that needs to be understood. There have been multiple cell types, mediators and cytokines associated with AD and discussed in this chapter is a snapshot of how the field of AD research has progressed.

1.9 Thesis Aims

Some of above-mentioned cell types and cytokines in this chapter namely; TSLP, CRTh2 and IL-33 have been considered as potential biomarkers for AD [292]. This thesis aims to add to the current knowledge of the heterogeneity of AD, to the existing biomarkers, and contribute novel targets for treatment of AD. In an era where precision medicine seems to be the best treatment strategy, biomarkers can be classified into diagnostic, predictors of disease and targets for treatment [66]

- To establish a clinically relevant murine model of skin inflammation that displays the hallmarks of AD, using the vitamin D analogue MC903.
- To characterise the cells involved in this MC903 model of AD.
- To delineate the cytokines involved in the MC903 model of AD.
- To determine specific immune targets for the treatment and prevention of AD.
- To obtain mechanistic insight into the inflammatory cascade in the MC903 model of AD.

2 Materials and methods

2.1 Materials

2.1.1 Labware

Supplier/Manufacturer	Product
Axygen Scientific, Inc., Union City CA USA.	<ul style="list-style-type: none">• 0.5-20uL, 200ul, 1000uL Tips Racked and Pre-sterilised• 0.6, 1.7 and 2.0mL MAXYmum Recovery™ Microtubes
Becton Dickson Pty, Ltd Mountain View, CA USA.	<ul style="list-style-type: none">• BD Ultra-Fine™ & Ultra-Fine™ II Insulin Syringes - Bulk Packaged - Box/100 15mL, 50mL high-clarity polypropylene conical centrifuge tube• 5mL, 10mL, 25mL polystyrene serological pipet.• 12x75 mm, 5 mL polystyrene round bottom test tube. No cap. Non-sterile.• BD Falcon™ 175 cm² Cell Culture Flask, 750 mL, tissue culture treated, straight neck, black lined phenolic screw cap.• BD Falcon™ 75 cm² Cell Culture Flask, 250 mL, tissue culture treated, straight neck, black lined phenolic screw cap• BD Falcon™ 25cm² Cell culture Flask 50 mL, tissue culture treated, straight neck, black

	<p>lined phenolic screw cap</p> <ul style="list-style-type: none"> • BD Falcon™ 6-well Multiwell Plate. Tissue culture treated polystyrene, flat bottom, with low-evaporation lid. • BD Falcon™ 12-well Multiwell Plate. Tissue culture treated polystyrene, flat bottom, with low-evaporation lid. • BD Falcon™ 24-well Multiwell Plate. Tissue culture treated polystyrene, flat bottom, with low-evaporation lid. • BD Falcon™ Clear 96-well Microtest™ Plate. Tissue culture treated polystyrene, flat bottom, with low- evaporation lid. • BD Falcon™ Clear 96-well Microtest™ Plate. Tissue-culture treated polystyrene, U-bottom, with low- evaporation lid. • BD Plastipak™ Syringes 30 mL, Luer- Lok™ • BD Syringe 1mL, 2mL, 3mL, 5mL, 10mL Luer-Slip Tuberculin • Cellstrainer with 70 µm nylon mesh, white frame color. Sterile.
Millipore Sigma	<ul style="list-style-type: none"> • Millipore Pure Express™ Plus Stericup and Steritopp. 0.22µm non pyrogenic pre-sterilized. Vacuum driven.
Sefar Filter Specialist, Nelson, New Zealand	<ul style="list-style-type: none"> • 70µm nylon gauze • 80µm stainless steel mesh
Thermo Fisher Scientific, Waltham, MA, USA.	<ul style="list-style-type: none"> • Nunc CryoTube 1.0mL 1.8mL PP sterile Nunc-Immuno™ Plates

	<p>Polystyrene, without lids. 96 wells per plate. MaxiSorp®.</p> <ul style="list-style-type: none"> • Transfer pipette graduated 1mL large bulb •SecureSeal™ Thermal Adhesive Sealing Film for PCR application • Slide-A-Lyzer Dialysis Cassettes, 10000 MWCO • Glass Pasteur pipette 150mm (p/1,000) • Nanodrop 1000 Spectrophotometer
TESA® Micromaster®	<ul style="list-style-type: none"> • Micromaster® electronic micrometer with digital display • 0-30mm
Cortex Technology Smedevaenget Hadsund, Denmark	<ul style="list-style-type: none"> • DermaLab USB Single Parameter TEWL probe, SKINLABS Software
Sarstedt	<ul style="list-style-type: none"> • Microvette® 200 Z-Gel tubes. 200ul polypropylene tubes with clotting activator for serum.
Thermo Scientific™, New Zealand Ltd.	<ul style="list-style-type: none"> • Tissue Loc™ histological tissue embedding cassettes
Briscoes, Wellington, New Zealand	<ul style="list-style-type: none"> • Breville Fast slow cooker
Thermo Scientific™	<ul style="list-style-type: none"> • Citadal 2000 Carousel Tissue processor • HistoStar Embedding Center • HM 325 Rotary Microtome
Olympus, Wellington, New Zealand	<ul style="list-style-type: none"> • Brightfield BX51 Microscope
	<ul style="list-style-type: none"> • Laser Scanning Confocal Microscope FV1200®

2.1.2 Reagents and buffers

2.1.2.1 Culture Media Reagents

Foetal bovine serum (FBS)

FBS was purchased from Invitrogen (Gibco, Auckland, New Zealand, 12203C) and stored in 25mL aliquots at -20°C

Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM: F12)

DMEM was purchased from Invitrogen (Gibco Auckland, New Zealand, 11320-033) and supplemented with 100U/mL antibiotic - antimycotic from Invitrogen (Gibco Auckland, New Zealand, 15240-062) and 5% FBS. Media was filter sterilised and stored at 4°C for maximum of 14 days.

Cell dissociation buffer (enzyme-free)

Cell dissociation buffer was purchased from Invitrogen (Gibco Auckland, New Zealand, 1315-014).

Complete Iscove's modified Dulbecco's medium (cIMDM)

IMDM was purchased from Invitrogen (Auckland, New Zealand, 31980-097) and supplemented with 100U/mL Penicillin-Streptomycin from Invitrogen (Gibco, Auckland New Zealand, 15140-122), and 5% FBS. Media was filter sterilized and stored at 4°C for maximum of 14 days.

Ethylenediaminetetraacetic acid (EDTA)

Diluted EDTA (0.5M) was purchased from Invitrogen (Auckland, New Zealand, 15575-020) and stored at room temperature.

Trypsin-EDTA

Trypsin containing 0.05% Ethylenediaminetetraacetic acid (EDTA) and phenol red was purchased from Invitrogen (Gibco, Auckland, New Zealand, 25300062) and stored in 25mL aliquots at -20°C.

2.1.2.2 Enzyme and tissue processing

Bovine serum albumin (BSA)

BSA was purchased as a lyophilized powder from, Sigma-Aldrich, New Zealand (Auckland, New Zealand, A7888) and stored at 4°C.

Bicinchoninic acid (BCA)TM protein assay kit

BCATM protein assay kit was purchased from Thermo Scientific Ltd (Auckland, New Zealand) and stored at room temperature.

Dulbecco's phosphate buffered saline (dPBS)

dPBS is a powder containing no calcium or magnesium was purchased from Invitrogen (Gibco, Auckland, New Zealand, 21600-069) and dissolved in deionized water (dH2O).

Collagenase IV (Collagenase from histolyticum)

Collagenase type IV was purchased from Sigma-Aldrich, New Zealand Ltd (Auckland, New Zealand, C5138). The lyophilised powder was reconstituted in dPBS to the concentration of 50mg/mL and stored in aliquots at -20°C.

Liberase TL

Liberase TL was purchased from Roche Diagnostics New Zealand Ltd (Auckland, New Zealand, 5401020001). The lyophilised powder was reconstituted in IMDM to a concentration of 1mg/mL and stored in aliquots at -20°C for up to three months.

Protein assay kit

PierceTM BCA Protein Assay Kit was purchased from Life Technologies New Zealand Ltd (Auckland, New Zealand, 23225). The kit was stored at 4°C.

DNase 1

DNase 1 was purchased from Roche Diagnostics New Zealand Ltd (Auckland, New Zealand, 10104159001) as a lyophilised powder and dissolved in cIMDM to a concentration of 10mg/mL and stored at 20 °C.

Skin digestion buffer

Collagenase type IV at a final concentration of 2.5mg/mL is made from stock of 50mg/mL and DNase 1 at 120ug/mL from a stock of 10mg/mL were made up in cIMDM to the required volume.

Hydrochloric acid (HCl)

HCl was purchased from Global Science and technology Ltd (Auckland, New Zealand, 10125) and stored in an acid storage cupboard.

Protease inhibitor

Halt™ Protease Inhibitor Cocktail (100X) was purchased from Life Technologies Ltd (Auckland, New Zealand, 78430) and stored at 4°C.

Red blood cell lysis buffer

Red blood cell lysis buffer was purchased from Sigma-Aldrich, New Zealand Ltd (Auckland, New Zealand, R7757) and stored at room temperature.

Sodium Azide (NaN₃)

NaN₃ was purchased in powder form from Sigma-Aldrich, New Zealand, Ltd (Auckland NZ, S8032) and dissolved in dPBS to give a stock concentration of 5%. The solution was stored at room temperature.

Tween 20

Tween 20 was purchased from Sigma-Aldrich, New Zealand Ltd, (Auckland, New Zealand, P13789) and stored at room temperature.

2.1.2.3 Flow cytometry reagents

4,6-Diamidino-2-Phenylindole dihydrochloride (DAPI)

DAPI was purchased as a lyophilized powder from Invitrogen (Auckland, New Zealand D1306) and dissolved in sterile dH₂O to a concentration of 5mg/mL and stored at -70°C. Stock was further diluted in flow cytometry buffer to concentration of

200mg/mL in FC buffer and stored at 4°C.

Live/Dead[®] fixable dead cell staining kit

Live/Dead kit was purchased from Life Technologies New Zealand Ltd, Molecular Probes (Auckland, New Zealand L23105) and stored at -20°C. The lyophilized powder was dissolved in 50uL anhydrous DMSO (components of the kit) per vial before use and stored at -20°C for a maximum of 6 weeks.

Compensation beads

OneComp ebeads were purchased from eBioscience (San Diego, CA, USA, 0111142) and was stored at 4°C. These beads react with antibodies of mouse, rat and hamster origin.

Flow cytometry (FC) buffer

NaN₃, FBS and EDTA were added to IL dPBS to give final concentration of 0.01% NaN₃, 1% FBS and 1mM of EDTA. FC buffer was stored at 4°C.

Flow counting beads

AccuCount Blank Particles was purchased from Spherotech[™] (Lake Forest, Illinois USA, ACBP2010) and stored at 4°C.

Golgi Stop

GolgiStop[™], a protein transport inhibitor was purchased from Invitrogen (Auckland New Zealand) as a part of the fixation and permeabilization kit (554715).

Ionomycin

Ionomycin was purchased from Merk Millipore (407950), resuspended in sterile dPBS at stock concentration of 1µg/mL and stored at -20°C.

Intracellular cytokine kit

Fixation and permeabilization kit was purchased from Invitrogen (Auckland, New Zealand, 554715)

Phorbol 12-myristate 13-acetate (PMA)

PMA was purchased from Sigma-Aldrich, New Zealand Ltd (Auckland, New Zealand, P8139), resuspended in sterile dPBS at stock concentration of 50ng/mL and stored at -20°C.

2.1.2.4 Antibodies

2.1.2.4.1 Monoclonal antibodies used for *in vivo* studies

Rat anti - mouse CD4 (Clone GK1.5)

Anti - mouse CD4 was purchased from Bio X cell (West Lebanon, New Hampshire USA, BE0003-1). It was stored at shipping concentration at -70°C.

Rat anti - mouse IL-5 (Clone TRFK5)

Anti - mouse IL-5 was kindly provided by Abbvie. It was stored at shipping concentration at -70°C.

Rat anti - mouse IL-9 (Clone 9C1)

Anti - mouse IL-9 was purchased from Bio X cell (West Lebanon, New Hampshire USA, BE0181). It was aliquoted at a concentration of 1mg/mL and stored at -20°C.

2.1.2.4.2 Isotype controls

Rat anti - mouse IgG2a (Clone C1.18.4)

Anti - mouse IgG2a was purchased from Bio X cell (West Lebanon, New Hampshire USA, BE0085). It was aliquoted at a concentration of 1mg/mL and stored at -20°C.

Rat anti - mouse IgG1 (HRPN)

Anti - mouse IgG1 was purchased from Bio X cell (West Lebanon, New Hampshire USA, BE0088). It was aliquoted at a concentration of 1mg/mL and stored at -20°C.

Rat anti - mouse IgG2b (LTF-2)

Anti - mouse IgG2b was purchased from Bio X cell (West Lebanon, New Hampshire USA, BE0090). It was aliquoted at a concentration of 1mg/mL and stored at -20°C.

Rat anti - mouse CD16 (Fc γ RIII)/CD32 (Fc γ RIII) (Clone 2.4G2)

Alternatively known as Mouse Fc Block, was affinity-purified in house (Malaghan Institute, Wellington New Zealand) from hybridoma culture supernatants using Hi Trap protein G Sepharose columns.

2.1.2.4.3 Fluorescently conjugated antibodies

Table 2.1 – Flow cytometry antibodies

Specificity	Fluorophore	Clone	Company
Antigen 7/4	PE	7/4	Abcam
B220	BV650	RA3-6B2	BD Pharmingen
CD2	FITC	RM2-5	BD Pharmingen
CD3	PE CY7	2C11	eBioscience
	BV786	145-2C11	BD Horizon
CD4	Qdot 605	RM4-5	Invitrogen
CD11b	FITC	M1/70	BD Pharmingen
	BV650	M1/70	BD Pharmingen
CD45	APC CY7	30F11	BD Pharmingen
	BUV395	30F11	BD Pharmingen
CD49b	FITC	DX5	Biolegend
CD64	APC	X54-5/7.1	Biolegend
CD90.2	APC	53-2.1	Biolegend
CD117	APC	2B8	BD Pharmingen
	APC CY7	2B8	Biolegend
CD200R3	APC	Ba13	Biolegend

CD326	BV421	G8.8	BD Pharmingen
	BV711	G8.8	BD Pharmingen
FECR1a	PE	MAR-1	Biolegend
	APC	MAR-1	Biolegend
IgE	Biotin	R35-118	BD Pharmingen
IL-9	PE	RM9A4	Biolegend
NK1.1	BV650	PK136	BD Pharmingen
Siglec F	PE CF594	E50-2440	BD Horizon
T1 ST2	Biotin	DJ8	MD Bioproducts
TSLPR	PE		R & D systems
Fluorophore bound Streptavidin		Company	
SA- PE CY7		BD Pharmingen	

2.1.2.4.4 Immunohistochemistry (IHC) antibodies

2.1.2.4.4.1 Primary antibodies

Hamster anti - mouse Interleukin 9 (Clone D9302C12)

Biotin anti - mouse IL-9 was purchased from Biolegend, (Auckland, New Zealand, 504804) and stored at 4°C.

Rat anti - mouse interleukin 9 receptor (IL9R) (Clone 12F43)

IL9R monoclonal antibody was purchased from Acris Antibodies, Inc. (San Diego CA, USA, DM3569P) and stored at 4°C.

Mouse anti - mouse eosinophil peroxidase (EPX) (Clone MM25-82.2)

Eosinophils peroxidase monoclonal antibody was purchased from the Mayo Clinic (Phoenix, Arizona, USA) as lyophilized powder and at -70°C.

2.1.2.4.2 Secondary antibodies

Hamster IgG isotype control (Clone HTK888)

Purified Armenian hamster IgG isotype control was purchased from Biolegend, (Auckland, New Zealand, 400901) and stored at 4°C.

Donkey anti - rabbit IgG (Poly4064)

Polyclonal DyLight TM 594 donkey anti - rabbit IgG antibody was purchased from Biolegend, (Auckland, New Zealand, 406405) and stored at 4°C.

Rabbit anti - hamster IgG

Anti - hamster IgG biotin conjugate antibody was purchased from Novex, (Fredrick MD USA, A18899) and stored at 4°C.

Anti - rabbit IgG

Alkaline phosphatase anti - rabbit IgG was purchased from Vector Laboratories, Inc. (Burlingame CA, USA, AP-1000) and stored at 4°C.

2.1.2.5 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISAs were all performed to detect cytokine production in either serum samples and or in tissue lysate samples. ELISA kits were purchased from either BD Pharmingen (Auckland New Zealand), R&D Systems (Minneapolis Minnesota, USA) or eBioscience (San Diego, CA, USA). Reagents were all stored as per manufacturers' specifications.

Table 2.2 ELISA Kits for cytokine detection

Species	ELISA kit	Cat NO	Company
Mouse	IgE OptEIA	555248	BD Pharmingen
Mouse	IL-1 β	EBI 88-7013-88	eBioscience
Mouse	IL-4		In house

Mouse	IL-9	EBI 88-8092-88	eBioscience
Mouse	IL-13	EBI088-7137-88	eBioscience
Mouse	IL-17	DY421	R & D systems
Mouse	IL-25	EBI 88-7002-88	eBioscience
Mouse	IL-31	BMS6030	eBioscience
Mouse	IL-33	EBI 88-7333-88	eBioscience
Mouse	TGF- β 1	DY1679	R & D systems
Mouse	TSLP	DY555	R & D systems

Mouse IL-4 ELISA Reagents (in house)

Capture antibody- 11B11 (2 μ g/mL)

Detection antibody- α IL-4 - biotin (2 μ g/mL)

TMB substrate (BD Pharmingen, Auckland, New Zealand, 555214)

Streptavidin-conjugated horseradish peroxidase (HRP) (Biolegend, Auckland New Zealand 405210)

2.1.2.6 Allergens, peptides and drugs

Acetone

Acetone was purchased from Global Science and technology Ltd (Scharlau Auckland, New Zealand, ACS03112500) and stored at room temperature.

Anaesthetic

10x stock solution was diluted in sterile dPBS to 8.6mg/mL ketamine and 0.26mg/mL xylazine working solution.

Calcipotriol (MC903)

Calcipotriol (hydrate) > 98% was purchased from Cayman Chemicals (Ann Arbor, Michigan, USA, CAS10009599). Calcipotriol was diluted in absolute ethanol to a concentration of 1.6mM and stored at -20°C. Stock solution was diluted 1:10 in absolute ethanol to obtain a working solution.

CRTh2 antagonist (CRTh2^{ant})

CRTh2 antagonist was purchased as a lyophilized powder from Ferrier Institute (Wellington, New Zealand, GT145A). CRTh2^{ant} was reconstituted to a final concentration of 1mg/mL in 0.5% methylcellulose.

Diphtheria Toxin (DT)

DT was purchased as a lyophilized powder from Sigma-Aldrich, New Zealand Ltd, (Auckland, New Zealand, D2918). DT was reconstituted in dH₂O at a stock concentration of 2mg/mL. Stock aliquots of DT is further resuspended in 1ML of sterile dPBS to obtain a 20ng/g working solution.

Ethanol (EtOH)

Absolute ethanol was purchased from Pure Science (Porirua, New Zealand, N1034705).

Methylcellulose

Methylcellulose was purchased as a lyophilized powder, from Sigma-Aldrich, New Zealand Ltd, (Auckland, New Zealand, M-0512).

Olive oil

Olive oil was purchased from Sigma-Aldrich, New Zealand Ltd (Auckland, New Zealand, 01514) and stored at room temperature.

Protein- tyrosine kinase inhibitor

Imatinib (Glivec[®]) >87% purity, was purchased as a lyophilized powder from Ferrier Institute (Wellington, New Zealand, GT151A) [293].

Resorcinol

Resorcinol ASC .99.0% was purchased from Sigma-Aldrich, New Zealand Ltd, (Auckland, New Zealand, 398047), as a lyophilized powder and stored at room temperature.

Thymic stromal lymphopoietin (TSLP)

Recombinant TSLP (mouse) with purity of >95% was purchase from R & D Systems

(Minneapolis, Minnesota USA) in lyophilized form and stored at -20°C until reconstituted. rTSLP was reconstituted at 10g/mL in sterile dPBS and stored at -20°C for maximum of 3 months.

Trimethyl Anhydride (TMA)

1,2,4- Benzenetricarboxylic anhydride was purchased as a lyophilized powder, from Sigma-Aldrich, New Zealand Ltd, (Auckland, New Zealand, B4600). 5% and a 2% working solution of TMA was obtained by diluting TMA in an acetone: olive oil solution. The acetone: olive oil was made up as a ratio of 4:1.

2.1.2.7 Histological reagents

2.1.2.7.1 Histology processing

Formaldehyde

Pierce™ 16% Formaldehyde (w/v) methanol free was purchased from Life Technologies New Zealand Ltd (Auckland, New Zealand, PIE28908) and stored at room temperature. 16% formaldehyde is diluted in dPBS to obtain a working solution of 4%.

HistoClear

HistoClear II, clearing agent (xylene alternative) was purchased from Thermo Fisher Scientific New Zealand Ltd (Auckland, New Zealand, ASHC78/2-G) and stored in a chemical storage cupboard.

Paraffin Wax

Histoplast PE paraffin, an embedding medium for histology was purchased from Thermo Fisher Scientific New Zealand Ltd (Auckland, New Zealand, ALP8330) and stored at room temperature. Histoplast paraffin is melted at 56°C when in use at the embedding station.

2.1.2.7.2 Immunohistochemistry reagents

Antibody diluent

Antibody diluent with background reducing components was purchased from Dako North America Inc. (Carpenteria, USA, S3022) and stored at 4°C.

Bouins Solution

Bouins solution was purchased from Sigma-Aldrich, New Zealand Ltd, (Auckland, New Zealand, HT10132) and stored at room temperature.

DPX mountant

DPX was purchased from Ajax Fine Chemicals (Thermo Fisher, Auckland, New Zealand, 3197) and stored at room temperature.

Eosin Y

Eosin Y was purchased from Sigma-Aldrich, New Zealand Ltd (Auckland, New Zealand, 230251) and stored at room temperature.

Haematoxylin

Mayer's Haematoxylin was purchased as lyophilized powder, from Sigma-Aldrich, New Zealand Ltd (Auckland, New Zealand, H3136) and stored at room temperature. Haematoxylin was diluted in dH₂O to obtain a working solution was.

Methyl green counterstain

Methyl green was purchased as lyophilized powder, from Sigma-Aldrich, New Zealand Ltd (Auckland, New Zealand, M8884) and stored at room temperature.

Mouse on mouse alkaline phosphatase (AP) Polymer

Mouse on mouse AP polymer was purchased from Biocare Medical (Concord, CA, USA, MM624H) and stored at 4°C.

Mounting medium

Faramount aqueous mounting medium was purchased from Dako North America Inc. (Carpenteria USA, K0640) and stored at room temperature.

Naphtol AS-D Chloroacetate solution

Naphtol AS-D Chloroacetate was purchased from Sigma-Aldrich, New Zealand Ltd (Auckland, New Zealand, N0758) and diluted into N-N dimethyl formamide and stored at -20°C.

N-N dimethyl formamide

N-N dimethyl formamide was purchased from Sigma-Aldrich, New Zealand Ltd (Auckland, New Zealand, FSBD3840) and stored at -20°C.

New Fuchsin solution

New Fuchsin was purchased as lyophilized powder, from Sigma-Aldrich, New Zealand Ltd (Auckland, New Zealand, 72200) and diluted in hydrochloric acid (HCl) and stored at room temperature.

Normal rabbit serum

Rabbit serum was heat-treated and purchased from Vector Laboratories, Inc. (Burlingame CA, USA, S-5000) and stored at 4°C.

Normal goat serum

Goat serum was heat-treated and purchased from Vector Laboratories, Inc. (Burlingame CA, USA, S-1000) and stored at 4°C.

Normal rat serum

Rat serum was heat-treated and purchased from Invitrogen (Auckland, New Zealand, 10710C) and stored at 4°C.

Permanent red substrate

Permanent red substrate was purchased from Dako North America Inc. (Carpenteria USA, K0640) and stored at 4°C.

Pepsin Digest

Digest-All™ (Pepsin solution) digest was purchased from Invitrogen (Auckland, New Zealand, 003009) and stored at 4°C.

Rodent Block

Rodent block was purchased from Biocare Medical (Concord, CA, USA, RMB961L) and stored at 4°C.

Rodent decloaker

Rodent decloaking (10x) buffer was purchased from Biocare Medical (Concord, CA, USA, RD913L) and stored at room temperature. Rodent decloaking buffer diluted at a 1x concentration in dH₂O.

Sodium Nitrite solution

Sodium nitrite was purchased as lyophilized powder, from Sigma-Aldrich, New Zealand Ltd (Auckland, New Zealand, 237213), diluted in dH₂O and stored at room temperature.

Tris Buffer Saline (TBS)

TBSplus at a stock of 10x, was purchased from Biocare Medical (Concord, CA, USA, TBS942M) and stored at room temperature. TBS was diluted dH₂O a 1X working solution.

Trichrome staining kit

Masson's trichrome staining kit was purchased from Sigma-Aldrich, New Zealand Ltd (Auckland, New Zealand, H7151KT) and stored at room temperature.

2.1.2.8 Polymerase chain reaction reagents

Master Mix

TaqMan® Fast universal PCR master mix was purchased from Applied Biosystems™ (Life Technologies Ltd, Auckland, New Zealand, 4364103). The kit was stored at -20°C.

Reverse transcription kit

High-Capacity cDNA-to RNA™ kit was purchased from Invitrogen, (Auckland, New Zealand, 4387406) and stored at -20°C.

RNA extraction kit for sorted cells

Quick-RNA™ MicroPrep kit was purchased from Ngaio Diagnostics Ltd (Nelson, New Zealand, #R1051). Columns were removed from the kit stored at 4°C while the remainder of the kit was stored at room temperature.

Taqman Primers

Primers for interleukin 9, interleukin 9 receptor, vitamin d receptor and cyp24A1 (an active form of vitamin D) were purchased from Life Technologies Ltd (Auckland, New Zealand) and stored at -20°C. The assay ID's are as below:

Mm00434313_m1 Interleukin 9 receptor

Mm00434305_m1 interleukin 9

Mm00437297_m1 vitamin d receptor

Mm00487244_m1 cyp24A1

2.1.3 Mice

2.1.3.1 Ethics

Mice were bred and housed within the Biomedical Research Unit of the Malaghan Institute of Medical Research, under specific pathogen free conditions. Majority of the experiments involved mice between 6 - 8 weeks of age and mice were sex matched as best as possible. Experimental procedures were approved by the Animal Ethics Committee at Victoria University and carried out in accordance with guidelines of Victoria University of Wellington, New Zealand,

The mouse experiments in this thesis were under the following ethics protocols;

2014R16M: Understanding the cellular mechanism that drive allergic inflammation

22746: Identification of therapeutic approaches for allergy

2.1.3.2 Mouse strains

Balb/c mouse strain

Balb/c breeding pairs obtained from Jackson Laboratory (Bar Harbour, Maine, USA) and maintained as an inbred strain.

C57Bl/6 mouse strain

C57Bl/6 breeding pairs obtained from Jackson Laboratory (Bar Harbour, Maine, USA) and maintained as an inbred strain. Obtained from Prof William Paul (National Institute of Allergy and Infectious Diseases, National Institute of Health, Bethesda USA).

CRLF2KO (TSLPRKO) mouse strain

Breeding pairs were obtained from Prof William Paul (NIAID, NIH, Bethesda USA). The *CRLF2* gene encodes for TSLPR protein. In *CRLF2KO* mice; this is disrupted by neomycin resistance cassette, as such cells, in this strain of mice, do not express functional TSLPR [294].

IL4 reporter strain (both BALB/c and C57Bl/6 background)

Reporter mouse where green fluorescent protein (GFP) has been knocked – in to the IL-4 gene. Mice express GFP as surrogate for IL-4 expression. Bred as homozygous mice to obtain IL-4 deficient strain [295]. Mice are on a *BALB/c* (Line 185) and *C57Bl/6* (Line 166) background. Breeding pairs were obtained from Prof William Paul (NIAID, NIH, Bethesda USA).

IL-4/IL-13 Dual reporter (4C13R)

Dual reporter mice known as the 4C13R mice with transgenic expression of a cyan fluorescent protein, AmCyan, and a red fluorescent protein, DS Red. The AmCyan under the control of *Il4* regulatory elements and DS Red under the control of *Il13* regulatory elements [1]. Breeding pairs were obtained from Prof William Paul (NIAID, NIH, Bethesda USA).

Il25-LacZ-KI (IL-25KO)

IL-25KO breeding pairs were obtained from M. Kleinshek (Schering Plough Biopharm CA, USA). A LacZ construct was inserted into exon II and III disrupting the production of IL-25. *IL-25^{-/-}* are on *C57Bl/6* background [296].

C57.STAT6KO

Mice homozygous for Stat6^{tm1Gru} mutation have defect in generation of Th2 cells. Lymphocytes are unable to respond to IL-4 and mesenteric lymph nodes from *C57.STAT6KO* mice have reduced secretion of IL-4, IL-5, IL-13 and IFN –gamma. Breeding pairs obtained from Jackson Laboratory (Bar Harbour, Maine, USA) and maintained as an inbred strain [269].

iPHIL.B6

Inducible eosinophil-deficient knock-in mice (*iPHIL*) were generated via a knock- in strategy. Mice were obtained from Professor James Lee (Mayo Clinic, Scottsdale, Arizona). Transgenic-human diphtheria toxin receptor (DTR) has been inserted into endogenous eosinophil peroxidase (*epx*) genomic locus [297].

2.2 Methods

2.2.1 Mouse manipulations

2.2.1.1 Intragastric (i.g.) administration

Solutions were administered to mice in volume of 200-250 μ L via oral gavage using a 22G gavage feeding needle.

2.1.1.1.1 Intragastric administration of CRTh2 antagonist

The CRTh2 antagonist was resuspended at a concentration of 1mg/mL in 0.5% of methylcellulose and administered by i.g. daily from day 7 to day 18. The control group received 0.5% methylcellulose by i.g.

2.1.1.1.2 Intragastric administration of imatinib (protein tyrosine kinase inhibitor)

Dissolved in dPBS, 1mg/mL of this protein tyrosine kinase inhibitor was administered by i.g. daily from day 5 to day 18. The control group received dPBS by i.g.

2.2.1.2 Intraperitoneal (i.p.) administration

Solutions were administered to mice in volume of 200-250 μ L via using a 1ml syringe and 25 gauge needle.

2.2.1.2.1 Intraperitoneal administration of anti - IL-9

Treatment of 250 μ g/mL of anti - IL-9 and the isotype control antibody was administered by i.p. every alternate day from day -1 to day 19.

2.2.1.2.2 Intraperitoneal administration of anti - CD4

Treatment of 0.5mg/mL of anti - CD4 and the isotype control antibody was administered by i.p. evry 4 days from day -3.

2.2.1.2.3 Intraperitoneal administration of anti - IL-5

Treatment of 0.5mg/mL of anti - IL-5 and the isotype control antibody was administered by i.p. from day 12 every alternate day.

2.2.1.2.4 Intraperitoneal administration of resorcinol

Treatment of 25µg/mL of resorcinol was administered by i.p. daily from day 5 to day 19. The control group received dPBS.

2.2.1.2.5 Intraperitoneal administration of DT

Treatment of 20ng/g of DT was diluted to a working solution in dPBS and administered by i.p. every 3 days from day 0 (for the iPHIL mice).

2.2.2 Topical application models

2.2.2.1 MC903 topical application

All mice were between 6 - 8 weeks of age, and were sex matched. Mice are anaesthetized using ketamine/xylazine (200ul per mouse). Once under sedation 10ul of the 1nmol MC903 in 100% absolute EtOH (working solution) was topically applied using a pipette to both sides of the ear (20ul/ear in total). The control group was treated with 100% EtOH. Topical application of 1nmol MC903 was conducted three times a week for 19 days. A total of 8 applications with the final topical application of day 16 and mice were sacrificed on day 19.

2.2.2.2 Trimellitic anhydride (TMA) topical application

Mice between 6-8 weeks were anaesthetized and sensitized (topical application) with 50ul 5% of TMA (97%) on the shaved flank skin on day 0 and day 5. Topical application of 15ul 2%TMA was applied to the dorsum of both ears daily from day 6 to day 14. Mice were sacrificed on day 16. The control group received the

sensitization with 50ul of 5% TMA on the shaved flank but received vehicle. Vehicle was acetone: olive oil, 4:1[34].

2.2.3 Endpoint procedures

Experimental endpoint for the typical MC903 induced AD model was at day 19. The TMA-induced model of skin inflammation had an endpoint at day 16. However, this differed based on the experimental design.

2.2.3.1 Euthanasia

Mice were euthanized by CO₂ asphyxiation or cervical dislocation.

2.2.3.2 Ear thickness measurements

Ear thickness is measured (with a digital calliper) on each treatment day, just prior to topical application.

2.2.3.3 TEWL measurements

Transepidermal water loss (TEWL) was measured while mice were sedated, using the DERMALAB™ TEWL probe; at day 0 and at endpoint. TEWL was measured on both ears of the mice, at room temperature and results were recorded when TEWL regions stabilized. Two readings on each ear were taken and averaged for each mouse.

2.2.3.4 Itching frequency

Mice were recorded via time-lapse videography and itch events determined and quantified. This pathophysiological measurement was obtained from video observation of the relevant treatment groups 24 hours prior to experimental endpoint and this was conducted at the Mayo Clinic, Arizona as per specifications in Lee *et al* [34]. Mice were housed individually in containers and filmed from above for 24 hours. These video recordings were then watched and the evaluator assessed the itching

events. An itching event is defined as a deliberate scratching activity by either the front or hind paws. Itching events were represented as fold increase of the ear itching events over the contralateral control ear of that same mouse. The data was expressed as the mean $\pm$ SEM derived from these assessments.

2.2.3.5 Serum collection

At experimental endpoint, mice were sacrificed and cardiac puncture performed using syringe and 25 gauge needle. Blood sample was placed in a Microvette® gel tube and spun for 5 minutes at 12000rpm. Serum was transferred to 1.7ml Eppendorf tubes and stored at -20°C until further analysis.

2.2.3.6 Ear tissue collection for histology

At experimental endpoint, one ear tissue was carefully removed with a forceps and scissors, for histological analysis, and placed in a 6 well plate, in 4% PFA for 24 hours. Thereafter transferred into histological cassettes and placed in 70% EtOH until processed.

2.2.3.7 Ear tissue collection for ELISA

At experimental endpoint, one ear tissue was carefully removed with a forceps and scissors, for ELISA analysis. The tissue was then placed in cryotubes and snap frozen either in liquid nitrogen or on dry ice. Ear tissue was stored in -70°C until further processing.

2.2.4 Skin harvest and processing

2.2.4.1 Skin tissue processing for single cell suspension

Ears were excised, and using forceps the dorsal and ventral halves were carefully split. Samples were then placed in a shaking incubator in skin digestion buffer for 30 mins at 37°C. Digested tissue was mashed through a 70µm mesh cell strainer with a plunger from a 1mL syringe. Cells were washed through with IMDM and pelleted at 419 X g for four minutes. Cells were then resuspended in 2mL of flow cytometry buffer. A final volume of 100uL of cell suspension was removed from each sample for cell counts and transferred into microtiter tubes (cell counting 2.2.4.2).

2.2.4.2 Cell counting

AccuCount beads were given a vigorous vortex. A volume of 50uL of AccuCount beads (approximately 50000 beads) were added to the 100uL of skin cell suspension. Just prior to sample acquisition on the cytometer, 5uL of DAPI was added to each sample.

2.2.5 Cell re-stimulation and flow cytometry staining

2.2.5.1 PMA/ionomycin

A cell suspension of 2×10^6 cells were plated in 96-well plates, pelleted and centrifuged at 250xg for 2 minutes. Thereafter, the supernatant was removed and cells were resuspended in cIMDM containing 50ng/mL phorbol 12-myristate 13-acetate (PMA, P8139, Sigma-Aldrich, MO USA), 1µg/mL ionomycin (I0634, Sigma-Aldrich, MO USA) and 1µL/mL GolgiStop™ (554724, BD Bioscience, CA USA). As a

control group, cells were resuspended in cIMDM containing 1 μ L/mL GolgiStop™ and incubated at 37°C for 5 hours.

2.2.5.2 Cell surface staining

Cells were resuspended, into 5mL polystyrene round bottom tubes, at a volume of 2mL and filtered through a 70 μ m gauze. Thereafter, cells are pelleted at 419 X g for 4 minutes. Following this, cells were resuspended in 50 μ L of mouse Fc Block™ (Clone 2.4G2) and incubated for 10 minutes at 4°C. At this stage, 50 μ L of fluorophore bound antibody cocktail was added to the tubes and incubated for another 30 minutes at 4°C. Staining was halted by the addition of 4mL of flow cytometry buffer and spun at 419 X g for 4 minutes. Finally, samples were resuspended in 125 μ L of flow cytometry buffer and 125 μ L of DAPI solution was added. DAPI was added to the final cell suspension just prior to acquisition on the cytometer.

2.2.5.3 Intracellular cytokine staining (ICS)

Intracellular cytokine staining was performed with a BD Cytofix/Cytoperm™ kit (BD Biosciences, CA, USA) as per the manufactures instructions. Cells were stained with fixable viability dye (Invitrogen™ ThermoFiosher Scientific, MA USA) in dPBS for 30 minutes at 4°C. Thereafter, cells were washed twice in dPBS and stained for cell surface markers (as described in 2.3.5.2). Following this, cells were washed and incubated with 100 μ L Fixation/Permeabilization solution for 20 minutes at 4°C. At this stage cells were pelleted and washed with 1X Perm/Wash which was diluted in dH₂O. It is vital that cells are thoroughly rinsed and to aid this the cells were incubated for 15 minutes at 4°C in Perm/Wash solution. Next, cells were resuspended in 100 μ L Perm/Wash solution which also contained the diluted antibodies against cytokines of interest. Cells were then stained for 20 minutes at 4°C before being washed with Perm/Wash solution. As a precautionary step to avoid unnecessary background staining, cells were incubated for a further 20 minutes in Perm/Wash at 4°C. Finally, cells are pelleted and resuspended in FACS buffer and samples were transferred to microtubes for FACS analysis.

2.2.5.4 Fluorescence activated cell sorting

Cells were stained for cell surface markers and single stain controls were prepared as described in 2.2.5.2. After staining the cells were resuspended in sterile flow buffer with viability dye (DAPI) at a volume of 4×10^6 cell/mL. The voltages of each channel was set up, compensation adjusted using unstained cells and single stains. Cells were then sorted with help of staff of Hugh Green Cytometry Core (HGCC) on FACSInflux™ cell sorter, using DIVA software (Becton Dickson, San Jose, CA, USA).

2.2.6 Analysis of cells by flow cytometry

2.2.6.1 Acquisition of flow cytometry data

All FACS data was acquired on LSRFortessa SORP™ or LSRII SORP™ flow cytometer with FACS DIVA software (Becton Dickinson, San Jose, CA, USA). The staff of the HGCC at MIMR performed daily cytometer setup, and recorded the cytometer performance to ensure calibration of the cytometers was maintained. Photomultiplier tube (PMT) voltages and compensation was set for each experiment. To do this both unstained cells and OneComp eBeads (eBioscience, CA, USA) were stained with each fluorophore. Compensation was verified manually using fluorescence minus one (FMO) controls prior to sample acquisition.

2.2.6.2 Analysis of flow cytometry data

FACS data was analysed using FlowJo software (version 9.9; Treestar, Inc, CA, USA). Doublets were excluded based on forward and side scatter properties and with the use of viability dye; dead cells were excluded. Cells were gated based on fluorescence minus one (FMO) controls and or isotype controls where appropriate.

2.2.6.3 FACS data visualization and display

FACS data were visualized by utilizing plots generated from FlowJo software (version 9.9; Treestar Inc, CA, USA). All graphical representation was done using Graph Pad Prism software (version 5 for Mac OS X; Graph Pad Software, CA, USA).

2.2.7 ELISA

2.2.7.1 Skin lysate for ELISA

Homogenization buffer was made up of Halt™ protease inhibitor, 1% 0.5M EDTA in dPBS and kept at on ice. Several 50ml Falcon tubes are filled with 20ml cold dPBS. The tubes of dPBS were used to wash the homogenizer between samples and groups. Skin tissue is placed in 1.5mL of homogenizing buffer and homogenized. Homogenate samples were transferred to 1.5mL Eppendorf tubes using a pipette. Samples are then centrifuged at 15000g for 10minutes at 4°C. Aqueous phase was pipetted into a new Eppendorf tube and spun as above. After the second spin the aqueous phase was aliquoted into 0.6ml tubes and stored at -70°C until ELISAs were performed.

2.2.7.2 Total protein quantification

Total protein concentrations were quantified using Bicinchoninic acid BCA™ protein assay kits according to manufactures' specifications.

2.2.7.3 ELISA Kits

ELISAs were all performed to detect cytokine production in either serum samples and or in tissue lysate samples. Manufactures' protocols were followed for IL-1 β , IL-9, IL-13, IL-17, IL-25, IL-31, IL-33, TGF β and TSLP. Samples were either serially diluted to get optimal readings or 100uL of neat sample was loaded. The absorbance was read on a VersaMax™ plate reader according to the protocol and concentration was determined using the standard curve and dilution factors.

2.2.7.3.1 IL-4 (in house) ELISA

Briefly, 96 well Nunc Maxisorp plates were coated with 100uL of capture antibody (rat anti - mouse IL-4, 11B11). This capture antibody was at a concentration of 2µg/mL in 0.1M of carbonate buffer (pH = 9.6) and incubated overnight at 4°C. Plates were washed by submersion in a bucket of wash buffer (dPBS with 0.05% Tween20). To prevent non-specific binding, the plate was then blocked by adding 200uL of 10% FBS in dPBS and incubating for 2 hours at 37°C. Plates were washed again in wash buffer and 100uL of serially diluted samples and standards diluted in 10%FBS in dPBS were added. Plate was incubated overnight at 4°C.

Following this, the plates were washed again and incubated with 100uL of 2µg.mL biotinylated IL-4 in 10% FBS in dPBS for 2 hours at 37°C. Plates were washed again, and 100uL of streptavidin conjugated horseradish peroxidase (HRP) diluted at 1:1000 was added and incubated for 1 hour. After final wash, 100uL of TMB substrate was added and 10-30minutes later the reaction was stopped by adding 50uL of 2M sulphuric acid. The absorbance was read on the Versamax plate reader at 450nm. The concentration was determined using the standard curve and dilution factors.

2.2.8 Histopathology

2.2.8.1 Histology processing

Tissue was immersed in 4% PFA for fixation, immediately after removal from the mouse. After 24 hours in PFA, it was then placed in histological cassettes in 70% EtOH for a maximum of 14 days. Following this, samples were processed in a tissue processor overnight and then embedded in paraffin wax. The tissue processor involves 10 dehydration steps from 70% EtOH to Xylene and the final two steps ensure the tissue is fixed and infiltrated with paraffin wax. Tissue sections were serially sectioned on a microtome at 3-4µm onto adhesive microscope slides. Slides were de-waxed in xylene and then rehydrated using an ethanol concentration gradient from 100% to 70% prior to staining.

2.2.8.2 Histology Staining, Analysis & Microscopy

2.2.8.2.1 Haematoxylin and eosin (H & E) staining and analysis

Following rehydration, tissue sections were mordant in Bouin's solution at 60°C for one hour. Slides were rinsed in water until the water is completely colourless. Sections were stained in Mayer's haematoxylin for one minute and slides are washed in tap water until the water is clear. Sections were then dipped between 2-5 times in 2% ammonia, further washed in water and counter stained in eosin for 30-40 seconds. Lastly, slides were rinsed in water until all the excess stain was washed off, dehydrated through to xylene and cover-slipped with DPX.

Images of the tissue sections were taken on an Olympus Brightfield (BX51) microscope at 20x magnification to determine the acanthosis [11, 24] i.e. the epidermal thickening. Measurements of the epidermal layer were taken by Image J. To ensure consistency, three measurements were taken; at the beginning, midway and at the end of each tissue section. This was done on the thicker, more inflamed side of tissue. An average of the 3 measurements were taken over 10 images per ear section. Each data point on the graph represented an ear section i.e. the average of these 10 images. Further phenotype assessment was carried out to determine the presence of spongiosis i.e. intercellular edema and hyperkeratosis i.e. the presence of nuclei in the stratum corneum. This was done by evaluation under the microscope under 50X - 100X magnification.

Figure 2.1 Representation of ear tissue sectioning

Ears were excised and embedded on end, in paraffin wax. Serial sections were obtained in a cross-sectional manner from the apex to the base of the ear. Sections were cut at 4 μ m following every 10 revolutions to ensure the entire tissue was sectioned.

Figure 2.2 Fixed ear tissue stained with H & E to show cellular infiltration and skin pathology

H & E section at 40X magnification to illustrate the hyperkeratosis (black arrows), acanthosis (black line) and spongiosis (green arrows).

2.2.8.2.2 Chloroacetate esterase (CAE) staining and analysis

Following rehydration sections were soaked in dPBS for 5mins. A solution of 5mL of dPBS was added into a 15mL BD Falcon tube. A volume of 225uL of NaphtholAS-D Chloroacetate solution was added to the 5mL dPBS. The 15mL Falcon was mixed by inversion. A mixture of 12.5uL of New Fuchsin added to 12.5uL of sodium hydroxide was made and inverted until it turned yellow. 12.5uL of this yellow solution was then

added to the 15mL Falcon tube. Once inverted the solution immediately turned pink. Approximately, 1mL of the final solution was then dropped onto each slide and the stain was left to incubate for 45 minutes, in the dark, at room temperature. After 45 minutes, the stain was rinsed off and the slides were washed in dPBS for 5 minutes, following these sections were counterstained with haematoxylin for 30-40 seconds. Slides were rinsed in water until all the excess stain was washed off, dehydrated through to xylene and cover-slipped with DPX.

CAE staining was performed to detect the mast cells in red, in ear tissue sections. Images were taken on an Olympus Brightfield (BX51) microscope at 20x magnification. Mast cells are counted consistently on one side of the central cartilage across 10 random high-power fields (HPF). The average counts were determined per HPF per ear section. Each data point on the graph represented an ear section i.e. the average of these 10 HPFs.

Figure 2.3 Fixed ear tissue section stained with CAE to detect mast cells

CAE staining conducted on tissue sections to detect mast cells (red) and evaluated at a 20X magnification, with 10 random HPFs taken and assessed using Image J analysis. Mast cells counted on the more inflamed side of tissue on one side of the central cartilage (as indicated by the arrow).

2.2.8.2.3 Eosinophil peroxidase (EPX) staining and analysis

Following rehydration slides were rinsed in dH₂O for one minute. Slides were then immersed in a pressure cooker, filled with decloaking buffer, and it was set for four minutes. Once done, slides were left to cool for 10 minutes and rinsed in dH₂O. Sections were rinsed with 1 x TBS wash buffer. Pepsin solution was then added (200uL per slide) for 10 minutes. Following that, slides were rinsed in TBS three times, 200uL of rodent block was applied per slide, and further rinsing in TBS. EPX antibody was diluted into a 1:100 concentration in antibody diluent. The use of antibody diluent reducing background staining. EPX antibody was applied for 60 minutes and rinsed

in TBS three times. Mouse on mouse AP polymer was added for 15-20minutes and thereafter slides were rinsed in TBS three times. Permanent red was then added for 10 minutes, and slides rinsed in dH2O. Slides were counterstained in 0.1% methyl green for 20- 30 seconds. Finally, slides were rinsed in dH2O, air-dried and cover-slipped with clear mounting media [34]. EPX staining was performed to detect eosinophils, in pink, in ear sections. Images were taken on an Olympus Brightfield (BX51) microscope at 100x magnification. Eosinophils were assessed at 100x objective and this was done through 20 HPF's of view. In each HPF, the eosinophils were counted and assigned a degranulation score based on the scoring system below [34].

Figure 2.4 Fixed ear tissue stained with eosinophil peroxidase monoclonal antibody (EPX)

EPX staining depicted at 100X magnification showing eosinophil infiltration and the degree of degranulation. Below is a representation of the various degrees of degranulation.

Figure 2.5 EPX stained tissue depicting a degranulation score of 0.

Degranulation of 0 = Very faint staining. Intact eosinophil. Minimal EPX staining.

Figure 2.6 EPX stained tissue depicting a degranulation score of 1.

Degranulation of 1= Strong EPX staining, granules forming. Eosinophils were still intact

Figure 2.7 EPX stained tissue depicting a degranulation score of 2.

Degranulation of 2= Stronger staining, more diffuse i.e. eosinophils are starting to release EPX.

Both the eosinophil number and degranulation was plotted on a graph to indicate not just the number of eosinophils infiltrating the tissue but the proportion of degranulation that occurred.

2.2.8.2.4 Confocal microscopy staining

Whole mount staining was utilized to stain ear tissue with fluorescent antibodies [298] that was detected on the Olympus Laser Scanning Confocal microscope. Briefly, whole ear tissue was excised from the mouse and dorsal and ventral halves were gently separated with forceps. Each half of ear tissue was immersed in 4% PFA for fixation for 30 minutes. Tissue was then washed in 10% sucrose for 10 minutes. Fixed tissue was washed in dPBS; containing 5% FBS, 0.3% Triton X-100 and 2mM EDTA and incubated for 30 minutes at 37°C. Ear tissue was then blocked with 10% of the appropriate serum (i.e. rat, goat and or rabbit) in dPBS for one hour at room temperature. Block was flicked off the tissue and tissue stained with 200uL of the primary antibody, overnight at 4°C. The following day ear tissue was rinsed in dPBS three times and mounted onto a slide. Depending on the staining panel, either a clear mounting media was used or one that contained DAPI. For IL-9 staining on ear tissue, a secondary and tertiary antibody was used (see details below) [299]. Ear tissue was washed in dPBS three times between steps and finally mounted onto a slide using Faramount aqueous mounting media.

A

B

Figure 2.8 Tissue excision and imaging technique

The ears were split into dorsal and ventral halves A) the dorsal half was stained using whole mount technique. The internal surface of the ear is placed face down on the slide and visualized. B) Area that is imaged on the confocal is 50 μ m.

IL-9 staining panel was as follows:

Day 1 Primary IL-9 biotin stained overnight at 4°C.

Day 2 Secondary rabbit anti - hamster was stained for 2 hours at room temperature.

Tertiary Donkey Dylight 594 was stained for 4 hours at room temperature

Other primary antibodies:

All staining was done overnight at 4°C.

Table 2.3 Antibodies for confocal microscopy

Specificity	Fluorophore	Clone	Company
CD4	Qdot 605	RM4-5	Invitrogen
CD45	BV510	30F11	BD Pharmingen
CD90.1	AF647	OXO7	Biolegend
CD117	APC	2B8	BD Pharmingen
CD200R	APC	Ba13	Biolegend
CD326	BV421	G8.8	BD Pharmingen

2.2.8.2.4.1 Imaging and analysis

All data collected from confocal microscopy was analysed using Image J software. 3D reconstructions were recorded of the co-stained whole ear tissue, along the Z axis between 17-50µm. Z- stacks were recorded from bottom-top and assessed. Images in the results section (chapter 6) were a depiction of both the first 4µm and the last 4µm that was recorded [300, 301]. 6 slides of tissue were imaged per group in each experiment.

2.2.9 Data visualization and statistical analysis

All graphical representation of data was done using Graph Pad Prism software (version 5 for Mac OS X; Graph Pad Software, CA, USA). Statistical analyses used were stated in each figure legend and $p < 0.05$ was considered significant. For comparison between two similarly-sized groups, Student's t-test was used. Analysis examining a single parameter of three or more groups were compared with one-way ANOVA with Tukey's post-tests. Repeated measures tests were used in the experiments that compared the same biological sample. Unless specifically stated, symbols represent individual samples and errors bars represent mean $\pm$ the standard error of the mean (S.E.M.).

3 Characterisation of the MC903 model and delineating the cell populations involved in AD

3.1 Introduction

This chapter characterises an AD model, with the use of a vitamin D analogue MC903. I initially evaluated the clinical and disease parameters of AD and thereafter assessed inflammatory changes in the model. The purpose of the body of work described below was to delineate which immune cell population are involved with the initiation and maintenance of disease.

There have been many studies employing a murine model of AD induced by MC903 [89-91, 125, 127]. While these studies have contributed significantly towards the understanding of AD, there have been very few suitable targets that have emerged for the treatment and prevention of AD. To date, Dupilumab is our most promising candidate for the treatment of AD [102, 103]. Furthermore, many of these models demonstrated some caveats. Firstly, the immune cells and cytokines that were revealed in these experiments depended on the duration of MC903 treatment and concentration. Secondly, the involvement of some cytokines for e.g. TSLP is

transient. Thirdly, many of these murine models illustrate very high levels of circulating TSLP which is not a clinical feature seen in patients with AD [291].

This model of AD was originally described utilizing a high concentration of MC903 (4nmol), which was topically applied for 16 days. The initial study depicted, by confocal imaging, the expression of TSLP by keratinocytes and very high levels of TSLP in the serum. MC903 is a vitamin D analogue and exerts its activity by binding to the retinoid acid receptor which results in the activation of the TSLP gene. TSLP sets about a skin inflammatory cascade. Interestingly, when MC903 was applied to the ears of RAG1 knockout mice (mice that do not possess T and B cells), the skin inflammation persisted. Thereby suggesting that the mechanism by which MC903 was inducing skin inflammation was independent of T and B cells [89].

Prior to the commencement of my project, a previous investigator in our laboratory had attempted to recapitulate the MC903 experiment from Li *et al*, however the mice became quite ill and experiments were terminated (Ryan Kyle thesis). Thus, my initial experiments focus on titrating MC903, to ensure it was sufficient to induce skin inflammation, whilst ensuring the mice were kept healthy to endpoint.

The experiments conducted to determine itching intensity in figure 3.3A, 3.11G and 3.12F were carried out by Huijun Luo, based at the Lee lab at the Mayo Clinic in Arizona, USA. The MC903 utilized to induce inflammation was obtained from the same supplier, and used exactly as per my protocol and mice were accordingly age and sex matched. Itching was assessed using time-lapse videography [34] at day 19, prior to mice being culled.

3.1.1 Aims

The following experiments set out to establish a clinically relevant model of AD, with precisely characterised kinetics for the accompanying cell infiltration at the skin site. The model of choice in this project is the topical administration of MC903, a vitamin D analogue. Additionally, depletion strategies were employed to determine the precise roles of cells, in particular those usually associated with allergic disease namely mast cells, basophils and eosinophils.

My specific aims included:

- To characterise the phenotypic inflammatory changes in the epidermis
- To determine the accumulation of cellular infiltrate and cytokines in the dermis following skin exposure to MC903 over time.
- To evaluate if this MC903 model had the same impact in both C57Bl/6 mice and BALB/c mice.
- To assess the roles played by various cells in the MC903-induced model. I sought to establish this by using depletion strategies and/or specific mice.
- Mast cells would be analysed by administration of imatinib. This is a selective tyrosine kinase inhibitor which aims to block c-kit (a surface marker on mast cells).
- The contribution of CD4⁺ T cells will be assessed by depletion of CD4⁺ T cells with the use of anti - CD4.
- To evaluate eosinophils, I will utilize the inducible eosinophil (iPHIL) mice where eosinophils can be depleted by the treatment of diphtheria toxin. In addition to

this, experiments will be performed using resorcinol which specifically inhibits the activity of eosinophil peroxidase (EPO).

3.2 Results

3.2.1 Kinetics of skin inflammation in ears of MC903-treated mice

The concentration of MC903 was initially titrated to 1nmol, as opposed to the 4nmol concentration that was first published [89]. In addition, I topically applied the vitamin D analogue to both ears, on days 0,2,4,7,9,11,14,16 and 17, as I hypothesized this would allow the MC903 to induce effects on the skin while allowing the mice to survive for a longer period. The control group received 100% EtOH. C57Bl/6 mice were treated for 14 and 21 days respectively **(3.1A)** to determine the effects of 1nmol MC903 over time. Mice survived the 21 days of treatment and did not lose body weight. Ear thickness was measured as a symptom of inflammation in the skin and this revealed ear thickness began increasing at day 8 and continued to peak until day 18 following MC903 treatment compared to the control group. It appeared that ear thickness measurements plateaued between day 18 - day 21. The ear thickness of the control group remained unchanged **(3.1B)**. The accumulation of mast cells into the tissue was evaluated by chloroacetate esterase (CAE) staining of paraffin embedded fixed ear tissue **(3.1C)**. and there was an increase of mast cells over time following MC903 treatment as compared to EtOH-treated ear tissue **(3.1D)**.

In order to further investigate the kinetics of this MC903 model, C57Bl/6 mice were treated for 7, 14 and 19 days **(3.2A)**. The model was adjusted to 19 days, as opposed

to 21 days as this was observed to be the peak of inflammation. The appearance of the mouse ears revealed dryness and scaly lesions over time following MC903 application compared to EtOH-treated controls **(3.2B)**. Haematoxylin and eosin (H&E) staining of paraffin embedded fixed tissue exhibited the inflammation, and increase in cellular infiltrate **(3.2C)**. Ear thickness increased over time, beginning at day 7 and peaking at day 19 **(3.2D)** following MC903 treatment. Topical application of MC903 did induce skin inflammation but the plateau seen between day 18 – day 21 did suggest that the peak of inflammation was at day 19. The mouse skin lesions closely resembled the scaly lesions seen in patients with AD and the tissue sections revealed an accumulation of inflammatory cells.

A)

B)

C)

D)

Figure 3.1 Vitamin D analogue, MC903 induces skin inflammation

C57Bl/6 mice were topically administered with 1nmol MC903 or EtOH (vehicle control) for 14 and 21 days. **A)** Schematic of MC903/EtOH treatment regime. **B)** Ear thickness was measured from day 0 and at each point of treatment thereafter. Line graph shows mean $\pm$ SEM **C)** Chloroacetate esterase (CAE) staining was performed on ear tissue sections to detect mast cells **D)** Mast cells were quantified by Image J analysis. Data is representative of at least 3 independent experiment, with at least 5 mice per group. Statistics calculated by one-way ANOVA with Tukey post-test. ** $P < 0.01$, *** $0.01 < P < 0.001$, **** $P < 0.001$.

B)

day 19 EtOH

day7 MC903

day 14 MC903

day 19 MC903

C)

day 19 EtOH

day7 MC903

day 14 MC903

day 19 MC903

D)

Figure 3.2 MC903 application over time induced skin inflammation

C57Bl/6 mice were topically administered with 1nmol MC903 or EtOH (vehicle control) for 7,14 and 19 days. **A)** Schematic of MC903/EtOH treatment regime over time. Topical application of MC903/EtOH was every alternate day. **B)** Ear tissue was photographed at end point. **C)** H&E staining was performed to assess cellular infiltration over time. **D)** Ear tissue was measured from day 0 and at each point of treatment thereafter. Line graph shows mean $\pm$ SEM. Data is representative of at least 3 independent experiment, with at least 5 mice per group.

3.2.2 Determining the AD clinical parameters & inflammatory changes after MC903 skin exposure

To evaluate the inflammatory changes over time MC903 was topically applied and clinical parameters assessed at various time points (day 7, 14 and 19). The ear tissue was analysed by macroscopic, microscopic and immune diagnostic criteria for skin inflammation [11, 24]. The primary focus was on epidermal and dermal changes that occur in AD patient lesions. Pruritus (itch) was assessed by observing & counting the scratching behaviour of mice and itch events [34] were significantly increased by day 19 compared to EtOH-treated controls **(3.3A)**. Trans-epidermal water loss (TEWL) is an important readout in dermatology clinics to determine skin barrier dysfunction of AD patients. TEWL measurements increased after day 7, and peaked at day 14. The TEWL reading plateaued at day 19 **(3.3B)**. Histopathology of the epidermis (at 100X magnification) exhibited the hallmarks of AD **(3.3C)** such as hyperkeratosis (presence of nuclei in stratum corneum layer of skin) and spongiosis (intercellular spaces in epidermal tissue). Acanthosis (epidermal thickening) was assessed from H&E stained tissue by Image J analysis and this was quantified. There was an increase of acanthosis from day 7 and it peaked at day 14. Similar to the TEWL measurement, acanthosis plateaued at day 19 **(3.3C-D)**.

Following this, the dermis of the skin was evaluated and this revealed an increase of mast cells over time. Mast cells per high power field peaked following 19 days of

MC903 treatment **(3.4A)**. EPX-mAB based immunohistochemistry was used to determine eosinophil infiltration over time. Eosinophils were quantified and scored for degranulation, (eosinophils scoring method discussed in chapter 2 [34]). There was an increase of eosinophils observed infiltrating into the tissue and in terms of the proportion of degranulation over time **(3.4B)**. ELISA assays were performed for cytokine detection in the tissue lysate over time. This demonstrated an increase of IL-4 **(3.4C)** and IL-1 β **(3.4D)** over time. IL-17 levels in the tissue decreased until MC903 day 14 treatment group and plateaued at day 19 **(3.4E)**. There was no change in the levels of IFN γ observed in the tissue **(3.4F)**. Notably, the hallmarks of AD including itch, acanthosis, spongiosis, mastocytosis, and eosinophilia were revealed in MC903-treated tissue.

A)

B)

C)

D)

Figure 3.3 Phenotypic assessment in the epidermis following MC903 treatment

C57Bl/6 mice were topically administered with 1nmol MC903 or EtOH (vehicle control) for 7, 14 and 19 days. Phenotypic assessment was performed by evaluating macroscopic and microscopic criteria. **A)** Itch events were assessed for 24 hours by analysis of time lapse videography after last treatment of MC903 on day 19. Bar graph shows mean $\pm$ SEM. Statistics were calculated using unpaired Students t-test. *** $p < 0.001$. **B)** Trans-epidermal water loss (TEWL) was measured at endpoint. **C)** Haematoxylin and eosin (H&E) staining was performed. Histopathology of ear tissue was examined at 100X magnification i.e. spongiosis, acanthosis and hyperkeratosis **D)** Acanthosis was measured in tissue sections and quantified by Image J analysis. Statistics calculated by one-way ANOVA with Tukey post-test. Plots show mean $\pm$ SEM * $P < 0.05$, ** $P < 0.01$, *** $0.01 < P < 0.001$, **** $P < 0.001$, NS= non-significant. Data is representative of at least 3 independent experiment, with at least 5 mice per group.

Figure 3.4 Phenotypic assessment in the dermis following MC903 treatment

C57Bl/6 mice were topically administered with 1nmol MC903 or EtOH (vehicle control) for 7,14 and 19 days respectively. Phenotypic assessment was conducted by evaluating the cellular and cytokine infiltration in the dermis. **A)** CAE was performed to detect mast cells and these were quantified by Image J analysis. **B)** EPX-mAB based immunohistochemistry was used to assess eosinophil infiltration over time. Eosinophils were quantified and scored for degranulation. (Eosinophil scoring method discussed in chapter 2). Bar graphs show mean $\pm$ SEM **C)** IL-4 levels **D)** IL-1 β levels **E)** IL-17 levels and **F)** IFN γ levels were measured by ELISA in tissue. Statistics calculated by one-way ANOVA with Tukey post-test. Plots show mean $\pm$ SEM * $P < 0.05$, ** $P < 0.01$, *** $0.01 < P < 0.001$, **** $P < 0.001$, NS= non-significant. Each graph is representative of three independent experiments with at least 5 mice per group.

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3.2.3 Optimization of skin digestion for flow cytometry

The skin harbours the epithelial cells in the epidermis and the immune cells are mostly located in the dermis. This network of cells are anchored and tightly held together by collagen and elastin fibres [3]. In order to prepare suspensions of these cells for FACS analysis, the skin tissue needed to be effectively digested using the correct enzyme at the optimum concentration. There is a fine balance between either having too much digestion and destroying the cells and their surface markers for analysis and too little digestion leading to insufficient cell yields [302]. I optimized my skin digestion protocols using, two different concentrations of collagenase IV and as a control I included another digestion medium, i.e. Liberase TL **(3.5A)**. The innate cells of interest in this project were then quantified by flow cytometry. The groups for this experiment included 5mg/mL collagenase IV; 2.5mg/mL collagenase IV and 0.5mg/mL Liberase TL. The total cell numbers revealed no significant change between these groups **(3.5B)**, however there was an increase in both the number of leukocytes **(3.5C)** and frequency of leukocytes **(3.5D)** detected in the group with 2.5mg/mL of collagenase IV compared to the 5mg/mL and Liberase TL. There were more basophils, in terms of number **(3.5E)** and frequency **(3.5F)** when using the 2.5mg/mL collagenase IV compared to the higher concentration and the Liberase TL. An increase in the number **(3.5G)** and frequency **(3.5H)** of innate lymphoid cells group 2 (ILC2s) were seen in 2.5mg/mL collagenase IV compared to the higher concentration of collagenase IV and Liberase TL. Finally, the optimum skin tissue protocol was chosen based on the

protocol that obtained the maximum number of leukocytes, ILC2s and basophils. Cell populations were quantified as per the gating strategy in the appendix.

A)

Comparison of 19 days of MC903 treated ear tissue using 3 different digestion methods. 5mg/mL and 2.5mg/mL of collagenase IV and Liberase TL

B)

C)

D)

E)

F)

Figure 3.5 Optimization of skin digestion protocols for flow cytometry

C57Bl/6 mice were topically administered with 1nmol MC903 for 19 days. Cellular infiltration was assessed by flow cytometry under different skin digestion conditions. **A)** Conditions included 5mg/mL and 2.5 mg/mL of collagenase IV and 0.5mg/mL of Liberase TL. **B)** Total cell numbers of live cells. **C)** Total number of leukocytes (CD45⁺). **D)** Frequency of leukocytes. **E)** Total number of basophils. **F)** Frequency of basophils. **G)** Total number of ILC2s. **H)** Frequency of ILC2s. Statistics calculated by one-way ANOVA with Tukey post-test. * $P < 0.05$. Plots show mean $\pm$ SEM. Each graph is representative of two independent experiments with at least 5 mice per group.

3.2.4 Evaluating the impact MC903 has on C57Bl/6 compared to BALB/c mice and is there gender disparity in C57Bl/6 mice?

Previous investigations have revealed strain differences when employing the MC903 model, therefore I wanted to identify if this was also the case in our model [90, 91]. Furthermore, there have been studies showing gender disparities in the immunomodulatory response to vitamin D in humans. In order to determine if both genders of mice respond to the vitamin D analogue, MC903 in a similar manner male and female C57Bl/6 mice were treated with 1nmol MC903 or EtOH for 19 days. There was a significant increase in ear thickness in both C57Bl/6 males and females treated with MC903 for 19 days compared to EtOH-treated controls and there was no difference between the MC903-treated males and females **(3.6A)**. C57Bl/6 and BALB/c mice were topically administered with 1nmol MC903 or EtOH for 19 days. MC903 induced an increase in ear thickness in C57Bl/6 mice and BALB/c mice alike **(3.6B)**. TEWL measurements revealed an increase in both mouse strains compared to EtOH-treated mice. There was no difference between the TEWL measurements of the two different strains of mice **(3.6C)**. This vitamin D analogue induce skin inflammation in a similar manner in both C57Bl/6 and BALB/c mice and there is no gender disparity in C57Bl/6 mice.

A)

B)

C)

Figure 3.6 Assessing the impact of MC903 in C57Bl/6 compared to BALB/c mice and between male and female C57Bl/6 mice

Male and female C57Bl/6 mice were treated with 1nmol MC903 or EtOH for 19 days (n=10). **A)** Ear thickness was measured at endpoint. C57Bl/6 and BALB/c mice were topically administered with 1nmol MC903 or EtOH for 19 days. **B)** Ear thickness was measured at endpoint in C57Bl/6 and BALB/c mice. Ear thickness graphs shows two experiments pooled together. Bar graphs and plots show mean $\pm$ SEM. **C)** TEWL was measured at endpoint in C57Bl/6 and BALB/c mice. Statistics calculated by one-way ANOVA with Tukey post- test. NS= non-significant. Each graph is representative of two independent experiments with at least 5 mice per group.

3.2.5 Inhibition of mast cells in the MC903 model, by using imatinib treatment

Imatinib mesylate is a potent inhibitor of targeted protein tyrosine kinases, including platelet-derived growth factor receptor and c-kit. Furthermore, c-kit is the surface marker expressed of mast cells [303]. Imatinib, is frequently used to target mast cells, in the treatment of patients with gastrointestinal-stromal tumours and has been used to alleviate murine intestinal food allergy [304, 305]. I employed the MC903 model and treated a group of mice with imatinib. The control group received dPBS. This was administered by gavage daily from day 5 **(3.7A)**. Ear thickness was significantly reduced in MC903 + imatinib-treated mice compared to MC903 + dPBS group **(3.7B)**. Quantification of mast cells showed these cells to be significantly reduced in MC903 + imatinib-treated group **(3.7C)**. Significantly, the inhibition of mast cells in the skin resulted in a reduction of inflammation in the MC903 AD model.

Figure 3.7 Inhibition of mast cells by imatinib administration

C57Bl/6 mice were topically administered with 1nmol MC903 or EtOH (vehicle control) for 19 days. **A)** Schematic of imatinib i.g. administered administration daily from day 5. **B)** Ear thickness was measured at endpoint. **C)** CAE was performed to detect mast cells and these were quantified by Image J analysis. Bar graphs and plots show mean $\pm$ SEM. Statistics calculated by one-way ANOVA with Tukey post-test. * $P < 0.05$, *** $0.01 < P < 0.001$, **** $P < 0.001$. Each graph is representative of three independent experiments with at least 5 mice per group.

3.2.6 CD4⁺ T cells do not play a role in the MC903 model

Li *et al* has previously documented that when mice were treated with MC903 (a higher concentration of 4nmol for 16 days) in RAG1KO mice, these mice continued to develop skin inflammation. This suggested that this MC903 model was independent of T and B cells [89]. I sought to determine the contribution of CD4⁺ T cells by inhibiting the CD4⁺ T cells [306, 307] in this MC903 model (lower concentration and different treatment regime to Li *et al* [89]). Mice were treated with MC903 for 19 days and mouse anti - CD4 (clone GK1.5) was administered every 4 days from day - 3 **(3.8A)**. The control group received MC903 + isotype control antibody. MC903 + anti - CD4 treated mice displayed the same level of ear thickness as MC903 + isotype mice **(3.8B)**. There was no change in the mast cell numbers in these two groups **(3.8C)**. Interestingly, blocking of CD4⁺ T cells had no effect on the MC903 induced skin inflammation, suggesting no requirement for CD4⁺ T cells in this model.

Figure 3.8 Depletion of CD4+ T cells in the model of MC903

C57Bl/6 mice were topically administered with 1nmol MC903 or EtOH (vehicle control) for 19 days and anti-CD4 (clone GK1.5) was intraperitoneally administered every 4 days from day -3. **A)** Schematic of MC903 treatment and anti-CD4. **B)** Ear thickness was measured at endpoint. **C)** CAE was performed to detect mast cells and these were quantified by Image J analysis. Statistics calculated by one-way ANOVA with Tukey post-test., ** $P < 0.01$, *** $0.01 < P < 0.001$, NS= non-significant. Bar graphs and plot show mean $\pm$ SEM. This experiment has only been done once with 5 mice per group.

3.2.7 Assessment of ILC2s, eosinophils and basophils in skin of MC903-treated mice over time

It has been documented that ILC2s [91], eosinophils [89] and basophils [125] infiltrate the tissue in allergic skin inflammation when using MC903 treatment. However, these investigations have utilized different concentrations of MC903 topical application was for varied lengths of time (ILC2s-2nmol for 4 days, eosinophils- 4nmol daily treatment 16 days and basophils 1nmol 7 days respectively [89, 91, 125]). Thus, making it complex when trying to compare results. In light of this, I have assessed ILC2s and basophils over time in our MC903 AD model.

C57Bl/6 mice were topically administered with 1nmol MC903 or EtOH for 14 and 19 days. Flow cytometry was performed to assess cellular infiltration. The number of ILC2s seemed to decrease slightly by day 14 compared to both the control and day 19 treated groups **(3.9A)**. The frequency of ILC2s increased slightly in the day 19 MC903 group **(3.9B)**. Basophil numbers increased at day 14 and continued to increase until day 19 compared to control **(3.9C)**. The frequency of basophils peaked at day 14 and decreased by day 19 but was still above baseline **(3.9D)**. Eosinophils increased over time both in number **(3.9E)** and frequency **(3.9F)**. Both eosinophils and ILC2s accumulated over time in the tissue and peaked at day 19. Basophil data revealed that there were more basophils, in terms of numbers, at day 19 but a lower frequency. This is possibly due to there being more cellular infiltration at day 19 time-

point due to the inflammation in the tissue therefore basophils make up a smaller percentage of the leukocytes.

Figure 3.9 Characterisation of the accumulation of innate immune cells over time in the MC903 model

C57Bl/6 mice were topically administered with 1nmol MC903 or EtOH for 14 and 19 days. Flow cytometry was performed to assess cellular infiltration in the ear tissue. **A)** Total number of ILC2s. **B)** Frequency of ILC2s of CD45+. **C)** Total number of basophils **D)** Frequency of basophils of CD45+. **E)** Total number of eosinophils. **F)** Frequency of eosinophils of CD45+.. Bar graphs and plots show mean $\pm$ SEM. Statistics calculated by one-way ANOVA with Tukey post-test. ** $P < 0.01$, *** $0.01 < P < 0.001$. Each graph is representative of at least three independent experiments with at least 5 mice per group.

3.2.8 Dissecting the accumulation of eosinophils and assessing the effect of EPO inhibition in the MC903 model

Eosinophils were seen to accumulate in the tissue following MC903 treatment (as seen in figure 3.4B). To determine the precise time-point that eosinophils were beginning to severely degranulate in the tissue (Figure 3.4 suggested it was around day 14 that a degranulation score of 2 was seen) I evaluated eosinophils between day 7 and day 14 of MC903 treatment. C67Bl/6 mice were administered with 1nmol MC903 for 7, 9, 11 and 14 days **(3.10A)**. EPX-mAB based IHC was utilized to assess the eosinophil infiltration and degranulation [34]. The quantification revealed eosinophils begin to infiltrate into the tissue at day 7 however severe degranulation (a score of 2) begins at day 11 **(3.10B)**. These findings give us an indication that eosinophils begin degranulation at day 9 in this MC903 model.

Eosinophils release mediators during degranulation and one such mediator is eosinophil peroxidase (EPO). EPO can be inhibited by the use of resorcinol [308]. Resorcinol induces irreversible loss of EPO activity by covalently binding the catalytic site of the peroxidase [309]. C57Bl/6 mice were topically administered with 1nmol MC903 or EtOH for 19 days. After the previous results revealed that eosinophils begin degranulation at day 9 (Figure 3.10B) I decided that daily i.p. administration of resorcinol would be from day 5. Control group received dPBS **(3.11A)**. Ear thickness measurements revealed a significant reduction following resorcinol treatment **(3.11B)**.

There was no change in TEWL measurements following resorcinol treatment compared to the MC903-treated group **(3.11C)**. Acanthosis was markedly reduced following resorcinol treatment **(3.11D)**. Mast cell numbers were unchanged in the resorcinol-treated group compared MC903 only **(3.11E)**. Eosinophils were markedly reduced both in number and the proportion of degranulation in resorcinol-treated mice compared to MC903 only **(3.11F)**. Ear itching intensity was assessed on day 19, prior to mice being culled **(3.11G)**. This was done by time-lapse videography by collaborators at the Mayo Clinic. There was no significant difference between the itching in MC903-treated group compared to the MC903 + resorcinol group. Cumulatively, these results demonstrate that eosinophils contribute to AD pathology in the MC903 model, in an EPO dependent manner.

Figure 3.10 Determining the accumulation of eosinophils prior to day 14

C57Bl/6 mice were topically administered with 1nmol MC903 for 7, 9, 11 and 14 days. Eosinophils were evaluated at each time point by histopathology. **A)** Schematic of MC903 treatment. **B)** EPX-mAb based immunohistochemistry was used to assess eosinophil infiltration over 7, 9, 11 and 14 days. Eosinophils were quantified and scored for degranulation. Bar graphs show mean $\pm$ SEM. Data is representative of two independent experiments, with 5 mice in each group.

A)

B)

C)

D)

E)

F)

Figure 3.11 Assessment of the impact of EPO inhibition in the MC903 model

C57Bl/6 mice were topically administered with 1nmol MC903 or EtOH for 19 days and resorcinol (eosinophil peroxidase inhibitor) was intraperitoneally administered daily from day 5. **A)** Schematic of MC903/EtOH and resorcinol treatment. **B)** Ear thickness was measured at endpoint. **C)** TEWL was measured at endpoint. **D)** H&E staining was performed and acanthosis was measured in tissue sections and quantified by Image J analysis. **E)** CAE was performed to detect mast cells and these were quantified by Image J analysis. **F)** EPX-mAB based immunohistochemistry was used to assess eosinophil infiltration. Eosinophils were quantified and scored for degranulation. Bar graphs and plots show mean $\pm$ SEM. **G)** Ear itching (fold-increase over the contralateral ear control) was assessed on day 19. Statistics calculated by unpaired Students t test or a one-way ANOVA with Tukey post-test. ** $P < 0.01$, **** $P < 0.0001$. Each graph is representative of at least three independent experiments with 5 mice per group.

3.2.9 Evaluating the significance of eosinophils in the MC903 model using the iPHIL mice

At this stage of the project, I had established that eosinophils were an important part of the MC03 model of AD. The inducible eosinophil deficient mice (iPHIL) served as an excellent tool to evaluate the relevance of eosinophils to the development of AD in the MC903 model. These mice have the human diphtheria toxin (DT) receptor (DTR) inserted into the eosinophil peroxidase gene locus. This effectively turns “on” or “off” the eosinophils by i.p. administration of DT. iPHIL (inducible eosinophil deficient) and WT mice were topically administered with 1nmol MC903 or EtOH for 19 days. Mice received 20ng/g diphtheria toxin (DT) intraperitoneally every 3 days from day 0 **(3.12A)**. Ear thickness measurements were significantly reduced in MC903 iPHIL group compared to the WT **(3.12B-C)**. Furthermore, both TEWL **(3.12D)** and acanthosis **(3.12E)** of the MC903 iPHIL mice was markedly reduced when compared to MC903 WT. Ear itching intensity was assessed on day 19, prior to mice being culled. This was done by time-lapse videography by collaborators at the Mayo Clinic. Itching data was generated by pooling together two experiments (n=8). Itching significantly decreased in MC903 iPHIL compared to MC903 WT **(3.12F)**. Flow cytometry revealed a significant increase in the frequency of eosinophils in the both the spleen **(3.12G)** and ear tissue **(3.12H)** when WT mice (no DT) were treated with MC903. However, iPHIL mice when treated with both DT and MC903 demonstrated a drastic reduction in eosinophils in both the spleen **(3.12F)** and ear tissue **(3.12G)**.

This flow data is representative of 3 of the 5 samples assessed. Gated flow plots indicated an efficient depletion system in the iPHIL mice upon treatment with DT. Functional depletion of eosinophils resulted in an amelioration of disease, making eosinophils critical to the pathogenesis of AD in the MC903 model.

E)

F)

G)

WT MC903 Spleen

Gate name	Freq. of Parent
eosinophils	24.3

Gate name	Freq. of Parent
eosinophils	32.4

Gate name	Freq. of Parent
eosinophils	25.1

iPHIL MC903 Spleen

Gate name	Freq. of Parent
eosinophils	0.535

Gate name	Freq. of Parent
eosinophils	0.762

Gate name	Freq. of Parent
eosinophils	0.873

H) WT MC903 Ear

iPHIL MC903 Ear

Figure 3.12 Eosinophils are critical to the MC903 AD model

iPHIL (inducible eosinophil deficient) and WT mice were topically administered with 1nmol MC903 or EtOH for 19 days. Mice received 20ng/g diphtheria toxin (DT) intraperitoneally every 3 days from day 0. **A)** Schematic of MC903/EtOH and DT treatment regime. Ear thickness was measured at **B)** each treatment time point and then at **C)** endpoint. **D)** TEWL was measured at endpoint. **E)** H&E staining was performed and acanthosis was measured in tissue sections and quantified by Image J analysis. **F)** Ear itching (fold-increase over the contralateral ear control) was assessed on day 19 (data was pooled from two separate experiments $n = 8$). Eosinophils detected by flow cytometry in both WT and DT iPHIL MC903-treated **G)** spleen tissue and **H)** ear tissue. Flow plots are representative $n = 5$. Bar graphs and plots show mean $\pm$ SEM. Statistics calculated by either an unpaired Students t-test or one-way ANOVA with the Tukey post-test. ** $P < 0.01$, *** $0.01 < P < 0.001$, **** $P < 0.0001$. Each graph is representative of at least three independent experiments with at least 5 mice per group.

3.2.10 Evaluation of the effect of eosinophil depletion over time in the MC903 model

The precise timeframe that eosinophils were recruited into the tissue was assessed, by utilizing the DT eosinophil depletion strategy i.e. the iPHIL. The iPHIL and WT mice were topically administered with 1nmol MC903 or EtOH for 19 days. Mice received 20ng/g diphtheria toxin (DT) intraperitoneally every 3 days from either day 0, day 6 and day 12 respectively **(3.13A)**. Ear thickness measurements were substantially decreased in the group that received DT only in week 3 compared to WT. The most significant reduction in ear thickness measurements were in the group that received DT in week 2 and 3 compared to all other MC903-treated groups **(3.13B)**. TEWL **(3.13C)** and acanthosis **(3.13D)** was reduced the most in the iPHIL mice that received DT in week 2 and 3. However, flow cytometry was performed and demonstrated a depletion of eosinophils (data not shown) in both the spleen and ear tissue as described in figure 3.12. Intriguingly, when the depletion of eosinophils was restricted to the last 2 weeks of treatment (week 2 and 3) it was sufficient to significantly reduce inflammation and improve barrier function, thereby suggesting eosinophils as the central effector cells in the MC903 AD model.

Figure 3.13 Evaluating how eosinophil depletion over time impacts the MC903 model

iPHIL (inducible eosinophil deficient) and WT mice were topically administered with via i.p. every 3 days from day 0, 6 and 12. **A)** Schematic of MC903/EtOH and DT treatment regime. **B)** Ear thickness was measured at endpoint. **C)** TEWL was measured at endpoint. **D)** H&E staining was performed and acanthosis was measured in tissue sections and quantified by Image J analysis. Bar graphs and plots show mean $\pm$ SEM. Statistics calculated by one-way ANOVA with Tukey post-test. * $P < 0.05$, ** $P < 0.01$, *** $0.01 < P < 0.001$, **** $P < 0.0001$. NS= non-significant. Each graph is representative of at least three independent experiments with at least 5 mice per group.

3.3 Discussion

In this chapter, I have established a robust clinically relevant model of AD, using the topical application of MC903. After the kinetics of skin inflammation following MC903 treatment was assessed the optimum treatment regime was determined (1nmol as illustrated in figure 3.2A for 19 days). This treatment regime ensured the peak of skin inflammation, at day 19, was reached and the mice were healthy and survived the duration of the experiment. Assessment of AD clinical symptoms and inflammatory changes were demonstrated in this MC903 model. Our model closely resembles the AD phenotype in patients including inflammation, barrier dysfunction, itch, accumulation of mast cells and eosinophils and elevated levels of pro-inflammatory cytokines [5, 9, 25]. Histopathology assessment revealed the hallmarks of AD i.e. acanthosis, hyperkeratosis and spongiosis [24]. The presence of spongiosis is crucial in the diagnosis of AD as it is only present in AD and not in psoriasis. It is imperative that the distinction between these two skin inflammation diseases; psoriasis and AD is made as they both have very different immune responses. AD has been described as type two inflammatory setting while psoriasis is a type 17 (Th17) setting. The reduction over time of IL-17 as well as the lack of IFN γ presence in the tissue supports the phenotype we have induced [54, 55, 113]. Over the years, the caveats of these models have been discussed, in particular, that innate cells that were examined were in isolation of each other, concentrations of MC903 administered were variable, and mouse strain differences were seen. Thus, it was important to determine early on in the project that the 1nmol MC903 model was able to induce AD in both C57Bl/6 and

BALB/c mice alike. In addition, MC903 induced skin inflammation in a similar manner in both male and female C57Bl/6 mice.

Li *et al* elegantly portrayed, using the chronic model of MC903 (4nmol daily application for 16 days) to be independent of both T and B cells [89]. This was further emphasized in our model, as we found no role for CD4⁺ T cells following anti-CD4 treatment [306]. We did not assess the CD4⁺ T cells in the skin in this experiment but were confident of substantial depletion as the antibody was administered from day -3, thereby preventing a CD4⁺ T cell response in these mice [307]. Imatinib treatment suggested a possible role for mast cells however, the use of imatinib would inhibit all cells expressing c-kit [304] which included the melanocytes as well as the mast cells, and any other tyrosine kinases in the skin.

Furthermore, this chapter highlights the accumulation of innate immune cells in the skin during inflammation which included mast cells, eosinophils, basophils and ILC2s. Notably, eosinophils played an essential role in disease pathology. This was depicted both in the resorcinol experiments and with the use of the elegantly constructed iPHIL mice. In both of these experiments there was an amelioration of disease. Significantly, there was a substantial decrease in itching intensity in the MC903-treated iPHIL mice compared to MC903 WT. However, this was not the case in the MC903 + resorcinol-treated mice. These mice revealed no difference in itching compared to MC903 WT mice. This does imply an important role for eosinophils in the induction of itch in AD. In the resorcinol-treated mice, while eosinophil numbers are drastically reduced, they are not completely eradicated compared to the iPHIL mice. Perhaps, the presence of some eosinophils following resorcinol treatment is an explanation as to why itching was not reduced in these mice. However, the

accumulation of mast cells that developed as a result of AD remained unchanged following resorcinol treatment. Furthermore, eosinophils were exerting their effects in a EPO dependent manner. Interestingly, depleting eosinophils for merely 2 weeks was sufficient to ameliorate disease. Thus, elucidating eosinophils as key effector cells in the MC903 AD model.

3.4 Conclusion

This model of MC903 presents with the key clinical manifestations of AD, thus making it an excellent platform to identify specific therapeutic targets for the treatment AD in patients. The accumulation of basophils, mast cells, ILC2s and eosinophils were demonstrated and correlated with disease progression. Conversely, CD4+ T cells did not play a significant role in this model. Importantly, eosinophils drive disease severity and most notably itch. Accordingly, functional depletion of eosinophils resulted in an amelioration of disease. Therefore, eosinophils emerge as a promising therapeutic target for the treatment of AD.

4 Determining the cytokines and mediators that play a role in the MC903 model of AD

4.1 Introduction

In order to understand AD and implement disease management strategies, it is vital to delineate the role different cytokines play in the initiation and maintenance of AD. I sought to assess cytokines that have either been associated with AD in patients and/or have been described in other allergic contexts. In particular, IL-9 and IL-9 receptor gene polymorphisms have been associated with AD patients [236, 237]. IL-9 is also a cytokine implicated in allergic skin inflammation. Its specific role however remains unclear; it was therefore my intention to shed some light on IL-9 involvement in skin allergy. Furthermore, the epithelial derived cytokine, TSLP, has been described to be the master regulator of allergic disease [183]. TSLP has been shown to be crucial to the MC903-induced skin inflammation. MC903, exerts its action via the heterodimer formed between retinoid X receptor and vitamin D receptor and this results in the induction of the TSLP promoter [89, 199]. The kinetics of the expression levels of TSLP in this MC903 model of AD would prove insightful into the inflammatory pathway involved.

In addition, IL-25, has been shown to be important for type two directed immune responses in the pathogenesis of AD in murine models. Increased levels of il25 mRNA have been detected in patients with AD [93, 203]. There have been studies proposing that a compromised skin barrier could promote the activity of IL-25 in the skin [90]. Notably, the success of Dupilumab, as a treatment for AD, has highlighted a role for both IL-4 and IL-13 potentially in the pathomechanism of AD [102]. IL-4 and IL-13 both involve STAT6 signalling [257] and determining the requirement of STAT6 signalling in this model will be achieved by application of MC903 in STAT6 deficient mice.

In addition, a mediator of interest in the context of an allergic setting, is prostaglandin D2 (PGD2). It has been detected in the bronchiolar lavage of asthma patients, and the skin of AD patients [150]. PGD2 exerts its action via its receptor CRTh2 [153]. To explore whether this PGD2-CRTh2 axis is involved in the MC903 AD model, I will inhibit this activity with the use of a CRTh2 antagonist (CRTh2^{ant}). Moreover, it is important to determine the timing of the expression levels of these cytokines in this model in order to design experiments involving cytokine blockade. Knowledge of how these cytokines and mediators initiate or contribute to disease progression provides insight into the inflammatory pathway(s) that could be pursued as therapeutic targets for AD.

4.1.1 Aims

To evaluate the specific role of cytokines and mediators in the MC903 AD model.

These aims include:

- To evaluate the cytokines in the MC903 AD model over time by ELISA.
- To employ the MC903 AD model in different cytokine knockout mice to determine the roles of TSLP, IL-4, IL-25 and the IL-4/IL-13 axis.
- To employ the MC903 model together with blocking antibodies against IL-5 and IL-9 and thereby investigate their contribution in AD pathogenesis.

In addition, I sought to evaluate whether the PGD2-CRTh2 axis is involved in the MC903 model, by use of a CRTh2 antagonist.

4.2 Results

4.2.1 Evaluating key cytokines over time in the MC903 AD model

It was critical to assess the expression of different cytokines over time in the MC903 model. Cytokines were measured in the tissue lysate following MC903 treatment by ELISA. In the previous chapter (figure 3.4) I have assessed and described, IL-4, IL-1 β , IL-17 and IFN γ . There was a slight increase in IL-33 following day 14 of MC903 treatment however this decreased in the 19 day treatment group **(4.1A)** in the tissue. No change in the expression levels of IL-25 **(4.1B)** and IL-13 **(4.1E)** in the tissue could be observed. IL-9 levels increased over time in the tissue lysate **(4.1C)**, and in the serum IL-9 peaked at day 7 and decreased following 14 days of treatment **(4.1D)**. These findings suggest that IL-33 and IL-13 were being expressed constitutively in the tissue, while there was minimal expression of IL-25. Importantly, IL-9 was detected in both the serum and tissue which reflects what is seen in patients with AD [109]. This data together with figure 3.4 depicted that the MC903 model of AD had a Th2 cytokine profile, characterised by high expression IL-4 and IL-9 and low expression of IL-17 and IFN γ .

A)

B)

C)

D)

E)

Figure 4.1 Cytokine profile in model of MC903

C57Bl/6 mice were topically administered with 1nmol MC903 or EtOH (vehicle control) for 7, 14 and 19 days. Cytokine levels were measured in the tissue lysate and/or serum by ELISA. **A)** IL-33 **B)** IL-25 **C)** IL-9 in tissue. **D)** IL-9 in serum. IL-9 ELISA detection range is between 32-4000pg/mL. **E)** IL-13. Plots show mean $\pm$ SEM. Statistics calculated by one-way ANOVA with Tukey post-test. * $P < 0.05$, ** $P < 0.01$, *** $0.01 < P < 0.001$ *** $0.01 < P < 0.001$. Each graph is representative of at least three independent experiments with at least 5 mice per group.

4.2.2 TSLP is essential for the MC903 AD model

Many studies have documented TSLP to be the initiator of skin inflammation [183, 291] and this needed to be confirmed in this MC903 AD model. TSLP expression over time was determined by ELISA. In the tissue lysate, there was a slight increase of TSLP at day 7, it peaked by 14 days and decreased by day 19 **(4.2A)**. In the serum, there was a marked increase of TSLP at day 7 and this decreased at day 14, with a further decrease at day 19 **(4.2B)**. To further assess the role of TSLP in my MC903 AD model, I employed TSLPRKO mice. At endpoint (day 19), ear thickness significantly decreased in the MC903-treated TSLPRKO mice compared to the MC903-treated C57Bl/6 mice **(4.2C)**. Less mast cell accumulation was seen in the MC903-treated TSLPRKO mice than in MC903-treated C57Bl/6 mice **(4.2D)**. No change in the levels of IL-9 in the tissue lysate was detected **(4.2E)**. This indicates that TSLP is essential for the MC903 AD model and it is imperative to bear in mind that TSLP peaks at day 14 and decreases by day 19 in this model. Consistent with previous findings, the MC903 AD model is TSLP dependent [89].

Figure 4.2 TSLP is essential to the MC903 induced AD model

C57Bl/6 mice were topically administered with 1nmol MC903 or EtOH (vehicle control) for 7, 14 and 19 days. TSLPRKO mice were topically administered with 1nmol MC903 or EtOH (vehicle control) for 19 days. Cytokine levels were measured in the tissue lysate and serum by ELISA. **A)** TSLP levels in tissue over time. **B)** TSLP levels in serum over time. TSLP ELISA detection range is 10-1000pg/mL. **C)** Ear thickness was measured at endpoint. **D)** CAE was performed to detect mast cells and these were quantified by Image J analysis. **E)** IL-9 levels were quantified in the tissue lysate by ELISA. IL-9 ELISA detection range was 32-4000pg/mL. ELISA was done in duplicate and results pooled. Plots and bar graphs show mean $\pm$ SEM. Statistics calculated by one-way ANOVA with Tukey post-test. * $P < 0.05$, ** $P < 0.01$, *** $0.01 < P < 0.001$, **** $P < 0.0001$. NS= non-significant. Each graph is representative of at least three independent experiments with at least 5 mice per group.

4.2.3 IL-25 is not required in the model of MC903

The epithelial derived cytokine, IL-25, has been shown to be important for type two directed immune responses in the pathogenesis of AD in murine models [93, 203]. In my model of MC903, there was minimal IL-25 expression in the tissue as seen in figure 4.1B. To further validate the role of IL-25 in the MC903 model, I used IL-25KO mice. These IL-25KO mice were treated with MC903 for 19 days, and there was no change in both ear thickness measurements **(4.3A)** and mast cell counts **(4.3B)** at end point compared to MC903-treated C57Bl/6 mice, suggesting that IL-25 was not required for the MC903 AD model.

Figure 4.3 IL-25 is not required in the MC903 model

C57Bl/6 and IL-25 KO mice were topically administered with 1nmol MC903 or EtOH (vehicle control) for 19 days. **A)** Ear thickness was measured at endpoint. Bar graphs and plots show mean $\pm$ SEM. **B)** CAE was performed to detect mast cells and these were quantified by Image J analysis. Statistics calculated by unpaired Students t-test, NS = non-significant. This experiment was conducted once with 5-6 mice per group.

4.2.4 Determining the role of IL-4 in both MC903 treated BALB/c and C57Bl/6 mice

Studies have revealed that an increase of IL-4 levels in the dermis [107] and in the serum [272] correlated with the development of skin inflammation. In my AD model, IL-4 levels were elevated in the tissue upon MC903 treatment, as seen in figure 3.4. To further elucidate the role of IL-4 in this model, IL-4 deficient mice were used both on a C57Bl/6 as well as BALB/c background. The genetic deletion of IL-4 resulted in a significant decrease in the ear thickness measurements in both strains of MC903-treated IL-4 KO mice **(4.4A & 4.4C)**. Intriguingly, mast cell counts remained unchanged in the MC903-treated IL-4KO C57Bl/6 mice **(4.4B)**, however there was a marked reduction in mast cell numbers in the MC903-treated IL-4KO BALB/c mice **(4.4D)**. Both strains of IL-4KO mice demonstrate a role for IL-4 in the MC903 model, but the impact of mast cells differed across the two strains.

Figure 4.4 IL-4 plays a role in the MC903 AD model

C57Bl/6, IL-4 KO mice on a C57Bl/6 background (**4.4A- B**) and BALB/c and IL-4KO mice on a BALB/c background (**4.4 C-D**) were topically administered with 1nmol MC903 or EtOH (vehicle control) for 19 days. **A**) Ear thickness was measured at endpoint. **B**) CAE was performed to detect mast cells and these were quantified by Image J analysis. **C**) Ear thickness was measured at endpoint. Bar graphs and plots show mean $\pm$ SEM. **D**) CAE was performed to detect mast cells and these were quantified by Image J analysis. Statistics calculated by one-way ANOVA with Tukey post-test. ** $P < 0.01$, *** $0.01 < P < 0.001$, **** $P < 0.0001$. NS= non-significant. Each graph is representative of at least three independent experiments with at least 5 mice per group.

4.2.5 IL-5 may play a role in the MC903 AD model

Interleukin 5 has been linked to eosinophilic driven allergic diseases in particular in asthma and infantile AD [166, 281]. The relevance of IL-5 in the model was determined by administration of anti - IL-5 via i.p. every alternate day from day 12 **(4.5A)** and this resulted in no significant change in ear thickness measurements **(4.5B)** when compared to the MC903-treated C57Bl/6 mice. There was a small but significant reduction in the mast cell counts in the MC903 anti - IL-5 group **(4.5C)** as well as an increase in the levels of IL-4 in the tissue lysate **(4.5D)**. These are preliminary results which suggest that IL-5 might play a role in the MC903 model of AD. Unfortunately, due to a combination of lack of sufficient reagent and time constraints this experiment was only carried out once. Further investigations are required into the precise role IL-5 may play in the MC903 model of AD.

Figure 4.5 IL-5 may play a role in the induction of AD in the MC903 model

C57Bl/6 were topically administered with 1nmol MC903 for 19 days and anti - IL-5 (clone TRFK-5) /isotype control was intraperitoneally administered from day 12 every alternate day.

A) Schematic of MC903 treatment and anti - IL-5/isotype control. **B)** Ear thickness was measured at endpoint. Bar graphs and plots show mean $\pm$ SEM. **C)** CAE was performed to detect mast cells and these were quantified by Image J analysis. **D)** IL-4 levels were detected in the tissue lysate by ELISA. Statistics calculated by unpaired Students t -test *p <0.05, ns = non - significant. Data is representative of the experiment only being conducted once with 5 mice in each group.

4.2.6 STAT6 signalling is essential to the MC903 AD model

The success of Dupilumab, in the treatment of AD, [85] has indicated that the IL-4/IL-13 axis could play a fundamental role in the pathogenesis of AD. I hypothesized the IL-4/IL-13 axis could be important in the MC903 model of AD. To address this, I topically applied MC903 to STAT6KO mice. In these KO mice the signalling of both IL-4 and IL-13 is impaired via the deletion of the common downstream transcription factor STAT6 [269]. STAT6KO mice treated with MC903 showed a dramatic reduction in ear thickness measurements compared to the MC903 WT group **(4.6A)**. Trans-epidermal water loss (TEWL) **(4.6B)**, and acanthosis **(4.6C)** were significantly reduced in the MC903-treated STAT6KO mice as well. However, there were no changes in the mast cells counts in the MC903-treated STAT6KO mice compared to the MC903 WT groups **(4.6D)**. Finally, EPX-mAB based immunohistochemistry showed that there was a substantial reduction of eosinophils in the tissue of MC903-treated STAT6KO mice compared to the WT **(4.6E)**. In summary, the absence of IL-4 and IL-13 signalling leads to a significant reduction in AD phenotype, including a substantial loss of eosinophils.

Figure 4.6 STAT6 signalling is essential to the MC903 induced AD phenotype

C57Bl/6 mice and STAT6KO mice were topically administered with 1nmol MC903 or EtOH for 19 days. **A)** Ear thickness was measured at endpoint. **B)** TEWL was measured at endpoint. **C)** H&E staining was performed and acanthosis was measured in tissue sections and quantified by Image J analysis. **D)** CAE was performed to detect mast cells and these were quantified by Image J analysis. **E)** EPX-mAB based immunohistochemistry was used to assess eosinophil infiltration. Eosinophils were quantified and scored for degranulation. Plots and bar graphs show mean $\pm$ SEM. Statistics calculated by one-way ANOVA with Tukey post-test. * $P < 0.05$, ** $P < 0.01$, *** $0.01 < P < 0.001$, **** $P < 0.0001$. NS= non-significant. Each graph is representative of at least three independent experiments with at least 5 mice per group.

4.2.7 IL-9 blockade ameliorates AD phenotype

Previously in this chapter, it was demonstrated that MC903 application led to increased levels of IL-9, both in tissue and serum **(4.1C & D)**, consistent with clinical features of AD [109]. To further elucidate the role of IL-9 in the MC903 model of AD, mice were treated with MC903 and anti - IL-9 every alternate day for 19 days **(4.7A)**. Ear thickness **(4.7B)**, TEWL measurements **(4.7C)** and acanthosis measurements **(4.7D)** were all significantly reduced following IL-9 blockade. Furthermore, the dermis revealed a marked reduction in mast cell numbers **(4.7E)** and eosinophils infiltration and degranulation **(4.7F)**. This amelioration of AD-characteristic symptoms presents IL-9 as an attractive target for the clinical management of AD.

A)

B)

C)

D)

E)

F)

Figure 4.7 IL-9 blockade ameliorates AD phenotype

C57Bl/6 were topically administered with 1nmol MC903 or EtOH for 19 days and anti - IL-9 (clone 9C1) /isotype control was intraperitoneally administered every alternate day from day -1. **A)** Schematic of MC903/EtOH and anti - IL-9 treatment over time. **B)** Ear thickness was measured at endpoint. **C)** TEWL was measured at endpoint. **D)** H&E staining was performed and acanthosis was measured in tissue sections and quantified by Image J analysis. **E)** CAE was performed to detect mast cells and these were quantified by Image J analysis. **F)** EPX-mAB based immunohistochemistry was used to assess eosinophil infiltration. Eosinophils were quantified and scored for degranulation. Plots and bar graphs show mean $\pm$ SEM. Statistics calculated by one-way ANOVA with Tukey post-test. ** $P < 0.01$, *** $0.01 < P < 0.001$, **** $P < 0.001$, NS= non-significant. Each graph is representative of at least three independent experiments with 5 mice in each group.

4.2.8 IL-9 has an effect on AD phenotype in a shorter (14 day) MC903 model

Based on the amelioration of AD pathology that was seen in figure 4.7, I evaluated the impact of short-term anti – IL-9 treatment. MC903 was topically applied for 14 days and anti - IL-9 administered i.p. every alternate day **(4.8A)**. Anti-IL-9 treatment was sufficient to significantly reduce ear thickness measurements in a shorter MC903 model **(4.8B)**. Mast cells counts remained unchanged **(4.8C)**. Notably, TSLP levels in the serum decreased following IL-9 blockade after two weeks **(4.8D)**. (Important to note that day 14 was the peak of TSLP in this MC903 model). The early impact of IL-9 blockade suggests that IL-9 potentially contributes to the initiation of AD.

A)

B)

C)

D)

Figure 4.8 IL-9 has an effect on AD phenotype in a 14 day model of MC903

C57Bl/6 were topically administered with **A)** 1nmol MC903 or EtOH for 14 days and anti - IL-9 (clone 9C1) /isotype control was intraperitoneally administered every alternate day from day -1. **B)** Ear thickness was measured at endpoint. **C)** CAE was performed to detect mast cells and these were quantified by Image J analysis. **D)** TSLP levels detected in the serum of 2 week treated mice. Plots and bar graphs show mean $\pm$ SEM. Statistics calculated by one-way ANOVA with Tukey post-test. * $P < 0.05$, ** $P < 0.01$, *** $0.01 < P < 0.001$, NS = non-significant. Each graph is representative of at least three independent experiments with 5 mice in each group.

4.2.9 IL-9 blockade has an effect on skin inflammation in a BALB/c MC903 model

The contribution of different cytokines in murine models are sometimes mouse strain dependent. In particular, TSLP was documented to be the initiator of skin inflammation in a C57Bl/6 model of AD [90], while IL-25 and IL-33, and not TSLP, were the crucial factors driving inflammation in a BALB/c model of AD [91]. To assess whether the impact of IL-9 blockade was strain specific, I co-administered MC903 and anti – IL-9 in BALB/c mice for 19 days. There was a marked reduction in ear thickness measurements **(4.9A)** but no change in the TEWL **(4.9B)**. IL-9 blockade has therefore a similar effect on MC903-treated BALB/c mice, as compared to C57Bl/6 mice, in terms of inflammation, but without the improvement in barrier function.

IL-9 blockade for 3 weeks in Balb/c mice

Figure 4.9 IL-9 blockade has an effect on AD phenotype in a BALB/c MC903 model

BALB/c mice were topically administered with 1nmol MC903 or EtOH for 19 days and anti - IL-9 (clone 9C1) /isotype control was intraperitoneally administered every alternate day from day -1. **A)** Ear thickness was measured at endpoint. **B)** TEWL was measured at endpoint. Plots and bar graphs show mean $\pm$ SEM. Statistics calculated by one-way ANOVA with Tukey post-test. **** $P < 0.001$, NS= non-significant. Each graph is representative of two independent experiments with 5 mice in each group.

4.2.10 Minimal effect on ILC2s and basophils following IL-9 blockade

As shown in figure 4.7, IL-9 blockade effectively reduced the clinical parameters of AD. I therefore examined the effect of IL-9 blockade on ILC2s and/or basophils. C57Bl/6 mice were treated with MC903 for 19 days and anti - IL-9 was administered every alternate day, (as described in 4.7A). Ear tissue was digested and the number and frequency of ILC2s and basophils were determined by flow cytometry. Mice that received MC903 + isotype control antibody showed an increased number **(4.10A)** and the frequency **(4.10B)** of basophils compared to EtOH-treated controls. There was no significant difference in both the number **(4.10A)** and frequency **(4.10B)** of basophils in the mice that were treated with MC903 + anti - IL-9. Similarly, no change in the number of ILC2s was observed in the tissue when comparing EtOH controls, MC903 + isotype, and MC903 + anti - IL-9 groups **(4.10C)**. In terms of frequency of ILC2s, there was a decrease in the frequency in the MC903 isotype mice compared to EtOH-treated controls, but a slight increase in the IL-9 blockade groups compared with the MC903 isotype **(4.10D)**. The moderate increases of both ILC2s and basophil frequencies upon IL-9 blockade (from 2.5% and 4% of total CD45+ hematopoietic cells in MC903-treated mice to 9% and 4.5% with additional treatment of anti - IL-9, respectively) **(4.10 E)** suggests that efficacy of IL-9 blockade is independent from these two cell types.

Figure 4.10 IL-9 blockade resulted in no significant impact on ILC2s and basophils in the skin

C57Bl/6 were topically administered with 1nmol MC903 or EtOH for 19 days and anti - IL-9 (clone 9C1) /isotype control was intraperitoneally administered every alternate day from day -1. ILC2s and basophils was assessed by flow cytometry after skin digestion. **A)** Total number of basophils. **B)** Frequency of basophils of CD45+ cells. **C)** Total number of ILC2s. **D)** Frequency of ILC2s of CD45+ cells. **E)** Comparison of the frequency of ILC2s and basophils in both MC903 + isotype and MC903 + anti - IL-9 groups. Statistics calculated by unpaired Students t-test and one-way ANOVA with Tukey post-test. * $P < 0.05$, **** $P < 0.001$, NS= non-significant. Plots and bar graphs show mean $\pm$ SEM. Each graph is representative of at least three independent experiments with at least 5 mice in each group.

4.2.11 Basophils and ILC2s produce IL-4 and IL-13 respectively in the MC903 model

Previously in figure 3.4, I have shown that IL-4 does accumulate over time, upon MC903 application. In addition, the role of IL-4 has been established in figure 4.4, with the use of IL-4 KO mice. Therefore, exploration of the source of IL-4 was essential. Conversely, I was unable to detect changes in the levels of IL-13 in the MC903 model by ELISA. Previous studies have shown basophils [125] and ILC2s [1] to be responsible for the production of IL-4 and IL-13 respectively in the skin following MC903 treatment. To determine which cells were producing these cytokines in my MC903 model, I used 4C13R, dual reporter mice. These mice express the fluorochromes AmCyan as a reporter for IL-4 and DsRed as a reporter for IL-13 **(4.11A)**. Expectedly, I observed an increase of IL-4 AmCyan⁺ cells and IL-13 DsRed⁺ cells following topical treatment of MC903 **(4.11B & C)** as well as an increase in the frequencies of basophils ILC2s **(4.11 D & E-** and as previously reported in chapter three). Importantly, both the frequency and absolute number of IL-4-producing basophils increased in my model **(4.11F & G)**. Similarly, MC903 treatment induced an increase in IL-13-secreting ILC2s, however without altering their overall frequency **(4.11G & H)**. These observations suggest that basophils and ILC2s are indeed a significant source of IL-4 and IL-13, respectively, in my model.

A)

B)

C)

H)

I)

Figure 4.11 Basophils and ILC2s produce IL-4 and IL-13 respectively in the MC903 model respectively

4C13R dual reporter mice (on a C57Bl/6 background) were topically administered with 1nmol MC903 or EtOH for 19 days. These mice possess AmCyan as a reporter for IL-4 and DsRed as a reporter for IL-13. Ears were harvested, digested and FAC analysis conducted. **A)** Schematic of MC903/EtOH treatment in 4C13R mice. **B)** Frequency of the IL-4 (AmCyan)+ cells. **C)** Frequency of the IL-13 (DsRed)+ cells. **D)** Frequency of basophils of live cells. **E)** Frequency of ILC2s of CD45+ cells. **F)** in terms of numbers. Assessed the IL-13 producing ILC2s **G)** in terms of number. **H)** frequency of IL-4 producing basophils. **I)** frequency of IL-13 producing ILC2s. Statistics calculated by unpaired Students t-test, and Mann-Whitney post-test. * $P < 0.05$, ** $P < 0.01$, *** $0.01 < P < 0.001$, NS = non-significant. Each graph is representative of at least three independent experiments with 5 mice in each group.

4.2.12 CRTh2 antagonism therapeutically impacts AD phenotype

To determine whether PGD2 is involved in the pathogenesis of AD in my MC903-driven model, I made use of a small molecule CRTh2 antagonist (CRTh2^{ant}). This method has been successfully employed in the past, in pre-clinical allergy models [150, 252]. A similar approach was thus carried out by administering CRTh2^{ant} daily by oral gavage from day 5 (**4.12A**). The administration of the antagonist was at a time-point just prior to the initial accumulation of mast cells and eosinophils (as described in chapter 3) to try and prevent cellular infiltration in the tissue and possibly reducing AD phenotype. Ear thickness measurements were significantly reduced at endpoint (**4.12B**). In the epidermis, TEWL (**4.12C**) and acanthosis (**4.12D**) were remarkably decreased as well. The dermis revealed lower mast cells counts (**4.12E**), and lower eosinophils infiltration and degranulation (**4.12F**). However, there was no change in the serum levels of TSLP (**4.12G**), nor IL-4 and IL-9 levels (**4.12H& I**) in the tissue lysate. Overall, CRTh2 inhibition was highly effective in reducing AD pathology supporting a vital contribution of the PGD2-CRTh2 axis to the MC903 model.

A)

MC903/EtOH + methylcellulose

MC903 + CRTh2 antagonist (CRTh2^{ant})

1mg/mL of CRTh2 antagonist (CRTh2^{ant}) was administered daily by oral gavage from day 5.
Control group received methylcellulose

Figure 4.12 CRTh2 antagonism therapeutically impacts AD phenotype

C57Bl/6 were topically administered with 1nmol MC903 or EtOH for 19 days and CRTh2 antagonist /control was administered daily by oral gavage from day 5. **A)** Schematic of MC903/EtOH and CRTh2 antagonist treatment over time. **B)** Ear thickness was measured at endpoint. **C)** TEWL was measured at endpoint. **D)** H&E staining was performed and acanthosis was measured in tissue sections and quantified by Image J analysis. **E)** CAE was performed to detect mast cells and these were quantified by Image J analysis. **F)** EPX-mAB based immunohistochemistry was used to assess eosinophil infiltration. Eosinophils were quantified and scored for degranulation. **G)** TSLP serum levels **H)** IL-4 levels and **I)** IL-9 levels were detected in the tissue lysate by ELISA. Plots and bar graphs show mean $\pm$ SEM. Statistics calculated by one-way ANOVA with Tukey post-test. * $P < 0.05$, ** $P < 0.01$, *** $0.01 < P < 0.001$, **** $P < 0.001$, NS = non-significant. Each graph is representative of at least three independent experiments with 5 mice in each group.

4.2.13 CRTh2 antagonism has an effect on AD phenotype even after 10 days of administration

The therapeutic potency of CRTh2^{ant} was determined by restricting its administration to the last 10 days of treatment only **(4.13A)**. Interestingly, this resulted in a substantial decrease in ear thickness measurements **(4.13B)**, however without affecting mast cell numbers **(4.13C)**. Thus, inhibition of the CRTh2-PGD2 axis for merely one week was sufficient to have a positive impact on skin inflammation in the MC903 model.

Figure 4.13 CRTh2 antagonism has an effect on AD phenotype after 10 days of treatment

C57Bl/6 were topically administered with 1nmol MC903 or EtOH for 19 days and CRTh2 antagonist/control was administered daily by oral gavage from day 9. A) Schematic of MC903/EtOH and CRTh2 antagonist treatment over time. B) Ear thickness was measured at endpoint. C) CAE was performed to detect mast cells and these were quantified by Image J analysis. Plots and bar graphs show mean $\pm$ SEM. Statistics calculated by one-way ANOVA with Tukey post-test. ** $P < 0.01$, *** $0.01 < P < 0.001$, **** $P < 0.001$, NS = non-significant. Each graph is representative of at least three independent experiments with 5 mice in each group.

4.3 Discussion

The cytokine profile in the MC903 model clearly demonstrated the importance of type two (Th2) cytokines, such as TSLP, IL-4 and IL-9, for the phenotypic manifestation of AD. In human studies, AD has been characterised as either an acute or chronic presentation. The acute phase is associated with a biased Th2 immune environment while the chronic phase is characterised by a combined Th1/Th2 immune signature [310, 311]. This implies that this murine model of MC903 induced AD recapitulates the acute phase of the human disease. IL-5 appeared to not play a role in this model as I did not observe any change in ear thickness following anti-IL-5 treatment, however this was experiment will need to be repeated to verify this. Intriguingly, employing the model in STAT6KO mice resulted in an amelioration of AD, which demonstrated that the IL-4/IL-13 axis does indeed have a role in this MC903 model and inhibiting the signalling of the two cytokines has a far greater effect than just the deletion of IL-4 by itself. In addition, this would suggest investigating the role of IL-13 in the MC903 AD model could be beneficial. Furthermore, there was a dramatic reduction of eosinophils observed in the MC903-treated STAT6KO mice suggesting that STAT6 signalling was crucial for eosinophil recruitment into the tissue. However, the accumulation of mast cells that developed as a result of AD was the only disease phenotype that remained in the MC903 treated STAT6KO mice, suggesting that mast cell accumulation was independent of STAT6 signalling.

Consistent with previous findings [1, 125], I have confirmed that basophils and ILC2s are responsible for the production of IL-4 and IL-13 respectively, in the MC903 model. These two effector cytokines have been pursued as therapeutic targets for the

treatment of AD, leading to the FDA approval of Dupilumab (Dupixent®), a monoclonal antibody to the alpha subunit of the IL-4 receptor (engaged by both IL-4 and IL-13). However, not all AD patients responded to Dupilumab and it causes substantial side effects such as nasopharyngitis, conjunctivitis, and inflammation of the cornea [85, 86]. Thus, the discovery of other therapeutic targets for the treatment of AD is highly desirable.

Previous AD patient studies, have implicated IL-9 to be involved in the disease [109, 236], however little is known regarding the potential of IL-9 as a target for the treatment and/or prevention of AD. The IL-9 blocking experiments described in this chapter show a clear reduction in AD phenotype. Surprisingly, while IL-9 blockade reduced mast cell and eosinophil accumulation in the model, there was no reduction in the number of ILC2s and/or basophils. Moreover, anti - IL-9 administration for only 2 weeks was sufficient to reduce inflammation and TSLP serum levels. TSLP peaked day 14 in the MC903 model, and it was rather intriguing, that after 2 weeks of anti - IL-9 treatment the TSLP levels in the serum decreased. Conversely, when MC903 induced minimal inflammation in TSLPRKO mice, there remained a considerable level of IL-9 detected in the tissue. This would suggest IL-9 to be upstream of TSLP. In summary, the amelioration of AD-characteristic symptoms presents IL-9 as an attractive target for the clinical management of AD and the early impact of IL-9 blockade suggests that this cytokine potentially contributes to the initiation of AD.

PGD2 is a potent activator of allergic skin inflammation [150]. Interestingly, small molecule antagonists of the PGD2 receptor was strikingly effective in reducing AD phenotype. The PGD2-CRTh2 axis, appears to exert its action in the MC903

inflammatory pathway independent of cytokines. I have shown in this chapter no change in the serum levels of TSLP, and tissue levels of IL-4 and IL-9 following administration of a CRTh2^{ant}. There are two possible explanations for this 1) the accumulation of increased IL-4, IL-9 and TSLP occur early on in the initiation stage of the disease and CRTH2 blockade needs to be given at a higher concentration to have an impact on these cytokines i.e. given in a prophylactic setting from day -1 2) the PGD2-CRTh2 axis acts independently of cytokines. Through the implication of the above-mentioned cytokines and mediators, I was able to ascertain more knowledge as to the key players involved in the MC903-induced inflammatory pathway.

4.4 Conclusion

The presence of a Th2 cytokine profile (TSLP, IL-4 and IL-9) in this MC903 model closely resembles the acute manifestation of AD. Inhibition of IL-9 (prophylactically) and CRTh2 antagonism (therapeutically) both resulted in an amelioration of AD phenotype. Interestingly, a potential role for IL-9 as an initiator in the inflammatory cascade of AD has been demonstrated. Furthermore, blocking of STAT6 signalling revealed a remarkable reduction of disease severity therefore highlighting the unique contribution of the IL-4/IL-13 axis to AD.

5 Combination therapies, and the relationship between inflammation and remodelling in AD

5.1 Introduction

This chapter focuses on three separate but relevant aspects for the better understanding of AD pathophysiology with a view to informing improved treatment modalities. Firstly, I wanted to determine if, similarly to other immune mediated pathologies, combination therapies offer more therapeutic efficacy than monotherapies. This involved evaluating the potentially synergistic effect of the following therapeutic combinations: IL-9 blockade + resorcinol, IL-9 blockade +CRTh2^{ant}, and resorcinol + CRTh2^{ant}.

The second aspect of this chapter focused on gaining a better mechanistic understanding of the relationship between skin inflammation, and skin remodelling events that follow once inflammation is established. Tissue remodelling has been well characterised in asthma as a consequence of chronic airway inflammation and is

associated with a Th2 cytokine milieu. Similarly, AD is defined as a chronic relapsing inflammatory disease [5] as a consequence of barrier dysfunction and an immune dysregulation [61]. The typical hallmarks of AD in patients include epidermal hyperplasia, tissue remodelling and spongiosis [56]. As previously reported in chapter 3, the MC903 model of AD presents with acanthosis (epidermal thickening), hyperplasia and spongiosis. Accordingly, tissue remodelling was the next feature I set out to characterise in my MC903-treated skin. During this phase in the skin, collagen layers are deposited around the inflamed area giving it a thickened appearance [9]. I wanted to determine the cells and cytokines that played a role in the remodelling phase of the MC903 model, in the hope that specific biomarkers would emerge for the clinical management of AD, in particular to control chronic itch. The current paradigm of the pathogenesis of chronic itch in AD is once severe pathology has manifested, it is followed by chronic inflammation, hyper-proliferation of epithelial cells [57], large amounts of collagen deposition and fibrosis [60], this further disrupts the epidermal layer [58] and results in activation of neuropeptides such as substance P [63] thereby leading to chronic itching [34, 62]. These patients with AD enter into a vicious cycle of chronic inflammation, fibrosis, itch and risk of skin infection [312]. More recently, TSLP [313], IL-13 [108] and eosinophils [34] have been suggested to drive fibrotic remodelling in AD. These observations critically link acute and chronic immune events in the context of AD and warranted further investigations. In the experiments conducted to assess remodelling (section 5.2.4 and 5.3.5) I have referred to the period of time during which mice, after receiving MC903 treatment for 19 days, are set aside as the recovery phase. Disease parameters were evaluated to determine the resolution of inflammation and skin

lesions in these mice. The time period during which MC903 treatment is applied, I have referred to as the inflammatory phase.

Lastly, another model of skin inflammation was explored using the contact irritant, trimellitic anhydride (TMA) [289]. It has been demonstrated that eosinophils are the main drivers of skin inflammation and itch in a chronic TMA-induced model [34]. This is similar to my observations in the MC903 model revealing eosinophils to be crucial to AD disease symptoms, including itch. Moreover, TSLP has been shown to be a potent trigger for skin inflammation [178, 183] and most notably in MC903-induced models [89, 194], including my own observations described herein, therefore I thought it would be interesting to determine if TSLP also had a central role in the TMA-induced model of skin inflammation.

5.1.1 Aims

The specific aims of this chapter include:

- To assess the efficacy of combination therapies in the MC903 model
- (combining anti - IL-9, CRTh2^{ant}, resorcinol)
- To explore the potential links that may exist between skin inflammatory events, and the subsequent resolution and remodelling phases.
- To address this, the MC903 model was extended beyond 19 days and the application of MC903 exposure stopped once the clinical features of disease are established.
- To conduct a comparative study using the MC903- and TMA-induced models of skin inflammation and determine the role of TSLP in the latter.

5.2 Results

5.2.1 Inhibition of both EPO and IL-9 reduces AD phenotype in the MC903 model

I have previously described the therapeutic efficacy of resorcinol-mediated eosinophil peroxidase (EPO) inhibition (section 3.2.8). Resorcinol induces irreversible loss of EPO activity by covalently binding the catalytic site of the peroxidase [308, 309]. Since IL-9 blockade led to a mechanistically distinct but similarly impressive reduction in disease pathology, I hypothesized that these two compounds would have an additive effect and, in combination, further reduce disease phenotype. The topical application of MC903 was accompanied by i.p. administration of anti - IL-9 every alternate day from day -1 and daily co-administration of resorcinol from day 5 (figures 3.12A & 4.7A). This was compared to treatment of either monotherapies. Ear thickness measurements were significantly lowered in the group that received the combination treatment compared to the group with MC903 and anti - IL-9 and/or resorcinol by itself **(5.1A)**. CAE staining demonstrated the combination treatment group had a marked reduction in mast cell counts compared to resorcinol treatment alone. However, there was no significant difference in the mast cell counts between the combination treatment group and IL-9 blockade by itself. In addition, there was no difference in the mast cell counts of the combination treatment, the IL-9 blockade

and the EtOH-treated control group **(5.1B)**. Eosinophils were drastically reduced in the combination treatment group compared to both anti - IL-9 and resorcinol by itself **(5.1C)**. These results suggest that by simultaneously interfering with several effector pathways, this could provide additional benefit to the clinical management of AD.

Figure 5.1 Synergistic effect of IL-9 blockade and resorcinol results in further reduction of AD phenotype

C57Bl/6 were topically administered with 1nmol MC903 or EtOH for 19 days and anti - IL-9 (clone 9C1) /isotype control was intraperitoneally administered every alternate day from day -1. Mice also received daily i.p. administration of resorcinol from day 5. Combination treatment group was compared to MC903 + anti - IL-9 or resorcinol alone. **A)** Ear thickness was measured at endpoint. **B)** CAE was performed to detect mast cells and these were quantified by Image J analysis. **C)** EPX-mAB based immunohistochemistry was used to assess eosinophil infiltration. Eosinophils were quantified and scored for degranulation. Plots and bar graphs show mean $\pm$ SEM. Statistics calculated by one-way ANOVA with Tukey post-test. * $P < 0.01$, *** $0.01 < P < 0.001$, **** $P < 0.0001$. Each graph is representative of at least two independent experiments with at least 5 mice per group.

5.2.2 Synergistic effects of CRTh2 antagonism and IL-9 inhibition only slightly improved disease outcome

The potential synergistic effect of blocking both CRTh2 and IL-9 was assessed, as well as the combined effect of CRTh2^{ant} and resorcinol. A side-by-side comparison was conducted in the MC903 AD model. Ear thickness measurements demonstrated that co-administration of CRTh2^{ant} and anti - IL9 significantly reduced inflammation compared to CRTh2^{ant} + resorcinol treatment. There was no additive effect when combining CRTh2^{ant} and anti - IL-9 compared to anti - IL-9 alone **(5.2A)**. Surprisingly, when comparing the different combinations of blocking of CRTh2 and IL-9, blocking CRTh2 and resorcinol and IL-9 blockade alone, it appeared that the anti - IL-9 alone was the most effective intervention to reduce skin inflammation.

5.2.3 Extending the MC903 model beyond 19 days does not lead to disease exacerbation

In order to evaluate the inflammation beyond the peak of 19 days, mice were treated with MC903 for an additional 7 days. This entailed 11 topical applications of MC903 for 26 days (as opposed to the previously described model of 8 topical applications for 19 days). This was done to evaluate the inflammatory environment and to determine if I could exploit this model to investigate skin recovery and remodelling. Interestingly, there was no change in ear thickness measurements seen between the MC903-treated 26 day group in comparison to MC903-treated 19 day group **(5.3Ai & 5.3Aii)**. TEWL measurements indicated no difference among MC903 14 day, 19 day and 26 day treated groups **(5.3B)**. Essentially, application of MC903 beyond 19 days resulted in no further increase in ear thickness and TEWL. This period between day 19 and day 26 depicted a plateau in the inflammation and barrier dysfunction, which suggested that once the peak of disease was reached at day 19, continued application of MC903 led to the inflammation being maintained rather than disease exacerbation.

Figure 5.3 Extending the MC903 model up to 26 days does not lead to disease exacerbation

C57Bl/6 were topically administered with 1nmol MC903 for 7, 14, 19 and 26 days. **A) i)** Ear thickness was measured at endpoint. **ii)** Ear thickness measurements were taken at each time point before treatment. **B)** TEWL measurements were taken at endpoint. Plots and bar graphs show mean $\pm$ SEM. Statistics calculated by one-way ANOVA with Tukey post-test. NS = non-significant. Each graph is representative of two independent experiments with at least 5 mice per group.

5.2.4 Characterising the recovery phase in the MC903 model

To explore the skin recovery phase in the MC903 AD model, I restricted the topical application of MC903 to 19 days, at which point disease was established. Beyond day 19, mice were monitored (with no further exposure to MC903 topical application) for 1, 2 and 3 weeks respectively. As mentioned earlier in this chapter, I have referred to the period of time when mice are set aside, without any further MC903 exposure as the recovery phase whereas the period during which mice were treated with MC903 as the inflammatory phase. Disease parameters were determined throughout the duration of the experiment (inflammation and recovery phases). Ear thickness was significantly reduced following 1 week of recovery compared to the control (19 day treatment group no recovery). There was a small further reduction in inflammation following 2 weeks of recovery and a more substantial decrease in inflammation following 3 weeks of recovery (**5.4Ai & 5.4Aii**). TEWL measurements were taken at day 19 and thereafter at each respective endpoint (i.e. day 26, 32, and 40). There was a reduction in the TEWL following 2 weeks of recovery compared to control (no recovery). However, there was no further decrease in the TEWL between the 2 week recovery and 3 week recovery groups (**5.4B**). Acanthosis revealed a reduction following 1 week recovery and there was no further decrease thereafter (**5.4C**). CAE staining exhibited an increase in mast cell counts after 2 weeks of recovery which decreased after 3 weeks of recovery. However, there was no difference in the mast

cell counts between the control (no recovery) and the 3 weeks recovery group **(5.4D)**. Interestingly, there was a considerable decrease in eosinophils after 1 week of recovery which was maintained with just a slight drop after 3 weeks of recovery. **(5.4E)**. TSLP in the tissue was significantly reduced in the 1 week recovery group compared to the control group (no recovery) and this was maintained until the 3 weeks of recovery **(5.4F)**. By the 2 weeks recovery time point (endpoint at day 32) inflammation, TEWL, and acanthosis were observed to return to baseline. The mast cells were only reduced in the 3 weeks recovery period. Eosinophil numbers and TSLP levels dramatically reduced following 1 week of recovery but there was a steady number of eosinophils and a steady level of TSLP maintained (more than at baseline) until 3 weeks of recovery. Baseline of eosinophil numbers in the tissue are in the range of 0-15 (EtOH controls in chapter 3 figure 3.4). TSLP at baseline is not detectable (EtOH controls in chapter 4, figure 4.2).

A) i

A) ii

Figure 5.4 Evaluating the AD phenotype during the recovery phase of the MC903 model

C57Bl/6 were topically administered with 1nmol MC903 for 19 days. The endpoint for one group was at day 19 while the rest of the mice were set aside (no further exposure to MC903) and monitored for 1, 2, and 3 weeks respectively. This period has been defined as recovery whereas the control group (endpoint at day 19) was defined as no recovery. **A) i)** Ear thickness was measured at endpoint. **ii)** Ear thickness was measured throughout the duration of treatment and recovery. **B)** TEWL measurements were taken at the day 19 endpoint and then again at the respective endpoints. **C)** H&E staining was performed and acanthosis was measured in tissue sections and quantified by Image J analysis. **D)** CAE was performed to detect mast cells and these were quantified by Image J analysis. **E)** EPX-mAB based immunohistochemistry was used to assess eosinophil infiltration. Eosinophils were quantified and scored for degranulation. **F)** TSLP levels were detected in the tissue by ELISA. Plots and bar graphs show mean $\pm$ SEM. Statistics calculated by one-way ANOVA with Tukey post-test. * $P < 0.01$, ** $P < 0.01$, **** $P < 0.0001$ NS=non-significant. Each graph is representative of two independent experiments with at least 5 mice per group.

5.2.5 Assessing remodelling events in the MC903 model

To determine if remodelling was occurring during the recovery phase of my model (as defined in section 5.2.4 and figure 5.4), I assessed the amount of collagen deposition, a hallmark of remodelling and fibrosis in the skin [60]. Specifically, Masson's Trichrome staining and Image J analysis were used to detect and quantify collagen deposition in the tissue, respectively [108]. There was a clear increase of collagen (blue staining) in the 1 week recovery group and in the 2 week recovery group, compared to the control group (no recovery) **(5.5A)**. This was further quantified by Image J analysis and displayed as the percentage of collagen in the tissue. There was a significant increase in the amount of collagen in all recovery groups compared to the control (no recovery) group **(5.5B)**. Most notably, remodelling events, characterised by the collagen deposition peaked during the 2 weeks of recovery.

A)

B)

NS EtOH vs. day19
 **** EtOH vs. day19+1 recovery
 **** EtOH vs. day19+2 recovery
 **** EtOH vs. day19+3 recovery

Figure 5.5 Assessing remodelling in the recovery phase of the MC903 model

C57Bl/6 were topically administered with 1nmol MC903 19 days. Mice were then set aside and monitored for 1, 2 and 3 weeks respectively. Mice that were set aside were defined as recovery groups (as described in fig 5.4). Masson's trichrome staining was conducted to evaluate the collagen deposition in the tissue (collagen in blue) **A)** Tissue sections stained with Masson's trichrome demonstrating the presence of collagen. **B)** Area of collagen deposition is quantified by Image J analysis. Statistics calculated by one-way ANOVA with the Tukey post-test. ** $P < 0.01$, *** $0.01 < P < 0.001$, **** $P < 0.0001$ NS=non-significant. Each graph is representative of two independent experiments with at least 5 mice per group.

5.2.6 Characterising the impact of the TMA model of skin inflammation on C57Bl/6 and BALB/c mice

The contact irritant, TMA, has been previously used to induce skin inflammation in mice [289]. Our collaborator James Lee and his lab at the Mayo Clinic had been working on this chronic TMA model and we utilized their established protocol. This skin inflammatory model has been used to demonstrate an association between eosinophils and skin innervation and itch as well as collagen deposition [34]. In this study Lee *et al* has revealed a reduction of skin innervation due to a decrease of substance P in TMA treated-eosinophil deficient mice. Furthermore, TSLP has been shown to stimulate itch in the skin. Intradermal injections of TSLP into the skin of mice resulted in activation of sensory neurons. To address the role of TSLP in this TMA model, I used the TSLPRKO mice. However, it had to be first validated in both BALB/c mice as well as C57Bl/6 mice. This model was typically done in BALB/c mice however, the TSLPRKO mice were on a C57Bl/6 background, it was necessary to employ this model in C57Bl/6 mice to determine if inflammation was induced in a similar manner. This model involved a sensitization phase in which the mice were shaved on their flank and 50uL of 5% TMA was topically applied on day 0 and day 5. From day 6 - day 14, 15uL of 2% TMA was topically applied on the dorsal side of each ear. This topical application was performed daily. The vehicle control was a

solution of acetone : olive oil (4:1) **(5.6A)**. Ear thickness measurements demonstrated a substantial increase in inflammation in both the TMA-treated C57Bl/6 and BALB/c mice compared to controls. There was no difference between the ear thickness between the two strains of mice **(5.6Bi & 5.6Bii)**. TEWL measurements showed a significant increase in TMA-treated groups of mice compared to controls **(5.6C)**. TMA has a similar impact on inflammation and barrier dysfunction in both C57Bl/6 mice and BALB/c mice.

A)

TMA/Acetone : Olive oil (vehicle)

1 50ul 5%TMA on the flank on day0 & day5

2 15ul 2%TMA on dorsal side ear daily from day 6-day14

B)

C)

Figure 5.6 Characterising the impact of TMA model in C57Bl/6 and BALB/c mice

C57Bl/6 and BALB/c mice were sensitized on their flanks with 5%TMA on day 0 and day 5. Mice then received daily topical application of 2% TMA or vehicle control until day 14. Mice were sacrificed on day 16. **A)** Schematic of TMA treatment **B) i)** Ear thickness was measured at endpoint. **ii)** Ear thickness was measured throughout the duration of treatment. **C)** TEWL measurements were taken at endpoint. Plots and bar graphs show mean $\pm$ SEM. Statistics calculated by one-way ANOVA with Tukey post-test. ** $P < 0.01$, *** $0.01 < P < 0.001$, **** $P < 0.0001$ NS=non-significant. Each graph is representative of two independent experiments with at least 5 mice per group.

5.2.7 TSLP is required for the TMA model of skin inflammation but is not the only driver of inflammation

To delineate the role of TSLP in the chronic TMA model of skin inflammation, we used mice carrying a genetic deletion of the TSLP receptor (i.e. TSLPRKO mice). TMA-treated TSLPRKO mice were compared to TMA-treated C57Bl/6 mice and the respective controls. As expected, TMA-treated WT mice displayed a significant increase in ear thickness. In contrast, the ears of TMA-treated TSLPRKO mice revealed significantly less inflammation, although ear thickness was still statistically increased compared to controls (untreated TSLPRKO mice) **(5.7Ai & 5.7A ii)**. Similarly, TEWL measurements were significantly increased in TMA-treated C57Bl/6 compared to controls. TMA-treatment did not lead to any barrier dysfunction in TSLPRKO mice **(5.7B)**. TSLP levels detected by ELISA revealed no change across the TMA-treated groups compared to controls **(5.7C)**. To summarize, skin inflammation and barrier dysfunction were significantly reduced or completely blocked, respectively, in TMA-treated TSLPRKO mice, compared to the TMA WT controls. However, the TSLP expression levels detected were quite low in TMA-treated groups.

Figure 5.7 TSLP appears to be involved in the TMA model but is not the only driver of inflammation

TMA model was employed in C57Bl/6 and TSLPRKO mice as previously described. **A) i)** Ear thickness was measured at endpoint. **ii)** Ear thickness was measured throughout the duration of treatment. **B)** TEWL measurements were taken at endpoint. **C)** TSLP levels were measured by ELISA in the tissue lysate. Plots and bar graphs show mean $\pm$ SEM. Statistics calculated by one-way ANOVA with Tukey post-test. *** $0.01 < P < 0.001$, **** $P < 0.0001$ NS=non-significant. Each graph is representative of two independent experiments with at least 5 mice per group.

5.2.8 Evaluating the expression of TSLP at earlier time points in the TMA model

Surprisingly, the detection of TSLP in the tissue of TMA-treated mice was quite low (figure 5.7). I hypothesized that I may be missing the peak of TSLP expression in the TMA model and therefore determined TSLP levels at earlier time points, in particular days 9, 11 and 16. Ear thickness measurements revealed the initial increase in inflammation was at day 9, and there was a small but not significant trend to an increase at day 11 and the peak of inflammation was at day 16 **(5.8A)**. There was a small increase in TSLP observed at day 9, which appeared to plateau at day 16 **(5.8B)**. This data suggests that the peak of TMA-induced inflammation is at day 16 however, the TSLP levels observed by ELISA were quite variable in each group and therefore it was difficult to establish a peak of TSLP in this model.

A)

B)

Figure 5.8 Evaluating levels of TSLP at earlier time points in the TMA model

TMA model was employed in C57Bl/6 overtime. (day 9, 11, and 16) **A)** Ear thickness was measured at endpoint. **B)** TSLP levels were measure by ELISA in the tissue lysate over time. Plots and bar graphs show mean $\pm$ SEM. Statistics calculated by one-way ANOVA with Tukey post-test. *** $0.01 < P < 0.001$, **** $P < 0.0001$ NS=non-significant. Each graph is representative of two independent experiments with at least 5 mice per group.

5.3 Discussion

The knowledge obtained from the results in this chapter, while focusing on three separate aspects of AD, collectively assist in our understating of the pathophysiology and will help in providing more targeted therapy for AD patients.

5.3.1 Combination treatment

The central cells, cytokines and mediators that have been investigated in the MC903 AD model are eosinophils, IL-9, and PGD₂. Direct or indirect inhibition of each of these effectors have resulted in disease amelioration. This warranted the evaluation of blocking these above-mentioned targets in combination to assess if further reduction in disease severity could be achieved. My results suggested that concomitant blockade of eosinophil function as well as IL-9, using a combination of resorcinol and anti – IL-9, yielded the greatest reduction in disease phenotype. However, this was only slightly more effective than treating with anti - IL-9 alone. An important factor to be considered in the intervention strategy experiments is the duration and timing of the intervention. This gives us more understanding into the inflammatory cascade occurring in the AD model. For instance, anti-IL-9 treatment was used in a prophylactic setting (from day -1), while CRTh2^{ant} and resorcinol was used in a therapeutic setting (from day 5). Another vital consideration is, irrespective of which intervention strategy (combination therapies and/or monotherapies) I used, the ear thickness (as a measure of inflammation) was never reduced to baseline. Similarly, barrier function was improved in many intervention strategies, with the

exception of the CRTh2 antagonism, this did not return to baseline. Therefore, I deduce that in my murine model of AD, once the skin integrity has been compromised there will always be residual inflammation and barrier dysfunction cannot be fully restored. In order for these therapeutic interventions to translate into a human clinical setting we need to determine if the reduction in clinical parameters obtained in this murine model of AD, is adequately effective to alleviate symptoms in AD patients.

5.3.2 Remodelling and Fibrosis

Classically AD presents with chronic inflammation accompanied by a proliferation of keratinocytes, leading to epidermal hyperplasia [61]. AD patients experience relapsing flares [5] and typical hallmarks of their lesions include spongiosis (intercellular edema) and tissue remodelling [58]. The actual spongiotic defect is a morphological feature of the disruption of the epidermal barrier which in turn leads to the remodelling of the tissue [56]. This eventually leads to a thickened rubbery appearance of the skin known as lichenification [9]. The MC903-treated tissue displayed all the above-mentioned phenotypic features of AD, most notably the presence of remodelling in the skin (figure 5.5). Masson's trichrome staining is routinely used to determine collagen deposition in the tissue and the presence of collagen is the initial signs that remodelling is occurring [108]. The collagen deposition was markedly increased following 1 week of recovery and this peaked following two weeks in recovery. The recovery experiments demonstrated that inflammation is most substantially reduced in mice set aside to recover for 2 weeks following the 19 days of MC903 treatment. It is at this 2-week recovery time point, that other clinical parameters such as TEWL and acanthosis, substantially decline. The drastic

reduction of TSLP and eosinophils during this recovery phase suggested that eosinophils are not involved with remodelling in this model. However, this can be further validated by employing MC903 in the iPHIL mice and allowing these mice to recover. At the specific endpoint, eosinophil numbers between inflammation, recovery and remodelling phases can be compared. Unfortunately, I was unable to explore this aspect of the skin in more depth, due to time-constraints of my PhD. There remains a number of potential cytokines that are possibly driving remodelling in this MC903 AD model. Both IL-13 and TGF β have been well-established as pro-fibrotic factors in airway inflammation [314, 315] and IL-13 has been documented in skin remodelling in AD patient studies [108]. Furthermore, it was recently reported that IL-31 was responsible for skin remodelling that led to further skin barrier dysfunction in a murine model of skin inflammation [58]. Altogether, these cytokines have been reported to be crucial to the remodelling events in the skin. Overall, this recovery period in the MC903-induced AD model provides an excellent platform to study the cells and cytokines involved in fibrosis and remodelling in the skin.

5.3.3 An alternate skin inflammatory model

TMA, a contact toxicant, was used to induce skin inflammation [289]. Lee *et al* previously described this model to be dependent on eosinophils [34]. Based on my results eosinophils and TSLP play a crucial role in the MC903 model, I wanted to determine whether TSLP had a similar role in the TMA model. These experiments suggested that TSLP, did indeed have a role in the TMA model as TSLPRKO mice when treated with TMA did not develop skin inflammation to the extent as the TMA-

treated WT mice. In addition, the TMA-treated TSLPRKO mice have a reduced barrier function than the WT group. However, the expression levels of TSLP observed in the tissue, by ELISA did not corroborate the clinical readouts of inflammation. A possible explanation for the discrepancy in this data is perhaps the levels of TSLP observed on a protein level by ELISA is not reflective of the actual levels of TSLP released in the tissue and this may need to be assessed on an mRNA level by RT - qPCR. Studies have reported that TSLP protein can be post-translationally modified by endogenous proteases in the tissue [316]. Alternatively, it is possible that the repeated exposure of the skin to TMA has an effect on the TSLP that is released, and therefore TSLP on a protein level is unable to be properly detected by ELISA. Extensive characterisation of the cytokine profiles in a chronic model of TMA-induced skin inflammation was conducted and this study revealed that some of their cytokines such as IL-4 and IL-13 could either be detected on either an mRNA level or a protein level but not both [289]. Furthermore, the TMA-induced model of inflammation differs in comparison to the MC903 model of AD, in the sensitization process and it is highly plausible that TSLP has a vital role very early on in the sensitization phase. Alternatively, in the TMA model, TSLP could potentially be responsible for T cell priming in the lymph nodes, and if this is the case TSLP should be measured in the lymph nodes. Another possibility is TSLP could be very quick acting and taken up by the receptor as soon as it is released.

5.4 Conclusion

Intriguingly, the additive effect of IL-9 & EPO blockade resulted in the greatest reduction of AD phenotype. The MC903 AD model is beneficial to study the cellular changes from inflammation to remodelling and fibrosis in the skin. Furthermore, the greatest recovery correlates with the peak of remodelling (fibrosis and collagen deposition) in this model. This recovery period is 2 weeks following the removal of MC903 treatment. The rapid decline of eosinophils in the recovery phase suggests that in the MC903 model eosinophils are effector cells associated with inflammation and not remodelling. Moreover, while TSLP may be essential in driving inflammation in the TMA model, its role has not been fully established due to the fact that expression levels of TSLP observed in the tissue, by ELISA did not corroborate the clinical readouts of inflammation.

6 Determining the source of IL-9 in MC903 AD model

6.1 Introduction

The ability of IL-9 blockade to substantially reduce AD phenotype indicated that this cytokine had a critical part to play in the MC903 AD model. I hypothesized that IL-9 is a potent initiator of the MC903 induced inflammatory cascade in the AD model. This is due to the early peak of IL-9 in the serum of MC903 treated mice at day 7 which also corresponds to the peak of TSLP in the serum following MC903 treatment. Furthermore, a study has documented a correlation between the IL-9 and TSLP serum levels of infants with AD and suggested that perhaps there was a link between these two cytokines in allergic disease [317]. Therefore, I set out to ascertain the source of IL-9 in the skin and which cells were the responders. This led to the experiments described in this chapter. It has previously been documented that mast cells [233], T cell [225, 299], eosinophils [234], and ILC2s [229] were capable of producing IL-9 in various contexts. Many of these above-mentioned cells were upregulated in the MC903 AD model therefore I rationalized that one of these cells were the source of IL-9 production. Previous reports from Gerlach and colleagues, showed IL-9 and IL-9R fluorescent staining in the gut tissue [299]. I used their staining

techniques along with necessary optimization for staining in the skin. However, much to my disappointment, I was never able to successfully stain for IL-9R in the skin.

In order to determine the source of IL-9, I assessed the co-localization of CD4⁺ T cells, basophils and mast cells with IL-9 by whole mount staining (WMS) and imaging using confocal microscopy. Owing to the complexity of cytokine staining, and the autofluorescent nature of the skin tissue, multiple approaches seemed necessary. Intracellular cytokine staining (ICS) for IL-9 allowed for the evaluation of eosinophils, ILC2s and/or fibroblasts as being responsible for IL-9 production. In addition, FACS sorting experiments of the above-mentioned cells were performed, along with RT - qPCR assessment of the expression of *il9* and its receptor.

6.1.1 Aims

- To delineate which cell types produce IL-9 in the MC903 model.

Approaches include:

- Visualization by confocal microscopy. This technique comprises of whole mount staining (WMS) of MC903-treated ear tissue and staining with IL-9 antibody along with several different markers (namely c-kit, CD4+ T cells, CD45, CD90, and EPCAM)
- A fibroblast in vitro MC903 assay. The focus of these experiments are to culture murine fibroblasts and stimulate them with different concentrations of MC903.
- Intracellular cytokine staining (ICS) specifically staining for IL-9 in MC903-treated tissue. Cells subsets of interest are ILC2s, eosinophils and fibroblasts.
- FACS analysis of sorted populations of cells (eosinophils, and fibroblasts) and I will evaluate the levels of il9 and il9r by RT - qPCR.

6.2 Results

6.2.1 IL-9 is detected by fluorescent staining in the MC903 tissue, and is absent in IL-9KO skin

Tissue staining using a monoclonal IL-9 antibody was used to explore IL-9 expression in the skin. This revealed minimal IL-9 at baseline (EtOH-treated), and an increase in the staining in the day 19 MC903-treated group, which correlated with what was previously detected by ELISA. The main purpose of these experiments was to determine the specificity of the IL-9 staining, using the IL-9KO tissue as a comparison. C57Bl/6 mice were treated for 19 days with MC903 and/or EtOH. At endpoint, ear tissue from both the MC903 and EtOH, together with IL-9KO ear tissue were excised, split into dorsal and ventral halves and fixed in paraformaldehyde (PFA). WMS was then performed [298]. Skin tissue was stained with a panel of antibodies namely; primary IL-9 biotin antibody, a secondary rabbit anti-hamster and a tertiary dylight 594 and DAPI. This panel mentioned above has been used for the experiments below in sections 6.2.2-6.2.4. Ear tissue was then mounted on slides and imaged using a confocal microscope. The EtOH-treated tissue showed very little IL-9 staining as depicted in green, and there was co-staining of the nuclei using DAPI (blue) **(6.1Ai)**. An increase in the staining intensity of both the IL-9 (green) and DAPI (blue) in the MC903-treated tissue was seen **(6.1Aii)**. IL-9KO ears showed only DAPI

positive staining and no green staining **(6.1Aiii)**. This suggested that IL-9 antibody being used was potentially specific. The staining protocol was optimized and optimum dilution for the primary antibody was 1:100, while both secondary and tertiary were used at 1:200. Multiple images and Z-stacks were taken from 6 slides per group (3 whole ears). It is noteworthy that the IL-9KO tissue was not treated with MC903 and this should be considered when making deductions regarding antibody specificity. Expectedly, there would be little to no IL-9 present in non-treated tissue. This data suggests that the IL-9 antibody does not pick up unnecessary background staining.

A)

i) EtOH- IL-9 (green) + DAPI(blue)

ii) MC903- IL-9 (green) + DAPI(blue)

iii) IL-9KO - IL-9 (green) + DAPI(blue)

Figure 6.1 IL-9 is detected by fluorescent staining in the MC903-treated skin

C57Bl/6 mice were treated for 19 days with MC903 and/or EtOH. At endpoint ear tissue from both the MC903 and EtOH, together with IL-9KO ear tissue were excised, fixed and whole mount staining performed. **A)** Ear tissue was co-stained with IL-9 (green) and DAPI (blue) in **i)** EtOH-treated group **ii)** MC903-treated group and **iii)** IL-9KO group. All images were representative of either multiple single images and/or Z- stacks which were taken from 6 slides per group (3 whole ears). Experiments are representative of two independent experiments.

6.2.2 IL-9 does not co-localize with mast cells in MC903 tissue

The first candidate that was evaluated as being the source of IL-9 were mast cells. The surface marker used to detect mast cells in the skin was c-kit (CD117-APC). It is important to note that the melanocytes in the skin are also c-kit positive. MC903 was topically applied for 19 days and WMS performed. Staining for IL-9 was as previously described in section 6.2.1. Three-dimensional (3D) reconstruction showing 48 µm of MC903-treated tissue was recorded **(6.2Ai)** [300, 301]. This revealed that the IL-9 positive staining (green) did not co-localize with the c-kit⁺ cells (magenta). This was further elucidated by the Z-stacks **(6.2Aii& iii)** displaying from bottom to top that c-kit and IL-9 staining were in different layers of tissue. 3D reconstruction and Z-stacks were taken from 6 slides per group (3 whole ears). Scale bar 25µm. These findings demonstrated that mast cells, surprisingly, were not responsible for IL-9 production in the MC903 model.

A) MC903 treated tissue c-kit (magenta), IL-9 (green) DAPI (blue) Scale bar 25µm

i) 3D reconstruction 48µm Z axis

ii) Z-stack first 4µm bottom-top

iii) Z-stack last 4µm recording

Figure 6.2 IL-9 does not co-localize with mast cells in MC903-treated skin tissue

C57Bl/6 mice were treated for 19 days with MC903. At endpoint ear tissue from both the MC903 were excised, fixed and whole mount staining performed. **A)** Ear tissue was co-stained with c-kit (magenta), IL-9 (green) and DAPI (blue) **i)** 3D reconstruction 48µm recording of Z axis. Z-stack recorded bottom to top. **ii)** image from first 4µm **iii)** image from last 4µm. 3D reconstruction and Z-stacks were taken from 6 slides per group (3 whole ears). Scale bar 25µm. Experiments are representative of at least 5-6 independent experiments.

6.2.3 IL-9 does not co-localize with CD45+, nor CD4+ cells in the MC903-treated skin tissue

As an attempt to answer many questions regarding the potential IL-9 producers in the skin I decided to use markers that would allow classification of the IL-9 producer. To assess if T cells were responsible for IL-9 production, CD4 was selected. Moreover, to distinguish if the source of IL-9 was an immune cell, CD45 was selected. In addition, CD90 was stained to as both T cells and ILC2s express this marker but will also express CD45. In accordance with this rationale, CD4+ **(6.3A)**, CD45+ **(6.3B)** and CD90+ **(6.3C)** directly conjugated antibodies were used. MC903 was topically applied for 19 days and WMS performed. Staining for IL-9 was as previously described in section 6.2.1. 3D reconstruction of tissue (48µm Z axis) and Z-stacks revealed that both IL-9 and CD4+ staining were detected in MC903-treated tissue. However, the IL-9+ (green) did not co-localize with the CD4+ T cells (magenta) in the MC903-treated tissue **(6.3Ai)**. IL-9 positivity and CD4+ cells were difficult to visualize in the same field of view. Z-stacks recorded from bottom to top. These experiments showed expression of IL-9 in the first 4µm of the Z-stack **(6.3Aii)** while the CD4+ T cells were detected in the last 4 µm of the recording **(6.3Aiii)**. These images did not suggest that CD4+ T cells were the source of IL-9 production in this MC903 model.

3D reconstruction (37µm Z axis) of co-stained tissue with CD45 (magenta) and IL-9 (green) displayed no co-localization of the staining **(6.3.Bi)**. Z-stacks recording both the first 4µm **(6.3Bii)** and last 4µm **(6.3Biii)** further confirmed that the IL-9 producing cells were not CD45+ cells. However, there was a promising signal from the CD90 (magenta) and IL-9 (green) **(6.3C)** observed. 3D reconstruction (17µm Z axis) was recorded **(6.3Ci)** and this combined with the Z-stacks displayed that IL-9 producing cells could potentially be CD90+. In the first 4µm of the Z-stack IL-9 and CD90 visually seemed to be on the same cells **(6.3Cii)**. However, very little IL-9 was seen and no CD90+ cells detected in the last 4µm of the Z-stack **(6.3Ciii)**. 3D reconstruction and Z-stacks were taken from 6 slides per group (3 whole ears). All images described in figure 6.3 used a scale bar of 50µm. Intriguingly, IL-9 production was from a non-immune cell that was CD90+.

A) MC903 treated tissue CD4 T cells(magenta), IL-9 (green) DAPI (blue) Scale bar 50µm

i) 3D reconstruction 48µm Z axis

ii) Z-stack first 4µm bottom-top

iii) Z-stack last 4µm recording

B) MC903 treated tissue CD45 (magenta), IL-9 (green) DAPI (blue) Scale bar 50µm

i) 3D reconstruction 37µm Z axis

ii) Z-stack first 4µm bottom-top

iii) Z-stack last 4µm recording

A) MC903 treated tissue CD90 (magenta), IL-9 (green) EPCAM (yellow) CD45 (blue) Scale bar 50µm

i) 3D reconstruction 50µm Z axis

ii) Total Z projection showing only EPCAM and CD45

iii) Total Z projection showing CD90, IL-9, EPCAM

C) MC903 treated tissue CD90 (magenta), IL-9 (green) DAPI (blue) Scale bar 50µm

i) 3D reconstruction 17µm Z axis

ii) Z-stack first 4µm bottom-top

iii) Z-stack last 4µm recording

Figure 6.3 IL-9 producing cells are CD4⁻ and CD45⁻ cells, however there is potential signal from CD90 staining

C57Bl/6 mice were treated for 19 days with MC903. At endpoint ear tissue from both the MC903 were excised, fixed and whole mount staining performed. **A)** Ear tissue was co-stained with CD4⁺ (magenta), IL-9⁺ (green) and DAPI (blue). **i)** 3D reconstruction 48µm recording of Z axis. Z-stack recorded bottom to top. **ii)** image from first 4µm **iii)** image from last 4µm. **B)** Ear tissue was co-stained with CD45⁺ (magenta), IL-9⁺ (green) and DAPI (blue). **i)** 3D reconstruction 37µm recording of Z axis. Z-stack recorded bottom to top. **ii)** image from first 4µm. **iii)** image from last 4µm. **C)** Ear tissue was co-stained with CD90⁺ (magenta), IL-9⁺ (green) and DAPI (blue). **i)** 3D reconstruction 17µm recording of Z axis. Z-stack recorded bottom to top. **ii)** image from first 4µm. **iii)** image from last 4µm. 3D reconstruction and Z-stacks were taken from 6 slides per group (3 whole ears). Scale bar 50µm. Experiments are representative of at least 5-6 independent experiments.

6.2.4 IL-9 does not co-localize with keratinocytes

Previous confocal experiments (figure 6.2.3), highlighted the source of IL-9 was likely a non-immune cell (CD45-). Keratinocytes, the most abundant epithelial cell in the skin [4], is directly induced by MC903 to produce TSLP and proliferate [89] in this model of AD. Therefore, I needed to establish whether keratinocytes were a source of IL-9. Furthermore, I decided, that due to the IL-9 positive staining seen at close proximity to the CD90+ staining I included CD90 in this experiment. WMS was then performed on MC903-treated tissue and co-staining of IL-9, EPCAM (a keratinocyte marker), CD45, and CD90. 3D reconstruction of tissue (50µm Z axis) (**6.4Ai**) and Total Z projections (**6.4Aii & 6.4Aiii**) demonstrated co-localization of IL-9+ was with CD90+. There was no co-localization with EPCAM. However, with many colours there was a lot more auto-fluorescence thereby making visualization of all the markers extremely difficult. Two total Z projections were conducted of the same field of view. In the first total Z projection expression of CD90+ (magenta), IL-9+ (green), EPCAM+ (yellow) was assessed (**6.4Aii**) and thereafter EPCAM+ (yellow) and CD45+ (blue) was assessed (**6.4Aiii**). Images demonstrated CD90+ and IL-9+ were in a different plane as the CD45+ cells. Additionally, EPCAM+ cells did not appear to co-express IL-9 (as indicated by the arrows). Observations from these confocal experiments had suggested that the source of IL-9 were cells that CD45- EPCAM-. These observations demonstrate that keratinocytes were not the source of IL-9. In summary, the results thus far suggest that IL-9 is likely being produced by a non-immune cell (CD45-),

which is also CD90+. The only cell in the skin that displays the immune profile of being CD45- CD90+ are fibroblasts[318].

Figure 6.4 IL-9 is not produced by the keratinocytes in the MC903 model

C57Bl/6 mice were treated for 19 days with MC903. At endpoint ear tissue was excised, fixed and whole mount staining performed. **A)** Ear tissue was co-stained with CD90+ (magenta), IL-9 (green), EPCAM+ (yellow) CD45+ (blue). **i)** 3D reconstruction 50µm recording of Z axis. Total Z projections showing; **ii)** EPCAM+ and CD45 **iii)** EPCAM+, CD90+, and IL-9+. 3D reconstruction and total Z projections were taken from 6 slides per group (3 whole ears). Scale bar 50µm. Experiments are representative of at least 5-6 independent experiments.

6.2.5 Culturing murine skin fibroblasts, and confirming a homogeneous population of cells by flow cytometry

Following on from the confocal experiments previously performed (6.2.3-6.2.4), it was evident that the source of IL-9 was a non-immune cell (CD45-) that was not a keratinocyte (EPCAM-). I hypothesized that fibroblasts could be the potential source of IL-9 and set out to test if murine fibroblasts were capable of producing IL-9, in vitro. The first experiment was to successfully culture murine skin fibroblasts. I removed ear tissue from C57Bl/6 mice **(6.5Ai)**, and dissected them into 2 - 3mm squares. These were cultured in 10% DMEM : F12 + antibiotic/antimycotic **(6.5Aii)**. After, approximately, 2 weeks, cells began to 'walk out' of the skin pieces of tissue **(6.5B)**. These cells were fibroblast - like in appearance and when observed down the microscope they looked like a homogeneous population of cells. The next step was to determine whether the 'walk out' cells were a homogeneous population of fibroblasts by flow cytometry. Cultured cells were assessed by flow cytometry and this revealed there was no contamination of either keratinocytes or any other cells in the cultures **(6.5C)**. It was evident that these were a homogeneous population of murine skin fibroblasts.

A)

i)

C57Bl/6

ii)

coverslip

pieces of ear tissue cut into 2-3mm squares

DMEM F:12

ear tissue immersed in growth medium
under coverslip

approximately 2 weeks

B)

Figure 6.5. Culturing murine skin cells, and confirming a homogeneous population of cells by flow cytometry

Ai) Schematic of skin from excision, and **Aii)** culturing conditions. After 2-3 weeks cells ‘walked out’ of the skin and were evaluated under the microscope. **B)** Microscope images of skin tissue. Cultured cells were then analysed by flow cytometry. **C)** Representative FACS plots (Live/COI/CD45-/CD90+EPCAM-) of cultured population of cells. Experiments are representative of at least 5-6 independent experiments.

6.2.6 Murine fibroblasts may produce small amounts of IL-9 following MC903 treatment, in vitro

For in vivo experiments, MC903 is typically resuspended in EtOH [89], however for the in vitro experiments described below, it was resuspended in DMSO (as per manufacture's recommendation) [319]. The use of DMSO was more precautionary as I wanted to ensure the optimum cell growth. A starting concentration of 500 μ M (0.5mM) of MC903 was applied on cultured fibroblasts, and thereafter 2 -fold dilution in a 12-well plate. The final concentration was 0.486 μ M and this was incubated for 18 and 48 hours. 15ng of rTSLP was added to each concentration for 18 hours. Supernatant was collected and IL-9 ELISA performed. The detection range of the IL-9 ELISA is 32pg/mL-4000pg/mL. IL-9 was detected in the supernatant from 125 μ M - 0.49 μ M in the MC903 treatment for 18hours **(6.6Ai)**. The addition of rTSLP did not yield a greater level of IL-9 **(6.6Aii)**. IL-9 levels in the supernatant was not as consistent in every well compared following 48 hours of incubation **(6.6B)**. There was no IL-9 detected in media only, DMSO only, PMA/ionomycin only controls at both time points. rTSLP alone did not induce IL-9 production from fibroblasts **(6.6 Ai & B)**. Graphs are a representative of experiments done in triplicate. In each experiment, there were four wells treated with MC903 (n = 4) and three wells with rTSLP (n = 3). The data generated did not reflect a dose response of IL-9 to the MC903 stimulation. Overall, it was an intriguing finding that fibroblasts were capable of producing IL-9 in the supernatant.

Figure 6.6. Murine fibroblasts produce IL-9 following MC903 treatment, in vitro

Cultured fibroblasts were treated with 2-fold dilution of MC903 in DMSO from 500 μ M. IL-9 measured in supernatant by ELISA. IL-9 levels following MC903 treatment **Ai)** for 18hours **Aii)** with rTSLP for 18hours and **B)** for 48hours. Graphs are a representative of experiments done in triplicate. IL-9 ELISA detection range is 32pg/mL-4000pg/mL (indicated by red line). In each experiment, there were four wells treated with MC903 ($n = 4$) and one well with rTSLP ($n = 3$).

6.2.7 ILC2s are not the source of IL-9 production in the MC903 model of AD

To determine the primary producers of IL-9 in the skin, intracellular cytokine staining (PMA/ionomycin stimulation) for IL-9 was performed following 19 days of MC903 application. In order to obtain large amounts of cells, 3 separate groups were assessed (n=5) ILC2s, eosinophils and fibroblasts. This revealed the ILC2 subset were not the source of IL-9 **(6.7A)**. Eosinophils **(6.7B)** and fibroblasts **(6.7C)** had very faint IL-9 positive staining. There were only 1.26% of IL-9 from a population of eosinophils and only 1.76% of IL-9 from a population of fibroblasts. Representative FACS plots are depicted to illustrate specific cell subsets and IL-9 positive staining as the IL-9 staining was a very small percentage. Analysis of total IL-9 producing cells demonstrated that the production of IL-9 was heterogeneous. ILC2s appeared to be not responsible for producing IL-9 in the MC903 model. Furthermore, the levels of IL-9 expression, using the flow antibody did not reflect the IL-9 expression levels obtained during the IHC and ELISA assays.

A)

MC903 treated ear

EtOH treated ear

B)

MC903 treated ear

EtOH treated ear

C)

Figure 6.7. ICS revealed it is not the ILC2s that are the source of IL-9 in the MC903 model

ICS for IL-9 in the skin following 19 days of MC903. The following cell subsets were evaluated for expression of IL-9+ cells from MC903 and EtOH- treated ears; **A)** ILC2s, **B)** eosinophils **C)** fibroblasts. Representative FACS plots are depicted to illustrate specific cell subsets and IL-9+ staining. Three separate groups were assessed for each cell type and in each group, there were 5 ear samples (n = 5) for each group. Experiments are representative of at least 3 independent experiments.

6.2.8 Neither *il9* nor *il9r* expression could be detected from sorted fibroblasts and eosinophils

Observations from ICS experiments (figure 6.7) suggested that there was some IL-9 being produced by either eosinophils and/or fibroblasts but the results were not definitive. To further answer this question FACS sorted fibroblasts, eosinophils and a CD45- CD90- population of cells following MC903 treatment were assessed by RT-qPCR. I sorted 2000 cells from each subset and these were then valuated, for the expression of *il9*, and or *il9r*. Murine blood samples were assessed as a control for the eosinophil subset due to the fact that there are little to none eosinophils present in an EtOH-treated ear tissue. RT - qPCR demonstrated expression of Vitamin D receptor (*vdr*) and *cyp* genes, but no expression of *il9* (no data generated as there was no detection) and *il9r* (**6.8A**) from fibroblasts, eosinophils nor the CD45- CD90- population was observed. *Vdr* was detected on both skin fibroblasts and the CD45- CD90- subset but to a lesser extent on eosinophils (**6.8B**) and similarly *cyp24a1* was expressed in skin fibroblasts and CD45-CD90- subset, but not on eosinophils (**6.8C**). CYP24A1 is an active product expressed during VDR activation. Gene expression was normalized to the housekeeper gene, *gapdh* which showed CT values in all the evaluated populations (**6.8D**). Unfortunately, *il9* nor *il9r* could be detected from sorted populations of eosinophils or fibroblasts. Verification of the experiment was

confirmed by the fact that there were significant levels of *vdr* and *cyp24a1* detected in these sorted populations by RT - qPCR.

Figure 6.8. Il9 and il9r was not expressed by murine skin fibroblasts

MC903-treated tissue was digested, sorted into three cell populations by FACS; namely eosinophils, fibroblasts and CD45-CD90- subset. Cell subsets were then analysed by RT - qPCR for the expression of **A)** *il9r*, **B)** *vdr*, **C)** *cyp24a1* and **D)** *gapdh* (housekeeping- gene). Experiment was conducted once.

6.3 Discussion

The cells that are known to produce IL-9 were stained and these experiments clearly showed that mast cells, and CD4⁺ T cells were not producers of IL-9 in the MC903 model. Interestingly, confocal observations suggested that the source of IL-9 was a non-immune cell (CD45⁻), there was a potential signal from CD90⁺ cells but it was most likely not a keratinocyte (EPCAM⁻). This alluded to a non-immune cell in the skin as being potential producers of IL-9. However, the actual staining pattern of IL-9 in these confocal experiments were questionable as the staining appeared more membranous rather than cytoplasmic, as is expected with cytokine staining. The *in vitro* culture experiments demonstrated that fibroblasts were capable of producing small amounts of IL-9. Notably, there was no dose escalation effect of MC903 on the fibroblasts. Intracellular cytokine staining was the next approach, using a flow cytometry antibody for IL-9. This data demonstrated while ILC2s could be ruled out as sources of IL-9, there was a faint detection of IL-9 from eosinophils (1.26%) and fibroblasts (1.76%). However, the ICS detection was far lower than the levels detected by the IL-9 confocal antibody. Furthermore, PMA/ionomycin treatment involves restimulation of the cells and hence if there is cytokine within these subsets, this should amplify their expression. In summary, the ICS detection of IL-9 was not sufficiently convincing.

In a final attempt to delineate the source of the IL-9 production following MC903 treatment, eosinophils, fibroblasts and the above-mentioned non-immune subset (CD45⁻ CD90⁻) were FACS sorted and samples were assessed by RT - qPCR. Unfortunately, neither *il-9* nor *il-9r* expression could be detected in the sorted subsets

i.e. eosinophils, fibroblasts and CD45⁺ CD90⁻. Disappointingly, this gene expression data did not corroborate the IL-9 levels detected by confocal and ELISA assay.

6.4 Conclusion

Much to my dismay, I was unable to determine the source of IL-9 in the MC903 model. Observations from confocal, and in vitro data suggested fibroblasts to be a likely IL-9 producing cell, however, this could not be verified and corroborated with the gene expression levels. As this stage, the source of IL-9 in the MC903 AD model remains yet to be elucidated and this could be achieved by employing the MC903 model in the IL-9 fate reporter mice [229].

7 General Discussion

7.1 Establishing a clinically relevant model of AD

The principal aims of this thesis centred around describing and understanding the cellular events and inflammatory changes that are responsible for the pathogenesis of AD. Much of the early work of the project focused on establishing a robust, clinically relevant murine model of AD. This entailed characterising the clinical features of AD such as inflammation, itch, barrier dysfunction and the accumulation of pro-inflammatory cells and cytokines (figures 3.2, 3.4 & 4.1). It was vital to distinguish if the MC903 model was merely a model of skin inflammation or if the phenotype was consistent with AD. To achieve this, I assessed macroscopic, microscopic and immuno-diagnostic criteria for AD. In my model, I observed skin inflammation and increased barrier dysfunction as indicated by the increased TEWL. In addition, histopathological evaluation of MC903-treated skin exhibited hallmarks of AD, i.e. acanthosis, hyperkeratosis and spongiosis (figure 3.3). The presence of spongiosis, which is the intercellular oedema in the skin, is a diagnostic feature specific to AD [11, 24]. This MC903 AD model reflected a Th2 cytokine profile, characterised by high expression of IL-4, IL-9 and TSLP and low expression of IL-17 and IFN γ (figure 3.4, 4.1 & 4.2). The epithelial cytokine, TSLP has been described as

an early initiator of allergic disease [183, 198] and while many AD murine studies have reported high and prolonged systemic levels of TSLP [89, 90, 194] my model included its downregulation pass day 14. This is critical as circulating TSLP levels have not been found consistently elevated in human AD [185-187].

The disease pathology of AD patients is largely due to the innate immune response, in particular ILC2s and eosinophils [320], and there is a subset of AD that is also driven by T cells [310, 311]. Clinically, AD patients have been distinguished by an acute or a chronic manifestation of the disease. The acute presentation is associated with a biased Th2 immune environment while the chronic presentation is characterised by a combined Th1/Th2 immune profile [321]. Diagnosis of AD itself is complex as the histopathology closely resembles psoriasis. However, both skin diseases elicit a relatively distinct immune response. Historically, psoriasis has been associated with a Th17 immune response [53-55]. Interestingly, the MC903 model has been reported to be independent of T and B cells [89] and during my investigations, low levels of IL-17 and IFN- γ (figure 3.4) have been observed. The progression of AD research has facilitated the understanding that the immune dysfunction in AD patients is a lot more heterogeneous than a simple distinction between acute and chronic presentations. Clinical studies have proposed a role for Th17 cells and the presence of elevated tissue levels of IL-17 [112, 114] and IL-22 in AD [115, 321] has been documented. Conversely there have been reports to dispute this Th17 involvement in AD patients [115, 116]. It is difficult to tease this apart in mouse models of AD as in mice Th17 cells co-express IL-17 and IL-22. In contrast, however, in humans Th17 (producing IL-17) and Th22 cells (producing IL-22) seem to be distinct [322, 323]. This complex immune dysregulation together with the wide

spectrum of the clinical phenotype that manifests during AD contributes to the necessity of a precision medicine approach for the clinical management of this disease. The MC903 model of AD provides a novel platform to screen a panel of biomarkers for the prevention and treatment of AD (discussed in more detail in 7.6).

7.2 Delineating cellular effectors in the MC903 model of AD

In the MC903 AD model, which is being extensively discussed here, the peak of skin inflammation was observed at day 19 and this gradual development of disease phenotype provides a good platform for different intervention and/or depletion strategies. By determining the accumulation of the different cell types (chapter 3) and cytokines (chapter 4), over time, I was able to illustrate a better picture of the inflammatory pathway(s). Furthermore, this enabled me to deplete cells and cytokines, at the stage when their accumulation was detected, and thereby evaluate their precise action in the pathogenesis of AD. In patients, AD is mediated partially by T cells (as discussed in 7.1) and there is a considerable involvement of innate cells which contribute to the inflammatory response and disease progression [320]. Previous studies have shown that MC903 induced skin inflammation was independent of both T and B cells [89]. Consistent with these findings, I have demonstrated that CD4⁺ T cells are not required for the MC903 AD model (figure 3.8). This model is therefore particularly useful to study innate cells in allergic disease. The

results obtained during this project has highlighted the contribution of mast cells (figure 3.4), basophils, ILC2s (figure 3.10) and eosinophils (figure 3.4) to MC903-induced AD. Some of the most notable lessons learnt early on in this project were just how intertwined the innate arm of the immune system actually is. Therefore, I had to establish the individual kinetics of the key effector cells/molecules involved in this model of AD. Accordingly, interpretation of the results had to be carefully considered as these innate immune cells work in concert to initiate and maintain inflammation. Hence, timing of cellular interaction needed to be carefully considered when designing experiments.

7.2.1 Innate immune cells are driving AD phenotype

Accumulation of basophils and ILC2s have previously been documented in human AD skin lesions [324] as well as in MC903 murine studies [90, 91, 127]. Unfortunately, I have not explored the effects of depleting basophil recruitment into the skin during MC903 application as we did not have basophil knockout mice during the course of this project. Currently, the literature contains conflicting data as Schwartz *et al* documented increased inflammation when basophils were constitutively depleted [325] whereas Noti *et al* reported a significant reduction in inflammation using a DT-mediated basophil depletion [326]. It is likely that the discrepancy between these two studies lies in the different mouse strains used. Schwartz and colleagues used the Mcpt8-Cre mice [325] which constitutively depletes basophils due to Cre-mediated

toxicity [327]. Conversely, Noti and colleagues used the Bas-TRECK mice [326] where the diphtheria toxin receptor transgene is under the control of an IL-4 enhancer and in these experiments basophils were deleted following DT treatment [328]. However, we have recently acquired the Basoph8 mice which is a knock-in construct where the cells express Cre but are not spontaneously deleted. These mice will allow the depletion of basophils by treatment of DT [329]. Employing my MC903 AD model in these Basoph8 mice could clarify the role of basophils in this model.

Historically, it was assumed that mast cells contributed to skin inflammation as mastocytosis was often seen in AD murine models [197, 199] and skin lesions [158]. Mastocytosis is the abnormal proliferation and accumulation of mast cells, in which mediators are released. However, a study evaluating a cohort of Spanish allergy patients (including AD patients) showed that while these patients may exhibit increased mastocytosis it is not indicative of a higher risk of developing another atopic disease [330]. Furthermore, K14 stem cell factor (SCF)^{Tg} mice were generated and although these mice developed cutaneous mastocytosis they did not develop dermatitis [331]. Collectively this data suggests that mast cells are present as a consequence rather than a driver of skin inflammation. To confound matters, there has been some controversy surrounding the mast cell involvement in the induction of itch in AD. A study proposed that mast cells were responsible for the production of IL-31 to induce itch in AD in humans [156]. Dillon *et al* generated IL-31^{Tg} mice and these mice revealed AD-like lesions with mast cell infiltration. However, this same study injected IL-31 into mast cell deficient mice and they continued to develop AD-like lesions, thereby suggesting mast cells were not required for pruritus [155].

I have documented an accumulation of mast cells over time and a peak at day 19 however, it was following imatinib treatment (figure 3.7 targeting mast cells), CRTh2 antagonism and IL-9 blockade that mast cell numbers were significantly reduced. It was rather perplexing that when clinical AD parameters were reduced such as inflammation and acanthosis, the mast cell numbers remained unchanged. This occurred in both MC903 + resorcinol (figure 3.12) and in MC903-treated STAT6KO mice (figure 4.6). I would hypothesise that either mast cell infiltration is independent of the STAT6 signalling pathway or alternatively mast cells accumulate as a consequence of disease and act as a by stander.

7.2.2 Eosinophil involvement in the pathogenesis of AD

Eosinophils were shown to be necessary for AD disease pathology [332, 333] and exerted their effects in an EPO dependent manner (figure 3.12). Studies have implicated eosinophils to be associated with itch in the skin [34], however I have demonstrated that eosinophil depletion resulted in not just itch but TEWL, acanthosis and inflammation all being significantly reduced (figure 3.13). Importantly, depleting eosinophils in weeks 2-3 of the MC903 model was sufficient to significantly reduce disease phenotype (figure 3.14) which implies that the accumulated eosinophils in week 2 and 3 are contributing to most of the disease severity. This is consistent with the maximum accumulation of eosinophils detected at week 3 following MC903

treatment (figure 3.4). Notably, this establishes a role for eosinophils as effector cells in the MC903 AD model.

Conversely, when blocking EPO (resorcinol treatment), there was no change to the itching intensity. This finding can be justified by the small number of eosinophils (although substantially reduced) present in the resorcinol-treated mice, as opposed to the iPHIL mice that have almost a complete depletion of eosinophils. The eosinophils that remain in the resorcinol-treated mice are most likely sufficient to induce itch. Importantly, eosinophils emerge as an attractive therapeutic target for the clinical management of AD.

7.3 Delineating cytokine targets for the treatment and prevention of AD

7.3.1 TSLP, and the IL-4/IL-13 axis are necessary for the development of AD

There has been a lot of debate regarding the role of the three epithelial derived cytokines i.e. TSLP, IL-25 and IL-33 in the context of AD [125, 203, 218]. These cytokines, are seen as the first responders to the skin barrier breach and initiate an appropriate immune response. Consistent with the literature [89, 198], TSLP in my model of AD is essential to the progression of disease (figure 4.2). However, levels of IL-33 and IL-25 in the skin appeared to be unchanged throughout the model. Moreover, IL-25 KO mice treated with MC903 developed skin inflammation and mast cell accumulation suggesting that IL-25 was not required for AD development (figures 4.1 & 4.3). The variability of the level of responsiveness of TSLP, IL-33 and IL-25 in allergic disease was extensively discussed by a recent review [334]. This review highlighted that the dose and duration of treatment, affected the requirements for TSLP, IL-33 and IL-25. In accordance, there was a study that reported MC903 - induced skin inflammation in BALB/c mice was dependent on IL-25 [91] which conflicted with another study where *il25* or *il33* mRNA after MC903 application in BALB/c mice could not be detected (Han and Ziegler unpublished data) [334]. To

date, TSLP has been well documented to play essential a role in the pathogenesis of AD [106, 182, 188] but more work is required to fully validate the role of IL-25 and IL-33. There are certain levels of redundancy that may occur and for IL-25 and IL-33 to be potential targets for the treatment of AD we require more mechanistic insight into how they are involved in the initiation of AD [334].

Exploring the involvement of IL-4/IL-13 axis in the MC903 model was conducted by employing the MC903 model in STAT6KO mice. These mice are unable to respond to IL-4 and IL-13 signalling due to the deletion of the transcription factor STAT6 [269]. Intriguingly, MC903-treated STAT6KO mice demonstrated the most significant amelioration of disease (figure 4.6.). The importance of targeting both IL-4 and IL-13 was further highlighted as MC903-treated IL-4KO mice resulted in only a slight reduction in inflammation (figure 4.4). Furthermore, this data implies a potential role for IL-13 in the MC903 AD model. In addition, MC903-treated STAT6KO mice exhibited a drastic reduction of eosinophils in the tissue which suggests this STAT6 pathway is required for the recruitment of eosinophils. Knott *et al* has documented that IL-4/IL-13/ STAT6 pathway is critical for eotaxin production thereby driving eosinophil recruitment into the skin [335]. Moreover, increased levels of eotaxin and its receptor CCR3 have been reported in both AD mouse models [336, 337] and patients with AD [176]. Hence, I hypothesise that eosinophils are being recruited into the tissue via eotaxin and this is dependent on STAT6 signalling in the MC903 AD model. It would be interesting to assess eotaxin levels in MC903-treated STAT6KO mice compared to MC903 WT mice. In addition, evaluating eosinophils in MC903-treated STAT6KO mice that have been treated with recombinant eotaxin [338]. There have been several reports documenting CCR3 as being crucial for the recruitment of

eosinophils into murine skin and lung [336]. Therefore, by employing the MC903 model in CCR3 knockout mice we could potentially elucidate the manner by which eosinophil recruitment is occurring in the skin. It is noteworthy that apart from eotaxin CCR3 ligands include MCP-3 (CCL7) and RANTES (CCL5) [31, 339] and as such these chemokines need to be measured in the skin of MC903-treated CCR3 deficient mice, MC903-treated STAT6KO mice and the MC903-treated WT.

There have been reports documenting an increase of IL-5 in patients with AD [340] [341] and an association with skin innervation and itching following IL-5 recruitment of eosinophils [342]. However, given that much of the AD phenotype can be induced by IL-4 and IL-13 there has not been extensive work into the precise role played by IL-5 in AD [110]. My observations (chapter 4) suggested that IL-5 does not participate in the MC903 induction of AD as blocking IL-5 following MC903 treatment did not result in a reduction of skin inflammation. Although, I did not assess eosinophil infiltration in this experiment, I would hypothesize that based on no change in ear thickness and evaluation of H&E tissue sections (data not shown) that the eosinophil numbers would be similar in both the MC903+anti-IL-5 and MC903+isotype control groups. This experiment would need to be repeated to verify these findings.

More recently, a human monoclonal antibody to the alpha subunit of the IL-4 receptor (impairing the signalling of IL-4 and IL-13) [257], Dupilumab, has been successful in treating patients with AD. The positive impact of Dupilumab on AD patients has created more appreciation for the use of immunotherapy to treat AD [85, 103], and led to its FDA approval in March of 2017. However not all patients responded to Dupilumab and it caused substantial side effects such as nasopharyngitis,

conjunctivitis, and inflammation of the cornea and inflammation at injection site [86]. Thus, the discovery of other therapeutic options to treat AD is still imperative.

7.3.2 IL-9 is a potential initiator of AD

IL-9, an enigmatic cytokine, has been reported in several gene-wide and clinical studies to be associated with atopic diseases; in particular AD [236, 237] but the specific role of IL-9 in AD has yet to be elucidated. In my MC903 model, there has been considerable evidence to suggest a role for IL-9 in AD as IL-9 blockade resulted in a substantial amelioration of AD. Interestingly, the early impact of IL-9 blockade suggests that this cytokine contributes to the initiation of AD. However, IL-9 blockade did not affect the numbers of basophils or ILC2s (figure 4.10), indicating that the efficacy of this cytokine is independent of these two innate cells. I have demonstrated that the concomitant blockade of eosinophil function as well as IL-9, using a combination of resorcinol and anti - IL-9, yielded the greatest reduction in AD phenotype. However, this was only slightly more effective than treating with anti - IL-9 alone. The experiments outlined in chapter 6 were my attempt to determine which cells were the source of IL-9 in the skin, in the MC903 model of AD. This knowledge would have determined the precise contribution made by IL-9 in the AD inflammatory cascade. Unfortunately, neither confocal, ICS nor FACS sorting and RT-qPCR, provided me with a definitive source of IL-9. Based on my experiments, mast cells (figure 6.2), ILC2s (figure 6.7), CD4+ T cells (figure 6.3), keratinocytes (figure 6.4), fibroblasts (figure 6.7C & 6.8) and eosinophils (figure 6.7B & 6.8) did not appear to be the source of IL-9 in my model. Disconcertingly, the ICS expression levels of IL-9 did not corroborate the expression levels detected by the confocal staining. I did observe

that fibroblasts were capable of producing small amounts of IL-9 in vitro upon direct MC903 stimulation (figure 6.5), while rTSLP had no additive effect. However, I could not detect il9 mRNA in FACS sorted fibroblasts (figure 6.8). Altogether, this data suggests that a non-immune cell (CD45-), upon stimulation of MC903 in vivo is producing IL-9 and exerting its effects either directly or indirectly on TSLP [317] (figure 4.8), and indirectly on eosinophils [343] and mast cells [233, 236] in the skin (figure 4.7).

There was no change in the level of IL-9 expression in MC903-treated TSLPRKO mice compared to the WT (figure 4.2) observed by ELISA. Moreover, when mice were co-treated with MC903 and anti – IL-9 for 14 days (which is the peak of TSLP) this led to significant reduction in TSLP in the serum (figure 4.8). The literature has always suggested that TSLP is one of the first initiators of skin inflammation [183, 198] and data from figures 4.2 and 4.8, described above, proposes a novel role of IL-9 as an initiator of skin inflammation. I would hypothesise that IL-9 is produced either prior to TSLP or at the same time. In order to further verify the role of IL-9 in this model, IL-9 fate reporter mice [229] should be utilized and this would help in defining the source of IL-9 and confirm the contribution of this cytokine to AD.

7.3.3 CRTh2 antagonism could potentially ameliorate AD pathology

Prostaglandin D2 (PGD2) has been described as a potent chemoattractant for eosinophils, detected in the early phase of allergy [177]. PGD2 is specifically bound

to an orphan receptor CRTh2 [153] and is produced by activated mast cells and ubiquitously present on basophils and eosinophils [150, 151]. Notably, PGD₂-CRTh2 system has been implicated to have an essential role in chronic skin inflammation [154, 241]. We sought to block eosinophil recruitment by PGD₂ into the tissue. To address this, the Ferrier Research Institute synthesized a small molecule CRTh2 antagonist which had already shown efficacy in a mouse model of allergic rhinitis [252]. Remarkably, CRTh2 antagonism proved to be efficacious in treating AD in the MC903 model (figure 4.12). The CRTh2^{ant} was able to impressively reduce inflammation, TEWL, acanthosis, eosinophilia and mastocytosis in the MC903 AD model. Surprisingly, analysis of cytokine expression following CRTh2 antagonism revealed this axis was exerting its activity independent of cytokines (TSLP, IL-4 and IL-9) in this MC903 model. There are many CRTh2 antagonists that have passed through safety and efficacy trials for the treatment of eosinophil-driven diseases such as asthma [344] and EOE [345] and these hold promise for these patients with allergies [87]. Furthermore, Wambre *et al* have recently described a pathogenic Th2 subset of cells in allergic individuals that had a high expression of CRTh2, thereby suggesting that CRTh2 antagonism may be a mechanism worth exploring for treatment of allergic diseases [346]. In comparison to the prophylactic treatment (from day -1) of IL-9 blockade, the CRTh2 antagonism in the MC903 model was conducted therapeutically (from day 5). Thus, CRTh2^{ant} represents an additional treatment of AD.

7.4 Compromised skin barrier during AD may trigger the allergic march

This MC903 model was initially developed to study the allergic march, however due to time constraints of this PhD, I was unable to do so. This is the focus of current and future investigations within our laboratory.

Individuals that develop infantile AD between birth and two years of age are greatly predisposed to develop other atopic diseases (e.g. allergic rhinitis and asthma) during their childhood or later in their life. This disease progression is a phenomenon known as the allergic march [45, 47]. Many cross-sectional and longitudinal studies have demonstrated AD as the first step of the atopic march [44, 46, 347-350]. Kelleher *et al* elegantly reported that skin barrier dysfunction (measured by abnormal TEWL) in neonates was a prediction for the development of food allergy by the time these babies were infants [48]. The skin epidermis acts as a biosensor and a protective layer to the external environment. During AD, it is the defective skin barrier that has been thought to be the primary mechanism for the development of AD [44, 334, 351]. The current paradigm for the progression from AD to the allergic march is that the loss of barrier integrity, allows penetration of foreign antigens into the epidermis which possibly activates the antigen presenting cells (APC), specifically the Langerhans cells in the epidermis to promote Th2 skewing [49]. Another possible explanation could be that DC expansion leads to the activation of ILC2s [91], thereby promoting either a direct or indirect production of Th2 cytokines such as TSLP, IL-

13, and IL-4 [194, 277, 352]. Furthermore, IL-9 and IL-33 have been well-known for their association with food allergy, in particular peanut allergy. These cytokines have also been shown to be present at increased levels in paediatric patients with AD [114, 353].

AD pathogenesis includes; an increase in TEWL (both in lesional and non-lesional skin), a reduction in skin lipids and a proliferation of keratinocytes [41, 50]. Studies have verified that mutations or loss of the filaggrin gene (FLG), a crucial protein that maintains the integrity of the epidermis, results in AD [354]. Thus, children with AD are at higher risk of developing food allergies due to a weakened skin barrier [355, 356]. It has been reported that more than 50% of patients with moderate to severe AD develop food allergy by 1 year [357]. As a consequence, to the environmental insult, the keratinocytes produce the epithelial cytokine TSLP. There have been several links between keratinocyte-derived TSLP in triggering AD pathogenesis and resulting in the development of asthma [190, 352]. A review concluded approximately 70% of patients with severe AD developed asthma and/or allergic rhinitis later in life and those asthmatics with FLG mutations had more severe asthma exacerbations [358]. Interestingly, patients with Netherton syndrome which is caused by a mutation in *SPINK5*, have high expression levels of TSLP from both the keratinocytes and the airway epithelial cells. *SPINK5* encodes for a serine protease inhibitor Kazal type 5 and this protein is responsible for regulating protease activity and pH in the skin, thereby maintaining epidermal barrier integrity [359]. These individuals have recurrent AD, higher IgE levels, asthma and many of these individuals present with food allergy [191, 192]. This sequential acquisition of different allergic diseases has also been reported in patients with peeling skin syndrome 1 and SAM syndrome. In both these

conditions there is a mutation in the corneodesmosin (*CDSN*) and desmoglein 1 (*DSG1*) genes respectively [43, 360]. The *CDSN* and *DSG1* both form integral parts of the desosomes in the skin which are responsible for connecting the epidermal cells to the cytoskeleton. Once there are mutations in these genes the cell to cell adhesion is lost and the epidermal barrier is compromised [361]. Murine models have drawn attention to the fact that TSLP may provide the molecular link between AD and asthma [194, 362] and AD and food allergy [356]. Zhang *et al* used MC903 to induce AD and when these same mice were concomitantly sensitized with intraperitoneal ovalbumin (OVA), which resulted in these mice developing experimental asthma [194]. Nakajima *et al* have shown that Langerhans cells, in response to TSLP signalling, induce the production of antigen-specific IgE during epicutaneous sensitization to food allergens [363]. A study by Noti and colleagues modelled the allergic march from the skin to the gut. This study showed increased TSLP elicited basophils in promoting Th2 cytokines in the skin. In addition, it was reported that the TSLP- basophil axis was key in the regulation of intestinal inflammation [356].

Cumulatively, these studies strongly indicate that epidermal-derived TSLP is the key in orchestrating the allergic march. Multiple lines of evidence have suggested that increased TSLP in the skin results in AD and a Th2 inflammatory response. During this AD phase, there is an accumulation of Langerhans cells in the epidermis and innate cells in the dermis namely basophils, and ILC2s. These events result in setting up the allergic inflammatory environment including, the skin barrier dysfunction (allowing for easy access for other foreign antigens), the production of IL-33, IL-13,

IL-4 and higher IgE levels [194, 352, 356, 363]. Hence, the environment is set for the development of other allergic diseases namely, asthma and or food allergy [45, 47].

Finding specific targets involved in AD is not only beneficial for the treatment of AD but these targets could also be used to halt the allergic march. The model of MC903, which this thesis describes, is an excellent platform to try and model other allergic diseases. The duration of MC903 treatment in my model allows the systemic levels of TSLP to decrease by the peak of inflammation, thereby ensuring AD phenotype will be established and at this stage allergy at a peripheral site could be induced. It would be particularly interesting to induce airway inflammation once AD has been established using the MC903 model and compare the immune response across the skin and lung tissue.

7.5AD murine models; the benefits and the caveats.

A plethora of murine models of allergic skin inflammation have contributed to our current knowledge of the immune cells and cytokines involved with AD [276]. However, these models are accompanied by certain caveats which include a mechanical breach in the barrier by tape stripping, a presentation of skin inflammation that does not necessarily reveal all the hallmarks of AD. The tape stripping is an artificial method used to mimic itching in patients [277]. Itching is a vital clinical

symptom of AD which is concurrent with the inflammation in the skin, therefore therapeutic interventions are targeted to reduce itch [364]. In order to achieve a more organic skin barrier breach and to be able to determine how to restore this; studies are using chemical compounds to induce itch such as TMA [289]. Itching events are defined as a deliberate scratching of the ear using the paws [34] and this is recorded and quantified in mouse models of skin inflammation. The barrier dysfunction that occurs during the MC903 model is gradual compared to the artificial mechanical injury induced by tape stripping in other models. MC903-treated mice scratch their lesions over time enabling itching intensity to be measured. It is extremely useful to have a longer model when determining the kinetics of the accumulation of inflammatory cells and cytokines involved as this helps us determine when the peak of each of these effectors occur. Furthermore, this MC903 model has been evaluated for all the clinical hallmarks of AD i.e. inflammation, TEWL, acanthosis, the presence of spongiosis and hyperkeratosis, itch as well as increased levels of type two cytokines. It is imperative that a distinction is made in the murine models of skin inflammation whether the phenotype that manifests is actually AD or psoriasis or merely skin inflammation.

The spontaneous murine models of AD are good tools as they usually present with the clinical phenotype of the disease [276] for example the NC/Nga mice, and the K5 TSLP [195]. These NC/Nga mice, were initially established as an inbred Japanese strain [51], have exhibited immunological alterations that closely resemble patients with AD [51, 365]. However, the limitation of these models is the low rate of AD-like lesion development, or a late onset of AD. In the NC/Nga mice the onset is anywhere

between 8-17 weeks and these mice lose their phenotype if moved to specific pathogen free (SPF) conditions. This makes generating data and reproducibility difficult. The K5TSLP mice are induced by a tetracycline, doxycycline, to overexpress TSLP under the control of keratin 5 promoter in the skin [195, 197]. These mice develop skin inflammation following 2-3 weeks of doxycycline treatment [197]. Spontaneous manifestations of disease also run the risk of AD presenting to different extents in these mice. For example, you may have one mouse which presents with mild AD, and another with more severe AD [197, 276, 285]. These mice have been used extensively to dissect vital components of the pathophysiology of AD in patients rather than to assess potential therapeutic targets [366]. Hapten-induced AD models have the advantage of being highly reproducible, and these haptens such as 2,4,6-trinitrochlorobenzene (TNCB) or oxazolone (Ox) can be employed using different strains of mice [283]. Interestingly, this allows for the study of various types of inflammation and potentially reflects the heterogeneity in the way AD is clinically presented. Initially, TNCB was topically applied to BALB/c mice, which intrinsically skew to a Th2 phenotype [367]. Surprisingly, when Ox was employed in BALB/c mice it induced a mixed profile of Th1 and Th2. After repeated application of Ox in C57Bl/6 mice (intrinsically skewed to a Th1 phenotype) there was a distinct Th2 profile. This enables the different clinical manifestations of skin inflammation to be explored. These experiments were reproduced in several different labs and haptens were often applied on the ears of mice. This was due to the lack of hair on the ears which made application easier and avoided the need for shaving and causing damage to the skin surface. Furthermore, measuring total ear thickness became a reliable parameter of inflammation [368, 369].

The simple and less complex structure of the murine epidermis in comparison to human epidermal skin underplays the significant contribution that the epidermis plays in AD. There is a large interplay between the cellular components of the epidermis i.e. the tight junctions, epithelial cells, and the immune cells. Notably, data derived from AD mouse models have been extremely beneficial to our current understanding of the disease, however this data needs to be interpreted carefully and with these caveats in mind. The MC903 model was carefully chosen to improve on the current murine representation of atopic dermatitis. The contribution of this model is elaborated in greater detail below (7.6).

7.6 The contribution of this MC903 model to the understanding of AD

The vitamin D analogue, MC903, also known as Calcipotriol, is frequently utilized to treat patients with psoriasis. I was especially curious as to the reason a drug that is effectively treating psoriasis patients is capable of inducing AD in mice. After enlightening discussions with dermatologists, it was revealed that there is a cohort of psoriasis patients who develop AD lesions following their treatment with Calcipotriol. This adds to the validity of this murine model of AD. The method of action that has been characterized for MC903 activity is its association with vitamin D receptor (VDR). MC903 binds to the VDR and the retinoid X receptor (RXR) and these two receptors form a heterodimer. This RXR/VDR heterodimer that forms, induces activation of the

TSLP gene in murine skin [89, 199]. This topical model is similar to the hapten models, and allows the delineation of cell types and cytokines over time. There have been several other studies employing the MC903 model, however they differed in the duration of treatment, the concentration of MC903 used [1, 89, 106] and some reported transient involvement of cytokines as well as strain differences [90, 91]. I set out to establish a model using a long treatment schedule (treatment every alternate day for 19 days), and my primary goal was to explore a broad range of cell types and cytokines. The fact that the MC903 model was previously reported to be independent of T and B cells [89], I focused primarily on innate cell types. To my knowledge, all the AD clinical parameters (spongiosis, hyperkeratosis, acanthosis, TEWL, itch and immune dysfunction) have not been characterized in the MC903 model using the same treatment regime. The peak of inflammation in my model of MC903 (a concentration of 1nmol) was at day 19 and this specific model revealed all the clinical parameters of AD as well as the immunological changes that recapitulate the human condition. This 1nmol of MC903 does induce AD in both BALB/c and C57Bl/6 mice however I have conducted all the experiments in C57Bl/6 mice as this would be the most beneficial when conducting intervention strategies and using knockout strains. Detailed observations of the epidermal changes were made which included increased TEWL, hyper-proliferation of the keratinocytes, acanthosis (epidermal thickening), the presence of spongiosis and hyperkeratosis. Beyond the epidermal assessment covered in this thesis, it would be good to measure the levels of the tight junctions in the MC903 model, in particular claudin 1. Tight junctions are integral proteins that anchor adjacent keratinocytes to the stratum granulosum layer of the epidermis and are aberrant during AD [370, 371]. A dysfunction in the tight junctions result in a

dysregulation in the barrier of the stratum corneum [372]. Moreover, the length of this AD model allowed the discrimination between cells and cytokines that were involved in the initiation phase (e.g. IL-9) compared to the cells that were involved in the effector phase (e.g. eosinophils). TSLP proved to be a potent initiator of the inflammatory cascade and peaked following day 14 of MC903 treatment but there was a decrease in TSLP levels by day 19 (endpoint). This role of TSLP, as demonstrated in earlier chapters, emphasizes the relevance of this particular MC903 model.

Notably, whenever I employed the MC903 model in either cytokine knockout mice (e.g. IL-4KO or STAT6KO) or MC903 in conjunction with blocking antibodies (e.g. MC903 + anti - IL-9), we always obtained this intermediate phenotype. In most situations, there was a partial reduction of ear thickness and the most profound decrease was seen in the STAT6KO experiments. There are two possible explanations for this; 1) in the absence of one cytokine or cell type there are others that are recruited to maintain inflammation, 2) the intervention strategies have reached a certain threshold and this is the best reduction (in terms of inflammation) that will be obtained. If the latter is the case, then the pertinent question that follows is, is this intermediate phenotype adequate to ameliorate AD in patients? The most debilitating symptom of AD is the itch that patients experience as this leads to subsequent skin infections [6]. The skin barrier dysfunction and water loss both contribute to the patients' uncontrollable itch. Thus, the key clinical parameters of AD that potential treatments need to focus on is reducing itch and TEWL.

AD is defined as a chronic relapsing and remitting condition with intense itching. During patient flares of inflammation, the keratinocytes rapidly proliferate and this leads to the epidermal hyperplasia seen in AD skin lesion [5]. The pathogenesis of chronic itch in AD commences once AD pathology manifests, chronic inflammation follows, which leads to hyper-proliferation of keratinocytes [57] which in turn promotes further barrier dysfunction [58]. At this stage, large amounts of collagen fibers are deposited and the skin undergoes fibrosis [60] which results in activation of neuropeptides such as substance P [63] thereby leading to chronic itching [34, 62]. Unfortunately, from here the patient enters a vicious cycle of chronic inflammation, fibrosis, intense itching and subsequent skin infections [62, 312]. Upon evaluation of these lesions the histopathology reveals spongiosis (intercellular oedema-depicted in figure 3.3) and tissue remodelling (figure 5.5) [11, 24, 61]. This spongiosis is a defect that becomes more severe as the architecture of the epidermal tissue is disrupted both during skin inflammation and remodelling [56]. This eventually leads to the leathery appearance of the skin known as lichenification [9]. Accordingly, I felt understanding the cellular and molecular links between the phases of inflammatory, recovery and remodelling would be beneficial for a more 'in-depth' analysis of AD and most notably control chronic itch. In my MC903 model, I observed that after disease was established (at day 19), and mice were set aside, resolution of inflammation occurred after 7 days. During this time, there was a marked increase in collagen deposition which peaked after a 14 day recovery period. Interestingly, during this recovery period inflammation, TEWL, acanthosis, TSLP levels and eosinophils were severely reduced. The TSLP levels would suggest that it was not responsible for the fibrosis in the MC903 model. Interestingly, while eosinophil numbers were

seen to decrease dramatically in the recovery mice, there were still a small number present in the tissue. It would be worthwhile investigating if these smaller numbers of eosinophils would play a part in fibrosis in the skin. This could be assessed by employing the MC903 AD model in the iPHIL mice and comparing recovery in both an eosinophil sufficient and deficient context. Both TGF β and IL-13 have been associated with remodelling of the airways [314, 373] and IL-13 and periostin [312, 374, 375] have been described as pro-fibrotic factors in AD. Furthermore, IL-31 has been reported to be driving skin remodelling in a murine model of skin inflammation [58]. Therefore, it is possible any of these above-mentioned factors may be crucial to fibrosis and remodelling of the tissue in MC903-treated mice. This may enable us to develop targeted-therapy to either control skin inflammation or fibrosis or both, thereby alleviating chronic itch in AD patients.

7.7 AD, the road to precision medicine

AD is a particularly difficult disease to treat as it displays a wide spectrum of phenotypes and there is considerable heterogeneity [66]. Patients with AD comprise those that develop it from birth until their childhood [47], those children who proceed to develop food allergy [355, 356] or asthma later in life [349], and those patients who persist with only AD throughout their lifespan [67]. In terms of the immunological phenotype, many AD patients display IgE-mediated sensitization [376] while others possess genes associated with epidermal barrier dysfunction [11] e.g. FLG2 [377]. Furthermore, while the use of Dupilumab to treat patients with moderate to severe AD was successful in many patients there was still a portion of AD patients that did not respond to this treatment [85, 86]. Recently the field of skin allergy has begun to focus on the role of ILC2s in driving the pathogenesis of AD [378, 379], by promoting a potent type two immune response [90, 378, 380]. This MC903 model is independent of T and B cells [89, 199], however there is an upregulation of ILC2s and a strong type two inflammatory response, which is seen by the increased levels of IL-4, IL-9, TSLP and the accumulation of eosinophils (chapter 3 & 4). Therefore, this model demonstrates that AD skin pathology can occur in the absence of T cells which could have important implications for the treatment of AD.

Thus, a better stratification of this skin condition is required to ensure that there are treatment options for all patients. Accordingly, this makes AD perfect to benefit from precision medicine [381]. This would entail a tailored approach to treat subgroups of patients with AD and possibly classify them based on a panel of biomarkers. Recently, a distinct pathogenic subset of human Th2 cells were identified and this

study revealed these cells were unique to atopic individuals [346]. Accordingly, many of the effectors that this thesis has highlighted such as eosinophils and IL-4/IL-13 axis are highly desirable candidates to serve as biomarkers for AD subtypes. Indeed, this would allow us to distinguish AD patients who have a Th2 associated immune response [382] compared to a Th1 immune response and therefore enable them to receive the appropriate treatment. Previous studies have proposed TEWL, as predictive biomarker in new-borns at high risk for AD [48]. Similarly, it has been discussed that TSLP [362], and FLG [13, 358] would determine predisposition for the development of AD and subsequently the allergic march. Understanding the specific manifestation of AD would certainly lead to optimum treatment options for patients. Further exploration of the targets implemented in this study, in particular, eosinophils could potentially be of diagnostic and therapeutic advantage (Figure 7.1).

Figure 7.1 Proposed schematic of an inflammatory pathway in the MC903 AD model

Topical application of MC903 causes the keratinocytes to proliferate, and produce TSLP. The inflammatory pathway induced by MC903 is T cell independent. TSLP acts on the basophils and ILC2s to produce IL-4 and IL-13 respectively, and in a STAT6 dependent manner eosinophils are recruited into the tissue via eotaxin. There is potentially an epithelial cell producing IL-9, upon MC903 stimulation, and this leads to mast cells and eosinophil recruitment. TSLP has either an in direct or a direct effect on IL-9 and/or eosinophils. The events in this inflammatory cascade results in the atopic dermatitis pathology and clinical phenotype.

7.8 Future perspectives

I believe this MC903 model can be beneficial in contributing to the field of allergic disease, in particular to the form of AD that is T cell independent. This model resembles a form of AD that is driven by ILC2s and presents with a strong type two inflammatory response. Furthermore, it can be utilized to screen potential targets for the prevention and treatment of AD. Exploitation of this MC903 model can help in the understanding of the allergic march, by moving sensitization of the skin to another barrier site such as the lung. Promising therapeutic options that this thesis has provided are eosinophils and IL-9. However, further work is required to dissect the specific inflammatory pathway of this model prior to its clinical translation. Importantly, the source of IL-9 needs to be determined by employing this model in IL-9 fate reporter mice [229]. Exploration of the skin recovery and remodelling events can also be studied using this MC903 model and the roles of TGF β , periostin [374, 375] and IL-13 need to be determined [314]. Furthermore, a vital part of the MC903 inflammatory cascade that should be uncovered is whether eotaxin is responsible for recruiting eosinophils into the skin in a STAT6 dependent manner. Another fundamental biomarker that I have not investigated is histamine. Histamine and its 4 receptors are expressed on the skin. Both the secretion of histamine from Th2 cells, the responsiveness to histamine by Th2 cells have been well described [383, 384]. This included T cells, basophils, mast cells, and eosinophils [385-387]. Recently, a lot of attention has been given to histamine 4 receptor (H4R) [388]. Notably, H4R has been shown to mediate Th2 inflammation and anti - H4R treatment has proved efficacious in reducing inflammation and pruritus in experimental models of AD [389,

390]. The knowledge from this thesis could be further elucidated in AD patient studies to determine if eosinophils, IL-9, STAT6, and CRTh2 could potentially be a part of the biomarker panel for AD.

7.9 Key findings

- The 1nmol MC903 model is clinically relevant and depicts the hallmarks of AD namely: itch, skin barrier dysfunction, spongiosis, hyperkeratosis and acanthosis. It is a presentation of AD that is non-T cell dependent and has a strong type two inflammatory response.
- There is an accumulation of mast cell, eosinophils, basophils, and ILC2s in this MC903 AD model.
- To our knowledge, this is the first model to recapitulate the involvement of
- IL-9 in AD pathogenesis, as seen in patients.
- Eosinophil depletion in the MC903 model, resulted in amelioration of disease, most notably itch. Therefore, making eosinophils potentially attractive targets for the treatment of AD.
- Eosinophils are the effector cells that contribute to AD pathology work in an EPO dependent manner.
- The PGD2-CRTh2 axis was revealed to be vital to the MC903 model, as such CRTh2 antagonists are additional targets for the treatment of AD.
- STAT6 signalling (the IL-4/IL-13 axis) is critical in the MC903 AD inflammatory pathway.

8 Appendix

This includes the gating strategy for the following cell subsets;

- A. Eosinophils (CD45+, CD11b^{high} , SiglecF+)
- B. ILC2s (CD45+, CD90+ , CD3-, lin neg (B220 NK1.1), CD2-, ST2+)
- C. Fibroblasts (CD45-, CD90+ , C326-) CD326 aka EPCAM
- D. Basophils (CD45+, FcεRα+, c-kit-, SiglecF-, CD49b+)

FACS Gating Strategy

A) Eosinophils

B) ILC2s

continued....

...ILC2s

C) Fibroblasts

D) basophils

continued..... basophils

basophils
(CD45⁺, FCεRα⁺,
CD49b⁺, c-kit⁻,
SiglecF⁻)

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