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**ENVIRONMENTAL CONDITIONS
CAN HAVE A PROFOUND EFFECT
ON THE TRAJECTORY OF DISEASE
IN THE *maflg^{ft}/maflg^{ft}* MODEL OF
SKIN BARRIER DEFICIENCY AND
ATOPIC DERMATITIS**

A thesis submitted for the degree of PhD

2012

Caoimhe Máire Róis Fahy

TRINITY COLLEGE DUBLIN



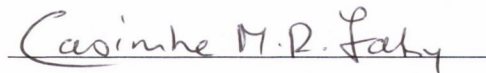
Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and is entirely my own work.

It is the work of the candidate except where clearly indicated. In particular, murine lung function assessment and bronchoalveolar lavage, were accomplished by Mr Sean P.

Saunders and Dr Hendrik Nel, respectively. In addition, the cell culture and bronchoalveolar lavage cytospin work detailed herein were undertaken in a collaborative manner with Dr Sylvie Amu and Dr Emily Hams respectively.

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Caoimhe M.R. Fahy

Summary

The prevalence of atopic disease has increased in recent decades. The hygiene hypothesis postulates that cleaner environments contribute to this observed increase in atopy. The cutaneous manifestations of atopic dermatitis (AD) are thought to be the beginning of the atopic march. Approximately one third of patients with AD go on to develop allergic rhinitis and half will develop asthma. It is recognised that epidermal barrier dysfunction is a causal factor in the pathogenesis of AD. Loss-of-function mutations in the gene for the epidermal protein, filaggrin, convey the strongest genetic risk for AD. It has been shown that the flaky tail (*maflg^{fl}/maflg^{fl}*) mouse is a model of skin barrier deficiency.

This study examines the effects of differing environments on skin barrier deficiency. *maflg^{fl}/maflg^{fl}* mice were bred in 2 specific-pathogen-free housings; individually ventilated (IVC) and conventional open (CON) cages. Controlled air delivery in IVC was the only difference in husbandry. A group of *maflg^{fl}/maflg^{fl}* mice were moved at 1 week from CON to IVC facility. A group of *maflg^{fl}/maflg^{fl}* mice were administered systemic antibiotics during the 1st 4 weeks of life. Wild type mice bred in both environments were used as controls. Clinical dermatitis scoring, lung function tests, skin/lung histology, and antibody ELISAs and spleen and skin-draining lymph node cell cytokine expressions were recorded at 1, 4, 8, 20 and 32 weeks.

The *maflg^{fl}/maflg^{fl}* mice in the IVC and CON-IVC groups had significantly less severe phenotypes in comparison with the *maflg^{fl}/maflg^{fl}* mice in the CON group. Total IgE was higher in the CON group compared to the IVC and CON-IVC groups.

Lung compliance findings indicated the development of a restrictive pattern in the *maflg^{fl}/maflg^{fl}* mice compared to their WT controls. There were reduced cutaneous and lung histological inflammatory changes in the *maflg^{fl}/maflg^{fl}* mice IVC, CON-IVC and CON A/B groups in comparison to the *maflg^{fl}/maflg^{fl}* mice in the CON group.

This study suggests, that in the context of murine skin barrier deficiency, environmental conditions have an important influence on the development and severity of cutaneous inflammation. The research implies that such conditions will also affect systemic spontaneous atopy with long-term consequences for the lung phenotype in skin barrier deficiency. The thesis explores the interaction of the environment with the skin barrier and also highlights how altering the microbiome can influence and increase spontaneous atopy. The research discussed in this thesis illustrates how the *maflg^{fl}/maflg^{fl}* mouse is a robust model of AD and is a useful tool for future research into the pathogenesis and treatment of this disease.

**Environmental conditions can have a profound effect
on the trajectory of disease in the *maflg^{ft}/maflg^{ft}*
model of skin barrier deficiency and atopic dermatitis**

Caoimhe M.R. Fahy

Trinity College Dublin, PhD thesis, 2012

Abstract

Atopic dermatitis (AD) is one of most common skin disorders seen in childhood. It is recognised that epidermal barrier dysfunction is part of the complex aetiology. The flaky tail (*maflg^{ft}/maflg^{ft}*) mouse is a model of skin barrier deficiency with, among other genetic mutations, an analagous mutation to the loss-of-function mutations in the human *FLG* gene, the most robust genetic risk for AD.

The effects of different environmental conditions on skin barrier deficiency are investigated in this study. Two different environmental conditions; individually ventilated (IVC) and conventional open (CON) cages, were used to house *maflg^{ft}/maflg^{ft}* and control WT mice. Interventions involving altering conditions and antibiotic treatments were assessed with respect to phenotype severity. Clinical dermatitis scoring, lung function tests, skin/lung histology, and antibody/cytokine ELISAs were recorded at 1, 4, 8, 20 and 32 weeks.

This thesis dissects part of the complex aetiology of inflammation in AD. It illustrates the profound role that environmental conditions have on the severity of inflammation and distal consequences of skin barrier deficiency. The pathogenesis of AD is further explored by using antibiotic treatment with this model. Questions are raised with regards to the role of the microbiome in the aetiology and management of disease. The *maflg^{ft}/maflg^{ft}* mouse model is an invaluable tool for investigating the pathogenesis and treatment of the complex disease of AD.

Acknowledgements

It has been a privilege to begin my research career with this thesis under the supervision of Professor Alan D.Irvine and Professor Padraic G.Fallon. I feel very fortunate to have benefitted from having Prof. Irvine as a supportive, encouraging supervisor and mentor in clinical and academic dermatology. I am very grateful that he introduced me to this research. Prof Fallon's lab is a stimulating, supportive 'environment' in which to do research and I am so appreciative that I had the opportunity to work in Lab 1.06. I greatly appreciate that Prof WH Irwin McLean was a collaborative supervisor for this research and valued his input over the course of the project. Professor Dermot Kelleher was a very encouraging mentor before and throughout the course of the research.. I have learned so much from PhD training and am looking forward to always having research in my career because of my experiences in Trinity College Dublin.

I am so very grateful for all the support, guidance and encouragement given by my laboratory colleagues. I am very lucky to have worked with you and am very glad that to have made such good friends. Thank you to Seán Saunders, Dr Sylvie Amu, Dr Emily Hams, Dr Seán Barry, Dr Julia Fahel, Chris Cluxton, Dr Aoife Smyth, Dr Hendrik Nel, Bina Mistry, Graeme Ireland, Alan Nolan, and Rosie Fallon. Thank you to Nuala, my other colleagues in OLCHC and the patients and their parents in OLCHC, who taught me much about real life with AD. Thank you to the consultants who have provided the greatest support and encouragement to me - Dr Rosemarie M. Watson, Prof Louise Barnes, and Dr Patrick Ormond. Thank you also to Dr J.F. Lyons, Dr J.F. Bourke and Dr M. Murphy who were there at my first job – Dermatology intern in the SIVUH.

Thank you so very much to my husband Daithí Ó'Donnabháin for his loving support in everything, including the many weekends in TCD! Daithí is always there for me in every aspect of my life and was so encouraging for me, especially at the start of dermatology.

I will never be able to say thank you enough to my family. My parents, Aidan and Rosarie Fahy, have been so encouraging of me at every step in my education and career. Their support has been immense. It is unparalleled. My sisters, Dr Aodhnait Fahy and Cranait Fahy, like our parents, give amazing encouragement to always do my best, no matter what the task.

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Abbreviations

AD	Atopic dermatitis
AHR	Airway hyper-responsiveness
AMPs	Antimicrobial peptides
APOC	Apolipoprotein C
BAL	Bronchoalveolar lavage
BCA	Bicinchoninic assay
BSA	Bovine serum albumin
CASP	Caspase enzyme
CCE	Cornified cell envelope
CD	Cluster of differentiation antigen
Chr	Chromosome
CON	Conventional cage
DC	Dendritic cell
dH ₂ O	deionised H ₂ O
DMSO	dimethylsulfoxide
DNFB	1-fluoro-2,4-dinitrobenzene
Dp	<i>Dermatophagoides pteronyssinus</i>
EDC	Epidermal differentiation complex

ELISA	Enzyme linked immunosorbent assay
FCS	Foetal calf serum
FITC	fluorescein isothiocyanate
<i>FLG</i>	Filaggrin gene (human)
<i>Flg</i>	Filaggrin gene (murine)
GCR	Glucocorticoid receptor
GINIplus	German Infant Nutritional Intervention plus environmental and genetic influences on allergy development study
GM-CSF	Granulocyte-macrophage colony stimulating factor
H&E	Haematoxylin and eosin
HPFs	High powered fields
HRP	Horseradish peroxidase
IDECs	Inflammatory dendritic epidermal cells
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ISAAC	International Study of Asthma and Allergies in Childhood
IV	Ichthyosis vulgaris
IVC	Individually ventilated cage
kD	kiloDalton

KLK	Kallikrein
LCE	Late cornified envelope
LCs	Langerhans cells
LEKTI	Lymphoepithelial Kazal-type-related inhibitor
LISApplus	Life-style factors on the development of the Immune System and Allergies in East and West Germany plus the influence of traffic emissions and genetics
mAB	monoclonal antibody
MHC	Major histocompatibility complex
mRNA	messenger ribonucleic acid
MPO	Myeloperoxidase
MTB	Masson's trichrome blue
MWRH	Mid Western Regional Hospital, Dooradoyle, Limerick
NF- κ B	Nuclear factor κ B
NMF	Natural moisturising factor
NOA	Naruto Research Institute Otsuka Atrichia
OLCHC	Our Lady's Children's Hospital, Crumlin, Dublin
OVA	Ovalbumin
PARSIFAL	Prevention of Allergy – Risk Factors for Sensitization in Children Related to Farming and Anthroposophic Lifestyle
PAS	Periodic Acid Schiff

PAMPs	Pathogen associated molecular pattern molecules
PBS	Phosphate buffered saline
PFTs	Pulmonary function tests
PMA	Phorbol myristate acetate
PSR	Picro Sirius red
RANTES	Regulated on activation normal T cell expressed and secreted
R _L	Lung resistance
RNA	Ribonucleic acid
SCCE	Stratum corneum chymotryptic enzyme
SCORAD	Scoring atopic dermatitis
SE	Staphylococcal enterotoxins
SPF	Specific-pathogen-free
SPINK5	Serine protease inhibitor Kazal-type 5
SPPR	Small proline-rich region
TAB	hexadecyltrimethylammonium bromide
TCS	Topical corticosteroids
TCI	Topical calcineurin inhibitor
TEWL	Transepidermal water loss
TNF	Tumour necrosis factor
Th1	Type helper 1

Th2	Type helper 2
TLR	Toll-like receptor
TSLP	Thymic stromal lymphopoeitin
TSST	Toxic shock syndrome toxin
TV	Tidal volume
WT	Wild-type

Chapter 1

Introduction

Atopic Dermatitis

Introduction

Atopic dermatitis (AD), also known as atopic eczema, is a chronic relapsing inflammatory skin disorder and it is the most common type of dermatitis in childhood (Moffatt et al., 2005). The majority of patients receive the initial diagnosis in infancy or early childhood. Stereotypical features of AD include dry and itchy skin with a disease pattern characterized by a chronic nature and frequent relapses. However, the clinical picture at presentation can vary and factors such as a patient's age, ethnicity, duration and clinical course of disease, exacerbating environmental factors and infection can all affect the morphology, severity and distribution of the skin lesions. Wise and Sulzberger introduced the concept of AD in 1933 (reviewed in James *et al.* (James et al., 2006)) as a cutaneous aspect of an atopic diathesis. Prior to 1931, what was thought to be a nondescript itching was termed 'eczema' or 'prurigo diathésique de Besnier' by physicians as early as the 18th and 19th centuries (Barnes, 2010).

1.1.1 Epidemiology of atopic dermatitis

Understanding the epidemiology and prevalence of AD is important at an individual, national and international level and many studies worldwide have analysed this topic in depth. Atopic dermatitis affects approximately 13.2 % of children (13-14 years) in Ireland (Kabir et al., 2011) and up to 25 % of children in the UK (Shamssain, 2007, Kay et al., 1994). A considerable proportion of affected infants (45 %) develop AD

within their first 6 months, while up to 60 % of cases begin during the first year and approximately 85 % of cases develop AD before 5 years of age (Bieber, 2008).

Approximately 70 % of affected children have a spontaneous resolution of symptoms and signs before adolescence (Bieber, 2008).

There is significant variation in the prevalence of AD throughout the world. The International Study of Asthma and Allergies in Childhood, (ISAAC), Phase One (1992-1997), described the prevalence of symptoms of AD in 154 centres across 56 countries, using standardized epidemiologic techniques (Williams et al., 1999). This study conducted a survey on random samples of schoolchildren aged 6-7 and 13-14 years, on whether they had an itchy, relapsing skin rash in the skin folds, over the previous 12 months. When published in 1999, ISAAC Phase One showed that AD prevalence ranged from 0.6 % to 20.5 % of the study population (Williams et al., 1999). The children between 6-7 years who reported AD symptoms ranged in prevalence from less than 2 % in Iran to over 16 % in Japan and Sweden (Williams et al., 1999). Less than 1 % prevalence was noted in Albania, for the 13-14 year age group, ranging up to 17 % prevalence in Nigeria (Williams et al., 1999).

The prevalence of AD has been increasing within the past decades. Phase Three of the ISAAC study in 2009 explored this and demonstrated an increase in prevalence over the course of a decade suggesting that environmental factors may be an important regulator of disease expression in AD. In the Irish ISAAC study, the prevalence of AD in 13-14 year old Irish teenagers increased between 1995 and 2007 (Table 1.1) (Williams et al., 1999, Manning et al., 1997, Kabir et al., 2011). From a prevalence of 9.4 % (N=2,671) in 1995, this figure rose to 14.4 % (N=2,894) in 2002 (Kabir et al.,

2011). There was a slight decline in AD prevalence in 2007 (13.2 %, N=2,809) (Kabir et al., 2011).

Just as the ISAAC study, Phase One and Three, demonstrated geographic variability in AD prevalence, the 2003 National Survey of Children's Health in the US also showed a trend in geographic variability between east coast, central and southwestern states in the USA. Furthermore, within this study, while there was an 11 % national prevalence of eczema (AD) in children less than 18 years, urban living and Afro-Caribbean race were significantly associated with increased prevalence of AD (Shaw et al., 2011). In addition, third-level education in a household was associated with a greater prevalence of eczema (Shaw et al., 2011).

The epidemiology of AD reveals that it affects children across the world. The underlying factors for the differences in prevalence seen within and between countries are as yet not fully defined, however, environmental factors, industrialisation and urban living are associated with the rise in AD and atopic disease.

ECZEMA SYMPTOMS, –IRELAND*			PREVALENCE RATIOS	
Year	Prevalence (%)	Study number	Unadjusted (95% C.I.)	Adjusted (95% C.I.)
1995	9.4	2,671	1	1
1998	10.4	2,273	1.11 (0.93-1.33)	1.06 (0.89-1.27)
2002	14.4	2,894	1.54 (1.32-1.8)**	1.4 (1.16-1.69)**
2007	13.2	2,809	1.41 (1.2-1.65)**	1.31 (1.07-1.59)**

Table 1.1 Prevalence of symptoms of eczema among Irish schoolchildren aged 13-14 years, 1995-2007 (n=10,647). Exponentiated log regression coefficients were used to derive prevalence ratios. Poisson distribution computed adjusted prevalence ratios. Confidence intervals of 95% were analysed based on standard error estimates (Kabir et al., 2011).

*Adjusted simultaneously for sex; medications; pet exposure; current active smoking status alone; second hand smoke exposure alone; hay fever symptoms ever; clustering effect within schools; wheeze past 12 months; symptoms of severe asthma

**statistically significant in comparison to previous assessment year.

1.1.2 Clinical features of atopic dermatitis

Clinical features of AD tend to vary with age (Kunz and Ring, 2005). Manifestations which are common to all age-groups include pruritus, xerosis and erythematous lesions with active disease (James et al., 2006). In infancy, the face and scalp are usually the initial locations for lesions, which develop as erythematous macules evolving into small papulovesicular lesions with a tendency to ooze and form crusts (Kunz and Ring, 2005). This eruption can then progress to involve the neck, upper trunk, limbs and peripheral extensor surfaces. This is demonstrated in Figure 1.1.2.1, which shows images of a patient reviewed by the candidate in the Paediatric Dermatology Outpatient's Department, Our Lady's Children's Hospital, Crumlin, (OLCHC), Dublin. Vesicles on an erythematous background are apparent on the infant's cheeks (a), while excoriated flaking skin is also seen on the limbs (b). The clinical picture changes as the infant develops the capacity to scratch. At this point, lesions become more varied and poorly demarcated scaly, erythematous patches are present with linear and punctate crusted excoriations. Secondary infections occur following persistent scratching and rubbing (Kunz and Ring, 2005).



a



b

Figure 1.1.2.1 Infantile atopic dermatitis. Erythematous, flaking patches of active AD on the cheeks (a), forearms and lower limbs (b) of a 6 month old infant.
Source of image: Dr C.M.R. Fahy, Prof Alan D. Irvine, Department of Paediatric Dermatology, OLCHC, Dublin.

The classic flexural disease that is easily recognisable as AD begins from 2-3 years and progresses through childhood (Kunz and Ring, 2005). Typical lesions are often lichenified and indurated and involve flexures, the dorsal neck, and the dorsal aspects of the limbs. Figure 1.1.2.2 demonstrates 2 patients reviewed by the candidate in the Paediatric Dermatology Outpatient's Clinic in OLCHC, Dublin. The image highlights how the skin may become involved in a generalised pattern affecting the trunk and limbs and that in the 14 year old patient the limbs are particularly affected.

There is often a coexistence of different types of lesions at varied distributions on the body. The sites of affected skin alter as a child ages. The extensor and flexor aspects of the extremities become involved in disease as the frequency of involvement of the face and head decrease (Kunz and Ring, 2005). If there is persistent rubbing and scratching at affected chronic sites of disease, lichenification develops with accentuated skin furrows. This is highlighted in Figure 1.1.2.2 (b and c) which demonstrates lichenification at the anterior ankles of a 14 year old patient, with associated eroded excoriated skin at the peripheries of the lichenified plaques as well poorly defined erythematous, excoriated patches on the dorsal arms and elbows (Figure 1.1.2.2 (d and e)).

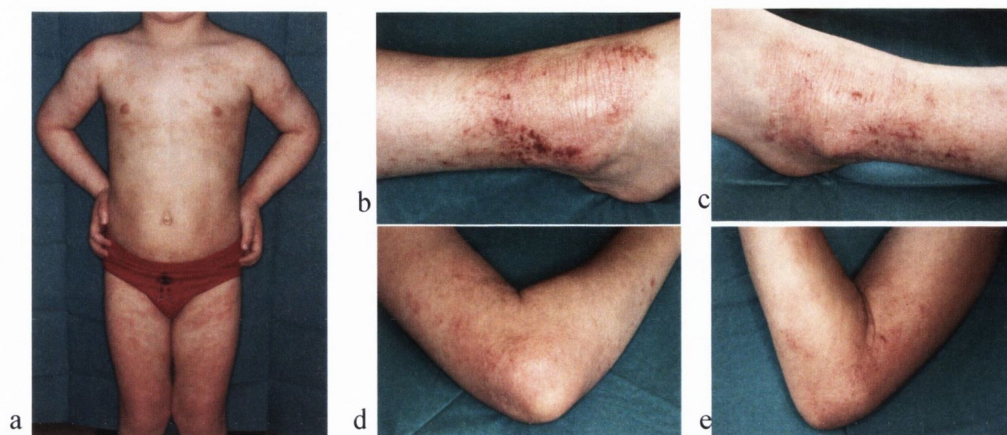


Figure 1.1.2.2 Childhood and adolescent atopic dermatitis. Photographs of affected locations of a 5 year old (a) and 14 year old (b – d). Source of image: Dr. C.M.R. Fahy, Prof Alan D. Irvine, Department of Paediatric Dermatology, OLCHC, Dublin.

In adolescents and adults affected with AD, lesions may occur as localised erythematous scaly papular exudative or lichenified plaques. These can be widely distributed and are shown by the patient reviewed by the candidate in the Dermatology Department Outpatient's Clinic in the Midwestern Regional Hospital (MWRH), Dooradoyle, Limerick (Figure 1.1.2.3 a and b). These photographs highlight excoriations on a background of poorly defined erythematous plaques on the back, neck and scalp of an adult patient. The adolescent phase involves increased scaling, microvesiculation, erosions and excoriations. Chronic hand dermatitis can particularly be a feature of AD in older affected adults (Kunz and Ring, 2005) as demonstrated in Figure 1.1.2.3c which shows that in particular the dorsal distal interphalangeal joints are subject to fissuring and erythema as well as lack of growth of nail cuticles. Typical flare factors for eczema include heat, sweating, stress, extremes of cold, external irritants and skin surface bacterial colonisation (Huang et al., 2009).

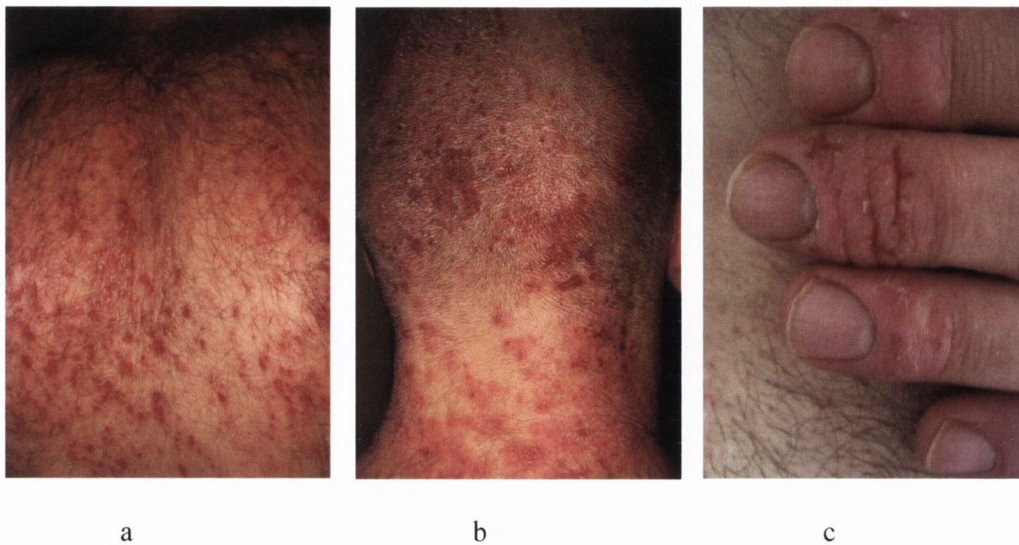


Figure 1.1.2.3 Adult atopic dermatitis. A male affected with AD is photographed on his back (a), neck and scalp (b), and hand (c). Source of image: Dr. C.M.R. Fahy, Dr B.Ramsey, Department of Dermatology, MWRH, Dooradoyle, Limerick.

1.1.3 Impact of AD

A cross-sectional study using the Children's Life Quality Index showed that AD had a greater impact on Health Related Quality of Life than cystic fibrosis, asthma, diabetes mellitus or epilepsy (Beattie and Lewis-Jones, 2004). This highlights the impact that AD has on an affected child's life and leading on from this, the quality of life of their parents and family overall.

1.1.4 Current management of AD

There are two approaches to the management of AD. One approach highlights the importance of the maintenance of skin barrier function (emollients and avoidance of detergents); the other places importance on the modulation of the cutaneous inflammatory reaction (reducing exposure to antigens and cutaneous or systemic anti-inflammatory drugs). The success of these two arms in the treatment of AD highlights the dual importance of skin barrier and immunology in AD. Emollient therapy is a large component of the maintenance of the skin barrier and enhancing its protective and preventative functions in treatment of AD (Bingham, 2005). Reducing exposure to obvious initiating factors such as increased indoor heating, poor ventilation, use of soaps and detergents are important basic steps that should be taken when managing AD (Cork et al., 2009).

The important role for anti-inflammatory therapy in the treatment of AD highlights the key role that immunology plays in the aetiology of the disease. These therapies often prove vital for stabilisation of skin surface immunology and improving eczematous skin lesions (Leung et al., 2004). As explored further below, anti-inflammatory treatments inhibit cytokine release by T cells, mast cells and other cells (Leung et al., 2004). They

decrease T cell activation, and reduce the numbers and activity of Langerhans cells (Leung et al., 2004). Inflammatory reactions decrease the skin barrier function by reducing production of filaggrin, so anti-inflammatory treatments may also indirectly improve barrier function (Irvine et al., 2011).

1.1.4.1 Topical corticosteroids (TCS)

Topical corticosteroids (TCS) accompanied by emollients have been the mainstay of the treatment of AD since their introduction in the 1950s (Bingham, 2005). They help control both acute and chronic skin inflammation (Leung et al., 2004). The anti-inflammatory effect of TCS is mediated through a cytoplasmic glucocorticoid receptor (GCR) in target cells (Leung et al., 2004). After ligand binding, the corticosteroid/GCR complex translocates to the nucleus where it binds to various transcription factors including nuclear factor (NF) - κ B to inhibit the transcriptional activity of proinflammatory genes encoding cytokines such as IL-1, IL-4, IL-5, IL-13, TNF α as well as to downregulate chemokines, monocyte chemotactic proteins, eotaxin and adhesion molecules (Leung et al., 2004).

1.1.4.2 Topical calcineurin inhibitors (TCIs)

Other topical anti-inflammatory agents include the macrolactam derivatives topical calcineurin inhibitors (TCI) such as tacrolimus or pimecrolimus that acts as a steroid-sparing agent (Grassberger et al., 1999). They inhibit inflammatory cytokine transcription in activated T cells and other inflammatory cells through inhibition of calcineurin (Grassberger et al., 1999). Topical calcineurin inhibitors do have the potential for local immunosuppression, however, clinical trials have shown no increase in

systemic or local skin infections (Kawashima et al., 1996, Alaiti et al., 1998, Fleischer et al., 2002).

1.1.4.3 Anti-infective therapy

Infection by *Staphylococcus aureus* (*S.aureus*) in AD is often secondary to high levels of *S.aureus* colonisation, thus bacterial growth suppression is considered an important treatment in AD (Huang et al., 2009). Antiseptic baths, with addition of diluted sodium hypochlorite to the bath water, greatly decrease the bacterial load, reducing the potential for infection from persistently increased colonisation by typically *S.aureus* (Huang et al., 2009). Weeping, crusting flares of AD are usual features of secondary bacterial infections. Intermittent oral antibiotic therapy or occasional use of prolonged oral antibiotic therapy may be necessary to reduce the number and severity of infective eczematous flares (Bingham, 2005).

1.1.4.4 Systemic therapies

Despite the usual success of topical therapies at gaining control of a patient's AD, there are a minority of patients for whom systemic therapy is a necessary next step in AD management (Bingham, 2005). Systemic approaches include steroids (for their immunomodulatory capacity explored above), as well as azathioprine, methotrexate, cyclosporine and recombinant IFN γ , which act as steroid-sparing immunosuppressants (Berth-Jones et al., 2002, Hanifin et al., 1993, Schmitt et al., 2010, Schram et al., 2012). Figure 1.1.4.4 show improvement in AD in a patient of the Department of Dermatology, MWRH, Limerick, Ireland. The resolution of his AD is shown in the example of his hands, which had reached a disabling level of severity of AD activity. Systemic therapy

with azathioprine (3mg/kg) was required when topical therapy alone was insufficient for disease control (Figure 1.1.4.4).



Figure 1.1.4.4 The effect of systemic therapy on AD. Progressive improvement in AD on the patient's hands after systemic azathioprine therapy (3mg/kg) was begun when topical therapy proved insufficient for adequate control of the patient's AD. Source of image: Dr. C.M.R. Fahy, Dr B.Ramsey, Department of Dermatology, MWRH, Dooradoyle, Limerick.

1.2 Aetiology of atopic dermatitis

The aetiology of AD was, for many years, associated with and attributed primarily to immune dysregulation, however, it is now widely established that the aetiology of AD is of a multifactorial nature (Elias and Steinhoff, 2008). It was thought that dysregulation of adaptive immune system was largely the cause of AD with T cell disruption, IgE synthesis, dendritic cell signalling dysregulation, mast cell signalling and hyperactivity (Leung et al., 2004). Treatment was thus directed towards resolving Th2-mediated pruritus and inflammation (Elias and Schmuth, 2009, Proksch et al., 2006). Although tendencies towards development of secondary infections and deficiencies in the skin barrier were recognised as being features of AD, it was assumed that these abnormalities illustrated the consequences of the immunologic dysregulation and were not causative factors (Seidenari and Giusti, 1995, Proksch et al., 2006, Sugarman et al., 2003, Chamlin et al., 2002, Baker, 2006, Taieb, 1999).

More recently, the importance of skin integrity in the pathogenesis of AD was emphasised by the association of loss-of-function mutations in the filament-aggregating protein (filaggrin) *FLG* gene with AD as discussed in more detail in Sections 1.3.1, 1.3.2, and 1.3.2.2. This indicated that epidermal barrier dysfunction is a primary aetiological phenomenon in AD rather than a consequence of disrupted immunology (Palmer et al., 2006).

Xerosis and an increased permeability barrier in the epidermis can drive inflammation, in the “outside-to-inside” theory on AD aetiology (Elias 1999). However, the “inside-to-outside” hypothesis, wherein increased inflammatory changes facilitate barrier breakdown in response to allergens and irritants has also been investigated (Leung 2004).

1.2.1 Immunology in the aetiology of atopic dermatitis

Dysregulation of innate and adaptive immune responses is a large component of AD (Bos, 2005). The skin's innate immune system includes keratinocyte-derived pathogen associated molecular pattern (PAMP) molecules, innate cytokines, the inflammasome and antimicrobial peptides (AMPs) (Oyoshi et al., 2009a). Damage-associated molecular pattern (DAMP) molecules are preformed endogenous molecules that are released following cell death and injury (Carta et al., 2009, Krysko et al., 2011). DAMPs induce pattern-recognition receptors (PRRs) that include TLRs which trigger recruitment of professional cells to clear the offending agent or promote immune activation (Carta et al., 2009). They also influence the function of cell types such as eosinophils, mast cell and neutrophils (Krysko et al., 2011). PAMPs such as Toll-like receptor 2 (TLR2) are vital for inducing a host defence sequence following recognition of organisms including Gram-positive bacteria, fungi and staphylococcal cell wall components (Oyoshi et al., 2009a). Gram-negative bacterial components are detected by TLR4 (Baker, 2006). The defensive sequence of events associated with PAMPs occurs via NF- κ B which leads to expression of proinflammatory cytokines such as Interleukin-1 (IL-1), TNF α , IL-12, AMPs and inducible enzymes in the skin (Baker, 2006, Oyoshi et al., 2009a). The inflammasome is a cytosolic protein complex, which regulates the activation of the enzyme caspase-1 that cleaves the proinflammatory cytokines IL-1 β and IL-18 into their active forms (Lamkanfi and Dixit, 2009). Expression of these innate immune cytokines by keratinocytes is upregulated following cutaneous mechanical injury such as excoriation which is a hallmark clinical feature of AD (Oyoshi et al., 2009a).

Increased keratinocyte cytokine production is another facet of the maladaptive immunology of AD (Oyoshi et al., 2009a, Akdis et al., 1999). Changes in the epidermal barrier can increase sensitivity to general irritants and thus proinflammatory cytokines are

formed. These include IL-1, IL-6, IL-8 and TNF α (Bos, 2005). Autocrine effects of the cytokines on keratinocytes include the upregulation of adhesion molecules which will bind leukocytes (Bos, 2005). Paracrine effects of the cytokines on papillary dermal endothelial cells increase adhesion molecules which then increases the presence of leukocytes such as monocytes and lymphocytes (Bos, 2005).

Cutaneous lesions of AD are characterised by activated CD4⁺ Major Histocompatibility Complex (MHC) Class 2 memory/effector T cells, eosinophils, mast cells, dendritic cell (DC) subsets and macrophages that may be coated with IgE (Oyoshi et al., 2009a, Leung et al., 1983, Leung et al., 1987). Th2 cells play an important pathogenic role in AD which is supported by the presence of blood eosinophilia and increased serum IgE levels in most individuals with AD (Oyoshi et al., 2009a).

Genetic factors are important in atopic dermatitis: concordance for AD is higher in monozygotic twins (77%) than dizygotic twins (15%) (Bieber, 2008). Several candidate genes involved in the regulations of IgE synthesis have been identified in AD. They include IL-4, IL-5, IL-12, IL-13 and granulocyte-macrophage colony-stimulating factor (GM-CSF). IL-4, IL-5 and IL-13 are produced by Type 2 helper T cells (Th2) and upregulate the production of IgE. Mutations in the promoter region of the lymphocyte-attracting chemokine RANTES and gain-of-function polymorphisms in the α subunit of the IL-4 receptor on Chr 16q12 have been found in patients with AD (Bieber, 2008). Another route for activating a Th2-dominated immune response is pruritus which causes an itch-scratch cycle (Oyoshi et al., 2009a). Spergel *et al.* demonstrated in a murine model that application of antigen to skin with a skin barrier defect caused scratching,

which then elicited a downstream Th2-response and skin inflammation with many features of AD (Spergel et al., 1998).

The Th2-inflammatory nature of acute skin lesions of AD is characterised by dermal infiltration of CD4⁺ T cells, eosinophils and increased cutaneous expression of Th2 cytokines including IL-4, with little IFN γ (Leung et al., 2004). Multiple studies report the involvement of IL-4 in the pathogenesis of cutaneous inflammation in AD (Baker, 2006, Chan et al., 2001, Kurahashi et al., 2008, Oyoshi et al., 2009a). Chan *et al.* suggested a pathogenic role for IL-4 in AD by demonstrating that keratinocyte overexpression of IL-4 in transgenic mice resulted in AD-like cutaneous lesions (Chan et al., 2001). Applying IL-4 to skin with a skin barrier defect, further disrupted the permeability barrier function of skin in a murine model of skin barrier deficiency (Kurahashi et al., 2008). IL-4 also impedes the enhancement of ceramide synthesis after barrier disruption (Kurahashi et al., 2008).

The immunopathology of the acute and chronic stages of AD skin lesions are distinct. Acute skin lesions have increased numbers of IgE-bearing Langerhan cells (LCs) and macrophages with eosinophils and a significantly greater number of IL-4, IL-5 and IL-13 expressing cells (Leung et al., 2004). In contrast, lesions from the skin of a patient with chronic AD have significantly fewer IL-4 and IL-13 expressing cells with greater numbers of IL-12 and IFN γ mRNA expressing cells (Leung et al., 2004). IL-12 and IFN γ are formed by Type 1 helper T cells (Th1) which suppress the production of IgE and stimulates production of IgG antibodies (Bieber, 2008) and this change from a Th2 to a Th1 driven pathology as AD progresses from acute to chronic forms is noteworthy. IL-18 contributes to this change towards Th1 polarization in chronic disease (Oyoshi et al.,

2009a). Polymorphisms of the gene encoding this cytokine have been postulated to promote an imbalance between Th1 and Th2 immune responses in AD (Novota et al., 2005). The switch from a Th2 response to a Th1 response is also thought to be reliant on the local formation of IL-12 from infiltrating eosinophils and/or inflammatory dendritic epidermal cells (IDECs) (Leung et al., 2004). It has been shown that allergen-induced skin lesions such as those aggravated by house dust mite have an initial responsive phase with IL-4 producing Th2 cells being the predominant cell infiltrate and then a subsequent phase characterised by IFN γ -producing Th1 cells (Leung and Bieber, 2003).

Th17 cells are essential for the first-line defence of the human organism (Eyerich et al., 2009). It is thought that a possible mechanism for defence against microorganisms by IL-17 in the skin, is the upregulation of antimicrobial peptides in keratinocytes (Liang et al., 2006). The role that IL-17-secreting Th17 cells, play in skin inflammation has been investigated in a number of studies (He et al., 2007, Eyerich et al., 2009, Oyoshi et al., 2009a, Oyoshi et al., 2009b, Boguniewicz and Leung, 2011). Extracutaneous sensitisation resulted in local and systemic Th17-driven response when mice were exposed to ovalbumin (OVA) (He et al., 2007). Filaggrin-deficient mice were shown to manifest Th17-driven skin inflammation and eczematous changes and there were higher levels of IL-17 production in the epidermis (Oyoshi et al., 2009b). Acute rather than chronic AD cutaneous lesions preferentially expressed IL-17 in a study of the differences in cytokine expression in acute and chronic AD (Toda et al., 2003). Eyerich *et al.* demonstrated that in an atopy patch test model, IL-17-secreting T cells infiltrate acute AD skin lesions and trigger β defensin-2 production by keratinocytes (Eyerich et al., 2009). Additionally, He *et al.* showed that extracutaneous sensitisation not only increased IL-17 cutaneous inflammation but also induced a systemic Th17 response to

antigen and resulted in airway inflammation, which was characterised by expression of IL-17 and an associated neutrophil infiltration after inhalation challenge (He et al., 2007).

1.2.2 Role of infection in the aetiology of atopic dermatitis

The skin plays host to multiple microbial communities. Grice *et al.* analysed 16S ribosomal RNA gene sequences from distinct skin sites of healthy humans to reveal that similar bacterial populations reside on physiologically comparable skin locations (Grice et al., 2009). While the skin microbiome does contain pathogens, commensal microbes provide direct benefit and also prevent fellow more harmful microbes from establishing residence (Kong and Segre, 2012). Resident microbes are found routinely on the skin and they re-establish themselves after disturbance (Kong and Segre, 2012). Transient microbes come from the environment and only persist on the skin for hours to days (Kong and Segre, 2012). Patients with AD have an increased susceptibility to bacterial, fungal and viral infections (Oyoshi et al., 2009a). Microbes are predicted to have a pathophysiological role in many dermatoses, which have a predilection for specific skin sites such AD, which is typically found in the antecubital and popliteal fossae (Grice et al., 2009). These body locations were shown to have similar ranges of microbes with better preserved community membership than structure (Grice et al., 2009). It has recently been shown that microbial communities at sites of disease predilection were very different in patients with AD than controls (Kong et al., 2012). Increases in the proportions of various bacteria were noted during disease flares, especially *S. aureus* (Kong et al., 2012). It is thus important to consider the skin microbiome when investigating AD and it's management. Because the environment can affect the viability of microbes it is also appropriate to consider environmental conditions in AD research.

The many structures and layers of the skin act as hosts to microbes that include commensal and pathogenic bacteria that have a role in human health and disease (Grice et al., 2008). The exact mechanism for increased colonisation in AD is not fully known but proposed ideas include the defective skin barrier, reduced levels of protective endogenous antimicrobial peptides and bacterial superantigens inducing the release of proinflammatory cytokines all further exacerbating AD (Huang et al., 2009, Ong et al., 2002). As outlined above, antimicrobial peptides such as β -defensins and cathelicidins are important in the skin defence against colonisation and infection. (Ong et al., 2002). However, it has been shown that the cathelicidin LL-37 and β -defensins are deficient in skin lesions from patients with AD (Ong et al., 2002). The levels of β -defensins expression are also inhibited by Th-2 cytokines such as IL-4 and IL-13 (Ong et al., 2002). These Th2-cytokines are well established to be increased in the skin lesions of AD and thus may further increase the susceptibility of AD to skin infections (Ong et al., 2002).

Both normal-looking and lesional skin in AD is extensively colonised by bacteria, in particular *S. aureus*, or fungi such as *Malassezia furfur* (Bieber, 2008). Such colonisation can lead to low-grade inflammation, leaving the skin even more susceptible to inflammatory and infective exacerbations (Bieber, 2008). Th2 cytokines such as IL-4 increase expression of fibrinogen and fibronectin receptors in atopic skin, which mediate adhesion of *S.aureus* to the stratum corneum (Cho et al., 2001). Cutaneous lesions of AD will flare under the influence of bacterial superinfection, mediated by the release of staphylococcal enterotoxins (SE) or superantigens such as SEA, SEB and toxic shock syndrome toxin (TSST)-1 (Baker, 2006). Correlation between staphylococcal superantigen-specific IgE antibodies and the severity of AD and serum IgE level has been shown (Mrabet-Dahbi et al., 2005). Superantigens add to an exacerbation of AD by activating mast cells, eosinophils, LCs and basophils (Oyoshi et al., 2009a).

The *Malassezia* yeasts are components of the normal human cutaneous flora, found in 75-80% of healthy adults (Yim et al., 2010). Colony-formation begins immediately in the early neonatal period with increased levels on areas of high sebum production (Yim et al., 2010). *Malassezia*-specific IgE antibodies and T cell reactivity to the organism is present in 30-80% of individuals with AD (Scheynius et al., 2002). The internalisation of *Malassezia* has leads to the development of proinflammatory and immunoregulatory cytokines favouring the initiation of a Th2 response (Baker, 2006). Using anti-fungal agents to treat patients who are mostly affected by AD on the head and neck areas has been shown both to reduce *Malassezia* colonization and to reduce the severity of AD lesions (Yim et al., 2010). This suggests that the *Malassezia* species have a role in the induction and/or the exacerbation of AD (Johansson et al., 2002, Scheynius et al., 2002).

1.2.3 Systemic atopy in the aetiology of atopic dermatitis

The atopic disorder is a systemic condition involving asthma, food allergy, allergic rhinitis and the cutaneous manifestation of AD (Leung et al., 2004). Increased levels of serum IgE and peripheral eosinophilia are found in these related conditions. Atopic dermatitis is often thought to be the first step in the “atopic march” progressing to allergic rhinitis and asthma in a considerable number of patients (Langan et al., 2006, Leung et al., 2004). Within the atopic diathesis, abnormalities in total and specifically abnormal IgE production regulation exist (Spergel et al., 1998). In experimental models of AD, the epicutaneous induction of allergic skin inflammation can increase the systemic allergic response and exacerbate the airway hyperreactivity characteristic of asthma (Spergel et al., 1998). IgE-mediated sensitisation occurs several weeks to months after initial lesions appear in early-onset AD (Bieber, 2008). Although 20-30 % of patients with AD have an “intrinsic” form which is without IgE-mediated sensitisation, while 70-80 % of patients with “extrinsic” AD have associated IgE-mediated sensitisation (Novak and Bieber,

2003), both forms of AD have associated eosinophilia (Leung et al., 2004). Epidermal dendritic cells (DCs) in AD bear IgE and express its high-affinity receptor (FcεRI) (Bieber et al., 1992) and as part of their antigen-presenting capacity, they contribute to the polarisation of the Th response. Inflammatory epidermal DCs lead to Th1 polarisation by producing IL-12 and IL-18 and releasing proinflammatory cytokines (Bieber, 2008). Recruitment of T cells into the skin is thus orchestrated by a complex network of mediators that contribute to chronic inflammation (Bieber, 2008).

1.2.4 Hygiene hypothesis in aetiology of atopic dermatitis

Since the middle of the last century, there has been an increase in the incidence of chronic inflammatory disease that mirrors the increase in urban living and increase in industrialisation (Eder et al., 2006). The diseases on the rise include atopic diseases such as asthma, allergic rhinoconjunctivitis, food allergies and AD as well as autoimmune disorders such as Type 1 diabetes mellitus, inflammatory bowel disease and neurodegenerative disorders (Renz et al., 2011). The hygiene hypothesis postulates that microbial contact in the early postnatal period permits the development of Th1-type responses, and that enhancing Th1 stimulation down-regulates the Th2-specific responses and protects against atopic and autoimmune disease states (Strachan, 2000). It was noted in the British national cohort study in 1970 which was investigating risk factors for childhood AD, that there was an increased prevalence of reported AD in advantaged socioeconomic groups (Williams et al., 1994). It was established by Strachan *et al.* that this finding did not represent a reporting bias but may instead have reflected an environmental influence on allergic sensitisation (Strachan et al., 1996).

Epidemiological evidence supports the hypothesis that a combination of environmental and genetic risk factors has led to this rise in atopic and inflammatory disease, as genetics alone cannot explain the elevation in such disease (Renz et al., 2011). A study by Strachan demonstrated that the most striking associations with occurrence of hay fever were those of family size and position in the household during childhood (Strachan, 1989). This study suggested that a number of factors are associated with the more widespread clinical expression of atopic disease including: declining family size, improvements in household amenities, higher standards of personal cleanliness and a reduced opportunity for cross-infection (Strachan, 1989).

A recently published work confirmed the association between increased exposure to multiple environmental allergens in early life with decreased risks of atopy via two cross-sectional studies in Germany, where children living on farms were compared with children in a control group, with respect to the prevalence of asthma and atopy (Ege et al., 2011). In both studies; Prevention of Allergy – Risk Factors for Sensitization in Children Related to Farming and Anthroposophic Lifestyle (PARSIFAL) and Multidisciplinary Study to Identify the Genetic and Environmental Causes of Asthma in the European Community Advanced Study (GABRIELA), the children who lived on farms had lower prevalences of asthma and atopy (Ege et al., 2011). They were exposed to a greater variety of environmental microorganisms than the children in the reference group (Ege et al., 2011).

Many environmental factors have altered as atopy has been on the rise, including a dramatic increase in the amount of soap and detergent wash products purchased between 1981-2011, exceeding that which would be expected from population growth (Cork et al.,

2009). In addition, over the past 40-50 years there has been much change in the infrastructure of domestic housing, with alterations and improvements to heating, ventilation, insulation and floor coverings of houses in the developed world. These infrastructural changes not only create optimal conditions for the house dust mite but also increased heating and insulation have become well-established environmental flare factors in AD (Cork et al., 2009).

1.2.5 The skin barrier in the aetiology of atopic dermatitis

The creation of a competent protective barrier at the interface of the body to the environment is a fundamental function of skin (Madison, 2003). The skin barrier, at the lower part of the stratum corneum, is created following a complicated series of events which are completed in a process of cell death by terminally differentiating keratinocytes which become flattened and are referred to as corneocytes (O'Regan et al., 2008). The barrier is maintained by the cornified envelope (CE), which is a tough, insoluble layer that replaces the plasma membrane of the epithelial keratinocytes. The water-resistant lipid lamellae encase the corneocytes and help prevent water loss and penetration of water-soluble materials (Candi E., 2005, Cork et al., 2009). There is also an antimicrobial aspect to the defensive function of the skin barrier and this encourages the colonisation of nonpathogenic flora and resists the development of microbial pathogens (Elias and Steinhoff, 2008). Strid *et al.* demonstrated that epicutaneous immunisation to protein antigens is induced following cutaneous mechanical barrier disruption (Strid et al., 2004). The immune responses induced were of a Th2 nature, with secretion of IL-4 by T cells from the draining lymph nodes as well as high levels of protein specific IgE and IgG₁ with no IgG_{2a} (Strid et al., 2004). Rapid migration of Langerhans cells from the epidermis to the draining lymph nodes was observed on exposure to protein antigen following removal of the stratum corneum (Strid et al., 2004). However, removal of the

stratum corneum alone induced only maturation and increased expression of Langerhan cell MHC class II, CD86, CD40, CD54 and CD11c (Strid et al., 2004). Figure 1.2.5 summarises how the structure of the skin barrier interacts with local immunology to permit the development of inflammation and the control of same, in the skin's reparative function following inflammation.

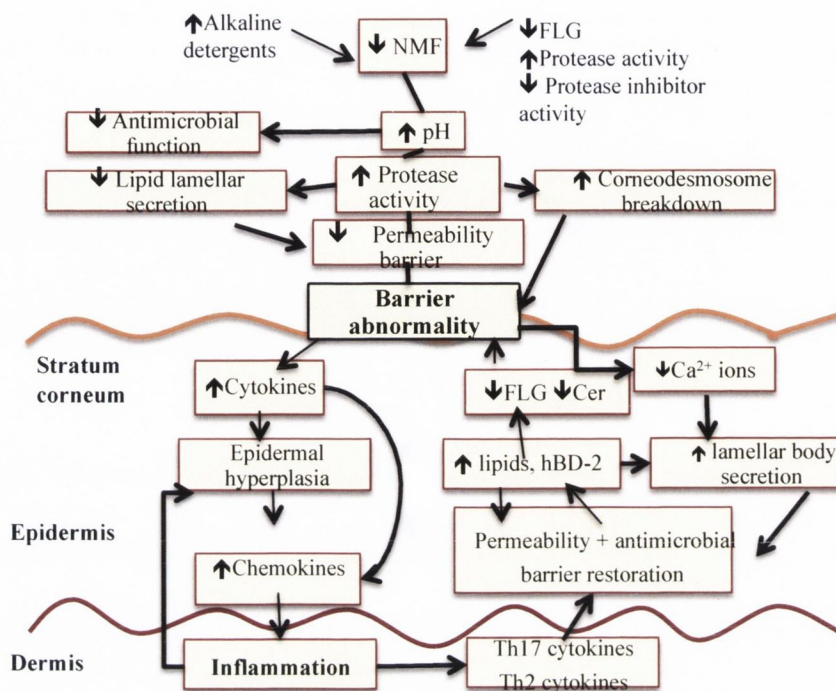


Figure 1.2.5 Interaction of the skin barrier with cutaneous immunology.

The skin barrier is the site of interactions between various parts of immune responses with the external environment and is where the consequences of these interactions are induced. NMF (natural moisturising factor), FLG (filaggrin protein), Cer (ceramides), hBD-2 (human β defensin-2). Adapted from (Elias and Steinhoff, 2008).

1.2.5.1 Infrastructure of the skin barrier

As is shown in Figure 1.2.5.1, the upper layer of the skin is the epidermis, which delivers the important protective barrier function of the skin and the stratum corneum modulates the permeability factor (Elias and Steinhoff, 2008). It consists of keratinocytes in various stages of differentiation within the ascending layers of the basal, spinous and granular layers. The stratum corneum rests above these and is composed of terminally differentiated keratinocytes, known as corneocytes. In corneocytes the plasma membrane is replaced by the cornified envelope (CE). Development of the CE involves the sequential expression of major structural proteins (Cookson, 2004). Filaggrin is a key protein in the structural reinforcement of the CE and is one of the final proteins to be added to the barrier (O'Regan et al., 2008). Filaggrin and other structural proteins such as loricrin, involucrin and small-proline-rich proteins are crosslinked by transglutaminases. They act as a scaffold for the attachment of a layer of lipids which are covalently bound to the extracellular surface, forming an outer lipid envelope (Cork et al., 2009). Two loss-of-function mutations in the *FLG* gene were initially identified as strong predisposing factors for AD (Palmer et al., 2006). Additional mutations in *FLG* linked to manifestation of inflammatory skin diseases were subsequently identified (Sandilands et al., 2007, Nomura et al., 2007). Many of the key proteins involved in cornification and terminal differentiation are encoded for in the epidermal differentiation complex (EDC) (Section 1.2.5.4), a region on chromosome 1q21 with significant linkage with AD and psoriasis (Cookson W.O., 2001) (Figure 1.2.5.4).

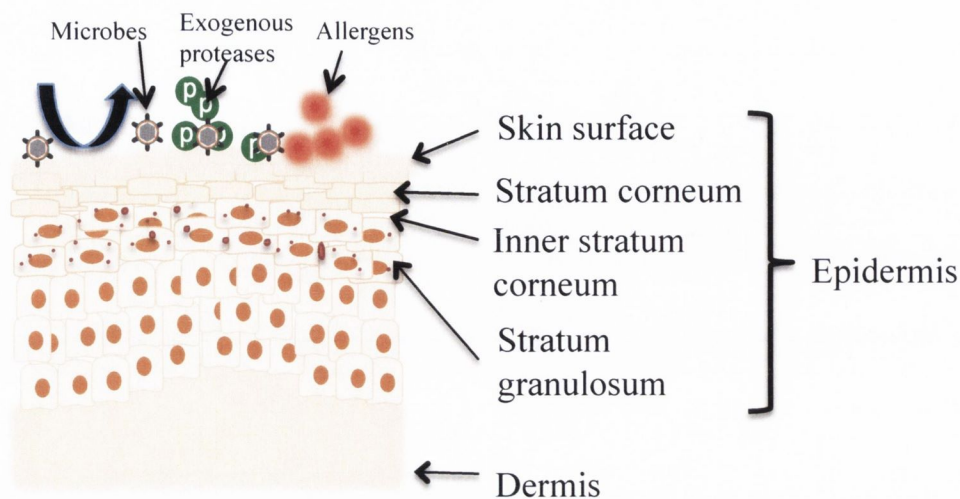


Figure 1.2.5.1 Normal epidermis in skin. The layers of the epidermis are pictured ranging from the basal layers through the stratum granulosum, containing the keratohyalin F granules, to the inner and outer layers of the stratum corneum up to the skin surface. Microbes (grey stars), exogenous proteases (green discs), and allergens (red discs) are examples of exogenous agents that can penetrate the skin barrier and thus interact with local and systemic immunological pathways. Adapted from Irvine and McLean, NEJM, 2011.

1.2.5.2 Lipids in the skin barrier

The lipid bilayer plays a role in barrier function in the stratum corneum. Lamellar bodies are cytoplasmic inclusions in the granular epidermal layer and they are packed in multi-layered stacks (Oyoshi et al., 2009a). At the interface of the stratum corneum lamellar body exocytosis forms the lipid lamellae. The lipid lamellar layer, which is enriched in ceramides, cholesterol and free fatty acids, forms around the strong scaffolding of the corneocytes providing a water-resistant aspect to the barrier (Oyoshi et al., 2009a, Elias and Steinhoff, 2008). Abnormality in the lipid composition of the corneal layer has been identified in the xerosis of AD (Hara et al., 2000). Alterations in essential fatty acid metabolism may be related to changes in the epidermal barrier linked to dry skin.

1.2.5.3 Proteases in the skin barrier

Proteases also have an important role to play in the processing of the skin barrier where a balance between proteolytic and antiproteolytic activities in cutaneous homeostasis is achieved (Rawlings and Harding, 2004). Proteases are involved in desmosome breakdown and corneocyte desquamation (Hansson et al., 1994, Sato et al., 1998, Simon et al., 2001). The enzymatic activity of these proteases are tempered by protease inhibitors which include lymphoepithelial Kazal-type-related inhibitor (LEKTI), encoded by the *SPINK5* gene (Shimomura et al., 2005). Altered activity of proteases and protease inhibitors have been observed in individuals with AD (Oyoshi et al., 2009a).

1.2.5.4 Epidermal Differentiation Complex (EDC)

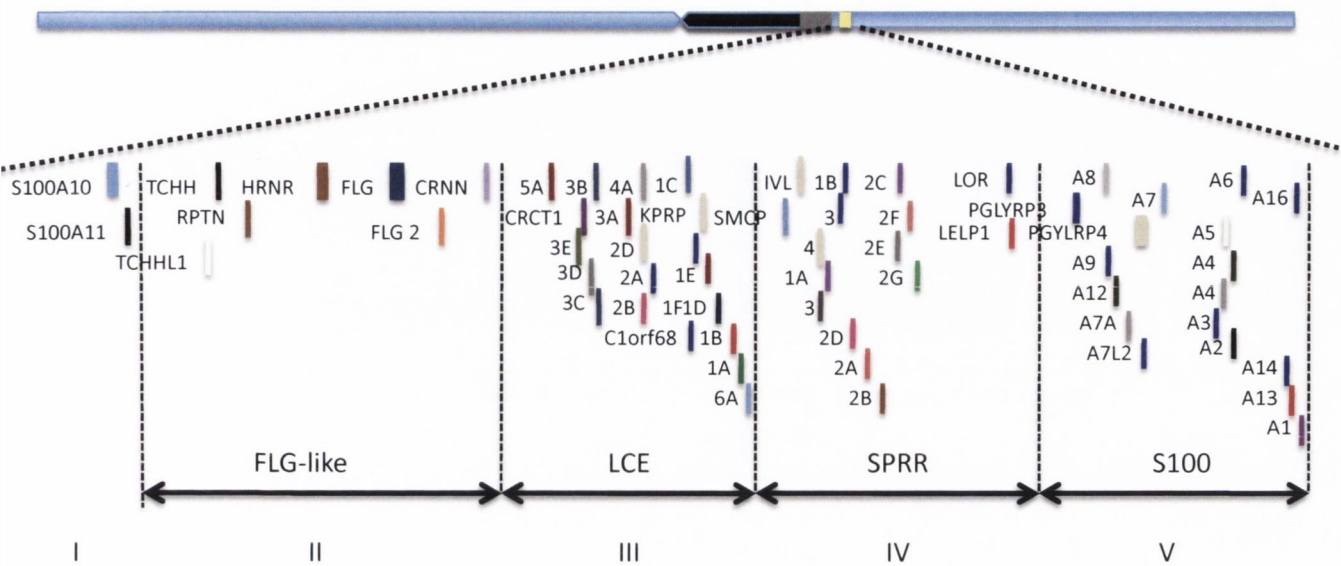
The Epidermal Differentiation Complex (EDC) contains 4 groups of gene families that are associated with skin barrier integrity (Figure 1.2.5.4). The EDC is on human chromosome 1q21 (1.6Mb). The gene families include Filaggrin (*FLG*)-like, Late Cornified envelope (*LCE*), Small Proline Rich Region (*SPRR*) and the *S100* genes (de Guzman Strong et al., 2010). The first 3 gene families encode the structural proteins that are cross-linked as part of the epidermal barrier at the skin surface. It has been suggested that the EDC contributes to the aetiology of inflammatory skin disorders with altered barrier function such as AD and psoriasis, as genetic linkage to the EDC was confirmed for both of these inflammatory skin conditions (Cookson et al., 2001). *FLG* is one of the subclusters of genes encoding 7 fused S100 proteins. The proteins (filaggrin, filaggrin-2, hornerin, trichohyalin, trichohyalin-like 1, cornulin and repetin) all share a common protein-domain organisation, with an S100 calcium-binding motif at the N-terminal and a highly repetitive, extended tail (Irvine et al., 2011). Specific loss-of-function mutations in the *FLG* gene were identified in ichthyosis vulgaris (IV) and in AD (Palmer et al., 2006, Rodriguez et al., 2008, Smith et al., 2006).

1.2.5.5 Homology between murine EDC and human EDC

The EDC proteins are highly conserved across primates (de Guzman Strong et al., 2010). Murine EDC on mouse chromosome 3 differs in size from the human EDC locus by just 3.1 Mb (de Guzman Stron et al., 2010). The variation is due to a 'gene desert' insertion which is centromeric to the *FLG* genes and telomeric to *HRN* genes (de Guzman Strong et al., 2010).

a

Human Chr 1q21.3: E.D.C.



b

Murine Chr 3: E.D.C.

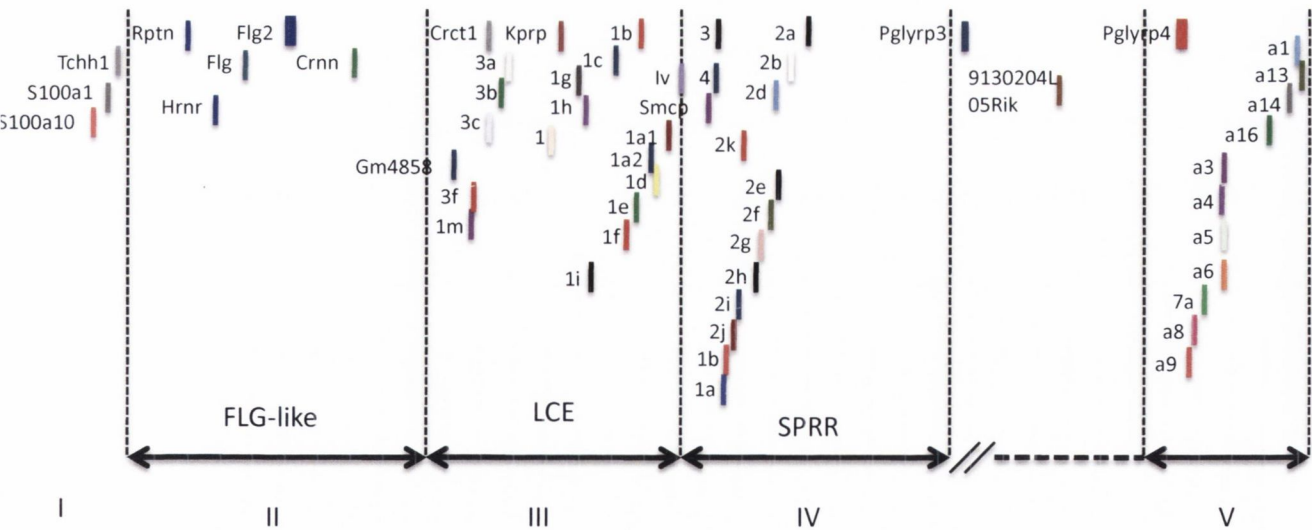


Figure 1.2.5.4 Epidermal differentiation complex (EDC). Human EDC is composed of tandemly arranged gene families on Chr 1q21.3 (a). Mouse EDC, on Chr 3, comprises 4 gene families, similar to human EDC. Adapted from de Guzman Strong *et al*, 2010)

1.3 Filaggrin

Filaggrin is a key structural protein in the epidermis (Irvine et al., 2011). The 37kDa filaggrin protein was initially identified as a highly insoluble, histidine-rich protein which co-purified with keratin intermediate filament proteins in epidermal extracts (Dale, 1977). Keratin intermediate filaments were condensed and aligned *in vitro* by this purified protein, leading to its name 'filaggrin' for filament aggregating protein (Steinert et al., 1981). The high-molecular-weight proprotein, profilaggrin was identified in a similar manner and it is now known to have a mass of greater than 400 kD (Smith et al., 2006). Profilaggrin is a major constituent of the keratohyalin granules in the granular layer of the interfollicular epidermis. This molecule is mostly composed of 10-12 repeats of the filaggrin sequence after a short, unique N-terminal domain (Smith et al., 2006). Once the cells of the granular epidermal layer undergo terminal differentiation, profilaggrin is proteolytically cleaved into approximate 37 kD filaggrin peptides and the S100-like calcium domain within the N-terminal domain (Smith et al., 2006). As outlined above, the filaggrin gene (*FLG*) is located in chromosome 1q21, in the EDC (Figure 1.2). *FLG* comprises 3 exons (Figure 1.3). Exon 1 is non-coding, exon 2 encodes part of the S100 domain, and exon 3 encodes almost the entire profilaggrin protein (Sandilands et al., 2009). Southern blot analysis of population samples confirmed that there are common size-variant alleles in the general population with 10-12 repeats of filaggrin within the *FLG* gene (Figure 1.3).

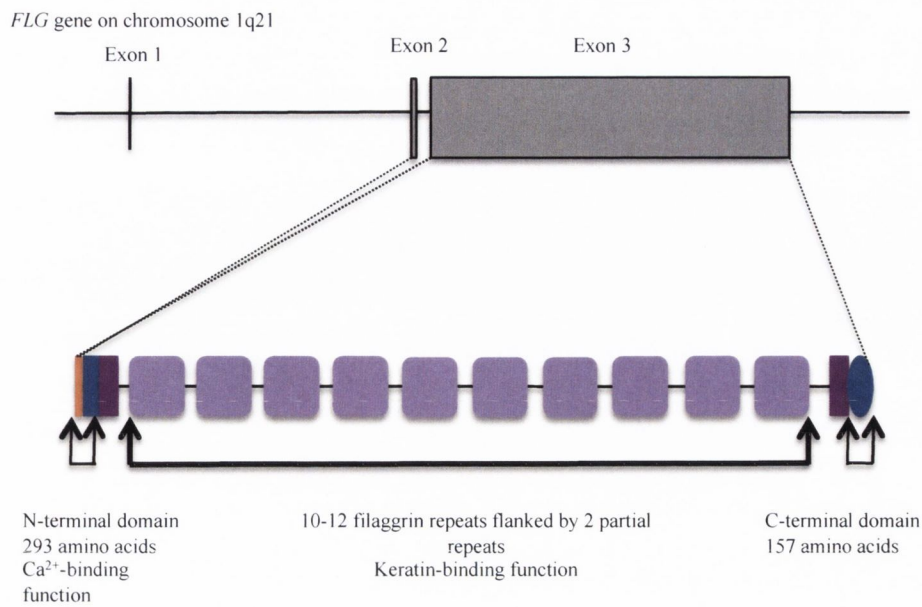


Figure 1.3 Profilaggrin and filaggrin (*FLG*) structure. The *FLG* gene has 3 exons and 2 introns. Exon 3 encodes most of the profilaggrin protein, which has a variable number of filaggrin units. Adapted from Sandilands *et al.* Journal of Cell Science 2009 (122), 1285-1294.

1.3.1 Role of filaggrin in the skin barrier

The identification of *FLG* null alleles in up to 50 % of patients with moderate-to-severe AD shows a fundamental role for barrier homeostasis in aetiology of this disease (Smith et al., 2006, Sandilands et al., 2006, Rodriguez et al., 2009, Palmer et al., 2006, Baurecht et al., 2007). During terminal differentiation of keratinocytes, the epidermal granular cells are collapsed into flattened squames following the aggregation of the keratin filamentous cytoskeleton by the free filaggrin peptides (Sandilands et al., 2009). The macrofibrils resulting from the aggregation cause the alignment of the intermediate filaments in tightly packed parallel arrays. These steps contribute to cellular compaction (Sandilands et al., 2009). Transglutaminases extensively crosslink the keratin cytoskeleton while the cornified cell envelope (CCE) is being formed (Smith et al., 2006). This molecular process is a large part of the formation of the protective barrier that is a major function of the skin, and more specifically, the stratum corneum. As corneocytes undergo desquamation, filaggrin is degraded by posttranslational modification enzymes (peptidylarginine deiminase 1 and isoforms) into substituent modified amino acids (urocanic acid, pyrrolidone carboxylic acid and alanine (O'Regan et al., 2008). The pool of amino acids, their metabolites and various ions make up the Natural Moisturising Factor (NMF) (Rawlings and Harding, 2004). NMF plays a part in maintenance of stratum corneum hydration due to its hygroscopic capacity (O'Regan et al., 2008). As well as filaggrin providing much of the contents of the NMF, it may also indirectly have a role in the regulation of key biochemical events of the skin by maintaining an acid pH at the skin surface due to the acidic factors produced by its breakdown to NMF (O'Regan et al., 2008). The biochemical functions which may be influenced by the skin pH in this way include protease activity, barrier permeability and cutaneous microbial defence which are all linked in skin barrier homeostasis (O'Regan et al., 2008).

1.3.2 Consequences of filaggrin deficiency in the skin barrier

Two independent mutations in *FLG* carried by 9 % of those of European origin result in complete loss of processed functional filaggrin in the epidermis (Smith et al., 2006, Palmer et al., 2006). These genetic mutations have been shown to impair epithelial barrier formation and function (Gruber et al., 2011). Recurrent and family-specific mutations in *FLG* lead to ichthyosis vulgaris (IV) (Smith et al., 2006) and AD (Palmer et al., 2006). Distinct profiles in *FLG* mutations stratify into certain ethnic groups and have been well studied in European and Asian populations to date (Irvine et al., 2011). Patients with AD who have *FLG* mutations have more persistent disease, an increased risk of asthma, a greater incidence of cutaneous infections, higher risks of multiple allergies, greater associations with irritant contact dermatitis and nickel allergy (Irvine et al., 2011).

1.3.2.1 Ichthyosis vulgaris – effective semi-dominant genodermatosis underscored by filaggrin deficiency

Ichthyosis vulgaris (IV) is a common genetic skin disorder with a prevalence of 1 in 250 persons (Wells and Kerr, 1966) (Figure 1.3.2.1). It usually presents within the first few months of life with fine, dry scaling of cutaneous surfaces, particularly those of extensor areas. Palmar and plantar hyperlinearity are features of the condition (Figure 1.3.2.1). There is an accompanying predisposition towards AD and asthma in those affected with IV (Sandilands et al., 2009). It is inherited in an autosomal semi-dominant pattern where carriers of a single mutation have mild or no disease (Smith et al., 2006). Loss-of-function mutations in *FLG* underlie IV (Smith et al., 2006). Since the initial 2 mutations (R501X and 2282del4) were identified, 47 other mutations have been reported (Sandilands et al., 2006). All mutations in *FLG* thus far identified predict loss-of-function with no translated protein. As well as the clinical characteristics of those with

IV, histopathological features include markedly reduced or absent filaggrin protein in skin and/or keratinocytes in immunoblotting studies (Fleckman et al., 1987).



a



b



c



d

Figure 1.3.2.1 Ichthyosis vulgaris. The palmar (a, b) and plantar (c, d) surfaces of a 6 month old infant, with IV, displaying hyperlinearity which has been shown to be associated with IV. IV is also associated with keratosis pilaris (KP) and is a well-recognised association with AD. Source of image: Dr. C.M.R. Fahy, Prof Alan D. Irvine, Department of Paediatric Dermatology, OLCHC, Dublin.

1.3.2.2 Filaggrin: a major genetic risk for atopic dermatitis

Palmer *et al.* showed that 49 % of their AD study population carried 1 or 2 *FLG* null alleles (Palmer *et al.*, 2006). Further studies confirmed this finding in Scottish, Irish, Danish (Palmer *et al.*, 2006), German (Weidinger *et al.*, 2008) and English (Barker *et al.*, 2007) populations. These associations have been replicated in other white European study populations where these mutations are prevalent (Ruether *et al.*, 2006, Marenholz *et al.*, 2006, Morar *et al.*, 2007, Stemmler *et al.*, 2007). A Japanese study has demonstrated that analogous loss-of-function mutations in *FLG* have also been found to be significantly associated with AD, further illustrating that this gene contributes to AD worldwide (Nomura *et al.*, 2007). A longitudinal birth cohort from the UK was studied and it was shown that *FLG* alleles strongly predispose to AD at all ages; they are associated with both atopic and non-atopic AD; the association was stronger with higher odds ratios for AD than for nonatopic AD ($P = 0.0023$) and are also associated with persistent AD (Henderson *et al.*, 2008). Overall odds ratios for associations of AD with *FLG* mutations range from 3.12 to 4.78 (Irvine *et al.*, 2011). Patients with AD who carry *FLG* mutations may have a predisposition for more persistent disease, a higher incidence of eczema herpeticum and a greater risk of multiple allergies (Irvine *et al.*, 2011).

1.3.2.3 Association of asthma with filaggrin deficiency and atopic dermatitis

A recent systematic review of prospective studies showed that 1 in 3 young children with AD go on to develop asthma at the age of 6 years or older (van der Hulst *et al.*, 2007). Ireland has the fourth highest prevalence of asthma in the world (Kabir *et al.*, 2011), and the prevalence of asthma is increasing (Table 1.3) (Kabir *et al.*, 2011).

Mutations in *FLG* are associated with the increased risk of asthma (overall and in the presence of AD) and with airway hypersensitivity in allergic rhinitis (Weidinger *et al.*,

2008). Studies of large cohorts of patients with AD reveal an overall risk of asthma of between 1.48 to 1.79 in the presence of *FLG* loss-of-function mutations (Weidinger et al., 2008, Henderson et al., 2008). It is established that those with AD and *FLG* mutations are at an increased risk of asthma (McLean et al., 2008, Henderson et al., 2008, Spergel and Paller, 2003, Weidinger et al., 2008). In patients with asthma, those that have *FLG* mutations have increased frequency of exacerbations and a more difficult course (Palmer et al., 2007, Basu et al., 2008, Li et al., 2011).

SEVERE ASTHMA* (IRELAND)			PREVALENCE RATIOS	
Year	Prevalence %	Study number	Unadjusted	Adjusted
1995	12	2,671	1	1
1998	11.4	2,273	0.95 (0.8-1.11)	0.9 (0.76-1.07)
2002	8.7	2,894	0.72 (0.61-0.85)**	0.76 (0.62-0.92)**
2007	15.3	2,809	1.27 (1.1-1.47)**	1.39 (1.14-1.69)**

Table 1.3 Prevalence of symptoms of severe asthma among Irish school children aged 13-14 years, 1995-2007 (n=10,647) who had 4 or more attacks of wheeze in the last year or had 1 or more nights per week sleep disturbance from wheeze in the last year or had wheeze affecting speech in the last year. Exponentiated log regression coefficients were used to derive prevalence ratios. Poisson distribution computed adjusted prevalence ratios. Confidence intervals of 95% were analysed based on standard error estimates (Kabir et al., 2011).

* Adjusted simultaneously for sex; medications; pet exposure; current active smoking status alone; secondhand smoke exposure alone; allergies (hayfever and eczema ever) [not for inhaler usage]; clustering effect within schools; wheeze alone [not for inhaler usage].

** statistically significant from the previous assessment year.

1.3.2.4 Association of food allergy with atopic dermatitis

Food allergy in children with AD is accepted to be approximately 30 % (Suh, 2010). Food allergy in children overall is estimated to have a prevalence of approximately 4-10 % (Suh, 2010). The greatest prevalence occurs in the first few years of life and decreases during the first decade as tolerance develops (Suh, 2010). Peanut allergy has been shown to be a heritable condition and there is monozygotic concordance of 64 %, compared to 7 % in dizygotic twins (Sicherer et al., 2000). A single study has demonstrated that *FLG* mutations bestow an overall odds ratio of 5.3 for peanut allergy (positive food challenge) with a residual odds ratio of 3.8 when corrected for AD (Brown et al., 2011).

Strid *et al.* analysed cutaneous exposure to peanut protein allergy, it was found that epicutaneous exposure to peanut protein induced Th2-type immune responses with high levels of IL-4 and serum IgE (Strid et al., 2005). Epicutaneous peanut exposure prior to a tolerogenic feed prevented oral tolerance and allergic sensitisation was further enhanced (Strid et al., 2005). In contrast, mice with existing oral tolerance to peanut were partly sensitised to peanut after epicutaneous exposure on barrier disrupted skin, where higher levels of IL-4 were produced from skin-draining lymph nodes and peanut-specific T cell proliferation was enhanced in tolerant animals (Strid et al., 2005).

1.3.2.5 Environmental interactions with filaggrin deficiency

The risk of development of AD is increased by interplay between environmental factors and *FLG* mutations (Bisgaard et al., 2008, Cramer et al., 2010). The skin barrier deficiency brought about by *FLG* mutations has been postulated to exacerbate the effect of environmental exposures and to alter the immunology of the skin response to those exposures (Table 1.2) (Bisgaard et al., 2008). Bisgaard *et al.* illustrated that there was a

significant interaction between *FLG* loss-of-function mutations and neonatal cat exposure on the risk of developing early life AD (Bisgaard et al., 2008). Cramer *et al.* examined the effect of *FLG* mutations on the risk of development of AD in children in two studies – the Life-style factors on the development of the Immune System and Allergies in East and West Germany plus the influence of traffic emissions and genetics study (LISApplus) and the German Infant Nutritional Intervention plus environmental and genetic influences on allergy development study (GINIplus) (Table 1.1) (Cramer et al., 2010). These cohorts were followed until they were 6 years old, diagnosis of flexural AD was determined if the child had visible flexural dermatitis at 6 years and at least 1 parental report of AD-like symptoms in the previous 6 years (Cramer et al., 2010). The number of siblings at birth of the index subject was reported and other variables were collected by questionnaires including: sex of infant, parental education, parental allergy, contact with dog/cat in the 1st year, maternal age at childbirth > 30 years, maternal smoking during pregnancy, day care attendance during the first 2 years of life and study region (Cramer et al., 2010). The previously asserted hypothesis of the protective effect of elder siblings was not reported in children with *FLG* mutations, neither did daycare attendance provide a protective effect on children with *FLG* loss-of-function mutations (Cramer et al., 2010). To avoid any confounding variables between children with elder siblings and early day care attendance, when analysis was restricted to children who did not attend day care there was a significant interaction between *FLG* mutations and elder siblings (Cramer et al., 2010). Day care also enhanced the effect of *FLG* mutations on AD (Cramer et al., 2010). These interaction analyses indicate that *FLG* mutation and environmental exposure are interactive factors in the aetiology of AD.

1.3.2.6 Filaggrin breakdown products affect *S.aureus*

As discussed in Section 1.2.2 colonisation and infection of skin lesions by *S.aureus* occurs in individuals with AD. Colonisation by *S.aureus* exacerbates inflammation by a number of mechanisms. It has been shown by Miajlovic *et al.* that urocanic acid (UCA) and pyrrolidone carboxylic acid (PCA), which are both breakdown products of filaggrin, can *in vitro*, reduce growth rates of *S.aureus* and decrease the final density of the bacteria (Miajlovic et al., 2010). This illustrates the important role of skin pH in the protective aspect of the skin barrier and how filaggrin is associated with this aspect of the skin barrier function. Increased pH of atopic skin compared to normal skin in the presence of skin barrier deficiency secondary to *FLG* mutations will also increase the risk for these individuals to develop more frequent and severe infective flares of AD.

1.4 Animal models of atopic dermatitis

Animal models of human disease allow detailed analysis and investigation of molecular and cellular pathological processes and further our understanding of disease aetiology and pathogenesis. They are also used in the research and development of diagnostic tools and therapeutic approaches. Just as the incidence of AD has been rising so too has the interest in animal models of AD been increasing (Shiohara et al., 2004). A number of murine models of AD have been developed which include: genetically engineered models; models induced by injury, haptens or superantigens; models induced by oral immunisation; spontaneous models (Oyoshi et al., 2009a).

Mouse models of AD that have been derived from genetic engineering are described in Table 1.4 (Oyoshi et al., 2009a). Variable expression of combinations of these chemokines, cytokines and enzymes results in a spectrum of phenotypes ranging from

some to all of the signs of AD including: xerosis, pruritus, inflammatory skin lesions, conjunctivitis, and infection. They also represent responses to flares that result in inflammation, as well as the initial causative insult.

STRAIN	GENE EXPRESSION	SKIN / HISTOLOGY	SKIN IMMUNOLOGY	SYSTEMIC RESPONSE
IL-4 tg (Chan et al., 2001)	K-specific (K14 promoter)	Xerosis, cutaneous inflammation, S.aureus infection*, conjunctivitis	IL-4, CCL27	↑ serum IgE, IgG1 ↓ serum IgG2a
IL-13 tg (Hamid et al., 1996)	K-specific (Tetracycline- inducible K5 promoter)	Xerosis, pruritus, erythema, excoriation, crusting, bacterial pyoderma, lichenification, skin remodelling, alopecia	TSLP, Eotaxin, CCl17/TARC, CCL27, MCP-1, CCL22/MDC, RANTES, TGFβ- 1	↑ serum IgE, IgG1 ↑ IL-4, IL- 13 by CD4 ⁺ T cells (from lymphoid tissues and PBMCs)
IL-31 tg (Dillon et al., 2004)	L-specific (Em-Lck promoter) or ubiquitous (EF1a promoter)	Pruritus, alopecia, hyper- /parakeratosis, conjunctivitis, acanthosis		Normal levels of IgE, IgG
Caspase-1 tg (Yamanaka et al., 2000)	K-specific (Human K14 promoter)	Pruritus, erosions, hyper- /parakeratosis, acanthosis, spongiosis, papillomatosis	IL-18, IL-1β	↑ serum IgE, IgG1 ↑ splenic neutrophils ↑ splenic IL- 4, IL-5 ↑ serum IL- 18, IL-1β ↑ plasma histamine
IL-18 tg (Konishi et al., 2002)	K-specific (Human K14 promoter)	Pruritus, erosions, marked lichenification, hyper-/parakeratosis, acanthosis, spongiosis, papillomatosis,		↑ serum IgE, IgG1 ↑ IL-4, IL-5 ↑ splenic neutrophils ↑ serum IL- 18, ↑ plasma histamine

STRAIN	GENE EXPRESSION	SKIN / HISTOLOGY	SKIN IMMUNOLOGY	SYSTEMIC RESPONSE
Cathespin E ko (Tsukuba et al., 2003)		Pruritus, erythema, erosions, crusting, alopecia, epidermal hyperplasia after S.aureus infection,		↑ serum IgE ↑ splenic IL- 4, IL-5 ↑ splenic IL- 4, IL-5 ↑ serum IL- 18, IL-1β
RelB ko (Weih et al., 1997) (Barton et al., 2000)		Hyper-/parakeratosis, alopecia, epidermal hyperplasia, alopecia	IL-4, IL-5, IFNγ, CCL11/Eotaxin, CCR3, TGF-β, IL-1β	↑ serum IgE
Foxp3 ko (Fontenot et al., 2003, Lin et al., 2005)		Erosion, scarring, alopecia, keratosis		↑ serum IgE, IgG1 ↓ serum IgG2a ↑ serum IL- 4, IL-5, IL- 10, IFNγ, TNF-α ↑ splenic IL- 4, IL-5, IL- 13
TSLP tg (Yoo et al., 2005)	K-specific (Tetracycline- inducible K5 promoter)	Xerosis, erythema, erosions, crusting, hyper- /parakeratosis, acanthosis, spongiosis	IL-4, IL-5, TNF-α	↑ serum IgE, IgG1 ↓ serum IgG2a IL-4 producing CD4 T cells
MAIL/IkBz ko (Shiina et al., 2004)		Pruritus, erosions, alopecia, scarring, hyper- /parakeratosis,acanthosis, partial spongiosis		↑ serum IgE
SCCE tg (Hansson et al., 2002)	K-specific (SV40 enhancer and promoter)	Hyper-/parakeratosis, epidermal hyperplasia, acanthosis		

STRAIN	GENE EXPRESSION	SKIN / HISTOLOGY	SKIN IMMUNOLOGY	SYSTEMIC RESPONSE
APOC1 tg (Jong et al., 1998, Nagelkerken et al., 2008)	Liver and skin (APOC-1 promoter)	TEWL, alopecia, scarring, hyper- parakeratosis, spongiosis, hyperlipidaemia		↑ serum IgE ↓ serum cholesterol, triglycerides, free fatty acids No subcutaneous fat No atrophic sebaceous glands

Table 1.4 Murine models of atopic dermatitis developed from genetic engineering. tg, transgenic; TSLP, thymic stromal lymphopoietin; SCCE, stratum corneum chymotryptic enzyme; APOC1, apolipoprotein C1; K-specific, keratinocyte-specific; L-specific, lymphocyte-specific.

Models of AD that involve epicutaneous sensitisation with a hapten or superantigen, demonstrate the response of inflammation secondary to sensitisation, while other approaches, which rely on the instigation of injury, demonstrate the consequences of deliberate skin barrier deficiency (Table 1.5). However, the barrier defects are artificial and cannot completely replicate the situation of human skin barrier deficiency although the insights provided from the inflammatory changes direct further investigation. A model of AD that develops in orally immunised mice after epicutaneous antigen challenge is important for the link between AD and food allergy and the immunopathology of this association.

CLINICAL FINDINGS	STRAIN	SKIN INFLAMMATION	CYTOKINES	SYSTEMIC RESPONSE	CYTOKINES	REF
Allergic skin inflammation model in WT	WT	↑ Eosinophils, ↑ CD4 ⁺ ↑ thickening	IL-4, IL-5, IL-13	↑ OVA-IgE ↑ OVA-IgG1 ↑ OVA-IgG2a	IL-4, IL-5, IL-13, IFN-γ	(Spergel et al., 1998)
Exaggerated skin inflammation	C3aR ^{-/-}	↑ Eosinophils		↑ OVA-IgG1	↑ IL-4, ↑ IL-5, ↑ IL-10	(Kawamoto et al., 2004)
	COX2 ^{-/-}	↑ Eosinophils ↑ CD4 ⁺	↑ IL-4	↑ OVA-IgE ↑ OVA-IgG1 ↓ OVA-IgG2a	↑ IL-4, ↓ IFN-γ	(Laouini et al., 2005)
Decreased skin inflammation	IL-4 ^{-/-}	↓ Eosinophils ↑ CD4 ⁺	↓ IL-5, ↑ IL-2, ↑ IFN-γ	↓ OVA-IgE ↓ OVA-IgG1 ↑ OVA-IgG2a	↓ IL-13, ↑ IFN-γ	(Spergel et al., 1999)
	IL-5 ^{-/-}	↓ Eosinophils, ↓ thickening				(Spergel et al., 1999)
	IL-10 ^{-/-}	↓ Eosinophils	↓ IL-4, ↓ IL-5 ↓ Eotaxin, ↑ IFN-γ	↓ OVA-IgE ↓ OVA-IgG1 ↑ OVA-IgG2a	↓ IL-4, ↑ IFN-γ	(Laouini et al., 2003)
	IFN-γ ^{-/-}	↓ thickening	↑ IL-4,	↑ OVA-IgE ↑ OVA-IgG1 ↓ OVA-IgG2a	↑ IL-4	(Spergel et al., 1999)
	IL-21R ^{-/-}	↓ Eosinophils ↓ CD4 ⁺ ↓ thickening	↓ IL-4, ↓ IL-13 ↓ Eotaxin, ↓ TARC	↓ OVA-IgE, ↓ OVA-IgG1, ↓ OVA-	↓ IL-4, IL-13, IFN-γ ↓	(Jin et al., 2009b)

				IgG2a		
CLINICAL FINDINGS	STRAIN	SKIN INFLAMMATION	CYTOKINES	SYSTEMIC RESPONSE	CYTOKINES	REF
Decreased skin inflammation	TSLPR ^{-/-}	↓ Eosinophils ↓ thickening	↓ IL-4 ↓ IL-13	↓ OVA-IgE ↓ OVA-IgG1		(He et al., 2008)
	CCR3 ^{-/-}	↓ Eosinophils, ↓ thickening				(Ma et al., 2002)
	C3 ^{-/-}	↓ Eosinophils	↓ IL-4, ↓ IL-5	↓ OVA-IgE ↓ OVA-IgG1 ↓ OVA-IgG2a	↓ IL-4, ↓ IL-5, ↓ IL-13, ↓ IFN-γ	(Yalcinda g et al., 2006)
	TCRα ^{-/-}					(Woodward et al., 2001)
	Rag 2 ^{-/-}					(Woodward et al., 2001)
No effect on skin inflammation	IgH ^{-/-}					(Woodward et al., 2001)
	IgE ^{-/-}					(Spergel et al., 1999)
	TCRδ ^{-/-}					(Woodward et al., 2001)
	CD40 ^{-/-}			↓ OVA-IgE ↓ OVA-IgG1 ↓ OVA-IgG2a		(Woodward et al., 2001)
	J-Kit ^w /Kit ^{w-v}		↑ IFN-γ	↓ OVA-IgE		(Alenius et al., 2002)
	CD1d ^{-/-}					(Elkhal et al., 2006)
	TLR2 ^{-/-}	↓ thickening	↓ IFN-γ	↓ OVA-IgG2a	↓ IFN-γ	(Jin et al., 2009a)

Table 1.5 Murine models of atopic dermatitis derived from epicutaneous sensitisation by hapten, superantigen or by injury. Comparison to WT control: ↑ Increased, ↓ Decreased.

Spontaneous murine models of AD may reflect human AD more closely because of recent, replicated evidence in human and animal studies, that genetically defective skin barrier function is an important primary causal factor for AD (Table 1.6).

STRAIN	CLINICAL FEATURES	HISTOLOGY	CYTOKINES	REF
NC/Nga mice (Chr 9, derm1 locus)	Pruritus, excoriation, erythematous erosive lesions, (CON SPF conditions) ↑ TEWL, ↑ excoriation (CON non-SPF conditions)	Eosinophils, mononuclear cells, hyper-/parakeratosis, hyperplasia, spongiosis	↑ serum IgE ↑ IL-18, IL-12, CASP1 ↑ IFN-γ	(Kohara et al., 2001, Matsumoto et al., 2001, Yagi et al., 2002)
DS-nonhair mice	Erythema, S.aureus colonization (CON&SPF conditions)	Mast cells, Eosinophils, CD4 ⁺ T cells, CD 11b ⁺ Mphages	↑ serum IgE	(Watanabe et al., 2003, Hikita et al., 2002, Haraguchi et al., 1997)
NOA mice	Pruritus, erosive lesions, alopecia	Mast cells,	↑ serum IgE	(Kondo et al., 2005)
<i>maft^{fl}/maflg^{fl}</i> mice (Chr 3, EDC locus)	Xerosis, flaking skin, erythema, alopecia, neonatal tail and paw constrictions	↑ Ephils ↑ Lcytes ↑ Mononuclear cells Attenuated epidermal granular layer, ↓ CCE filaggrin, hyperkeratosis, acanthosis	↑ serum IgE, ↑ OVA-IgE ↑ OVA-IgG ↑ IL-17 ↑ IL-6 ↑ IL-23	(Presland et al., 2000, Fallon et al., 2009, Oyoshi et al., 2009b, Scharschmidt et al., 2009, Moniaga et al., 2010)

Table 1.6 Spontaneous murine models of atopic dermatitis. NC/Nga mouse, Nishiki Nezumi Cinammon/Nagoya University mouse; DS - nonhair mouse, Develops Spontaneously - nonhair mouse; NOA mouse, Naruto Research Institute Otsuka mouse;

EDC, Epidermal differentiation complex; TEWL, Transepidermal water loss; CON, conventional housing; SPF, specific pathogen free; Mphages, macrophages; Ephils, eosinophil; Lcytes, lymphocytes; CASP1, Caspase-1; CCE, cornified cell envelope; OVA, ovalbumin.

In 1957 NC/Nga mice were developed as an inbred strain in Japan and became the first reported mouse model of AD (Matsuda et al., 1997). When these mice are kept in conventional conditions they develop cutaneous disease that is similar to human AD. However, when this strain of mouse is maintained in specific-pathogen-free (SPF) conditions the cutaneous lesions do not develop (Matsuda et al., 1997). This feature implies that environmental aero-allergen or microbiome exposure is an initiating factor in cutaneous inflammation. Various other associations have been made regarding this spontaneous model of AD including; that disease severity is related to cutaneous injury such as excoriation; that there are increased populations of eosinophils and mast cells in cutaneous lesions; increased serum total IgE, with high expression of Th2-specific chemokines, and Th differentiation toward Th2 cells in the spleen (Yagi et al., 2002, Kohara et al., 2001, Matsumoto et al., 2001, Tanaka et al., 2001, Matsumoto et al., 1999).

The DS-nonhair model of AD is another Japanese spontaneous mouse model which was established in 1976 (Haraguchi et al., 1997). This model also develops AD-like disease and elevated levels of serum total IgE in a conventional environment and these clinical features are consistently less common while maintained in SPF conditions. Another spontaneous mouse model of AD is the Naruto Research Institute Otsuka (NOA) mouse, which has some aspects of the spectrum of cutaneous inflammation seen in patients with AD, including a pruritic dermatitis, cutaneous mast cell infiltrates with increased serum IgE and alopecia.. However the NOA mice do not develop cutaneous lymphocytic infiltration or the typical histological features of human AD (Kondo et al., 2005).

The flaky tail or *maflg^{fl}/maflg^{fl}* mouse has been shown to manifest skin barrier deficiencies and act as a valuable model of AD (Lane, 1972, Presland et al., 2000, Fallon

et al., 2009, Oyoshi et al., 2009b, Scharschmidt et al., 2009, Moniaga et al., 2010). The *maflg^{fl}/maflg^{fl}* mouse is the result of genetic mutations including the matted (*ma*) mutation, the nonagouti (*a*) mutation and a mutation in the filaggrin gene that is analogous to the common loss-of-function mutations in human *FLG*. This complex model may closely represent the cutaneous inflammation that occurs in AD (Fallon et al., 2009). It can be used to further investigate the complex interactions of specific skin barrier dysfunction with other changes from immunological over/under-expression of chemokines and cytokines, epicutaneous sensitisation without mechanical skin disruption and oral immunisation, in order to elucidate the complex immunopathogenesis of AD.

1.5 *maflg^{fl}/maflg^{fl}* (Flaky tail) mouse

The spontaneous recessive mouse mutant, the flaky tail mouse, arose in 1958 on the background of an existing nonagouti (*a*) and matted (*ma*) recessive hair phenotype which had been crossed with strain C57BL/6J (Lane, 1972). It was noted that among the progeny of the cross between strains some mice had smaller ears than their sibling and it was shown that this was due to a new recessive mutation (Lane, 1972). It has since been maintained on the mixed strain. Studies have demonstrated that the genetic mutation mapped to mouse chromosome 3, in the approximate location of murine EDC (Figure 1.2.5.4) (Presland et al., 2000). Presland *et al.* demonstrated that the flaky tail mouse expressed an abnormal profilaggrin polypeptide, which was no longer processed to filaggrin peptides (Presland et al., 2000). It was proposed that this animal could be used as a model for the filaggrin-deficient IV due to the defective skin barrier resulting from absent filaggrin in the stratum corneum (Presland et al., 2000).

A homozygous frameshift mutation in the murine *Flg* was detected using a shotgun sequencing method (Fallon et al., 2009). In *maflg^{fl}/maflg^{fl}* mice, the exon 3 of *FLG* lacks one repeat, owing to a 750-bp in-frame deletion, as well as the pathogenic frameshift mutation 5303delA (Fallon et al., 2009). This mutation is analogous to common human *FLG* mutations and results in dry, flaky skin and annular tail and paw constrictions in the neonatal period in *maflg^{fl}/maflg^{fl}* mice secondary to the deficiency of processed filaggrin (Presland et al., 2000). The defective physical barrier in the epidermis, as with this model, is a key mechanism in the initiation of cutaneous inflammation in humans with filaggrin-related atopic disease (Fallon et al., 2009).

1.5.1 *maflg^{fl}/maflg^{fl}* mouse exhibits increased percutaneous sensitization

Fallon *et al.* demonstrated that topical application of a widely studied and clinically relevant allergen, such as OVA, to these mice, resulted in increased cutaneous inflammatory infiltrates (Fallon et al., 2009). When assessing for a systemic effect, percutaneous exposure of the allergen to the intact skin of the *maflg^{fl}/maflg^{fl}* mice generated exaggerated OVA-specific Th2 (IL-4, IL-5, IL-13), Th1 (IFN γ), regulatory (IL-10) and Th17 (IL-17) cytokine expression (Fallon et al., 2009).

1.5.2 *maflg^{fl}/maflg^{fl}* mouse has Th17-dominated inflammation

The *maflg^{fl}/maflg^{fl}* mouse has been studied by other groups. Oyoshi *et al.* highlighted that the *maflg^{fl}/maflg^{fl}* mouse model exhibited Th17-dominated skin inflammation as demonstrated by increased cutaneous expression of IL-17, IL-6, IL-23 mRNA, accompanied by Th2 associated IL-4 mRNA and Th1 (IFN γ) cytokine mRNA (Oyoshi et al., 2009b). Ovalbumin application to the intact shaved skin of *maflg^{fl}/maflg^{fl}* mice resulted in the production of IL-17, IL-4, IL-13 and IFN γ , by splenocytes in response to

in vitro stimulation with ovalbumin (Oyoshi et al., 2009b). This study followed the clinical progress of *maflg^{fl}/maflg^{fl}* mice and demonstrated that *maflg^{fl}/maflg^{fl}* mice develop eczematous, AD-like cutaneous lesions with age (Oyoshi et al., 2009b). All *maflg^{fl}/maflg^{fl}* mice were affected at 32 weeks of age. They had scaly, pink, eczematous cutaneous lesions on the face and periauricular areas, periorbital swelling and patches of AD-like skin on the neck and trunk, with thinning of the overlying hair (Oyoshi et al., 2009b).

1.5.3 *maflg^{fl}/maflg^{fl}* mouse demonstrates decreased inflammatory thresholds secondary to epicutaneous barrier abnormality

Scharschmidt *et al.* used the ultrastructural cytochemistry of lipase activity as a marker for epidermal lamellar bodies, permitting the assessment of the efficacy of organelle secretion in the study on the *maflg^{fl}/maflg^{fl}* mouse model (Scharschmidt et al., 2009). In the intact epidermis, enzyme activity localises both to lamellar body contents and to within the stratum corneum extracellular domains. *maflg^{fl}/maflg^{fl}* mice epidermis showed a similar localisation of enzyme activity to lamellar bodies but there was evidence of reduced secretion of enzymes at the interface between the stratum granulosum and the stratum corneum (Scharschmidt et al., 2009). The consequences of impaired lamellar body secretion include that the quantities of extracellular lamellar bilayers decreased in the stratum corneum of *maflg^{fl}/maflg^{fl}* mice. Another consequence is that the processing of newly secreted lamellar body content into mature bilayers is delayed in *maflg^{fl}/maflg^{fl}* mouse skin (Scharschmidt et al., 2009). The low-molecular-weight tracer, colloidal lanthanum nitrate (4%) in Karnovsky solution, was used to determine the outward permeation of water-soluble markers (Scharschmidt et al., 2009). In WT mice, the tracer did not breach the stratum granulosum – stratum corneum interface. However in *maflg^{fl}/maflg^{fl}* mice, the tracer breached this interface as well as extending several layers

outward into the stratum corneum (Scharschmidt et al., 2009). *maflg^{fl}/maflg^{fl}* mice exhibit a background level of low-grade cutaneous inflammation while acute cutaneous inflammation develops secondary to allergen sensitisation in the *maflg^{fl}/maflg^{fl}* mouse (Scharschmidt et al., 2009).

1.5.4 *maflg^{fl}/maflg^{fl}* mouse exhibits increased inflammation secondary to topical dust mite extract

Moniaga *et al.* confirmed that AD-like cutaneous lesions develop spontaneously in the *maflg^{fl}/maflg^{fl}* mouse model (Moniaga et al., 2010). Skin barrier deficiency was confirmed using fluorescence studies to assess the penetration of fluorescein isothiocyanate isomer (FITC) applied to the shaved skin of the mice. The epidermis of the *maflg^{fl}/maflg^{fl}* mice contained increased levels of FITC compared to the epidermis of the WT controls (Moniaga et al., 2010). Severe AD-like lesions were induced on the skin of *maflg^{fl}/maflg^{fl}* mice by applying: phorbol myristate acetate (PMA), 0.5 % 1-fluoro-2,4-dinitrobenzene (DNFB), OVA, and 0.5 % *Dermatophagoides pteronyssinus* (Dp) in white petroleum. Extract of dust mite is a clinically relevant allergen and has been used in other murine models of allergic cutaneous inflammation (Moniaga et al., 2010). Applying the Dp ointment to the shaved skin of the WT mice did not initiate any cutaneous inflammation. However, the same application to the *maflg^{fl}/maflg^{fl}* mice induced AD-like cutaneous lesions suggesting that the known skin barrier deficiency of these mice led to a secondary exaggerated cutaneous inflammatory response (Moniaga et al., 2010).

1.5.5 Summary of *maflg^{fl}/maflg^{fl}* strain as a model of atopic dermatitis

This *maflg^{fl}/maflg^{fl}* model serves as a validated animal model of human AD and emphasises the importance of filaggrin in skin barrier integrity and in the pathogenesis of AD (Fallon et al., 2009, Scharschmidt et al., 2009, Moniaga et al., 2010, Oyoshi et al., 2009b). Associated with clinically relevant skin lesions are histopathological cutaneous changes as well as systemic differences compared to WT controls, including elevated total serum IgE (Fallon et al., 2009). On cutaneous exposure to various allergens including ovalbumin, DNFB, and Dp, antigen-specific cytokine production is observed from splenocyte culture (Oyoshi et al., 2009b, Moniaga et al., 2010). Detailed studies on the integrity of the skin barrier such as assessing inward and outward movement of tracer markers and lamellar body organelle secretion, respectively demonstrated that skin barrier deficiency exists in the *maflg^{fl}/maflg^{fl}* mouse model (Scharschmidt et al., 2009).

1.6 Thesis Aim

The aim of this thesis is to investigate further the interplay between environmental factors and filaggrin deficiency in a murine model of AD.

1. This study seeks to confirm the *maflg^{fl}/maflg^{fl}* mouse as a model of AD and to investigate the importance of the ambient environment in these skin barrier deficient mice.
2. As part of the investigation of the role of external environment on phenotype, the study analyses the effect that changing conditions has on the development of phenotype in skin barrier deficiency.

3. This study further explores the role of bacterial colonisation and infection on the clinical phenotype of AD through investigation of the difference between *maflg^{fl}/maflg^{fl}* mice with or without antibiotic administration.

4. The role of filaggrin and environmental exposure in airway hypersensitivity further studied to characterise the role of the skin barrier in airway hyper-responsiveness. The distal effects of the phenotype in cutaneous inflammation in skin barrier deficiency are also assessed by more detailed analysis of the lung phenotype including histopathology and assay assessment of inflammation and infection.

Chapter 2

Materials and Methods

2.1 Mice and animal husbandry

Flaky tail ($maflg^{fl}/maflg^{fl}$) mice (Jackson Laboratory, Bar Harbor, Maine, U.S.A.) were backcrossed to C57BL/6J (WT) strain mice (Jackson Laboratory, Bar Harbor, Maine, U.S.A.). Brother/sister siblings were separated into 2 animal facilities at TCD to establish genetically identical colonies. They were maintained on the C57BL/6J background and bred in-house. Mice were housed in specific pathogen-free (SPF) cages. They were fed an irradiated diet (Harlan, UK), with food and water supplied *ad libitum*. All animal experiments were done in compliance with the Irish Department of Health and Children regulations and approved by the Trinity College Dublin's BioResources ethical review board.

Populations of $maflg^{fl}/maflg^{fl}$ and WT mice were bred in SPF facilities in two different settings: individually ventilated cages under positive pressure (Techniplast, Buguggiate, VA, Italy) (IVC) and conventional open cages (CON) (Figure 2.1). Living conditions including room and cage temperature (19 - 23°C) and humidity (55 % + / - 10 %), 12 – hour light/dark cycle, autoclaved bedding, water and irradiated food were similar in both housings. The cages in the CON conditions were open to the laboratory atmosphere and the individually ventilated cages were administered with filtered air under positive pressure (Figure 2.1).

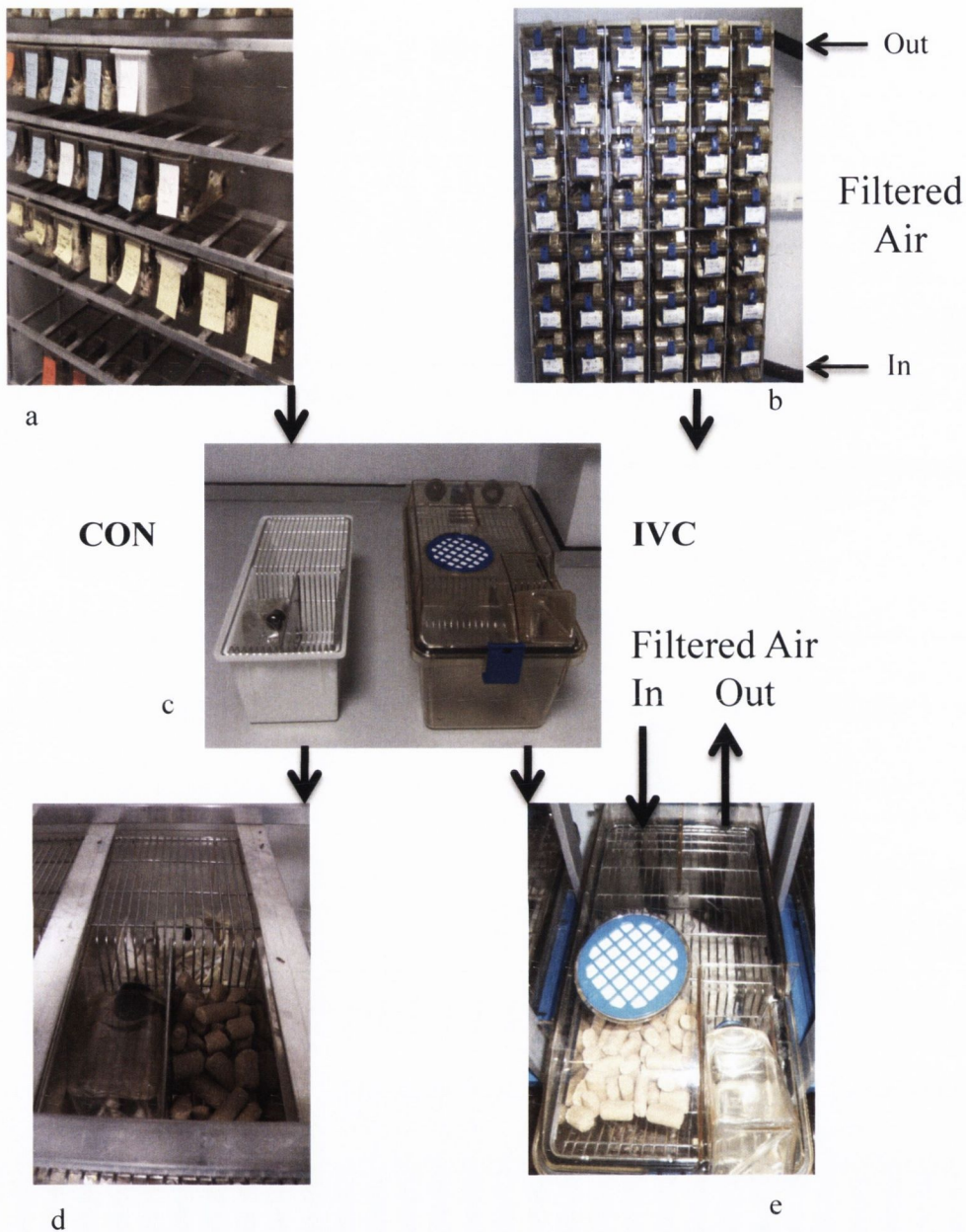


Figure 2.1: Mouse cages used in experiments. All housing was treated similarly with regard to room/cage temperature and humidity, food, water, bedding and cage cleaning cycle. Conventional caging (CON) (a,d) is pictured above, with a comparison between a CON cage (left) and IVC cage (right) (c), and individually ventilated sealed caging (IVC) (b,e).

2.1.1 Interventions in environmental conditions and antibiotic administration

Groups of WT (N= 2 each groups) and *maflg^{fl}/maflg^{fl}* mice (N = 6 each group) were maintained in both the CON and IVC environments and selective culls were done at 1, 4, 8, 20 and 32 weeks.

A litter of 7 female *maflg^{fl}/maflg^{fl}* mice was transferred at 1 week from CON to IVC housing and maintained to 32 weeks (CON-IVC group). Control *maflg^{fl}/maflg^{fl}* mice (N = 6) and WT mice (N = 2) were bred and housed in the CON and IVC environments at the same time.

Two pairs of breeding *maflg^{fl}/maflg^{fl}* mice in the CON facility had administration of oral antibiotics, diluted in drinking water (CON A/B group) (Table 2.1). Antibiotics were changed on a fortnightly rotational basis to avoid the development of antibiotic resistance in the experimental mice and their progeny. The antibiotics were chosen for their particular anti-bacterial effectiveness and skin penetrance. Antibiotic administration continued following birth of the litters, up to weaning at 28 days. The *maflg^{fl}/maflg^{fl}* mice (N = 8) used in experiments from these litters were maintained until 32 weeks. Control *maflg^{fl}/maflg^{fl}* mice (N = 3) were maintained simultaneously in the CON environment.

No specific sensitising agent was topically applied or injected intradermally to the mice.

ANTIBIOTIC REGIMEN	
Protocol 1 (1 L H ₂ O)	g/L
Metronidazole	500
Ciprofloxacin	12.5
Sucrose	20
Protocol 2 (1 L H ₂ O)	
Tetracycline	0.2

Table 2.1 Interventional antibiotic treatment of mice in CON facility (CON A/B group). Antibiotic protocols were alternated every 2 weeks from initial creation of 2 breeding *maflg^{fl}/maflg^{fl}* mice harems until weaning of *maflg^{fl}/maflg^{fl}* mice at 4 weeks.

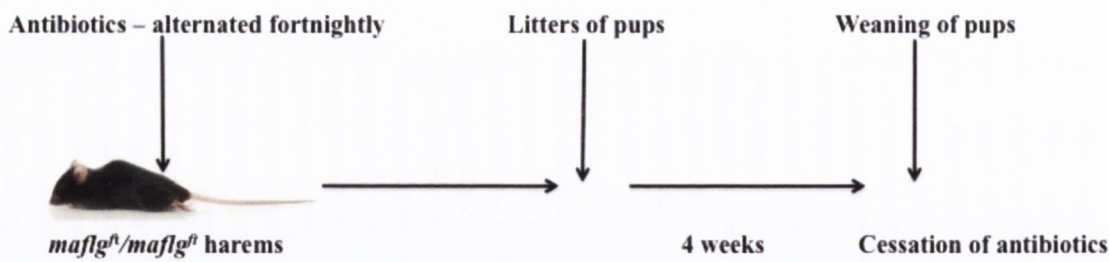


Figure 2.1.1 Timeline of administration of antibiotics intervention. Antibiotics diluted in H₂O (Table 2.1) were administered to harems of *maflg^{fl}/maflg^{fl}* mice. When

litters of pups were weaned at 4 weeks to gender separate caging they were transferred to normal drinking H₂O and irradiated food.

2.2 Clinical scoring

The clinical severity of skin lesions was recorded on mice at 32 weeks, using a scoring system based on the macroscopic diagnostic criteria developed for the NC/Nga mouse (Yamamoto et al., 2009). The total score for each subject was produced from the sum of individual scores, assessing different criteria involved in diagnosing dermatitis-like skin changes, maximum score possible is 16 (Table 2.2).

DISEASE CHARACTERISTICS	SCORE OF CLINICAL SIGN
Eyes	
No ocular changes	0
Periocular alopecia	1
Periocular alopecia, oedema	2
Periocular alopecia, oedema, infection	3
Tail	
No change	0
Blunt ended tail-tip	1
Skin	
Erythema	0=none, 1=mild, 2=moderate, 3=severe
Oedema	0=none, 1=mild, 2=moderate, 3=severe
Dryness/scaling	0=none, 1=mild, 2=moderate, 3=severe
Erosion	0=none, 1=mild, 2=moderate, 3=severe
Total Score	

Table 2.2: Clinical scoring of features of disease. Mice were scored according to the above parameters of severity of disease. Maximum score possible – 16. Adapted from (Yamamoto et al., 2009).

2.3 Pulmonary function

To assess pulmonary function mice that were 32 weeks old were anaesthetized with a combination of ketamine 75 mg/kg, metomidine 1 mg/kg and midazolam 1 mg/mg. The trachea was exposed and cannulated for tracheostomy. The cannula was secured within the trachea to ensure that no leak would occur during pulmonary function testing (PFTs). The mice were then inserted into a sealed chamber, where the tracheal cannula was connected to the ventilator and the pulmonary transducers (EMMS). The pulmonary transducers measured transpulmonary pressure as well as airflow measurement. Methacholine (Sigma, St.Louis, MO, U.S.A.) was inserted into the nebuliser attachment (Aero Neb Pro, Dangan, Galway, Ireland) to the ventilator. Methacholine causes bronchoconstriction, which results in decreased airflow and increased pulmonary pressure.

The average lung resistance (R_L) and lung compliance (C_L) were quantified for 3 minute periods at baseline and after exposure to nebulized endotoxin-free PBS (Sigma, St.Louis, MO, U.S.A) and acetyl-b-methycholine chloride (Sigma, St.Louis, MO, U.S.A) at 10, 30, 60 and 120 mg/ml (Figure 2.3). Compliance is an index of lung distensibility (Figure 2.3).

$$\text{Airways Resistance} = \frac{\text{Transairway pressure gradient}}{\text{Flow rate}}$$

$$\text{Dynamic Lung Compliance} = \frac{\text{TV}}{\text{PIP} - \text{PEEP}}$$

Figure 2.3 Airways resistance, Dynamic lung compliance.

TV, tidal volume; PIP, peak inspiratory pressure; PEEP, peak end expiratory pressure.

2.4 Tissue collection

Mice were administered a lethal anaesthetic (midazolam/fentanyl dilution in sterile H₂O, dilution 1:1:2, 10µl/g of anaesthetic solution). Following collection of blood, which was then left aside (Section 2.4.1), abdominal skin was sectioned and divided into 2 pieces – 1 piece, placed in a cryogenic vial (ThermoScientific, Waltham, MA, U.S.A.) was immediately snap-frozen in a canister of liquid nitrogen. The other piece was placed in a bijou (BD Biosciences, Franklin Lakes, NJ, U.S.A.) of 10 % (vol/vol) formaldehyde saline (Section 2.5.1). The frozen tissue was transferred to a -80 °C freezer for storage. The lungs were removed whole, and processed as per Section 2.7. The remaining organs (spleen and submandibular lymph nodes, as below) were then removed. Approximately half of each spleen was inserted into a cryogenic vial and snap frozen on liquid nitrogen and transferred to -80 °C for storage. The other half was placed in a tube of 1 ml of complete media (RPMI, 10 % (vol/vol) foetal calf serum, L-glutamine, Sigma, St.Louis, MO, U.S.A), on ice and processed as per Section 2.4.3. Two of the four submandibular

lymph nodes were snap frozen, as above, and the remaining pair was processed for cell culture (Section 2.4.3).

2.4.1 Sera processing

Tubes of harvested blood were allowed settle for 2 hours, SeraSieve (Hughes & Hughes, UK) was added to the tubes and they were centrifuged at 3,000 x *g* for 10 min and the serum supernatant was harvested and stored at -20 °C.

2.4.2 Bronchoalveolar lavage and cytopins

Bronchoalveolar lavage (BAL) fluids were collected by cannulating the trachea and lavaging the lungs with a 1 ml of ice-cold PBS in 15 ml tubes (Greiner, Frickenhausen, Germany) (Mangan et al., 2006). The lavage was centrifuged at 400 x *g* for 5 minutes at 4 °C. The supernatant was discarded and the cells in pellet-form were re-suspended in 200 µl of sterile PBS. Cell counting was performed using glass slides with standardised grids. Sterile PBS was added at a volume of 180 µl with 10 µl of sample and 10 µl of Trypan blue dye (Sigma, St.Louis, MO, U.S.A). A volume equivalent to 5×10^4 cells for each sample was added to PBS making up a total volume of 100 µl. Slides were inserted into the cytocentrifuge machine (Shandon, Pittsburgh, PA, U.S.A.) and 100 µl of the diluted cell samples were added to each appropriately labelled slide. Cytospins were fixed and stained using Diff-Quik Wright-Giemsa Stain kit (Thermo Shandon, Waltham, MA, U.S.A.). Differential cell counts were determined by counting a minimum of 100 cells per slide using morphologic criteria (Mangan et al., 2006).

2.4.3 Cell culture

Approximately half of each spleen and 2 skin-draining submandibular lymph nodes were processed to produce supernatants. Once the organs were harvested they were placed on ice, in tubes of 1 ml complete medium (RPMI (Sigma, St.Louis, MO, U.S.A), 10 % (vol/vol) foetal calf serum, L-glutamine). The tissue was then passed through a 70µm sieve using the top of a 1 ml syringe (BD Biosciences, Franklin Lakes, NJ, U.S.A.) through a cell filter (BD Biosciences, Franklin Lakes, NJ, U.S.A.) into 50 ml tubes (Greiner, Frickenhausen, Germany). The sieve was washed with complete media and centrifuged at 4,000 x g for 5 min at 4 °C. Presence of the pellet was checked before discarding the supernatant. Complete media was added to the spleen pellets (2 ml) and lymph node pellets (1 ml). Cell counting was performed as per Section 2.4.2 and the volume of supernatant required to insert 1 million cells into each culture well was determined.

2.4.4 Spleen and lymph node cell supernatant

Cell suspensions were cultured at 37 °C in 24 or 48 well plates at 1×10^6 per well in complete media with various stimuli for 24 and 72 hours. Cells were cultured and/or activated with complete media, phorbol myristate acetate (PMA-ionomycin (PMA/I) (2.5ng/ml/250ng/ml) and anti-CD3/CD28 (0.5µg/ml/4µg/ml) (BD Pharmingen, Franklin Lakes, NJ, U.S.A.). Supernatants were harvested after 24 or 72 hours incubation and inserted into appropriately labelled 24 and 48 well plates (Greiner Bio-One, Frickenhausen, Germany).

2.5 Histology

2.5.1 Cutaneous histology

Abdominal skin of age- and sex-matched mice (*maflg^{fl}/maflg^{fl}* IVC and CON, WT IVC and CON) was removed and fixed in 10 % (vol/vol) formaldehyde saline and sent for histological processing (AML Laboratories Inc., Baltimore, Maryland, USA). Paraffin-fixed skin sections were stained with haematoxylin and eosin (H&E) and toluidine blue stain. To quantify the individual inflammatory cells infiltrating the skin the total number of infiltrating cells and lymphocytes were counted in 20 high-powered fields (HPFs) (Olympus CX31 light microscope, Olympus Corporation, Shinjuku, Tokyo, Japan) (Fallon et al., 2009). Toluidine blue staining was used to detect mast cells in skin samples in 20 HPFs. Histological photography was performed using an Olympus DP70 light microscope with as DP Controller 2.1.1.183, Olympus Corporation, Shinjuku, Tokyo, Japan.

2.5.2 Lung histology

Lungs of age- and sex-matched mice (*maflg^{fl}/maflg^{fl}* IVC and CON and WT mice, IVC and CON and intervention groups of *maflg^{fl}/maflg^{fl}* mice) were removed and fixed in 10 % (vol/vol) formaldehyde saline and sent for histological processing (AML Laboratories Inc., Baltimore, Maryland, USA). Paraffin-fixed lung sections were stained with H&E. Inflammatory changes that occurred in the lung tissue and airways were scored on 10 areas in each H&E stained slide, using a system based on 4 easily identifiable features of inflammatory changes which were scored on a scale of 0-4 (maximum score = 16): 0 = normal; 1 = infrequent mild infiltrates; 2 = frequent mild or moderate dense infiltrates; 3 = severe, dense and diffuse infiltrates with extension into the alveolar space and septae; 4 = very severe, diffuse infiltrates with complete extension into the interstitial and

alveolar spaces. The 4 inflammatory processes were: 1) perivascular inflammation; 2) peribronchiolar inflammation; 3) alveolar inflammation; and 4) alveolar wall thickness.

An overall total score of inflammation was derived based on the summation of all scores from the 4 processes to generate a cumulative total histopathological score. All scores are presented as a mean $\pm$ standard error of the mean (SEM) generated from each group at the various time-points (Belperio et al., 2003).

To detect the deposition of collagen in the lung parenchyma and peribronchiolar locations Masson Trichrome Blue staining of the lung slides was used for identifying collagen and fibrin deposition (Le Saux et al., 2008). The grading scale of peribronchial trichrome stain was: 1 = marginal peribronchial and perivascular blue trichrome stain; 2 = slight increase in peribronchial and/or perivascular trichrome stain; 3 = increase in trichrome stain in all airways; 4 = dramatically increased stain in all airways.

The grading scale for parenchymal collagen deposition was: 0 = no to marginal stain; 1 = slight and consistent increase; 2 = increased, uniform stain throughout the parenchyma; 3 = dramatic increase. The final score for each sample reflected the grading using both scales and an average of the scores of 10 airways per lung was used, maximum score per airway = 6 (Le Saux et al., 2008).

Histological photographs were taken using an Olympus BX50 microscope and digital photography was done with an Olympus DP70 microscopic camera, with DP Controller; 2.1.1.183 computer software, and are presented at 40x magnification.

2.6 Enzyme Linked Immunosorbent Assay (ELISA)

96-well flat-bottomed microtitre medium binding plates (Greiner Bio-One, Frickenhausen, Germany)) were coated with capture monoclonal antibodies (2 µg/ml) in coating buffer (PBS) at 50 µl per well and incubated in a wet-box overnight at 4 °C. The plates were then returned to room temperature and washed x 5 in ELISA wash buffer (PBS with 0.5 % (vol/vol) Tween) and blotted dry. Blocking buffer (1 % (w/vol) BSA in PBS) was inserted into each well at a volume of 150 µl, and the plates were left in a wet-box, for 2 hours at room temperature. Plates were then washed x 5 in ELISA wash buffer and blotted dry. Antibody standards were prepared in incubation buffer (0.1 % (w/vol) BSA in PBS). Standard control antibodies were added in duplicate, to the first 2 columns of wells, in a 2-fold serial dilution from top to bottom of the plate leaving the last 2 wells as blank wells with only incubation buffer. Test samples were diluted in incubation buffer and added to wells according to a predetermined plate format. The volume of standard/sample in each well was 50 µl. Plates were left in a wet-box overnight at 4 °C. On the following day, plates were washed x 5 in ELISA wash buffer.

Biotinylated monoclonal antibody was added to each well at a concentration of 2 µg/ml in incubation buffer and a volume of 50 µl per well. This was left at room temperature for 2 hours. Plates were washed x 5 in ELISA wash buffer. Horseradish peroxidase-conjugated Streptavidin (BD Pharmingen, Franklin Lakes, NJ, U.S.A.) was added at a dilution of 1/1000 in PBS, at a volume of 50 µl per well. The plates were incubated, in the dark, at room temperature for 30 min then washed with ELISA wash buffer x 5 and blotted dry. Reaction development substrate (o-phenylenediamine solution), (OPD, Sigma, St.Louis, MO, U.S.A), was added to each well at a volume of 50 µl per well. This was incubated in the dark. The substrate was prepared by dissolving a 5 mg OPD tablet

in 25 ml of phosphate-citrate buffer (pH 5.0) with 18 μ l of hydrogen peroxide (H_2O_2) in a 50 ml Falcon (Greiner, Frickenhausen, Germany) tube that was kept in the dark. The development reaction was stopped by 25 μ l/well of 2M H_2SO_4 when a colour gradient became obvious in the duplicate columns of standard control antibody dilutions. The plates were placed in a microplate spectrophotometer (VERSA_{MAX} microplate reader, Molecular Devices, Berkshire, U.K.). Absorbance was read at 490 nm. The levels of antibodies were quantified by interpolation from a standard curve using Soft Max Pro 3.0 software. Data were transferred to Microsoft Excel for analysis.

2.6.1 Blood sera antibody ELISAs

Total serum IgE, IgG₁, and IgG_{2a} antibodies were assayed using sandwich ELISA. 96-well medium-binding plates were coated with monoclonal anti-IgE/anti-IgG₁/IgG_{2a} capture antibody (BD Pharmingen, Franklin Lakes, NJ, U.S.A.). Serum samples were diluted in 0.1 % (w/vol) BSA/PBS and added according to a predetermined plate format. Plates were incubated overnight. Bound antibody was detected using monoclonal biotin-labelled anti-IgE/IgG₁/IgG_{2a} detection antibodies and streptavidin HRP (R&D Systems, Minneapolis, ME, U.S.A.) as above. Concentrations were calculated using purified IgE/IgG₁/IgG_{2a} as standard controls.

2.6.2 Spleen cell and lymph node cell culture cytokine ELISA

Sandwich cytokine ELISAs were performed to quantify levels of specific cytokines (IL-4, IL-17, TSLP, IFN γ) in the supernatants from spleen and lymph node cell cultures. High-binding 96-well plates were coated with capture antibody diluted in incubation buffer (1 % (w/vol) BSA PBS). Reagents for capture and detection of IL-4 were from

BD Pharmingen, Franklin Lakes, NJ, U.S.A., IL-17, TSLP and IFN γ cytokines were assessed using DuoSet ELISA development kits and reagents (R&D Systems, Minneapolis, ME, U.S.A.). Concentrations were calculated using purified IL-4, IL-17, TSLP and IFN γ as standards.

2.7 Lung processing

Whole lungs were removed from the mice. The left lung was placed in formal saline for histology. The post-caval lobe of the right lung was separated from the other right lung lobes and all right lobes were snap-frozen. The larger portion of the right lung (superior, middle and inferior lobes) was homogenized. The lobes were placed in 300 μ l of ice-cold C-TAB buffer (2 % (vol/vol) foetal calf serum (FCS), 0.25 % (w/vol) hexadecyltrimethylammonium bromide (TAB), PBS) and cut into sections. The lobes were homogenized twice with a homogeniser. The tubes were then centrifuged at 500 x g for 15 min at 4 °C. Supernatant was collected and the quantity of protein was measured with a BCA assay kit (Thermo Scientific, Waltham, MA, U.S.A.) (Section 2.8).

2.7.1 Collagen assay

Sircol Collagen Assay Kit (Biocolor, Carrickfergus, Northern Ireland) was used according to the manufacturer's instructions to determine levels of collagen deposition in the lung sample (Amu et al., 2010). Extraction buffer (C-TAB, Sigma, St.Louis, MO, U.S.A) was used as the reagent blank in this assay. Aliquots of 5 μ g, 10 μ g, 15 μ g and 20 μ g of collagen reference standard were used. One hundred μ l of reagent blanks, standards and samples (diluted 1:10 with PBS) were added to 1 ml of Sircol Dye Reagent (Biocolor, Carrickfergus, Northern Ireland) and placed on a mechanical shaker for 30

min. During this time, a collagen-dye complex formed and precipitated out from the soluble unbound dye. The tubes were transferred to a microcentrifuge (Mikro 200R, Hettich, Tuttlingen, Germany) at 3,200 x g for 10 min and then drained. Ice-cold Acid-Salt wash reagent was added at a volume of 750 µl to each pellet to remove unbound dye and re-centrifuged at 3,200 x g for 10 min. The wash was discarded and 250 µl of Alkali Reagent was added to reagent blanks, standards and samples. The collagen bound dye was release back into solution using a vortex mixer (Clifton Cyclone, Electro Ltd., U.K.). When the bound dye was fully dissolved 200 µl of each sample/standard/blanks were transferred to a 96 well plate (Greiner Bio-One, Frickenhausen, Germany). The microplate reader (Molecular Devices, Berkshire, U.K.) was set to 555 nm to measure absorbance. The collagen concentrations were obtained from the standard curve.

2.7.2 Myeloperoxidase assay

Myeloperoxidase release in lung homogenates was measured as previously described (Mangan et al., 2007). A 2-fold standard curve of myeloperoxidase (MPO) (Sigma, St.Louis, MO, U.S.A) from 0.5 U/ml to 0.0078 U/ml was obtained by diluting standard MPO in phosphate citrate buffer. Homogenization buffer (C-TAB, Sigma, St.Louis, MO, U.S.A) was used as reagent blank for this experiment. The substrate was made by dissolving a tablet of 3,3',5,5'- tetramethylbenzidine (TMB, Sigma, St.Louis, MO, U.S.A) in 1 ml of dimethylsulfoxide (DMSO, Sigma, St.Louis, MO, U.S.A), 9 ml of phosphate citrate buffer and 2 µl of 30 % (vol/vol) H₂O₂. Resorcinol (Sigma, St.Louis, MO, U.S.A.) was added to a final concentration of 120 µM by adding 50 µl of 12 mM resorcinol to 4950 µl of TMB solution). Samples were diluted in phosphate citrate according to the plate format and 50 µl of each was inserted into a 96 well plate. 50 µl of substrate was added at left at room temperature. Once a colour gradient was apparent in

the standard wells the reaction was stopped with 25 μ l of 2 M H_2SO_4 . The plate was read at 450 nm in the microplate reader (Molecular Devices, Berkshire, U.K.) and the myeloperoxidase concentration was obtained from the standard curve.

2.8 Protein quantification

Protein quantification of samples was carried out using the BCA Protein Assay Reagent Kit (Thermo Scientific, Waltham, MA, U.S.A.) according to the manufacturer's instructions. Standards were prepared from a stock solution of 2 mg/ml BSA in dH_2O , ranging from 125 $\mu\text{g/ml}$ to 2,000 $\mu\text{g/ml}$. Standards and samples added to a 96 well plate (Greiner Bio-One, Frickenhausen, Germany) in a volume of 10 μ l and 200 μ l of BCA solution was added to each well. The plate was incubated at 37 $^\circ\text{C}$ for 30 min to allow the reaction to develop. Absorbance was measured at 562 nm in a microplate reader. The concentration of protein in the supernatants was then calculated from the standard curve generated with the BSA standards.

2.9 Statistical analysis

GraphPad Prism and GraphPad Instat software (La Jolla, CA 92037, U.S.A.) were used for data analysis. Differences between groups was determined by Student's t-test or, if nonparametric data, Welch corrected t-test. Differences were considered significant when $p < 0.05$, data was tested for normality by the Kolmogorov and Smirnov test.

2.10 Clinical photography

Clinical photography was done in the Departments of Dermatology in Our Lady's Children's Hospital, Crumlin (OLCHC), Dublin, Ireland and in the Midwestern Regional

Hospital (MWRH), Dooradoyle, Limerick, Ireland. Patient consent for photography and use of images in educational and medical publications was obtained. Clinical photography was performed using a Canon EOS 5D mark 2 camera (OLCHC, Dublin, Ireland) and a Canon Ixus 100 1S camera (MWRH, Limerick, Ireland)

Chapter 3

The effect of environmental conditions

on the *maflg^{fl}/maflg^{fl}* mouse phenotype

3.1 Introduction

Skin barrier deficiency is one of the multiple aetiological factors in AD; however the clinical phenotype is the result of a delicate interplay between skin barrier deficiency and many other factors including the environment, microbes and immunological background. It has been shown that the skin barrier defect in *maflg^{fl}/maflg^{fl}* enhances antigen transfer and alters IgE sensitization and cutaneous inflammation (Fallon et al., 2009 (Oyoshi et al., 2009b, Scharschmidt et al., 2009, Moniaga et al., 2010, Presland et al., 2000). The Flaky tail (current formal strain nomenclature: *maflg^{fl}/maflg^{fl}*) mouse developed in 1958 on the background of an existing nonagouti (*a*) and matted (*ma*) recessive hair phenotype which had been crossed to strain C57BL/6J (Section 1.5) (Lane, 1972). Since then this mouse has been used to explore the skin barrier defect, which is associated with filaggrin deficiency amongst other mutations as in this model.

This chapter characterises further the *maflg^{fl}/maflg^{fl}* phenotype as expressed under differing environmental conditions. The clinical phenotype is explored from neonatal life through to adulthood. Clinical investigations of disease manifestations of skin barrier deficiency in response to housing conditions that are either open cages (CON) or cages with filtered air (IVC) were undertaken. The inflammatory responses of the *maflg^{fl}/maflg^{fl}* mice housed in IVC or CON conditions are characterised at a histological

level and at a systemic immunological level through investigation of the antibody and cytokine responses. As described in Section 1.5, the *maflg^{fl}/maflg^{fl}* mouse characteristically has higher total serum IgE levels indicating increased levels of spontaneous atopy (Moniaga et al., 2010, Oyoshi et al., 2009b, Scharschmidt et al., 2009), and a systemic Th2-driven allergic response (Oyoshi et al., 2009b). Levels of serum antibodies are analysed in wildtype and *maflg^{fl}/maflg^{fl}* mice to confirm this assertion and to investigate the effect of basic environmental conditions on this immunophenotype.

3.2 *maflg^{fl}/maflg^{fl}* mouse clinical phenotype

3.2.1 Neonatal *maflg^{fl}/maflg^{fl}* mouse clinical phenotype

Features of the *maflg^{fl}/maflg^{fl}* mouse phenotype begin in early life and differences between *maflg^{fl}/maflg^{fl}* and control WT mice are evident at the neonatal stage. Control C57BL/6J (WT) mice did not have any abnormal features in the neonatal period (Figure 3.2.1 a). Neonatal *maflg^{fl}/maflg^{fl}* mice developed widespread, dry, erythematous, hyperlinear, flaky skin (Figure 3.2.1 b,c). This phenotype was similar to the results of previous studies (Presland et al., 2000). As highlighted in Figures 3.2.1 d-f, tail changes in the *maflg^{fl}/maflg^{fl}* model are characteristic and include flaking of tail skin, annular tail constrictions, tail-tip necrosis and tail-tip auto-amputation.

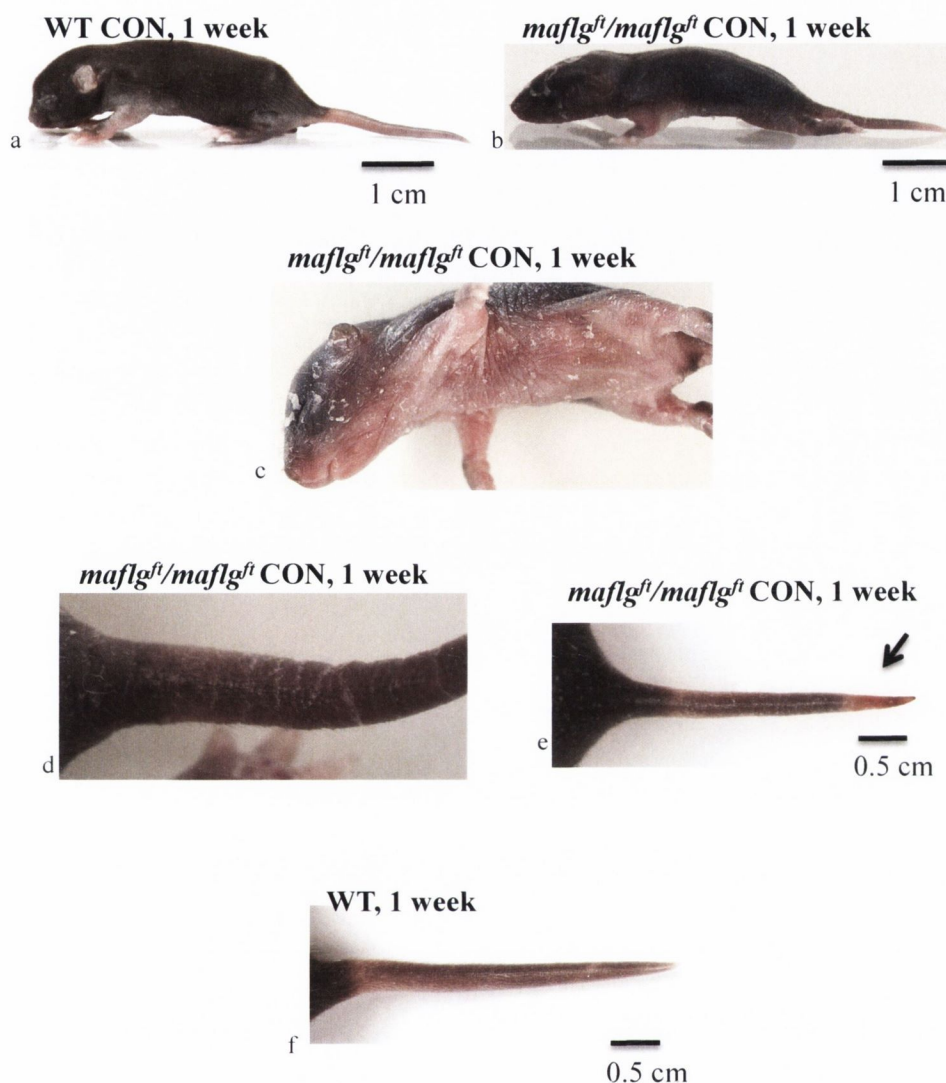


Figure 3.2.1 Neonatal phenotype in *maflg^{fl}/maflg^{fl}* mice. WT mouse at 1 week (a), the skin phenotype of *maflg^{fl}/maflg^{fl}* mice at 1 week (b, c). A magnified view of annular constrictions and flaking on the tail of a 1 week old *maflg^{fl}/maflg^{fl}* mouse is shown (d), the erythematous tip of the tail of a *maflg^{fl}/maflg^{fl}* mouse at 1 week old, about to undergo necrosis and auto-amputation (e). The tails of WT mice (c) were compared to *maflg^{fl}/maflg^{fl}* mice (f) both at 1 week. The photographs are representative of the mice analysed in each subset.

3.2.2 Tail changes in *maflg^{fl}/maflg^{fl}* mouse phenotype

Tail auto-amputation is one of the earliest features of the *maflg^{fl}/maflg^{fl}* phenotype. It occurs in the neonatal period with erythema and necrosis of the tail-tip (Figure 3.2.3). By adulthood there is 100% tail auto-amputation (Figure 3.2.3). Figure 3.2.3 compares neonatal and adult tails of WT control mice and *maflg^{fl}/maflg^{fl}* mice. The tail surfaces of the control mice are smooth and the tail-tips taper to narrow points. In the *maflg^{fl}/maflg^{fl}* phenotype, the tail-tip became erythematous at 1 week (Figure 3.2.3). The tapered tip of the neonatal *maflg^{fl}/maflg^{fl}* mouse tail crusted during the onset of auto-amputation that occurs within the first 3 weeks but usually within the first 14 days. By 32 weeks, the tail of the *maflg^{fl}/maflg^{fl}* mouse became dry with discrete scale and concentric rings. The tail tip has become attenuated and amputated (Figure 3.2.3).

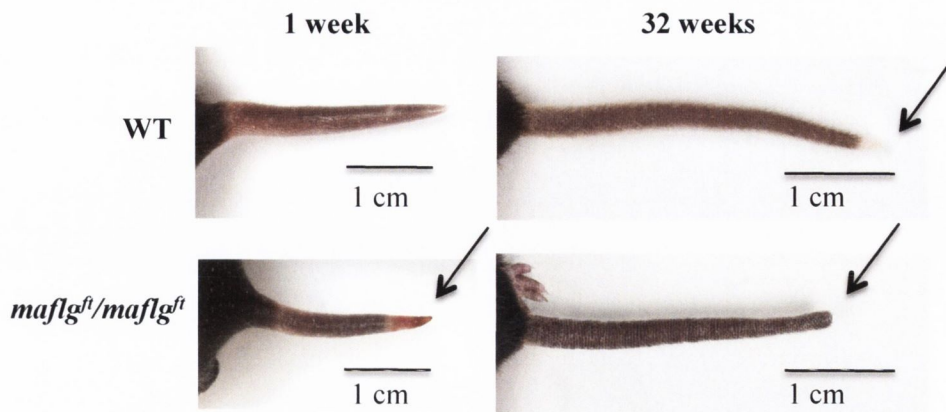


Figure 3.2.2 Tail auto-amputation in the *maf1g^{fl}/maf1g^{fl}* mouse. The tails of WT mice (top row) and *maf1g^{fl}/maf1g^{fl}* mice (bottom row) are compared at both 1 week (left column) and 32 weeks (right column). These photographs are representative of 6 mice per *maf1g^{fl}/maf1g^{fl}* groups and 4 mice per WT groups analysed.

3.2.3 Adult *maflg^{fl}/maflg^{fl}* mouse clinical phenotype

Differences in physical features of the *maflg^{fl}/maflg^{fl}* mice were seen to continue into adulthood at 32 weeks (Figure 3.2.3). The consequences of the genetic mutations involved in the *maflg^{fl}/maflg^{fl}* mouse included the development of alopecia at areas of skin inflammation (Figures 3.2.3 a, b). Hair pigmentation became lighter in the *maflg^{fl}/maflg^{fl}* mouse and changed from the black/brown colour of the WT normal sibling to a russet brown colour (Figures 3.2.3 c, d). The pinnae of the *maflg^{fl}/maflg^{fl}* mouse also emphasised the differences from WT subjects. As the ears were smaller than those of normal siblings in the neonatal period due to slower epidermal growth, they remained shortened in adulthood (Figures 3.2.2 e, f). Stunted pinnae are evident in comparison to WT control mice (Figure 3.2.2 e, f). A spectrum of periorbital inflammation also developed in the *maflg^{fl}/maflg^{fl}* model (Figures 3.2.3 e, f, Figure 3.2.4). The periorbital area became oedematous with associated alopecia, conjunctivitis and blepharitis (Figures 3.2.2 e, f, Figure 3.2.4 b).

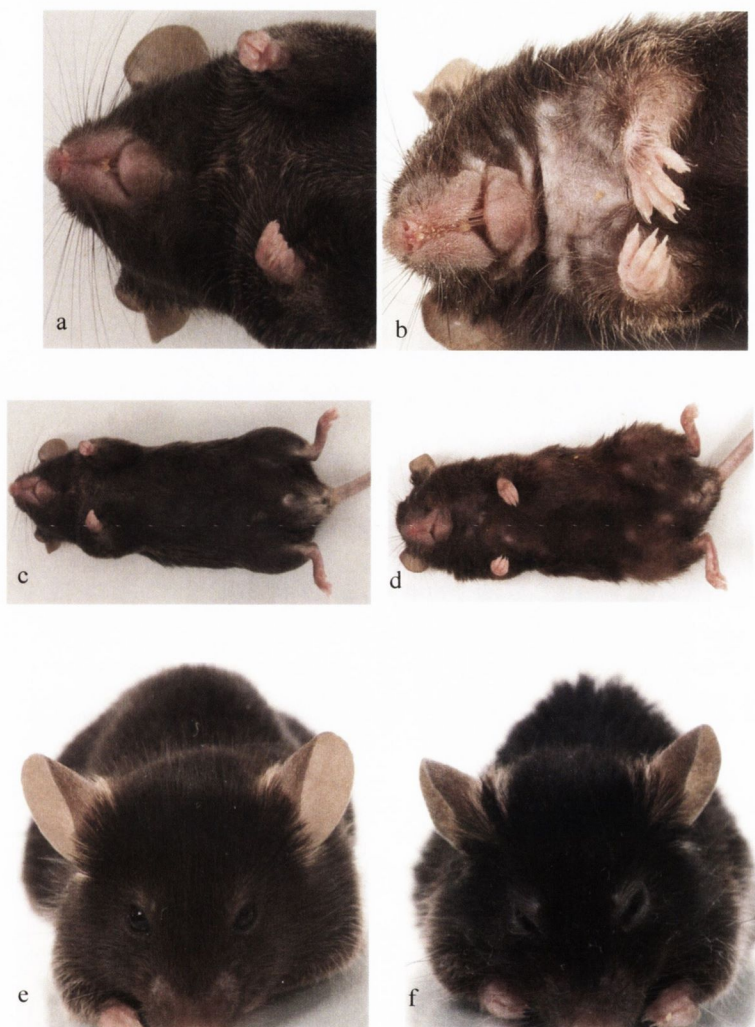
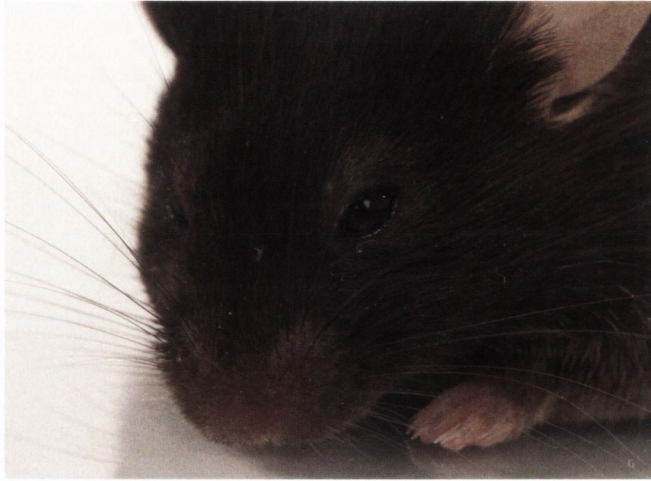
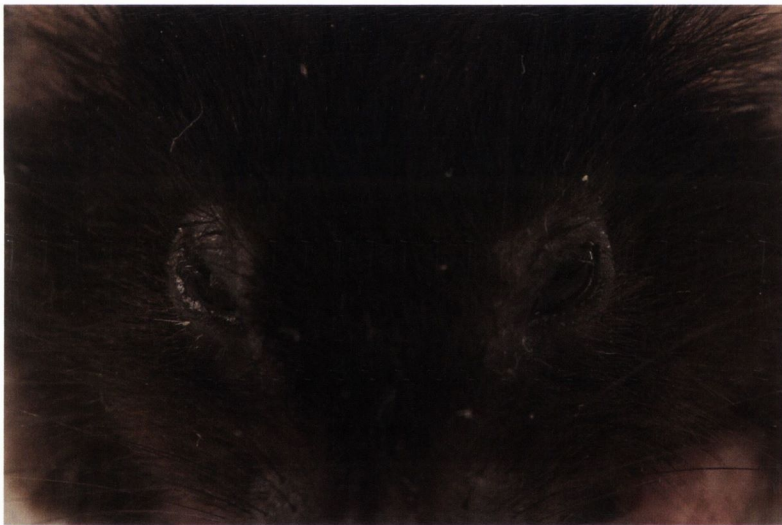


Figure 3.2.3 Adult phenotype in *maflg^{fl}/maflg^{fl}* mouse model. Hair growth on a 32 week WT mouse (a) is compared to a 32 week *maflg^{fl}/maflg^{fl}* mouse (b). Hair pigmentation on a 32 week WT (c) is compared to that of a *maflg^{fl}/maflg^{fl}* mouse (d). Pinnae and eyes of a 32 week old WT mouse (e), are compared to those of a 32 week old *maflg^{fl}/maflg^{fl}* mouse.



WT CON, 32 weeks



***maflg^{fl}/maflg^{fl}* CON, 32 weeks**

Figure 3.2.4 Periorbital area and eyes of a WT and a *maflg^{fl}/maflg^{fl}* mouse, CON conditions (32 weeks). The normal eyes and periorbital areas of the WT mouse are in comparison to the periorbital oedema, alopecia and infection in the eyes of the *maflg^{fl}/maflg^{fl}* mouse.

3.2.4 Alteration in *maflg^{fl}/maflg^{fl}* mouse clinical phenotype in different environments

When the *maflg^{fl}/maflg^{fl}* mice were imported to the Animal Unit in TCD from the Jackson Laboratory, Bar Harbor, Maine, U.S.A. they were back-crossed to C57BL/6J and then brothers/sisters were separated and genetically identical sibling pairs were bred in 2 SPF facilities – the transgenic breeding animal unit (CON) and the facility where the cages were administered filtered air under positive pressure (Techniplast, Buguggiate, VA, Italy) (IVC). It was noted when genetically identical *maflg^{fl}/maflg^{fl}* mice were then bred in both facilities that there were differences in severity of overt skin inflammation between the *maflg^{fl}/maflg^{fl}* mice in the 2 different conditions. Environmental agents feature as exacerbating factors of disease in AD. Populations of the *maflg^{fl}/maflg^{fl}* subjects and WT controls were bred in the 2 different environments: conventional, open caging (CON) and individually ventilated sealed caging (Techniplast, Buguggiate, VA, Italy) (IVC). This was to investigate if environmental conditions influence the development of phenotype in skin barrier deficiency.

3.2.4.1 Changes in neonatal *maflg^{fl}/maflg^{fl}* mouse clinical phenotype in different environments

Control mice in both locations had identical features when measured at 32 weeks, with no overt pathology. The *maflg^{fl}/maflg^{fl}* mice in the CON unit developed phenotypical features of the *maflg^{fl}/maflg^{fl}* profile of a more frequent and severe nature in comparison to those in the IVC housing. In the neonatal period, cardinal, gross early features of *maflg^{fl}/maflg^{fl}* mice as detailed above were evident within the first week in mice in both environments (Figure 3.2.4.1). The gross appearance of neonatal *maflg^{fl}/maflg^{fl}* pups (1 week) revealed more marked skin flaking and hyperlinearity in *maflg^{fl}/maflg^{fl}* mice in

CON conditions relative to *maflg^{fl}/maflg^{fl}* mice housed in the IVC environment as illustrated in Figure 3.2.4.1.

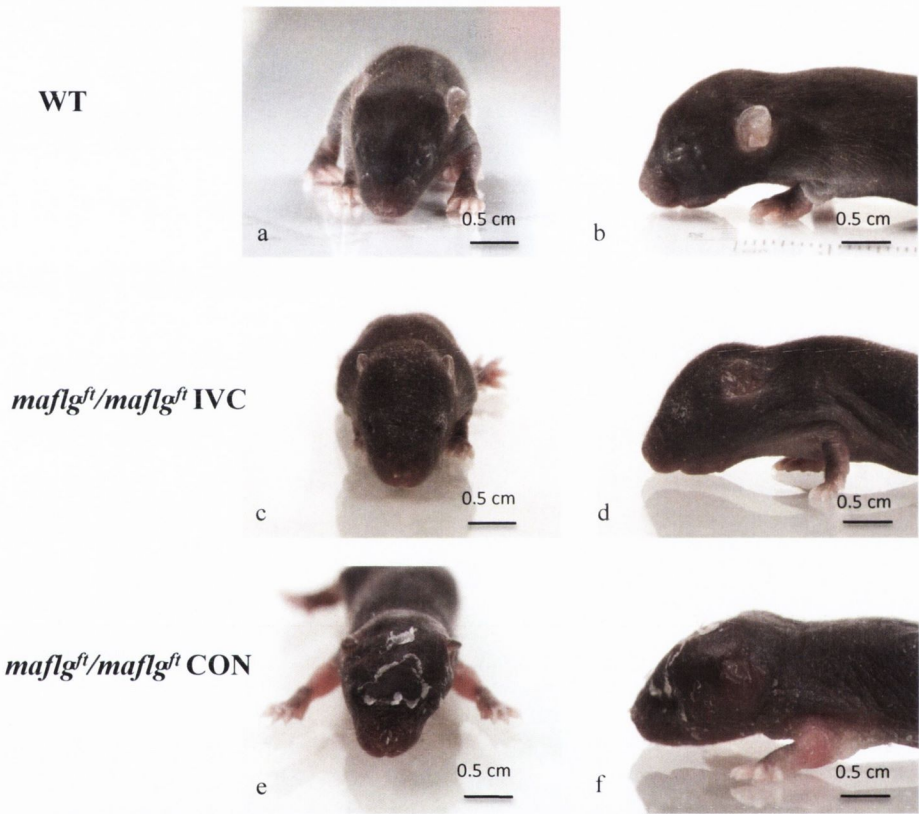


Figure 3.2.4.1: Changes in the neonatal *maflg^{fl}/maflg^{fl}* mouse clinical phenotype in different environment. Mice were housed in varying environments and their clinical phenotype was photographed at 1 week. The subsets included WT IVC (a, b), *maflg^{fl}/maflg^{fl}* IVC (c, d), *maflg^{fl}/maflg^{fl}* CON (e, f)

3.2.4.2 Tail changes in *maflg^{fl}/maflg^{fl}* mouse clinical phenotype in different environments

Tail-tip auto-amputation occurred in the populations of *maflg^{fl}/maflg^{fl}* mice in both environments. Tail changes such as tail erythema, constrictions, attenuation and amputation were noted weekly until weaning at 28 days. Tail auto-amputation was found to occur at a significantly earlier age in the CON environment when compared with the *maflg^{fl}/maflg^{fl}* population in the IVC unit ($p < 0.001$) (Figure 3.2.4.2).

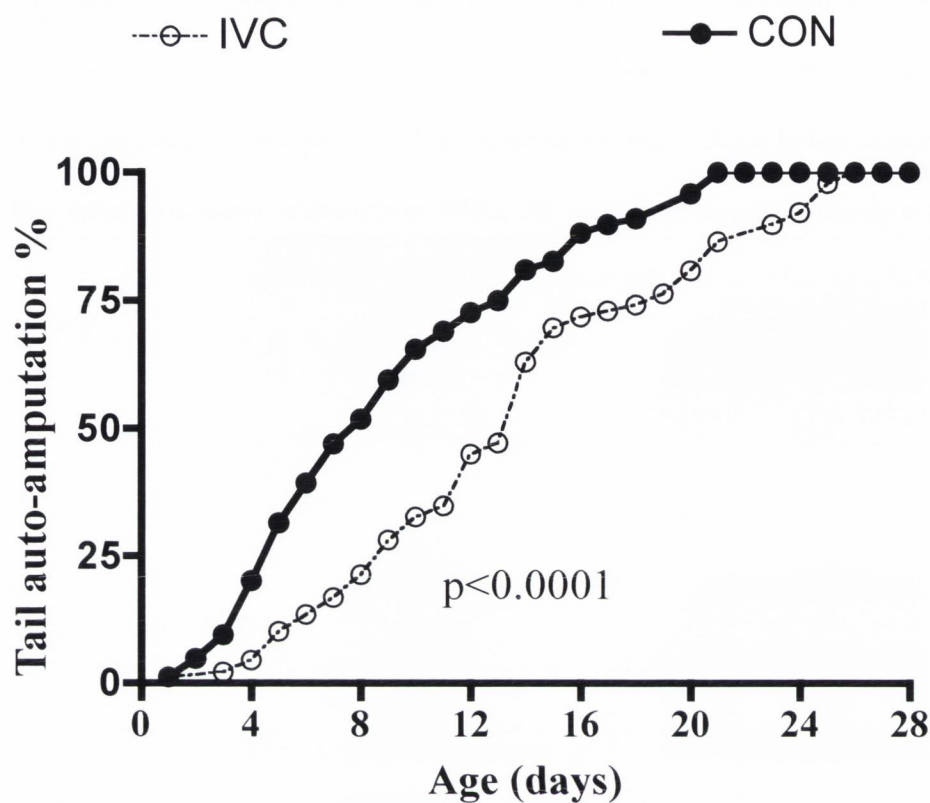


Figure 3.2.4.2 Timing of tail-tip auto-amputation in *maflg^{fl}/maflg^{fl}* mice bred in IVC and CON facilities. *maflg^{fl}/maflg^{fl}* mice bred in differing environmental conditions were analysed to determine the relative timing of tail-tip auto-amputation, and the data is plotted as days of age at which the tail-tip auto-amputation occurred. Statistical analysis was undertaken using the Spearman rank correlation, two-tailed p value as indicated.

3.2.4.3 Alteration in adult *maflg^{fl}/maflg^{fl}* mouse phenotype in different environments

The more marked inflammation in neonatal *maflg^{fl}/maflg^{fl}* mice in CON conditions, relative to IVC conditions, extended into adulthood. At 32 weeks *maflg^{fl}/maflg^{fl}* mice that had been bred and housed in the CON unit had more severe overt skin pathology. These features included dry skin, patches of alopecia, erythematous patches, fissuring and crusting at some patches of active inflammation, periocular alopecia, periorbital erythema, oedema and infection, with associated blepharitis and conjunctivitis (Figure 3.2.4.3). At 32 weeks, the clinical severity of any skin lesions of WT and *maflg^{fl}/maflg^{fl}* mice groups from both environments was assessed using a modified clinical scoring system (Yamamoto et al., 2009) which assessed cutaneous erythema; oedema; xerosis and scaling; and erosion; ocular alopecia, oedema and peri-ocular and conjunctival infection; tail-tip changes (Table 2.2). Clinical phenotypical changes were evident on general inspection in adult *maflg^{fl}/maflg^{fl}* mice (Figure 3.2.4.3). Figure 3.2.4.3 demonstrates that while these phenotypical changes occurred in *maflg^{fl}/maflg^{fl}* mice in both environments, a significantly higher level of dermatitis-like skin inflammation occurred in the *maflg^{fl}/maflg^{fl}* mice from the CON environment compared to the *maflg^{fl}/maflg^{fl}* mice bred in the IVC units. Data is represented by the mean score + the standard error of the mean and is representative of 2 separate experiments. Unpaired two-tailed t test, $p < 0.02$ (Figure 3.2.4.4).

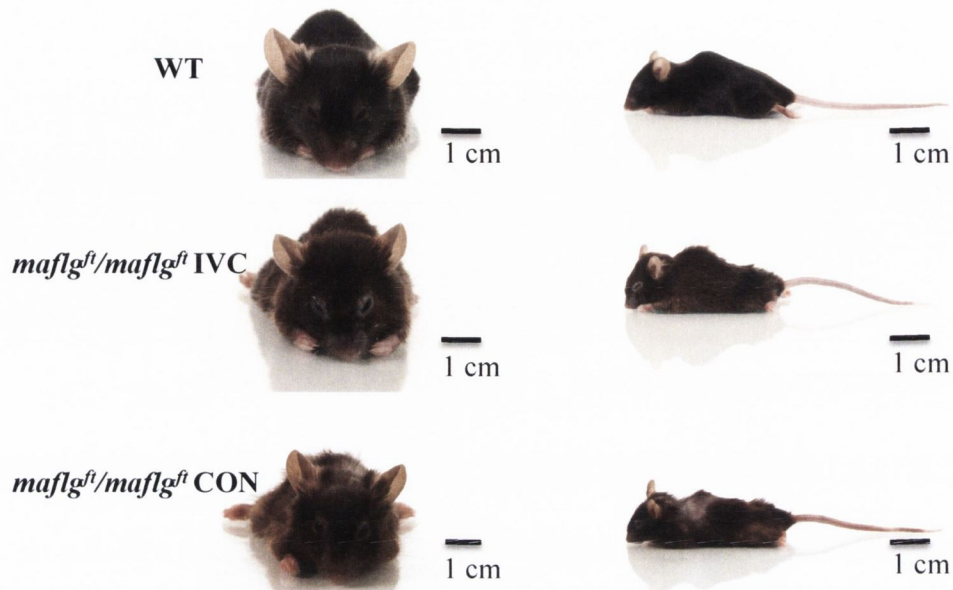


Figure 3.2.4.3 Alteration in adult *maflg^{fl}/maflg^{fl}* phenotype in different environments. Physical and phenotypical features of WT mice and *maflg^{fl}/maflg^{fl}* mice in IVC and CON conditions were analysed at 32 weeks and photographed.

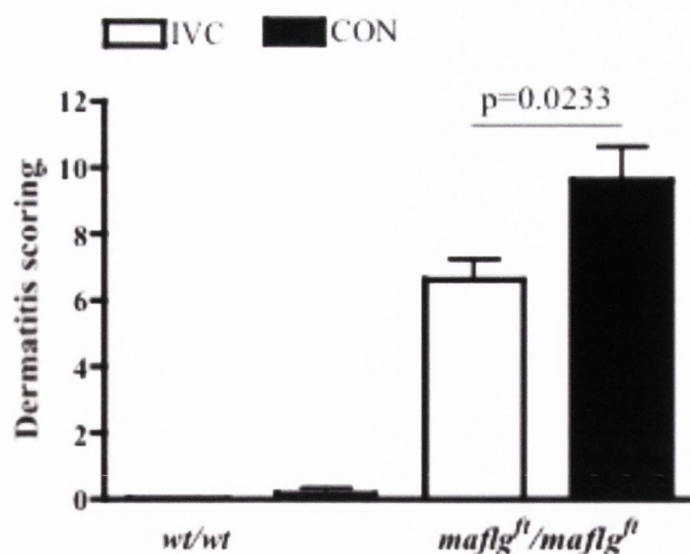
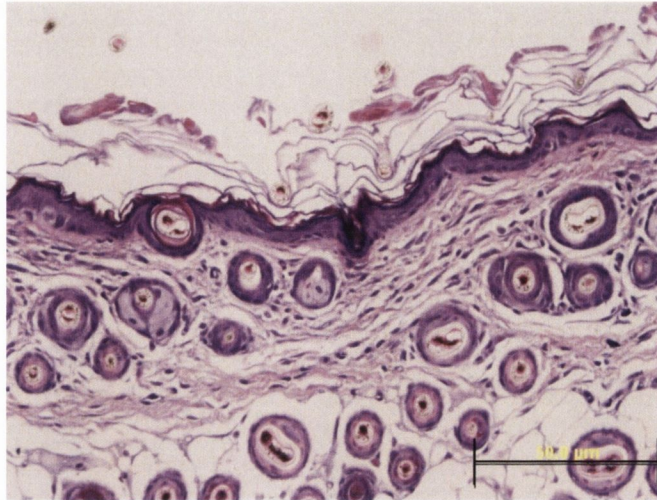


Figure 3.2.4.4 Clinical dermatitis scoring in *maflg^{fl}/maflg^{fl}* mice in IVC and OPEN units at 32 weeks. A comparison of clinical scoring between WT mice (N=2, IVC; N=2 CON) and *maflg^{fl}/maflg^{fl}* mice (N=6, IVC; N=6, CON) at 32 weeks is shown. Data is presented by the mean score and the standard error of the mean, and is representative of 2 separate experiments. Statistical analysis was undertaken using the unpaired student t-test, two-tailed *p* values as indicated.

3.3 Cutaneous histology of the *maflg^{fl}/maflg^{fl}* mouse phenotype

Skin samples from abdominal skin of the *maflg^{fl}/maflg^{fl}* mouse groups were biopsied. The paraffin-fixed skin sections were stained with haematoxylin and eosin (H&E), which illustrated inflammatory changes in the *maflg^{fl}/maflg^{fl}* mice from all environments, the quantification of these changes is detailed in Section 2.5.1. As described in previous studies, histological analysis of skin biopsies from *maflg^{fl}/maflg^{fl}* mice demonstrate diffuse orthokeratotic hyperkeratosis, foci of acanthosis, epidermotropism, an attenuated granular layer that lacks F-granules, dermal lymphocytic and mast cell infiltration at different magnitudes of severity (Presland et al., 2000, Fallon et al., 2009). Figure 3.3 highlights some of the features that were found in this experiment following examination of H&E stained WT and *maflg^{fl}/maflg^{fl}* mouse skin biopsies. Histological inflammatory signs imaged include: epidermotropism, spongiosis and dermal lymphocytic inflammatory infiltration.

WT



maflg^{fl}/maflg^{fl}

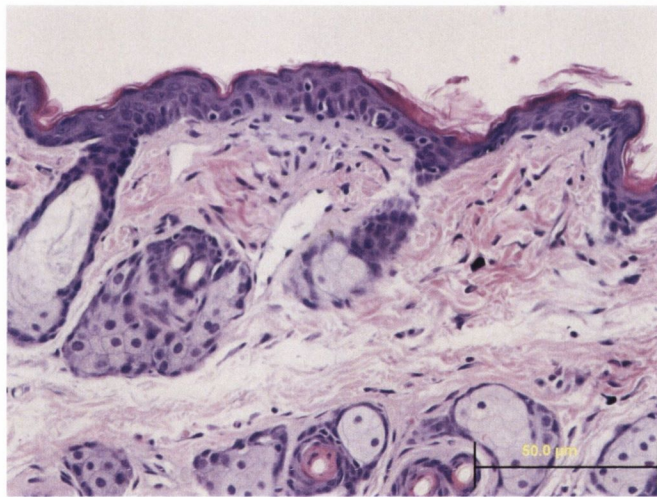


Figure 3.3 Histology of skin from WT and *maflg^{fl}/maflg^{fl}* mice.

Histology of skin biopsies of adult WT and *maflg^{fl}/maflg^{fl}* mice bred in the IVC environment. Stained with H&E. These images demonstrate some of the histological features of cutaneous inflammation including hyperkeratosis, dermal cellular infiltration including dermal lymphocytic and mast cell infiltration, epidermotropism and spongiosis. 50 μm scale as indicated, 20x magnification.

3.3.1 Cutaneous histology of the *maflg^{fl}/maflg^{fl}* mouse phenotype in different environments

Figure 3.3.1.1 shows H&E - stained skin biopsies from WT and *maflg^{fl}/maflg^{fl}* mice at 4 weeks, from IVC and CON units. Histology sections of this skin demonstrated typical cutaneous inflammatory changes in *maflg^{fl}/maflg^{fl}* mice with more marked parakeratotic hyperkeratosis, monocytic, lymphocytic and polynuclear cellular infiltration, in skin biopsies from *maflg^{fl}/maflg^{fl}* mice housed in CON conditions compared to IVC conditions. Skin biopsy histopathology from WT and *maflg^{fl}/maflg^{fl}* mice at 32 weeks is shown in Figure 3.3.1.2, which displays the range of cutaneous pathology on the H&E staining of the skin sections. Increased dermal inflammation was found at 4 weeks and 32 weeks in the skin samples of *maflg^{fl}/maflg^{fl}* mice from the CON environment compared to both WT controls and the *maflg^{fl}/maflg^{fl}* mice from the IVC conditions (Figures 3.3.1.1, 3.3.1.2). Mast cell infiltration is frequently associated with atopy, so sections of skin of both WT and *maflg^{fl}/maflg^{fl}* mice were stained with toluidine blue stain, to assess mast cell infiltration (Figure 3.3.1.3). Figure 3.3.1.3 demonstrates toluidine blue staining of skin biopsies from WT and *maflg^{fl}/maflg^{fl}* mice at 32 weeks, from both the IVC and the CON environments where increased mast cells were seen on microscopy in the *maflg^{fl}/maflg^{fl}* skin slides from the CON environment compared to *maflg^{fl}/maflg^{fl}* skin samples from the IVC unit and WT controls (Figure 3.3.1.3).

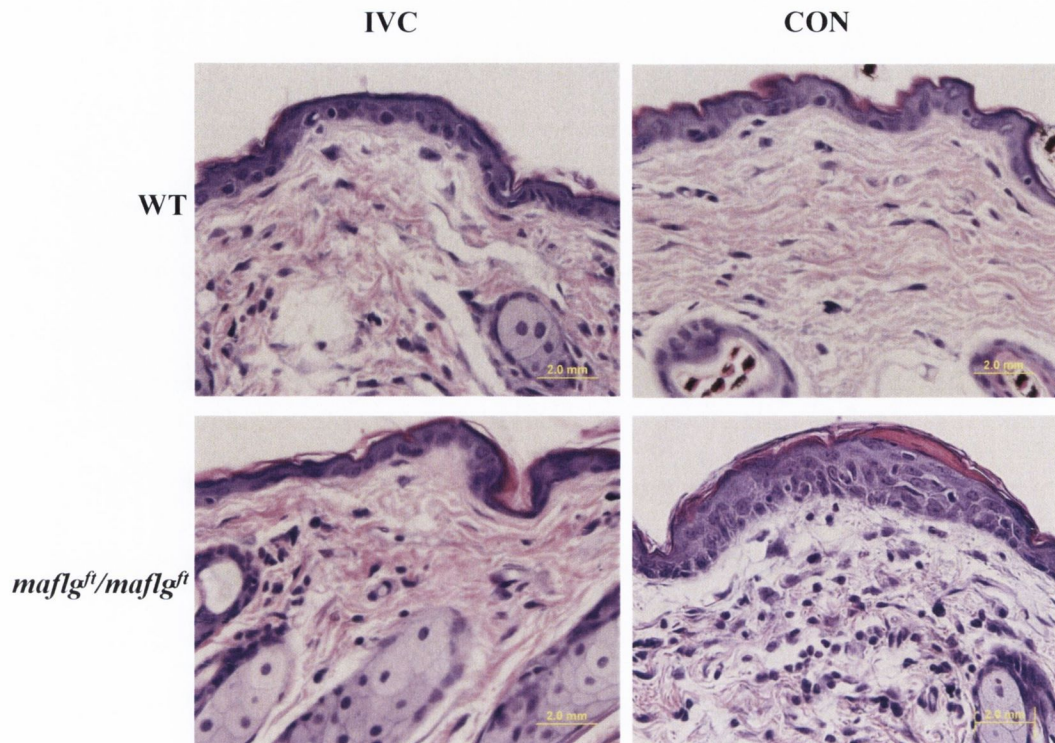


Figure 3.3.1.1 Histology of skin from WT and *maflg^{fl}/maflg* mice in different environments (4 weeks). Histology of skin biopsies stained with H&E of WT and *maflg^{fl}/maflg* mice at 4 weeks, from both IVC and CON environments highlighting increased inflammatory cell infiltrates in skin biopsy samples from *maflg^{fl}/maflg* mice from the CON environment. 2.0 mm scale as indicated, 40x magnification.

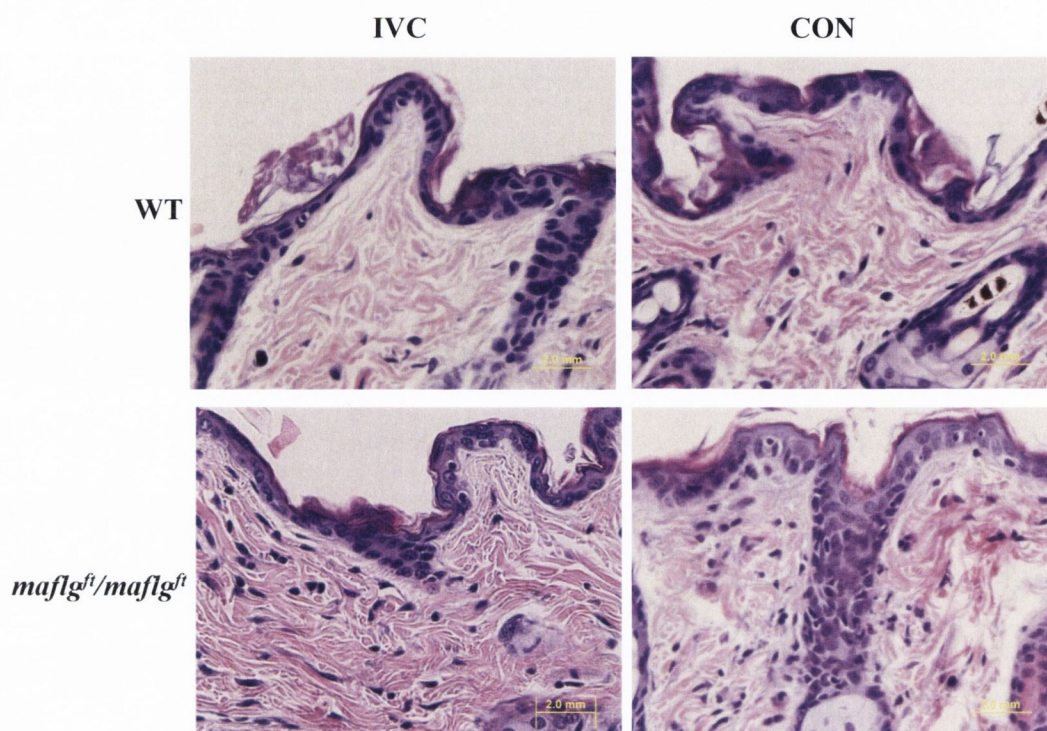


Figure 3.3.1.2 Histology of skin from WT and *maf1g^{fl}/maf1g^{fl}* mice in different environments (32 weeks). Skin biopsies, stained with H&E, were taken from WT and *maf1g^{fl}/maf1g^{fl}* mice from IVC and CON environments, at 32 weeks. Features of inflammation in the CON *maf1g^{fl}/maf1g^{fl}* specimens, in particular are: epidermotropism and spongiosis, dermal lymphocytic and polymorphonuclear cellular inflammatory infiltration. 2.0 mm scale as indicated, 40x magnification.

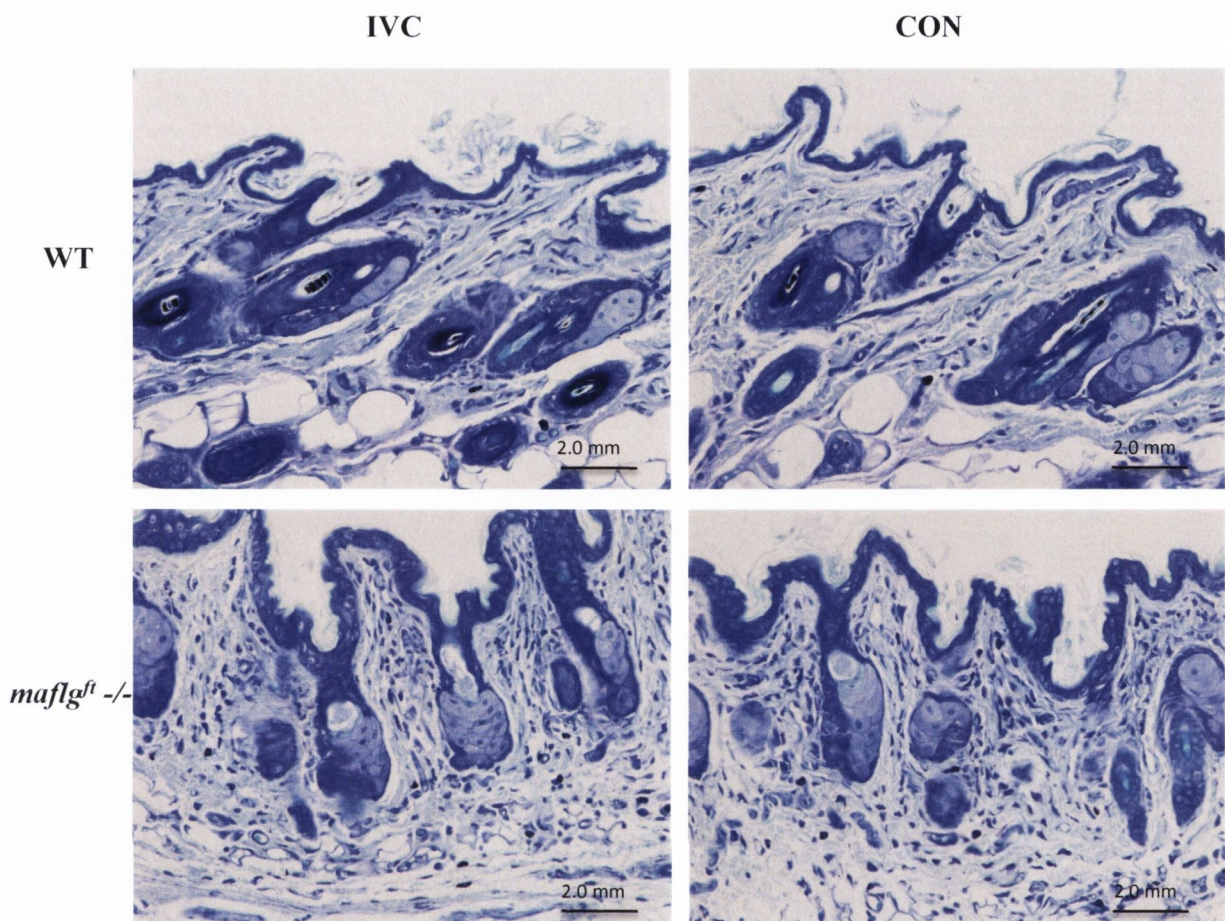


Figure 3.3.1.3 Histology of skin highlighting mast cell infiltration in WT and *maflg^{fl}/maflg^{fl}* mice at 32 weeks. Skin biopsies, stained with toluidine blue stain, were taken from WT and *maflg^{fl}/maflg^{fl}* mice from IVC and CON environments at 32 weeks. Mast cell infiltration is highlighted with this stain. 2.0 mm scale as indicated, 40x magnification.

3.3.2 Scoring of histological changes in *maflg^{fl}/maflg^{fl}* mouse phenotype in different environments

The H&E stained skin sections were further analysed at each time point (at 1, 4, 8, 20 and 32 weeks) to quantify inflammatory infiltration (15-20 HPFs per slide) (Section 2.5.1). Significant differences in cutaneous inflammation were identified between *maflg^{fl}/maflg^{fl}* mice and their corresponding WT controls at 4 weeks (Figure 3.3.2.1). Increased inflammatory cell infiltration, in particular, dermal lymphocytic inflammatory infiltration, as well as increased markers of inflammation including epidermotropism and spongiosis were seen in *maflg^{fl}/maflg^{fl}* mice samples from the CON environment compared to the IVC housing at 4 weeks, (unpaired T-test, two-tailed *P* - value = 0.0002) (Figure 3.3.2.1). The *maflg^{fl}/maflg^{fl}* mouse samples from the CON environment continued to have higher levels of inflammation at older ages although the significant difference between the groups of *maflg^{fl}/maflg^{fl}* mice waned with age (Figure 3.3.2.1).

Epidermal and dermal lymphocytes were counted per slide (20 HPFs per section) as another measure of cutaneous inflammation in the IVC and CON environments. A significant increase in lymphocytic cellular infiltration in *maflg^{fl}/maflg^{fl}* skin biopsies from the CON unit compared to those from the IVC environment was shown at all age-groups analysed, unpaired two-tailed t-test *p* values: 4 weeks (*p* < 0.01), 8 weeks (*p* < 0.05), 20 weeks (*p* < 0.05), 32 weeks (non-significant) (Figure 3.3.2.2).

There was a significant increase in mast cell infiltration in *maflg^{fl}/maflg^{fl}* mice from the CON environment compared to their WT controls in the same unit at 4 weeks (unpaired T-test, two-tailed *p* - value = 0.05) (Figure 3.3.2.3). A significant increase in mast cells

in samples from $maflg^{ft}/maflg^{ft}$ mice from the CON environment compared to $maflg^{ft}/maflg^{ft}$ mice from the IVC unit was seen also at 20 weeks (unpaired t-test, two-tailed p value = 0.0064) (Figure 3.3.2.3).

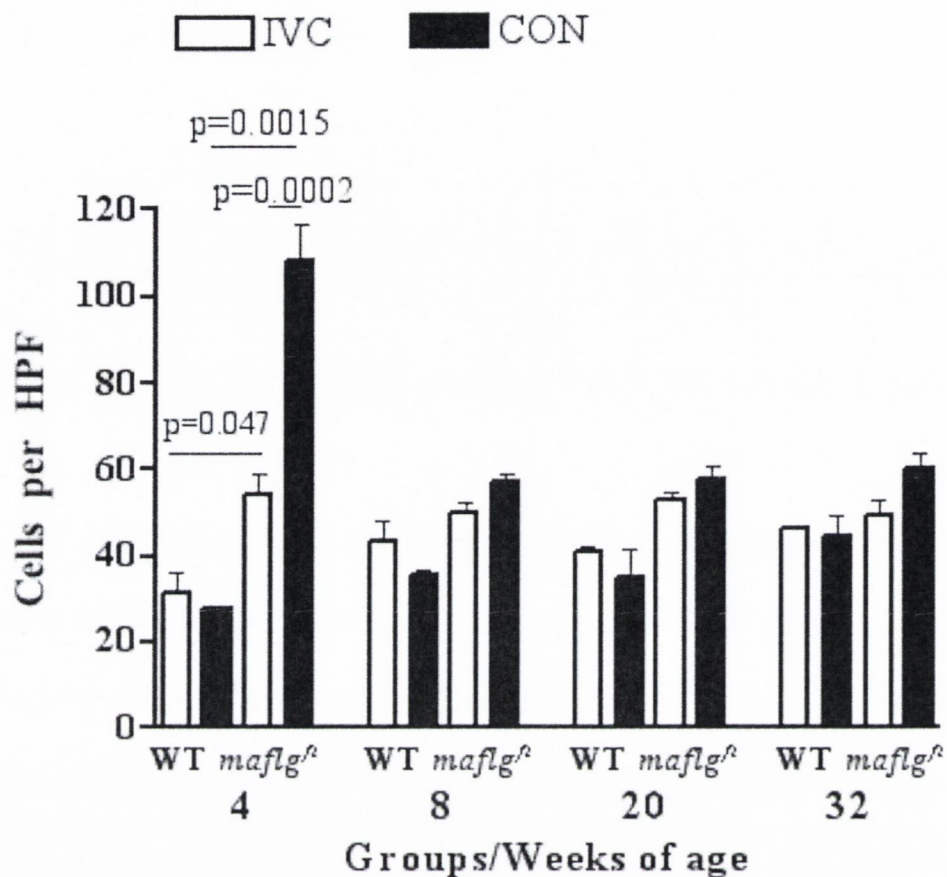


Figure 3.3.2.1 Total inflammatory cell count scoring of histological changes in WT and *maflg^{fl}/maflg^{fl}* mice in different environments. Skin biopsy analysis of WT and *maflg^{fl}/maflg^{fl}* mice from IVC and CON environments, at 4, 8, 20 and 32 weeks. Data is presented as the mean + / - SEM and is representative of 2 (WT) to 6 (*maflg^{fl}/maflg^{fl}*) mice. Statistical analysis was undertaken using the unpaired student t-test, two-tailed *p* values as indicated.

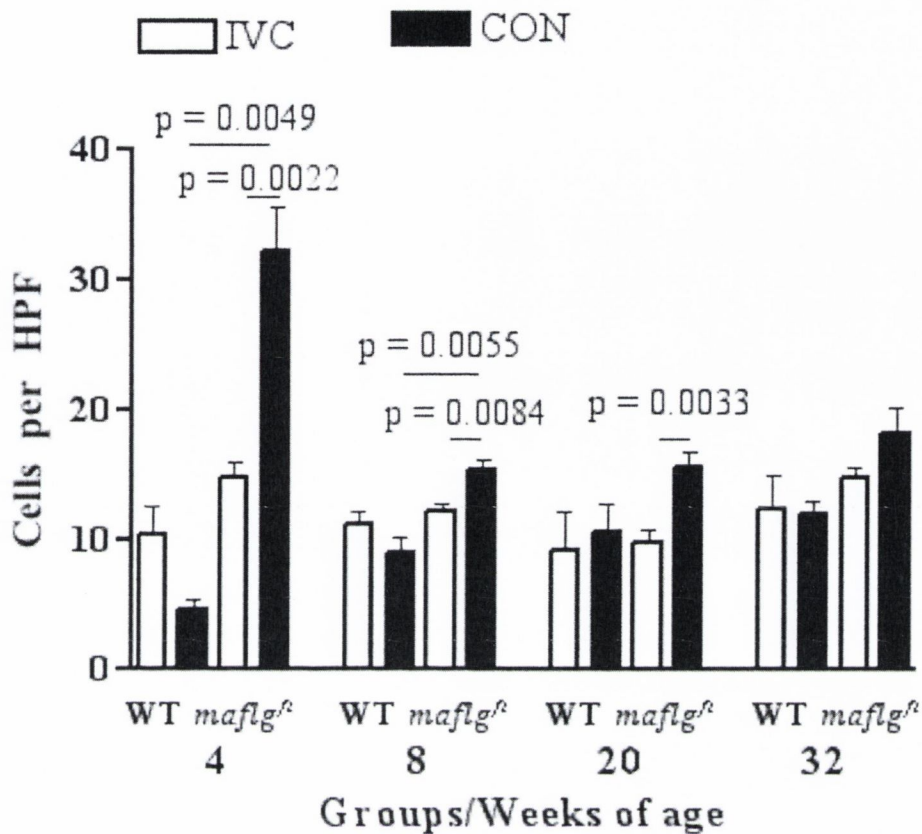


Figure 3.3.2.2 Cutaneous lymphocytic infiltrative histological scoring in WT and *maf1g^f/maf1g^f* mice in different environments. Cellular lymphocytic infiltration of H&E stained skin sections of WT and *maf1g^f/maf1g^f* mice from both environments at 4, 8, 20 and 32 weeks. Data is presented as the mean + / - SEM and is representative of 2 (WT) to 6 (*maf1g^f/maf1g^f*) mice. Statistical analysis was undertaken using the unpaired student t-test, two-tailed *p* values as indicated.

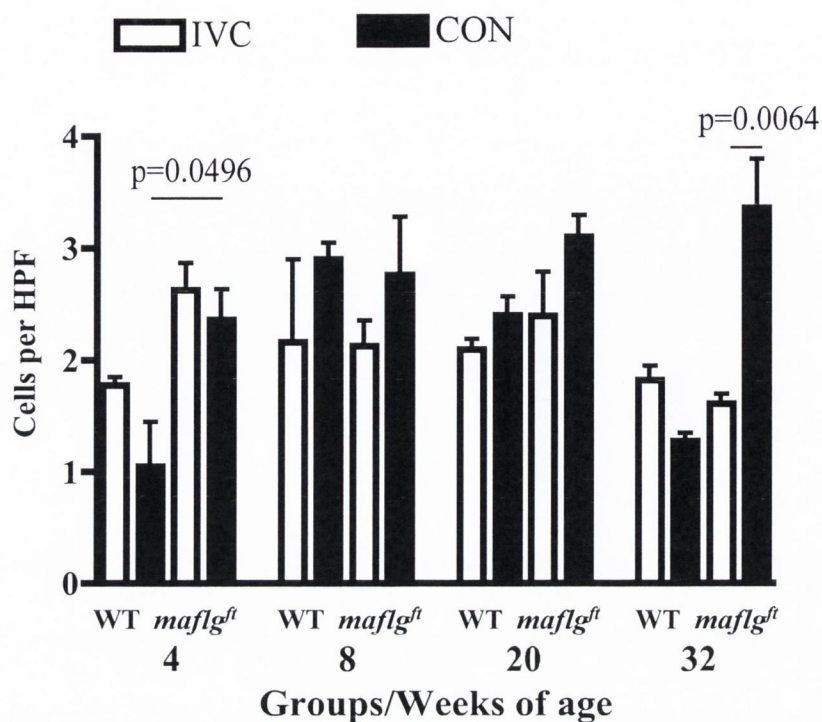


Figure 3.3.2.3 Cutaneous mast cell infiltration scoring in WT and *maf1g^{fl}/maf1g^{fl}* mice in different environments. Mast cell infiltration of toluidine blue stained skin biopsies, of WT (N = 2 per subset) and *maf1g^{fl}/maf1g^{fl}* (N = 6 per subset) mice, from both environments, at 4, 8, 20 and 32 weeks. Slides were scored by mast cell count per HPF, scoring 20 HPFs per slide. Statistical analysis was undertaken using the unpaired student t-test, two-tailed *p* values as indicated.

3.4 Antibody responses in the *maflg^{fl}/maflg^{fl}* mouse phenotype in different environments

The serum antibody levels in mice exposed to both environmental conditions were analysed from weeks 4 – 32, as detailed in sections 2.6 and 2.6.1. Levels of total IgE, IgG₁ and IgG_{2a} were investigated by sandwich ELISA. Significance was assessed with the unpaired t-test, two-tailed *p* value. *maflg^{fl}/maflg^{fl}* mice in both CON and IVC housing showed spontaneously elevated levels of total serum IgE from 8 weeks. However, the *maflg^{fl}/maflg^{fl}* mice from the CON caging had significantly higher levels of IgE from 20 weeks when compared to *maflg^{fl}/maflg^{fl}* mice from IVC housing [unpaired t-test, two-tailed *p* value < 0.0006 (20 weeks), *p* value < 0.0233 (32 weeks)] (Figure 3.4). Total serum IgG₁ and IgG_{2a} were measured as these antibodies are components of the Th2 and Th1 responses, respectively. At 32 weeks, IgG₁ expression was significantly higher from the *maflg^{fl}/maflg^{fl}* mice compared to WT mice (unpaired t-test, two-tailed *p* value = 0.0463). IgG_{2a} was seen to increase in level as the mice became older and were of elevated values compared to WT, (unpaired t-test two-tailed *p* value = 0.0074, 8 weeks; *p* = 0.0003, 20 weeks) (Figure 3.4). No significant differences between *maflg^{fl}/maflg^{fl}* mice groups from the CON and IVC environments were found on analysis of IgG₁ expression (Figure 3.4). Expression of IgG_{2a} was found to be significantly increased at 8 weeks and 20 weeks from the *maflg^{fl}/maflg^{fl}* mice bred in the CON environment compared to the *maflg^{fl}/maflg^{fl}* population in the IVC housing (Figure 3.4). Although there was higher expression of IgG_{2a} at 32 weeks from the *maflg^{fl}/maflg^{fl}* mice from the CON environment compared to the *maflg^{fl}/maflg^{fl}* samples from the IVC unit, this was not found to be significant (Figure 3.4).

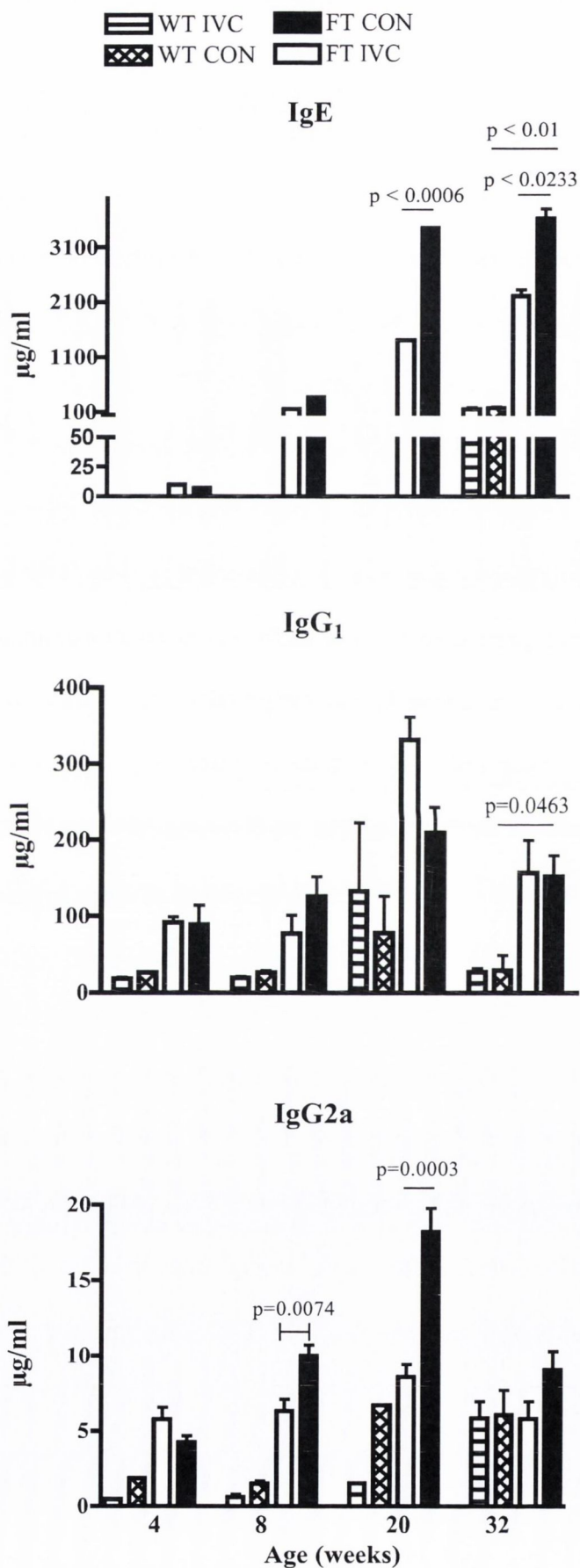


Figure 3.4 Total serum antibodies expression of WT and *maflg^{fl}/maflg^{fl}* mice in different environments. At 4, 8, 20 and 32 weeks, serum was extracted and analysed from *maflg^{fl}/maflg^{fl}* mice, from the CON and IVC environments. Levels of total IgE, IgG₁ or IgG_{2a} were quantified. Data is presented as mean = / - SEM and is representative of 2 (WT) to 6 (*maflg^{fl}/maflg^{fl}*). Statistical analysis was undertaken using the unpaired student t-test, two-tailed *p* - values were considered significant if *p* < 0.05.

3.5 Spleen cytokine responses in the *maflg^{fl}/maflg^{fl}* mouse phenotype in different environments

To further define the altered atopic responses in *maflg^{fl}/maflg^{fl}* mice the spleens were removed from 32 week-old mice. Cells were extracted as per Section 2.4.3, counted (Section 2.4.2) and the cells were stimulated either in a general stimulatory fashion with PMA/I (2.5ng/ml/250ng/ml) or by T-cell specific stimulation with anti-CD3 mAb/CD28 (0.5µg/ml/4µg/ml) as per Section 2.4.4. The production of Th1 (IFNγ), Th2 (IL-4) and Th17 (IL-17) cytokines analysed (Section 2.6.2). Cytokine production by spleen cells showed a similar trend to that of lymph node cells, however this result was not statistically significant (Figure 3.5). A consistent pattern of IFNγ expression is found on assessment of spleen and where elevated levels of expression are seen from cell culture expression from *maflg^{fl}/maflg^{fl}* mice from the CON environment in comparison to the *maflg^{fl}/maflg^{fl}* samples from the IVC environment. Interleukin-4 levels were equivalent between the mice when spleen cells were stimulated (Figure 3.5).

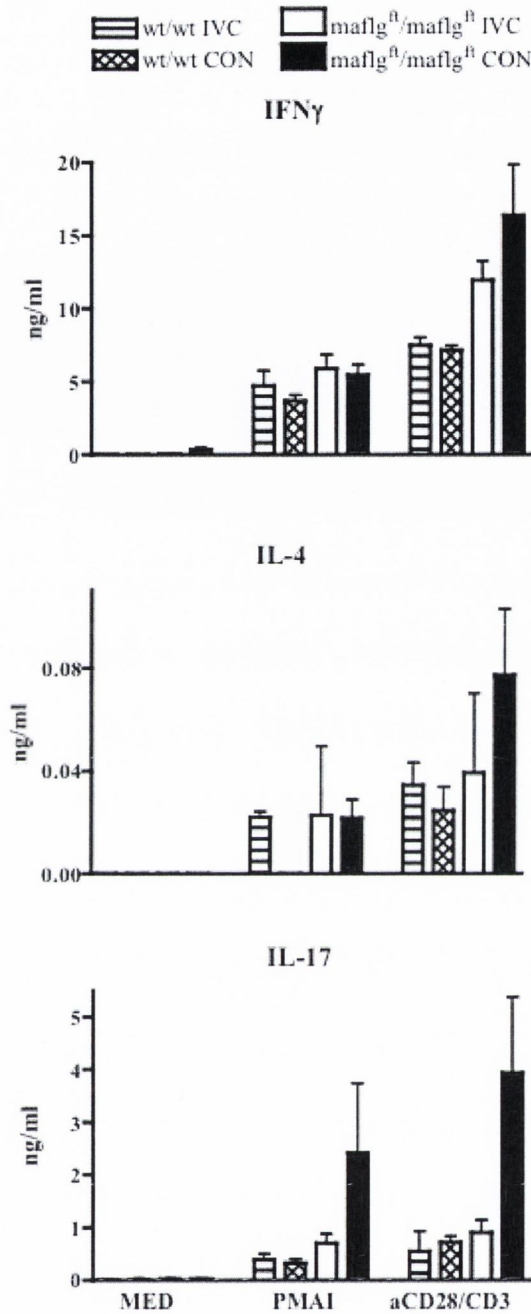


Figure 3.5 Spleen cell expression of cytokines from WT and *maflg^{fl}/maflg^{fl}* mice in different environments. Whole spleens were removed from WT and *maflg^{fl}/maflg^{fl}* mice, housed in IVC and CON environments, at 32 weeks. Cytokine expression from 24-hour (IL-4) and 72-hour (IL-17, IFN γ) cell cultures of these lymph nodes, were measured. Data is presented as the mean + / - SEM and is representative of 2 (WT) to 6 (*maflg^{fl}/maflg^{fl}*) mice. Statistical analysis was done with the unpaired student t-test, two-tailed *p* values as indicated.

3.6 Skin - draining lymph node cytokine responses in the *maflg^{ft}/maflg^{ft}* mouse phenotype in different environments

To investigate the development of immune responses secondary to the local cutaneous immunology to the draining lymph nodes skin-draining submandibular lymph nodes were removed from 32 week-old mice. Cells were extracted as per Section 2.4.3, counted (Section 2.4.2) and the cells were stimulated either in a general stimulatory fashion with PMA/I (2.5ng/ml/250ng/ml) or by T-cell specific stimulation with anti-CD3/CD28 (0.5µg/ml/4µg/ml) as per Section 2.4.4. The production of Th1 (IFN γ), Th2 (IL-4) and Th17 (IL-17) cytokines analysed (Section 2.6.2). Lymph node cells from *maflg^{ft}/maflg^{ft}* mice had greater production of all cytokines relative to cells from WT mice irrespective of polyclonal (PMA/I) or T cell-specific (anti-CD3/CD28) stimulation. However, there was greater production of IFN γ , IL-4 and IL-17 from cells from *maflg^{ft}/maflg^{ft}* mice in CON conditions, relative to *maflg^{ft}/maflg^{ft}* mice housed in IVC conditions, with IL-17 production being particularly enhanced ($p = 0.0199$ (general stimulation), $p = 0.0258$ (T cell specific stimulation)), (Figure 3.6). A consistent pattern of IFN γ expression is found on assessment of skin-draining lymph node T cells where elevated levels of expression are seen from cell culture expression from *maflg^{ft}/maflg^{ft}* mice from the CON environment in comparison to the *maflg^{ft}/maflg^{ft}* samples from the IVC environment. There was a significant increase in values of IFN γ expressed by lymph node cells from the *maflg^{ft}/maflg^{ft}* mice from the CON caging (unpaired t-test, $p = 0.0289$) (Figure 3.6). Interleukin-4 levels were equivalent between the mice when lymph node cells were stimulated (Figure 3.6).

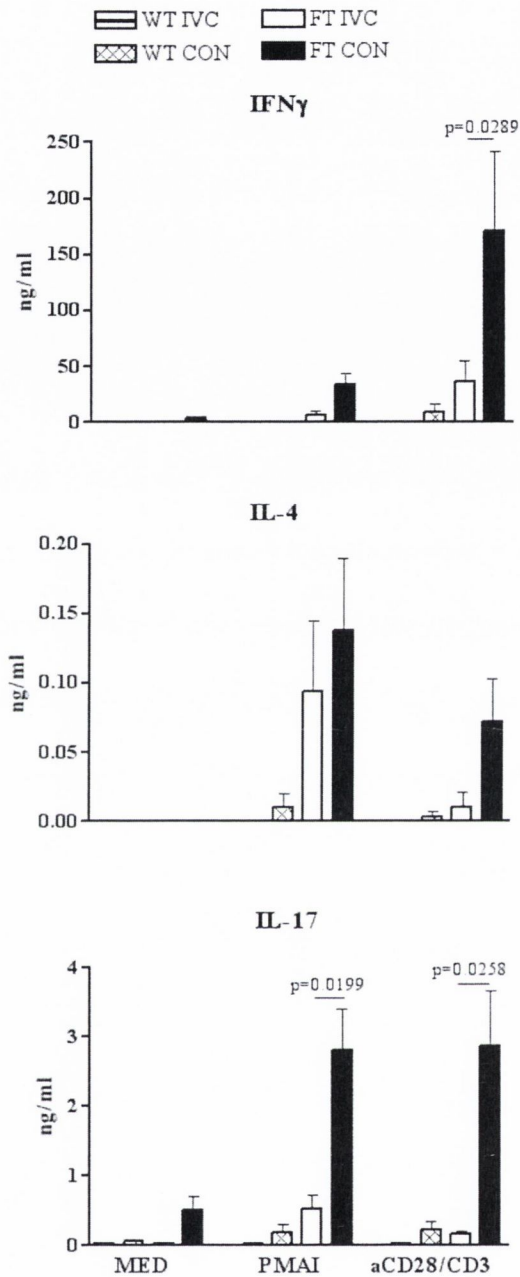


Figure 3.6 Skin-draining lymph node cell expression of cytokines from WT and *maflg^{ft}/maflg^{ft}* mice in different environments. Skin-draining submandibular lymph nodes were removed from WT and *maflg^{ft}/maflg^{ft}* mice, housed in IVC and CON environments, at 32 weeks. Cytokine expression from 24-hour (IL-4) and 72-hour (IL-17, IFN γ) cell cultures of these lymph nodes was measured. Data is presented as the mean $\pm$ SEM and is representative of 2 (WT) to 6 (*maflg^{ft}/maflg^{ft}*) mice. Statistical analysis was done with the unpaired student t-test, two-tailed p - values as indicated.

3.7 Discussion

The results of this chapter further assert what has been shown in other studies, that the *maflg^{fl}/maflg^{fl}* mouse is a model of skin barrier deficiency and AD (Fallon et al., 2009, Moniaga et al., 2010, Oyoshi et al., 2009b, Presland et al., 2000, Scharschmidt et al., 2009). A spectrum of AD-like disease was noted in *maflg^{fl}/maflg^{fl}* mice in both CON and IVC environments. The physical phenotypical features identified correspond to the results of previous studies (Moniaga et al., 2010, Oyoshi et al., 2009b). It was evident from neonatal timepoints through to adulthood that *maflg^{fl}/maflg^{fl}* mice from the CON environment develop the phenotypical inflammatory responses of the *maflg^{fl}/maflg^{fl}* profile of a more frequent and severe nature compared to their littermates in the IVC environment. As in Moniaga *et al.*, a scoring system (Yamamoto et al., 2009) was used to objectively rate the level of clinical signs in the *maflg^{fl}/maflg^{fl}* mice. Assessing the clinical features with a scoring system that has also been used in other models of dermatitis-like inflammation permitted a reliable, quantifiable method to demonstrate what is obvious on clinical inspection: that *maflg^{fl}/maflg^{fl}* mice in the CON unit had more severe features of prolonged cutaneous inflammation into adulthood, with increased and more severe dermatitis-like changes on clinical scoring of signs of inflammation. The scoring system was adapted to take account of particular features of the *maflg^{fl}/maflg^{fl}* mice. Combining tail changes and in particular, the inflammatory changes that occurred in the eyes and periorbital areas in the *maflg^{fl}/maflg^{fl}* profile allowed the scoring system to be thorough in accounting for the various aspects that become affected with inflammation secondary to the skin barrier defect associated with the *maflg^{fl}/maflg^{fl}* phenotype.

The results of the clinical scoring are in line with the structural findings from other studies on *maflg^{fl}/maflg^{fl}* mice (Moniaga et al., 2010, Scharschmidt et al., 2009). Scharschmidt *et al.* investigated organelle secretion by ultrastructural cytochemistry of lipase activity and used it as a marker of skin barrier deficiency in *maflg^{fl}/maflg^{fl}* mice (Scharschmidt et al., 2009). Moniaga *et al.* investigated penetrance of a fluorescein isomer to evaluate and demonstrate skin barrier deficiency in *maflg^{fl}/maflg^{fl}* mice.

The results of the histological analysis presented in this chapter highlighted that there is increased inflammation in the skin of the *maflg^{fl}/maflg^{fl}* mice, which corresponds to previous study of the dermatopathology of the *maflg^{fl}/maflg^{fl}* mouse (Moniaga et al., 2010, Oyoshi et al., 2009b, Scharschmidt et al., 2009). The result of assessment of H&E stained skin sections of *maflg^{fl}/maflg^{fl}* mice from the CON environment showed more evidence of inflammation compared to *maflg^{fl}/maflg^{fl}* mice of the IVC units. Although the mice did not have any specific experimental sensitisation, the cutaneous inflammatory cellular infiltrate was analysed in the *maflg^{fl}/maflg^{fl}* mice and an increased level of cellular infiltration in *maflg^{fl}/maflg^{fl}* mice was noted at the 4 week timepoint. At this stage of life the mice are approximately one-third to half the weight and size of their adult littermates. Their skin is thinner and has less hair growth. This may suggest that transcutaneous penetration of proteins and allergens occurs more readily, stimulating elevated immune responses, which abate in non-lesional adult mouse skin.

The *maflg^{fl}/maflg^{fl}* mouse characteristically has higher levels of total serum IgE indicating increased levels of spontaneous atopy (Moniaga et al., 2010, Oyoshi et al., 2009b, Scharschmidt et al., 2009) and a systemic Th2-driven allergic response (Oyoshi et

al., 2009b). Levels of serum antibodies analysed in WT and *maflg^{fl}/maflg^{fl}* mice as per Section 3.4.1 confirmed this assertion. *maflg^{fl}/maflg^{fl}* mice in both CON and IVC housing developed spontaneous atopy, as analysed at 8 and 32 weeks. These data demonstrated the propensity of *maflg^{fl}/maflg^{fl}* mice to spontaneously develop antibody profiles consistent with an atopic Th2 biased immune responses. *maflg^{fl}/maflg^{fl}* mice in CON conditions had markedly elevated IgE levels relative to *maflg^{fl}/maflg^{fl}* mice in IVC conditions. This suggests that environmental conditions may have a role to play in the aetiology and severity of inflammation in the *maflg^{fl}/maflg^{fl}* mouse. Overall IgE, IgG₁ and IgG_{2a} antibody levels were elevated in the samples of *maflg^{fl}/maflg^{fl}* mice from the CON environment relative to *maflg^{fl}/maflg^{fl}* mice from the IVC environment. The expression of IgG_{2a} in both environments in *maflg^{fl}/maflg^{fl}* mice relative to wild-type mice suggests that a Th1-type response occurs with age, in the presence of skin barrier deficiency. This may indicate a transition from an early Th2-type allergic response to the increased ingress of allergens/pathogens in the context of a skin barrier defect towards a later exaggerated Th1-type response profile to allergen/pathogen exposures.

Specific experimental sensitisation of the *maflg^{fl}/maflg^{fl}* mouse with ovalbumin results in increased expression of IL-17, IL-4, IL-13 and IFN γ as shown by Oyoshi *et al.* (Oyoshi *et al.*, 2009b). The variation in systemic responses of unstimulated *maflg^{fl}/maflg^{fl}* mice in different environments, as in this thesis, highlights the influence of varying environmental exposure to aeroallergens in the presence of a skin barrier defect. Cytokine analysis, in particular that from the skin-draining lymph nodes, shows that *maflg^{fl}/maflg^{fl}* mice developed atopic responses, with elevated production of Th1/Th2 and Th17 cytokines that was more apparent in *maflg^{fl}/maflg^{fl}* mice housed in CON conditions compared to their IVC littermates.

3.8 Conclusion

This chapter initially sought to further confirm that the *maflg^{fl}/maflg^{fl}* mouse is a model of skin barrier deficiency. By assessing the progression of inflammation from early life to adulthood at various levels the results support that the *maflg^{fl}/maflg^{fl}* mouse is a model of skin barrier deficiency and human AD.

It is well established that the aetiology of AD is complex and that both skin barrier deficiency and immunology are part of this. The results of this chapter demonstrate that environmental conditions can also have an important influence on the nature of phenotype that develops and the severity of subsequent inflammation. Because increased immune responses were noted clinically, serologically and systemically in the *maflg^{fl}/maflg^{fl}* mice bred in the CON environment compared to those bred in the IVC conditions, these results also confirm that aetiological factors of skin barrier deficiency and immunology are inextricably linked.

This chapter suggests that basic environmental conditions are important in the pathogenesis of inflammation in the context of skin barrier deficiency. This implies that alterations in environment may lead to consequential differences in severity of inflammatory disease.

In this chapter, I have shown that environmental conditions can have a lasting effect on the trajectory of disease in a model of skin barrier deficiency and AD. The results of this chapter open questions of numerous aspects regarding the pathogenesis of AD and the contribution that influences on the skin barrier have on the severity of inflammation.

In further chapters in this thesis I will exploit the environmental conditions further with regard to changing these conditions and the effect of treatment with antibiotics to alter disease progression in a murine model of AD that is associated with mutations in filaggrin and other genes.

Chapter Four

Changing environmental conditions affects the development

of phenotype in the *maflg^{fl}/maflg^{fl}* mouse model of atopic dermatitis

4.1 Introduction

Multiple studies have suggested that in the presence of skin barrier deficiency, derived from a genetic change, increased environmental exposure leads to an increased risk of the development of an inflammatory cutaneous phenotype (Bisgaard et al., 2008, Cramer et al., 2010, Renz et al., 2011). By evaluating the effect of interventions on the environment in which the *maflg^{fl}/maflg^{fl}* mouse is bred, the interaction of the environment with the genetic mutation associated with the skin barrier defect can be assessed. As differences in severity of inflammation were noted in skin barrier deficiency when exposed to 2 different environments, as per Chapter 3, the hypothesis for this chapter, was that changing environmental conditions in early life may be associated with changes in overall severity of inflammation in the context of skin barrier deficiency.

This chapter aims to delineate more thoroughly the interplay between the mutations in the *maflg^{fl}/maflg^{fl}* mice and the immunological and clinical phenotype that develops when the environment conditions are changed. The clinical phenotype demonstrated by *maflg^{fl}/maflg^{fl}* mice was assessed under a range of environmental conditions and stressors. By comparing the *maflg^{fl}/maflg^{fl}* mice to wildtype mice, insight into interactions between them and the environment with respect to the development of AD can be analysed. By transferring a group of *maflg^{fl}/maflg^{fl}* mice at 1 week of age from a

CON environment to an IVC environment and repeating clinical assessments, serological, organ and lung investigations, the impact that changing environmental conditions in early life has on consequential inflammation in the context of skin barrier deficiency can be examined.

4.2 *maflg^{fl}/maflg^{fl}* mouse model clinical phenotype following an environmental intervention

The observations in the *maflg^{fl}/maflg^{fl}* mice of the spontaneous development of differences in disease severity when housed in two different environmental conditions afforded the opportunity to explore if a change in environment could alter disease trajectory. Neonatal *maflg^{fl}/maflg^{fl}* mice (< 7 days of age), born in CON conditions, were transferred to IVC caging (referred to as CON-IVC), with age-matched neonatal *maflg^{fl}/maflg^{fl}* reference control *maflg^{fl}/maflg^{fl}* mice maintained in CON and IVC conditions also established. All groups of mice were observed on a weekly basis. The development of disease was examined and scored to give a quantitative comparison between the groups at 32 weeks of age.

4.2.1 Transfer to different environmental conditions alters the clinical/phenotypical features in skin barrier deficiency

The features analysed included: cutaneous erythema; oedema; xerosis and scaling; and erosion; ocular alopecia, oedema and periocular and conjunctival infection; tail-tip auto-amputation (Section 2.2, Table 2.2). On general inspection, clinical features of cutaneous inflammation were reduced in the *maflg^{fl}/maflg^{fl}* mouse CON-IVC intervention group compared to the control *maflg^{fl}/maflg^{fl}* mouse CON group (Figure 4.2.1). *maflg^{fl}/maflg^{fl}* mice that were moved from CON to IVC had comparable dermatitis-like clinical scores

to IVC mice, and features were less marked than mice that had remained in CON housing (Figure 4.2.2). Specifically, mice that were housed continuously in a CON environment had increased erythema, patchy alopecia, cutaneous xerosis and scaling compared to the findings of the *maflg^{fl}/maflg^{fl}* mice from the IVC and CON-IVC groups. Cutaneous erosions were found to be of increased severity on the mice from the CON unit in comparison to both other groups. The ocular manifestations including periocular oedema, erythema, alopecia and conjunctival infection were all found to be present and more severe in the *maflg^{fl}/maflg^{fl}* mice from the CON environment, where only periocular alopecia and oedema were present in the *maflg^{fl}/maflg^{fl}* mice from the IVC environment and the *maflg^{fl}/maflg^{fl}* mice of the CON-IVC group.

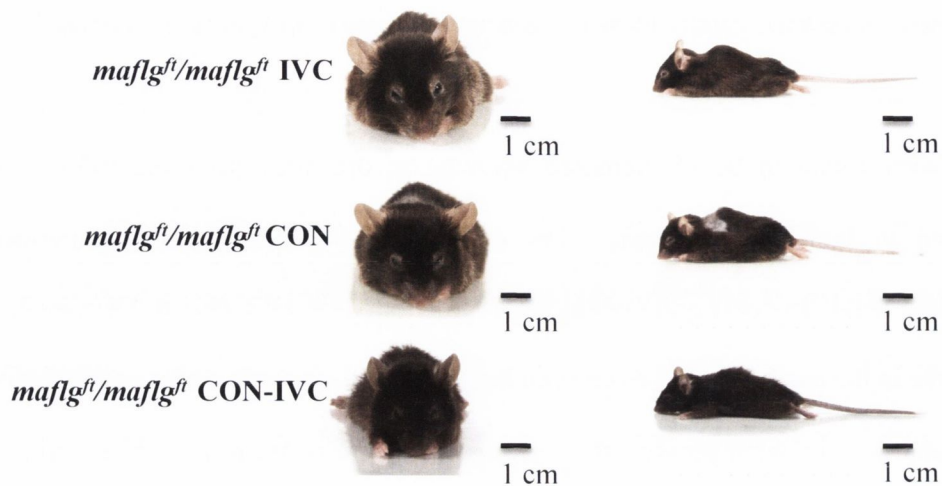


Figure 4.2.1 Transfer to an environment with altered environmental conditions improves the clinical phenotype in skin barrier deficiency.

Representative *maflg^{fl}/maflg^{fl}* mice bred in the IVC and CON environments, and a *maflg^{fl}/maflg^{fl}* mouse born in the CON environment and transferred to the IVC unit at 1 week (CON-IVC intervention group).

All mice were 32 weeks of age

4.2.2 Dermatitis-like scoring of inflammatory changes in the *maflg^{fl}/maflg^{fl}* mouse model following environmental intervention

Using a severity scoring system, as previously described in Table 2.2, scoring of clinical signs of cutaneous inflammation at 32 weeks in the *maflg^{fl}/maflg^{fl}* mice groups – CON, IVC, CON-IVC is shown in Figure 4.2.2. Factors included specifically disease characteristics in the eyes, tail and skin. The results of this chapter confirm the findings of Chapter 3 on multiple levels. Cutaneous inflammation in the *maflg^{fl}/maflg^{fl}* mice from the CON environment was significantly increased compared to the *maflg^{fl}/maflg^{fl}* mice from the IVC caging, unpaired two-tailed t test, $p = 0.0065$ (Figure 4.2.2). Significantly elevated cutaneous inflammation was also seen when comparing the *maflg^{fl}/maflg^{fl}* mice from the CON environment to their littermates transferred from the CON environment to the IVC housing at < 1 week of age, unpaired two-tailed t test, $p = 0.0228$ (Figure 4.2.2). No significant difference was found between the clinical severities of cutaneous inflammation of the *maflg^{fl}/maflg^{fl}* mice from the IVC group compared to the *maflg^{fl}/maflg^{fl}* mice from the CON-IVC group ($p = 0.0995$) (Figure 4.2.2).

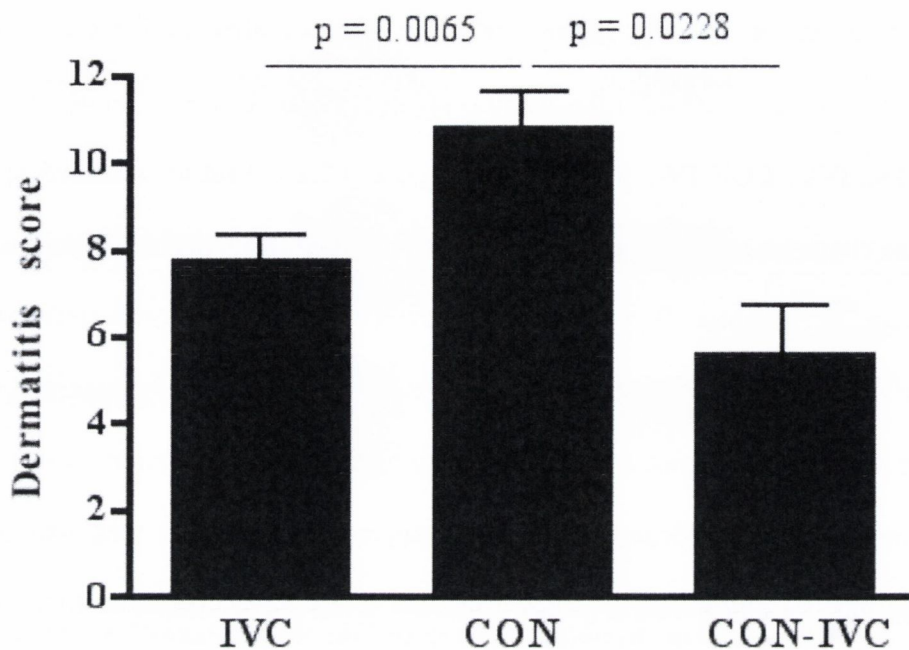


Figure 4.2.2: Clinical scoring of dermatitis-like inflammatory changes in the

***maflg^{fl}/maflg^{fl}* mouse model following transfer between environments.** *maflg^{fl}/maflg^{fl}* mice from the CON and IVC environments and the *maflg^{fl}/maflg^{fl}* mice in the CON-IVC group were assessed for clinical manifestations of cutaneous inflammation at 32 weeks using a scoring system examining changes in the eyes, tails and skin of all groups. Comparison of clinical scoring the *maflg^{fl}/maflg^{fl}* mice groups from the CON environment, the IVC environment and the group of *maflg^{fl}/maflg^{fl}* mice transferred from the CON to the IVC environment showed the differences in frequency and severity of cutaneous inflammation between the groups. Data is presented as mean +/- standard error of mean of 7-8 mice per group. Statistical analysis was undertaken using the unpaired student t-test, two-tailed *p* - values as indicated.

4.3.1 Analysis of the cutaneous histology of the *maflg^{fl}/maflg^{fl}* mouse phenotype following transfer between environments

At 32 weeks skin samples from abdominal skin of the above experimental *maflg^{fl}/maflg^{fl}* mouse groups were biopsied and paraffin-fixed skin sections were stained with haematoxylin and eosin (H&E), which illustrated inflammatory changes in the *maflg^{fl}/maflg^{fl}* mice from all environments as detailed in Section 2.5.1. Figure 4.3.1 shows H&E stained skin biopsies from *maflg^{fl}/maflg^{fl}* mice from the IVC and CON units and the group of *maflg^{fl}/maflg^{fl}* mice, which were transferred at < 1 week of age from the CON to the IVC environment. All mice were at 32 weeks of age. A range of cutaneous pathology is evident on H&E staining of the skin sections and this correlates with the clinical dermatitis-like scoring. Cutaneous infiltrates on H&E-stained skin sections were analysed in a quantitative manner using the scoring systems described in Section 2.5.1. Differences were noted between *maflg^{fl}/maflg^{fl}* mice from the different environments, where it was seen that skin samples from *maflg^{fl}/maflg^{fl}* mice bred in the CON environment had higher levels of inflammatory cellular infiltration compared to *maflg^{fl}/maflg^{fl}* mice skin samples from the IVC unit however these results were not statistically significant (unpaired t-test, two-tailed p - value = 0.1797) (Figure 4.3.2). Elevated cellular inflammation was found in the skin sections from *maflg^{fl}/maflg^{fl}* mice bred in the CON environment compared to the group of *maflg^{fl}/maflg^{fl}* mice transferred from the CON to the IVC environment, again without statistical significance (unpaired t-test, two-tailed p - value = 0.6198) (Figure 4.3.2). Intensity of inflammatory cellular infiltrate on skin sections between the IVC and CON-IVC groups was comparable (unpaired t-test, two-tailed p - value = 0.3139) (Figure 4.3.2).

Epidermal and dermal lymphocytes were counted per slide (20 HPFs per section) as another measure of cutaneous inflammation in the IVC and CON environments where an increase in lymphocytic cellular infiltration in *maflg^{fl}/maflg^{fl}* skin biopsies from the CON unit was found when compared to the samples from the IVC environment ($p = 0.2403$) and those in the CON-IVC group ($p = 0.2949$), however these results were not statistically significant (Figure 4.3.2). In a similar pattern to total cell count, a trend was shown in lymphocyte count, where decreased levels were found in the *maflg^{fl}/maflg^{fl}* samples from the CON-IVC group compared to the *maflg^{fl}/maflg^{fl}* CON samples. The reduced levels in the CON-IVC group were not as low as the *maflg^{fl}/maflg^{fl}* IVC group ($p = 0.6282$) (Figure 4.3.2).

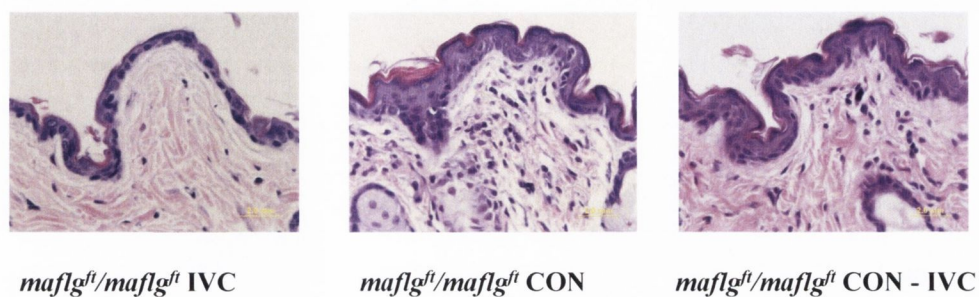


Figure 4.3.1 Histology of skin of the *maflg^{fl}/maflg^{fl}* mouse phenotype following transfer between environments. At 32 weeks, skin biopsies were taken from *maflg^{fl}/maflg^{fl}* mice from IVC and CON environments and the group of *maflg^{fl}/maflg^{fl}* mice in the CON-IVC group. Staining with H&E highlighted inflammatory cellular infiltration. Increased inflammatory changes are evident in the *maflg^{fl}/maflg^{fl}* skin sample from the CON environment compared to the *maflg^{fl}/maflg^{fl}* sample from the IVC unit and the sample from the *maflg^{fl}/maflg^{fl}* mouse in the CON-IVC group. 2mm scale as indicated, 40x magnification.

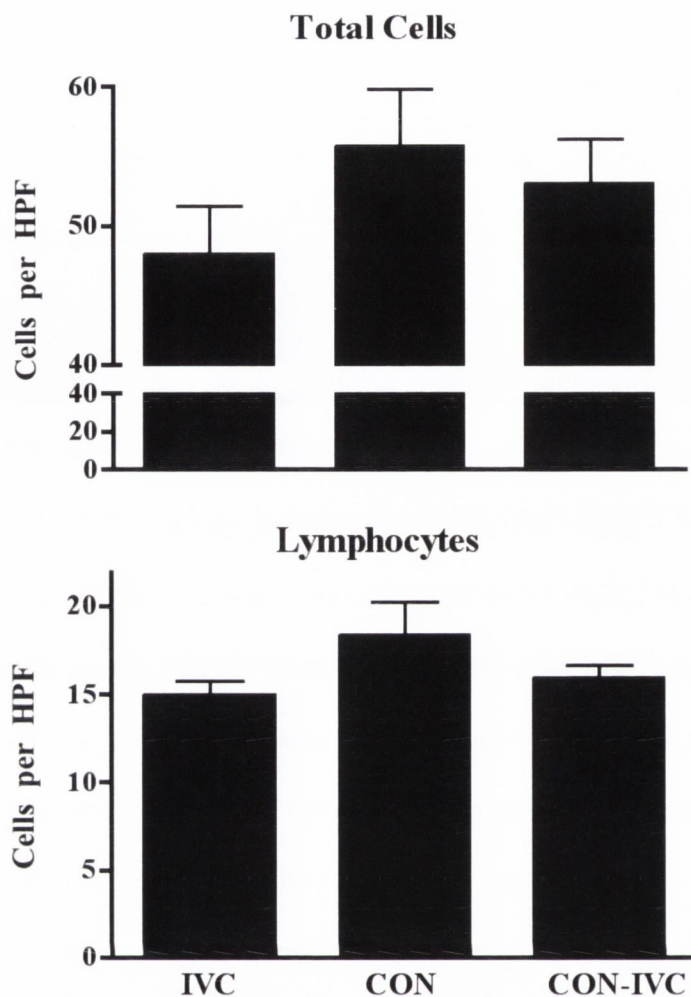


Figure 4.3.2: Scoring of cutaneous histological changes in *maflg^{fl}/maflg^{fl}* mice following transfer between environments. Skin biopsy analysis of *maflg^{fl}/maflg^{fl}* mice from IVC and CON environments and of *maflg^{fl}/maflg^{fl}* mice that were transferred from the CON environment to the IVC environment at 1 week (CON-IVC group). Total cell count and lymphocyte count are shown, Significance was not found. Data is presented as mean +/- SEM and is representative of 7-8 mice per group. Statistical analysis was undertaken using the unpaired student t-test, two-tailed *p* - values were considered significant if *p* < 0.05.

4.4 Analysis of the systemic and local responses in the *maflg^{fl}/maflg^{fl}* mouse phenotype following transfer between environments

To evaluate further if these groups of *maflg^{fl}/maflg^{fl}* mice replicated the findings of chapter 3 with regards to systemic and local immunological responses for those mice bred either in the CON or IVC environment systemic response was assessed by measuring serum total antibody responses, spleen cell and skin-draining lymph node cell expression of cytokines. The same parameters were used to assess the group of *maflg^{fl}/maflg^{fl}* mice that were transferred between environmental conditions in neonatal life.

4.4.1 Influence of environment on serum antibody responses in the *maflg^{fl}/maflg^{fl}* mouse

Serum antibody responses were analysed as detailed in sections 2.6 and 2.6.1. Serum levels of IgE, IgG₁ and IgG_{2a} were investigated by sandwich ELISA. Significance was assessed with the unpaired t-test, two-tailed *p* – value. Serum levels of IgE were noted to be lower in mice from the IVC environment relative to mice from the CON housing (IVC:CON *p* = 0.1781). Mice from the CON-IVC group (CON: CON-IVC *p* = 0.4651, IVC:CON-IVC *p* = 0.1437) showed serum IgE levels at an intermediate level (Figure 4.4.1), however, this difference was not significant. Expression of total IgG₁ or IgG_{2a} was consistent throughout the groups (IVC:CON *p* = 0.7939, CON: CON-IVC *p* = 0.2377, IVC:CON-IVC *p* = 0.133) (Figure 4.4.1).

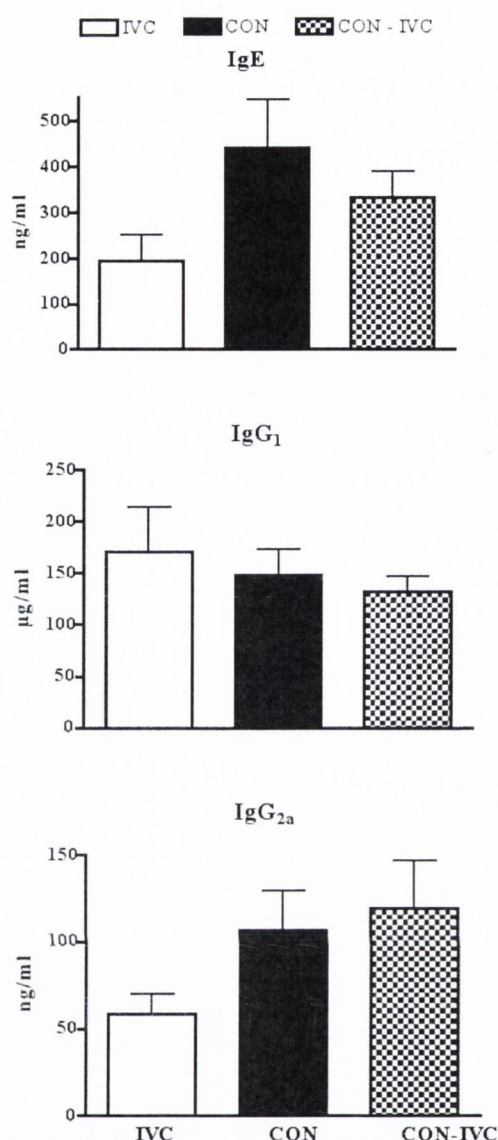


Figure 4.4.1: Total serum antibody levels of *mafIg^{fl}/mafIg^{fl}* mice following transfer between environments. At 32 weeks, serum was recovered and analysed from *mafIg^{fl}/mafIg^{fl}* mice, from the CON and IVC environments and the group of *mafIg^{fl}/mafIg^{fl}* mice that were transferred at 1 week old, from the CON environment to the IVC housing. Levels of total IgE, IgG₁ or IgG_{2a} were quantified. No significant difference was found between the antibody levels in the groups. Statistical analysis was undertaken using the unpaired student t-test, two-tailed *p* - values were considered significant if *p* < 0.05.

4.4.2 Environmental influences on spleen cell cytokine responses in the

maflg^{ft}/maflg^{ft} mouse

Spleens were isolated from *maflg^{ft}/maflg^{ft}* mice at 32 weeks in the CON, IVC and CON-IVC groups. Cells were extracted as per Section 2.4.3, counted (Section 2.4.2) and the cells were stimulated either in a general stimulatory fashion with PMA/I (2.5ng/ml/250ng/ml) or by T-cell specific stimulation with aCD3/aCD28 (0.5µg/ml/4µg/ml) as per Section 2.4.4. Levels of IL-4, IFN-γ and IL-17 released were then analysed.

The levels of IL-4 released by spleen cell stimulation were comparable in all three groups of *maflg^{ft}/maflg^{ft}* mice (Figure 4.4.2). When cells extracted from spleens were stimulated in a general fashion, higher levels of IL-17 were seen in the CON group relative to the IVC or CON-IVC groups (Figure 4.4.2). However, when these spleen-derived cells were stimulated in a T-cell-specific manner, the levels of IL-17 in the IVC and CON-IVC groups were comparable (Figure 4.4.2). Elevated levels of IFN-γ were released after T-cell-specific stimulation of spleen cells in *maflg^{ft}/maflg^{ft}* mice from the CON environment in comparison to the *maflg^{ft}/maflg^{ft}* samples from the IVC environment and the *maflg^{ft}/maflg^{ft}* mice from the CON-IVC group however this was not found to be significant (Figure 4.4.2).

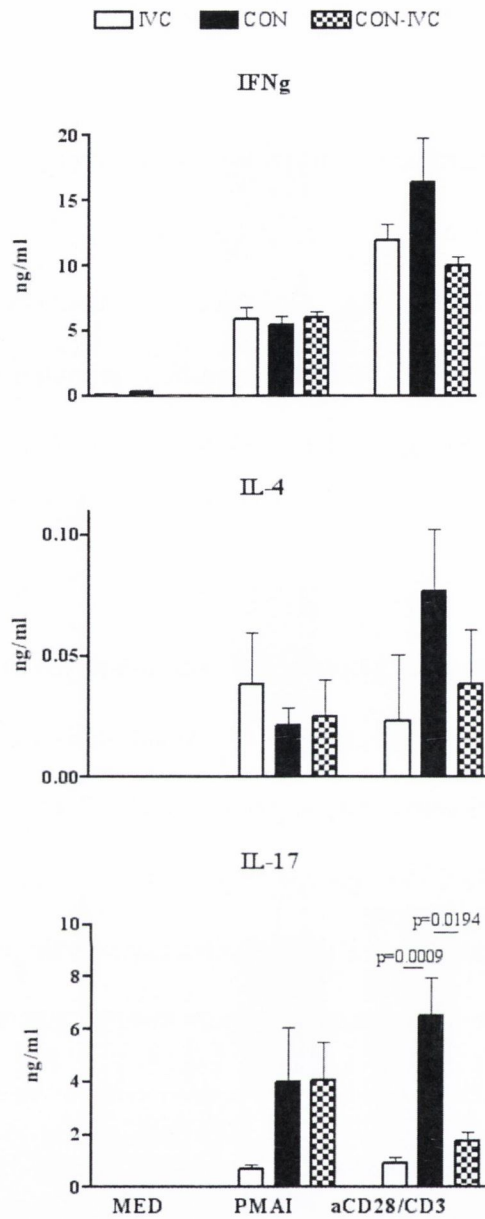


Figure 4.4.2 Cytokine expression from stimulated spleen cells harvested from *maflg^{ft}/maflg^{ft}* mice in varying environmental conditions. Whole spleens were removed from *maflg^{ft}/maflg^{ft}* mice at 32 weeks, housed in IVC and CON environments and from *maflg^{ft}/maflg^{ft}* mice in the CON-IVC group. Cytokine expression from 24- (IL-4), and 72-hour (IL-17, IFN γ) cell cultures of these organs were measured. Data is presented as mean $\pm$ SEM of 7-8 mice per group. Statistical analysis was done by unpaired t-test, two-tailed p - values as indicated.

4.4.3 Environmental influence on skin-draining lymph node cell cytokine responses in the *maflg^{fl}/maflg^{fl}* mouse

Skin-draining lymph nodes were isolated from *maflg^{fl}/maflg^{fl}* mice at 32 weeks in the CON, IVC and CON-IVC groups. Cells were extracted as per Section 2.4.3, counted (Section 2.4.2) and the cells were stimulated either in a general stimulatory fashion with PMA/I (2.5ng/ml/250ng/ml) or by T-cell specific stimulation with aCD3/aCD28 (0.5µg/ml/4µg/ml) as per Section 2.4.4. Levels of IL-4, IFN-γ and IL-17 released were then analysed. There was significantly ($p < 0.05$) elevated T-cell derived IL-4 released from skin-draining lymph node cells from the *maflg^{fl}/maflg^{fl}* mice in CON conditions and mice moved (CON-IVC) relative to the IVC group of *maflg^{fl}/maflg^{fl}* mice (Figure 4.4.2.1).

When cells extracted from lymph nodes were stimulated in a general or T-cell-specific fashion, IL-17 was expressed at significantly higher levels in cells from *maflg^{fl}/maflg^{fl}* mice from the CON environment compared to those from the IVC unit or the CON-IVC group (Figure 4.4.3).

Levels of IFN-γ expressed after T-cell-specific stimulation of cells extracted from skin-draining lymph nodes indicated that elevated levels of IFN-γ are seen in *maflg^{fl}/maflg^{fl}* mice from the CON environment in comparison to the *maflg^{fl}/maflg^{fl}* samples from the IVC environment and the *maflg^{fl}/maflg^{fl}* mice from the CON-IVC group. The levels of IFN-γ released from stimulated T cells from skin-draining lymph nodes between the CON environment and the other groups showed a statistically significant increase (CON:IVC $p = 0.0289$, CON:CON-IVC $p = 0.0298$) (Figure 4.4.3).

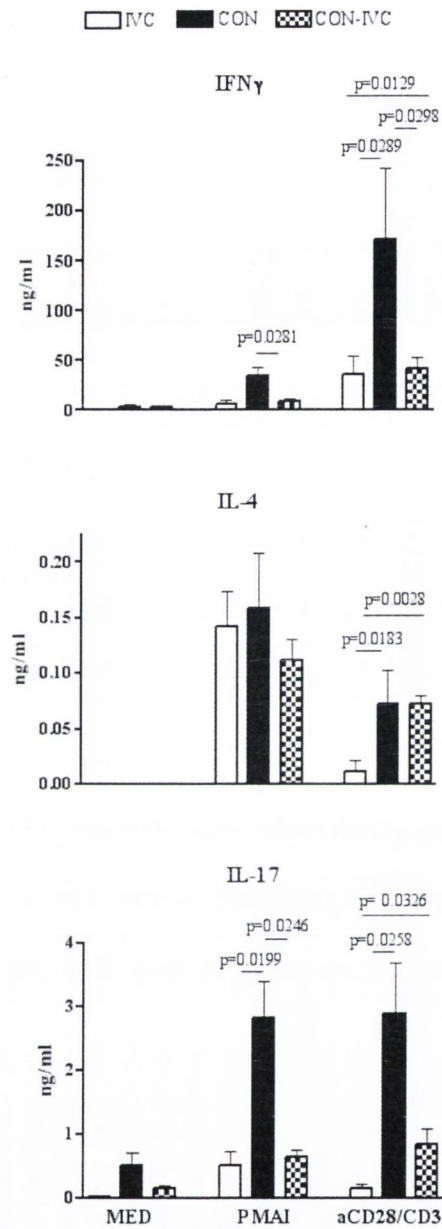


Figure 4.4.3: Skin-draining lymph node cell-cytokine expression of *maflg^{f1}/maflg^{f1}* mice. Skin-draining submandibular lymph nodes were removed from *maflg^{f1}/maflg^{f1}* mice bred in IVC and CON environments and from *maflg^{f1}/maflg^{f1}* mice in the group, which was transferred from the CON to IVC conditions at < 7 days. Cytokine expression from 24- (IL-4) and 72-hour (IL-17, IFN γ) cell cultures of these lymph nodes, were measured.

Data is presented as mean +/- SEM of 7-8 mice per group. Statistical analysis was done by unpaired t-test, two-tailed *p* - values as indicated.

4.5 Discussion

In this chapter the influence of environmental conditions on the resultant phenotype was further investigated. The findings reported in chapter 3 were replicated. *maflg^{fl}/maflg^{fl}* mice from the CON environment significantly developed more severe phenotypical inflammatory responses of the *maflg^{fl}/maflg^{fl}* profile, compared to their littermates housed in the IVC environment. To explore the interaction of the environment with the genetic mutations associated with skin barrier deficiency, a neonatal group of *maflg^{fl}/maflg^{fl}* mice was transferred at 1 week from the CON environment to IVC conditions, which has not previously been explored.

The neonatal change of environment resulted in some significant differences in inflammatory phenotype, confirming that environmental exposures play a role in aetiology of inflammatory disease in skin barrier deficiency. Scoring the clinical features of cutaneous inflammation in all groups of *maflg^{fl}/maflg^{fl}* mice (CON, IVC, and CON-IVC) highlighted that the *maflg^{fl}/maflg^{fl}* mice from the CON environment have significantly more severe features of prolonged cutaneous inflammation compared to the other *maflg^{fl}/maflg^{fl}* groups. What was also demonstrated was that this increase was not entirely ameliorated by a switch to the IVC environment at 7 days of life (Figures 4.2.2).

The results of histological analysis demonstrated non-significant differences between the *maflg^{fl}/maflg^{fl}* mouse groups. These differences suggest that the skin of *maflg^{fl}/maflg^{fl}*

mice from the CON environment showed increased cutaneous inflammation compared to *maflg^{fl}/maflg^{fl}* mice of the IVC unit and those from the CON-IVC group, without any specific experimental sensitisation. A marked trend towards higher levels of inflammatory changes was found in the *maflg^{fl}/maflg^{fl}* mice from the CON environment compared to the IVC group and the group of *maflg^{fl}/maflg^{fl}* mice, which were transferred from the CON to the IVC environment.

As previously reported *maflg^{fl}/maflg^{fl}* mice develop spontaneous atopy (Moniaga et al., 2010, Oyoshi et al., 2009b, Scharschmidt et al., 2009) and this was demonstrated in this chapter. Expression of total serum IgE showed a similar pattern to that of cutaneous inflammation, where increased levels of total IgE occurred in the *maflg^{fl}/maflg^{fl}* mice from the CON environment compared to those from the IVC environment. The expression of IgE from the *maflg^{fl}/maflg^{fl}* mice from the CON environment was elevated compared to the CON-IVC group, and this group showed levels of IgE, which fell between the levels of IgE of the *maflg^{fl}/maflg^{fl}* mice of both the CON and IVC groups (Figure 4.4.1). The levels of total serum IgG₁ or serum IgG_{2a} were equivalent between the groups of *maflg^{fl}/maflg^{fl}* mice in varying environmental conditions.

There was a trend towards increased levels of IL-4 from T cells extracted from the *maflg^{fl}/maflg^{fl}* mice from the CON group compared to the other groups (Figure 4.4.2). It has also been highlighted that the *maflg^{fl}/maflg^{fl}* mouse is associated with Th17-dominated skin inflammation (Oyoshi et al., 2009b). A significant increase was noted in expression of IL-17 from stimulated inflammatory cells of skin-draining lymph nodes in the *maflg^{fl}/maflg^{fl}* mice from the CON environment compared to both the *maflg^{fl}/maflg^{fl}*

mice from the IVC environment and those that had CON-IVC transfer in early life (Figure 4.4.3). The significant elevation of expression of IL-17 in *maflg^{fl}/maflg^{fl}* mice from the CON environment in comparison to those from the IVC unit strongly suggests the involvement of a Th17-type immune response to transcutaneous antigen entry. Such a pattern in IL-17 expression was not found from spleen cells exposed to general stimulation although there was a significant difference in the levels of IL-17 expressed in spleen cells stimulated by anti-CD3/CD28 antibody from *maflg^{fl}/maflg^{fl}* mice samples from the CON environment compared to the IVC environment and those in the CON-IVC group, indicating that T cells are the primary driving force behind the variance in IL-17 levels.

IFN γ expression after stimulation of T cells from skin-draining lymph nodes showed a pattern similar to lymph node IL-17 expression. Significantly increased levels of IFN γ expression were seen from the stimulated T cells from lymph nodes of *maflg^{fl}/maflg^{fl}* mice samples from the CON environment compared to the IVC environment and those in the CON-IVC group (Figure 4.4.3). No such pattern was evident when assessing the expression of IFN γ from stimulated spleen cells (Figure 4.4.2).

While human studies have been performed investigating the association of particular environmental and domestic organic and chemical allergens with AD, few studies examine the influence of indoor environment and air pollutant levels and AD symptoms (Lee et al., 2011). A study carried out using a low pollutant room, which was designed to have low levels of dust, house dust mite, micro-organisms and indoor air pollutant, showed that using the well-validated scoring system; the SCORing Atopic Dermatitis (SCORAD) Index as a measure of disease severity, the overall SCORAD was

significantly reduced following hospital admission ($p < 0.001$) (Lee et al., 2011). Some of these patients continued to show improved symptoms at their first outpatient clinic visit which was 1 month following discharge. This study demonstrated that in patients with AD a change of environments can alter and improve the severity of symptoms (Lee et al., 2011). The results of changing environmental conditions in early life, in an animal model of skin barrier deficiency highlights that the phenotype that develops can be altered and improved if the environment is changed. The results of transfer in this model of skin barrier deficiency, implies that environmental factors interact with genetic and immunologic factors in the aetiology, extent and severity of cutaneous inflammation. This further confirms that environmental factors are not just associated with exacerbation of flares in skin barrier deficiency but have an important role in aetiology of inflammation secondary to skin barrier defects. In skin barrier deficiency, having less environmental factors in early life, may improve the overall outcome of cutaneous and systemic inflammation.

In this chapter I have shown the effect that changing environmental conditions can have on the trajectory of disease and severity of inflammation in this model of skin barrier deficiency and AD. This raises questions with regards to the profound effect that the environmental conditions at birth and early neonatal life has on disease severity and progression throughout life. This suggests that changing conditions in a variety of ways in early life can alter disease in a model of skin barrier deficiency and AD.

Chapter Five

Effects of systemic antibiotic intervention

on cutaneous inflammation in the *maflg^{fl}/maflg^{fl}* mouse

5.1 Introduction

The many structures and layers of the skin act as hosts to microbes including commensal and pathogenic bacteria that have a role in skin health and disease (Grice et al., 2008). In AD, both normal skin and lesional skin are frequently extensively colonised by bacteria, in particular *Staphylococcus aureus* (*S.aureus*), or fungi such as *Malassezia* (Bieber, 2008) and AD is frequently complicated by recurrent infections of skin lesions (Ong et al., 2002). Resultant cycles of infection and inflammation have deleterious effects on the barrier function of the skin, further reducing the protective capacity of the skin. Alteration of the skin flora has been shown to affect cutaneous inflammation, highlighting the role of the microbiome in the skin barrier (Arck et al., 2010).

Recent studies have demonstrated how pathogens, such as bacteria like *S. aureus*, have an important role to play in inflammation in the skin barrier where colonisation leads to low-grade inflammation, rendering the skin more susceptible to inflammatory and infective exacerbations (Cogen et al., 2010a, Cogen et al., 2010b, Grice et al., 2008, Huang et al., 2009, Ong et al., 2002). The important role of *S. aureus* was first highlighted in a study by Leyden *et al.* where the severity of skin disease in patients with atopic dermatitis without superinfection, was reduced by treatment with a combination of

anti-staphylococcal antibiotics and topical corticosteroids (Leyden and Kligman, 1977). My hypothesis was that early modification of the skin microbiome, through the use of systemic antibiotics could influence the downstream severity of the inflammatory phenotype in flaky tail mice. To address the hypothesis of the inflammatory response in skin of *maflg^{fl}/maflg^{fl}* mice exposed to an antibiotic regimen in early life was compared to that of mice without antibiotic administration. A number of different inflammatory measures are quantified including cell infiltration, cytokine responses and serum antibody levels in the various cohorts of mice.

5.2 *maflg^{fl}/maflg^{fl}* mouse model clinical phenotype following an anti-bacterial intervention in early life

Two pairs of breeding *maflg^{fl}/maflg^{fl}* mice in the CON facility were administered doses of oral antibiotics diluted in drinking water, as detailed in section 2.1 (CON A/B group) (Table 2.1). The antibiotics administered included a metronidazole/ciprofloxacin combination and tetracycline. The antibiotics were chosen for their differing antibacterial activity to ensure broad spectrum cover for cutaneous colonisation and/or infection during treatment. Ciprofloxacin has gram-positive and gram-negative activity, metronidazole has high activity against anaerobic bacteria and protozoa while tetracycline has broad spectrum cover for gram-positive and gram-negative bacteria as the alternating antibiotic during the cycles (British Medical Association, 2012, Wolverton, 2007). Administration courses were alternated on a fortnightly basis so as to minimise resistance. Antibiotic administration continued following birth of the litters, up to weaning at 4 weeks, so the *maflg^{fl}/maflg^{fl}* mice studied had the effects of the antibiotic treatment *in utero*, and then from the nursing dam up until weaning. A group of 8 female *maflg^{fl}/maflg^{fl}* mice from these litters were maintained on normal diet and water until 32

weeks. The control group for this experiment was a group of *maflg^{ft}/maflg^{ft}* mice bred in the same CON facility, without any antibiotic administration.

5.2.1 Alteration in skin microbiome at early life improves clinical scoring in the *maflg^{ft}/maflg^{ft}* mouse model

When the above groups of *maflg^{ft}/maflg^{ft}* mice reached 32 weeks, the clinical severity of skin lesions was scored (Table 2.2). As seen in Figure 5.2.1, comparison of clinical scoring between the groups showed that the severity of cutaneous inflammation in the antibiotic administered group of *maflg^{ft}/maflg^{ft}* mice (CON A/B group) was not statistically lower than that of mice without antibiotics (CON group).

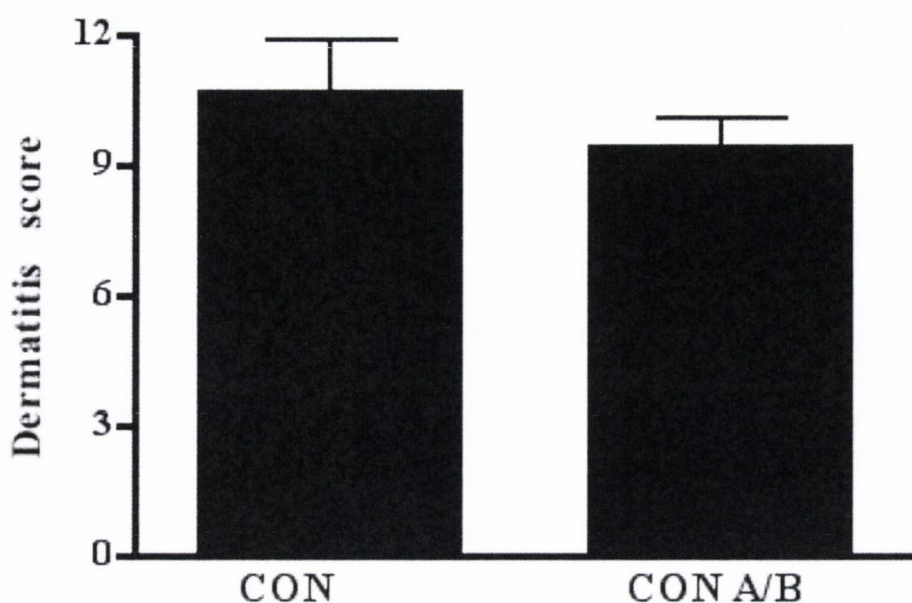


Figure 5.2.1 Clinical scoring of cutaneous inflammatory changes in the *maflg^{fl}/maflg^{fl}* mouse model following microbiome intervention in early life. *maflg^{fl}/maflg^{fl}* mice from the CON environment and *maflg^{fl}/maflg^{fl}* mice in the CON A/B group were assessed for clinical manifestations of cutaneous inflammation at 32 weeks, using a scoring system examining changes in the eyes, tails and skin of the groups. Data is presented as mean + / - SEM and is representative of 3-8 mice per group. Statistical analysis was undertaken using the unpaired student t-test, two-tailed p - value = 0.6326.

5.3 Cutaneous histology of the *maflg^{fl}/maflg^{fl}* mouse phenotype following antibiotic pre-treatment

Skin samples from abdominal skin of the *maflg^{fl}/maflg^{fl}* mouse groups at 32 weeks, were biopsied and paraffin-fixed skin sections were stained with haematoxylin and eosin (H&E) as detailed in Section 2.5.1. Quantification of inflammatory infiltration was done by total cell and lymphocyte counting of each skin sample (20 HPFs per section) (Section 2.5.1).

5.3.1 Alterations in microbiome affect cutaneous inflammation as seen by histological analysis in the *maflg^{fl}/maflg^{fl}* mouse model

Inflammatory infiltration was quantified by total cell count (20 high-powered fields (HPFs) per section) of the slides stained with H&E in the group of *maflg^{fl}/maflg^{fl}* mice exposed to antibiotics for the first 4 weeks of life and in the control group of *maflg^{fl}/maflg^{fl}* mice bred in the CON environment without any antibiotics. Significantly less cutaneous cellular infiltrative inflammation at 32 weeks was noted in the *maflg^{fl}/maflg^{fl}* mice exposed to antibiotics compared to the control group of *maflg^{fl}/maflg^{fl}* mice (Figure 5.3.1). To investigate this difference in inflammation further, epidermal and dermal lymphocytes were counted per slide (20 HPFs per section). The difference between groups was not significant although the group of *maflg^{fl}/maflg^{fl}* mice in the CON A/B group had a trend of lower levels of lymphocytic inflammatory infiltration compared to the control *maflg^{fl}/maflg^{fl}* mice from the CON environment (Figure 5.3.2).

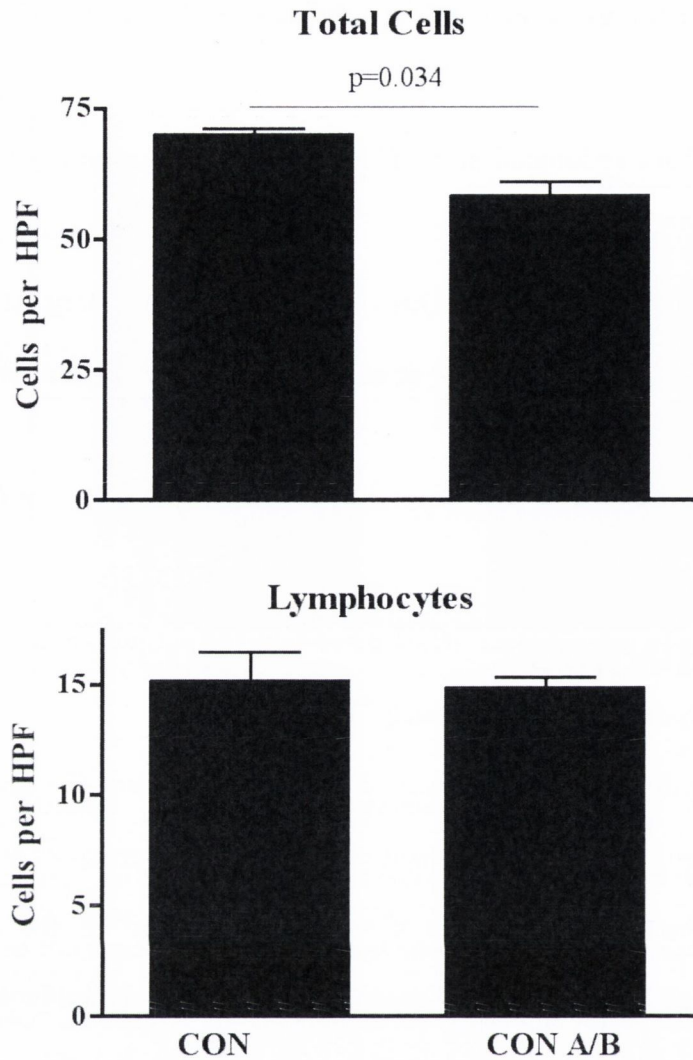


Figure 5.3.1 Cutaneous histological scoring in skin barrier deficiency in the *maflg^{fl}/maflg^{fl}* mouse model, following microbiome intervention in early life. Skin biopsies, stained with H&E, were taken from *maflg^{fl}/maflg^{fl}* mice from the CON environment and from the group of *maflg^{fl}/maflg^{fl}* mice in the CON A/B group. All mice were 32 weeks. Total cell count and lymphocyte count are shown. Data is presented as mean +/- SEM and is representative of 3 - 8 mice per group. Statistical analysis was undertaken using the unpaired student t-test, two-tailed *p* - values were considered significant if *p* < 0.05.

5.4 Systemic response in skin barrier deficiency in the *maflg^{fl}/maflg^{fl}* mouse model following antibiotic intervention

Total serum antibody responses, spleen cell and skin-draining lymph node cell expression of cytokines were measured in both groups of *maflg^{fl}/maflg^{fl}* mice at 32 weeks. This was to evaluate if antibiotic treatment interacted with skin barrier deficiency with regards to systemic and/or local immunological responses.

5.4.1 Serum antibody responses in skin barrier deficiency in the *maflg^{fl}/maflg^{fl}* mouse phenotype following antibiotic intervention

The sera of *maflg^{fl}/maflg^{fl}* mice treated or not treated with antibiotics were analysed to determine whether there was a difference in levels of IgE, IgG₁ and IgG_{2a} expression between the cohorts, as described in section 2.6 and Section 2.6.2. Total serum IgE was higher in the group of *maflg^{fl}/maflg^{fl}* mice that had been exposed to antibiotic administration (CON A/B group) in comparison to IgE levels in the CON group however this was not significant (Figure 5.4.1).

Levels of serum IgG₁ were also analysed in antibiotic-administered mice. As total serum IgG₁ expression is associated with a Th2-type immune response, a similar pattern to IgE expression would be expected, however, this was not found. Total serum IgG₁ levels were non significantly different in the *maflg^{fl}/maflg^{fl}* mice with antibiotic administration (CON A/B) relative to control *maflg^{fl}/maflg^{fl}* mice (CON) ($p = 0.0267$) (Figure 5.4.1).

There was a significant increase in serum IgG_{2a} expression in the *maflg^{fl}/maflg^{fl}* mice from the CON A/B group in comparison to the control *maflg^{fl}/maflg^{fl}* mice from the CON unit (Figure 5.4.1).

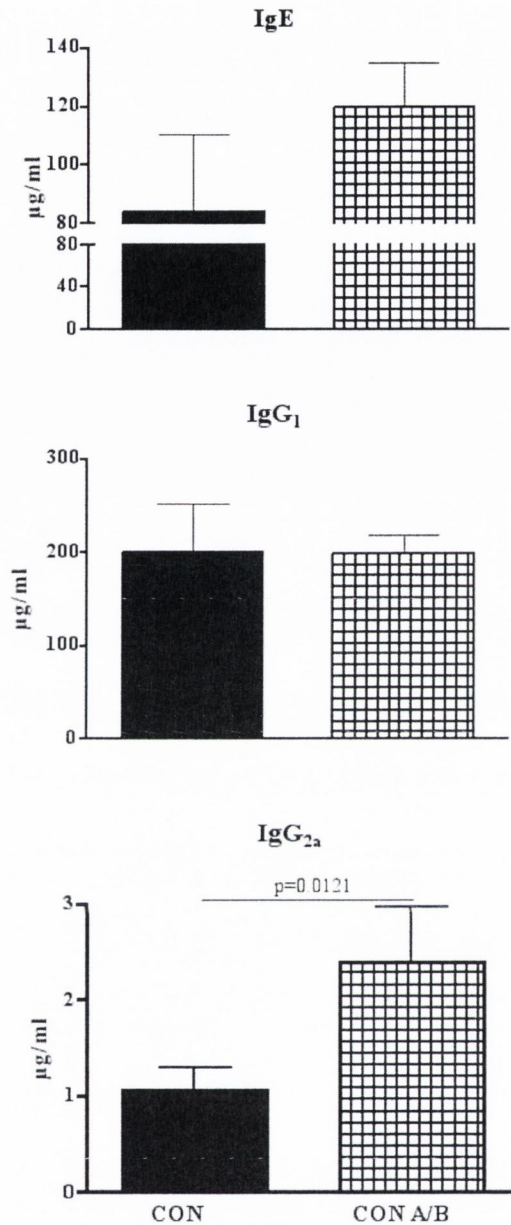


Figure 5.4.1 Total serum antibody expression from *maflg^{fl}/maflg^{fl}* mice following microbiome intervention in early life. At 32 weeks, serum was recovered from whole blood samples from the *maflg^{fl}/maflg^{fl}* mice in the CON and CON A/B groups and analysed. Total serum IgE, IgG₁, IgG_{2a} levels were quantified. Data is presented as mean + / - SEM and is representative of 3-8 mice per group. Statistical analysis was undertaken using the unpaired student t-test or the Mann-Whitney test with non-parametric data, two-tailed p = values as indicated.

5.4.2 Spleen cell cytokine responses in skin barrier deficiency of the *maflg^{fl}/maflg^{fl}* mouse phenotype following intervention in the microbiome in early life

To investigate the systemic inflammatory response after antibiotic administration, spleens were harvested from the antibiotic-administered mice as well as from the control group, as detailed in Section 2.4. Cells were stimulated either in a general stimulatory way with PMA/I (2.5ng/ml/250ng/ml) or by T-cell specific stimulation with anti-CD3/CD28 mAbs (0.5µg/ml/4µg/ml) as per Section 2.4.4. The expression of a number of cytokines (IL-4, IFN γ , IL-17) was analysed by sandwich ELISA, as detailed in Sections 2.6 and 2.6.2.

On assessing both generally stimulated cells and T cell stimulated release of IFN γ , levels of expression from the *maflg^{fl}/maflg^{fl}* mice treated with the antibiotic regimen did not show significant differences when compared to the *maflg^{fl}/maflg^{fl}* mice that were not treated (Figure 5.4.2). The levels of splenic cell expression of IL-4 on stimulation of inflammatory cells was seen to be comparable in the *maflg^{fl}/maflg^{fl}* mice group from the CON environment compared to the *maflg^{fl}/maflg^{fl}* mouse CON A/B group (Figure 5.4.2).

The levels of IL-17 expressed after inflammatory cell stimulation and T-cell-specific stimulation from cells harvested from spleens were analysed. Samples harvested from *maflg^{fl}/maflg^{fl}* mice housed in CON environment, IVC environment and mice transferred between the two as well as from *maflg^{fl}/maflg^{fl}* mice administered antibiotics were compared. Levels of IL-17 were non-significantly higher in the *maflg^{fl}/maflg^{fl}* mice that had not been treated with antibiotics compared to the group that had received this treatment (Figure 5.4.2). Interleukin-17 expression from T-cell-specific stimulation was significantly elevated in the *maflg^{fl}/maflg^{fl}* samples from the CON environment compared to the CON A/B group ($p = 0.0262$) (Figure 5.4.2).

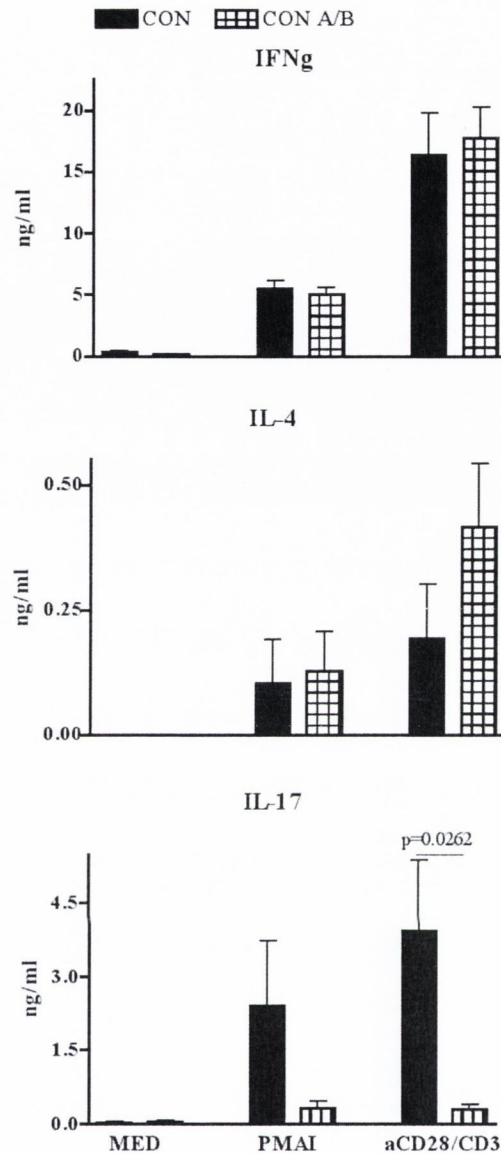


Figure 5.4.2 Cytokine expression from stimulated spleen cells harvested from *maflg^{fl}/maflg^{fl}* mice following alteration in the microbiome in early life. Whole spleens were removed from *maflg^{fl}/maflg^{fl}* mice housed in the CON environment and from *maflg^{fl}/maflg^{fl}* mouse group exposed to antibiotics in early life. All mice were 32 weeks. Cytokine expression from 24-hour (IL-4), and 72-hour (IL-17, IFN γ) cell cultures of these organs were measured. Data is presented as mean + / - SEM and is representative of 3-8 mice per group. Statistical analysis was done with unpaired t-test, two-tailed *p* - values as indicated.

5.4.3 Skin-draining lymph node cell cytokine responses in skin barrier deficiency of the *maflg^{ft}/maflg^{ft}* mouse phenotype following intervention in the microbiome in early life

To investigate the local inflammatory response after antibiotic administration, lymph nodes were harvested from the antibiotic-administered mice as well as from the control group, as detailed in Section 2.4. The skin-draining lymph nodes were processed. Cells were stimulated either in a general stimulatory way with PMA/I (2.5ng/ml/250ng/ml) or by T-cell specific stimulation with anti-CD3/CD28 (0.5µg/ml/4µg/ml) as per Section 2.4.4. The expression of a number of cytokines (IL-4, IFN γ , IL-17) was analysed by sandwich ELISA, as detailed in Sections 2.6 and 2.6.2.

It was found that generally stimulated lymph node cell expression of IFN γ was comparable between the groups of *maflg^{ft}/maflg^{ft}* mice whether they received antibiotic treatment or not (Figure 5.4.3). There was an increased expression of IFN γ when assessing T cell stimulated release from the *maflg^{ft}/maflg^{ft}* CON group compared to the *maflg^{ft}/maflg^{ft}* group that had been treated with antibiotics, however, this difference was not significant (Figure 5.4.3).

On analysis of IL-4 levels, there was increased expression of IL-4 from both generally stimulated cells and T cell stimulated cells in the *maflg^{ft}/maflg^{ft}* mice that had not been treated with antibiotics, compared to the group that had received treatment. These differences were not significant (Figure 5.4.3).

The levels of IL-17 expressed after inflammatory cell stimulation and T-cell-specific stimulation from cells harvested from skin-draining lymph nodes were analysed. Levels of IL-17 were higher when measuring expression from generally stimulated cells of the

maflg^{fl}/maflg^{fl} mice that had received antibiotic treatment in comparison to the *maflg^{fl}/maflg^{fl}* CON group but this was not significant (Figure 5.4.3). Interleukin-17 was expressed at comparable levels in both of these groups when measuring expression from T cell specific stimulation (Figure 5.4.3).

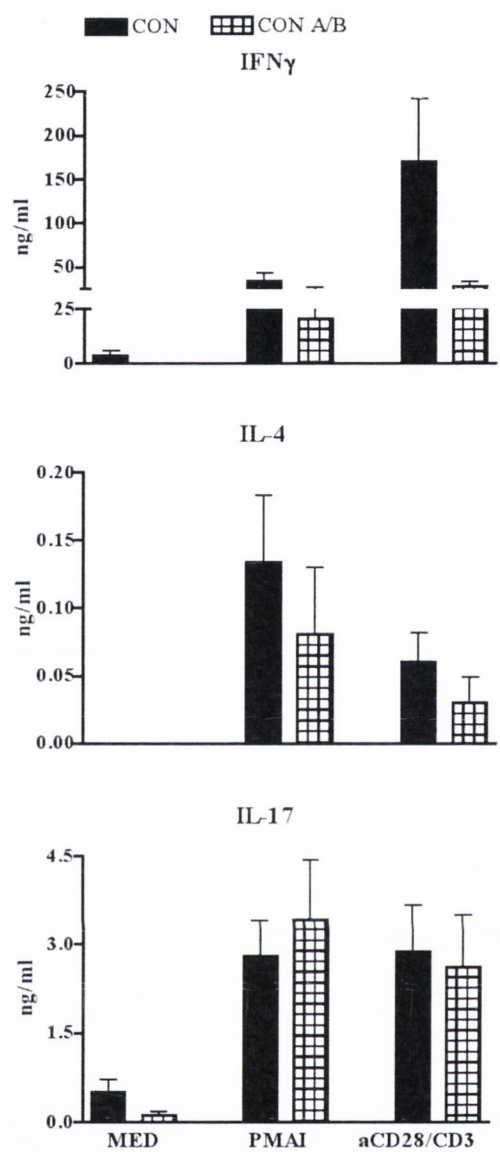


Figure 5.4.3 Skin draining lymph node cell-cytokine expression of *maflg^{fl}/maflg^{fl}* mice following alteration in microbiome in early life. Skin-draining submandibular lymph nodes were removed from *maflg^{fl}/maflg^{fl}* mice housed in the CON environment and from *maflg^{fl}/maflg^{fl}* mice that were exposed to antibiotics in the 1st 4 weeks of life. All mice were 32 weeks. Cytokine expression from 24-hour (IL-4) and 72-hour (IL-17, IFN γ) cell cultures of these lymph nodes, were measured. Data is presented as mean + / - SEM and is representative of 3-8 mice per group. Statistical analysis was done with unpaired t-test, two-tailed *p* – value was considered significant if *p* < 0.05.

5.5 Discussion

This chapter characterises the role of modification of the bacterial skin flora in the inflammatory phenotype of AD in the context of skin barrier deficiency associated with the *maflg^{fl}/maflg^{fl}* mice. There is no precedent of examining the effects of experimental alteration of the microbiome in this model of AD. A recent study on paediatric patients with AD highlighted how high amounts of *S. aureus* correlated to clinically worse AD lesions (Travers et al., 2012). The study went on to demonstrate how treatment with topical TCS and cephalexin resulted in significant improvement in lesional and total severity scores (Travers et al., 2012). The study by Travers *et al.* and others illustrate how the skin microbiome plays a key role in the nature of and severity of disease exacerbations in AD (Huang et al., 2009, Kong et al., 2012, Travers et al., 2012). This chapter seeks to evaluate the effects of treating bacterial colonisation and/or infection on the severity of cutaneous and systemic inflammation in the *maflg^{fl}/maflg^{fl}* model of AD.

The results of this chapter demonstrated that the clinical phenotype of the *maflg^{fl}/maflg^{fl}* mice was changed when the microbiome was altered. The *maflg^{fl}/maflg^{fl}* mice had been treated with antibiotics from conception, nursing through to weaning and thus had altered microbiome from birth. Non-significant decreased signs of cutaneous inflammation were found on scoring of dermatitis-like changes (Figure 5.2.1). Of note, the mice that were treated with antibiotics had decreased severity of erythematous skin lesions and eye changes when compared to their littermates that had not been treated with antibiotics. The lack of significance in clinical scoring may be secondary to the numbers of mice assessed and repeating this analysis in a larger study may be more insightful. Overall, this result suggests that bacterial colonisation and/or infection play a role in the severity of inflammation in the *maflg^{fl}/maflg^{fl}* model of AD in a similar way to the results of human studies.

Overall cutaneous histological inflammation was also decreased in the *maflg^{fl}/maflg^{fl}* mice that were treated with antibiotics in the 1st 4 weeks of life in comparison to the *maflg^{fl}/maflg^{fl}* mice maintained treatment-free in the CON environment. Total cell count was significantly decreased in the *maflg^{fl}/maflg^{fl}* mice treated with antibiotics in comparison to the control *maflg^{fl}/maflg^{fl}* mouse group (Figure 5.3.1). The lymphocyte counts were comparable between the groups.

In the previous 2 chapters the results of clinical scoring of disease had similar patterns to the expression of total serum IgE antibody in the *maflg^{fl}/maflg^{fl}* mice in the different groups (Figure 3.2.4.4, Figure 4.2.2). Total IgE was at increased levels in the *maflg^{fl}/maflg^{fl}* mice bred in the CON environment compared to the *maflg^{fl}/maflg^{fl}* mice bred in the IVC environment (Figure 3.4) and compared to the *maflg^{fl}/maflg^{fl}* mice that were transferred in neonatal life from the CON conditions to the IVC conditions (Figure 4.4.1). In this chapter, expression of total serum IgE was increased in mice treated with antibiotics in comparison to the *maflg^{fl}/maflg^{fl}* mice bred in the CON environment (Figure 5.4.1). This was in contrast to the results of clinical scoring where the *maflg^{fl}/maflg^{fl}* mice bred in the CON conditions and not treated with antibiotics had non-significantly increased clinical scores in comparison to the *maflg^{fl}/maflg^{fl}* mice that had received antibiotics in early life (Figure 5.2.1). Total serum IgG₁ was measured as analyzing another component of the Th2 response. Levels of expression of IgG₁ from the *maflg^{fl}/maflg^{fl}* mice in the CON A/B group were found to be at similar levels to that expressed by the *maflg^{fl}/maflg^{fl}* mice in the CON environment. These results suggest that an increased Th2-type immune response is active in the inflammatory response in skin barrier deficiency both at baseline states and under experimental antibiotic intervention. The results of this chapter are in line with recent work by Hill *et al.* who demonstrated that serum IgE was elevated in antibiotic-treated mice (Hill et al., 2012). Assessment of

levels of IgE antibody in this chapter support the notion that depletion of bacterial communities is associated with an increase in IgE concentrations and exaggerated Th2 cell responses and allergic inflammation (Hill et al., 2012).

Expression of IgG_{2a} part of Th1 immune responses was found to be elevated in the *maflg^{fl}/maflg^{fl}* mice in the CON A/B group compared to mutant mice bred solely in the CON environment. This may indicate, in the context of skin barrier deficiency in the *maflg^{fl}/maflg^{fl}* model, that by the adult stage of 32 weeks a Th1 inflammatory response has begun to be part of chronic disease and inflammation.

The expression of IFN γ , the Th1 cytokine, from both general cell and T cell specific stimulated spleen cells, was not found to differ significantly between the groups of *maflg^{fl}/maflg^{fl}* mice regardless of antibiotic treatment (Figure 5.4.2). While the levels of IL-4 were comparable on general cell stimulation, it is noteworthy that there was a non-significant increase in IL-4 on T cell specific stimulation of spleen cells (Figure 5.4.2). This aligns with the results of total serum IgE, as above, and suggests that Th2 inflammatory responses are induced with the use of antibiotic treatment in a *maflg^{fl}/maflg^{fl}* model of AD.

The expression of IL-17 from spleen cells on general cell stimulation suggests that there is increased expression in *maflg^{fl}/maflg^{fl}* mice bred in the CON conditions without antibiotic treatment when compared to *maflg^{fl}/maflg^{fl}* mice bred in the same environment but treated with antibiotics, although this result was not significantly raised in this chapter (Figure 5.4.2). There was, however, significantly raised expression of IL-17 from spleen cells on T cell specific stimulation in *maflg^{fl}/maflg^{fl}* mice that were not treated with antibiotics compared to those that did receive the antibiotics regimens. This suggests that

the antibacterial effect in the treated *maflg^{fl}/maflg^{fl}* mice suppressed a Th17 response. Without the antibiotics, the results of the T cell specific stimulation of spleen cell expression of IL-17 imply that systemic IL-17 expression was induced in this *maflg^{fl}/maflg^{fl}* model of skin barrier deficiency in a CON environment.

The non-significantly increased expression of IFN γ on T cell specific stimulation of skin-draining lymph node cells suggests that a Th1 response may be induced in the *maflg^{fl}/maflg^{fl}* mice that were not treated with antibiotics (Figure 5.4.3). On analysis of IL-4 levels, there was increased expression of IL-4 from both generally stimulated cells and T cell stimulated cells in the *maflg^{fl}/maflg^{fl}* mice that had not been treated with antibiotics, compared to the group that had received treatment. These differences were not significant (Figure 5.4.3). This implies that a Th2 immune response is being induced at a local level although it is in contrast to the Th2 response that is suggested by the increased levels of serum IgE in the *maflg^{fl}/maflg^{fl}* mice treated with antibiotics compared to the non-treated *maflg^{fl}/maflg^{fl}* mice.

The levels of IL-17 expressed from skin-draining lymph node cells were comparable between the groups of *maflg^{fl}/maflg^{fl}* mice. This may suggest that the effect of the antibacterial action of the antibiotics does not alter the induction of IL-17 expression at a cutaneous level. This may be associated with the specific action of the antibiotic regimen in this chapter and that IL-17 levels remained at a comparable level to that expressed in the untreated *maflg^{fl}/maflg^{fl}* mice to be part of the first-line defence against microorganisms.

The development of a Th2 cytokine - mediated allergic inflammatory response is evident when the *maflg^{fl}/maflg^{fl}* model is treated with antibiotics. These results together

suggest that alteration of the commensal bacteria may influence the inflammatory response in a complex fashion that is expressed both systemically and cutaneously.

In this chapter, I have shown that by influencing the microbiome through the use of systemic antibiotic treatment in early life, the trajectory of disease in the context of skin barrier deficiency can be altered. The long-term severity of clinical disease is improved, even at the adult stage, following antibiotic treatment in early life in the *maflg^{fl}/maflg^{fl}* model of skin barrier deficiency. The results of the chapter support the studies, which have demonstrated that an allergic Th2 cell inflammatory response can be induced at a systemic level via the administration of antibiotics (Hill et al., 2012, Liang et al., 2006). It is noteworthy that this allergic effect of antibiotic alteration of commensal bacteria can have an effect even when only administered in early life. This raises questions about the use of systemic v's topical antibiotics when treating AD in affected patients. The use of sodium hypochlorite baths as a method of suppressing bacterial colonisation of skin in AD may be an important anti-bacterial part of treatment of AD. The results of this chapter reinforce the notion that skin barrier deficiency is key in the development of the inflammation in AD and the systemic responses that occur as disease progresses. The responses that are part of the inflammatory response in skin barrier deficiency are complex and are not restricted to local cutaneous cells but also have systemic consequences.

Chapter Six

The effects of skin barrier deficiency on the lungs

in the *maflg^{fl}/maflg^{fl}* mouse model

6.1 Introduction

Atopic dermatitis is frequently found to be associated with other forms of atopy including asthma, food allergy and allergic rhinitis (Spergel, 2010). A greater ingress of allergens through a defective skin barrier is thought to hasten and/or intensify the activation of the immunologic changes of asthma so patients with asthma in conjunction with *FLG* defects may experience asthma of greater severity with less effective overall control of symptoms and increased airway obstruction (Palmer et al., 2007). Strid *et al.* demonstrated how potent epicutaneous immunisation to protein antigens occurred in the context of skin barrier deficiency in a mouse model, where the stratum corneum was removed (Strid et al., 2005). The results of that study explored the notion that antigen presentation to LCs gives predominantly Th2 immune responses accounting for allergic sensitisation to some antigens (Strid et al., 2005). Studies in mouse models highlighted that epithelial barrier defects can initiate systemic sensitisation and increase the risk of allergic airway inflammation and other allergic diseases (Akei et al., 2006, Lee and Flavell, 2004, Spergel et al., 1998). While mice do not develop the human disease of ‘asthma’, these *in vivo* studies opened the possibilities to further investigate disease pathogenesis in the atopic march (Spergel, 2010). By assessing the lung function and inflammatory markers of a mouse model of skin barrier deficiency such as the *maflg^{fl}/maflg^{fl}* mouse, the effect of epithelial barrier deficiency on the lungs can be explored to further increase our understanding of the pathogenic manifestations of it and

its role in atopic disease. Within this chapter, the lung function of *maflg^{fl}/maflg^{fl}* housed in varying environments is compared to WT mice.

6.2 *maflg^{fl}/maflg^{fl}* mouse lung function

Variations in lung function are one of the consequences of changes secondary to asthma. Although the mouse lung is not an ideal model of the human lung, changes such as bronchoconstriction occur and can be measured by lung function assessment in the mouse lung. Following anaesthesia and tracheostomy, average lung resistance and lung compliance for each mouse was measured as described in Section 2.3. All mice were at 32 weeks. No significant differences were found in airway resistance between WT and *maflg^{fl}/malfg^{fl}* mouse groups, between the *maflg^{fl}/malfg^{fl}* mouse groups from the 2 different environments or between the *maflg^{fl}/malfg^{fl}* mouse group from the CON environment and the *maflg^{fl}/malfg^{fl}* mouse CON-IVC group (Figure 6.2.2). Lung compliance was assessed and showed significantly reduced lung compliance (C_L) in *maflg^{fl}/malfg^{fl}* mice relative to WT mice ($p < 0.05$ at 60 and 100 mg/ml of methacholine) (Figure 6.2.1) in all subsets of mice. While the increased compliance was most marked in *maflg^{fl}/malfg^{fl}* mice in CON housing and the *maflg^{fl}/malfg^{fl}* mice transferred from CON to the IVC environment at 1 week, it was not significantly different between any of the *maflg^{fl}/malfg^{fl}* animal subsets (i.e. those housed in CON, in IVC or transferred CON-IVC) (Figure 6.2.2).

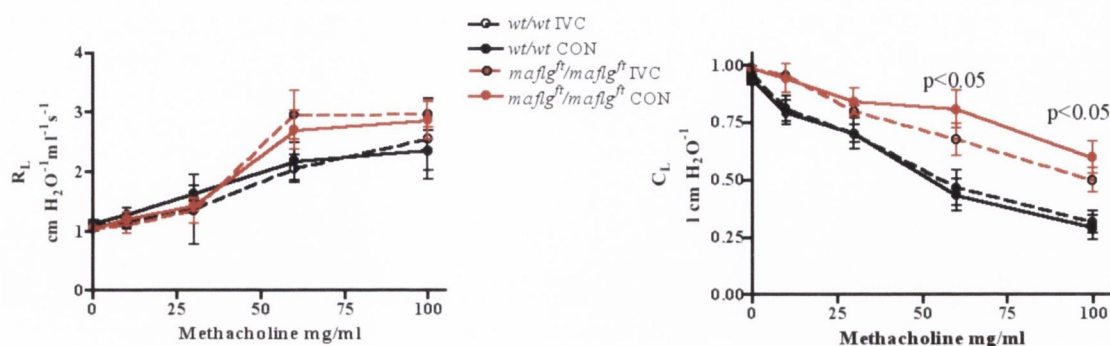


Figure 6.2.1 Measurements of lung resistance and lung compliance of WT and *maflg^{fl}/maflg^{fl}* mouse groups as part of overall lung function investigations. Lung resistance (a) and compliance (b) were measured by whole body plethysmography in WT and *maflg^{fl}/maflg^{fl}* mouse groups; between *maflg^{fl}/maflg^{fl}* mouse groups from the CON environment and the IVC environment. Lungs were stimulated by methacholine at varying concentrations and the compliance measured. Data is representative of 6 mice per *maflg^{fl}/maflg^{fl}* mouse groups and 2 mice per WT groups. Significance was assessed by student's t-test, *p* values (between WT and *maflg^{fl}/maflg^{fl}* mice) as indicated.

The lung resistance and compliance were also measured for the *maflg^{fl}/maflg^{fl}* mice transferred from CON to the IVC environment in neonatal life. While the reduced lung compliance was most marked in *maflg^{fl}/maflg^{fl}* mice in CON housing and the *maflg^{fl}/maflg^{fl}* mice transferred from CON to the IVC environment at 1 week, it was not significantly different between any of the *maflg^{fl}/maflg^{fl}* animal subsets (i.e. those housed in CON, in IVC or transferred CON-IVC) (Figure 6.2.2).

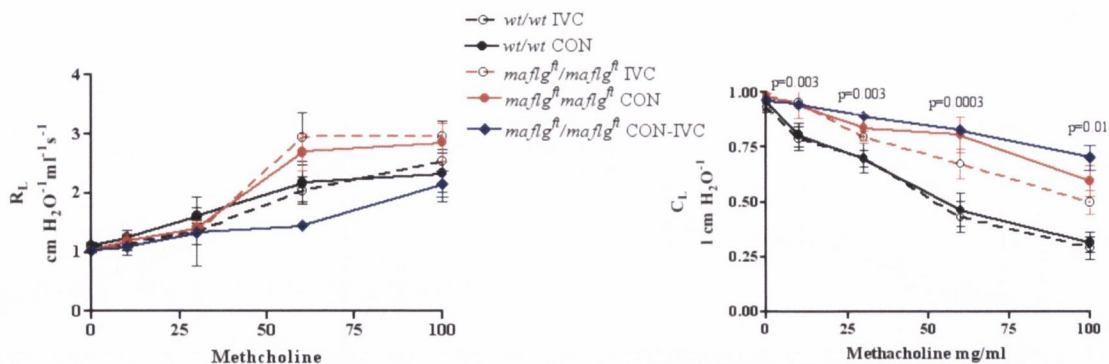


Figure 6.2.2 Measurements of lung resistance and lung compliance of WT and $\text{maf1g}^{\text{fl}}/\text{maf1g}^{\text{fl}}$ mouse groups including $\text{maf1g}^{\text{fl}}/\text{maf1g}^{\text{fl}}$ mouse CON-IVC group. Lung resistance (a) and compliance (b) was measured by whole body plethysmography in WT and $\text{maf1g}^{\text{fl}}/\text{maf1g}^{\text{fl}}$ mouse groups; between $\text{maf1g}^{\text{fl}}/\text{maf1g}^{\text{fl}}$ mouse groups from the CON environment and the IVC environment. Lungs were stimulated by methacholine at varying concentrations and the compliance measured. Data is representative of 6-7 mice per $\text{maf1g}^{\text{fl}}/\text{maf1g}^{\text{fl}}$ mouse groups and 2 mice per WT group. Significance was assessed by student's t-test, p values (between WT and $\text{maf1g}^{\text{fl}}/\text{maf1g}^{\text{fl}}$ mice) as indicated.

6.3 Cellular infiltration of airways in skin barrier deficiency of the *maflg^{fl}/maflg^{fl}* mouse model

Bronchoalveolar lavage (BAL) fluids were collected as detailed in Section 2.4.2 and cells within the BAL were analysed initially by quantifying as detailed in Section 2.4.2. Cell counts, assessments of infiltration of neutrophils, lymphocytes, monocytes and eosinophils, were performed on BAL fluids that were harvested after lung function measurements (Figure 6.3). No significant differences in counts of lymphocytes, monocytes and eosinophils were found between WT and *maflg^{fl}/maflg^{fl}* mouse groups, between the *maflg^{fl}/maflg^{fl}* mouse groups from the CON and IVC environments or between the *maflg^{fl}/maflg^{fl}* mouse CON or IVC groups and the *maflg^{fl}/maflg^{fl}* mice in the CON-IVC group. Neutrophils were significantly increased in the *maflg^{fl}/maflg^{fl}* CON group compared to the *maflg^{fl}/maflg^{fl}* CON-IVC group ($p = 0.0234$, Figure 6.3). No other significant differences were shown between the other *maflg^{fl}/maflg^{fl}* and WT groups on neutrophil count (Figure 6.3).

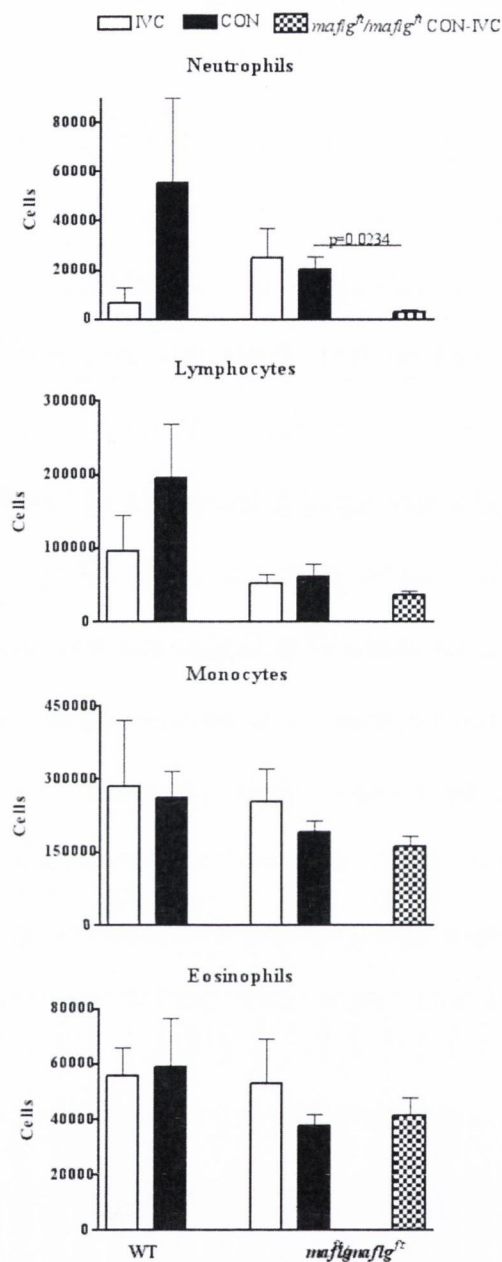


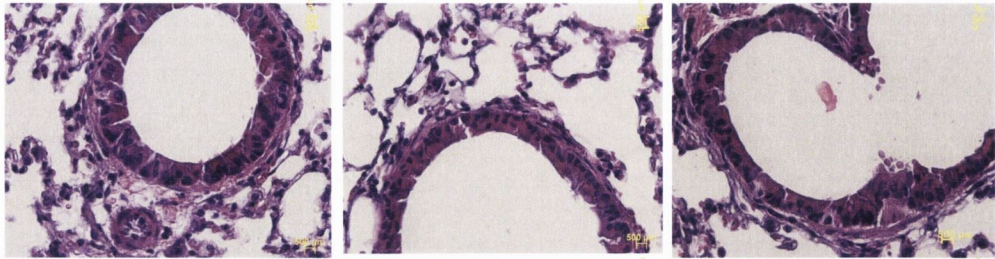
Figure 6.3 Cellular findings in bronchoalveolar lavages (BALs) of WT and *maf1g^{fl}/maf1g^{fl}* mouse groups. Cell counts of neutrophils, lymphocytes, monocytes and eosinophils were performed on BALs that were harvested after lung function measurements. Data is representative of 2 (WT) – 7 (*maf1g^{fl}/maf1g^{fl}* mice) mice per group. Significance was analysed by student's t-test and *p* values are indicated.

6.4 Lung histopathology in the *maflg^{fl}/maflg^{fl}* mouse model

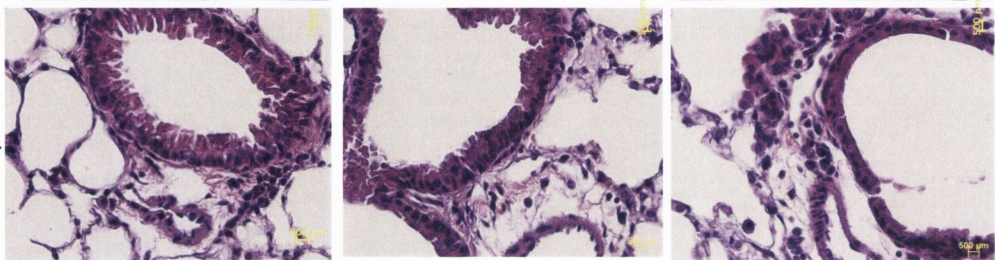
As per Section 2.5.2, the lungs of age - and sex - matched mice (WT and *maflg^{fl}/maflg^{fl}* from the IVC and CON environments, and *maflg^{fl}/maflg^{fl}* mice from the CON-IVC group and the CON A/B group) were removed, placed in 10% (vol/vol) formaldehyde saline and sent for histological processing (AML Laboratories Inc., Baltimore, Maryland, USA). Paraffin-fixed lung sections were stained with H&E and used for histological analysis. Increased inflammatory changes were noted in the H&E lung slides of *maflg^{fl}/maflg^{fl}* mice from any environmental condition in comparison to WT mice housed in the same units, (Figure 6.4.1).

IVC

WT

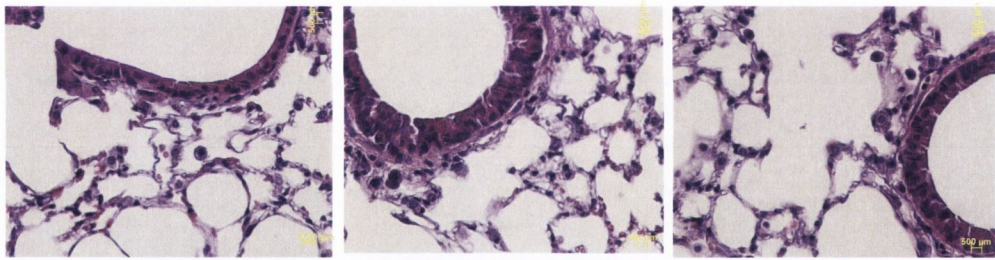


maflg^{fl} -/-



CON

WT



maflg^{fl} -/-

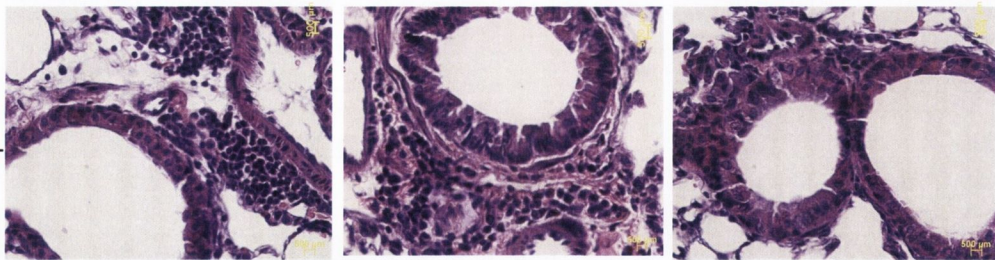


Figure 6.4.1 Lung sections (stained with H&E) from WT and *maflg^{fl}/maflg^{fl}* mouse groups from IVC and CON environments. Lung sections from groups of WT (N = 2 per group) and *maflg^{fl}/maflg^{fl}* mice (N = 6 per group) housed in IVC and CON environments were analysed by H&E staining. Analysis was undertaken to assess inflammatory changes including peribronchiolar, perivascular and alveolar inflammation. Scale as indicated, at 40x magnification.

Inflammatory changes that occurred in the lung airways and parenchymal tissue were scored to have a quantifiable measure of lung inflammation occurring in the *maflg^{fl}/maflg^{fl}* mouse model which was not specifically sensitised, as detailed in Section 2.5.2. Inflammatory changes were scored on 10 areas of each lung section slide and 4 patterns of inflammation (notably peribronchiolar, perivascular and alveolar infiltration) were scored on a scale of 0-4. On quantitative analysis, as outlined above, there was no significant difference found between the WT groups in the IVC and CON conditions. Inflammatory changes in the lungs were significantly elevated in *maflg^{fl}/maflg^{fl}* mice from any environmental condition relative to WT mice housed in the same units (Figure 6.4.2). There was also a significantly higher incidence of inflammation found in the H&E stained lung slides of *maflg^{fl}/maflg^{fl}* mouse groups from the CON environment compared to the *maflg^{fl}/maflg^{fl}* mice bred in the IVC environment (Figure 6.4.2).

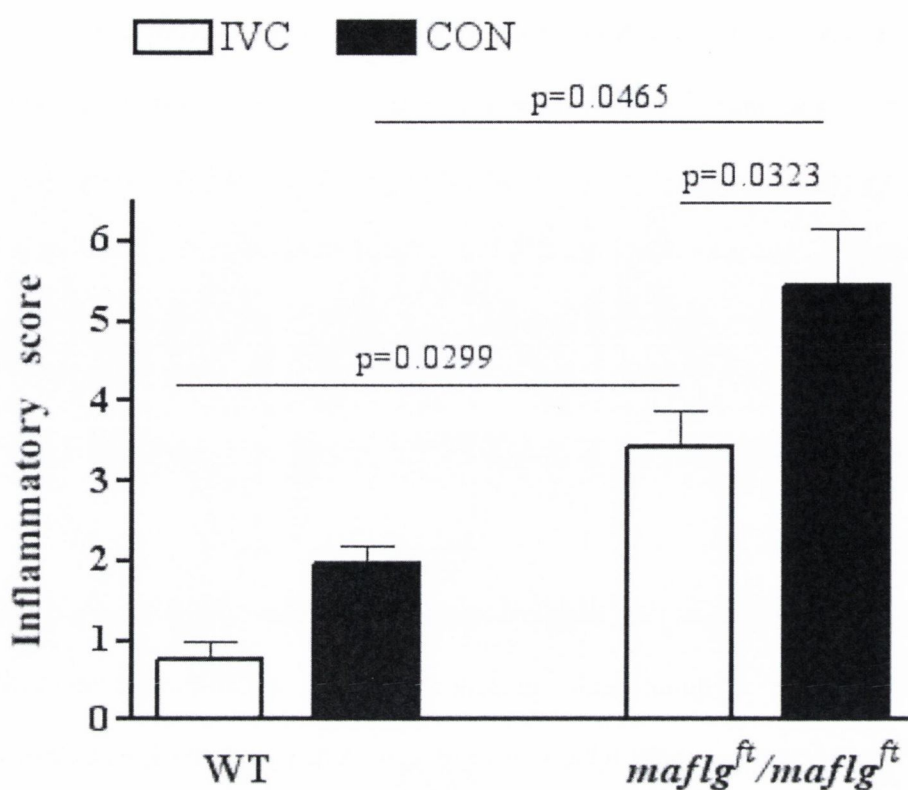


Figure 6.4.2 Scoring of lung inflammatory changes in WT and *maflg^{ft}/maflg^{ft}* mouse groups from IVC and CON environments. Inflammatory changes that occurred in the lungs of WT and *maflg^{ft}/maflg^{ft}* mouse groups from the IVC and CON environments were scored using a gradation system as per Section 2.5.2. Data is presented as mean + / - SEM and is representative of 2-6 mice per group. Significance was analysed with student's t-test and p values are indicated.

The *maflg^{fl}/malfg^{fl}* mice in the CON-IVC group also showed significantly reduced lung inflammation relative to continuous CON housing, almost rendering the lung inflammation as low as continuous housing in IVC (Figure 6.4.3). Significantly increased inflammatory scores were also noted in *maflg^{fl}/malfg^{fl}* mice lung sections from the CON environment relative to the IVC environment in this part of the study (Figure 6.4.3).

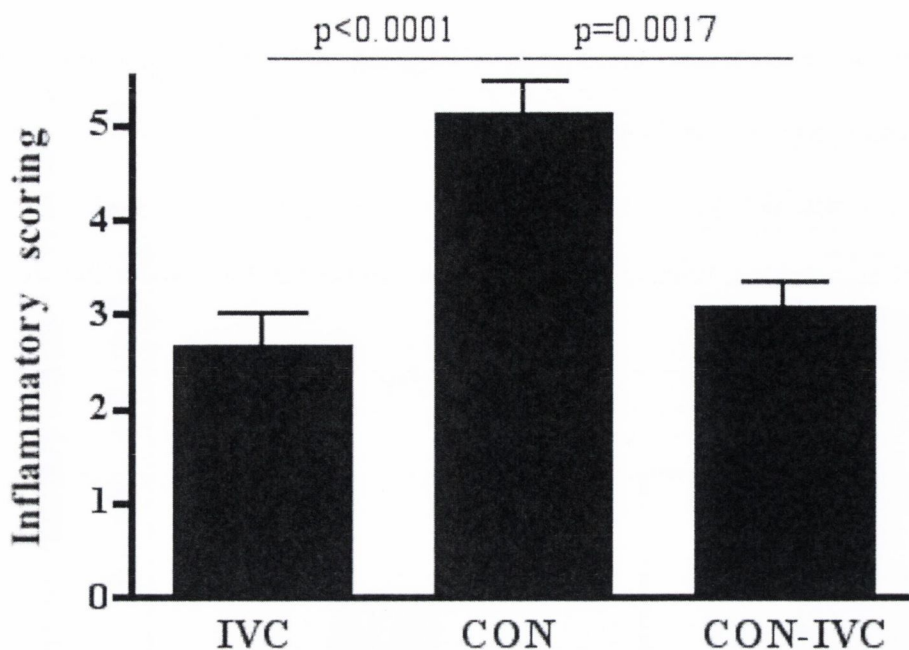


Figure 6.4.3 Scoring of lung inflammatory changes in *maflg^{fl}/maflg^{fl}* mouse groups from IVC, CON, CON-IVC groups. Inflammatory changes in lungs harvested from *maflg^{fl}/maflg^{fl}* mouse groups from IVC, CON, CON-IVC and CON A/B groups were analysed. Data is presented as mean + / - SEM and is representative of 4-7 mice per group. Significance was determined using the student's t-test, *p* values are as indicated.

Administering systemic antibiotics, however, did not alter lung inflammation in the *maflg^{fl}/maflg^{fl}* mouse CON A/B group when compared to the *maflg^{fl}/maflg^{fl}* mice that did not receive antibiotic treatment (Figure 6.4.4). Comparable levels of inflammation were found between these groups.

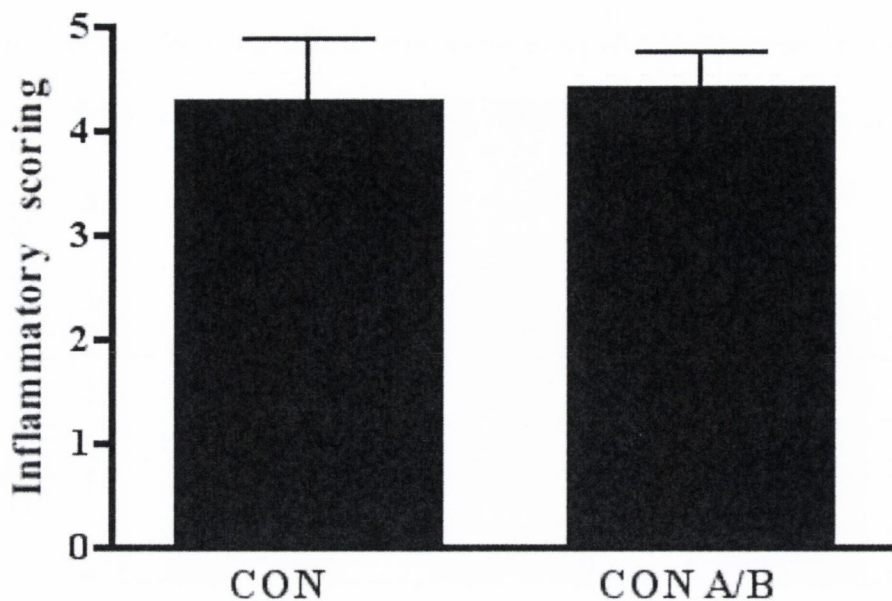


Figure 6.4.4 Scoring of lung inflammatory changes in *maflg^{fl}/maflg^{fl}* mouse groups from CON and CON A/B groups. Inflammatory changes in lungs harvested from *maflg^{fl}/maflg^{fl}* mouse groups from the CON and CON A/B groups were analysed. Data is presented as mean + / - SEM and is representative of 4-8 mice per group. Significance was determined using the student's t-test and *p* value was considered significant if *p* < 0.05.

6.5 Peribronchiolar and parenchymal lung changes in the *maflg^{fl}/maflg^{fl}* mouse model

Deposition of collagen in the lung is associated with chronic lung inflammation and fibrosis. To assess if there was increased deposition of collagen, altering the lung parenchyma, lung sections from *maflg^{fl}/maflg^{fl}* mice were compared to WT mice housed in the different environmental conditions. The results of the scoring of collagen deposition in lung sections from the *maflg^{fl}/maflg^{fl}* mice in the IVC and CON conditions were also compared to each other and to the interventional *maflg^{fl}/maflg^{fl}* mouse groups (CON-IVC and CON A/B).

The lung slides were stained with Masson's trichrome blue (MTB) stain, as per Section 2.5.2, which detects deposition of collagen in peribronchiolar and parenchymal locations (Figure 6.5.1).

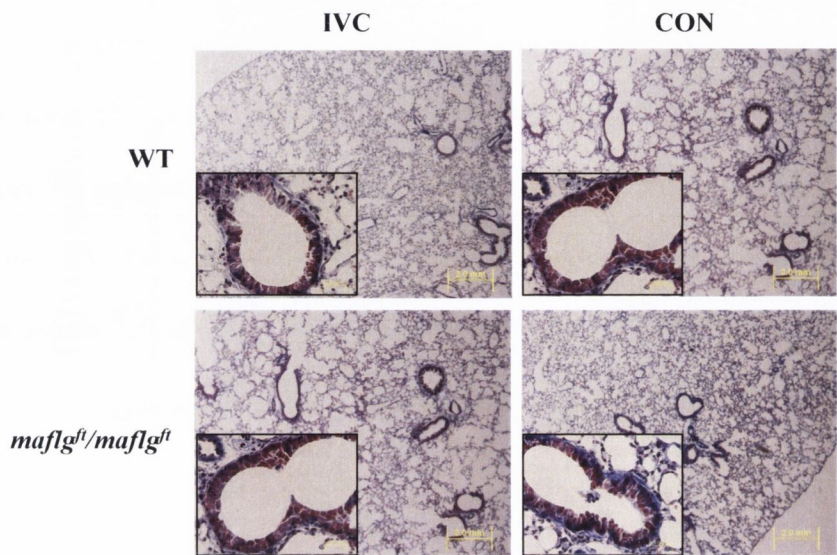


Figure 6.5.1 Lung sections from WT and *maflg^{fl}/maflg^{fl}* mouse groups from IVC and CON environments were stained with Masson's trichrome blue (MTB) stain. Staining of non-sensitised WT control and *maflg^{fl}/maflg^{fl}* mouse lungs from IVC and CON environments with MTB stain. Scale as indicated, 40x magnification. ice in the CON-IVC group.

Masson's trichrome blue staining was used for quantification of lung collagen deposition in WT and *maflg^{fl}/maflg^{fl}* lung samples, from both these locations. The final score for each sample reflected the grading using both peribronchiolar and parenchymal scores. Significantly increased deposition was noted in both the lung parenchymal tissue and in a peribronchiolar location in *maflg^{fl}/maflg^{fl}* mice bred in the CON housing compared to littermates bred in the IVC environment (Figure 6.5.2). Significantly higher levels of collagen and inflammation were seen when *maflg^{fl}/maflg^{fl}* mice from CON housing were compared to *maflg^{fl}/maflg^{fl}* mice from the CON-IVC group of mice (Figure 6.5.2). Levels of collagen deposition were comparable between the *maflg^{fl}/maflg^{fl}* mice from the IVC group and the CON-IVC group (Figure 6.5.2).

There were significantly higher levels of collagen deposition found on histological scoring when *maflg^{fl}/maflg^{fl}* mice from CON housing were compared to *maflg^{fl}/maflg^{fl}* mice from the CON A/B group (Figure 6.5.3).

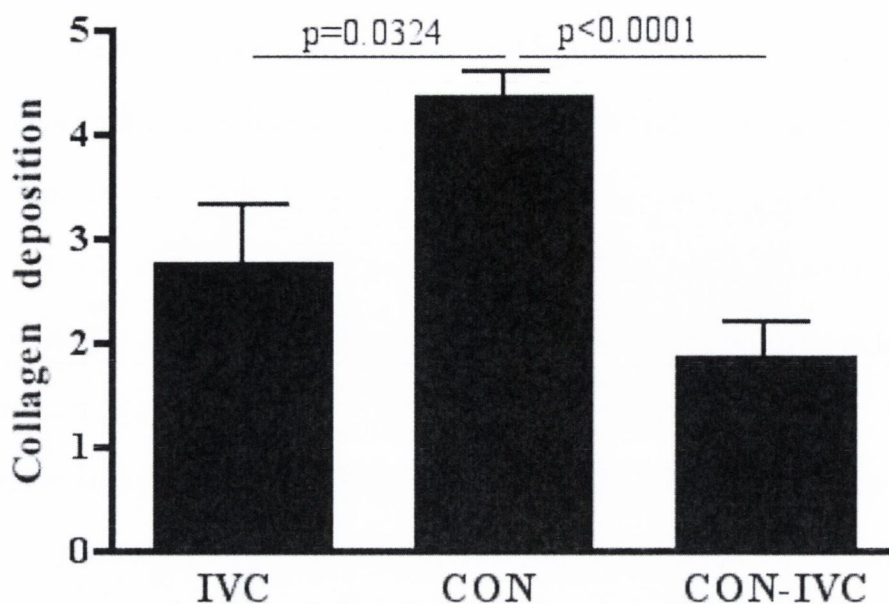


Figure 6.5.2 Scoring of MTB staining on lung sections from *maflg^{fl}/maflg^{fl}* mouse groups from IVC and CON environments and from the CON-IVC group. Significantly increased deposition of MTB was found in the *maflg^{fl}/maflg^{fl}* mice bred in the CON housing compared to their *maflg^{fl}/maflg^{fl}* mice littermates in the CON-IVC group. Data is presented as mean + / - SEM and is representative of 6-7 mice per group. Significance was analysed using a student's t-test, *p* values as indicated.

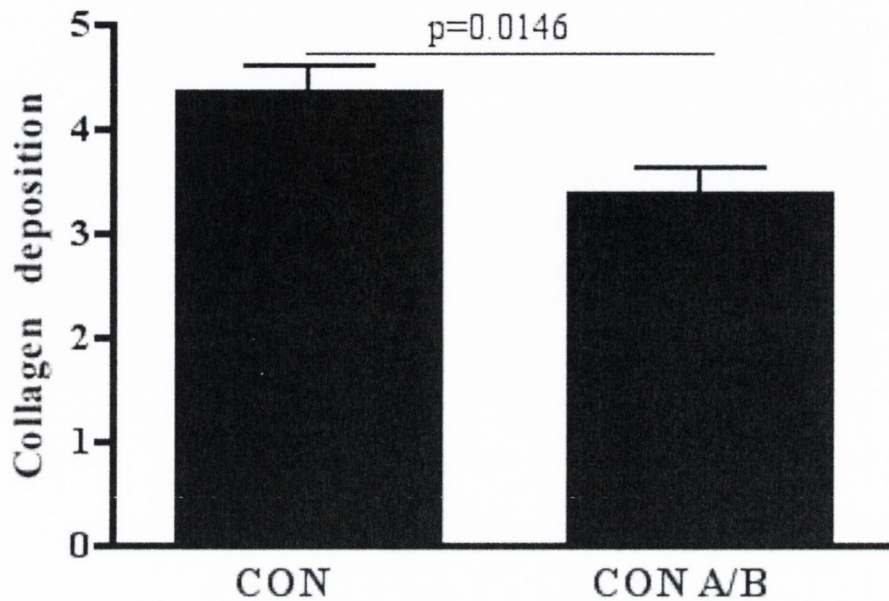


Figure 6.5.3 Scoring of MTB staining on lung sections from *maflg^{fl}/maflg^{fl}* mouse groups from the CON environment, untreated controls (CON) and treated (CON A/B). Significantly increased deposition of MTB was found in the *maflg^{fl}/maflg^{fl}* mice bred in the CON housing compared to their *maflg^{fl}/maflg^{fl}* mice littermates in the CON A/B group. Data is presented as mean + / - SEM and is representative of 4-8 mice per group. Significance was analysed using a student's t-test, *p* value as indicated.

6.6 Assay of collagen to assess lung changes in the *maflg^{ft}/maflg^{ft}* mouse model

A collagen assay kit was used to further assess deposition of collagen in the lungs as another method of quantifying increased changes that may be secondary to the skin barrier defect in the *maflg^{ft}/maflg^{ft}* mice and if this was associated with different environments (Section 2.7.1). The results of this assay demonstrate that the extraction of collagen from the lungs of *maflg^{ft}/maflg^{ft}* mouse IVC, CON and CON-IVC groups was comparable to those found in WT mouse lungs (Figure 6.6). However, the levels found in the *maflg^{ft}/maflg^{ft}* mice from the CON environment were non-significantly higher than those of WT and those from *maflg^{ft}/maflg^{ft}* mice from the IVC environment (Figure 6.6).

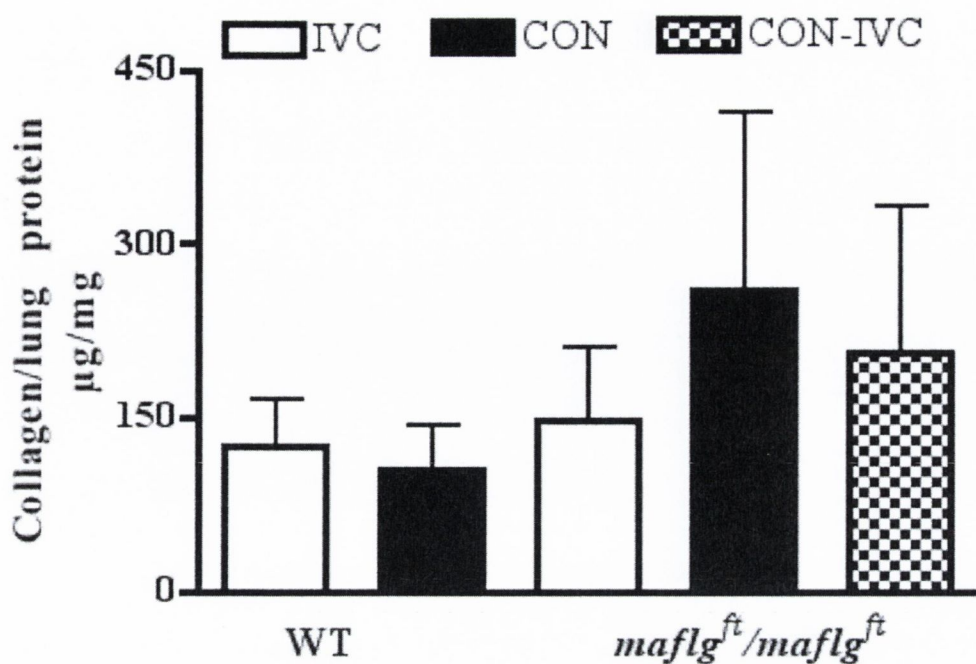


Figure 6.6 Collagen levels in lung homogenates. Collagen was extracted from lung homogenates from WT and *maflg^{ft}/maflg^{ft}* mouse groups from the IVC and CON environments and the *maflg^{ft}/maflg^{ft}* mouse CON-IVC group and quantified by ELISA analysis. Data is presented as mean + / - SEM and is representative of 6-7 mice per group. Statistical analysis was performed with an unpaired t-test two-tailed. Data was considered significant if $p < 0.05$.

6.7 Assay of lung tissue myeloperoxidase (MPO) production

During extensive inflammation neutrophils undergo necrosis, which can lead to release of MPO. Myeloperoxidase can damage surrounding tissue and cells. To further investigate distal effects in the lung of skin barrier deficiency an assay of MPO production from the lung samples homogenates of the WT and *maflg^{ft}/maflg^{ft}* mice groups were processed and analysed as detailed in Section 2.7.2. Levels of MPO from the *maflg^{ft}/maflg^{ft}* IVC, CON and CON-IVC groups were not found to be significantly higher than those found from WT lung homogenates (Figure 6.7).

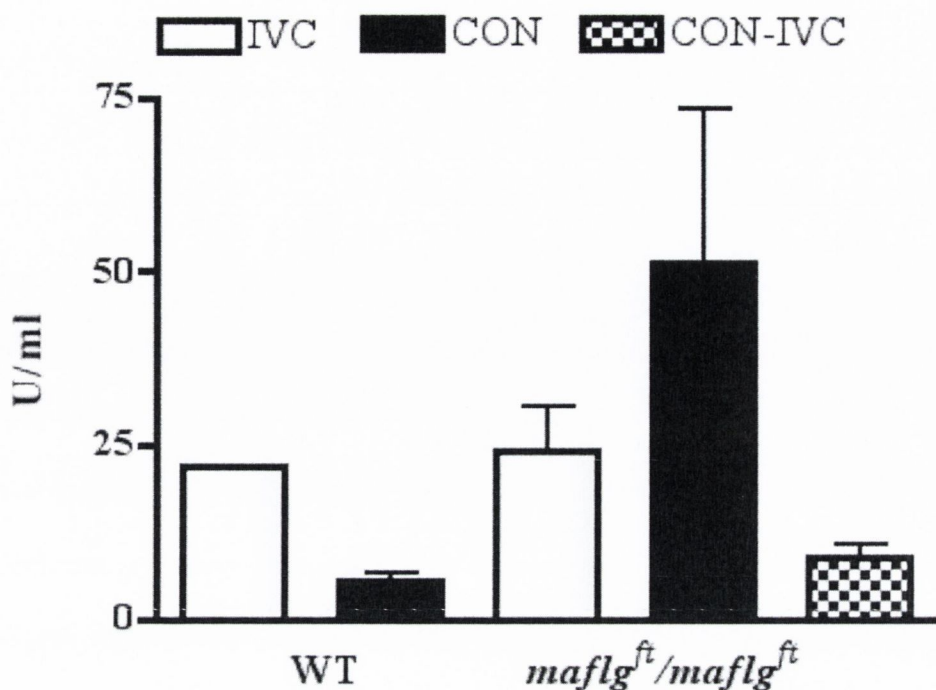


Figure 6.7 MPO assay of lung homogenates of WT and *maflg^{ft}/maflg^{ft}* mice from the IVC and CON environments and the *maflg^{ft}/maflg^{ft}* mice from the CON-IVC group. MPO was extracted from lung homogenates from WT and *maflg^{ft}/maflg^{ft}* mouse groups from the IVC and CON environments and the *maflg^{ft}/maflg^{ft}* mouse CON-IVC group and quantified by ELISA analysis. Data is presented as mean + / - SEM and is representative of 6-7 mice per group. Statistical analysis was performed with an unpaired t-test two-tailed. Data was considered significant if $p < 0.05$.

6.8 Discussion

The associations between AD and other atopic diseases such as allergic rhinitis and asthma are complex and multifactorial. In large population studies, *FLG* mutations conferred an overall risk of asthma ranging from 1.48 to 1.79 (Henderson et al., 2008, Weidinger et al., 2008). When the barrier properties of the stratum corneum are diminished, as in AD, harmless environmental agents may cause irritation, inflammation and sensitisation (Strid et al., 2004). As discussed in Section 6.1, epicutaneous exposures with protein antigens induce Th2-like immune responses with high production of IgE in mice and airway hyper-responsiveness (Wang et al., 1996, Spergel et al., 1998). This chapter seeks to analyse the effect of skin barrier deficiency in the *maflg^{fl}/maflg^{fl}* mouse model on the lungs when the mice are bred in different environmental conditions. It has previously been shown that airway hyperresponsiveness does not occur in the *maflg^{fl}/maflg^{fl}* mouse model (Fallon et al., 2009). The results of this chapter aim to further assess inflammation in the lung parenchyma. Lung function was assessed and lung resistance and compliance were analysed. There was no difference in lung resistance between the groups of mice analysed, however, there was significantly decreased lung compliance in *maflg^{fl}/maflg^{fl}* mice compared to the WT groups (Figure 6.2.1). Lung compliance was shown to be comparable between the *maflg^{fl}/maflg^{fl}* mice in the IVC, CON and CON-IVC groups. (Figure 6.2.2). This change in lung compliance reflects a restrictive process in the lung parenchyma.

Characterisation of the inflammatory cell infiltrate in the BAL indicated that there was no variation in cell infiltration into the lungs in *maflg^{fl}/maflg^{fl}* mice relative to WT mice. There was no elevation of cells such as eosinophils, which are associated with allergic reactions and asthma. Of note, there were no significant differences in inflammatory

cellular content of BAL between *maflg^{fl}/maflg^{fl}* mice housed in different environmental conditions or transferred between environments (Figure 6.3).

The above results indicated that airway hyper-responsiveness (AHR) was not part of the downstream effects of skin barrier deficiency in the *maflg^{fl}/maflg^{fl}* mouse model. They also suggest that the changes in lung compliance tend towards a restrictive lung pattern as is found in chronic lung changes such as fibrosis (Le Saux et al., 2008). Examining the histopathology of lung sections stained with H&E from *maflg^{fl}/maflg^{fl}* mouse groups emphasised that although acute allergic-like disease does not appear to occur in this model of skin barrier deficiency, increased chronic lung inflammation does occur (Figure 6.4.1, Figure 6.4.2). Histopathology scoring reflects the changes found on cutaneous histology analysis in Chapters 3 and 4, where there was increased inflammation found in the *maflg^{fl}/maflg^{fl}* mice in comparison to WT controls. What was also replicated was that the inflammatory changes were increased in the *maflg^{fl}/maflg^{fl}* mice from the CON conditions in comparison to those in both the IVC and CON-IVC groups (Figure 3.3.2.1, Figure 4.3.2).

As described above, collagen deposition is associated with chronic lung changes such as fibrosis. Deposition of collagen in the lungs of the WT and *maflg^{fl}/maflg^{fl}* mice was assessed. Histological analysis demonstrated that there was significantly increased deposition of collagenous fibrotic material in the peribronchiolar and parenchymal regions in the lungs of the *maflg^{fl}/maflg^{fl}* mice from the CON group in comparison to the *maflg^{fl}/maflg^{fl}* mice in the IVC and the CON-IVC (6.4.2). There was no difference found in collagen deposition between the *maflg^{fl}/maflg^{fl}* mice CON and CON A/B groups

(Figure 6.4.3). The measurement of collagen by assay did not show significant differences although a similar trend was observed as between the histology collagen scoring in the *maflg^{fl}/maflg^{fl}* mouse groups (Figure 6.6). These results suggest that skin barrier deficiency, as in the *maflg^{fl}/maflg^{fl}* mouse models may be associated with lung changes that occur over an extended period of time, resulting in increased inflammation, increased peribronchiolar deposition of collagen and alteration in the lung tissue structure with increased deposition of collagen in the lung parenchyma.

No significant differences were found between the *maflg^{fl}/maflg^{fl}* mouse groups and WT or between the *maflg^{fl}/maflg^{fl}* mice bred in the CON conditions compared to those bred in the IVC environment or transferred from CON-IVC on MPO assay (Figure 6.7). The trend that developed followed that of the collagen assay and suggests that increased MPO may be released in the *maflg^{fl}/maflg^{fl}* mice compared to WT. These results also imply that *maflg^{fl}/maflg^{fl}* mice in the CON environment have higher levels of MPO release than their littermates in the IVC conditions or those that were transferred in neonatal life (Figure 6.7). This suggests that inflammation occurs in the lungs of this murine model of skin barrier deficiency. Inflammation of this nature may be damaging for the lung tissue and further exacerbate the distal effects of skin barrier deficiency.

While the *maflg^{fl}/maflg^{fl}* mouse model cannot be used as a completely analogous model of the interaction of *FLG* mutations with the risk of chronic lung changes as in humans, the results of this chapter develops the notion that skin barrier deficiency is a risk factor for altered lung responsiveness. This chapter also highlights that even within the context of skin barrier deficiency, environmental changes play a very significant role in the

inflammatory lung pathology secondary to the initiating cutaneous inflammation. These results indicate that disease pathogenesis in the lung, secondary to AD, may involve more types of inflammation than solely allergic lung inflammation as in allergic rhinitis and asthma. Skin barrier deficiency may have increased chronic effects on the lung including lung fibrosis. These results demonstrate the important influence that different environmental conditions had on lung inflammation of this nature.

Chapter 7

General Discussion

Atopic dermatitis has a complex aetiology involving multiple genetic and environmental interactions (Irvine et al., 2011). This work has sought to explore the environmental aspect of the multifactorial nature of the inflammatory pathology in this condition. Skin barrier deficiency models have provided important details as to the complex immunology of AD. This work characterises further the *maflg^{fl}/maflg^{fl}* mouse, one relevant murine model, given that these mice carry, in addition to the matted and nonagouti mutations, an analogous filaggrin mutation to that found in human disease. This thesis seeks to assesses the impact of differing environmental conditions on the inflammatory phenotype of the *maflg^{fl}/maflg^{fl}* mouse and how interventions involving alterations in such conditions and also in the microbiome impacts on inflammation.

In human disease inverse associations between atopy and various environmental factors, including daycare attendance, sibling order (lower rank, i.e. more elder siblings) and farming lifestyle support the hygiene hypothesis, which is an important tenet in atopic research (Taieb, 1999, Cork et al., 2009, Candi et al., 2005, Zutavern et al., 2005). The effects of these factors were consistent with a protective influence of postnatal infection against the programming of allergic immune responses and that the critical period for protection extends beyond the 1st month of life and the 1st postnatal exposure to an allergen (Strachan et al., 1996). However, many environmental factors such as climate temperature and humidity; domestic temperature and humidity; ventilation; insulation; low sibling order; daycare attendance and neonatal cat exposure have a role to play in exacerbating AD and acting as flare factors for AD as the disease progresses (Langan and

Williams, 2006, Bisgaard et al., 2008, Cork et al., 2009, Langan et al., 2009, Cramer et al., 2010). Recent studies have demonstrated that in an individual with known skin barrier deficiency, secondary to *FLG* mutation, the risk of developing AD is increased if they are exposed to certain environmental factors, such as older siblings, neonatal cat exposure and daycare attendance (Bisgaard et al., 2008, Cramer et al., 2010). This is in contrast to the hygiene hypothesis as it implies that increased exposure and infection in early life increases the development of AD and possible general atopy in the specified case of skin barrier deficiency. This work investigates the interplay between genetic and environmental factors that play a role in disease.

In this thesis, *maflg^{fl}/maflg^{fl}* mice (with their pre-existing skin barrier defect) were used to investigate the impact of the environmental conditions in which animals are housed on the spontaneous development of skin inflammation, the severity of inflammation and on the generation of distal inflammation in the lungs.

Initially, this study confirmed that the *maflg^{fl}/maflg^{fl}* mouse model demonstrated spontaneous phenotypical cutaneous inflammatory changes displayed previously (Presland et al., 2000) and that a spectrum of disease severity occurred which is consistent with the spectral nature of AD in humans. The work described in this thesis then goes on to explore the interaction between environmental factors and skin barrier deficiency in depth, predominantly through the use of two alternate housing conditions – the individually ventilated caging which is supplied with controlled, filtered air and the conventional caging which is, in essence, in full communication with external air. In summary, the IVC caging restricts external aeroallergens and aerosolised microbes from the laboratory ambient atmosphere from coming into contact with the *maflg^{fl}/maflg^{fl}* mice. In contrast, CON caging allows any environmental agents, aeroallergens or

aerosolised microbes in the laboratory atmosphere and from other animal cages to penetrate the cages. The significant differences in cutaneous and systemic responses of *maflg^{fl}/maflg^{fl}* mice in the 2 different environments highlights the importance of the influence of environmental conditions on inflammation in the presence of a skin barrier defect.

The *maflg^{fl}/maflg^{fl}* mice that were bred in the IVC unit developed less severe clinical features than their littermates in the CON environment as they aged. There was similar significance in the differences between the IVC and CON groups of *maflg^{fl}/maflg^{fl}* mice with regards to histological inflammation scored on analysis. The *maflg^{fl}/maflg^{fl}* mice in the CON conditions had significantly increased lymphocytic cellular inflammation in comparison to their littermates in the IVC environment (Figure 3.3.2.2). Differences were not found between the groups of WT mice in the IVC and CON units, implying that the aetiological factor in the increased inflammation was not related to the environment. This suggests that the causal agent is related to the *maflg^{fl}/maflg^{fl}* mice while they are bred in the different conditions. It is also further suggested that in the context of skin barrier deficiency, general environmental exposures play a key role in the initial responsive inflammation.

When a group of *maflg^{fl}/maflg^{fl}* mice were switched from CON to IVC conditions in neonatal life to determine this allowed investigation of how much the skin barrier deficiency inflammatory response was determined by early programming, and how much was simply brought about by changes in environmental factors. In transferring the mice from CON to IVC, the clinical inflammatory outcome was improved and this was seen in the reduction in clinical and histopathological severity of phenotypical inflammatory

changes. The fact that these results were comparable but not as low as the inflammatory score for the IVC controls suggests that the initiation of a more severe inflammatory response began before the 1st week of life. At birth, the humidity of the environment changes rapidly from *in utero* and the pH of the skin has not yet reached the acidic pH that is necessary for normal function (Cork et al., 2009). It may be that at this point that an early inflammatory response is instigated. However, the transfer experiment also suggests that reducing environmental exposures in early life can ameliorate inflammatory consequences of skin barrier deficiency. This is in line with the study by Lee *et al.* where severity of disease was reduced by admission of the paediatric patients to a low pollutant room (Lee et al., 2011).

Serological analysis of antibody development demonstrated that there was an early Th2-type response, in the presence of skin barrier deficiency, which then switched to a Th1-type response in more chronic disease, which supports our understanding of the Th2/Th1 switch in AD (Oyoshi et al., 2009a, Spergel et al., 1999). This early Th2-type response was increased in the *maflg^{fl}/maflg^{fl}* mice from the CON environment compared to the *maflg^{fl}/maflg^{fl}* mice from the IVC environment, which further illustrates that in a more stimulated environment, the immunopathology is more dramatic and the excessive stimulation with allergen leads to more marked Th2 and Th1 responses. This is in contrast to what would have been expected if the hygiene hypothesis held sway in the context of skin barrier deficiency. Total serum IgE continued to increase in expression in the *maflg^{fl}/maflg^{fl}* mice as they aged which suggests that a Th2-type response to external factors remained a significant part of the immunological responses in this model of skin barrier deficiency even in the *maflg^{fl}/maflg^{fl}* mice bred in the CON environment regardless of the extent of environmental exposures, which according to the hygiene

hypothesis should lead to decreased atopy with increased exposure. It would thus suggest that the *maflg^{fl}/maflg^{fl}* mice bred in the IVC unit, with more controlled atmospheric conditions would have increased severity of disease, which did not occur in this experiment. This study implies that skin barrier deficiency alters the parameters by which constitutional immune responses are programmed for an individual and increased exposures tend to be associated with increased inflammation and severity because of what occurs secondary to the increased ingress of antigens and allergens.

The cytokine analysis of skin-draining lymph nodes and spleens from *maflg^{fl}/maflg^{fl}* mice shows that *maflg^{fl}/maflg^{fl}* mice developed inflammatory and atopic responses, with elevated production of Th1, Th2 and Th17 cytokines that was more marked in mice housed in CON conditions compared to the IVC environment. The increased environmental exposures that were present in the CON environment resulted in an increased IFN γ or Th1 response from the *maflg^{fl}/maflg^{fl}* mice in the CON environment compared to the *maflg^{fl}/maflg^{fl}* mice bred in the IVC caging. The pattern of IL-4 expression, which was equivalent in either environment, may indicate that a complete Th2-type allergic response abates by week 32 in the *maflg^{fl}/maflg^{fl}* mice (in the absence of specific epicutaneous sensitisation or challenges).

It is well established that Th17-type responses are part of the first-line defence against microorganism (Liang et al., 2006). The significant elevation of expression of IL-17 in *maflg^{fl}/maflg^{fl}* mice from the CON environment compared to those from the IVC unit, suggests the involvement of a Th17-type immune response to transcutaneous antigen entry at any age. This may indicate that while Th2 responses are part of an early response with allergic inflammation markers or found in acute flares of disease and Th1 responses are associated with chronic disease in AD, Th17 responses are part of a

response to antigen initiation of inflammation in skin barrier deficiency regardless of age of subject or chronicity of disease. This implies that the influence of environmental conditions is of considerable importance when constituent immune responses are being stimulated and re-activated when necessary.

Both IFN γ and IL-17 from skin-draining lymph nodes in the *maflg^{fl}/maflg^{fl}* mice from all 3 environmental groups showed patterns of expression that were similar to those of clinical and histological inflammation. The importance of a Th17-type response was further demonstrated by the increased expression from the lymph nodes of the *maflg^{fl}/maflg^{fl}* mice from the CON environment compared to those from the IVC unit and in the CON-IVC group. There was no consistent pattern of cytokine expression demonstrated from stimulated spleen cells. This may be because there was a limited development of a systemic response in an unstimulated mouse model of inflammation.

It has been widely acknowledged that the microbiome is a key part of the skin barrier and that alterations in the skin microbiome are associated with increased inflammatory disease in the skin (Grice et al., 2009, Kong, 2011, Kong et al., 2012). Antibiotics were administered to *maflg^{fl}/maflg^{fl}* mice in the CON environment and the pups that were assessed at 32 weeks had received treatment in utero and for the 1st 4 weeks, until weaning. This was to determine the inflammatory and immunological consequences of modifying the cutaneous microbiome in early life in a model of skin barrier deficiency. Also, from the results of the previous chapters, this is the time period when it was suggested that inflammatory responses are set up in skin barrier deficiency. The clinical and histological severity of cutaneous inflammation of *maflg^{fl}/maflg^{fl}* mice that had antibiotic administration in early life (CON A/B) was less than that of *maflg^{fl}/maflg^{fl}* mice bred in the CON environment without treatment although the results of the clinical

scoring were not significant. This suggests that alteration of the bacterial flora improved the cutaneous manifestation of disease but it did not impact on the level of severity as much as reducing the environmental exposures in which the *maflg^{fl}/maflg^{fl}* mice were housed. It is thus implicated that exposure to aerosolised bacteria can only be partly responsible for an increased inflammatory response in the conventional environments and that external factors have as much of an impact on the long-term cutaneous inflammatory outcome in skin barrier deficiency as does altering the cutaneous microbiome. This may be because the genetic mutations associated with skin barrier deficiency in AD, have multiple functions in the skin barrier and altering the microbiome alone will not replace the effect of decreasing external influences.

As mentioned above, Th17 cells are essential for first-line defence and chronic skin infection is associated with an impaired or absent IL-17 response. IL-17 responses are also associated with triggering by bacteria-derived superantigens (Eyerich et al., 2009). The results from this study between the effects of different environments in skin barrier deficiency, imply that there are external factors such as allergens and organisms (bacteria, yeasts) present at an increased level in the CON environment compared to the IVC environment, and systemic alteration of the cutaneous microbiome did not counteract the impact of environmental conditions enough, to allow cytokines to be expressed at similar levels in the CON unit, as those found from the *maflg^{fl}/maflg^{fl}* mice bred in the IVC unit.

It is also important to note that antibiotic treatment has been shown to induce Th2-cell responses secondary to alterations in commensal bacteria (Hill et al., 2012). This was shown in the results of this thesis and this factor further emphasises the complex nature of

inflammation in AD. Treatment of colonisation and/or infection is known to ameliorate disease as with the improved clinical scoring in the *maflg^{fl}/maflg^{fl}* mice in the CON A/B group, however, alteration of the microbiome also increased the Th2 nature of the responses, which was seen in the increased levels of total serum IgE in these *maflg^{fl}/maflg^{fl}* mice.

AD is often associated with other atopic disorders such as allergic rhinitis and asthma (Bieber, 2008). Thus, the lungs of the *maflg^{fl}/maflg^{fl}* mouse model were assessed for any potential lung changes that may have occurred secondary to the skin barrier defect and the associated inflammation. Decreased lung compliance was demonstrated in the *maflg^{fl}/maflg^{fl}* mouse groups compared to WT controls. The significant differences between *maflg^{fl}/maflg^{fl}* and WT lung compliance suggest that the increased lung inflammation may be associated more with parenchymal lung tissue changes, perhaps collagen deposition, rather than airway abnormalities. The decrease in compliance in the *maflg^{fl}/maflg^{fl}* mouse CON-IVC group was comparable to the decrease in the *maflg^{fl}/maflg^{fl}* mice bred in the CON unit. This suggests that the basic inflammatory responses had already been set up in these mice with skin barrier deficiency and that the changes to lung function that occurred in this CON-IVC group followed a “blueprint” of lung changes of *maflg^{fl}/maflg^{fl}* mice born into and bred in a CON environment.

Increased histological evidence of inflammation was noted in the *maflg^{fl}/maflg^{fl}* mouse lung slides relative to WT mice, with greater inflammatory changes in the *maflg^{fl}/maflg^{fl}* from the CON housing compared to those from the IVC environment. Deposition of collagen (a marker of chronic inflammation and fibrosis) was found to be decreased in the *maflg^{fl}/maflg^{fl}* mouse CON A/B group compared to the *maflg^{fl}/maflg^{fl}* mice from the

CON group. However deposition of collagen remained lower in the *maflg^{fl}/maflg^{fl}* mouse IVC group and the CON-IVC group than in the group of *maflg^{fl}/maflg^{fl}* CON A/B or CON mice. These results mirror the inflammatory responses outlined earlier – i.e. that reducing environmental exposure reduces inflammatory responses (CON versus IVC and CON-IVC) or by marginally reducing bacterial load with systemic antibiotics (CON A/B) leads to a concomitant reduction in the severity of disease manifestation.

Future work

To address the question of whether increased cellular infiltration in early life as seen in this study was associated with increased protection against responses later in life a future experiment would be to include a sensitisation arm of an experiment with *maflg^{fl}/maflg^{fl}* mice, sensitising with ovalbumin, using the same time-points as in this study and bred in both environments. By using a sensitising agent, inflammation would be expected to develop in a model of skin barrier deficiency, which would give a more predictable inflammatory response in all groups of *maflg^{fl}/maflg^{fl}* mice. However, because there would still be 2 different environments, the evolution of the inflammatory responses in the different environments can be more accurately examined, as there would be increased uniformity with regards to the source of inflammation, thereby giving increased reliability to the results.

The variables that would be examined include: clinical scoring at all timepoints using an adapted clinical scoring system for neonatal pups. Assessment of histological changes would be from areas of sensitised disease-active skin and areas of non-active skin using H&E and Toluidine blue staining. The hygiene hypothesis postulates that enhanced exposure in early life promotes the development of a controlled immune response to

allergens and antigens. The above proposed study would also allow investigation if early inflammatory cellular infiltration in skin barrier deficiency has a “protective” influence with age and decreases the risk of chronic inflammation or if inherent skin barrier deficiency will have altered responses because of its decreased protective and preventative functions.

In this work, mice that were exposed to the IVC conditions and kept in a more controlled ambient environment, developed fewer features of inflammatory skin disease. It is relevant in treatment advice to patients in the clinical setting – while it is implausible to have individually ventilated houses for the general patient with chronic mild disease, perhaps acute and significant flares of AD may find some relief in isolation in rooms where the air flow can be more closely controlled. In countries where air conditioning is more usual and rates of AD are significant (e.g. USA), it may be worth investigating modifications to air conditioning systems that allow for the provision of allergen-refined air delivery (Ostro et al., 2010). Given that this substantial change in environmental conditions had such a marked effect on AD inflammatory phenotypes in skin barrier deficiency, it would be prescient to investigate the multicomponent nature of that alteration. Changing environmental factors in a more marginal fashion by using IVCs with altered humidity, air-flow, temperature and dust may further characterise the aspects of the aerosolised exposures that manifest a greater inflammatory response.

What is also of great interest is that WT mice did not show a similar response to the alteration in environmental conditions. This further emphasises the pivotal role that the skin barrier or lack thereof, has on the development of a cutaneous and systemic inflammatory phenotype. An intact skin barrier protects against environmental agents and aerosolised aeroallergens as well as preventing inflammatory responses to

commensal or colonised organisms. Equally, this prompts further questions about the interplay between the skin barrier deficiency and the conditions in the CON housing, that are allowed to stimulate a response by virtue of the defects.

Because these responses are so exaggerated in mice with skin barrier deficiency, this may lead to a frameshift in our understanding and our treatment of the disease. There may exist two competing approaches that will have a varying prominence depending on the patient genotype. In the context of a characterised skin barrier deficiency, it may be more important to decrease further exposures either by modifying the environment or by modifying the skin barrier (through consistent specifically tailored emollients as detailed in section 1.1.4). However, in the context of patients with intact skin, the modification of the inflammatory response may be more important as the exposure to allergens is the same as controls, but the response they stimulate is excessive. This would place an emphasis in this patient subtype on anti-inflammatory and immunomodulatory medications as detailed in Sections 1.1.4.1, 1.1.4.2 and 1.1.4.4. It is most probably that each patient with AD demonstrates an element of skin barrier deficiency and an element of excessive immune responses, but characterising each patient's phenotype would allow a more appropriate personalised balance in their treatment approach and provide optimal treatment based on their individual pathology. The use of the *maflg^{fl}/maflg^{fl}* mouse model is thus particularly appropriate, considering the polygenic nature of the mutant mouse and the complex nature of the pathogenesis of AD.

As discussed above and in Chapter 4, a group of *maflg^{fl}/maflg^{fl}* mice were switched from CON to IVC environs early in life to determine how much the skin barrier deficiency inflammatory response was determined by early programming, and how much was simply brought about by intercurrent environs. This is particularly important in the

context of the hygiene hypothesis. This dogma intimates that early exposure to a range of antigens programs the immune response towards increased tolerance and results in the reduced manifestation of inflammatory diseases such as asthma or AD (Ege et al., 2011, Spergel and Paller, 2003, Strachan, 1989, Strachan, 2000, Strachan et al., 1996). This hypothesis has been used to explain the marked increase in inflammatory disease in children housed in less antigen-rich environments – for example, city versus farm life, and increased exposures to other children early in life (Ege et al., 2011, Spergel and Paller, 2003, Strachan, 2000, Flohr et al., 2006). What remains unclear, however, within this hypothesis is when the early exposure is optimal to prime the immune system. Furthermore, the precise nature of the optimal early exposure is also yet to be defined.

The mice either bred in the IVC environment or transferred to this environment after the 1st week of life had decreased clinical and histological inflammation and decreased spontaneous atopy compared to their littermates bred solely in the CON environment. This study implies that skin barrier deficiency alters the parameters by which constitutional immune responses are programmed for an individual, and increased exposures tend to be associated with increased inflammation and severity because of what occurs secondary to the increased ingress of antigens and allergens. The hygiene hypothesis was not developed according to the genetic background of patients and these experiments with the *maflg^{fl}/maflg^{fl}* mouse model of skin barrier deficiency, suggest that careful genotype/phenotype definition is required when applying such hypotheses with regard to predicting inflammatory outcome for individuals.

The work herein infers that in the context of skin barrier deficiency, this hygiene hypothesis (i.e. early extensive exposure leading the reduced later inflammation) does not hold true. Mice initially housed in the CON environment, with less controlled air

delivery, consistently showed a greater immune response than those housed in the IVC environment with controlled, filtered air delivery, even after they were also transferred to that environment. In fact, their inflammatory response never reduced to the level of the mice that were constantly housed in the IVC environment, implying if anything the reverse of the hygiene hypothesis. However significant caveats remain.

Firstly, it is appropriate to query the timing of the exposure more precisely and to monitor whether that changes in an age dependent fashion, including whether *in utero* exposure is already part of any pre-programming involved in the immunophenotype of mammals. Precise timing experiments would include transferring at a variety of ages including prenatal, birth and at 1 day intervals after that point, and characterising any change in subsequent inflammatory response to see at what point allergen exposure influenced later inflammatory phenotype. Equally, comparing this phenomenon in wild type and in skin barrier deficient mice is particularly appropriate. One might expect that if the hygiene hypothesis relies on early exposure to alter and programme reduced later inflammatory responses, mice with skin barrier deficiency should in fact show an exaggerated hygiene hypothesis type response and have reduced levels of later inflammation. As detailed above, the opposite was in fact true. This raises the question whether the alterations we saw were prompted by the skin barrier defect or by early exposure that exceeded that which produces a positive response and in fact triggered the offset of a non-remitting excessive inflammatory response. Thus having a more precise way of altering the original extent of exposure – perhaps by housing mice in IVC and adding individual stimuli gradually – would expand on our knowledge of this facet of the pathology, and might allow us to more thoroughly understand or even update the mechanism of the hygiene phenomenon. The variables measured would include all of those assessed in this study with the additional investigations as described above. As

indicated above, it is difficult to determine how much of any immunophenomenon is brought about by the skin barrier defect, and how much was the programmed inflammatory response. It would be judicious to run all experiments in parallel between skin barrier deficient and wild type mice so as to monitor whether it may also be that just the timing of the exposures varies in the context of a skin barrier defect, and that by stimulating for too long, we have masked the immunophenotype.

Another hypothesis that could be explored in a future experiment, which develops from this work, would be a transfer between the environments in the reverse direction, from the IVC environment in early life to the CON environment. This experiment would add to our knowledge about when immune responses are set up – with confirmed skin barrier deficiency, does being in an environment with controlled air delivery at birth and in the immediate neonatal period improve the long-term clinical, histological and systemic outcomes? The disease markers assessed in the work should be used to investigate this hypothesis as well as using the additional investigations as discussed in this chapter.

As discussed in Chapter 5, antibiotics were administered to determine the effects of modifying the cutaneous microbiome in early life in skin barrier deficiency both to determine whether early modification of inflammation influenced disease manifestation, and to determine whether this method of early alteration of the distinct exposures led to an altered later inflammatory responses in skin barrier deficiency. The use of early antibiotics altered the levels of inflammatory responses seen. However, this reduction was not as effective as reducing the aeroallergen exposure by altering the housing. It is also important to note that environmental factors such as climate and hygiene can have considerable effects on microbial communities (Kong and Segre, 2012). This may be because filaggrin, which is associated with the skin barrier deficiency in the

maflg^{fl}/maflg^{fl} model, has multiple functions in the skin barrier and altering the microbiome alone will not replace the effect of decreasing external influences.

The administration of antibiotics is, however, a particularly appropriate intervention as it is one that could be readily translated into clinical practice. Given that bacteria and other microbials such as fungi are known to play a role in both the modulation of the immunophenotype as well as in exacerbations of the inflammatory phenotype, this area of research may lead to particularly interesting developments. Firstly, future investigations will need to characterise specifically the alterations brought about in the microbiome by the use of systemic antibiotics. It may be worthwhile to take skin scrapings, in future experiments with the *maflg^{fl}/maflg^{fl}* mice, and determine whether these antibiotics have rendered changes in the skin microflora. When taking skin cultures it would also be important to take multiple swabs from different locations on a *maflg^{fl}/maflg^{fl}* mouse. It has been shown that, just as different bacteria tend to reside in different body sites on human skin, the same applies with skin microbiome in *maflg^{fl}/maflg^{fl}* mice (unpublished data, personal communication from the Fallon *et al.* group).

It would also be prescient to compare whether the topical application of antibiotics led to similar reductions in inflammation as the systemic route, as the side effects engendered with systemic use make their clinical application less appealing. Also, as demonstrated in this thesis, antibiotic treatment increased spontaneous atopy by increasing levels of total IgE in the *maflg^{fl}/maflg^{fl}* mice that were treated with the antibiotic regimens. Further experiments are necessary to determine whether the timing of antibiotic administration may be particularly valuable in altering the immunophenotype. This again could have particular clinical relevance as it could provide a window wherein children with high risk factors for atopic disease (e.g. strong family history of atopy) or genetic characterisation

indicating the same, may be treated for a short amount of time with antibiotics which would lead to a downregulated subsequent immunophenotype. The precise mechanism of the changes rendered by antibiotic administration are characterised by detailed investigation of the cellular and cytokine responses. It would be appropriate to investigate whether antifungals had alternative effects, or whether the eradication of particular bacterial subgroups led to particular immunoprogramming.

The predisposition towards forming IgE antibody responses to common environmental and protein antigens, or atopy is strongly associated with AD (Boguniewicz and Leung, 2011). An incidence of approximately 80% has been demonstrated in infants with AD (de Benedictis et al., 2009). In future work, measurement of total serum and antigen-specific IgE, IgG₁ and IgG_{2a} at all timepoints would show how the development of the cutaneous immunology had systemic inflammatory influences in skin barrier deficiency and further define the evolution of how immunological responses are programmed in skin barrier deficiency.

Spleen cells from *maflg^{fl}/maflg^{fl}* mice in the study by Fallon *et al* produced OVA-specific Th2 (IL-4, IL-5, IL-13), Th1 (IFN γ), regulatory (IL-10) and TH17 (IL-17) cytokines (Fallon et al., 2009). This demonstrated that a general allergen-specific cytokine response occurred in these mice with skin barrier deficiency and it was not just a Th2 skewed response (Fallon et al., 2009). This increased overall response in the *maflg^{fl}/maflg^{fl}* mice when exposed to an environment with more exposures, highlighted that an increased severity in inflammatory markers develops in skin barrier deficiency. This suggests that “protection” does not develop with increased exposures in the situation of inherent skin barrier deficiency, as is postulated in the hygiene hypothesis.

By examining these inflammatory markers from skin-draining lymph nodes and spleen cells in a sensitisation experiment with *maflg^{fl}/maflg^{fl}* mice which were bred in both the CON and IVC environments, detailed analysis of the evolution of inflammatory responses, resulting from the interaction of a defective skin barrier with the environment will be done. A sensitisation experiment using both environmental facilities will give information regarding specific inflammation secondary to the sensitising agent to the defective skin barrier. The data regarding cytokine expression would be of further analytical interest because inflammation would be expected and thus the pattern that would develop would more specifically show the pattern of inflammation in skin barrier deficiency secondary to environmental exposures.

Analysing cutaneous mRNA expression of IFN γ , IL-4, IL-17, IL-1 β , and TSLP from the sensitised areas of skin and areas of non-active disease would expand the information about the local cutaneous immunology and add to results that would be derived from the skin-draining lymph nodes. It has been recently shown that IL-1 β expression is upregulated in the skin and keratinocytes of *maflg^{fl}/maflg^{fl}* mice (Kezic et al., 2012). IL-1 β mRNA is also upregulated in primary epidermal keratinocytes in the proliferative and terminal differentiation states in the stratum corneum (Kezic et al., 2012). Additionally this study demonstrated that the stratum corneum in AD is associated with an increased IL-1 cytokine profile (Kezic et al., 2012). It has been shown that expression of TSLP in murine skin keratinocytes locally initiates AD but also leads to aggravation of OVA-induced asthma-like lung inflammation (Zhang et al., 2009). This correlates with human studies where it was highlighted that the epidermis in lesional skin in AD has increased levels of TSLP expression than that of uninvolved skin or in skin from healthy normal control subjects (Ziegler and Artis, 2010).

Taking whole skin-draining lymph nodes and spleens would allow measurement of cell cytokine expression (measuring IFN γ , IL-4, IL-17, IL-1 β) on *maflg^{fl}/maflg^{fl}* mice from 4 - 32 weeks). While this would investigate cutaneous and systemic inflammatory changes that occur as the model ages and the nature of immunological responses evolve, the assessment of pulmonary changes would involve the same techniques used in this study. Measuring lung homogenate expression of IFN γ , IL-4, IL-17, IL-1 β and TSLP could supplement the cutaneous and systemic results. This would allow full analysis of the evolution of immunological responses as the *maflg^{fl}/maflg^{fl}* mice aged. This may give a better understanding of the transitions from either Th1 or Th2-type responses to the other and if increased early inflammatory infiltration enhances overall inflammation with age.

AD is often associated with other atopic disorders such as allergic rhinitis and asthma (Bieber, 2008). Thus, the lungs of the *maflg^{fl}/maflg^{fl}* mouse model were assessed for any potential lung changes that may have occurred secondary to the skin barrier defect and the associated consequential inflammation. Increased lung compliance was demonstrated in the *maflg^{fl}/maflg^{fl}* mouse groups compared to WT controls. The significant differences between *maflg^{fl}/maflg^{fl}* and WT lung compliance suggest that the increased lung inflammation may be associated more with parenchymal lung tissue changes, perhaps collagen deposition, rather than airway abnormalities. The increase in compliance in the *maflg^{fl}/maflg^{fl}* mouse CON-IVC group was comparable to the increase in the *maflg^{fl}/maflg^{fl}* mice bred in the CON unit. This suggests that the basic inflammatory responses had already been programmed in these mice with skin barrier deficiency and that the changes to lung function that occurred in this CON-IVC group followed a “blueprint” of lung changes of *maflg^{fl}/maflg^{fl}* mice born into and bred in a CON environment.

The significance of investigating lung changes that occur in a murine model of skin barrier deficiency is because of the proportion of those patients with *FLG* mutations that develop further atopy including allergic rhinitis and asthma (Irvine et al., 2011). If skin barrier deficiency causes both cutaneous and non-cutaneous inflammation then morbidity for the patient increases considerably. Exacerbations of other diseases including asthma and infections of other organs all exacerbate AD (Kunz and Ring, 2005). Thus minimising the effects of skin barrier deficiency leading to cutaneous inflammation may very positively improve a patient's overall morbidity and in an infant with skin barrier deficiency may improve the long-term prognosis of atopic disease.

While the mouse lung is not an exact model of the human lung organs, the consequential lung inflammation and fibrosis guides human studies on the non-cutaneous influences of the skin barrier. Various lung conditions such as emphysema, pulmonary fibrosis and chronic obstructive airway disease have increased prevalence at older adult ages and are associated with many types of environmental exposures such as smoking, residential and occupational exposures to dusts, chemicals and metals and recurring pulmonary infections. The assessment of lung changes in *maflg^{fl}/maflg^{fl}* mice at adult ages of 4, 8, 20 and 32 weeks should be included in an experiment where there were changing environmental conditions in a marginal fashion by using IVCs with altered humidity, air-flow, temperature and dust. This may further characterise the aspects of the aerosolised exposures that manifest a greater inflammatory and/or fibrotic lung response and collection at the various adult timepoints would show the development of the lung phenotype in skin barrier deficiency.

The translation from this study to human AD occurs as environmental exposures are trigger factors in AD and can be important parts of simultaneously using different avenues for treatment, such as lifestyle changes, emollient regimes, as well prescribed anti-inflammatory topical therapies. Impacting on environmental changes in early life may impact on the progress of disease as a child ages. Also if a patient has a characterised skin barrier defect then the direction of treatment from birth will be biased towards barrier maintenance to protect against environmental agents.

Skin barrier deficiency leads to a reduction in the composition of the lipid lamellar layer and the NMF as discussed in Section 1.2.5.2. If the composition of the corneal layer was determined by assaying the contents of tape-strippings from *maflg^{fl}/maflg^{fl}* mice then a therapeutic experiment involving a murine model of skin barrier deficiency such as the *maflg^{fl}/maflg^{fl}* mice could be designed. An emollient designed specifically for the deficient murine skin could be used in a double-blinded, controlled study, in both sets of environments. As well as assessing clinical, histological and microbial cutaneous changes, serological antibody and organ cytokine expression as above, clinical, histological and microbial analysis of lung samples could be performed. An arm of a therapeutic study would need to involve groups of *maflg^{fl}/maflg^{fl}* and WT mice in both environments that had specific cutaneous sensitisation to fully assess the therapeutic responses to specific, controlled inflammation.

In skin barrier deficiency, as in the *maflg^{fl}/maflg^{fl}* mouse model neither transferring to conditions with increased air control at 1 week, nor altering the cutaneous microbiome had the impact on the clinical cutaneous outcome, systemic responses or distal effects, that being in a controlled environment from birth, had on overall inflammatory consequences. This work illustrates further the importance of considering the impact of

external exposures when contemplating the immunology of AD and anti-inflammatory treatments. Eliminating environmental exposures would be very difficult to do for every patient with skin barrier deficiency, who would be at an increased risk of developing AD, allergic rhinitis and asthma. This study confirms that therapeutic research must continue to have a large focus on improving the skin barrier in skin barrier deficiency, which would also have anti-inflammatory and anti-microbial effects (Elias, 2008). Improvement of the barrier in skin barrier deficiency via disease-specific, barrier-corrective topical treatments such as ceramide-dominant mixtures with barrier lipids, would down-regulate pro-inflammatory signalling mechanisms involved in barrier repair, the ingress of further haptens which drive Th2-type responses would be increasingly blocked by skin barrier improvement and the lipid mixture would give an acidic pH on the skin surface adding to the barrier function and blocking the activation of proinflammatory serine proteases (Elias, 2008).

It is evident from clinical studies that infection plays an important part in disease aggravation in patients with AD. Resident skin bacteria maintain a healthy skin environment and are involved in the protection against pathogenic bacteria (Kong, 2011). Changing the cutaneous microbiome via systemic antibiotics in early life altered the cutaneous clinical outcome, serum antibody and skin-draining lymph node cytokine expression and lung inflammation in *maflg^{fl}/maflg^{fl}* mice, with skin barrier deficiency. This study implies that further delineation of bacterial colonization in skin barrier deficiency is important in assessing the molecular associations of the skin microbiome with the skin barrier and in particular structural skin barrier proteins such as filaggrin. Future therapeutic research in AD should involve examining this inflammatory skin disease from the aspect of aligning both the microbiology of skin and the structural barrier itself, both pivotal parts of the function of the skin barrier.

This study highlighted that when designing studies to assess the response to various treatments in both animal and human studies that it is necessary to consider the impact of the skin barrier on lung function, inflammation and overall prognosis. Cutaneous inflammation and non-cutaneous consequences of same, particularly in relation to skin barrier deficiency, cannot be mutually exclusive and must be considered at all stages of disease, investigation and treatment.

The results of this thesis revealed differences in genetically identical specimens based on controlled environmental accommodation. It showed the spectral nature of AD. The profound effect of environmental conditions on the inflammation that results from skin barrier deficiency and on severity was demonstrated by the differences described in the *maflg^{fl}/maflg^{fl}* mice in the different environments. The thesis also explored the association of environmental conditions in the pathogenesis of inflammation in skin barrier deficiency by in a transfer experiment with the *maflg^{fl}/maflg^{fl}* mice. Another intervention into the influences on the skin barrier employed the antibiotic treatment to assess if alteration of the microbiome was associated with change in disease. Although transfer in neonatal life to the environment with controlled air delivery and alteration of the cutaneous microbiome improve clinical cutaneous outcome in skin barrier deficiency, such steps did not match the positive effect that controlled conditions from birth has on the protective and preventative functions of the skin.

This study suggests that skin barrier deficiency is of vital importance when immune responses are being developed in early life. It raises questions with regards to the impact of other factors such as the microbiome on the skin barrier and suggests that further research is required to answer these questions fully. This research is important because it not only will answer questions with regards to the effect of the microbiome on the

inflammatory responses that are set up in skin barrier deficiency that may then lead to dermatitis-like disease. Also, treating or suppressing the microbiome if there is inappropriate and inflammatory colonisation of bacteria may be an important tool in AD therapy.

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Presentations

Oral Presentations

1. A mouse model of atopic dermatitis: environmental influences on skin barrier deficiency. **C.M.R.Fahy**, W.H.I McLean, P.G.Fallon, A.D.Irvine. Jacob Medal Presentations, Royal Academy of Medicine of Ireland, Dermatology section, March 2011
2. Variation in the development of phenotype: a murine model of atopic dermatitis. **C.M.R.Fahy**, W.H.I McLean, P.G.Fallon, A.D.Irvine. Annual Meeting, British Society of Investigative Dermatology, April 2011
3. A mouse model of atopic dermatitis: environmental influences on skin barrier deficiency. **C.M.R.Fahy**, W.H.I McLean, P.G.Fallon, A.D.Irvine. Second prize in Burrowes Cup, Annual Meeting, Irish Association of Dermatologists, May 2011,
4. A mouse model of atopic dermatitis: environmental influences on skin barrier deficiency. **C.M.R.Fahy**, S.P.Saunders, S.Amu, E.Hams, W.H.I McLean, P.G.Fallon, A.D.Irvine. Summer Course 2012, European Association of Dermatology and Venereology/European Society for Dermatological Research, July 2012

Poster Presentations

1. Environmental influences on a model of atopic dermatitis. **C.M.R.Fahy**, S.P.Saunders, S.Amu, E.Hams, H.Nel, W.H.I McLean, A.D.Irvine, P.G.Fallon. Annual Symposium, Institute of Molecular Medicine, Trinity College Dublin, November 2010

2. Environmental influences on a model of atopic dermatitis. **C.M.R.Fahy**, S.P.Saunders, S.Amu, E.Hams, H.Nel, W.H.I McLean, A.D.Irvine, P.G.Fallon. Annual Meeting, Society of Investigative Dermatology, May 2011
3. A murine model of atopic dermatitis: variation in phenotype development in skin barrier deficiency. **C.M.R.Fahy**, S.P.Saunders, S.Amu, E.Hams, H.Nel, W.H.I McLean, A.D.Irvine, P.G.Fallon. Annual Symposium, Molecular Medicine Ireland, June 2011
4. Environmental influences on a model of atopic dermatitis. **C.M.R.Fahy**, S.P.Saunders, S.Amu, E.Hams, H.Nel, W.H.I McLean, A.D.Irvine P.G.Fallon. Postgraduate Study Day, School of Medicine, Trinity College Dublin, September 2011
5. A murine model of skin barrier deficiency and atopic dermatitis: development of phenotype can be influenced by environments. Tercentenary Symposium, Institute of Molecular Medicine, School of Medicine, Trinity College Dublin, November 2011

Manuscript prepared

Environmental influences on a murine model of skin barrier deficiency **C.M.R.Fahy**, S.P.Saunders, S.Amu, E.Hams, H.Nel, W.H.I McLean, A.D.Irvine, P.G.Fallon