DEVELOPMENT OF TH2 IMMUNE RESPONSE
MODELS TO EVALUATE ALLERGENICITY OF
MILK PROTEINS

by

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Abstract

Food allergy, defined as an adverse immune response to food, is increasing in prevalence. It can be broadly separated into phases of sensitization, in which allergy-triggering Immunoglobulin E (IgE) is generated, and the post-sensitization allergic response, in which the allergic response is triggered by sensitizing allergen. While much is known about the specific mediators that cause allergies, the immune processes that underlie disease progression are less clear. This project has employed mouse models of Th2 immunity to clarify the factors involved in the initiation and maintenance of allergic disease.

At the centre of allergic disease is the Interleukin (IL)-4-producing CD4+ T helper type 2 (Th2) cell. One of the key inducers of Th2 cell development *in vitro* is IL-4, but its involvement in Th2 cell development *in vivo* is controversial. In our studies, we saw that Th2 cell development could be initiated *in vivo* by primary, adjuvant-free allergen immunisation in the absence of IL-4. However, Th2 cells were more frequent in IL-4-sufficient conditions. We also determined that genetic lesions that result in loss of one, or both, IL-4 alleles impaired the Th2 cell-mediated allergic process, such that IL-4-heterozygous mice can be considered haplo-insufficient for IL-4 in allergic disease contexts.

In addition to the generation of IgE antibody, Th2 cells are implicated in the post-sensitization phase of allergy. Multiple oral challenges of sensitized mice induces elevations in Th2-associated cytokines and elevates intestinal mast cell frequencies. It was the second aim of this project to clarify the role of CD4+ T cells in the post-

sensitization intestinal allergic process. We demonstrate a key role for CD4+ T cells in this jejunal mast cell recruitment, and identify that this is required in addition to their established contribution to IgE production. Our investigations also reveal a previously unappreciated role for the CD4+ T cell-derived cytokine IL-3 in oral food allergy. These findings suggest that intestinally localised mast cell-inducer Th2 (Th2m) cells are required for allergic responses generated in the intestine.

We also investigated whether specific components of ruminant milks influence the allergic process. While goat and cow milks share significant protein homology, goat milk has lower sensitizing and response-evoking capacity, or allergenicity, than cow milk, in numerous experimental systems. In this project, we compared dominant allergens purified from cow and goat milks for their ability to initiate Th2 cell development. We also examined the ability of one of these allergens to initiate the intestinal allergic process. In these studies, we observed similar Th2 cell development and intestinal mast cell activity in response to both cow and goat milk proteins. These responses indicate that the intrinsic allergenicity of the proteins analysed is not sufficient to explain the differential allergenicity attributed to cow and goat milk.

These studies examine the endogenous and exogenous factors that contribute to the development of allergic disease. This project clarifies the role of IL-4 in *in vivo* Th2 cell development, identifies functional segregation of CD4+ Th2 cells in the intestinal allergic process and further illustrates some of the similarities in the allergenicity of isolated cow and goat milk proteins. Collectively, these studies uncover fundamental aspects of the allergic process which may be useful targets for disease intervention in both prophylactic and therapeutic settings.

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Marcus Robinson,

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Abbreviations

Standard International Unit abbreviations were used throughout this thesis.

+/+	Wild-type mouse
+/-	IL-4 heterozygous mouse
-/-	IL-4-deficient mouse
4get	IL-4-GFP enhanced transcript
5HT	5-hydroxytryptamine; serotonin
7-AAD	7-amino actinomycin D
AEC	3-amino-9-ethylcarbazole
APC	Antigen presenting cell
ANOVA	Analysis of variance
anti-IFN γ	antibody against IFN γ
anti-IgD	antibody against murine IgD
anti-IL-4	antibody against IL-4
BAL	bronchoalveolar lavage
bBLG	cow β -lactoglobulin
BCA	Bicinchoninic acid
BCF-1	IL-4
BRU	Biomedical Research Unit of the Malaghan Institute of Medical Research
BSA	Bovine serum albumin
BLG	β -lactoglobulin
CAE	Chloroacetate esterase
cBLG	(caprine) goat β -lactoglobulin
CD	cluster of differentiation
CD40L	CD154
cIMDM	base culture medium
DAPI	4,6-diaminidino-2-phenylindole
DELTA BLAST	Domain enhanced lookup time accelerated Basic local alignment search tool

EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbant assay
Flow buffer	2% FCS, 0.01% Sodium azide in PBS
FCS	Foetal Calf serum
FPLC	Fast (low) pressure liquid chromatography
FTY720	Sphingosine-1-phosphate Receptor inhibitor
G4	IL-4 reporter allele in which GFP is produced under the control of the IL-4 promoter
G4/+	G4 heterozygous mouse with one wild-type IL-4 allele
G4/G4	IL-4-deficient dual G4 reporter mouse
GFP	enhanced green fluorescent protein
haplo-insufficiency	Occurs when a genetic deficiency in one allele prevents development of the normal wild-type phenotype
hCD2	inactive human CD2 used to identify IL-4-producing cells in the KN2 reporter
HDM	<i>Dermatophagoides pteronyssinus</i> crushed house dust mite extract
HPLC	High pressure liquid chromatography
HS	hypersensitivity site
ICOS	Inducible T cell co-stimulator; CD278
i.d.	intradermal
i.g.	intra gastric
i.v.	intravenous
<i>iFABP_p</i> -IL-9	IL-9 transgene inserted under the control of the Rat fatty acid binding protein
IFN γ	Interferon- γ
Ig	Immunoglobulin
Igs	polyclonal Immunoglobulins
IL	Interleukin
IMDM	Iscoe's modified Dulbecco's medium
IRES	Internal Ribosomal entry site
i.p.	intraperitoneal
KLH	Keyhole Limpet Hemocyanin
KN2	IL-4 gene knock-in human CD2

KN2/+	KN2 reporter mouse with one wild-type IL-4 allele
KN2/KN2	IL-4-deficient dual KN2 reporter mouse
KN2/G4	IL-4-deficient reporter mouse carrying two separate IL-4 reporter alleles
L	Ligand
LPS	Lippopolysaccharide
major allergen	protein against which more than 50% of food allergic individuals demonstrate IgE-binding
mMCP-1	murine mast cell protease-1
MWCO	molecular weight cut-off
Nb	dead infective-phase <i>i</i> L3 <i>Nippostrongylus brasiliensis</i> larvae
nTg-/-	IL-3-deficient mouse sib-matched to Tg-/- mice
Oral potentia- tion	post-sensitization phase of experimental allergy that precedes diarrhoea. Characterized by elevated Th2 profile, intestinal mast cell infiltration and increased IgE levels.
OVA	Ovalbumin
PAF	Platelet activating factor
PBS	Dulbecco's phosphate buffered saline
PCR	polymerase chain reaction
PCV	Packed cell volume; haematocrit
PD-1	Programmed cell death-1; CD279
pTh2 cell	Th2 cell that develops in response to primary adjuvant free immunisation
R	receptor
r	recombinant
SEM	standard error of the mean
SCF	Stem cell factor (cKit ligand)
TCR	T cell receptor
TFH cell	follicular T helper cell
TGF- β	Transforming growth factor- β
Tg+/-	Mouse carrying the <i>i</i> FABP p -IL-9 transgene and two wild-type IL-3 allele

Tg+/-	Mouse carrying the <i>iFABP</i> <i>p</i> -IL-9 transgene and a single wild-type IL-3 allele
Tg-/-	IL-3-deficient mouse carrying the <i>iFABP</i> <i>p</i> -IL-9 transgene
Th cell	T helper cell
Th0 cell	Unpolarised activated Th cell
Th1 cell	IFN γ producing Th cell
Th2 cell	IL-4, -5, or -13 producing Th cell. In this thesis, <i>in vivo</i> Th2 cells are considered reporter-expressing cells. <i>in vitro</i> Th2 cells are CD4+ T cells that have been cultured for five or more days in the presence of IL-4.
Th2m cell	Mast cell-inducer Th2 cell
Th9 cell	IL-9-producing Th cell
Th17 cell	IL-17-producing Th cell
TCA	Trichloroacetic acid
TMB substrate	3,3',5,5'-Tetramethybenzidine substrate
Treg cell	Regulatory Th cell
TSLP	Thymic Stromal Lymphopoeitin
U	Units
WT	Wild-type

Chapter 1

General Introduction

1.1 Thesis overview

Food allergy remains a life-threatening problem in society today. One in twenty children will have an allergic episode before they turn three, and the majority of these will be invoked by a common dietary protein. Over 90% of these will be induced by one of six foods: cows milk, egg, peanut, tree nuts, wheat or soy. It is still unclear why these six foods are common allergens, and further more, why only some people will develop an allergy at all. This project aimed to clarify the basic immune processes that result in allergic disease.

Research over the past hundred years has made great advances in the field of allergic disease research. The key mediators of the allergic response have been elucidated; the cell types involved in the response are known; and a number of risk factors have been identified. However, our current understanding of the allergic process is inadequate. We don't know the first steps that lead to allergic sensitization; we are not able to predict with reasonable certainty which children will become allergic; we don't know how to prevent an allergy from developing; and most critically, we don't know how to turn an established allergy off.

This thesis begins with a general introduction into the field of allergy. In this introduction, knowledge of the role of mast cells and the immunological processes that underlie allergic sensitivity are outlined. Some of the epidemiology of allergy is covered and is also used as evidence that supports the concept of intrinsic allergenicity, which is subsequently investigated for some common allergens. As interleukin(IL)-4 producing, CD4+ T helper type 2 (Th2) cells are heavily implicated in the allergic process and are a major focus of this thesis, their development and contribution to the allergic process are discussed in depth. Chapter two provides details of the meth-

ods that were used during this project. Chapters 3-6 present the project findings regarding Th2 cell development and function in allergic immunity.

The focus on the Th2 cell arose from previous studies in our lab that broke the historically accepted view that IL-4 is required to initiate Th2 cell development[1]. From these earlier studies, questions pertaining to the true contribution of IL-4 to Th2 cell development naturally evolved and were accordingly investigated. These studies, in turn, gave rise to the question of how genetic defects in IL-4 production affect not only Th2 cell development, but also their function. These concepts are the focus of Chapter four. Chapter five aims to clarify the role of CD4+ T cells in the post-sensitisation phase of food allergy, and in the final results chapter, a series of tests are performed that aim to clarify why goats milk is a less allergenic food than cows milk. Future directions and potential implications of our findings are covered at the end of each chapter, and a general overview of the concepts contained within are discussed in Chapter seven.

My dissertation covers the reasons that I think allergies are still problematic. It outlines our attempts to clarify unknown facets of Th2 cell development and function, and in this, specifically addresses some fundamental aspects of the allergic process. Understanding the immunological processes that lead to the physiological allergic response has great potential to reduce the burden of allergic disease in society today.

1.2 Allergy and food induced allergic reactions

1.2.1 What is allergy?

Allergy, or type I hypersensitivity, is an acute adverse immune response to a generally innocuous protein or substance. The current paradigm of allergy links back to the 1800s[2], with the first direct tests carried out 100 years ago in dogs[3]. Portier and Richet[3] determined that when dogs are immunised with two additively non-lethal doses of poisonous *Physalia* man o' war tentacle extract, if the second injection is given two weeks after the first, the dogs will collapse, develop cyanosis and in many cases, die. These studies gave rise to the concept that underpins allergy as it is understood today. More general allergic symptoms include vomiting, diarrhoea, wheal and flare, urticaria, wheezing and bronchospasm and some or all of these symptoms can be triggered simultaneously during an allergic response[4–6]. Fatal allergic anaphylaxis is usually attributed to complications involving the respiratory and circulatory system, caused by either hypovolaemic systemic shock and inadequate organ perfusion or difficulties with breathing[5–8]. Food allergy is defined as an adverse immune response to food[9, 10]. To manage an allergy, people are advised to practise allergen avoidance[10, 11]. However, the rapid onset and potential severity of an allergic episode make it necessary to develop new strategies to deal with allergies. This is particularly important because one in four cases of fatal anaphylaxis is caused by unintended allergen ingestion, and immediate adrenalin administration, which is the best current intervention, does not prevent death in every case[7, 8]. There is currently no treatment available that is entirely sufficient for the prevention of anaphylactic death[5, 7, 8]. Food allergy is thought to be increasing in prevalence[9, 10, 12] and understanding ways to subvert the immune processes that precede allergic responses are key directions of current medical research.

1.2.2 IgE is at the centre of allergic disease

Food allergies are distinguished from food intolerances because an allergy is caused by an active immune response against a specific food[10]. Food intolerances can be

caused by specific food components, but are not immune mediated. For example, an inability to absorb and breakdown fructose or lactose is associated with gastrointestinal discomfort and diarrhoea that disappears with elimination of the sugars from the diet[13–15]. Toxins in foods and pharmacologically active molecules like caffeine can also cause food allergy-like symptoms[5]. The food allergy response is also acute, which helps distinguish it from other chronic forms of gastrointestinal disorder, like inflammatory bowel disease or functional diarrhoea[16–18]. The current view of allergy is that on first allergen exposure, either *in utero* or soon after birth[19, 20], sensitization occurs such that subsequent exposures can trigger an allergic response. The culmination of the sensitization phase is the generation of allergen-specific Immunoglobulin E (IgE) antibody. Fifty-sixty percent of food allergies can be attributed to IgE dependent processes[11, 21, 22].

IgE was first discovered and designated as the reaginic antibody in a series of studies by Ishizaka and Ishizaka[23–25]. In their studies, serum from people with ragweed pollen allergy was depleted of the major known antibody isotypes (IgM, IgD, IgG and IgA) and shown to retain its ability to passively sensitize skin[23, 25]. These investigations further determined that it was possible to remove the reaginic activity from 10/10 studied sera by depleting a component that specifically recognised the ragweed allergen[25]. This component had detectable antibody light chains but none of the IgM, IgD, IgG or IgA isotypic regions, and had distinct electrophoretic properties to the other antibody isotypes[23, 24, 24]. The isotype class was proposed and accepted as γ E antibody, or IgE.

Although the specificity of the allergic response is provided by IgE, the allergic response is caused by vasoactive mediators released by IgE-bound mast cells and basophils. IgE is bound to the surface of mast cells and basophils by direct interactions with the alpha subunit of the high-affinity IgE receptor, FcεRI[26–29]. When cell-bound IgE is ligated by allergen, the cells release granules containing vasoactive mediators that cause the allergic symptoms[5, 30]. Clinical allergic symptoms are generally more severe, and allergies are far less likely to resolve in individuals with high titre IgE[31–35]. In experimental systems, the severity of the allergic response correlates with the amount of IgE and level of mastocytosis[36–39]. In some murine experimental systems, alternate cell types and an alternate antibody isotype, IgG1,

are now known to play important roles in antigen-specific anaphylactic and allergic responses[40–42]. However, IgE-mediated experimental anaphylaxis initiated by the intravenous or the oral route is attributed to mast cell activity[37, 43, 44]. Therefore, IgE and mast cells are central to the allergic response.

1.2.3 Physiological changes that occur during systemic anaphylaxis

The vasoactive mediators released by mast cells are diverse. These include prostaglandins, leukotrienes, mast cell proteases, histamine, serotonin and platelet-activating factor (PAF)[5, 30, 37, 45]. Histamine and proteases are preformed mediators, whereas PAF, leukotrienes and prostaglandins are synthesised after activation[30]. Appropriately activated mast cells are also reported to produce a number of cytokines, including Interleukin (IL)-4 and IL-13[30]. The mediators generally act in a co-ordinated fashion, but some manifestations of allergy can be prevented by treatment with specific mediator antagonists and this has been examined in terms of the inhibition of the physiological allergic response.

Experimental systemic anaphylaxis induces measurable physiological changes mediated predominantly by histamine and PAF. IgE-mediated mast cell-dependent[43] systemic anaphylaxis causes smooth muscle contraction and vascular leak[46, 47], resulting in loss of plasma volume, loss of blood pressure and a drop in core body temperature[43, 48]. Isotonic fluid perfusion of anaphylactic mice is sufficient to prevent haemaconcentration, ameliorate temperature loss and prevent anaphylactic death[48], indicating that hypovolaemia is a major cause of anaphylactic death in mice. The systemic drop in temperature in mice can be prevented by administration of anti-histamines or combined histamine and PAF antagonism, as the temperature nadir is reduced and vascular leak is reduced when these pathways are inhibited[37, 48, 49]. Conversely, serotonin inhibitors, and 5-lipoxygenase inhibitors that prevent prostaglandin synthesis are unable to prevent allergen-specific systemic anaphylaxis in mice[37, 49]. This indicates that histamine and PAF mediate the systemic allergic reaction. Histamine- and PAF-induced anaphylaxis are also exacerbated by IL-4 and IL-13[48, 50]. Additionally, PAF-induced anaphylaxis causes more

haemaconcentration when the amount of mediator introduced induces equivalent hypothermia, suggesting that distinct mechanisms may be involved in each part of the anaphylactic response[48]. However, both PAF and histamine can cause anaphylactic death and are important for mast cell- and IgE-mediated experimental systemic anaphylactic responses.

Currently, there is no universal clinical definition of anaphylaxis[4, 6]. In humans, anaphylaxis is generally considered severe when cyanosis is observed, when the individual collapses, or when soluble oxygen levels in the blood fall below 92 percent saturation[6]. More general changes include a drop in blood pressure, and a range of physical symptoms indicating respiratory or gastrointestinal involvement[4, 6]. Intramuscular adrenalin administration is the best recommended treatment for anaphylaxis[6], and its delayed or improper administration is a common cause of anaphylactic death[7, 8, 10, 11]. One of the most frequent presentations of food allergy, and a secondary diagnostic feature of anaphylaxis, is diarrhoea[4] and some of the physiological mechanisms of allergic diarrhoea induced by intestinal allergen exposure have been investigated.

1.2.4 Physiological processes during fasting and allergic intestinal responses

The normal physiological motility of the intestine is disrupted during an intestinal allergic response. In the fasted state, the major contractions of the intestine are periodic coordinated phasic contractions that begin in the stomach and pass along the length of the small intestine to maintain uni-directional gastric transit[51]. This process, called peristalsis, occurs in most mammals[51]. Introduction of allergen to the small intestine of sensitized rats disrupts fasting peristaltic movements and induces a rapid increase in phasic contractile frequency of both smooth muscle layers along the entire length of the small intestine, causing increased allergen transit through the small intestine[52, 53]. Similarly, when specific allergen is deposited onto colonic sections from sensitized rats, a tonic contraction of both smooth muscle layers is induced and the colonic contents are transported more rapidly *in vivo*[44, 54]. Not only is motility increased, but net sodium, chloride and water absorption are impaired and

concomitant active luminal secretion of water and ions increased following allergen exposure[55, 56]. Oral allergen exposure in sensitized mice also causes acute loss of blood plasma volume analogous to that occurring during systemic anaphylaxis in sensitized intravenously-challenged mice[48, 57], suggesting that the net change in intestinal fluid secretion can affect homeostatic blood volume as well. Collectively, these processes expedite allergen transit and ultimately result in profuse watery diarrhoea soon after intestinal allergen exposure[37, 44, 53, 58, 59].

The processes occurring in allergic humans are similar to those in experimental systems and have been concisely summarised by Hugh Sampson[60]. In the stomach of allergic individuals, allergen transit is slowed, but once in the small intestine, intestinal motility is enhanced, peristalsis is interrupted, intestinal vasodilatation and mucus hypersecretion occur[60]. Diarrhoea and sometimes vomiting follow[5, 60]. Mice and rats are non-emetic species[61] so diarrhoea is the major presentation of intestinal allergic responses in rats and mice. Much of the physiology of the intestinal allergic response is known.

1.2.5 IgE and mast cells mediate intestinal allergen-induced allergic responses

Investigations have demonstrated that intestinal allergic responses are IgE- and mast cell-dependent. Mast cell degranulation is observed upon intestinal allergen exposure[37, 54]. The response is mediated by IgE, as demonstrated by colonic contractile responses being specific for the sensitising antigen and passively transferrable to naive intestinal sections via immune sera, but like passive skin immunisations[62], this activity is lost if sera is first heat-inactivated[44, 54]. Similarly, treating mice with an anti-IgE antibody, ablating mast cell (and the intestinal pace-maker interstitial cells of Cajal) populations with anti-cKit antibodies, cKit-inhibiting drugs[63] or monitoring allergic disease development in FcεRI-deficient mice, all result in reduced diarrhoea incidence[37]. In humans, positive oral food challenges also cause intestinal mast cell degranulation and in greater than 95 percent of cases this correlates with antigen-specific skin prick sensitization[60], indicative of antigen-specific IgE sensitization. Collectively, the data provide strong evidence that oral allergen-induced diarrhoea is

mast cell- and IgE-mediated.

The mast cell products that cause diarrhoea differ from those that cause systemic anaphylaxis. Colonic circular muscle contraction can be induced by exogenous administration of a range of mast cell vasoactive mediators: 5-hydroxytryptamine (5-HT; i.e., serotonin) and leukotriene D4 are particularly potent, and contractility can be partially induced by histamine, PAF or prostaglandin D2[44]. However, the antigen-specific response of both smooth muscle layers along the entire length of the intestine is prevented by mast cell stabilization with doxantrazole, and some responses with disodium cromoglycate, but histamine antagonists have no effect[44, 54, 64, 65]. Leukotriene D4 antagonists and PAF blockade prevent contraction of both layers of the colon in the rat[44, 54], whereas, 5-HT1 and 5-HT2 receptor blockade and indomethacin, which inhibits the cyclooxygenase pathway and inhibits prostaglandin synthesis, prevent allergen-induced jejunal smooth muscle contractility[64, 65]. Additionally, in live animals, prostaglandins and serotonin have been implicated in altering jejunal contractility by modifying neuronal innervation[52]. Concomitant blockade of multiple mediators is the best means to inhibit antigen-induced colonic contraction and diarrhoea[37, 54]. Specifically, concomitant blockade of PAF and serotonin can inhibit allergic diarrhoea in mice[37]. In addition to these vasoactive mediators, cytokines can also influence the intestinal allergic response: IL-4 and IL-13 directly enhance intestinal smooth muscle contractility[50, 66]. IL-4 can also affect intestinal ion flux and tissue responsiveness to mast cell vasoactive mediators[50]. Thus, while histamine is a key trigger for systemic anaphylaxis, histamine- and histamine receptor-antagonists do not prevent intestinal anaphylaxis, whereas, PAF, serotonin, leukotrienes and prostaglandins are implicated in the intestinal response[37, 47, 54].

In summary, while both intestinal and systemic allergic responses are mediated by mast cells, and systemic and intestinal anaphylactic responses are exacerbated by IL-4 and IL-13[48, 50, 66] when antigen is deposited in the intestine, distinct mediators are implicated in the response. The similarities and differences that are attributed to systemic and intestinal anaphylactic responses are summarised in figure 1.1. Histamine and PAF are key to the systemic anaphylactic response; PAF, serotonin, and potentially prostaglandins and leukotrienes are involved in intestinal responses[37, 52, 64].

There is also evidence that systemic and intestinal manifestations of disease can exist independently of each other, even when allergen is administered by the oral route[37, 67, 68]. Therefore, it is possible that the immunological mechanisms that permit allergic responses in the intestine are distinct from those that drive systemic anaphylaxis.

Anaphylaxis	Response:	Major Mediators
Systemic	Vascular smooth muscle contraction ¹ Vascular leak ^{1,2} Plasma volume↓ ^{3,4} Blood pressure ↓ ⁴ hypoxia ⁵	Histamine + PAF ^{1-3,15,17}
Gastrointestinal	Intestinal smooth muscle contraction ⁶⁻¹¹ Fluid absorption↓ ¹² Luminal ion/water secretion↑ ¹³ plasma volume↓ ¹⁴ Intestinal permeability ^{14,15} mucus hypersecretion ¹⁶	PAF + Serotonin ^{6-8,10,11,15} prostaglandins & leukotrienes

Figure 1.1: **Symptoms and mediators of systemic and intestinal anaphylaxis.** Mast cell-dependent responses that result in systemic or intestinal (not including the stomach) allergic responses in rodents and/or humans. Superscript numbers denote references, which are listed in the table-specific bibliography in the appendices.

1.2.6 Management of food allergy

The current advice given for the prevention of allergic responses in sensitized individuals is allergen avoidance[10, 11], however, a number of alternative strategies are also used. Therapies can target end-stage effects, for example, interrupting the signaling of the products of mast cells and basophils such as the cysteinyl leukotriene and histamine antagonists monteleukast and loratidine[69] or aim to inhibit mast cell degranulation using local administration of disodium cromoglycate[70, 71]. Clinicians have also explored the use of allergen desensitization by giving escalating doses of allergen sublingually, orally or subcutaneously[72–74]. Desensitizing immunotherapy can take months to years to be effective and is generally incomplete, however, treatment usually increases the tolerated allergen dose and reduces the severity of the allergic symptoms evoked by chance or experimental allergen exposure[74, 75]. During the desensitization process, patients can experience mild allergic symptoms[75] and a rare but major risk of desensitization therapy is allergic anaphylaxis[76]. To limit the risk of anaphylaxis during immunotherapy, desensitization strategies have explored, with some success, the use of peptide fragments of allergens, rather than whole allergens for therapy[77]. One of the most promising therapies to date uses allergen non-specific anti-IgE antibodies. This approach has reduced the severity and incidence of allergic responses and allergic asthma in the clinic setting[78–81] and is also shedding light on the processes that permit and perpetuate allergic responses once established[82–85]. While allergic responses in the lung are not a focus of this thesis, allergic asthma also affects a number of New Zealanders, and thus its management deserves a brief mention. To manage allergic asthma, inhaled glucocorticosteroids and when necessary, leukotriene modifiers and $\beta 2$ adrenergic agonists are the most commonly prescribed preventative therapies, and $\beta 2$ adrenergic agonists are also used to reverse acute asthma attacks[81]. These, however, do not reverse sensitisation, and aim to limit the severity and frequency of allergic asthma. The major current therapies, therefore, try to reverse sensitization or diminish the effects of the mast cell products. To date no single treatment has had complete success and each has associated maladies and contraindications. Understanding the steps that lead to sensitization and perpetuate allergies is key to reducing the incidence of allergic disease.

1.2.7 Identification of food allergy

Not only are food allergies difficult to manage, they are difficult to distinguish from other gastrointestinal disorders. Some diseases exist that present with similar symptoms to food allergies[86–89] and cases have been identified in which the response is likely to be IgE-mediated but systemic IgE and peripheral disease manifestations are not detected[90]. The identification of a specific causative food allergen is made even more difficult as the majority of allergic individuals are sensitized to multiple foods and often to multiple proteins contained in a single food[32, 33, 35, 91, 92]. This can make it difficult to appropriately exclude the allergen without unnecessarily restricting the diet, which is especially important for infants where dietary exclusion could significantly impact on normal development and growth[86, 93]. Thus, identifying the presence of a food allergy, particularly in the paediatric population, requires careful evaluation.

Standardised processes for food allergy determination are improving disease identification. IgE assays with high positive predictive values have now been established for some major allergens in the US, including milk and egg[31]. However, allergies to other foods still benefit from confirmation by the double-blind placebo controlled oral food challenge test. As self/parentally-reported food allergy incidence is inflated by several-fold[11], it is now advised that before allergen elimination is trialed, food allergies are diagnosed based on a combination of medical history, skin-prick testing, serum antigen-specific IgE titres and when it is not ill-advised, a double-blind placebo controlled oral food challenge[11, 94]. Thus, simple quantitative disease readouts enable rapid identification of potential allergens and support the qualitative observations used to identify a specific food allergy.

1.2.8 Hereditary risk factors for food allergy

Numerous risk factors for the development of allergic disease have been identified[19, 95]. Current reviews of risk factors[19, 95] and genes associated with allergy and asthma[96] are available.

First, there are hereditary and genetic predispositions. Allergies, asthma and eczema

encompass the atopic disorders[97], and are associated with polymorphisms in some genes. Generally, the interaction of a number of genes with low penetrance are thought to compound to increase disease risk, and these are revealed in carefully controlled, genome-wide association studies[96]. For example, high frequencies of single nucleotide polymorphisms in the *IL4* or *IL-4R α* genes are associated with increased risk or severity of asthma and allergic disease[98–100]. There are also specific mutations that can be associated with disease phenotypes. For example, loss-of-function mutations in the filaggrin gene, which is associated with epidermal barrier defects, predisposes infants to eczema, and increases risk of asthma and allergic rhinitis[19, 101, 102]. People of certain ethnicities, like african americans, are more likely to develop allergies than caucasian americans[19, 103]. Collectively, allergies are considered heritable[104], but there is significant gene-environment interaction that makes it difficult to predict disease development and the specific type of atopic disease that will present[96].

1.2.9 Diet and the intestinal microbiota influence disease risk

A risk factor that is not limited to the allergic disease setting, relates to the intestinal microbiota. A balanced and diverse intestinal microbial community protects from numerous diseases. Reduced intestinal microbial diversity in infants is associated with increased risk of allergic disease[105], and diet supplementation with selected probiotics has been associated with reduced incidence of allergies[106, 107]; similarly, drinking unpasteurized milk is associated with reduced skin prick test positivity in farm children[108] and this may relate to the effect of raw milk on intestinal microbiota composition. In terms of early-life exposure, birth by casearian section has, in some cases, been shown to be a risk factor for allergic sensitization[109, 110]. It has been suggested that this may be influenced by the type of microbes to which the infant would be exposed through the birth canal (i.e., vaginal and intestinal commensals), or without it[109–111]. Treating mice with antibiotics has been shown to alter the diversity and composition of the resident intestinal microbiota for long periods[112], and also to promote IgE production, basophil hyper-responsiveness and to increase allergic airways inflammation in mice[113]. The resident microbiota is now considered to significantly influence the intestinal immune system, and examining the changes in

population dynamics that are observed, and potentially influence human health and disease will continue to be an important focus for balanced human health.

Nutrition is both affected by, and thought to affect allergic disease progression. Aside from the obvious, that exclusion of allergens from the diet improves infant growth[114], levels of essential dietary metabolites, like vitamin A and D are associated with allergic disease risk[115–118]. Food allergies are associated with numerous subsequent developmental problems, including rickets, failure-to-thrive and some psychosocial maladies[114, 119–121]. More recently, the links between diet, microbiota and allergic-type immunity has been linked: Maslowski *et al.*[122] demonstrated that the short-chain fatty acids released by the break-down of dietary fibre by the intestinal microbiota limit allergic and enteric disease severity[122]. Thus, nutrition and commensal microbiota are inter-related disease risk factors.

1.2.10 Environmental exposure as a risk factor for allergy

There are also environmental risk factors involved in the development of food allergies. In a seminal study David Strachan showed that family position influenced the risk of developing an allergic disease and argued that this might relate to a 'dirty' environment[123]. Sensitization to environmental allergens has been associated with the levels of allergens found in the home[124]. Similarly, environmental sensitization has been proposed as a potential mechanism for sensitization to stable food allergens. This follows evidence that demonstrates an association between eczema, asthma, and allergic disease. Infants who develop atopic dermatitis at a young age are at increased risk of developing asthma and some forms of allergy in later life[97]. In a series of studies, Lack *et al.* examined the incidence of peanut allergies in infants in early life. In these studies, oral ingestion of peanuts was associated with protection from disease, whereas high household consumption in infants not fed peanuts, or exposed to peanut products by skin application through moisturisers or eczema creams, were associated with increased risk of peanut sensitization[125–127]. These data suggest that environmental, rather than intestinal exposure to some stable allergens may increase the risk of food sensitization; however, these studies are not all-encompassing. Maternal exposure to house dust mite allergens during pregnancy is associated with increased

cord blood IgE levels[128], which indicates that sensitization may occur *in utero*. The range of potential mechanisms of sensitization have lead to the removal of the advice that allergens should be avoided, in both the USA and the UK[19, 129].

1.2.11 Oral tolerance

The default response to ingested food proteins is oral tolerance. Oral tolerance is an active regulatory immune process that acts to reduce subsequent immune responses to specific antigens[130]. Feeding mice with OVA prior to sensitisation, either by gavage or indirectly through the breastmilk prevents sensitization and the subsequent generation of allergic responses in the intestine or lung of mice[130–136]. In humans, similar observations have been made. In an oral feeding study, Husby *et al.*[137] immunised individuals subcutaneously with keyhole limpet hemocyanin (KLH), half of whom had previously been fed numerous low doses of KLH. When individuals were subsequently challenged with KLH, IgM, IgG and IgA antibodies and antibody-secreting cells were detected systemically, but individuals that had ingested KLH eventually demonstrated diminished T cell proliferative responses and reduced frequencies of delayed-type (type IV) hypersensitivity reactions[137]. KLH can also induce IgE-mediated responses in humans, because skin wheal-and-flare responses can be induced by intradermal KLH injection in healthy and atopic individuals, if they are first immunised with KLH and alum[138]. However, oral exposure in some circumstances can result in sensitization. If mice are fed with protein in the presence of cholera toxin, this will result in IgE generation, intestinal mast cell infiltration and allergic sensitization permissive of allergic responses[139, 140]; guinea pigs can be sensitized through oral exposure in the absence of adjuvant[141]. In mice and humans, ingestion of proteins usually induces active immune processes that suppress subsequent immune responses[130, 142]. Therefore, the development of an allergy involves not only sensitisation, but a corresponding break in oral tolerance.

Work has investigated the processes involved in oral tolerance. Treg cells (that suppress other T cell responses) are involved in oral tolerance in rodents[131, 134, 143]. Similarly, Grindebacke *et al.*[144] recently demonstrated that for the first 18 months of life, Treg cells express the intestinal homing molecule $\alpha 4\beta 7$ [142] at in-

creased frequencies[144], indicating that the infant immune system may be preconditioned to support oral tolerance. The uptake and processing of the food proteins is also thought to be important. Particulate antigens, like bacteria and caseins are taken up by specialised M (microfold) cells in the follicle-associated epithelium overlying Peyer's patches[130, 139, 142]. These cells take up antigen and deposit the particulate matter, undigested, in an invagination below the M cell for uptake and presentation by other antigen presenting cells. This processing is thought to contribute to the tolerance process to intestinal bacteria[130]. Dendritic cells in, and directly beneath, the intestinal epithelial layer can extend dendrites into the intestinal lumen to sample antigens[142, 145]. Tolerance to soluble antigens involves dendritic cell antigen carriage[130], their migration to the mesenteric lymph nodes and induction of Treg cells that could subsequently migrate into the intestinal lamina propria[130, 134, 142, 143]. IL-10 producing T cells in Peyer's patches are also implicated in oral tolerance[146, 147]. There are also some other potential mechanisms of tolerance generation. Epithelial cells in orally fed naive rats have also been shown to produce antigen-loaded MHC-II 'tolerosomes' that disperse systemically and inhibit delayed-type hypersensitivity reactions[130, 142, 148]. This tolerizing capacity was lost if it was first MHC-II-depleted[148], suggesting that the tolerosomes specifically were required for tolerance development. Further, depletion of antigen by removing antigen-bound MHC-II is not a sufficient explanation for the loss of activity. While allergen is absorbed systemically following oral ingestion[149], and can induce allergic responses in passively-sensitised skin[150], the tolerizing capacity of serum does not relate to the amount of antigen contained in the transferred serum[151]. Therefore, in addition to specialised antigen presenting cells that are implicated in the generation of Treg cells, systemically dispersed tolerosomes may also contribute to the oral tolerance process.

Collectively, while much is known about the disease phenotype, the underlying physiological mechanisms that cause the allergic response, and the factors that increase disease risk are being elucidated, current treatments are inadequate. Better understanding of the allergic disease process is required to improve quality-of-life for allergy sufferers. Many allergic responses require the coordinated interaction of IgE, allergen and mast cells, so factors that promote allergic sensitization, beginning with the role

of the allergen itself, in the allergic process, are important.

1.3 Allergenicity and the epidemiology of allergic disease

1.3.1 Allergy and allergenicity

Proteins that have been identified as allergens in five or more people are now classified by the World Health Organization and International Union of Immunological Societies Allergen Nomenclature Sub-committee. Allergens are named based on the source species, and the order in which they were identified[152, 153]. For example, bovine serum albumin was the sixth identified allergen sourced from the common dairy cow *Bos taurus*, formerly *Bos domesticus*, and is referred to in the allergen nomenclature as Bos d 6. All classified allergens are listed at <http://www.allergen.org>[152]. This and similar allergen databases are proving useful in the identification of common properties of allergens[154].

In terms of what substances evoke allergic responses, not all proteins are equal. In 2008, Radauer *et al.*[155] observed that when allergens, including food, environmental and industrial allergens, are classified by structure only 2% of structural families contained allergens[155]. An equivalent distribution of randomly selected proteins from the same source taxa incorporated 5% of families[155]. The findings of Radauer *et al.* provide evidence that some allergens share common structural features. This was further evidenced in their analyses because specific protein families were enriched for allergens. Prolamins, profilins and EF hand domain-containing proteins were the most common plant allergens; tropomyosins, EF hand domain-containing proteins, lipocalins and PR-1 proteins were the most common animal-derived allergens[155]. With similar observations made by other groups[154], the findings of Radauer *et al.*[155] suggest that there is some intrinsic feature that makes a protein or food more likely to be an allergen.

This intrinsic property is not simply proportional to the amount of protein to which an individual is exposed. Small contaminants in highly purified proteins can provide the major sensitizing capacity of a food protein[156]; autoadjuvant properties of allergens can contribute to their sensitizing potential[157, 158], trace and non-protein antigens can evoke allergic responses[91, 158]; and cooking and sterilization processes can alter an allergen, rendering it innocuous or more hazardous[139, 159–162]. For example,

pasteurizing purified milk proteins increases the severity of the allergic response they induce in mice[139], but baked products containing milks are often well tolerated[162]. It is important to keep in mind that while studies like those of Radauer *et al.*[155] indicate some protein structures are more likely to be found in allergens, every food has the potential to be an allergen[10]. Similarly, it is thought that certain protein folds or structures are more likely, rather than are the only folds that are immunogenic and/or allergenic[163]. The term 'allergenicity' was coined to describe the intrinsic capacity of a substance to sensitise and trigger an allergic response. Allergenicity conveys a sense of the 'relative allergic risk' of a given protein or food, indicating its likelihood of being problematic in allergy-susceptible individuals[164]. Unravelling what gives an allergen allergenic properties has been a key question for a number of years[104], and is likely to continue as an important question for a number of years to come.

1.3.2 Molecular properties of allergens, with specific reference to food allergens

Numerous excellent reviews regarding the common features of allergens are available, and these identify some general properties common to food allergens (e.g., [153, 154, 164]). First, food allergens are usually small proteins, ranging in molecular weight from 10-70 kDa[153, 164, 165]. Bovine beta-lactoglobulin is an 18 kDa protein[56]; the egg allergens OVA and ovomucoid are 45 and 28 kDa, respectively[156]. However, the almond storage protein amandin (Pru du 6), is a hexameric protein with molecular weight estimates of 200, 360 and 427 kDa[166–168], and bovine immunoglobulin (Bos d 7) when fully assembled is 160 kDa and is ingested in milk[169]. Thus, there are examples of large food allergens.

One often overlooked feature of food proteins in general, is that proteins generally evolve with a purpose. Many allergens have been characterized as possessing a specific biological function, such as proteolytic activity[154]. Taking the most common allergen structures determined by Radauer *et al.*[155] as examples, plant allergens were enriched in the profilins, EF hand domain and tropomyosin families[155]. Profilins are involved in the regulation of signal transduction and actin polymerisation, and some

of these are food allergens[154]; polcalins are two EF hand-containing calcium-binding proteins that can be aeroallergens[154, 170, 171]; Tropomyosins are common food allergens, and are muscle proteins found in invertebrates[154]. Some food allergens are energy storage proteins, like albumins[154] and the almond allergen amandin (Pru du 6)[166, 168]. Nonspecific lipid transfer proteins cause cross-reactive fruit allergies to peach and apple[154, 172, 173] and were identified[155], and the lipocalin BLG binds a number of molecules, including fatty acids, cholesterol and vitamin D2[174–177]. Whether fatty acid transport is its natural function is not established[174, 175]. However, proteins with protease and enzymatic function, calcium, ligand- or metal-binding activity are enriched in allergens and this is also true of food allergens[154, 155].

The third property common to many food allergens is that they are often resistant to proteolytic digestion at acidic pH[153, 178]. Proteolysis-resistant proteins are typical class 1 food allergens[153], and can trigger responses in the intestine. When incubated in simulated gastric fluid at low pH (less than 3), ovalbumin (Gal d 2), beta-lactoglobulin (Bos d 5) and the apple allergen Mal d 2 all remain intact for at least sixty minutes[178–180]. It has been shown that the IgE binding capacity of BLG can be removed if it is de-stabilised and rendered susceptible to digestion[181]. The apple allergen Mal d 3 that is stable to proteolytic digestion causes cross-reactive fruit allergies[154, 172, 173]. Many response-evoking food allergens are therefore resistant to digestion.

Again, while resistance to digestion is a property of some food allergens, digestibility does not preclude a food from being an allergen. Food proteins that are digested by pepsin in less than two minutes, like caseins[178], can be major allergens in humans[91, 182, 183]. After extensive incubation of protein in digestive enzymes, antigenic epitopes can remain intact[184]. Foods can also contain both digestible and undigestible allergens[180, 185] and cross-reactive allergens from different food sources can be differentially sensitive to gastric digestion[186]. Class 2 food allergens are allergens that do not have sensitizing capacity, but are cross-reactive with aeroallergens[153]; the class 2 apple allergen Mal d 1 is readily degraded by pepsin at gastric pH[186], but can still trigger allergic responses in the upper gastrointestinal tract, skin and face[187]. Additionally, gastric transit time and pH are not always slow and low, and can also be compromised. For example, in newborns, the average gastric

transit time is short (around 25 minutes[188]) and their gastric pH is generally higher than in adults (pH 5 or more[189]). Proton pump inhibitors that prevent gastric acid secretion and raise the stomach pH are also prescribed to some infants[114], which could limit the opportunity for gastric enzymes to cleave ingested proteins. In terms of the allergic response, these processes could result in 'digestible' allergens passing through to the small intestine intact. However, not all individuals have allergic responses; clearly, exposure to a highly allergenic, stable protein does not automatically render an individual allergic. Understanding not only the features of the allergen that permit allergies, but also elucidating why only some individuals become sensitized requires further investigation.

The many exceptions to the general features of a food protein that can make it allergenic suggest that we do not fully comprehend what contributes to allergenicity. In part, I think that under the appropriate inflammatory conditions, any protein could be rendered allergenic. Adjuvants are employed with the intention of increasing the immunogenicity of antigens used to immunize and similar adjuvant effects could be argued to increase allergenicity. Even in these contexts, however, I would expect the longevity of the induced allergy-type immune response to differ between common and uncommon allergens. I think the immune system can sometimes work out when its response is misdirected. As such, rather than discrete populations of allergens and non-allergens, food proteins are likely to comprise those that are high-risk for allergy and those that are of lower risk. As multiple factors contribute to the development and maintenance of allergic sensitization (e.g., exposure frequency, exposure mass, route of first exposure, age of first exposure, inflammatory environment surrounding first exposure, route of second exposure), examining the ability of foods to induce allergic immunity, as well as the host-derived and environmental factors that increase the likelihood of sensitization and/or an allergic response need to be explored synchronously and considered together.

1.3.3 The epidemiology of food allergy

The allergenicity of food proteins has largely been established from epidemiological data. Similar to the restricted set of protein families that contain most allergens, most

food allergies can be attributed to a small number of allergens[60]. Food proteins have different likelihoods of sensitising infants, and have different resolution rates as children age[9, 33, 35, 190]. During infancy, milk allergies account for half of all food allergies, occurring in 2.5% of children, while the next most frequent allergen, egg, causes a response in 1.5% of children[9]. For milk and egg allergies, 70 to 80% resolve by the age of sixteen[4, 33, 35]. While less frequent[9], soy allergies follow a similar prognosis, with 70% resolving by age ten[32]. In contrast, although allergies to peanuts, tree nuts, fish and shellfish are also less frequent in infants[9], they are unlikely to resolve[4]. Allergies that persist into adulthood are associated with high IgE levels[32, 34] and for milk, peanuts and eggs, allergen-specific IgE titres with greater than 95% positive predictive value for an oral response have been determined empirically in some clinics[31].

1.3.4 Sensitization to multiple foods

An allergy to one protein is also highly likely to co-present with allergies to other foods[191] and this is a particular problem in milk allergic infants. While most milk allergies resolve, one major complication for milk allergic infants that cannot be breast-fed, is that there are few alternatives for feeding. In cow milk allergic individuals, cross-sensitization to other milks is common[169, 191]. Greater than 90% of cow milk allergic infants are cross-sensitized to goat milk[192], 40% of milk allergics are co-sensitized to soy[35], and 80% of soy allergic infants are also allergic to cow milk[32]. There are also rare cases of IgE cross-reactivity to homologous human milk proteins in cows milk-allergic infants[193–197]. Often, the best nutritional source for milk allergic infants is an extensively hydrolysed milk-based formula or a synthetic single amino-acid formula[114, 120]. Although they have poor palatability and different physico-chemical composition to normal milks[93], they are considered nutritionally complete. Further, the makeup of milks from different species is also different[169]. Food allergies, particularly milk allergies, during infancy may put infants at risk of nutritive deficiencies[121], failure to thrive[60, 93, 120] and impact the quality of life of allergic individuals and their families[97, 119]. Elucidating the molecular changes that result in sensitization, particularly milk protein sensitization, may provide novel

ways to interrupt the allergic process.

1.3.5 Atypical mechanisms of sensitisation

While epidemiological data shed some light on the 'relative allergic risk' of a number of proteins, defining an all-encompassing allergenicity hierarchy is not straightforward because sensitization to specific allergens can be contextual. During the first three years of life, most allergies that develop are to food proteins, whereas the prevalence of environmental allergen sensitization increases after this time[97, 198]. However, this does not preclude allergies to foods developing later in life, as they can form part of a normal diet during infancy but become problematic after cross-sensitization to an environmental allergen. This frequently occurs with class 2 fruit allergens, to which a large number of people are only sensitised after allergies develop to birch pollen during the teen years[187]. For example, exposure to Bet v 1 from birch can sensitize individuals to Mal d 1 in apple[186, 187, 199, 200]. Chronic workplace allergen exposure can also result in adult sensitisation, for example, in woodmill workers, fish packers, and bakers continually exposed to wheat flour[201–203]. Healthcare workers that regularly use latex gloves are similarly at increased risk of developing cross-reactive latex allergies than the general populace[76, 204].

Recently a unique subtype of IgE-mediated allergy has also been identified. The carbohydrate antigen galactose- α -1,3-galactose induces allergic responses to meats in adults[205]. This carbohydrate antigen is present on a number of mammalian meats, and results in an allergic phenotype presenting three to six hours after eating meat. This is thought to be because the meat needs to be digested to trigger mast cell degranulation. The symptoms of this type of allergy, contrasting with many allergies, involve the organ systems of the intestine, skin and sometimes systemic anaphylactic responses, but are not associated with allergic asthma[206]. This adult-onset allergy has also been shown to be caused by IgE cross-reactivity with *Amblyomma americanum* ticks, and to occur almost exclusively in tick-sensitized individuals[207]. Galactose- α -1,3-galactose also causes cross-reactions with a monoclonal antibody (the specificity of which is epidermal growth factor receptor[208], but this is irrelevant for the reaction) that contains the galactose- α -1,3-galactose sugar moiety in its

heavy chain[209, 210]. While these findings also suggest that tolerance can be broken in special circumstances, it is clear that the sensitising capacity and aetiology of allergy is different for different proteins and foods. The mechanisms that explain these differences, and why only some people develop allergies to allergenic substances are only now becoming apparent.

1.3.6 Physical and immunological differences in cow and goat milks

Whole milk is a complex mixture of protein, lactose, oligosaccharides, a range of fatty acids, lipids, non-protein nitrogen, vitamins and minerals[211–218]. The protein comprises of two main fractions: The casein fraction and the whey fraction. Casein was originally defined by its precipitation at pH=4.6 at 20 °C[211], whereas the whey proteins remain soluble. There is significant species-to-species variation in the absolute composition of milk (Table 2). Different to other mammals, humans consume the milk of other species. For example, cow, goat and sheep milks are frequently included as part of the normal infant diet in different parts of the world[219, 220]. However, the consumption of milk from other species allows the possibility of allergy development to other species’ milks. Allergies to cow, goat and sheep milk have all been reported, and milk allergies are not specific to western countries[34, 219, 221].

Breast milk is considered the gold standard in infant nutrition[222]. It is also important for the physiological development and maturation of the infant immune system (for review and discussion see[223, 224]). The main proteins in human milk are β -casein, κ -casein, α -lactalbumin, lactoferrin, immunoglobulins, serum albumin and secretory component[193, 225, 226]. Approximately 60% of the total protein content of human milk comprises the more soluble whey proteins[227]. β -casein is the dominant human milk protein, followed by α -lactalbumin[193]. The protein α s1-casein is present at low-to-undetectable concentrations[193, 228]. There are also a number of other trace proteins[229].

There are some significant differences in the composition of human and cow milks. In contrast to human milk, 80% of the protein in cow milk is casein[169, 230]. The

casein fraction in cow milk comprises 40 - 50% α s1 casein plus three other casein proteins: α s2-casein, κ -casein and β -casein[169, 211]. The other 20% of cow milk protein is the more soluble whey fraction. The predominant whey protein is BLG (60 - 80% of whey), with lower amounts of α -lactalbumin, immunoglobulins, serum albumin and traces of lactoferrin[169, 211, 231]. In comparison to human milk, cow milk has additional proteins (α s2-casein and β -lactoglobulin[193, 228]); a different whey to casein ratio; less non-protein nitrogen[223] and more concentrated protein, at 3 g/100 mL[169, 211] compared to approximately 1.6 g in colostrum, and $\approx$ 1 g /100 mL in at later times for human milk[223, 225].

For cow and goat milk, there are some differences and similarities in their composition. Cow and goat milks have similar total protein content, similar β -lactoglobulin content, and similar casein:whey balances[141, 169, 211, 231–235]. However, goat milk has lower levels of α s1-casein and higher β -casein levels[232, 234–236]. Like human milk, β -casein is the dominant protein in goat milk[233, 237]. One of the other differences between cows and goats, is that some breeds of goat produce milk with higher total fat content, and goat milk invariably has higher medium-chain fatty acid content than cow milk[235, 237]. The increased medium-chain fatty acid content renders goat milk fats more digestible by lipase activity[237]. Goat milk proteins are also more heat-labile[238] and more digestible by human- and simulated-gastric fluids than those of cow milk[185]. There are also differences in non-protein nitrogen content and composition between the two milks[215]. Mineral and vitamin content can differ between the species, but there is significant seasonal and study-to-study variation (*c.f.*[217, 235, 239]). The reported differences between cow and goat milks relate primarily to protein homology, α s1 casein content and the amount of medium-chain fatty acids present in the milks.

When individual proteins are examined, goat and cow milk proteins are more similar than cow and human homologues. Any given goat milk protein shares 85-95% homology with its cow milk counterpart[169]. In humans, the most conserved protein, α -lactalbumin, shares 76-78% sequence identity and almost 90% homology with cow α -lactalbumin[169, 193, 240], but other proteins are less homologous. Bovine and human β -casein are 47-57% homologous[169, 196]. Human α -s1 casein shares only partial ($\approx$ 32%) sequence homology with cow α -s1 casein[241, 242]. While it is absent

from human milk[228], BLG shares structural homology with serum retinol binding protein[177, 243]. It is also related to other human lipocalins, the glycodefin precursor protein[244], and human placental protein-14[245](44% by DELTA-BLAST; see methods section). Allergens often share sequence similarity with host proteins[246], and the similarities in cow and human milk proteins may contribute to the frequent incidence of cow milk allergy in humans[246].

Table 2: Composition and homology of human, goat and cow milk

	Human	Goat	Cow
Protein (%)	0.8-1.25	4.3	3.2-3.6
Caseins (%)	≈ 40	≈ 80	≈ 80
Whey (%)	≈ 60	≈ 20	≈ 20
Dominant milk protein	β -casein	<i>beta</i> -casein	α s1-casein
α s1-casein	0.8 g/L	0-3.5 g/L	12-15 g/L
α s2-casein	Not present	3.8-3.9 g/L	3-4 g/L
β -lactoglobulin	Not present	2.9-3.4 g/L	2-4 g/L
Homology (% to cow)			
α s1-casein	31.9	87.9	100
α s2-casein	N/A	88.3	100
β -casein	56.5	91.1	100
β -lactoglobulin	N/A	94.4	100

Proportions and concentrations are compiled from [141, 169, 193, 211, 225, 227, 228, 233, 235–237]; Homology data are taken from [169].

Evidence suggests that while goat and cow milk proteins are largely homologous, they have distinct properties as allergens. In one study, some cow milk allergic individuals tolerated goat milk, and in those that did not, as much as five times more goat milk was required to cause an allergic response compared with cow milk[192]. Further, the IgE-binding repertoire of sera from cow milk-allergic individuals generally binds to a comparably reduced set of goat milk proteins[192, 247, 248]. In terms of its ability to sensitize and evoke an allergic response in orally-sensitized mice, goat milk is less allergenic than cow milk[234]. Oral feeding of goat milk to guinea pigs results in less frequent challenge-induced anaphylaxis than cow milk[141]. Further, the allergenicity of ruminant milk correlates closely with its α s1 casein content, both in terms of IgE cross-reactivity in sera from cow milk allergic individuals, and in terms of its sensitizing and allergic response-evoking capacity in experimental systems[141, 232, 234, 236, 249]. Low α s1-casein levels are more typical of goat milks and demonstrate reduced allergenicity in mice and guinea pigs[233, 236, 249, 250]. Evaluating whether these differences can be attributed to differences in intrinsic allergenicity, or more predominantly to digestibility of the milk, remains to be addressed.

1.4 Predicting allergenicity

1.4.1 *In silico* prediction

As well as understanding the epidemiology of food allergy, predicting allergenicity and cross-reactivity are becoming more important in the modern world. Novel proteins are being introduced into the food chain, such as those from genetically-modified crops, and these need to be assessed for their allergic potential. Generally, cross-reactive allergens share more than 70% sequence homology with each other[154], so *in silico* comparisons to known allergens can be used as an early step in the screening process (e.g., [251, 252]). In fact, the second step in the World Health Organisation decision tree for the evaluation of allergic potential, after assessing whether a protein is derived from an allergenic source, is to examine sequence homology using protein databases[253, 254]. However, *in silico* analyses are only a start point. Even if allergenic potential is not identified in structural screens, serum screens, animal models

and screening for pepsin resistance are recommended by the guidelines[253, 254]. If a protein is able to sensitize animals, this is considered a significant risk factor for it to be a sensitizing allergen in humans[253, 254]. *In silico* screens also have the potential to reveal features of allergens of biological importance that may be missed in specific bioassays[152]. Allergenicity screening is beginning to incorporate *in silico* methodologies as an adjunct to biological testing (e.g.,[255]).

1.4.2 Biological evaluation of allergenicity and cross-reactivity

Examining allergenicity in biological systems utilises a variety of approaches. Classical analyses have examined peptide recognition of sequential epitopes, and in some cases these have identified certain B cell epitopes that correlate with allergies that persist (e.g.,[256, 257]). Other approaches use surface plasmon resonance to examine the affinity and/or avidity of antibody for antigen, and this has been used to analyse IgE affinity distributions in polyclonal sera in some cases[258, 259]. Linear epitope analysis can be informative, particularly for T cell epitope detection, because T cells recognise short linear epitopes (generally 8-20mers); there are a significant number of allergens for which discontinuous B cell epitopes are thought to be key[260]. This is exemplified nicely in the study by Clement *et al.*[259], in which only 1 of more than 50 monoclonal antibodies recognised denatured BLG, but epitope masking was able to identify 12 antigenic clusters on the surface of native BLG[259]. When the first crystal structure of an IgE-bound allergen was solved, specific surface-exposed residues identified in the screen by Clement *et al.*[259] were also involved in IgE-binding to BLG[261]. Similarly, the majority of IgE binding to recombinant α -lactalbumin is predicted to bind to a cluster of surface-exposed residues[262]. Combining structural data with epitope analysis has been useful in the identification of discontinuous epitopes that would not necessarily be observed if the full structure of the protein was not examined.

A range of novel *in vitro* and *in vivo* animal methods for testing changes in the allergic potential of foods are being trialed. These include oral sensitisation using allergen in the presence or cholera toxin, followed by analysis of the T cell, B cell and allergen-induced mast cell response[263]. Measuring degranulation products of mast

cells, the antibody response and the impact of feeding on basophil populations has been examined in this system to evaluate changes in the allergenicity of hydrolysates in mice[264]. It has also been used to compare the epitope specificity of IgE generated in mice to the epitopes involved in human IgE binding[265], and similarly to examine specific allergens contained in whole foods against which antibody responses are directed[263]. Similar examinations have examined the proteins to which antibody binds in guinea pigs sensitized to whole milks[232, 249]. There are numerous mouse models that examine a range of different aspects of the allergic process. I site some examples for further reading[136, 266–269]. In a similar attempt to clarify what makes chemical a (type IV) contact allergen, Kimber *et al.*[270] examine the response in an isolated lymph node assay[270]. In an independently derived, but similar assay, our lab has developed a novel mouse assay of allergenicity (see next section).

Collectively, a range of methods have been, or are being applied to the evaluation of the allergenicity of foods. These assays are also used to improve our understanding of the properties of allergens that render them allergenic and/or cross-reactive. Further, these models can provide information about the basic mechanisms that control the allergic process. Employing multiple biological approaches, in addition to *in silico* prediction methodologies will continue to be key for the determination of the allergenic potential of foods.

1.4.3 Development of a novel assay of allergenicity and Th2 function

In addition to the various tools that have been developed to examine allergenicity, our lab has used the G4/+ reporter in an assay that measures allergenicity in direct proportion with Th2 cell development. In early studies by Belkaid *et al.*[271], intradermal administration with *L. major* promastigotes induced local inflammation isolated to the ear[271]. Our lab examined Th2 cell development induced by dead parasite antigens, known allergens, or bacterial products in the auricular (superficial parotid) lymph node[1, 57, 272]. In experiments using indian violet ink, our lab determined that this is the only lymph node to which antigen administered to the ear is drained (figure 1.2; photo and observation made by Graham Le Gros). By combining this sensitive, single lymph node assay with an IL-4-reporter mouse[273] IL-4-producing CD4+ T cells can be sensitively identified and counted. The inflammation can be used to examine immunogenicity and GFP+CD4+ Th2 cell numbers can be used to compare allergenicity. Numerous immunogens have been compared in this system (figure 1.3; data generated by Mali Camberis and Melanie Prout). The most potent Th2-polarising immunogen that has been observed in this system, so far, is antigen derived from dead *iL3 N. brasiliensis* larvae (figure 1.3). Dermatophagoides pteronyssinus house dust mite extract (HDM), also induces strong Th2 cell development (figure 1.3). The expansion of IL-4-producing Th2 cells, as reported by GFP expression in G4/+ mice is reproducible. In addition to the data shown (figure 1.3), my studies have recapitulated these original findings: Immunisation with dead parasite antigens, caused a 200-fold increase in Th2 cell numbers, and HDM generated four-fold fewer Th2 cells than dead *N. brasiliensis* larvae (figure 1.4B). In this manner, *in vivo* Th2 cell development can be examined and has the potential to be applied in the analysis of intrinsic allergenicity.

Figure 1.2: **Indian violet ink drains to a single lymph node.** Seven days after intradermal injection of 600 dead *iL3 N. brasiliensis* larvae mixed with indian violet ink, the tissue architecture around the head of a mouse was photographed. The same picture is shown twice, and in the right-hand photo the location of the auricular draining (superficial parotid[272]) and non-draining (mandibular and accessory mandibular[272]) lymph nodes are indicated. In all other experiments, indian ink was not included in the injection. A non-lymphoid, likely adipose, tissue deposit is also indicated for disambiguation. The contralateral auricular lymph node can be seen at the arrow tip just above the ensanguined area at the junction of the superficial temporal and maxillary veins.

Figure 1.3: **Th2 response to environmental and food antigens.** Seven days after intradermal injection of sample, the number of Th2 cells found in the draining lymph node was assessed. Antigenicity (x-axis) and allergenicity (y-axis) were evaluated in response to helminth extracts (red), dead mycobacteria (yellow), environmental, non-protein and food allergens (light and dark blue). Data were generated and analysed by Mali Camberis and Melanie Prout.

Figure 1.4: Th2 response to parasite and dust mite antigens. (A) Total live cells were examined by flow cytometry for CD4 and GFP expression in draining (DLN) and non-draining (nDLN) lymph nodes of +/+ and G4/+ mice. (B) The total number of GFP+CD4+ T cells induced by immunization with 600 dead *i*L3 *N. brasiliensis* larvae (L3) or 100 μ g HDM (HDM) in G4/+ mice were combined from all experiments described in this thesis hereafter. The control (ctrl) group includes naive, PBS-injected and non-draining nodes of G4/+ mice. Symbols show individual responses of (n=72) N.b.-immunised, (n=27) HDM-immunised, and (n=76) control lymph nodes. Data were analysed by One-way ANOVA with Tukey's post-test. ****p<0.0001.

1.5 The immunological mechanisms of allergic disease

1.5.1 The generation of IgE

IgE is central to the allergic response, therefore, the processes that lead to IgE production are a key process in the allergen-directed immune response. IgE class switching is CD4+ T cell dependent[274, 275]. With some exceptions, isotype switching occurs in lymph nodes concomittant with affinity maturation[276–280]. The enzyme Activation-Induced Cytidine Deaminase is central to both processes[281]. Prior to isotype class switching, naive B cells excise the μ and δ heavy chains and begin to transcribe downstream heavy chain transcripts (γ , ϵ or α)[282, 283]. This means that naive B cells are IgM+IgD+, while isotype class-switched B cells will lose IgM and IgD expression and express IgG, IgE or IgA. When B cells mature into plasma cells, the class-switched B cell receptor is secreted as antibody of the class-switched isotype (for an example see [284]).

The cues required for B cell isotype switching to IgE have been determined and IL-4 is the most critical factor. In the 1980s it was discovered that when the protein BCF-1, now known as IL-4, was added to cultures of mitogen-activated B cells or human peripheral blood B cells they class-switched to produce antibodies of the IgE and IgG1 isotypes (or IgE and IgG4 in humans)[285, 286]. In mouse studies, it was shown that blocking IL-4 signaling by sequestering secreted IL-4[287] or by blocking IL-4 receptor signaling[288], IgE production was impaired by greater than 95 percent. The advent of IL-4 knockout mice confirmed the central role of IL-4 in IgE production[289]. In response to infection with the rodent helminth *N. brasiliensis* that usually generates high titres of IgE and IgG1, IL-4-deficient mice generated no detectable IgE and exhibited an 80 percent defect in IgG1 production[289]. The cellular source of IL-4 that provokes IgE production is known to be the CD4+ T cell[274, 275] and CD4+ T cell expression of CD40-ligand (L) is also critical for the CD4-dependent isotype class switch[290–292]. Thus, by producing IL-4 and expressing CD40L, CD4+ T cells promote IgE class switching.

1.5.2 Additional roles of IL-4

IL-4 influences not only IgE production, but also a number of other aspects of the allergic process. IL-4 increases mast cell FcεRI-expression[293] and potentiates non-haematopoietic cells to respond to vasoactive mediators of allergy[48, 50]. IL-4 signaling is required for experimental allergic airways disease[294] and for oral allergen-induced mastocytosis that follows the first few oral challenges of sensitized mice[295]. Clinically, IL-4 and T helper type 2(Th2)-associated cytokine mRNA transcript numbers are elevated in bronchoalveolar lavage lymphocytes of asthmatics and in the jejunal tissue of food allergic individuals[90, 296] indicating that IL-4 may evoke local tissue effects during allergic and asthmatic responses. These Th2-associated cytokines include IL-4, and also IL-5 and IL-13. All three of these cytokines are observed at increased levels following oral allergen challenge in sensitized mice[37, 58].

1.5.3 Mice are rendered susceptible to intestinally-triggered allergic responses in a post-sensitization oral potentiation phase

The elevation in Th2-associated cytokines is an important aspect of the intestinal allergic process. While experimental intestinal allergic responses are IgE- and mast cell-dependent, after sensitization, multiple oral exposures are usually required before severe diarrhoea is observed; it is in this phase that precedes disease in which Th2 cytokines first become elevated[37]. In sensitized wild-type BALB/c mice, multiple oral challenges are required to generate sufficient intestinal mast cell infiltration to permit oral allergen-induced diarrhoea development[36, 37, 67]. Further, passive sensitization does not permit diarrhoea without multiple previous oral challenges[67]. It has, however, been demonstrated that once mice have been orally challenged multiple times with OVA, passive sensitization with IgE against the trinitrophenyl (TNP) hapten permits diarrhoea development upon the first feeding of TNPylated-bovine serum albumin[67]. This suggests that both peripheral sensitization and intestinal mast cell infiltration are required, but these are discrete and likely independent processes that permit allergic diarrhea to be triggered by oral allergen challenge. As this local Th2-

type inflammatory process precedes observable diarrhea, I will refer to this process as oral potentiation.

IL-4 is key to oral potentiation, but other Th2-associated cytokines also become elevated during oral potentiation. In orally-challenged mice, IL-5 levels become elevated and increased intestinal eosinophil influx is observed[37, 58, 297]. There is no role for eosinophils in IgE-mediated diarrhoea[37], but they are implicated in villus shortening in oral-allergen challenged mice[42]. While beyond the scope of this thesis, eosinophils are also involved in other IgE-associated diseases, like eosinophilic esophagitis[11]. In terms of disease-relevant Th2 cytokines, IL-13 levels also increase in orally challenged mice[295] and blockade of IL-13 signaling decreases disease incidence in mice without significantly impairing mast cell infiltration[295]. This may be due in part to the effects of IL-13 on the smooth muscle tissue itself[50, 66]. The CD4+ T cell-derived cytokine, IL-9, is also implicated in the expansion of intestinal mast cells[57]. IL-4, IL-5 and IL-13 can all be produced by CD4+ T cells, so in addition to their role in IgE generation, there are a number of additional allergic processes that are associated with CD4+ T cell activity, including the oral potentiation that permits triggering of intestinal anaphylaxis.

1.5.4 CD4+ T cells are implicated in oral potentiation

Studies in three similar systems implicate intestinally-localised CD4+ T cells in the oral potentiation process[37, 57, 58, 298]. Prior to diarrhoea onset, sequential intra-gastric OVA challenges induces proliferation of- and $\alpha 4\beta 7$ expression on- OVA-specific CD4+ T cells[38], IL-4 production by intestinal CD4+ T cells[57, 58], and intestinal transcription of CCL20[299]. Additionally, adoptive transfer of *in vitro* restimulated OVA-specific CD4+ T cells from diarrhetic mice transfers disease susceptibility to naive mice[38]. As outlined previously, this oral potentiation period is also associated with increases in intestinal mastocytosis. The involvement of the IL-4/-13 STAT-6 signaling pathway in experimental food allergy is unequivocal[37, 57, 58, 295], however the distinct effects of CD4-derived IL-4 on IgE production and tissue-specific mast cell activity remain unknown. There is also evidence that after sensitization, CD4+ T cell recirculation is not required for disease to develop[299]. This indicates

that either peripheral processes (like IgE generation), or intestinally-localised CD4+ T cells are sufficient for oral potentiation. It has also been demonstrated that after oral potentiation, disease ensues when IL-4 is neutralised[295]. The processes involved in oral potentiation and those that maintain disease susceptibility may therefore be mediated by distinct cytokines and different cell types. In a similar vein, cells other than CD4+ T cells, namely basophils, eosinophils, *i*NK T cells, mast cells and B cells have been shown to produce IL-4, and could therefore contribute to the oral potentiation process[300–305]. The correlation between Th2 cell infiltration and IL-4 production implicate CD4+ Th2 cells in the allergic process, but whether they are required, or simply sufficient, for the induction of intestinal anaphylaxis in mice requires clarification. The involvement of CD4+ T cells in disease maintenance is also unclear: addressing the role of CD4+ T cells in the maintenance of disease is required. Close inspection of the role of CD4+ T cells in the post-sensitization phase of food allergy may establish unknown subtleties of the allergic process that facilitate better long-term disease alleviation.

1.5.5 Cytokine requirements for intestinal mastocytosis

Mast cells are key effector cells in IgE-mediated allergies. However, there is evidence that allergic responses that are induced by challenge in different tissues are mediated by different effector molecules. For some responses, passive sensitization through the transfer of IgE-containing serum is sufficient to permit immediate, local, type I hypersensitivity reactions. For example, injection of purified antigen-specific IgE, or antigen-specific IgE-containing serum into the ears of mice is sufficient to permit acute mast cell-dependent type I responses in the ears of mice[43, 306]. Passive sensitization can also permit systemic anaphylaxis in mice[41, 48]. Passive sensitization is possible in humans, too. Intradermal injection of reagenic serum to non-sensitized humans, followed by oral allergen ingestion, induces wheal-and-flare reactions in the skin without intestinal disease manifestations[150]. In mice, passive sensitization alone does not permit severe intestinal allergic responses without the oral potentiation phase[67]. Therefore, while IgE alone is sufficient to permit cutaneous and systemic disease, additional cues are required to promote intestinal mast-cell dependent allergic ana-

phylaxis.

There are a number of mast cell-active cytokines, and three of these are critically involved in inflammatory mast cell responses in mice. It is known that stem cell factor (SCF) is required for the presence of mast cells in the intestine at baseline[307]. Mutations in the gene encoding cKit render mice mast cell-deficient, in intestinal, peritoneal, and cutaneous tissues[43, 307, 308]. In contrast, while mast cells and their activity can be observed in mice deficient in IL-3, IL-4, or IL-9[50, 57, 309, 310], these three cytokines amplify intestinal mast cell infiltration caused by parasite infection[311–313]. These are the three cytokines that are most important for inflammatory mast cell responses. Exogenous administration of these cytokines can also expand intestinal mast cell populations[309, 314]. IL-3, is a particularly potent mast-cell active cytokine, and can increase intestinal mast cell progenitor numbers in the presence of SCF to a greater extent than IL-4 and IL-9 combined[314]. *In vitro*, IL-6 and IL-10 can also contribute to mast cell development[314]; however, each of IL-3, IL-4 and IL-9, has been demonstrated to expedite parasite expulsion and are therefore important in intestinal Th2 responses[50, 309, 311–313, 315]. Further, production of IL-3, IL-4 and IL-9 can be attributed, at least in part, to CD4+ T cell activity[316–318]. Roles for IL-4 and IL-9 in experimental oral allergen-induced mastocytosis and diarrhoea have been demonstrated[57, 295] but the potential involvement of IL-3 in the intestinal allergic process is currently unknown.

1.5.6 Intestinal mastocytosis heightens allergic disease susceptibility

Intestinal mast cells also contribute to additional aspects of the allergic disease process. High basal mast cell activity increases gut permeability, and this renders mice more susceptible to allergic sensitization and anaphylaxis[57, 67]. This is associated with a corollary decrease in blood plasma volume and impaired basal intestinal barrier function at baseline[57, 67]. Individuals with food allergies also have increased intestinal permeability, or a leaky gut[319]. Long-term changes in permeability are also observed in orally-primed wild-type mice[37, 57, 149]. It has also been demonstrated that hypothermia and shock can be induced by oral feeding of allergen, but only if

the allergen can cross the intestinal epithelial barrier and get into the blood[67, 68]. Correlations have also been drawn between mast cell numbers and orally-induced anaphylactic severity[36, 39]. Intestinal uptake of allergen is expedited by sensitization in a mast-cell dependent manner[308, 320], and the allergic leaky gut results in a 200-fold increase in allergen levels in the blood of allergic mice fed allergen compared to non-allergic mice[149]. Oral allergen challenge has also been shown to enhance systemic anaphylactic responses[37] and exacerbate lung inflammation to unrelated allergens[321]. These findings collectively suggest that intestinal mast cell activity can exacerbate not only intestinal allergic responses, but can also permit allergen dissemination and amplify systemic allergic responses. Processes that increase intestinal mast cell activity therefore have the potential to promote allergic responses, and this may be an important aspect of the clinical allergic process.

1.6 Th2 cells and the functional Th paradigm

While the full role of CD4+ T cells in allergic disease requires continued investigation, impetus already exists to investigate the signals that promote their development into Th2 polarised cells. Since 1986, IL-4 production by a CD4+ T cell has identified it as part of the Th2 subset, and much is now known about Th2 cell development *in vitro*; much still remains to be determined regarding their development in *in vivo* allergic disease contexts. In order to understand the early events that induce naive CD4+ T cells to attain Th2/IL-4-producing function the host-derived signals that lead to Th2 development have been investigated in detail.

The designation of the Th2 cell came, originally, from studies by Coffman and colleagues[322] in which CD4+ T cells were shown to develop into two mutually exclusive subsets based on function and their cytokine production profile. Long-term T cell clones that produced IL-2 and IFN-g were termed Th1 clones, whereas those that produced IL-4 (then called BCF-1), IL-5 and IL-10, were designated Th2. Th2 clones were the only clones capable of enhancing IgE and IgG1 switching during polyclonal B cell stimulation[322] and Th2 clones also permitted mast cell generation (both of these responses were identified as IL-4-dependent processes[322]). Further, irrespective of the strain of the mouse used, immunization with chicken red blood cells gave rise only to Th1 clones; in contrast, Th2 clones exclusively were generated by fowl gamma-globulin immunization. Thus, from the first demonstration of Th subset polarization, the concept was inherently tied to that of protein allergenicity. While Th2 cells are implicated in allergic disease and anti-parasite responses[38, 57, 58, 323], Th1 cells are effector cells that are important for the control of intracellular pathogens[324]. There are now a number of proposed Th subsets, including Th9, Th17, Treg (regulatory) and TFH (follicular helper) cells, and multifunctional populations that do not segregate clearly into any one particular subset[324, 325], however, Th2 cells remain an important allergy-associated cell. The involvement of Th17 cells in some allergic processes have been gaining interest[158], and in terms of the oral potentiation process, IL-9 derived from Th9 cells may also play an important role[57, 326]. However, the central role of IL-4 in the allergic process means that IL-4-producing Th2 cells remain the focus of this discussion.

If Th2 cells promote allergy, and allergenic immunizations promote their development, the question arises, what are the immunological changes triggered by an immunization that promote the differentiation of a CD4+ T cell toward any specific, and particularly a Th2 pathway? Commitment to a given Th subset is determined by lineage-specifying gene activation, and silencing of lineage-inappropriate cytokine genes[324, 327]. The master regulator of Th2 commitment, GATA-3[328] acts in conjunction with STAT-6 for the full elicitation of Th2 development and function *in vivo*[329–331], and simultaneously silences Th1 commitment[332]. T-bet is the master Th1 specifying transcription factor[333], and acts in collaboration with STAT-1[334]. Th17 cells are under the control of the master transcription factor ROR γ t[335], and are cross-antagonised by Foxp3 which specifies Treg development[336]. TFH cells, which are specialized B helper cells[275, 337, 338], are now known to develop under control of BCL-6[339–341]. In allergic disease IL-4, IL-5 and IL-13 producing Th2 cells are the most important Th subset and understanding the events that initiate and enhance their development are key current investigations.

1.6.1 The early and late events specifying Th2 polarization in experimental systems

The correlation between Th2 cells and allergic disease is clear, however, the events that permit their development remain incompletely defined. In addition to the role of the allergen in the induction of the allergic process, significant effort has gone into investigating the host-derived proteins that permit the acquisition of polarised Th function. Some work has been done *in vivo*, but the majority of understanding comes from *in vitro* studies.

In vitro studies have been important in elucidating the factors that promote Th2 cell development. Early studies identified IL-2 and IL-4 as important factors for *in vitro* Th2 cell differentiation[342–344]. Studies have now established that early IL-4 transcription is triggered by TCR ligation, CD4 co-receptor stimulation and IL-2R-signaling, and only later is IL-4R signaling required for continued Th2 development[345–349]. When naive CD4+ T cells are stimulated by plate-bound anti-CD3/CD28 or antigen presenting cells loaded with cognate antigen, increases in IL-4 mRNA can be

detected as early as one hour after stimulation, but in the absence of IL-4R signaling this is not maintained, and Th2 cell development thereafter is impaired[347–349]. Similarly, studies using an IL-4-linked conditional toxic gene construct identified that naive CD4+ T cells will die upon initial stimulation, but if they are first polarized to the Th1 subset they will not die[327, 350]. This indicated that the IL-4 gene is transiently expressed by naive T cells, but an important part of differentiation is silencing of alternate lineage cytokines thereafter[327, 347, 350]. These studies identified that Th2 development and IL-4 expression *in vitro* begins in an IL-4-independent manner, and this is followed by a period in which IL-4 specifies Th2 lineage commitment[347]. The importance of this IL-4-independent phase of *in vitro* Th2 cell development is only beginning to be appreciated, but has lead to a number of investigations into the Th2-initiating processes. More recently, tissue-derived cytokines have been demonstrated to promote Th2 cell development by modulating dendritic cell function *in vitro*[351]. In addition to the cytokine milieu and involvement of IL-4 in Th2 development, TCR affinity also influences Th polarization[349] and increased cellular stress is associated with Th2 polarization[352].

The conditions that lead to Th2 cell development *in vivo* are more complex. There is evidence that demonstrates Th2 immunity is amplified by the innate-type cytokines IL-25, IL-33 and TSLP[353–356], but these cytokines are not required for the development or function of CD4+ Th2 cells[357–359]. The role of IL-4 in Th2 development *in vivo* shares similarities with the *in vitro* data but is less clearly understood and far less rigorously validated. While IL-4 can maintain the population of cells transcribing IL-4 *in vivo*[360–362], IL-4 signaling is not required for early Th2 generation[1, 360, 362, 363]. Studies over the last 15 years have investigated the role of IL-4 in Th2 development *in vivo* and determined that the early generation of Th2 cells is equivalent in IL-4Ra^{-/-} and WT mice[362]; memory Th2 numbers are similar in doubly IL-4 and STAT-6 deficient mice[1]; IL-4 mRNA accumulation is equivalent in early immune responses[360]; and naive IL-4Ra deficient CD4+ T cells that have received TCR ligation will produce IL-4 on *ex vivo* TCR stimulation[363]. However, when IL-4Ra deficient mice are injected in the hind footpad with OVA plus alum adjuvant, draining lymph node IL-4 transcription is induced, but transcript levels decay relative to wild-type mice concomitant with lymph node atrophy as the im-

mune response progresses[360, 361]. Thus, IL-4 can influence the amount of IL-4 transcribed in an isolated lymph node *in vivo*, at least in an adjuvant based immunization regimen. Further, when IL-4 signaling is interrupted in mice responding to helminth infection, full Th2 effector function is diminished, as bronchoalveolar lavage (BAL) eosinophilia, which is a normal attribute of the anti-helminth response, and IgE switching are lost[1, 289], *ex vivo* T cell IL-4 production is curtailed[363], and the maintenance of Th2 cell and B cell populations is impaired[362]. It is becoming apparent that IL-4 is not required for Th2 development *in vivo*, but ascertaining the influence, rather than dependence, of IL-4 on Th2 cell development in primary Th2 contexts is becoming an increasingly important direction of current allergy research.

As such, studies investigating the role of IL-4 in allergen-induced Th2 development require further investigation. The *in vivo* studies that demonstrate a potential role for IL-4/STAT-6 signaling in Th2 development have examined mRNA transcript levels[1, 360, 361] and more recently an mRNA-tagged IL-4 reporter[362] either in response to live parasite infection[1, 362] or following adjuvant-based immunization[360, 361]. Other studies of allergenicity in an adjuvant-free context have had to rely on the B cell response as the primary readout of Th2 development[268]. Understanding the involvement of IL-4 in *in vivo* Th2 development as a function of allergenicity is an important, but unexplored area. In particular, close dissection of the role of IL-4 in Th2 development, and the relationship between Th2 cells and the other identified *in vivo* IL-4-producing cell, the TFH cell, induced in an adjuvant-free allergic context may provide new insight into the aetiology and initiation of the allergic disease process.

1.6.2 Are there multiple populations of IL-4-producing CD4+ T cells?

In 2009, Reinhardt *et al.*[275] along with two other groups[362, 364], provided evidence that lymph node IL-4 derives predominantly from CD4+CXCR5+PD-1-hi TFH cells located in follicular regions of lymph nodes. These works followed the identification of TFH cells that have transcribed IL-4 mRNA during T-dependent B cell

responses[337]. Subsequently it was shown that the molecule GL-7, which is expressed on activated T cells[365] and germinal centre B cells[338], demarcates germinal centre TFH that contain particularly high levels of IL-4 mRNA[366]. IL-4 producing Th2 cells that do not have the TFH phenotype or produce the hallmark TFH cytokine, IL-21, can also be found in the tissues[275, 317]. It is possible that these tissue-localised Th2 cells have come from the TFH cell pool[367], however, evidence is accumulating that lymph node versus tissue effector fate can be dictated early during lymph-node T cell activation[368, 369]. Additional data shows that IL-4 production by TFH is not strictly limited to Th2 immunisation, because IL-4 production by TFH cells is observed not only in response to Th2 stimuli such as live *N. brasiliensis*, *H. polygyrus bacheri*, *L. major* and *S. mansoni* infection[275, 362, 364], but also following lymphocytic choriomeningitis virus (LCMV) infection[366] and in lymph node- but not lung-localised CD4+ T cells responding to experimental influenza virus infection[370]. Thus, while IL-4 production is typically associated with Th2 immunity and allergy, understanding the function and eventual fate of the developing IL-4-producing CD4+ T cell may have implications for the potential disease phenotype of a given immunisation strategy.

1.6.3 Measuring IL-4 production using IL-4 reporter mice

One of the difficulties in monitoring Th2 development and IL-4 production *in vivo* is the short half-life of IL-4[371]. To facilitate better detection of IL-4 production, four different IL-4 reporter constructs have been reported in the literature[273, 372–374], and the use of three of these is still commonplace. The three widely used reporters are the 4get, the KN2 and the G4 reporter constructs (Figure 1.5). Each differs slightly in the way it has been inserted into the *IL4* gene and they provide complementary, but context specific, information about the regulation of IL-4 production.

First, the 4get construct is an IL-4 mRNA tagged enhanced green fluorescent protein (GFP) construct that adds the sequence for GFP protein with an internal ribosomal entry site (IRES) construct to the 3' region of the gene encoding IL-4[372]. Thus, the 4get construct remains IL-4 sufficient, has the 5' untranslated region (UTR) intact and at least 5 kb of the 3' UTR of the IL-4 gene remaining as part of the replaced gene segment[372]. The 4get construct remains IL-4 sufficient because the eGFP gene is placed directly downstream of the full-length IL-4 sequence. However, it has been shown that GFP expression in 4get cells does not demarcate cells actively producing IL-4, but rather identifies cells with the potential to produce IL-4[373], such that GFP+ 4get cells are considered 'Th2 competent' cells[275]. The 4get construct is generally used in conjunction with the IL-4 knockin human CD2 (hCD2) construct, abbreviated to 'KN2'[373].

The KN2 reporter replaces the first two exons, plus the intronic region between exons 1 and 2, of the murine IL-4 gene with the gene encoding hCD2 without disrupting other IL-4 promoter/enhancer regions (see supplementary data of[373]). The murine CD4 enhancer is included in the construct to amplify the hCD2 signal to detectable levels[373, 375]. KN2/KN2 mice, homozygous for the KN2 allele, are IL-4 deficient[373]. However, when KN2 cells are provided with Th2 stimulation, cells will express hCD2 on the surface, and this can be detected by flow cytometry[352, 373]. The advantage of the KN2 reporter is that in *in vitro* KN2/+ heterozygote Th2 cultures up to 80 percent of IL-4-producing cells express hCD2[373]. Further, Th2 immunizations that induce IL-4 production *in vivo* also induce hCD2 expression, and the longer half-life of hCD2 makes it simpler to detect IL-4 producing cells than using

conventional methods[373]. However, not all hCD2+ cells express IL-4, so while the KN2 construct is a useful tool for studying IL-4 synthesis *in vivo*, there remain some nuances of this reporter construct that will need clarification in the future.

The G4 construct inserts the gene encoding (enhanced) GFP into the IL-4 gene, replacing exon 1 and 178 nucleotides of the first intronic region of *IL4*[273]. The position of the insertion renders the G4 allele IL-4 null, like the KN2 construct, such that G4/G4 homozygous mice lack IL-4-dependent features of Th2 immune responses[1]. The reporter mouse developed by Littman and colleagues[374] was similar to the KN2 reporter, but is no longer used.

The methods used to generate the knock-ins of all three reporters were similar. The Littman group originally designed the hCD2 construct for insertion into the CD4 gene[375], but by altering the target site they were able to insert the construct into the IL-4 gene but with low detection levels in Th2 cells[374], so they included the murine CD4 enhancer sequence with the hCD2 knockin, such that higher levels of hCD2 were observed in conditions under which IL-4 would normally be expressed[374]. The hCD2 protein was modified to render it non-functional when expressed, and is therefore considered to operate solely as a 'reporter'[375]. The construct also included a neomycin resistance cassette which was used to identify successfully incorporated transgenes. The KN2 construct used the same reporter construct, but with an additional lox-cre site included to remove the neomycin resistance cassette after positive selection. The G4 construct also contained a lox-cre neomycin resistance cassette but does not include the CD4 enhancer site. Mohrs *et al.* used transient cre recombinase electroporation[373], and Hu-Li *et al.* crossed reporter mice to C57BL/6-cre mice in the F1[273] to remove the neomycin resistance cassette. The stem cells used for electroporation for the constructs were from strain 129, strain 129/SvJ, and E14 embryonic stem cells for the G4, KN2 and Littman constructs, respectively. All three were transferred to C57BL/6 blastocysts to yield chimeric mice, and then outbred for ten generations. Lastly, the G4 construct additionally contains the herpes simplex virus thymidine kinase gene 5' of the reporter, which allowed gancyclovir sensitivity screening assays to be used in the selection process. Both the G4 and KN2 reporters therefore were introduced in slightly different manners, and disrupt the IL-4 gene at different sites.

Figure 1.5: **Insertion sites of *4get*, *KN2* and *G4* reporter constructs.** (A) Wildtype *Il4*, (B) Addition of the eGFP gene with an internal ribosomal entry site was inserted upstream of the polyadenylation site of the full-length IL-4 cDNA for the *4get* construct. (C) The *KN2* construct replaces exon 1, intron 1 and exon 2 of the murine *Il4* gene. (D) The *G4* construct replaces exon 1 and the first 178 nucleotides of intron 1 of the *Il4* gene. IL-4 protein is not produced from either the *KN2* or the *G4* allele. Data are modified from [273, 372, 373, 375].

1.6.4 The regulation of IL-4 production

Epigenetic changes influence cytokine gene accessibility

The development of the polarised IL-4-producing Th2 cell phenotype is now established to involve many regulatory processes. With increasing proliferative cycles, Th2 cells become more likely to produce IL-4 and lose their capacity to produce IFN- γ [347, 376]. During this process, the chromatin structure of the Th1 genes becomes less accessible, and that around the Th2 locus becomes more accessible[347, 377]. This process involves changes to the chromatin structure that are the result of epigenetic modification.

Epigenetic modification is important in Th2 cell development. This is exemplified clearly in an early study by Bird *et al.*[378]. In this work, STAT-6-deficient CD4+ T cells cultured in IL-4 did not subsequently produce IL-4; however, if the cellular methylation/acetylation apparatus were interrupted, 16% of STAT-6-deficient CD4+ T cells produced IL-4 upon restimulation[378]. Numerous studies have confirmed the importance of changes in gene access and chromatin modification to the development of the Th2 phenotype[377, 379–384]. Regulation of gene access is now recognised as an important part of Th2 regulation (For review, see[324, 385]).

Probabilistic processes in biology

In addition to epigenetic regulation, some biological processes occur through stochastic, or probabilistic mechanisms. The immune system is no exception. T cells activated under Th2-polarising conditions become increasingly likely to produce IL-4 with increased proliferative cycles[376] and are progressively more likely to inactivate Th1 genes with sequential divisions[347]. However, synchronously stimulated T cells do not divide synchronously: probabilistic regulation influences their rate of division and dictates the eventual Th cell phenotype that arises[376]. Probabilistic mechanisms have also been shown to underlie B cell population expansion, by balancing proliferation, isotype class-switching and death[386]. In this manner, 'chance' processes can

affect the eventual biological response.

Probabilistic gene expression

Probabilistic regulation can account for the expression of some genes. Probabilistic regulation was proposed, based on the observation that in response to a given stimulus, many cells will produce a large amount of a gene product, and others will remain inactive[387]. A good example of this is seen in cells retrovirally transfected to express *Lacz*, rather than produce retrovirus: At baseline, very few cells will express β -galactosidase, reflecting *Lacz* activity, but those that do, exhibit significant activity[387]. The proportion of cells that exhibit activity increases when the 'signal strength', or exogenous stimuli that promote transcription increases. This is argued to be an attribute of an error-prone process, the success of which was increased by the multiplicative success of activation of enhancers[387]. In cases like this, the amount of a gene product produced by a clonal cell population will be controlled in an on/off manner, with cells producing either a lot, or no, product (figure 1.6B). This contrasts with the conventional, deterministic view, in which the 'signal strength' influences the transcriptional activity or amount of a gene product from each cell (figure 1.6A). In this manner, stochastic processes can regulate gene expression.

Probabilistic regulation of IL-4 gene expression

Probabilistic regulation of the Th2 locus has been proposed based on numerous studies identifying monoallelic expression of IL-4. When Th2 cells are activated *in vitro*, or expanded from clones, in a substantial proportion of cells, only one IL-4 allele will be detected. In the G4 reporter system, when *in vitro*-generated G4/+ Th cells are stimulated with antigen-loaded APCs in the presence of IL-4, approximately 7% will produce IL-4, approximately 7% will express GFP and less than 1% of cells will produce both IL-4 and GFP[1, 273, 388]. In other studies, IL-4 mRNA from only one allele was detected in approximately half of cloned Th2 cells[389]. It has also been observed that differences in IL-4 production between different strains of mice relates to the frequency of CD4+ T cells that produce IL-4, rather than regulation based on

the amount produced by each cell[390]. These observations have lead to the proposal of probabilistic monoallelic regulation as an advantageous means by which to regulate IL-4 production[381].

However, some equivocality exists in the predominant expression profile and definitive determination of whether cytokine genes, including IL-4, are regulated at the single allele level[352, 373, 391]. While induced by similar stimuli as IL-4 protein and the G4 allele, the KN2 reporter operates differently to the G4 construct in one key aspect - Where G4 GFP expression occurs mutually exclusive with IL-4 protein in 80-90% of *in vitro* Th2 cells[1, 273, 388], hCD2 expression occurs in 80% of IL-4 producing KN2/4get Th2 cells *in vitro*[373]. Further, where approximately equivalent proportions of GFP and IL-4-producing cells have been observed soon after TCR restimulation of G4/+ cells[1, 273] and also at times when IL-4 mRNA levels are at their peak[388], in the KN2/4get system a greater proportion of Th2 cells express hCD2 than IL-4, such that 60% of hCD2+ cells do not produce IL-4 protein[373]. More recent studies have, however, shown a less complete dominance of hCD2 over IL-4 expression in KN2/+ cultures[392]. Together, these data indicate that our understanding of the regulation of IL-4 production is incomplete and requires further clarification, both *in vitro* and *in vivo*.

Figure 1.6: **Probabilistic and deterministic mechanisms of gene regulation.** Two mechanisms can explain production of a set amount of factor 'x' in a clonal population of cells receiving identical signal of strength 'z'[387]. (A) In the deterministic view (xxz), increasing signal strength will cause a proportional increase in the amount of factor produced by all cells in that population ($z=4$; $x=4/\text{cell} \times 100 \text{ cells}=400 \text{ U}$; $z=6$; $x=6/\text{cell} \times 100 \text{ cells}=600 \text{ U}$). (B) In the probabilistic model, genes will be either in an 'on' or an 'off' state. Increasing signal strength will increase the proportion of a clonal population producing the same amount of factor 'x' ($z=4$; $x=100 \text{ U/cell} \times 4 \text{ cells}=400 \text{ U}$; $z=6$; $x=100 \text{ U/cell} \times 6 \text{ cells}=600 \text{ U}$). Model in (A) redesigned from Ross *et al.*[387], (B) redesigned from Guo *et al.*[381].

1.7 Themes of this thesis

The primary goal of this thesis was to clarify the key factors involved in the allergic process. This resulted in targeted investigations into the development and function of IL-4-producing CD4⁺ Th2 cells in allergic Th2 immune response models. Three questions pertaining to Th2 cell function and development were examined during this work, and relate, first to the controversial role of IL-4 in Th2 cell development *in vivo*; second, to their involvement in tissue-specific intestinal allergies; and third, to the way allergens initiate Th2 cell development and can be compared allergenically. After a short investigation characterising a novel reporter mouse used in our *in vivo* studies, these concepts form the focuses of chapters 4-6. The relevant background, and an overview of the concepts tested in each section are introduced at the beginning of each chapter.

The over-arching aims of this thesis are:

- 1) To clarify whether IL-4 alleles are regulated in an allele-specific, probabilistic monoallelic process *in vitro*;
- 2) To determine the role of IL-4 in *in vivo* allergen-induced primary Th2 cell development;
- 3) To examine the influence of IL-4 heterozygosity on Th2-dependent allergic immunity;
- 4) To clarify the role of CD4⁺ T cells in the oral potentiation of allergic disease;
- 5) To determine whether there is an immunological basis to the differential allergenicity of cow and goat milk components.

Chapter 2

Methods

2.1 Special reagents and miscellaneous procedures

2.1.1 Allergens

House dust mite (HDM) was purchased from Greer laboratories (Lenoir, NC, USA). Albumin from chicken egg white (OVA) was purchased from Sigma (St Louis, MO, USA). Bovine serum albumin (BSA) was purchased from Sigma. *i*L3 *Nippostrongylus brasiliensis* larvae were cultured in house according to established protocols[393] and were killed by three freeze-thaw cycles at -80°C . The whole casein fraction from cow and goat milks were prepared as described[394] and were provided by Ali Hodgkinson, AgResearch, Ruakura. Briefly, milk from a Hamilton dairy goat farm was heated to 45°C and centrifuged at 1650 g for 30 minutes and then had the fat removed. The milk was reheated to 45°C , the pH was adjusted to 4.2 using 2 M HCl, stirred for 30 minutes and centrifuged at 1650 g for 15 minutes to recover the precipitated caseins. These were resuspended four times in 10 times the original milk volume and stirred for 60 minutes before recentrifugation at 1650 g for 15 minutes. After the washes, the casein was freeze-dried, vacuum packed and stored at 4°C until further use. Upon use, the caseins were dissolved in Dulbecco's phosphate buffered saline (PBS; Invitrogen, Auckland, New Zealand) at 37°C for 60 minutes and then sonicated to further solubilise them. The final LPS concentrations were in the range 150-300 EU/mg for each. α s1 casein was purified from milk from the same source by high pressure liquid chromatography. Bovine β -lactoglobulin and caprine β -lactoglobulin were also provided by Ali Hodgkinson, and were purified by Trichloroacetic acid (TCA) precipitation.

2.1.2 Mice

Mice were aged 6-12 weeks at the start of experiments. BALB/c mice were from two sources. Early experiments in BALB/c mice used mice originally sourced from the Wellington School of Medicine maintained at the Biomedical Research Unit (BRU). The BALB/c breeding colony was replaced with BALB/c-ByJ mice sourced from The Jackson Laboratory (Bar Harbor, ME) in 2011 and these were used in later experiments. KN2 mice[373] were donated by Richard Locksley (University of California at San Francisco, CA). G4 mice[273] were donated by William Paul (National Institutes of Health, Bethesda, MD). IL-3^{-/-} mice have been described[309, 395, 396] and were donated by Booki Min (The Cleveland Clinic Foundation Biological Resources Unit, Cleveland, OH). Mice with an IL-9 transgene under the control of the intestinal rat fatty acid binding protein promoter (*iFABPp*-IL-9)[57] were a gift from Klaus Matthaei (John Curtin School of Medical Research, Australian National University, Canberra, Australia). All strains were backcrossed to a BALB/c background prior to arrival at the BRU, except *iFABPp*-IL-9 mice which were generated using BALB/c blastocysts[57]. KN2/KN2 and G4/G4 homozygotes were crossed to BALB/c-ByJ mice to generate KN2/+ and G4/+ mice, respectively. KN2/G4 mice were the F1 progeny of a KN2/KN2 by G4/G4 cross. To generate *iFABPp*-IL-9-IL-3^{-/-} mice, IL-3^{-/-} mice were first crossed with an *iFABPp*-IL-9 homozygote. F1 mice were crossed to IL-3^{-/-} and F2 pups that had no detectable IL-3 gene and carried the transgene were used as *iFABPp*-IL-9-IL-3^{-/-} parental mice. These F2 mice interbred with IL-3^{-/-} mice were used as the source of *iFABPp*-IL-9-IL-3^{-/-} and IL-3^{-/-} sib-matched controls in the F3. P1 *iFABPp*-IL-9 mice were used as *iFABPp*-IL-9-IL-3^{+/+} mice in these experiments. Experimental mice were maintained in a 14/10 hour light-dark cycle at the BRU at the Malaghan Institute of Medical Research, Victoria University of Wellington. Mice were euthanised by either cervical dislocation or carbon dioxide asphyxiation. Mice were used in accordance with the permissions of the Victoria University of Wellington Animal Ethics Committee.

2.1.3 Genotyping

Tail tips or ear clips of mice were lysed in 75 μ L lysis buffer (25 mM sodium hydroxide, 0.2 mM Ethylenediaminetetraacetic acid (EDTA; Invitrogen) in deionised H₂O for 60-120 minutes at 95 °C. Lysis was stopped by addition of 75 μ L 40 mM Tris-HCl. Debris was removed with a quickspin in a benchtop centrifuge and genomic (g)DNA was quantified by nanodrop (A260/280) and stored at 4 °C until being run through the polymerase chain reaction (PCR). Primers against wild-type *Il9* and the *iFABP*-IL-9 transgene were purchased from Sigma and were as follows: iFABP (forward) construct: GTGGACAGGACCGAATCTCTGC. *Il9* gene (forward): CAGGTTTGAGGGAAGCTGGTCC; 3' *Il9* reverse primer: GCCTTTGCATCTCTGTCTTCTGG. PCR amplification was performed using the iTaqTMDNA polymerase pre-mix kit (BioRad, Hercules, CA, USA). A master reaction mix consisting of 1x Magnesium-free master mix buffered with 2 mM MgCl₂, 400 nM iFABP (forward), 70 nM *Il9* gene (forward), 400 nM *Il9* reverse primer, 200 μ M dNTPs, 0.2 μ L iTaqTMDNA polymerase to which 300 ng template DNA were added to a total volume of 25 μ L. Primer aliquots were stored as 100 μ M stocks and pre-diluted such that 0.5 μ L would give the appropriate concentration (20 μ M, 3.5 μ M and 20 μ M respectively). The PCR cycle was as follows: Stage 1: 94 °C 3 minutes; Stage 2: 40x 94 °C 20 s 64 °C 20 s, 72 °C 20 s; Stage 3: 72 °C 3 minutes followed by an infinite hold at 4 °C. Amplified products were run on a gel made from 1-1.5% agarose (Invitrogen) and the presence of the *iFABP*-IL-9 transgene was detected as an approximately 400 bp product. PCR success was evaluated by the presence of a 200 bp WT band.

The same lysis conditions were used for the *Il3* gene. Primers (Sigma) against the gene encoding murine IL-3 amplified a were as follows: Forward primer: 5'GAACCT-GAACTCAAAACTGAT3' Reverse primer: 5'CTGAAGATTGGACTTGATAAC.

A 1x master mix was made to give a final reaction volume of 20 μ L containing 500 nM of the forward and reverse primer and 0.2 μ L iTaqTM. 200-500 ng DNA were added to each tube and the amplification cycle was as follows: Stage 1: 94 °C 2 minutes; Stage 2: 94.0 °C 20 s, 56.1 °C 10 s, 72.0 °C 30 s; stage 3: 72 °C 5 minutes followed by an infinite hold at 4 °C. The reaction was predicted to yield an 1131 bp fragment and IL-3-/- mice were identified as those in which the wild-type IL-3 band was not detected.

The success of the PCR in IL-3^{-/-} mice was further confirmed by the presence of the wild-type IL-9 gene. In the F2, IL-3^{+/-} and IL-3^{-/-} mice were born in approximate Mendelian ratios.

2.1.4 Protein quantification

Protein was quantified by nanodrop or using the Bicinchoninic acid BCATM protein assay kit (Thermoscientific, Rockford, IL, USA) according to the manufacturers recommendations with the following modifications. Samples, standards and reagents were added in 30 μ L volumes. After 30 minutes incubation at 37 °C plates were removed from the incubator and read at 562 nm on a Versamax plate reader.

2.1.5 Trypan blue exclusion test

Samples were diluted 1:1 with 0.4% trypan blue (Invitrogen). Between 30 and 200 cells were counted and these were back-calculated to the total volume for numbers.

2.1.6 Prediction of sequence homology using DELTA BLAST

The sequence for cow β -lactoglobulin was taken from Alexander *et al.*[397]. A protein-protein DELTA-BLAST search[398] was performed using the <http://blast.ncbi.nlm.nih.gov/> search engine and search species were Homo sapiens (taxid:9606), Mus musculus (taxid:10090) and Bos taurus (taxid:9913). Similar homologies to those reported by Restani *et al.*[169] were observed for α s1 and α s2 caseins (not shown). The query sequence for BLG was ALT CGA QALIVTQT MKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGDLEILLQKWENDECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLVCMENSAEPEQSLVCQCLVRTPEVDDEALEKFDKALKALPMHIRLSFNPTQLEEQCHI. The glycodelin[244] and human placental precursor protein-14[245] proteins, both of which have been previously identified as β -lactoglobulin-related proteins[244, 245], each returned 44% homology.

2.1.7 Statistical analysis and graphics

Data were graphed and analysed in Graphpad Prism 5.0. Statistical analyses used are stated in figure legends and $p < 0.05$ was considered as significant. When multiple individual group comparisons yielded similar significance values (i.e., were commonly < 0.05 , < 0.01 etc.), the significance value is indicated for all connected lines. In comparisons between two similarly-sized groups, Student's t -test was used. Analyses examining a single parameter of three or more groups were compared with One-way ANOVA with Tukey's post-test. For experiments examining two or more groups, Two-way ANOVA with Bonferroni's post-test were used. Repeated measures tests were used in cases in which the same biological sample was compared. Figures showing data combined from multiple experiments were first normalised such that baseline values were similar across combined experiments. Unless otherwise stated, symbols represent individual mice and error bars represent mean $\pm$ the standard error of the mean (SEM).

pTh2 assays were designed to detect 2-3-fold differences with 80% power ($1 - \beta$; $\beta = 0.2$) at $\alpha = 0.05$ in experiments using one or more experiments including groups of 5-10 mice. The calculation employed was:

$$n = (Z2\alpha + Z\beta) \times (Z2\alpha + Z\beta) \times ((SD1)^2 + (sd2)^2) / (ED)^2;$$

Where, 'n' is the number of mice required per group, 'ED' is the expected difference ($\mu_1 - \mu_2$), 'SD1' is the empirical SD for the responding immunogen, and 'sd2' is the sd of the group for comparison. For experimental groups, 'sd2' was approximated as half the expected μ_2 . Empirical values attained using mice ($n = 298$) immunised intradermally with 600 dead *iL3 N. brasiliensis* larvae give $\mu = 226379 \pm 133931$ Th2 cells; ($n = 27$) HDM-immunised G4/+ nodes $\mu = 87021 \pm 39275$; control lymph nodes ($n = 43$) $\mu = 2018 \pm 1444$. To fit with historical data and reduce the occurrence of type-1 errors, pTh2 data were not log-transformed prior to analysis. An example derivation for HDM power is shown:

To detect an x -fold difference to an allergen response on a similar scale to HDM immunization in 80/100 experiments ($\beta = 0.2$):

$$(x = 1.2, n = 71.8);$$

($x=2$, $n=8.4$);
($x=3$, $n=4.1$);
($x=4$, $n=3.1$);
($x=5$, $n=2.6$);
($x=10$, $n=2.0$) mice/group.

Figures were prepared in Adobe®Illustrator®version 5.1.

2.2 Cell culture

During preparation, cells were handled in Iscove's modified Dulbecco's medium + Glutamax (IMDM; Invitrogen). Base culture medium consisted of 5% or 10% foetal calf serum (Sigma), 100 U/mL Penicillin/100 μ g/mL Streptomycin (Invitrogen), 55 μ M β -mercaptoethanol (Invitrogen), in IMDM (cIMDM). Standard incubation conditions of 37 °C humidified in 5% CO₂ in air were used.

2.2.1 Hybridoma culture

GK1.5 (process similar for other antibodies made in house) anti-CD4 monoclonal antibody was prepared in house from the culturesupernatant of the GK1.5 hybridoma[399]. The GK1.5 hybridoma was grown by culture in 10% cIMDM and antibody was isolated from the supernatant by fast pressure liquid chromatography using Hitrap protein G affinity columns (GE Healthcare) used according to the manufacturer's instructions. After isolation into neutralising buffer, antibody was dialysed for 2-4 hours in 2L PBS using 10000 molecular weight cut-off (MWCO) cassettes (Pierce). The PBS was changed and antibody was dialysed overnight. The next day, dialysed protein was extracted from the cassette and concentration was determined by nanodrop absorption at 280 nm. Antibody was concentrated to 2.5 mg/mL in 10000 MWCO or 30000 MWCO vivaspin microtubes (GE Healthcare). Heavy and light chain expression was confirmed by sodium dodecyl sulfate gel electrophoresis, and in some cases, *in vivo* depleting capacity was assessed. Mice received 0.5 mg (200 μ L) antibody i.p. with each injection according to standard protocols[400], and in maintenance experiments, antibody was readministered every four days. On days of challenge plus monitoring

for peripheral CD4+ T cell content, monitoring was done prior to gavage and prior to GK1.5 administration. Mock-depleted mice received 0.5 mg polyclonal Rat IgG purified from naive rat sera purified in house by Hitrap protein G affinity or was purchased from Sigma.

2.2.2 Bone marrow cell culture for IL-3- and TSLP-elicited basophils

Bone marrow cells were isolated as described in mouse manipulations. Bone marrow cells were seeded at 1×10^6 /mL in 24-well plates. Cells were cultured in the presence of 10 ng/mL IL-3 (Peprotech, Rocky Hill, NJ, USA), 1 μ g/mL TSLP (R&D Systems, Minneapolis, MN, USA), 10 ng/mL IL-3 plus 1 μ g/mL TSLP, or an equivalent volume of 1% BSA/PBS as medium alone controls. After three days, half of the medium was removed without disrupting the cells and replaced with fresh medium containing 10 ng IL-3, 1 μ g TSLP, 10 ng IL-3 plus 1 μ g TSLP or an equivalent amount of 1% BSA/PBS as seeded. On day five, non-adherent cells were harvested, counted by trypan blue exclusion, and analysed by flow cytometry. By day five, adherent cells in IL-3-containing cultures had reached confluence and the medium had changed colour. TSLP containing cultures were predominantly non-adherent, however, some sparse adherent cells were observed extending pseudopods; these were also observed occasionally in medium-only cultures. Fc ϵ RI+cKit⁻ cells comprised approximately 0.8% of bone marrow cells on day 0, and at seeding of G4/+ cultures, 60% of these cells were GFP⁺.

2.2.3 Th cell polarization

Twenty-four-well plates (BD) were coated overnight at 4 °C, or for 2 hours at 37 °C with anti-CD3 in PBS (2C11, 1 μ g/mL, 300 μ L/well; in house). On the day of the assay, plates were washed twice with 500 μ L IMDM and 1×10^6 splenocytes from naive mice of the specified genotype were added to each well in a 1 mL culture volume. Cultures were left untouched for three days, at which point wells were resuspended and half of the cells were removed and discarded and 500 μ L of 2x conditioning medium was

again added to wells. On day 4, 500 μL was removed without resuspension and cells were fed as on day 3. For restimulation assays, cells were removed from wells on day 5, washed twice in IMDM and replated in wells containing 100 U/mL IL-2 and anti-IL-4 (11B11, 10 $\mu\text{g}/\text{mL}$; prepared in house) as the final culture conditions. During the rest period, half of the medium was replaced every day and if the medium reached pH7.0 or less in any culture, determined by a change in phenol red colouring, the contents of the wells were split in two. Separate wells were maintained for individual mice from the start of culture throughout the process, such that data points represent biological replicates unless explicitly stated. For initial conditioning, Th0 medium contained 100 U/mL IL-2, 10 $\mu\text{g}/\text{mL}$ anti-IL-4 and 10 $\mu\text{g}/\text{mL}$ anti-IFN- γ (AN.18; prepared in house). Th1 medium contained 100 U/mL recombinant (r)human IL-2, 10 ng/mL rIL-12p40 and 10 $\mu\text{g}/\text{mL}$ anti-IL-4. Th2 medium contained 100 U/mL IL-2 and 1000 U/mL chinese hamster ovary (CHO)-mIL-4 (IL-4 activity was compared by ELISA using standards originally calibrated using the CT.4S cell line as described[342]. One unit is equivalent to approximately 0.5 pg IL-4/mL[342]). During primary stimulation, all cultures contained 20 $\mu\text{L}/\text{mL}$ anti-CD28 (37.51; grown and titrated in house without purification) that was not replaced during subsequent feeds.

2.2.4 In vitro restimulation of Th cells

After conditioning, Th cells were transferred to wells coated with anti-CD3 at 10 $\mu\text{g}/\text{mL}$ as per vanPanhuyt *et al.*[1]. Restimulation medium included 100 U/mL IL-2 and 20 $\mu\text{L}/\text{mL}$ anti-CD28. In experiments examining IL-4 protein production, anti-IL-4 was also included in the restimulation medium. For the detection of intracellular cytokines, 4 μL GolgistopTMcontaining monensin (BD) was combined with 16 μL IMDM and 20 μL was added to each well two hours before the cells were harvested. In experiments that simultaneously examined cytokine production, parallel cultures to which monensin was not added were used.

2.2.5 IgG1 spot assay

MAIPSWU10 0.45 μm hydrophobic Immobilon P membrane 96-well ELISpot filter plates (Millipore, Billerica, MA) were pre-soaked for 90 seconds with 50 μL 70% ethanol. After removing the ethanol, plates were washed five times with 200 μL PBS and incubated overnight at 4°C with 10 $\mu\text{g}/\text{mL}$ of the coating antigen. The following day plates were washed five times with 200 μL PBS and were left at room temperature with 10% cIMDM to block unbound sites on the plate. After processing, spleen and/or bone marrow cells were resuspended in 10% cIMDM, and were serially diluted and transferred to spot assay plates. Plates were given a soft spin (50 g for 60 s) and overlaid with a further 50 μL assay medium and incubated for 4 hours at 37°C. After incubation, plates were washed five times with 200 μL PBS and then incubated with 0.91 $\mu\text{g}/\text{mL}$ biotinylated rat anti-mouse IgG1 (A85-1; BD) overnight at 4°C. Plates were washed a further four times with PBS and incubated with 1/1000 SPX for 1 hour at room temperature in the dark and detected with the 3-amino-9-ethylcarbazole AEC Chromogen staining kit (Sigma) resuspended in deionized water according to the manufacturers instructions and 40 μL was added to each well. Forty-five minutes later, plates were washed with tap water and put at 37°C in air until dry. Spots formed a red precipitate and were enumerated by eye and validated using images acquired on an ELISpot reader. Concentrations at which densities were too numerous to count were not included in quantitations; otherwise all dilutions were included in frequency calculations.

2.3 Mouse manipulations

2.3.1 Primary Th2 (pTh2) assay

After anaesthesia with 200 μL anaesthetic solution (100 mg/Kg ketamine, 3 mg/Kg Xylazine), mice were intradermally injected in the outer ear pinna (generally the left ear) with 30 μL of immunogen in PBS. The draining auricular lymph node was later excised and examined for live cells by trypan blue exclusion and for proportional GFP expression in CD4+ T cells by flow cytometry. Generally, analyses were performed

seven days after inoculation. When data were compiled from multiple experiments the proportion of GFP+ cells or hCD2+ cells were normalised to $0.04 < x < 0.05\%$ of CD4+ T cells in wild-type (+/+) mice immunized with either dead *iL3 N. brasiliensis* larvae or HDM. In cow and goat milk comparisons, casein samples had low solubility in PBS, therefore three separate injections were required and administered in the centre of the pinna, then on then in two additional injections perpendicular to the centre of the pinna. Controls also received 3 x 30 μ L injections in these experiments. In pTh2 assays that also examined B cell responses, the total number of IgG1+IgD-B220+ cells was determined by multiplying the proportion of live cells that were IgG1+IgD-B220+, as determined by flow cytometry, by the total number of live cells found in lymph nodes.

2.3.2 Oral allergen-induced experimental anaphylaxis (diarrhoea) model

A model of oral allergen induced diarrhoea was employed as established[37]. Mice were sensitized intraperitoneally (ip) twice, two weeks apart with 50 μ g ovalbumin (OVA; Sigma) + 1 mg Alu-gel-S (Serva Electrophoresis GmbH, Heidelberg, Germany) in 200 μ L using a 1 mL tuberculin syringe (BD) and 25-gauge needle (BD). Thrice-primed mice received an additional ip injection on day minus 14. From day 28, mice were fasted for four hours prior to gavage and subsequently challenged intragastrically three times a week with 50 mg OVA (200 mg/mL) in PBS. Mice were monitored for diarrhoea development 60 minutes later. For serum analyses, mice were bled two hours after challenge. Mice were bled by the submandibular route using a 4 mm lancet (Ables scientific, Welshpool, Western Australia) or were pre-heated under a heat lamp followed by lateral tail vein bleeding using a #10 carbon steel surgical blade (Swann-Morton, Sheffield, England). When applicable, CD4+ T cells were depleted with ip injections of 0.5 mg GK1.5 on days 25, 26 and 27, or the three days following challenge ten. Mock-depleted mice received polyclonal IgG purified from Hooded Lister rat serum. For long-term depletions, booster injections were administered every four days.

2.3.3 Active systemic anaphylaxis

Mice were warmed with a heatlamp for 3 minutes and then received 100 μg OVA in PBS intravenously through a 29-gauge needle (BD). Intravenous challenges were done two or three days after mice received their final gavage challenge. Temperatures were monitored using a RET-3 rectal temperature probe attached to a digital thermometer (Coherent Scientific, Hilton, South Australia).

2.3.4 Packed Cell Volume (PCV)

Haematocrit was taken directly from a tail nick and was most pronounced 45-60 minutes after challenge. 75 mm heparinized capillary tubes (Drummond scientific, Broomall, PA) were used to take haematocrit and were plugged with plasticine. Tubes were spun at 8000 rpm for 5 minutes on a benchtop centrifuge. PCV was assessed using a sliding scale and represents proportion of the total volume that were red blood cells.

2.3.5 Serum isolation

Blood was left to clot overnight at 4°C. Blood was spun for 3 minutes at 4 degrees ($\text{RCF}_{max}=1523$), serum removed and frozen at -20°C until analysed by ELISA.

2.3.6 Tissue isolation

After euthanasia, mice were rinsed with 70% ethanol. Depending on the requirements, the spleen, mesenteric and auricular lymph nodes were isolated by blunt dissection with forceps. Bone marrow was removed as outlined below. Colons and caecae were excised afterwards and the jejunal tissue was removed by cutting away the mesenteries. The first 1.5 cm of the intestine proximal to the stomach was deemed the duodenum. The next 1-1.5 cm of jejunal tissue was used for histological sections, and for cell isolation the next 5 cm section of jejunum was used. Jejunal tissue for

cell analyses were flushed with 5 mL Ca^{2+} and Mg^{2+} -free Hanks' Balanced Salt Solution (HBSS; Invitrogen) using an 18-gauge needle (BD) and were stored in Ca^{2+} and Mg^{2+} free HBSS on ice. All other tissues were stored in IMDM on ice until subsequent processing.

2.3.7 Processing of jejunal tissue

After vigorous shaking with forceps to dislodge mucus and remaining gut content, Peyer's patches were excised and put on ice. Jejunal sections were transferred to 6-well plates (BD) containing 5 mL stripping buffer (5 mM EDTA), 25 mM HEPES (Sigma), 10% FCS in Ca^{2+} and Mg^{2+} free HBSS) pre-heated to 37°C . Tissues were incubated in air at 37°C with shaking (100 rpm) for five minutes. Tissues were vigorously shaken with forceps to remove stripped epithelial cells. The process was repeated two more times for a total of 15 minutes stripping. Tissues were rinsed in 5 mL 4°C Ca^{2+} and Mg^{2+} free HBSS and placed on tissue paper to remove moisture. Jejunal tissue was chopped into pieces small enough to pass through a 1 mL pipette tip and incubated with shaking for 30 minutes in 5 mL 2.4 mg/mL collagenase A from *Clostridium histolyticum* (Roche Diagnostics GmbH, Mannheim, Germany) in IMDM. The solution was subsequently passed through a 21-gauge needle (BD) with pressure on the bottom of the plate and incubated for a further 15 minutes at 37°C with shaking. Debris was removed by passing through two layers of cotton gauze (Sefar Filter Specialists Ltd., Te Papapa, Auckland, NZ) and rinsed with 10 mL cIMDM or flow buffer and then through a $70\ \mu\text{m}$ cell strainer (BD). Cells were then processed as per standard flow cytometric procedures, but all isolated cells were stained for flow cytometry.

2.3.8 Bone marrow preparation

The left femur was used for bone marrow isolation. The leg was washed with 70% ethanol and the surrounding muscle was removed. The tibia was disconnected by dislocating the knee and then using forceps, the bone was removed by cutting under the hip socket. Bones were cleaned and stored in IMDM on ice until processing. To

process bones, both ends were cut and a 22-gauge needle (BD) was inserted into the bone tip. Five mL IMDM was flushed through the bone to isolate marrow, which was spun down and resuspended in red blood cell lysis buffer (Sigma) for 2 minutes at room temperature. Lysis was stopped by addition of 13 mL 5% cIMDM. Cells were again spun and then resuspended in 10% cIMDM for the spot assay, 5% cIMDM for mast cell culture, and were subsequently transferred to flow buffer (2% FCS, 0.01% w/v Sodium Azide (Sigma) in dPBS) for staining.

2.3.9 Splenocytes

After excision from the mouse, spleens were placed on a 70 μm cell strainer and dissociated with pressure against the strainer using the rubber end of a 1 mL tuberculin syringe (BD) and rinsed with IMDM. Cells were spun down and resuspended in 2 mL red blood cell lysis buffer for 2 minutes at room temperature. The reaction was quenched with 13 mL 5% cIMDM or flow buffer and then tubes were spun down for 4 minutes at 1400 rpm (419 x g). Spleen cells were then resuspended in 10 mL buffer for counting. Cells were respun and resuspended for future use.

2.3.10 Lymph node cells

Mesenteric, auricular lymph nodes and Peyer's patch cells were liberated by crushing lymph nodes through a 70 μm cell strainer with the rubber end of a 1 mL tuberculin syringe (BD) and rinsed with flow buffer for flow cytometry. Cells were spun and resuspended in 4 mL buffer for counting. Red blood cells were ignored during counting and were identified as small, dense, spherical cells with a darker hue than lymphocytes. For flow staining, cells were transferred through 70 μm gauze and stained for flow cytometry.

2.3.11 Jejunal mast cell quantification

Jejunal tissue sections were removed from mice and put in 10% formalin (4% w/v; Sigma) and stored at 4 °C until embedded in paraffin. Tissues were processed into 4

μm sections using standard histological techniques by Jane Anderson of the Wellington School of Medicine. Slides were stained with chloroacetate esterase (CAE) stain and lightly counterstained with hematoxylin as described[37, 57]. Mast cells were identified as CAE positive cells, which were easily identified as bright red cells as previously described[37, 57]. Four randomly selected high power fields (550 μm /field; 400 x magnification using 0.22 mm Field Number 10x ocular and 40x objective lens) were examined from each mouse.

2.4 Flow cytometry

2.4.1 Flow Cytometry reagents

A full list of the flow cytometry reagents is included in the appendices. The following viability dyes were used: 4,6-diaminidino-2-phenylindole (DAPI; Molecular Probes); 7-Amino Actinomycin D (7-AAD; BD Pharmingen). For intracellular staining, Live/Dead® fixable blue (Invitrogen) was used in place of DAPI.

Antibodies against the following molecules, and secondary reagents were purchased from BD Pharmingen, unless otherwise specified (clone): B220-V450 (RA3-6B2); B220-PE-Texas Red®(Ra3-6B2); CD3-AF488 (500A2; Invitrogen Molecular Probes); CD4-APC (RM4.5); CD4-APC-H7 (GK1.5); CD4-FITC (GK1.5; Prepared in house); CD4-PE (GK1.5); CD4-Percp (RM4.5); CD4-Qdot605® (RM4.5; Invitrogen Molecular Probes); CD8a-AF700(536.7; Biolegend); CD8a-PE/Cy7 (53-6.7); CD19-APC-H7 (ID3); CD44-PE (IM7); CD45-Pacific blue™ (30F11; Biolegend); CD62L-Biotin (MEL-14); CD62L-Pacific blue (MEL-14; Biolegend); CD117(cKit)-APC (2B8); FcεRI-PE (Mar-1; eBioscience); FcγRII/III-FITC (2.4G2); human CD2-APC (RPA2.10); Ia/Ie-PE/Cy7 (M51114.15.2; Biolegend); IL-3-PE (MP2-8F8); IL-4-APC (11B11); IL-4-PE (11B11); IL-5-PE (TRFK5); IL-10-PE (JES5-16E3); IL-13-PE (eBio13A; eBioscience); IgD-PE (11-26c.2a); IgE-biotin (R35-118); IgG1-biotin (A85-1); Pan NK-PE/Cy7 (DX5; eBioscience); Streptavidin-APC; Streptavidin-FITC; Streptavidin-PE; Streptavidin-PE-TexasRed®.

2.4.2 Surface staining

After tryan blue exclusion, 1.25×10^6 cells were transferred to 5 mL flow-compatible tubes through 70 μm gauze. Flow buffer containing 50 μL anti-CD16/32 antibody (24G2) made in house was added to each tube for five minutes which was then incubated with 50 μL containing the primary antibody mix. Samples were incubated for 30 minutes at 4 °C and then washed with 4 mL flow buffer (2% FCS, 0.01% sodium azide in PBS) and resuspended for flow. For secondary antibody incubation, cells were instead resuspended in 100 μL containing optimally diluted secondary reagent.

2.4.3 Intracellular staining

Cells were harvested and transferred to flow-compatible tubes containing 3 mL 4 °C PBS. Tubes were spun and resuspended in 1 mL PBS for counting. Cells were respun and incubated with 1 mL 1/1000 Live/dead fixable blue in PBS for 30 minutes at 4 °C in the dark. Cells were washed with 3 mL flow buffer and were subjected to normal surface staining methods. After staining, cells were fixed and permeabilized using the Fix & Perm® cell permeabilization kit (Invitrogen) according to the manufacturers instructions with the following modifications. Cells were fixed for 15 minutes using 'Reagent A' at room temperature, followed by quenching with 4 mL room temperature flow buffer. Samples were split in two at this stage and spun down at 1400 rpm for 4 minutes at room temperature. Intracellular stains for analysis and in some cases, isotype controls, were added diluted to 100 μL volumes of reagent B. After spinning down the cells, Intracellular stains or isotypes were added to samples and incubated for 20 minutes at room temperature. The staining was stopped by addition of flow buffer and samples were spun down then resuspended for flow cytometry.

2.4.4 Flow cytometers

A table of cytometers, lasers and filters used to detect specific fluorophores is included in the appendices.

Samples were analysed on an LSR-II special order product (BD) equipped with the following lasers. Fluorophores were detected as specified. Numbers indicate filter and the letter denotes the position of the detector in the flow cube. Ultraviolet laser (355 nm): DAPI 450/50B, Live/Dead fixable blue 450/50B. Violet laser (405 nm): Brilliant Violet 421 450/50F, Pacific Blue 450/50F, Horizon V450 450/50F, Qdot-605 605/40C. Blue laser (488 nm): PerCp 705/70A, GFP B515/20B, FITC B515/20B, Alexafluor488 B515/20B, Side Scatter 488/10C. Green laser (532 nm): PE-Cy7 780/60A, 7-AAD 660/40C, PE-Texas Red® 610/20D, PE G575/26E. Red laser (633/640 nm): APC-Cy7 780/60A, APC-H7 780/60A, Alexafluor-700 710/50B, APC 670/14C. In some experiments a BD FACSCalibur was used. For these experiments 7-AAD was used as the live/dead stain in place of DAPI. The configuration for these experiments was as follows: Blue laser (488 nm): One of FITC, Alexafluor-488 or GFP on the 530/30 FL1; PE 585/42 FL2; 7-AAD 670LP FL3; APC 661/16 FL4. Sorting was done on a FACSDiva equipped with a Blue (488 nm) laser with a 530/30 FL1 filter; 585/42 FL2 filter and an FL3 670LP filter. Red laser (633 nm): 661/16 FL4, 780/60 FL5. UV laser (355 nm) FL6 450/50, FL8 620LP. Sorting used DAPI in FL6 PE from FL2, and APC in FL4.

2.5 ELISAs

2.5.1 Antigen-specific IgG1, IgG2a and polyclonal Ig ELISA

Ninety-six well Immunosorp Maxi plates (Nunc) were coated overnight with 100 μ L/well, 10 μ g/mL HDM (Greer laboratories), OVA, bovine or caprine BLG in pH=9.6 0.1 M carbonate buffer. The next day, plates were washed with 0.05% Tween-20 (Sigma) in PBS (pH=7.4) on an automated plate washer (Skatron) and blocked for one hour with 200 μ L/well 10% FCS/PBS (blocking buffer). Blocking buffer was removed and replaced with 100 μ L sera diluted in blocking buffer and incubated for two hours at room temperature in the dark. Wells were washed then incubated for 60 minutes with 100 μ L/well of 91 ng/mL biotinylated anti-mouse IgG1 (A85-1; BD) or 91 ng/mL biotinylated anti-mouse IgG2a (R19-15; BD) or 33.3 μ g/mL biotinylated polyclonal goat anti-mouse Igs (cat #553999; BD) diluted in blocking buffer, followed

by a wash and incubation with streptavidin-conjugated horse radish peroxidase (GE Healthcare, Auckland, New Zealand) for 60 minutes. After a final wash step, 100 μ L TMB substrate (BD Pharmingen) was added and 5-20 minutes later the reaction was stopped with 50 μ L 0.5 M sulphuric acid. The absorbance was read at 450 nm on a Versamax plate reader. Serum titres were calculated in Graphpad's Prism 5.0 and were defined as the EC50 O.D. of serum dilution curves, constrained to plateau at 0.0, and a shared group maxima based on the average highest O.D. value in the series. EC50s were calculated for each mouse and their reciprocal value is shown as the titre.

2.5.2 IgE

For total and OVA-specific IgE, plates were coated overnight with 100 μ L/well 0.5 μ g/mL anti-mouse IgE (6HD5; in house). Plates were washed with 0.05% Tween 20 in PBS (pH=7.4) and then incubated with 10% foetal calf serum (FCS) in PBS. Plates were washed and samples and standards were incubated for two hours at room temperature in the dark. In some experiments plates were instead left overnight at 4°C. Plates were washed and then incubated with 100 μ L/well 3 μ g/mL biotinylated Rat anti-mouse IgE (R1E4; in house; total IgE) or 100 μ L 20 μ g/mL biotinylated ovalbumin for 90 minutes at room temperature in the dark. Plates were subsequently washed and incubated with 100 μ L streptavidin horse radish peroxidase for 60 minutes. After a final wash step, 100 μ L TMB substrate was added and 10-20 minutes later the reaction was stopped with 50 μ L 0.5 M sulphuric acid. The absorbance was read at 450 nm on a Versamax plate reader.

2.5.3 Murine mast cell protease-1 (mMCP-1).

mMCP-1 was analysed according to the manufacturers instructions. Initially kits were purchased from Moredun Scientific (Midlothian, Scotland) but became unavailable in 2011. mMCP-1 kits were subsequently purchased from eBioscience (San Diego, Ca) and again used according to the manufacturers instructions. Similar absolute values in the range of 10-40 ng/mL for naive mice and 5000-10000-fold increases in mice with

active diarrhoea were attained using both kits.

2.5.4 Total IgG1 and Total IgG2a

Total IgG1 and IgG2a reagents were purchased (Bethyl Laboratories, Montgomery, Texas) and analysed according to the manufacturers instructions with some modifications to the recommended buffers. Plates were coated overnight with 100 μ L of a 1/100 dilution of antibody in pH=9.6 0.1 M carbonate buffer. The next day plates were washed with 0.05% Tween 20 in PBS (pH=7.4) using an automated plate washer and then incubated with 10% foetal calf serum (FCS) in PBS. Plates were washed and samples and standards were incubated for two hours at room temperature in the dark. Plates were washed and then incubated with 100 μ L/well horse radish peroxidase-conjugated goat anti-mouse IgG1 or IgG2a diluted as recommended. After a final wash step, 100 μ L TMB substrate was added and 10-20 minutes later the reaction was stopped with 50 μ L 0.5 M sulphuric acid. The absorbance was read at 450 nm on a Versamax plate reader.

Chapter 3

Does the *in vitro* IL-4 allelic expression profile reflect functional segregation of IL-4-producing Th2 cell subsets?

3.1 Introduction

3.1.1 Monoallelic regulation of the IL-4 gene

Studies over the past fifteen years have aimed to establish the molecular events that generate stable IL-4-producing CD4⁺ T cells. During these studies, the observation was made that many cells produce IL-4 from a single allele (between 52 and 85%)[273, 374, 389]. To explain the observations, in 2005, Guo, Hu-Li and Paul argued for potential advantage of a probabilistic regulation mechanism, operating at the single allele-level for the IL-4 gene[401]. However, these investigations have been undertaken exclusively in an *in vitro* context, and will benefit from elaboration *in vivo*.

In order to evaluate IL-4 production *in vivo*, IL-4 reporter systems have been developed. In our lab, we have recently combined two of these, the KN2 reporter[373] and the G4 reporter[273]. This combined reporter has the potential to record expression of each IL-4 allele individually, through the expression of two distinct reporters. In this chapter, we sought to characterise the behaviour of the KN2/G4 reporter *in vitro* to clarify its potential utility for *in vivo* investigations into the role of IL-4 in Th2 immunity. In this chapter, we used established *in vitro* culture methods, and IL-4 reporter systems to determine the allelic expression profile of IL-4 *in vitro*. We further

test whether conditions that lead to IL-4-producing Th2 cell development *in vitro* also induce reporter expression in KN2/G4 cells.

3.2 Aims

The experiments in this chapter were designed to evaluate the allelic expression profile of IL-4 in KN2/G4 cells *in vitro*.

Our specific aims were:

- 1) To test whether hCD2 and GFP are expressed in conditions known to promote IL-4 production;
- 2) To assess if IL-4 is produced from one or both alleles in KN2/G4 mice;
- 3) To evaluate whether IL-4 alleles are regulated independently in KN2/G4 reporter mice.

3.3 Results

3.3.1 Examining IL-4 reporter expression during primary culture

Our first experiments were designed to establish whether conditions that promote IL-4 protein production also result in hCD2 and GFP expression in KN2/G4 cells. IL-4 promotes IL-4-producing Th2 cell development *in vitro*[342], so we began by culturing CD4+ T splenocytes on anti-CD3-coated plates in titrated doses of IL-4. Three days after seeding we used flow cytometry to examine the expression of hCD2 and GFP in KN2/G4 reporter cultures. KN2/G4 cells are IL-4-deficient, so in these experiments, the only potential source of IL-4 is exogenous.

When KN2/G4 cells were seeded in wells without any IL-4, expression of hCD2 and GFP were low (Figure 3.1A and C). CD4+ T cell numbers were similar at all concentrations of IL-4 (Figure 3.1B), but the number of cells expressing hCD2 and/or GFP increased in an IL-4 dose-dependent manner above 10 U/mL (Figure 3.1C). Therefore, IL-4 promoted reporter expression by KN2/G4 CD4+ T cells.

Our next step was to evaluate the relative expression of each allele. When we examined the allelic expression of IL-4, we observed that the majority of reporter was expressed from a single allele at all IL-4 concentrations assessed (Figure 3.2E). However, while monoallelism predominated, we also observed disparity between the absolute expression of hCD2 and GFP, because hCD2 was expressed on up to 60% of CD4+ T cells, whereas GFP was detected in a maximum of 30% of KN2/G4 cells (Figure 3.2G). While having different absolute frequencies does not preclude monoallelic regulation, to conform to an independent probabilistic regulatory mechanism, acquisition of biallelism would need to be a function of the likelihood of the expression of each individual allele. Based on this premise, independent probabilistic regulation would result in $P_{biallelic} = (\%hCD2^+ * \%GFP^+) * 100$, where $\%hCD2^+$ is the total percent of CD4+ T cells expressing hCD2 (Q1 + Q2, depicted in figure 3.2C) and $\%GFP^+$ is the total percent of CD4+ T cells expressing GFP (Q2 + Q3, depicted in figure 3.2D). By comparing the actual proportion of cells that were biallelic (Q2, depicted in figure 3.2C), to the predicted proportion based on the expression of the individual

reporters, we observed that at IL-4 concentrations above 25 U/mL, biallelism was observed more frequently than would be expected if the two alleles were expressed entirely independently (Figure 3.2F). Therefore, while monoallelic expression of IL-4 predominated the response, biallelic expression was more frequent than was expected for independently-expressed alleles. Therefore, in the presence of 25 or more IL-4 U/mL, the expression of one reporter was associated with an increased likelihood of co-expressing the alternate reporter allele during primary stimulation.

Figure 3.1: KN2/G4 cells cultured in IL-4 express IL-4 reporters. Splenocytes from KN2/G4 mice were stimulated on anti-CD3 coated plates for three days in the presence of 100 U/mL IL-2, anti-CD28 and titrating amounts of IL-4. (A) shows representative plots of cells cultured in the presence of 0 or 1000 units (U) of IL-4 and identifies quadrants 1-3 (Q1-3) used to define reporter-expressing cells. (B) The total number of live CD4⁺ cells and (C) proportion that were GFP⁺ and/or hCD2⁺ (Q1+Q2+Q3), were determined by trypan blue exclusion and flow cytometry. Data points show mean $\pm$ SEM of (n=3) individually cultured spleens from one of three similar experiments. Data were compared using one-way ANOVA with Bonferroni's post-test for increases above wells that did not contain IL-4; *p<0.05, ***p<0.001.

Figure 3.2: Culture in IL-4 increases biallelic IL-4 production above predicted levels. Cells were stimulated as in figure 3.1 for three days. The red boxes show the quadrants used to identify the total proportion of CD4+ T cells expressing (A) either hCD2 or GFP (monoallelic) (B) both hCD2 and GFP (biallelic), (C) hCD2, or (D) GFP. The same plot is shown in (A-D) and was generated in a culture containing 50 U/mL IL-4. (E) The total proportion of Th2 cells expressing IL-4 from one (monoallelic as in (A)) or both (biallelic as in (B)) alleles were compared; Data points were compared by two-way repeated measures ANOVA with Bonferroni's post-test and all data points were significant to $p < 0.0001$. (F) The proportion of CD4+ T cells expressing both hCD2 and GFP observed experimentally (black circles; as in (B)), or predicted by $P_{biallelic} = (\%hCD2_{total}^{+} * \%GFP_{total}^{+}) * 100$ (white circles, predicted), were compared. (G) The total proportion of CD4+ T cells expressing hCD2 (black; as in (C)) or GFP (white; as in (D)) were determined by flow cytometry. Data points show mean+SEM of ($n=3$) individually cultured spleens from one of three similar experiments. Across experiments, both female and male mice were used and mice born to G4/G4 and KN2/KN2 mothers were assessed with similar differential expression profiles and biallelism responses irrespective of mouse gender or paternity. (F,G) Data were compared by Two-way repeated measures ANOVA with Bonferroni's post-test; * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$ at the same dose of IL-4.

3.3.2 Examining IL-4 production in differentiated Th2 cells

We observed that primary culture in the presence of more than 25 U/mL IL-4 resulted in an increased proportion of cells expressing hCD2 and GFP biallelically (Figure 3.2F) and were interested to determine if this biasing was maintained in imprinted Th2 cells. Th2 cells can remain stably polarised to the Th2 subset after the removal of IL-4 and TCR stimuli for at least 3-7 days[1, 343, 388]. Therefore, in the next set of experiments, our aim was to determine the allelic expression profile of rested Th2 cells upon reactivation.

In this experiment, KN2/G4 and +/+ cells were stimulated with anti-CD3/anti-CD28 in the presence of anti-IL-4 or 1000 U IL-4 for five days, and then transferred to uncoated culture wells containing anti-IL-4 and 100 U/mL IL-2. Three to four days later, cells were restimulated on anti-CD3-coated plates in the presence of anti-CD28 and IL-2 for 5-6 hours. While the frequency of reporter positive cells under Th0 conditions was low and these assays may have limited statistical reliability, when we examined the proportion of cells that expressed both hCD2 and GFP prior to restimulation, less than 10% of reporter+ cells expressed both alleles, under both anti-IL-4 and plus IL-4 culture conditions (Figure 3.3A and 3.3C). When cells were restimulated, we observed proportional increases in hCD2 and GFP expressing cells from cells cultured under both anti-IL-4 and plus IL-4 culture conditions (*c.f.* figure 3.3A and 3.3B). In cells cultured with anti-IL-4, while biallelic expression was four-fold higher than prior to stimulation, the ratio of mono:biallelic expression was unaltered (Figure 3.3C). In contrast, in IL-4-conditioned cells we observed a more pronounced 100-fold increase in biallelic expression, and an altered mono:biallelic expression ratio (Figure 3.3C). While the low frequencies of biallelic cells under anti-IL-4 culture conditions may be statistically limiting and will require follow-up validation, these data indicate that enrichment in biallelic expression was observed in restimulated IL-4-conditioned cells.

In an additional effort to examine the allelic expression profile of Th2-conditioned cells, we asked whether monoallelic expression predominated only in KN2/G4 cultures. Therefore, we examined the frequency differential of single or dual IL-4 allele expression in KN2/+ and G4/+ cultures (Figure 3.4). In these experiments, spleno-

cytes were activated in the presence of IL-4 for five days, subsequently rested for 5-6 days in the presence of anti-IL-4 and restimulated in the presence of anti-IL-4. Monensin was added two hours prior to harvest at each timepoint. At each timepoint, cultures were normalised such that hCD2 and GFP proportions were less than 0.1% in +/+ cultures, and the IL-4+ population was less than 0.1% in KN2/G4 cultures. This is why the gates in the figures differ at the two timepoints.

When we examined expression of hCD2 and GFP after three hours, the majority of Th2 cells expressed a single IL-4 allele in KN2/G4, KN2/+ and G4/+ cultures (Figure 3.4A-F). By six hours, the proportion of Th2 cells that were biallelic had increased in all three strains (Figure 3.4D-F). The biallelic population in KN2/G4 cultures plateaued at its maxima (Figure 3.4D); in KN2/+ and G4/+ cultures, IL-4 production was not maintained and resulted in a diminution of the biallelic population concomitant with the loss of IL-4 (Figure 3.4E and 3.4F). We compared the observed frequency of biallelism to the expected frequency as calculated previously (Figure 3.2F). In all three strains, statistically significantly for the KN2/+ and G4/+ strains at the six hour timepoint (peak of the response), the proportion of cells detected as biallelic was greater than predicted (Figure 3.4G-I). These studies indicate that while IL-4 production derived predominantly from a single allele, biallelism was more frequent than predicted. This was a feature of both primary and secondary Th2 cell stimulation.

Figure 3.3: Culture in IL-4 potentiates cells to produce IL-4 biallelically upon secondary stimulation. After culture on anti-CD3-coated plates for five days in the presence of 100 U/mL IL-2, anti-CD28 and (Th0) anti-IL-4 or (Th2) 1000 U/mL IL-4, spleen cells were rested for three to four days in uncoated plates containing 100 U/mL IL-2 and then restimulated with anti-CD3 in the presence of anti-CD28 and 100 U/mL IL-2. (A) Cells were examined prior to or (B) 6 hours after restimulation. (C) The proportion of cells that expressed both hCD2 and GFP were compared in cells prior to (pre) and after (re) restimulation. Bars show mean $\pm$ SEM of single spleens cultured in triplicate from one of two similar experiments. Data were analysed by Two-way ANOVA with Bonferroni's post-test; **p<0.01.

Figure 3.4: Biallelic IL-4 production exceeds predicted frequencies in restimulated KN2/+ and G4/+ cells. Splenocytes culture in the presence of 1000 U/mL IL-4 as in Figure 3.3 were rested for five days. Cells were then left unstimulated or restimulated on anti-CD3 in the presence of 100 U/mL IL-2, anti-CD28 and anti-IL-4 and examined for hCD2, GFP and IL-4 production. Unstimulated cells were cultured with monensin for 2 hours and then stained, and all other cultures had monensin added for the last two hours of culture. (A) KN2/G4 (B) G4/+ and (C) KN2/+ cells were examined for GFP, hCD2 and intracellular IL-4 without stimulation (0 hours) or after 3 hours (3 hours). The proportion of Th2 cells that were biallelic in (D) KN2/G4, (E) G4/+ and (F) KN2/+ cultures was determined. The predicted (determined as in Figure 3.2) and observed frequencies of CD4+ T cells that were biallelic were compared in (G) KN2/G4, (H) G4/+ and (I) KN2/+ cultures. Data show mean $\pm$ SEM of (n=2-3) individually cultured spleens from one of three similar experiments. (G-I) Data were compared using Two-way repeated measures ANOVA with Bonferroni's post-test; *p<0.05, ****P<0.0001.

3.3.3 Testing for interactions between IL-4, KN2 and G4

In our studies, hCD2 expression was more frequent than GFP expression, and up to 75% of GFP+ cells co-expressed hCD2 (Figure 3.1A). Therefore, we were interested to determine if hCD2 was out-competing the G4 allele for transcription factors, resulting in a skewed hCD2:GFP ratio as we had observed (Figure 3.2G). If increases in the expression from one allele were an impediment to the expression of the other, we predicted that IL-4 production by G4/+ and KN2/+ cultures would differ; similarly, GFP expression in KN2/G4 cultures would be different from G4/+ cultures, and likewise for hCD2 expression in KN2/G4 and KN2/+ cultures. We used Th2 conditioned spleen cells rested for 5-6 days, and then restimulated in the presence of anti-IL-4 to compare hCD2, GFP and IL-4 production (by intracellular cytokine staining) in the three different strains of mice. Monensin was added for the final two hours of culture. We also included wild-type (+/+) mice in the experiments.

When we restimulated the cells, the proportion of cells producing IL-4 was similar in KN2/+ and G4/+ cultures, and 50-65 percent of +/+ cultures (Figure 3.5A). Up to 30% of +/+ cultures produced IL-4, whereas the two heterozygous cultures peaked at $\approx 20\%$. We also observed similar expression of hCD2 in KN2/G4 and KN2/+ cultures, and similar expression of GFP in KN2/G4 and G4/+ cultures (Figure 3.5B and C). These relationships were maintained at all timepoints examined after restimulation. The kinetics of IL-4 production varied between experiments, but in all cases, intracellular IL-4 expression was maximal between 6 and 12 hours, as shown (Figure 3.5A). Proportional hCD2 and GFP expression were maintained for much longer periods than intracellular IL-4, such that the proportion of cells expressing hCD2 or GFP plateaued and were maintained out to 24 hours, and this occurred similarly in IL-4-sufficient and IL-4-deficient heterozygotes (Figure 3.5B and C). Thus, we conclude that while the absolute levels of hCD2 and GFP expression were different, this was not a result of transcription factor competition between the constructs. Therefore, while GFP and hCD2 expression were not independent events in IL-4-conditioned cells, neither were they cross-antagonistic.

3.3.4 Comparison of IL-4 production in homozygous and heterozygous reporter strains

Previous investigations examining the allelic expression profile of IL-2 *in vitro* compared IL-2+/+ and IL-2+/- cultures, and observed a 50-65% differential between the wild-type and heterozygous cultures[402]. Our observation of a 50-65% differential between +/+ and the two heterozygous strains (Figure 3.5A) lead us to ask if, despite being expressed to different absolute maxima, similar differentials may be observed between reporter heterozygotes and homozygotes. In the next experiments, we cultured cells in the presence of IL-4 or anti-IL-4, and examined hCD2 and GFP expression three days later. In these experiments, we used spleen cells from +/+, KN2/+, KN2/KN2, KN2/G4, G4/+ and G4/G4 mice. Quadrants for hCD2 and GFP were positioned to below 0.1% in +/+ cultures. Representative plots from which data were generated are shown in figure 3.6 and figure 3.7.

We began by examining hCD2 and GFP expression in cells cultured in anti-IL-4. After three days of primary culture in medium containing anti-IL-4, we observed that the proportion of KN2/G4 CD4+ T cells that expressed hCD2 was 50-65% the frequency of KN2/KN2 cultures (Figure 3.8A). Similarly, when we examined GFP expression, we observed a similar proportional reduction in KN2/G4 cells compared to G4/G4 cells (Figure 3.8B). G4/+ and KN2/+ cultures demonstrated similar reductions, although these differences were not statistically significant. We conclude that similar relative decreases in hCD2 and GFP expression in heterozygous versus homozygous reporters occurred in the absence of IL-4 signalling.

When we examined hCD2 and GFP expression in our IL-4-containing cultures, the relationship was different. GFP expression in KN2/G4 cells remained close to the predicted range differential (50-65% of G4/G4 mice; figure 3.8D), but the addition of IL-4 induced almost equivalent frequencies of hCD2+ cells in KN2/G4 and KN2/KN2 cultures (Figure 3.8C). KN2/+ cultures expressed hCD2 at 80% the level of KN2/KN2 cultures and G4/+ cultures expressed GFP at $\approx 70\%$ of G4/G4 cultures (Figure 3.8C and 3.8D). These data may indicate that the KN2 allele is more responsive to IL-4 than the G4 construct, but this will require more investigation at a molecular level. Our studies from these experiments indicate that the behaviour of the KN2 and the

G4 allele were similar under anti-IL-4 conditions, but may differ following culture in the presence of IL-4.

Collectively our experiments suggest that monoallelic expression of IL-4 predominates in KN2/G4 cultures. Our data also indicate that hCD2 and GFP reflect the production of IL-4 *in vitro*. We identify that the KN2 and G4 alleles are not cross-antagonistic, and in the absence of IL-4, behave similarly to the wild-type IL-4 gene. Additionally, we observed biallelism more frequently than was predicted, which may indicate that biallelic IL-4 expression is a unique feature of a specialised population of Th2 cells.

Figure 3.5: IL-4 production is equivalent in heterozygous reporter cells. Cells were treated as in Figure 3.4 and restimulated on anti-CD3-coated plates in medium containing anti-IL-4, anti-CD28 and 100 U/mL IL-2. Time zero wells were transferred to uncoated wells in the same medium and all wells had monensin added 2 hours before harvest, including the unstimulated (time zero) culture. Cells were stained intracellularly and analysed by flow cytometry to determine live CD4+ T cell (A) IL-4 production, (B) hCD2 expression or (C) GFP expression after the stated times. Data points represent mean $\pm$ SEM of (n=3) individually cultured spleens from one of three similar experiments. Data were analysed by Two-way repeated measures ANOVA with Bonferroni's post-test. Different lowercase letters indicate statistically significant differences comparing groups at the same time points; $p < 0.05$.

Figure 3.6: **Reporter expression by homozygous and heterozygous reporter cells cultured in anti-IL-4.** Cells from mice of the indicated genotype were cultured on anti-CD3-coated plates in medium containing 10 $\mu\text{g}/\text{mL}$ anti-IL-4, anti-CD28 and 100 U/mL IL-2 for three days and examined for reporter expression by flow cytometry. Plots are shown from one experiment used to generate data in Figure 3.8.

Figure 3.7: Reporter expression by homozygous and heterozygous reporter cells cultured in IL-4. Cells from mice of the indicated genotype were cultured on anti-CD3-coated plates in medium containing 1000 U/mL IL-4, anti-CD28 and 100 U/mL IL-2 for three days and examined for reporter expression by flow cytometry. Plots are shown from one experiment used to generate data in Figure 3.8.

Figure 3.8: hCD2 and GFP behave similarly in the absence of IL-4 signalling. Total splenocytes received three days of primary anti-CD3/anti-CD28 stimulation in medium containing 10 $\mu\text{g}/\text{mL}$ anti-IL-4, or medium containing 1000 U/mL IL-4 as indicated. CD4+ T cells were examined for hCD2 (black bars) or GFP (white bars) as gated in Figure 3.2C and 3.2D. Data were normalised relative to expression in KN2/KN2 homozygotes (A, C) or G4/G4 homozygotes (B, D). Data represent mean+SEM of (n=7) anti-IL-4 and (n=8) plus IL-4 individual spleen replicates combined from three (anti-IL-4) or four (plus IL-4) experiments. In each experiment anti-IL-4 cultured homozygote reporter expression was between 2 and 10%, and IL-4 cultured homozygote reporter expression was 30 to 90% of total CD4+ T cells. The area between 50 and 65% is shaded in grey to reflect the predicted difference based on the difference in IL-4 production in restimulated IL-4-conditioned cells shown in figure 3.5. Data were analysed by Kruskal-Wallis with Dunn's post-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.4 Discussion

The manner in which IL-4 production is regulated is an important focus of allergic disease biology. Over the past 15 years considerable advances in understanding have been made regarding the mechanisms that control its expression, conditions under which it is upregulated, and the epigenetic changes that stably maintain access to the Th2 locus to permit its production. One of the observations, now made by numerous groups, is that many IL-4-producing cells transcribe only one of their two IL-4 alleles. We have added to these studies and monitored allelic IL-4 production in primary and secondary *in vitro* culture settings in two reporter systems. We found that in KN2/G4 cells, most reporter-expressing cells expressed only one of the two reporter constructs. We observed that biallelism was more frequent, as a proportion of the Th2 cell population, in cells cultured in the presence of IL-4 than in its absence, and was also more frequent than we predicted for a gene with two independently regulated alleles. We further observed that, compared to wild-type, or reporter homozygote cultures, heterozygous cultures produced only 50-65% as much IL-4. Taken together, these studies support the concept that monoallelic *IL4* gene expression is a frequent feature of Th2 cells.

3.4.1 Probabilistic monoallelic regulation of IL-4

Monoallelic IL-4 production has been observed numerous times in the literature. It was first reported by Bix and Locksley[389]. These authors demonstrated that approximately 50% of Th2 clones had transcripts that were detectable from only one of two alleles; this was a stable, heritable phenotype and could be transcribed from either allele[389]. These were followed by studies from the same group demonstrating that the proportion of CD4+ T cells producing IL-4, rather than the amount of IL-4 produced per cell resulted in biased IL-4 production by BALB/c compared with C57BL/6 CD4+ T cells[390]. Subsequently, studies in two separate reporter mice demonstrated that IL-4 was expressed predominantly monoallelically. Rivière et al.[374] used a precursor reporter to the KN2 system, and observed, for the most part, two distinct populations of hCD2-expressing or IL-4-producing cells[374]. How-

ever, they also demonstrated that the strength of TCR mediated signalling could increase the proportion of cells that were biallelic, under both anti-IL-4 and plus IL-4 conditions[374]. These early findings indicated that the strength of stimulus, in this case TCR stimulation, could enhance biallelic expression of IL-4. Hu-Li *et al.*[273] added to these works and investigated the longevity of monoallelic expression. By cloning individual T cells, they found that irrespective of the mono- or bi-allelic status of a given cell, when passaged multiple times, the allelic expression profile of a population of daughter progeny would recapitulate the original profile[273]. These findings indicated that while there was clone-to-clone variation in the likelihood of expressing one, other or both alleles, for a given outgrown clone, the probability of expression was a predictable event.

The clearest illustration of monoallelic IL-4 production came from subsequent work by Guo, Hu-Li & Paul[381]. Guo *et al.*[381] used RNA fluorescence *in situ* hybridisation to analyse IL-4 expression on a per-allele basis[381]. In these studies, 538 cells expressing IL-4 were examined, and only 15.4% of these cells were expressing IL-4 from both alleles[381]. When cells expressing only one of the two IL-4 alleles were examined, there was a strong change in histone acetylation at an IL-4 enhancer, but only on the favoured allele[381]. There is an overwhelming body of literature that establishes the potential for monoallelic expression of IL-4 *in vitro*, and strong arguments that attribute this to a probabilistic regulatory process[273, 381, 388–390, 403].

Advantages of allele-specific IL-4 production

The next point to consider is why such a process would evolve. Generally, natural selection results in the accumulation of beneficial traits. IL-4 can have major deleterious effects if it is produced inappropriately[404, 405]; control of IL-4 production through probabilistic regulation has been argued as a potentially advantageous regulatory mechanism[381, 390, 401]. In this respect, probabilistic rather than deterministic regulation could provide tighter control of IL-4-dependent events; the example of B cell IgE class-switch recombination and macrophage type 2 activation were proposed by Guo *et al.*, because controlling the number of IL-4-producing cells, rather than the amount produced by an individual cell, would make it easier to balance the strength-

of-stimulus with an appropriate strength of outcome ([381, 401]; reproduced in figure 3.9). In light of recent data indicating that *in vivo*, IL-4 can induce STAT-6 phosphorylation in all non-Th2 cells in an inflamed lymph node[334], one additional context in which probabilistic regulation could be conceivably advantageous is in limiting the frequency with which IL-4-exposed T cells begin to produce IL-4. The germinal centre of the lymph node contains high frequencies of IL-4-producing TFH cells[275, 362], which means germinal centre TFH cells are likely to be surrounded by IL-4 producing cells in high frequency. Plausibly, a probabilistic regulatory mechanism could prevent exponential amplification of IL-4 production through a positive feedback loop acting on the IL-4 producing cells in close proximity to each other. This does, however, require that IL-4 influences Th2 cell development *in vivo*, which is currently a controversial topic. However, there are potential advantages in regulating IL-4 production in a probabilistic process that can result in the expression of protein from only a single allele.

In this context, the question arises, why do we see biallelic cells? In our studies we saw that there was variability in the expression profile of each allele because some cells in the KN2/G4 system expressed only one allele, but a reasonable proportion expressed both alleles (Figure 3.6 and 3.8). As IL-4 expression is thought to result from probabilistic regulation in which access to each allele is conferred independently[401] it is reasonable to infer that biallelic cells arose from two independently regulated, but not mutually exclusive, individually expressed alleles. In part, biallelic expression supports the concept of a probabilistic monoallelic regulation mechanism. One of the attributes of probabilistic regulation is that it can result in a bimodal expression pattern[387, 388]: Genes are either expressed or they are not. For hCD2 and GFP we observed reasonably bimodal populations (Figure 3.6 and 3.8). In earlier work, bimodal expression of IL-4 was observed[388] and in some of our experiments we observed sufficient separation of the IL-4+ and IL-4- populations to suggest that IL-4, too, was expressed in a similar manner. Thus the presence of the biallelic population does not preclude a probabilistic regulatory mechanism that acts at the allele-specific level from operating for IL-4. However, we also observed that biallelic IL-4 expression occurred at higher-than-expected frequencies (Figures 3.2 and 3.4), which may indicate that biallelic expression reflects a small predisposition among the

Th2 cell population to express *both* IL-4 containing chromosomes simultaneously and this point deserves some specific discussion, as follows.

Is there evidence of functional subtypes of Th2 cells based on their allelic expression profile?

It is important to consider potential reasons that underlie the higher-than-expected occurrence of biallelic IL-4-producing cells. In our studies, we observed that biallelism was increased by exposure to IL-4; previous studies have demonstrated that biallelic IL-4 expression can be increased if the level of TCR stimulation is increased[374]. It may be that once signal strength reaches a certain threshold, biallelism becomes more likely, which could explain the data and relate our findings to those of Rivière *et al.*[374]. This may provide the cells with a particular niche, and provides the exciting possibility that biallelic cells are a specific functional subset of Th2 cells. An alternative explanation relates to the differentiation of the Th2 population. As a population, Th2 cells that have divided more than four times produce on average more IL-4[347, 376]. Biallelism may be enriched in cells at a certain stage of development, or in cells that have proliferated a set number of times. This could be addressed by examining biallelism in cells labelled with proliferation dyes at the start of the experiment. STAT-6-deficient cells have also been shown to develop IL-4-producing capacity and this is associated with eventual accumulation of increased levels of GATA-3[348]. Examining GATA-3 levels in KN2/G4 cells may be a simple means to identify a signal that is associated with biallelic expression. It may also relate to pre-existing Th2 cells that were present in our culture at the start. Our experiments used whole spleen cells from naive mice, but cells with mixed surface phenotypes were present in these cultures; it is possible that the higher-than-expected biallelic frequency is due to a small number of *in vivo*-generated pre-existing Th2 cells in which IL-4 production is regulated in a different manner to the bulk cultured population. Using sorted naive populations in future experiments could be used to address this appropriately. Further, in this light, investigation of the biallelic potential of Th2 cells *in vivo* may identify novel aspects of Th2 cell development that are important for allergic responses and protection from parasites.

Reporter independence

The independent regulation of each reporter observed in our *in vitro* experiments (figure 3.5) suggests that cross-antagonism of reporters is unlikely to occur. When we examined KN2/+ and G4/+ cultures, we observed that these cells produced equivalent amounts of IL-4 (Figure 3.5). These findings indicate that G4 and KN2 were equally permissive of IL-4 production. Further, the behaviour of each reporter, relative to homozygotes was similar under anti-IL-4 conditions (Figure 3.8). Comparing the absolute frequencies of hCD2 or GFP+ Th2 cells in KN2/G4, KN2/KN2 and G4/G4 mice may allow us to gain a preliminary view of the influence of a single allele on IL-4 production *in vivo*. The disparity in absolute expression, however, requires further discussion.

The insertion sites of the KN2 and G4 reporter constructs were similar, but not identical. The KN2 reporter allele replaces the first two exons, and the intronic region between the two genes. Similarly, the G4 allele replaces the first intron and 178 base pairs of the subsequent intronic region of the gene[273]. Differences in these knock-in sites may inadvertently influence the success of their transcription. Second, the KN2 reporter included a CD4 enhancer cassette, which was inserted to amplify its expression and improve detection[373–375]. This may improve the likelihood of KN2 transcription without interfering with the counterpart allele. Examining similarly sized mRNA (cDNA) products for KN2 and G4 may allow us to determine whether there are differences in the amount of transcription, that could contribute to the differential reporter expression. Similarly, examining mRNA stability with the actinomycin D stability assay[406] may allow us to compare the two constructs and see if they are equivalently stable, which could also influence their absolute expression. These or similar investigations will be informative in future studies to explain the differences in absolute expression of the two constructs.

Collectively, our investigations have established the behaviour of the KN2/G4 reporter system *in vitro*. These data provide evidence of the utility of the mouse for *in vivo* studies of Th2 immunity. Our studies indicate that the majority of IL-4 is derived from a single allele *in vitro*, and provide some novel directions for future work. Future experiments will be required to determine how IL-4 expression is regulated *in*

vivo.

Figure 3.9: Probabilistic regulation may provide tighter regulation of IL-4-dependent responses. According to Guo *et al.*[381], probabilistic regulation would result in tighter control of the relationship between Th2 signal strength and the activity of the Th2 locus, than would a deterministic mechanism. In part, this would relate to less variability in the amount of IL-4 produced by each IL-4-producing CD4+ T cell. Adapted from Guo *et al.*[381].

3.5 Summary

We find that the majority of IL-4-producing cells *in vitro* express only a single IL-4 allele. Biallelic IL-4 production was more frequent in IL-4-conditioned cells. Higher-than-expected frequencies of cells producing IL-4 biallelically than is predicted for independently regulated alleles were observed. Expression from one IL-4 allele does not influence expression from the counterpart allele; these events are independent events, but are induced by the same stimuli.

Chapter 4

Evaluating the contribution of IL-4 to Th2 cell development and function *in vivo*

4.1 Introduction

4.1.1 Establishing how genetic lesions in IL-4 affect allergic Th2 cell development and function *in vivo*

The development of IL-4-producing CD4⁺ Th2 cells is a critical aspect of the allergic process. One of the key factors that promotes Th2 cell development *in vitro* is exposure to IL-4. Studies have investigated the role of IL-4 in *in vitro* Th2 cell development in great depth, however, there is limited information addressing whether, and to what extent, IL-4 is required for *in vivo* Th2 cell development. Understanding the cascade of events that leads to the development of an allergen-responsive Th2 cell *in vivo* may identify new ways to subvert the allergic process before it can manifest as disease.

There is evidence that IL-4 is not required for *in vivo* Th2 cell development. Prior to the advent of IL-4 reporter mice, it was shown that if anti-IL-4 antibody was injected before a primary *N. brasiliensis* infection, IgE production was impaired by greater than 99%[288]. However, if IL-4 production was not blocked during a secondary infection of these same mice, IgE was produced faster and with greater magnitude than was observed during a primary infection[288]. As IgE production induced by secondary *N. brasiliensis* infection is CD4⁺ T cell dependent[407] and IL-4 dependent[289], these early findings suggested that Th2 cells can develop without IL-4 signaling.

Previous studies in our lab investigated the role of IL-4 signaling in *in vivo* Th2 cell development more directly[1]. These studies employed an IL-4 reporter mouse, in which the gene encoding GFP is inserted under the control of the IL-4 promoter[273]. This is useful because IL-4 has a very short half-life and cannot be detected directly *ex vivo*. In this manner, IL-4-producing CD4+ T cells, which are typically classified as 'Th2 cells'[324, 325], can be identified directly *ex vivo*[1]. In the work done by our group, G4/+ mice, which remain IL-4-sufficient through the '+' allele, were compared to IL-4-deficient G4/G4 double reporter mice, or to G4/+ mice deficient in STAT-6 signalling. This was done because STAT-6 is key for IL-4R α signalling[329–331], and is known to be important for optimal Th2 cell development *in vitro*[329]. The observations are outlined briefly below.

In response to live parasite infection, IgE generation and bronchoalveolar eosinophil influx were absolutely dependent on IL-4 and STAT-6, but no requirement for IL-4 or STAT-6 in the generation of Th2 cells *in vivo* was identified. In G4/+ and G4/G4-STAT-6-/- mice, Th2 cells developed with similar kinetics; the induction of IL-5 and IL-13 mRNA in Th2 cells was similar in IL-4 deficient and sufficient mice; and memory Th2 cell expansion was similar in IL-4 sufficient and deficient mice[1]. These, and other findings, provide evidence that Th2 cells can develop in the absence of IL-4. A small number of other studies have assessed the role of IL-4 in Th2 cell development *in vivo*, however, none have examined the role of IL-4 in Th2 cell development to allergen in an adjuvant free context[1, 360–363, 408]. Additionally, a limited number of studies have attempted to modulate the level of IL-4. Instead, they generally examine Th2 cell function in IL-4-deficient mice. Therefore, we were interested to closely evaluate the involvement of IL-4 in allergen-induced Th2 cell development and function *in vivo*.

In addition to the G4 reporter, an additional system that has been used to record Th2 cells by marking IL-4 producing cells, is the KN2 reporter mouse[373]. In this reporter, the gene encoding a non-functional version of the human CD2 gene replaces IL-4 but remains under the control of the IL-4 promoter[373]. In this reporter mouse, Th2 cells have also been observed to develop *in vivo* in the absence of IL-4[275]. In the studies described in this chapter, we have used both reporters, and additionally crossed homozygous KN2/KN2 and G4/G4 mice to generate KN2/G4 reporter mice. These

KN2/G4 mice are IL-4 deficient, and can be used to examine Th2 cells expressing a single allele of IL-4. In the previous chapter we characterised the behaviour of the KN2/G4 mouse *in vitro*. In this chapter, we employ IL-4 reporter systems, including the KN2/G4 mouse, to ascertain how IL-4 contributes to Th2 cell development, and subsequently, Th2 cell function.

4.2 Aims

Our specific aims were:

- 1) To test whether IL-4 contributes to primary Th2 cell generation following adjuvant-free allergen immunization;
- 2) To determine how the lack of one IL-4 allele affects allergen-specific Th2 cell-dependent responses.

4.3 Results

4.3.1 Testing whether IL-4 is required for *in vivo* Th2 cell development

The study by van Panhuys *et al.*[1] and other studies[275, 360] indicate that *in vivo* Th2 cell development does not require IL-4. Our first aim was to confirm these previous findings. We immunised G4/+ and G4/G4 mice with 600 dead *iL3 N. brasiliensis* larvae. Seven days after intradermal immunization, we observed similar proportional shifts, and similar 1000-fold increases in the numbers of Th2 cells in immunized G4/+ and G4/G4 lymph nodes (Figure 4.1B and C). Therefore Th2 cells indeed developed in the absence of IL-4 and to similar levels in G4/+ and G4/G4 mice. These data recapitulate previous findings[1] and indicate that there is no absolute requirement for IL-4 in Th2 cell development *in vivo*.

In this experiment, we observed that similar numbers of Th2 cells developed in G4/G4 mice and G4/+ mice (Figure 4.1C). G4/G4 mice have two reporter alleles, but G4/+ mice have only one. If IL-4 does not contribute to Th2 cell generation, Th2 cell numbers should have been more frequent in G4/G4 mice compared to G4/+ mice. Therefore, it is possible that there is a partial effect of IL-4 on Th2 cell development. Further, this experiment may have been underpowered to reveal a difference. However, it is not possible to distinguish between IL-4-dependent monoallelic IL-4 production and IL-4-independent biallelic production by comparing these mice. Therefore, an alternate system was required to distinguish between these two mechanisms.

In our *in vitro* studies, we determined that each allele in a KN2/G4 mouse was activated independently. The two alleles were induced by the same stimulus, but neither inhibited the level of expression from the counterpart IL-4 allele (Figure 3.5A-C). Therefore, the KN2/G4 mouse provides an IL-4-deficient, single-allele reporter mouse, which would allow us to distinguish between IL-4-dependent and -independent Th2 cell development.

Figure 4.1: **Th2 cells develop in the absence of IL-4 *in vivo*.** G4/G4 or G4/+ mice (n=5/group) were injected intradermally with 600 dead *iL3 N. brasiliensis* larvae or 30 μ L PBS. Seven days later draining lymph nodes were excised and live cells were examined for GFP expression by flow cytometry. (A) Flow plots show CD4 and GFP expression in live-gated cells from PBS- or dead *N. brasiliensis*-immunized G4/G4 mice. (B) The proportion of CD4⁺ T cells expressing GFP was evaluated by flow cytometry. (C) Total GFP+CD4⁺ numbers were calculated by multiplying the proportion of live cells that were GFP+CD4⁺ by the total live cell count, as determined by trypan blue exclusion. Data points represent individual mice, and lines represent group mean $\pm$ SEM, from one of four similar experiments. Data were compared using One-Way ANOVA with Tukey's post-test; ***p<0.001.

4.3.2 Evaluating the influence of IL-4 on primary, adjuvant-free *in vivo* Th2 cell development

To use the KN2/G4 reporter in our investigations, we next examined the Th2 response of single-allele reporter mice, that were either IL-4-sufficient or IL-4-deficient. In these studies, we used GFP+CD4+ T cell numbers to identify Th2 cells when comparing G4/+ and KN2/G4 mice; we used hCD2+CD4+ T cell numbers to identify Th2 cells when comparing KN2/+ and G4/+ mice.

We immunised G4/+, KN2/G4 and G4/G4 mice with 100 μ g HDM and examined the development of Th2 cells 3, 7 and 10 days later. We also examined Th2 cell numbers in naive mice on day 0. Prior to injection on day 0, we observed similar, low level, baseline numbers of Th2 cells in G4/+, KN2/G4 and G4/G4 strains (Figure 4.2B and 4.2C). Three days after intradermal injection of 100 ug HDM, we observed that the proportion and number of GFP+CD4+ T cells in G4/+ and KN2/G4 mice was similar, and approximately 50-60 percent of that observed in G4/G4 mice (Figure 4.2B and 4.2C). When we examined the response seven and ten days after immunization, the proportion and number of GFP+CD4+ cells in G4/+ mice was higher than in KN2/G4 mice (Figure 4.2B). Therefore, in the presence of IL-4, Th2 cell development and/or survival was increased. We also noted that the proportional difference in Th2 cell numbers between G4/G4 and KN2/G4 mice was maintained on day seven, and the same trend held for day ten (Figure 4.2B and 4.2C). Further, while there was some variability at early timepoints, it may be important to note that the GFP median fluorescence intensity generally correlated with the relative proportion of GFP+ cells observed. In all cases, on day 10 the median fluorescence intensity of GFP in the KN2/G4 cells trended toward being lower than those from the respective G4/+ mice (Appendix Figure 8.3).

We next examined the response of KN2/+, KN2/G4 and KN2/KN2 mice. Similar, almost identical observations were made in the KN2 reporter system. We observed that KN2/+ lymph nodes contained more hCD2+CD4+ T cells than KN2/G4 nodes on day ten (Figure 4.3B and 4.3C), and the average numbers of hCD2+CD4+ T cells detected in KN2/G4 lymph nodes were lower than numbers detected in KN2/KN2 nodes at all timepoints. We found that these differences were statistically significant

on days three and seven (Figure 4.3B and 4.3C). The median fluorescence intensity of hCD2 also trended toward being lower on KN2/G4 cells in all day 10 experiments (Appendix Figure 8.3). Therefore, in both the G4 and KN2 reporter settings, IL-4 sufficiency increased the number of detectable Th2 cells induced by HDM after day three.

We considered it important to clarify the role of IL-4 in the Th2 response to parasite antigens. We intradermally immunised G4/+, KN2/G4 and KN2/+ mice with 600 *i*L3 *N. brasiliensis* larvae and examined the draining lymph node response 7 days later. When we compared hCD2 expression in KN2/G4 and KN2/+ mice, hCD2+ cells were more frequent in KN2/+ lymph nodes (Figure 4.4D and 4.4E, middle panels). We observed that GFP+ cells were more frequent in G4/+ than KN2/G4 mice (Figure 4.4D and 4.4E, left panels). When we determined the total number of Th2 cells expressing one, other, or both IL-4 reporter alleles, both the proportion and number of Th2 cells present in KN2/G4 lymph nodes was lower than in G4/+ or KN2/+ heterozygotes (Figure 4.4D and 4.4E, far right panels). Therefore, Th2 cell development was increased in the presence of IL-4 when parasite antigens were used as the immunogen. Further, when we examined the allelic expression profile of IL-4 in KN2/G4 mice, we observed that approximately 25 percent of Th2 cells expressed both hCD2 and GFP (Figure 4.4A). Therefore, Th2 cells in KN2/G4 mice were able to simultaneously express both IL-4 reporter alleles.

Figure 4.2: IL-4-independent and dependent phases of Th2 development revealed in IL-4 reporter mice. Mice were intradermally immunized with 100 ug HDM and examined for CD4 and GFP expression by flow cytometry 3, 7 or 10 days later. (A) Shows representative plots of total live cell CD4 and GFP expression from mice of each indicated strain. (B) The proportion of CD4⁺ T cells expressing GFP and (C) total number of GFP⁺CD4⁺ T cells in draining lymph nodes were compared. Data points represent individual mice, and lines represent group mean $\pm$ SEM; for Day 3 and 10, of n=11-25 mice combined from 3-5 experiments; for Day seven n=9-10 mice combined from two experiments, with the exception of G4/G4 mice (n=3, one experiment). Data were normalised such that the GFP⁺ population was <0.05 percent of total CD4⁺ T cells in +/+ mice at each timepoint. Data were compared using One-way ANOVA with Tukey's post-test; *p<0.05, ** p<0.01, ***p<0.001.

Figure 4.3: IL-4-independent and dependent phases of Th2 development confirmed in alternate reporter mice. Mice were intradermally immunized with 100 ug HDM and examined for CD4 and hCD2 expression 3, 7 or 10 days later. (A) Shows representative plots of total live cell CD4 and hCD2 expression from mice of each indicated strain. (B) The proportion of CD4⁺ T cells expressing hCD2 and (C) total number of hCD2⁺CD4⁺ T cells in draining lymph nodes were compared. Data points represent individual mice, and lines represent group mean $\pm$ SEM; n=9-15 mice combined from 2 to 3 experiments at each timepoint. Data were normalised such that the hCD2⁺ population was <0.05 percent of total CD4⁺ T cells in +/+ mice at each timepoint. Data were compared using One-way ANOVA with Tukey's post-test; *p<0.05, ** p<0.01, ***p<0.001.

Figure 4.4: **IL-4 enhances Th2 response to parasite allergens.** Six hundred dead *iL3* *N. brasiliensis* larvae were injected into the dermal ear flap of mice of the indicated genotype and 6 days later hCD2 and GFP expression in live CD4⁺ T cells was examined by flow cytometry. (A) For each genotype, the proportion of cells in each quadrant was used to generate proportions and numbers shown in (D) and (E). (B) Total and (C) CD4⁺ T cell numbers isolated from draining lymph nodes of mice of the indicated genotype were quantified. (D) The proportion and (E) number of CD4⁺ cells expressing GFP (left), hCD2 (middle) and GFP and/or hCD2 (right) was assessed. Data points represent individual mice and lines represent group mean $\pm$ SEM, $n=3-5$ mice from one of two similar experiments. Data were compared using One-Way ANOVA with Tukey's post-test; * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

4.3.3 Establishing the role of STAT-6 in primary, adjuvant-free *in vivo* Th2 cell development

STAT-6 is important for IL-4R signaling[329–331, 347]. As a final means by which to test the influence of IL-4 on Th2 cell development, we compared Th2 cell development in G4/+ and G4/+STAT-6-/- mice. Based on the impairment we observed in our comparisons of KN2/G4 mice and IL-4 sufficient heterozygotes, we predicted that Th2 cells would be less frequent in G4/+STAT-6-/- than in G4/+ lymph nodes. In this experiment, we immunised G4/+ and G4/+STAT-6-/- mice intradermally with 100 μ g HDM and examined Th2 cell development in the draining lymph node 7 days later. When we compared Th2 cell development in G4/+ and G4/+STAT-6-/- mice, we observed similar numbers of CD4+ T cells (Figure 4.5A), but Th2 cells were less frequent in the G4/+STAT-6-/- mice (Figure 4.5B and 4.5C). Naive G4/+ and G4/+STAT-6-/- lymph nodes contained similar numbers of CD4+ T cells and Th2 cells (Figure 4.5D-F). Therefore, Th2 cell development in response to primary HDM immunization was impaired in the absence of STAT-6 signaling.

Taken together, these data suggest that IL-4 signaling was not required for early Th2 cell development, but IL-4 signaling through STAT-6 amplified *in vivo* Th2 cell development. IL-4, therefore, contributed to Th2 cell generation over and above the ability of antigens to initiate Th2 cell development. Furthermore, the proportional reduction in hCD2-expressing Th2 cells in KN2/G4 mice compared to KN2/KN2 mice, in GFP-expressing Th2 cells in KN2/G4 mice versus G4/G4 mice and the observation that approximately 75 percent of cells expressed only one of the two reporters in KN2/G4 mice, also suggests that biallelic IL-4 expression *in vivo* occurs, but is rare.

Figure 4.5: IL-4-dependent Th2 response requires STAT-6. Mice of the specified genotype were injected intradermally with 100 ug HDM and seven days later assessed for (A) Total CD4⁺ T cell generation, (B) GFP expression by CD4⁺ T cells and (C) the total number of Th2 cells generated. In a separate experiment naive mice of the specified genotype were euthanised and the number of (D) CD4⁺ T cells, (E) proportion of CD4⁺ T cells that expressed GFP and (F) total number of CD4⁺GFP⁺ T cells were examined. Data points represent individual mice and lines represent group mean ± SEM. Data in (A-C) and (D-F) are from separate single experiments. Data were compared using Student's *t*-test; *****p* ≤ 0.0001.

4.3.4 Examining the influence of IL-4 on Th2-dependent immune responses

Investigating the function of Th2 cells in B cell switch responses *in vivo*.

We observed reduced Th2 cell generation in IL-4-deficient mice relative to IL-4-heterozygous mice. To more fully understand the effect of IL-4 on Th2 cell development, we were next interested to determine whether genetic deletion of one IL-4 allele, in IL-4-heterozygous mice, affected Th2 cell development relative to wild-type mice. However, with no current access to an IL-4 reporter mouse with two intact IL-4 alleles (IL-4^{+/+}), we cannot directly examine Th2 cell development directly using our reporter systems. Rather, we sought to investigate the influence that the loss of one IL-4 allele provides to the Th2 response by examining known IL-4-dependent responses in allergic disease contexts. The B cell class-switch response to IgG1 is an IL-4 dependent process[289], and intestinal anaphylaxis is an IL-4-dependent model of egg allergy[295]; therefore, we next sought to determine if IL-4-heterozygosity impairs either of these two processes. The experiments in this section of the chapter investigated how the absence of one IL-4 allele affects Th2 cell function. These experiments also sought to clarify the phenotype and function of Th2 cells in the pTh2 assay.

Assessing the phenotype of Th2 CD4⁺ T cells induced by intradermal immunization

Th2 cells have been shown to function in different capacities in lymph nodes and tissues[275, 317]. TFH cells support IgG1 antibody class-switch recombination[275], however TFH function has not yet been evaluated in the pTh2 assay. We began this set of investigations by examining whether pTh2 cells had the same phenotype as the B cell-helper TFH cell. We examined Th2 cells for expression of molecules associated with TFH cell function (CXCR5, PD-1 and ICOS[337, 338, 368, 409–411]) and for activation molecules previously observed on *N. brasiliensis* induced mediastinal

lymph node Th2 cells (CD44, CD62L, and CD69)[1]. Our objective was to determine whether auricular lymph node Th2 cells were likely to support B cell class-switch responses.

Initially, we immunized G4/+ mice with HDM and examined expression of the early T cell activation molecule, CD69, three days later. In a separate group of mice, we examined CD44 and CD62L expression seven days after HDM immunization. Lastly, we determined levels of molecules expressed by TFH cells, CXCR5, PD-1 and ICOS seven days after injection of 600 dead *iL3 N. brasiliensis* larvae. As established previously [1], Th2 cells upregulated CD44, decreased expression of CD62L and had variable, but generally elevated CD69 expression (Figure 4.6A and 4.6B). Consistent with previous reports[275, 362, 364], we observed that PD-1 and ICOS were expressed by almost all Th2 cells, and the Median fluorescence intensity of CXCR5 on the total Th2 cell population was increased over total CD4+ T cells (Figure 4.6C).

We next examined the development of TFH cells. In this experiment, mice were immunized intradermally with 600 *iL3 N. brasiliensis* larvae. Mock-challenged controls received 30 μ L PBS. Fourteen days later, we examined draining lymph node CD4+B220- T cells for the expression of CXCR5 and PD-1. When we examined wild-type mice after intradermal immunization, we observed TFH cell development in immunized, but not mock-immunized lymph nodes (Figure 4.7A and B). We also observed that TFH cells developed in G4/+ mice (Figure 4.7A and 4.7B) and were substantially enriched for IL-4 producing Th2 cells (Figure 4.7C). In all experiments, between 10 and 60 percent of GFP-expressing Th2 cells in G4/+ mice induced by intradermal immunization expressed PD-1 and CXCR5 and fell in the TFH cell gate ([275, 337]; Figure 4.7D). While the difference between TFH frequency between G4/+ and +/+ mice was not statistically significant (by Mann-Whitney U-test) this trend was reproducible in multiple experiments. This may relate to the role of IL-4 in the expansion or maintenance of the Th2 population (covered earlier in this chapter and alluded to in [362]), or could also be a result of a decrease in IL-4-dependent B cell responses that contribute to TFH cell selection, as B cells have been shown to support TFH cell expansion in some studies[364]. Irrespective of the influence of IL-4 on the TFH population expansion, these results demonstrate that a substantial proportion of Th2 cells were TFH cells.

Figure 4.6: Th2 cells exhibit an activated TFH cell phenotype. Following intradermal challenge of G4/+ mice (n=5-6), GFP+CD4+ T cells (solid black lines) and GFP- CD4+ T cells (grey histograms) were examined for surface phenotype. Isotype staining on GFP+CD4+ T cells for CXCR5 is represented by the dashed black line. Molecules associated with (A) early T cell activation, (B) effector T cell activation, and (C) TFH cells were examined. A single representative plot is shown from (A) one of two experiments done three days after HDM injection, (B) one experiment seven days after HDM injection and (C) three or more experiments seven days after dead *iL3 N. brasiliensis* larvae injection. Each experiment included 5-6 G4/+ mice.

Figure 4.7: IL-4 production is common in TFH cells. Mice were intradermally challenged with 600 dead *iL3 N. brasiliensis* larvae (Nb) or PBS and assessed for TFH cell development. (A) Representative plots of CXCR5 and PD-1 expression in live CD4+B220- cells. (B) Shows the proportion of CD4+ cells that fell in the canonical TFH cell gate. (C) Shows the proportion of CD4+ T cells and TFH cells that expressed GFP. (D) Shows representative CXCR5 and PD-1 expression in GFP+ and GFP- CD4+ T cells. Data shown are from one of three similar experiments. Data were compared using Student's *t*-test; **p*<0.05, *****p*<0.0001.

Investigating biallelism as a specific feature of Th2 TFH cells

Reinhardt *et al.*[275] took advantage of reporter mice with one KN2 allele and the IL-4-sufficient GFP-tagged 4get mRNA reporter on the other, and observed that hCD2+4get+ cells were enriched in the TFH population. In the KN2/4get mouse, transcriptional access to the IL-4 gene is reported by 4get expression and IL-4 production occurs predominantly in 4get+ cells co-expressing hCD2[352, 373]. As the B cell switch-inducing Th2 TFH cells were recorded as hCD2+/4get+ TFH cells, this also means that the doublet population expressed, at least at the transcriptional level, both alleles of IL-4 simultaneously. This led us to consider the possibility that biallelism is a unique property of TFH cells, and the small population of cells we observed expressing both IL-4 alleles in KN2/G4 mice (Figure 4.4) were exclusively TFH cells.

To evaluate whether biallelic cells were specifically TFH cells, we intradermally immunized KN2/G4 and +/+ mice with 100 μ g HDM extract. Ten days later we examined the expression of CXCR5 and PD-1 in draining lymph node CD4+ T cells. When we examined TFH cell development in KN2/G4 mice, we found that all Th2 cell populations (hCD2+GFP-, hCD2+GFP+ and hCD2-GFP+) contained TFH (Figure 4.8B). Further, we also observed that hCD2 expression, rather than biallelism was the strongest predictor of TFH cell status (Figure 4.8B). Therefore, TFH cells were not exclusively biallelic. The difference in TFH frequency in hCD2+ and GFP+ cells may also indicate that the G4 and KN2 reporters demarcate T cells with separate functions, at different stages of development, or with different fates. Future experiments will be required to determine the relevance of the differential population frequencies.

Figure 4.8: IL-4 can derive from one or both alleles in TFH cells. (A) Lymph nodes from mice of the indicated genotype were examined for CXCR5+PD-1hi TFH cells after immunization with 600 dead *iL3 N. brasiliensis* larvae. (B) Ten days after immunization with 100 ug HDM, CD4+ T cells from KN2/G4 mice expressing neither hCD2 nor GFP (hCD2-GFP-), only GFP (hCD2-GFP+), only hCD2 (hCD2+GFP-) or both hCD2 and GFP (hCD2+GFP+) were assessed for the proportion of cells in that quadrant that fell in the CXCR5+PD-1Hi TFH gate. A representative plot is shown on the left, and coordinates were set to <0.05 percent in each quadrant in CD4+ T cells in similarly immunized +/+ mice. Bars in the graph shown in (B) represent mean $\pm$ SEM of n=12 mice combined from three experiments.

The GL-7+ germinal centre TFH population includes a significant number of IL-4-producing cells

We reasoned that if the pTh2 response initiated IL-4-dependent B cell responses, we would observe germinal centre formation in immunized lymph nodes. GL-7 is expressed by both germinal centre B cells[338] and germinal centre TFH cells that contain high levels of IL-4 mRNA[366]. We next used GL-7 expression to determine if GC B and Th2 TFH cells are present in draining auricular lymph nodes.

In these experiments, we intradermally immunized G4/+ and +/+ mice with 100 μ g HDM antigen. Seven days later, we harvested draining lymph nodes, and non-draining contralateral lymph nodes and examined GL-7 expression on B cells and CD4+ TFH cells. We found that the proportion of GL-7-expressing B cells in the lymph node was increased in draining lymph nodes (Figure 4.9B). Therefore, GL-7-expressing B cells developed in response to immunization. Similarly, GL-7 expression by CD4+ T cells was predominantly by PD-1-expressing cells (Figure 4.9C), and we observed that a large proportion of TFH cells expressed GL-7 (Figure 4.9D and 4.9E). We observed only minor differences in the proportion of GL-7+ and GL-7- TFH cells that expressed GFP (Figure 4.9F). This suggests that GL-7- TFH have also been induced to produce IL-4. These findings indicate that auricular pTh2 cells have the potential to support IL-4-dependent B cell isotype class-switching.

Figure 4.9: **IL-4 is produced by GL-7+ (germinal centre) TFH cells.** (A) Seven days after intradermal immunization with 100 μ g HDM, B cells were stained with an IgM isotype (left) or anti-GL-7 antibody (right) to assess germinal centre B cell development. The gates shown on the plots define GL-7+ and GL-7- cells analyzed in (B) and (E-G). (B) The proportion of live B220+ cells expressing GL-7 was compared in non-draining contralateral (nDLN) and draining (DLN) lymph nodes. (C) CD4+ T cells were examined for PD-1 and GL-7 expression by flow cytometry. (D) GL-7 expression was examined in TFH cells (black line; gated as shown in Figure 4.8) and compared to isotype stained TFH (grey histogram). (E) The proportion of CD4+ T cells and TFH cells expressing GL-7 was examined. (F) Representative flow plots of GFP expression in GL-7- and GL-7+ TFH cells (left), (G) in which the proportion of cells expressing GFP (percent in Q2) were compared. Data points represent individual mice; bars and lines represent group mean $\pm$ SEM. Data are shown from one (n=3-5 mice) of four similar experiments, two using HDM and two using dead *iL3 N. brasiliensis* larvae as the immunogen. Data were compared using Student's *t*-test; *** p <0.001, **** p <0.0001.

Intradermal immunization induces IgG1 isotype class switching

Germinal centre Th2 TFH cells developed in mice immunized with HDM in the absence of exogenous adjuvant. Our next objective was to examine whether IgG1 isotype class-switching could be observed without adjuvant.

In these experiments, we intradermally immunized $+/+$ mice with either 100 μg HDM antigen or 600 dead *iL3 N. brasiliensis* larvae. Sixteen days later, we examined IgG1 and IgD expression on B220+ B cells in draining and contralateral non-draining lymph nodes. We observed a distinct population of IgG1+IgD-B220+ cells in draining, but not non-draining lymph nodes of both immunized groups (Figure 4.10A - C). When we compared the HDM and parasite antigens, we observed that twice as many IgG1+IgD-B220+ cells developed in response to the parasite antigens (Figure 4.10C). Overall, these data suggest that parasite antigens and HDM both induce IgG1 class switching in the absence of exogenous adjuvant.

In a separate experiment, we examined the kinetics of IgG1+IgD-B220+ cell development. After performing optimisation experiments with a new batch of HDM antigen (data not shown), in draining lymph nodes of mice immunized with 200 μg HDM, the number of IgG1+IgD-B220+ cells increased with time and became significantly elevated on day 16 (Figure 4.10D). These data indicate that IgG1+IgD- B cells develop with the kinetics expected of an affinity matured, CD4+ T cell-dependent germinal centre response.

We were also interested to determine if we could observe systemic antibody production following intradermal immunization. In parallel with the assays described (Figure 4.10), we bled mice and froze serum samples prior to, seven and sixteen days after intradermal immunization. We then tested for HDM-specific IgG1 antibody in HDM- and parasite antigen-immunized mice. We detected HDM-specific IgG1 in day 16 sera, but not in day 0 or 7 sera (Figure 4.11A). We did not detect HDM-specific IgG1 in sera from dead *iL3 N. brasiliensis*-immunized mice (Figure 4.11B). These data suggest that the intradermally-induced auricular lymph node IgG1 response is antigen-specific.

Figure 4.10: TFH cell function is reported by IgG1+ B cell development. (A) Sixteen days after intradermal immunization with 100 ug HDM live auricular lymph node B220+ cells were examined for IgD and IgG1 expression by flow cytometry in draining (DLN) and non-draining (nDLN) lymph nodes. The proportion (B) and total number (C) of B220+ cells that stained for surface IgG1 and not IgD was compared in mice immunized with 100 ug HDM (black bars) or 600 dead *iL3 N. brasiliensis* larvae (white bars) (n=3/group). (D) The kinetic generation of IgG1+IgD-B220+ cells was measured in mice immunized with 200 ug HDM antigen in a separate experiment. Data shown in (A-C) are from one of three similar experiments. The data shown in (D) was performed and analysed in a single experiment. Data were compared by One-Way ANOVA with Tukey's post-test; **p<0.01, ****p<0.0001.

Figure 4.11: **IgG1 class-switch B cell response is antigen-specific.** (A) Prior to (crosses) and seven (circles) and sixteen (squares) days after intradermal injection of 100 ug HDM, mouse serum was analysed by ELISA for HDM-specific IgG1. (B) Sixteen days after intradermal challenge with 100 ug HDM (white squares) or 600 dead *i*L3 *N. brasiliensis* larvae (black squares), mouse serum was analysed by ELISA for HDM-specific IgG1. Data points show mean $\pm$ SEM of groups of (n=3) mice from one of three similar experiments. Data were analysed using (A) Two-way repeated measures ANOVA with Bonferroni's post-test versus day 0 and day 7 sera, or (B) Two-way ANOVA with Bonferroni's post-test versus L3 Nb; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

4.3.5 The influence of a single IL-4 allele on primary, adjuvant-free IgG1-class switch recombination

Parasite-induced IgG1 class-switching is reduced by approximately 80 percent in IL-4 deficient mice[289] and also requires STAT-6[330], however, signals other than IL-4[274, 287, 288], specifically CD40:CD40 ligand (L) interaction[291, 292, 412–414], contribute to isotype class-switching[274, 415]. Further, specific types of immunization induce STAT-6- and IL-4-independent IgG1 production[274, 329], and in special circumstances, even IgE can be generated in the absence of IL-4 or CD4+ T cell help[416–418]. Therefore, it was possible that we were observing IL-4-independent IgG1 class-switching in the auricular lymph node. We next designed experiments to assess the involvement of IL-4 in the B cell IgG1 class-switch response. To do this we used mice deficient in STAT-6 or IL-4 and examined the influence that the loss of a single IL-4 allele has on the B cell class-switch response.

In these experiments, we immunized $+/+$, STAT-6 $-/-$, G4/ $+$ or G4/G4 mice with 100 μ g HDM. Fourteen days later, we tested draining and non-draining lymph nodes for IgG1+ B cells. Further, because MHC-II is upregulated on activated B cells in an IL-4- and STAT-6-dependent manner[329, 330, 419], we also examined the expression of MHC-II on the B cells.

As expected, STAT-6-deficient mice exhibited impaired IgG1 switching (Figure 4.12A). Correspondingly, B cells in these draining lymph nodes of STAT-6-deficient mice did not respond to IL-4, because expression of MHC-II was lower than observed in draining lymph nodes of IL-4-sufficient mice (Figure 4.12B). When we examined Th2 cell development in G4/G4-STAT-6 $-/-$ mice, we observed a minor elevation in the proportion of GFP+CD4+ T cells present in the draining, but not the non-draining lymph node (Figure 4.12C). This indicates that Th2 cells developed, but were not able to support IgG1 switching in STAT-6-deficient conditions. While the MedFI of I-A/I-E on the B cells in the non-draining $+/+$ lymph node was higher than others, comparison to naive lymph nodes (which were not included) would be required to determine whether this reflects dissemination of the B cell response or not. Therefore, while Th2 cells developed without either IL-4 or STAT-6, IgG1-switched B cell hyperplasia induced by intradermal immunization was STAT-6 dependent.

Figure 4.12: **IgG1 class-switch B cell response requires STAT-6.** Mice were intradermally immunized with 100 ug HDM and fourteen days later draining (black bars/symbols) and non-draining (white bars) lymph nodes were excised and analysed by flow cytometry for IgG1 expression by B220+ cells and GFP expression by CD4+ cells. (A) The proportion of B220+ cells expressing IgG1 was examined. (B) the expression of MHC-II (I-A/I-E) on the B220+ population was compared in mice of different genotypes. (C) The proportion of CD4+ T cells that expressed GFP was evaluated in the different groups. Data are shown from a single experiment (n=3-5 mice/group) and were analysed by Two-way repeated measures ANOVA with Bonferroni's post-test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

While STAT-6 was required for the adjuvant-free IgG1 class-switch response, low levels of IgG1 and IgE have been observed in IL-4- and IL-4R α -deficient mice[418]. To examine the influence of each IL-4 allele on IgG1 class-switching, we examined class-switching in IL-4-deficient G4/G4 and IL-4-heterozygous G4/+ mice. Fourteen days later, we examined mice for IgG1+ B cells, the expression of MHC-II, and Th2 cell development.

We observed that Th2 cells developed in G4/G4 and G4/G4-STAT-6-/- mice (Figure 4.13C). This indicates that Th2 cells were able to develop in IL-4-deficient and IL-4 heterozygous mice. However, we observed that IgG1+IgD- B cells did not develop in G4/G4 mice (Figure 4.13A). Additionally, we observed that MHC-II expression was lower in G4/G4 and G4/G4-STAT-6-/- IgG1+ cells than in IL-4 sufficient G4/+ and +/+ draining lymph node IgG1+ B cells (Figure 4.13E). Therefore while Th2 cells developed in the absence of IL-4 (Figure 4.13) IL-4 was required for adjuvant-free B cell IgG1 class-switch recombination. Similarly in G4/+ mice, an increase in Th2 cell number was observed in draining lymph nodes but not non-draining lymph nodes (Figure 4.13C). When we examined the B cell response, we observed that a single IL-4-sufficient allele was sufficient to permit B cell MHC-II upregulation (Figure 4.13B), but was not sufficient to permit normal IgG1 induction, because these mice demonstrated a two-fold reduction in proportional switching relative to +/+ mice (Figure 4.13A). Thus, a 50% decrease in the number of functional IL-4-producing alleles impaired the IL-4-dependent B cell class-switch response. Taken together, these data indicate that the loss of a single IL-4 allele in mice impairs either Th2 cell development or TFH cell function relative to wild-type mice.

Figure 4.13: The IgG1 class-switch response is IL-4 allele dose-dependent. Fourteen days after intradermal injection of 0.2 mg HDM antigen, draining (DLN) and non-draining (nDLN) auricular lymph node B cells were examined for IgG1 and IgD expression (A) and CD4+ cells were examined for GFP expression (C). The expression of MHC-II (I-A/I-E) on IgG1+B220+ LN cells was compared (B). Data are shown from one of two similar experiments (n=2-3 mice/group). (A-C) Data were analysed by Two-way repeated measures ANOVA with Bonferroni's post-test; **p<0.01, ***p<0.001, ****p<0.0001.

4.3.6 The influence of IL-4 heterozygosity on the intestinal allergic response

As a final investigation of the effect of IL-4 heterozygosity on Th2-type allergic immune responses, we examined the development of oral allergen-induced intestinal anaphylaxis in IL-4-heterozygous mice.

In these experiments, mice were intraperitoneally immunized with 50 μ g OVA in the presence of 1 mg alum adjuvant, twice, two weeks apart. Mice were then bled, and examined for serum IgE levels. In one experiment, mice received two further fortnightly intraperitoneal immunizations. Following this sensitization phase, mice were orally gavaged with 50 mg OVA, and monitored for diarrhoea development. Mice were bled after the 9th challenge to measure haematocrit, and were bled after the 10th challenge to examine murine mast cell protease-1 (mMCP-1) and antibody titres in the serum. In these comparisons the phenotype of G4/+ and KN2/+ mice were essentially the same, so I will discuss these mice collectively as +/- mice. In figures the response of mice from each genotype are shown.

When we examined IgE generation in mice immunized twice with OVA and alum, we observed 90% less IgE in +/- mice than in wild-type +/+ mice (Figure 4.14A). When we examined IgE levels in mice that had received a total of four fortnightly i.p. sensitizations, and then ten oral OVA challenges, we still observed a 90% decrease in serum IgE levels (Figure 4.15A), and also lower levels of OVA-specific IgE in +/- compared with +/+ mice (Figure 4.15B). These data indicate that allergen-specific IgE production is impaired in IL-4-heterozygous mice.

When we challenged mice orally with OVA, only +/+ mice exhibited diarrhoea (Figure 4.16A). Blood haematocrit was higher in diarrhoetic +/+ mice than +/- mice (Figure 4.16B). These data demonstrate that +/- mice did not develop an intestinal allergic response. When we examined mast cell activity in these mice, we observed that mMCP-1 titres were lower, and jejunal mast cells were less frequent in OVA challenged +/- mice than +/+ mice (Figure 4.16C and 4.16D). While we did not examine basal mMCP-1 levels in IL-4 +/- mice and will require confirmation, basal levels in wildtype mice are usually approximately 20-40 ng/mL, indicating some increase in intestinal

mast cell activity may have occurred in the orally challenged +/- mice. These data indicate that both IL-4 alleles are required for normal oral allergen-induced mast cell activity.

Taken together, these data suggest that both IL-4 alleles contribute to IgE development. Mice deficient in a single IL-4 allele exhibited deficient IgE generation, impaired intestinal mast cell activity and were protected from allergic diarrhoea. These data suggest that both IL-4 alleles are required for normal allergen-specific Th2 immune responses.

Figure 4.14: **IgE production is impaired in IL-4 heterozygous mice.** (A) Mice were sensitized intraperitoneally with 50 μ g OVA plus 1 mg alum and 28 days later were bled and serum was analysed for Total IgE. Data are shown from one of four separate experiments in +/+ and G4/+ mice (n=3-5 mice). KN2/+ mice (n=5) were analysed at this time-point once. Data were analysed by One-way ANOVA with Tukey's post-test; ***p<0.001.

Figure 4.15: **IgE production is impaired in hyper-primed, orally-challenged, IL-4 heterozygous mice.** Mice were sensitized intraperitoneally four times, every two weeks with 50 μ g OVA and 1 mg alum. Two weeks after the fourth i.p. prime, mice were then orally challenged with 50 mg OVA ten times as per the standard regimen. Sixty minutes after the final challenge, mice were bled for (A) Total IgE and (B) OVA-specific IgE. Data points in (A) show individual mice and in (B) data points represent mean $\pm$ SEM of these same (n=3-5) mice from a single experiment. Data were analysed by (A) One-Way ANOVA with Tukey's post-test, or (B) Two-way ANOVA with Bonferroni's post-test; ***p<0.001, ****p<0.0001, +/+ versus KN2/+ or G4/+.

Figure 4.16: IL-4 heterozygosity prevents oral-allergen induced intestinal anaphylaxis. Mice shown in Figure 4.15 were (A) monitored for diarrhoea development following challenges and (B) haematocrit (%PCV) 60 minutes after the ninth challenge. (C) mMCP-1 was measured from sera taken 120 minutes after the final gavage challenge. (D) Intestinal mast cell infiltration at the experimental end-point. Data are shown from a single experiment and were analysed using One-way ANOVA with Tukey's post-test; * $p < 0.05$, ** $p < 0.01$. mMCP-1 data were log-transformed prior to analysis. In two additional experiments, G4/+ mice ($n=5$ /experiment) that received two ip primes did not exhibit diarrhea with ten 50 mg OVA challenges.

4.4 Discussion

The aim of this chapter was to evaluate the role of IL-4 in Th2 cell development and function *in vivo*. In these studies, we employed a primary Th2 response assay to examine the involvement of IL-4 in Th2 cell development. We subsequently examined the phenotype of Th2 cells responding to intradermal allergen immunisation, and then examined their ability to support IgG1 class-switch responses. We observed significant impairments in Th2 cell development in IL-4-deficient conditions, and demonstrated that both IL-4 alleles were required for the generation of allergen-specific IgG1, IgE and for intestinal allergic responses. These studies illustrate the importance of IL-4 in the co-ordination of allergic Th2 immune responses.

4.4.1 The role of IL-4 in Th2 cell development

Th2 cell development was observed to occur in two phases in our experiments. We observed that similar numbers of GFP+CD4+ T cells developed in G4/+ and KN2/G4 mice three days after adjuvant-free HDM immunization, but after this time G4/+ lymph nodes contained approximately 50% more GFP+CD4+ T cells than KN2/G4 lymph nodes (Figure 4.2). Thus, there was an IL-4-independent initiation phase, followed by an IL-4-dependent amplification phase. Similar IL-4-independent and -dependent phases were observed when we compared hCD2+CD4+ T cell development in KN2/+ and KN2/G4 mice (Figure 4.3). We also observed a requirement for STAT-6 in Th2 cell expansion, because GFP+CD4+ T cells were fewer in G4/+STAT-6-/- mice than G4/+ mice seven days after immunisation (Figure 4.5). Collectively, these data indicated that Th2 cell development was initially IL-4-independent, but was then amplified in the presence of IL-4. These data support the accumulating body of literature that indicates that Th2 cell development can be initiated in an IL-4-independent manner.

Our studies examined adjuvant-free allergen-induced Th2 cell development, but similar conclusions have been reached using adjuvant-based protein and hapten immunisation regimens. Cunningham *et al.* examined the requirement for IL-4 signaling using mice deficient in IL-4R α [360]. In their studies, mice were immunised with

haptened fowl gamma globulin, alum and heat-killed *Bordetella pertussis*. To measure Th2 development, IL-4 mRNA transcript frequencies were compared in isolated draining lymph nodes of IL-4R α ^{+/+} and IL-4R α ^{-/-} mice. They observed similar increases in IL-4 mRNA transcript levels and lymph node expansion in both strains for the first three days[361]. After this time, in IL-4R α -deficient mice, lymph nodes atrophied and ϵ - and γ 1-switch responses were diminished[361]. IL-4 mRNA transcripts were, however, still detected[361]. Thus, the induction of IL-4 production occurred normally without IL-4R α , but IL-4-dependent B cell responses required IL-4 signaling. Similarly, Reinhardt *et al.* examined Th2 cell development in response to haptened-bovine serum albumin adsorbed to alum[275]. They observed that Th2 cells developed in both KN2/+ and KN2/KN2 mice, but hapten-specific IgG1 production was decreased in KN2/KN2 mice. van Panhuys *et al.* immunised mice with keyhole limpet hemocyanin and alum, and then gave them an airways challenge with keyhole limpet hemocyanin[1]. They observed that mediastinal Th2 cell numbers expanded similarly in G4/+, G4/G4 and G4/G4-STAT-6^{-/-} mice, and airways eosinophilia was unimpaired, but IgE production was ablated[1]. Our studies add to these findings and demonstrate that adjuvant-free Th2 cell development also does not require IL-4. Collectively these data indicate that there is no absolute requirement for IL-4 in antigen-induced Th2 cell development, but IL-4 remains an important molecule in Th2-mediated B cell responses.

Studies have also examined the role of IL-4 in other Th2 response models. van Panhuys, King, Noben-Trauth, Mohrs *et al.*[1, 362, 363, 420] examined the development of the Th2 cell phenotype in parasite-infected mice. Seven days after *H. polygyrus bakeri* infection, Th2 cell numbers were similar in IL-4R α ^{-/-}4get and IL-4R α ^{+/+}4get reporter mice, but by day 14, IL-4R α ^{-/-} lymph nodes contained fewer 4get+ cells and fewer B cells than IL-4R α ^{+/+} counterparts[362]. Similarly, when *N. brasiliensis* was used to infect G4/+ and G4/G4-STAT-6^{-/-} mice, Th2 cells developed with similar kinetics during primary infection, and were even observed to expand to higher absolute frequencies following a secondary challenge[1]. T cells from *N. brasiliensis*- or *Leishmania major*-infected IL-4R α -deficient mice have been shown to produce measurable amounts of IL-4 upon *ex vivo* restimulation[363, 420]. Th2 cell development has also been demonstrated to be unimpaired in anti-IgD treated mice deficient in

IL-4, IL-4R α or STAT-6[1, 421]. Additionally, it has also been demonstrated that dead parasite antigens induce Th2 cell development in intradermally immunised IL-4 and STAT-6 doubly deficient mice[1]. Collectively, there are numerous studies that indicate that Th2 cell development can occur in the absence of IL-4 *in vivo*.

IL-4-independent and -dependent phases of Th2 cell development have been reported not only *in vivo*, but also *in vitro*. While IL-4 enhances Th2 cell commitment, naive CD4⁺ T cells lack repressive epigenetic marks associated with the IL4 locus[324, 347, 377–380, 385], and contain low levels of GATA-3 that are thought to be sufficient for the early initiation of IL-4 production[325, 422]. *In vitro* studies have identified that within the first few hours of TCR ligation, IL-4 and GATA-3 mRNA transcription are observed, even in STAT-6-deficient cells[327, 347, 348, 350]. Multiple rounds of culture in Th0 conditions can permit 10-fold increases in IL-4 protein production by antigen-stimulated IL-4R α -/- lymph node cells[421]. Similarly, STAT-6-deficient T cells can be induced to produce IL-4 and stably polarise to the Th2 subset for a number of weeks[348]. These findings illustrate that in the absence of IL-4R α or STAT-6, naive CD4⁺ T cells can begin to develop Th2 function *in vitro*.

Some factors that contribute to this IL4-independent expression of Th2 genes have been identified. Weak TCR ligation in the presence of IL-2 triggers the phosphorylation of STAT-5, which induces transcription of IL-4 mRNA and production of IL-4[325, 349, 422]. GATA-3 remains important for IL-4-independent Th2 development *in vitro* because in its absence STAT-5-induced IL-4 production is defective[423]. Additionally, while STAT-6-deficient cells can become Th2 polarised, the Th2 cells that do develop Th2 function accumulate high levels of GATA-3 mRNA[348]. Therefore, STAT-5 promotes IL-4-independent IL-4 production in the presence of GATA-3. The ability of low-level TCR stimulation to promote CD4⁺ T cell IL-4 production is also observed in polyclonal systems, because when T cells with high-affinity for antigen are removed from culture, IL-4 production induced by high-dose antigen stimulation is increased[424]. Therefore, IL-2 and weak TCR stimulation induce IL-4-independent Th2 cell development *in vitro*.

During primary *in vitro* stimulation, the IL-4-independent phase of Th2 cell development is short. After the first 24 hours of TCR stimulation, exogenous IL-4 is required

to increase and maintain both IL-4 mRNA and GATA-3 mRNA transcript levels *in vitro*[349]. Additionally, while STAT-6-deficient cells can produce IL-4, and stably polarise to the Th2 subset, they do not reach the levels of polarisation that can be observed in STAT-6-sufficient Th2 cells[1, 348]. These *in vitro* studies indicate that while IL-4 is not necessary during the initial activation phase *in vitro*, it remains a potent Th2-polarising cytokine. Thus, the initial transcription of IL-4 mRNA that occurs *in vitro* occurs in an IL-4-independent manner, and is followed by an IL-4-dependent phase that amplifies *in vitro* Th2 polarisation.

4.4.2 Potential mechanisms that promote IL-4-independent Th2 cell development *in vivo*

Understanding the initial events in IL-4-independent Th2 cell development *in vivo* is only beginning to be investigated. For a long time, it was thought that IL-4 was required for the initiation of Th2 cell development *in vivo*. When naive CD4+ T cells are initially activated, they can produce minute amounts of IL-4[421]. Similarly, NK-T cells and basophils have been shown to produce IL-4 in an IL-4- and STAT-6-independent manner[357, 363, 421, 425]. Either of these cell populations could have been a source of early IL-4 that promoted Th2 cell development. However, numerous studies, including the current investigation, indicate that the initiation of Th2 cell development *in vivo* cannot be attributed to IL-4 alone. Therefore, rather than initiating Th2 cell development, these sources may instead contribute to the expansion of Th2 cells.

A number of potential Th2-initiating pathways have been proposed. Basophils have been implicated in the initiation of Th2 responses, both through their production of IL-4 and by presenting low levels of antigen-MHC-II[353, 354, 426, 427]. TSLP, IL-25 and IL-33 have also been shown to promote Th2 immunity through the development of previously uncharacterised basophil-like and lineage-negative Th2-cytokine producing populations[355, 428–433]. However, Th2 cells develop to normal levels in the absence of basophils[357–359] and the tissue-derived cytokines still enhance Th2 cytokine production in the absence of CD4+ T cells[432]. Epithelial cell production of IL-25 exacerbates allergic airways disease in mice and also promotes Th2 cell develop-

ment *in vitro*; however, the effect of IL-25 specifically on Th2 cells requires IL-4[356]. Therefore, further investigations are required to determine the role of tissue-derived cytokines and basophils in the initiation of Th2 cell development. Specialised dendritic cell subtypes, namely those expressing FcεRI or OX40L, have also been associated with the initiation of Th2 cell development[351, 359, 434]. In *in vitro* studies, OX40L also promotes CD4+ T cell IL-4 production[435, 436]. These processes may not require IL-4 to initiate Th2 cell development; this also requires further investigation. Investigating the earliest events that promote Th2 cell development may provide novel ways to interfere with the allergic process.

4.4.3 Potential implications on IL-4-dependent Th2 cell expansion

Understanding the manner in which IL-4 increases the number of GFP+ or hCD2+ cells may have important implications for the aetiology of allergic disease. IL-4 may act to increase the proportion of committed Th2 cells that produce IL-4; IL-4 may induce the expansion of the committed Th2 cells that developed in an IL-4-independent manner; or IL-4 may promote polarisation of uncommitted antigen-specific Th cells towards the Th2 phenotype.

If IL-4 increases the proportion of committed Th2 cells that produce IL-4, the composition of the Th2 population would not be altered. According to the probabilistic regulation theory, the relative 'strength' of the Th2-inducing stimulus will cause more frequent IL-4-production by cells in a Th2 cell population[273, 401]. In this case, it is possible that the presence of IL-4 increases the Th2-activating 'strength' of an immunogen, and thus causes more committed Th2 cells to produce IL-4. Therefore, the number of committed Th2 cells will not be altered, but our ability to detect them will (Figure 4.17A). Thus, the composition of the antigen-specific population may not be altered, and their polarisation toward the Th2 phenotype may not be influenced. IL-4 may simply increase the proportion of committed Th2 cells that produce IL-4.

IL-4 may alternatively have altered the expansion of the allergen-specific Th cell population. The bulk Th2 cell population at the peak of the response could have

developed from precommitted IL-4-independent Th2 cells (Figure 4.17B), or from previously uncommitted Th cells that became Th2 polarised (Figure 4.17C). These two scenarios will influence the clonal diversity of the Th2 cell population: If IL-4 polarises uncommitted Th cells, then the Th2 cell population will become more clonally diverse; in contrast, if IL-4 acts solely to expand pre-committed Th2 cells, then the clonality of the Th2 cell population will not change, but the bias of the Th population towards the Th2 phenotype will be increased. At first sight, this may seem a redundant distinction. However, IL-4 production by CD4+ T cells has been shown to be preferentially invoked by low-dose and weak antigen-TCR stimulation[349, 424]. If IL-4 polarises uncommitted Th cells to the Th2 subset, this provides the potential for Th2 polarisation of Th cells with higher affinity for the insulting antigen. This, in turn could promote IgE class-switching of the affinity-maturing B cell population and contribute to the generation of high-affinity IgE. If instead, the pre-committed Th2 population is expanded by IL-4, identifying the manner in which IL-4-independent Th2 cell development is initiated could identify previously unexplored avenues for therapeutic intervention.

Basic mechanistic studies offer support to both types of Th2 population expansion. Recent studies indicate that STAT-6 phosphorylation is observed in essentially every non-Th2 cell in draining lymph nodes of mice infected in the foot-pad with *H. polygyrus bakeri*[334]. IL-4Ra is downregulated on highly-activated Th2 cells[373], rendering them refractory to the phosphorylation of STAT-6 caused by IL-4 produced in a draining lymph node[334]. If IL-4 directly induces Th2 cell development, this could indicate that the IL-4 produced by Th2 cells induces IL-4 production by previously uncommitted cells. In the work by Perona-Wright *et al.*[334], when foot-pad-infected mice subsequently received OVA-peptide-loaded dendritic cells, OVA-specific CD4+ T cells in the foot-pad draining lymph node were preferentially polarised to the Th2 subset[334]. Therefore, Th2 polarisation of uncommitted Th cells is possible, and can induce Th2 polarisation of unrelated allergens.

While activated Th2 cells may be refractory to autocrine IL-4[334], their expansion may occur indirectly through interaction with accessory cells. As established by others[275, 362, 364], we observed that a large proportion of Th2 cells were TFH cells (Figure 4.7 and 4.8). It is known that CXCR5-expressing TFH cells migrate into

B cell follicles[337, 437], and that B cells can provide stimulatory signals to antigen-experienced CD4+ T cells[438]. Therefore, it is plausible that committed Th2 cells, despite being refractory to IL-4, interact with B cells, expand and continue to produce IL-4 (Figure 4.17A). Equally, it could be argued that TFH arise from the uncommitted Th population after they are exposed to IL-4. However, while TFH populations take time to expand fully[364], the bifurcation of cells that are likely to become TFH cells (and by proxy a reasonable proportion of Th2 cells) or non-TFH Th cells can be identified within three days of immunisation[368]. Interaction with dendritic cells alone is also sufficient to initiate TFH development[368, 439], and similarly to initiate IL-4 production[408]. In the current study, we identified no requirement for IL-4 for Th2 cell development in the first three days (Figure 4.2 and 4.3). Taken together, it is plausible that IL-4-independent Th2 cells may be preferentially selected as the Th2 cell population prior to the involvement of IL-4 in Th2 cell expansion.

One final point we must consider is that IL-4 signalling may influence the allelic expression of IL-4, inducing more biallelic expression. We have based our studies on comparisons made in heterozygous G4/+, KN2/+ and KN2/G4 mice. In our *in vitro* studies, we observed a disproportionate increase in biallelic IL-4 production in cells exposed to IL-4 (figure 3.3 and 3.4). In these comparisons, KN2/+ and KN2/G4 cells cultured in IL-4 had similar frequencies of hCD2 expression as KN2/KN2 cells, indicating that IL-4 may have influenced the allelic expression profile. While the differential was maintained in G4/+ and G4/G4 cultures *in vitro*, it is possible that IL-4 potentiated both G4/+ and KN2/+ Th2 cells to express IL-4 biallelically *in vivo*. However, the concordance between the two reporter systems suggest that even if IL-4 is 'potentiating' biallelic expression, this 'potentiation' of Th2 cells may be an important component of Th2 development. Future studies will be required to clarify the role of IL-4 in IL-4-producing Th2 cell development.

The manner in which IL-4 increases Th2 development may have important implications for allergic disease biology. If pre-committed Th2 cells expand or produce IL-4 in response to IL-4, understanding the processes that initiate IL-4-independent Th2 cell development may provide new prophylactic disease interventions. If IL-4 polarises uncommitted Th cells to the Th2 phenotype, then targeting antigen-responsive cells, rather than the Th2-polarising apparatus, may provide more therapeutic benefit.

Further, if IL-4-independent Th2 cells are long-lived, and remain IL-4-independent, then interfering with IL-4 signaling may limit, rather than reverse the disease phenotype. I think the most likely explanation is that IL-4 increases the polarisation of uncommitted Th cells to the Th2 phenotype; however, clarifying the mechanism that underlies IL-4-dependent Th2 cell expansion will be an important endeavour for future studies.

Figure 4.17: **Potential mechanisms of IL-4-dependent Th2 cell expansion.** (A) IL-4 may increase the proportion of committed Th2 cells that produce IL-4 without changing the proportion of Th2-committed cells. (B) The presence of IL-4 may result in the preferential expansion of committed Th2 cells without altering the balance of IL-4-producing and non-producing Th2 cells. (C) IL-4 may polarise uncommitted Th cells towards the Th2 phenotype.

4.4.4 A glimpse at the allelic expression of IL-4 *in vivo*

In addition to operating as an IL-4-deficient single-allele reporter, the KN2/G4 reporter mice also provided insight into the allelic expression profile of IL-4 *in vivo*. This provides novel understanding of the potential phenotype of the Th2 cell. In response to both dead *iL3 N. brasiliensis* and HDM immunisation, a small proportion of cells were positive for both hCD2 and GFP (Figure 4.4 and 4.8). Previous explorations have also identified that dual 4get/KN2+ cells develop in helminth-infected mice[275, 362] and in response to isolated helminth antigens[364]. Similarly, GFP+CD4+ T cells isolated from helminth-infected G4/+ cells contain elevated levels of IL-4 mRNA[1]. Thus, in two systems, biallelic IL-4 expression has been determined at the mRNA level. Our studies extend these findings and confirm that the Th2 cells that developed were able to produce IL-4 biallelically. We also observed that biallelism was rare. Our observation of predominant monoallelism *in vivo* is in accordance with our *in vitro* observations, and in agreement with previous *in vitro* studies[273, 352, 374, 388, 401, 403]. The mice that we monitored for the development of the biallelic population were IL-4-deficient, indicating that biallelic expression of IL-4 also did not require IL-4 to be produced. Therefore, Th2 cells that develop in the absence of IL-4 can produce IL-4 both mono- and bi-allelically *in vivo*.

The allelic expression profile of IL-4 *in vivo* is a potentially important aspect of the Th2 response, and we conceived that it may have demarcated functionally specialised Th2 cells. When we tested whether biallelism specifically demarcated TFH cells, while we observed enrichment in the biallelic population, we also observed strong enrichment in the hCD2+ monoallelic, and to a lesser extent, the GFP+ monoallelic population (Figure 4.8). This indicated that while both TFH development and biallelism were rare, biallelism was not a specific attribute of Th2 TFH cells. Future investigations will be required to determine whether there are functional differences between cells producing IL-4 mono- or bi-allelically.

4.4.5 Modulating IL-4 levels in Th2 immunity

Following our investigations into the role of IL-4 in Th2 cell development, we examined the influence of IL-4 heterozygosity on allergic Th2 immune responses. In these studies, we designed experiments to evaluate how the loss of one IL-4 allele affected IL-4-dependent immune responses.

In these experiments, we first explored the B cell-helper potential of pTh2 cells. First, we clarified the phenotype of pTh2 cells. Our studies demonstrated that 10 to 30% of pTh2 cells were TFH cells, and some of these had a germinal centre TFH phenotype ([366]; Figures 4.6, 4.7 and 4.9). Thus, pTh2 cells had the potential to support B cell class-switch responses. We then confirmed that germinal centre B cells expanded (Figure 4.9), and that IgG1 class-switching was observed in intradermally immunised mice (Figure 4.10). This was associated with the development of systemic antigen-specific IgG1 in mice after immunisation (Figure 4.10 and 4.11). Finally, we confirmed that the IgG1 class-switch response was indeed STAT-6- and IL-4-dependent, as would be expected based on established data ([1, 289, 329–331]; Figure 4.12 and 4.13). Our studies established that the pTh2 assay is a suitable assay in which to examine IL-4-dependent IgG1 class-switch responses.

Primary Th2 cell development and IgG1 class switch recombination

While it is not directly related to our investigations into the role of IL-4, our studies in wild-type IgG1 class-switch responses are worthy of a brief digression. In these studies, we observed concordance between Th2 cell development and the level of IgG1 switching induced by two different immunogens. When dead *iL3 N. brasiliensis* larvae were used as the immunogen, more IgG1-switched cells were detected in the local lymph node than when HDM was used (Figure 4.10). Similarly, more Th2 cells developed in response to dead *iL3 N. brasiliensis* larvae than to HDM (Figure 1.4). Taken together with the TFH phenotype established as IgG1-switch inducing T cells[275], this suggests that class switching occurs in proportion with the number

of Th2 cells an allergen causes to develop. Exploring this correlation with a range of immunogens will be required to validate these findings, but this suggests that at least two aspects of the Th2 response can be observed by primary intradermal immunisation: Th2 cell development and IL-4-dependent class switching.

In addition to IgG1 switching, it will be advantageous in the future to examine IgE switching in this setting. While both IgG1 and IgE require IL-4 for production, IL-21, which is produced by TFH cells[275, 367], can have reciprocal effects on these two isotypes[440–443]. In our studies, IgG1 titres were only elevated significantly to titres of 1/40 or 1/80 (figure 4.11). IgE is produced at 1000-2000-fold lower concentrations than IgG1[287], making it unlikely that we would detect systemic antigen-specific IgE in intradermally-immunised mice. Similarly, detecting IgE class-switch recombination is beyond the sensitivity of most current methodologies; however, newly developed IgE-reporter technologies are shedding light on the processes that lead to IgE production *in vivo*[444, 445]. These may permit the detection of the IgE-inducing capacity of immunogens in a modified pTh2 assay. Therefore, future investigations will therefore require examination of a larger range of allergens and will also need to determine if IgG1 and IgE correlate with Th2 cell development, or alternatively determine if they reflect alternate allergenic properties of immunogens.

4.4.6 IL-4 haplo-insufficiency in Th2 immunity

IL-4 heterozygous mice and IgG1 class-switch recombination

To evaluate the requirement for two intact IL-4 alleles in allergic Th2 immune responses, we examined the IgG1 class-switch response in the pTh2 assay, and subsequently examined the disease phenotype of +/- mice in an oral allergen-induced model of food allergy. When we examined IgG1 class-switching, we observed that the loss of one IL-4 allele decreased allergen-induced IgG1 class-switching by approximately 50% (Figure 4.13A). +/- mice were also shown to have a 90% impairment in adjuvant-based IgE generation, as observed previously ([1]; Figure 4.14). +/- mice were also protected from oral allergen-induced diarrhoea in the intestinal allergy model (Figure 4.16). These findings indicated that both alleles of IL-4 were required

for normal anti-allergen Th2 immune responses. IL-4 heterozygous mice are therefore haplo-insufficient for IL-4.

Current models of antibody production indicate that the manner in which IgE is generated differs from the other major isotypes. In vitro studies indicate that in contrast to B cells of the IgA, IgM and IgG isotypes which can expand 5-15-fold, IgE-producing B cells have essentially no capacity to expand once they are induced to become an IgE-producing cell[284]. These studies are also supported by recent in vivo studies suggesting that once B cells class-switch to IgE they rapidly differentiate into short-lived plasma cells at a reasonably conserved ratio across a progressing *N. brasiliensis* infection or following haptenated protein/alum immunization (PC:GC B cell ratio close to 1:1)[445]. This contrasts with IgG1 class-switched B cells, which become preferentially accumulated in the germinal centre niche (PC:GC B cell ratio initially approximately 1:5 and later approximately 1:5000)[445]. Furthermore, as studies of the secondary immune response using haptenated proteins or *N. brasiliensis* infection reveal that IgE producing cells arise from IgE-negative memory B cells in an IL-4 and T cell dependent manner[274, 287, 288, 306, 407], there is evidence that even in secondary immune response contexts, IgE+ B cells have limited ability to clonally expand.

The concept of a short-lived, poorly expanding IgE+ B cell also has implications for the processes that select a B cell into the IgE producing plasma cell population. While serum IgE affinity increases with time and following boost immunization[306], this is not necessarily because the IgE-switched B cells are undergoing clonal expansion, but could be a result of their continual loss and subsequent rederivation from IgE negative antigen-specific B cells. Following haptenated protein immunization, there is evidence of less affinity maturation in IgE+ plasma cells than IgG1+ plasma cells, but still a high frequency of the IgE+ cells contain germline transcripts associated with high antigen affinity, indicating that a substantial proportion are generated through the extrafollicular pathway[445]. However, there are temporal increases in the number of accumulated mutations and inferred affinity of IgE producing plasma cells[306, 445], and at later timepoints a substantial proportion of these high affinity IgE+ B cells contain sterile gamma-1 switch transcripts indicating that they have passed through an IgG1 intermediate state[279, 306]. Thus, current models suggest that

IgE+ B cells develop both via the extrafollicular route and during the germinal centre reaction.

In our studies, immunization with OVA/alum generated much less IgE in G4/+ mice than in wildtype mice (Figures 4.14 and 4.15). Further, intradermal immunization with HDM demonstrated that IgG1 class-switching was also decreased (Figure 4.13). Intriguingly, anti-IL-4 treatment inhibits T-dependent IgE production in both primary and secondary immunization contexts, but does not suppress secondary immunization-induced T-dependent IgG1 production[287]. Further, while IgE production following anti-IgD immunization is STAT-6 and IL-4 dependent, anti-IgD induced IgG1 production does not require STAT-6 or IL-4[287, 329, 331]. This indicates that the production of IgE is more sensitive to defects in IL-4 than is IgG1 production. It is possible that IL-4 is one of a number of key mediators of the IgG1 class switch, but it is an irreplaceable mediator of the IgE class switch. Mechanistically, this could relate to differences in the amount of IL-4 required to promote IgG1 versus IgE production. *In vitro*, lower concentrations of IL-4 are required to initiate IgG1 production than IgE production[446], which could indicate that a certain threshold level of IL-4 is required for IgG1 and a higher threshold level is required to generate IgE *in vivo*. In support of this, our id HDM immunization data, in which the magnitude of IgG1 production was approximately 100-fold lower than that induced by OVA/alum immunization (antigen-specific IgG1 titres of approximately 1/1000 vs. 1/100000) showed that draining auricular lymph node IgG1 class-switching was decreased in the G4/+ mice compared to wildtype mice. Alternatively, it is also possible that IL-4 heterozygosity reveals a role for IL-4 in the initial induction of IgG1 class-switching in germinal centre B cells, but the expansion of this population thereafter was of sufficient magnitude to mask any measurable effect following protein/alum immunization. As IL-4 signaling has established roles in IgG1 affinity maturation [275], it also remains possible that even with protein/alum immunization the absence of one IL-4 allele has some uncharacterized impact on the quality of the IgG1 response.

In contrast to the IgG1 class-switch, our data indicate that IgE production was sensitive to the amount of IL-4 available at all stages, because IgE production was impaired in G4/+ mice immunized with OVA/alum. As current models suggest that IgE class-switched B cells have limited capacity to expand, the heterozygous cells appear to

be unable to secrete sufficient IL-4 to induce B cells to class-switch to IgE at normal frequencies. As previously mentioned, this may relate to the sheer amount of IL-4 available, or alternatively, could indicate that IgE switching requires highly concentrated synaptic production of IL-4 at the interface between cognate conjugated CD4+ T cells and B cells (i.e., at the immunological synapse). In support of this latter concept, IL-4R-alpha heterozygosity impairs IgE production by approximately 90% yet does not impinge upon IgG1 production[447]. Together with our findings, this could indicate that B cells require either frequent or strong IL-4 receptor stimulation to class-switch to IgE and this can only be provided by CD4+ T cells with biallelic IL-4 producing potential.

While it seems likely from current models that the class-switching to epsilon is impaired in the +/- mice, there are some alternate explanations to the data. Class-switching does not guarantee a B cell will develop into an antibody producing cell[282, 283, 448]. Further, there are reported instances of IL-4-independent IgE production[416, 418] and elevated epsilon mRNA transcript frequencies in IL-4R-alpha-deficient mice[360]. While we consider it to be a less likely scenario, epsilon class-switching may occur normally in the IL-4 +/- mice but these cells may have impaired ability to enter the plasma cell pathway, or require high levels of IL-4 to survive. Finally, it is also possible that both alleles of IL-4 are required to generate the precursor population from which the IgE producing B cells derive. Again, because a substantial proportion of IgE+ B cells have passed through an IgG1 intermediate state[306] and IgG1 titres could be generated in the IL-4 +/- mice, this seems to be unlikely, but remains a possible source of the diminished IgE production we observe. Collectively, these findings have implications for the manner in which an IL-4-producing CD4+ T cell can facilitate IgE production.

Modulating IL-4 levels in other models of Th2 immunity

While the role of IL-4 has been studied many times in IL-4-deficient conditions, studies in which IL-4 levels are modified are less common. Over-expression studies indicated that IL-4 results in severe auto-immune pathologies, were associated with atopic dermatitis-like lesions, hyper-IgE production and IL-4- and CD4+ T cell dependent

self-reactive B cell responses[404, 405]. More recently, mutations in genes that result in over-production of IL-4 have been shown to induce similar atopic dermatitis-type phenotypes and hyper-IgE production[449]. Regulating the amount of IL-4 produced has key implications for the atopic/allergic process. The closest comparative study to the current investigation comes from work done by Kopf *et al.*, and the preceding body of work by vanPanhuyt *et al.* in IL-4-heterozygous mice[1, 450]. Kopf *et al.* examined the effect of IL-4 heterozygosity on *Leishmania major* infection in mice[450]. Despite no requirement for IL-4R α in Th2 cell development[420], in the absence of IL-4 protein Kopf *et al.* observed that footpad swelling in disease-susceptible BALB/c mice was minimised in response to footpad inject with *L. major* promastigotes[450]. Similarly, footpad swelling in +/- mice was reduced, but not ablated[450]. van Panhuys *et al.*, in addition to comparing Th2 cell function in +/+ and G4/+ mice, observed 50% less IgE in anti-IgD or *N. brasiliensis*-infected mice, and a 90% reduction in alum-induced IgE production[1]. Therefore, IL-4 heterozygosity resulted in reduced disease severity in *L. major*-infected mice, and decreased, but did not ablate IgE production in IL-4-heterozygous mice. We confirmed that IL-4-dependent Th2 responses require both IL-4 alleles.

Determining the amount of IL-4 available locally in IL-4-heterozygous mice

Assessing the amount of IL-4 available during local immune responses in IL-4 heterozygous conditions was recently investigated based on the expression of molecules by IL-4-exposed cells. In these studies, *in vitro* IL-4 concentrations above 15 pg/mL increased IL-4R α expression on cultured non-Th2 cells in a STAT-6-dependent manner[334]. However, when IL-4 concentrations exceeded 500 pg/mL, both STAT-6+/+ and STAT-6-/- cells downregulated IL-4R α expression[334]. By examining the expression of IL-4R α on non-Th2 cells in helminth-infected chimeric mice, they observed IL-4R α upregulation in STAT-6+/+ cells, but not downregulation in STAT-6-/- cells[334]. From these data, Perona-Wright *et al.* were able to conclude that the effective concentration of IL-4 in the local lymph node was equivalent to 15 to 500 pg/mL *in vitro* units[334]. In our local lymph node studies in +/- mice, it may be similarly possible

to use IL-4R α expression to approximate the effective interstitial IL-4 concentration. Alternatively, examining IL-4 mRNA transcript numbers, as done previously in IL-4R α -deficient conditions[360] may allow us to approximate the relative defect in IL-4 transcription in IL-4 haplo-insufficient conditions.

4.4.7 Mechanisms that may underlie the haplo-insufficient phenotype of IL-4-heterozygous mice

While we observed impaired IgG1 class switching in +/- mice, at least two mechanisms may underlie this phenotype. Polarised Th2 cell development may have been impaired in +/- mice relative to +/+ mice. Alternatively, a single IL-4 allele may have been sufficient to permit normal Th2 cell development, but their ability to produce IL-4 may have been compromised. Based on the current data, we cannot distinguish between the two.

Th2 cell development may have been compromised in +/- mice. Our studies comparing G4/+ and KN2/G4 mice determined that IL-4 was required for the normal expansion of the Th2 cell population(Figure 4.2 and 4.3). However, we are unable to compare +/- and +/+ mice in the same manner. Different aspects of the Th2 response are sensitive to differences in the concentration of IL-4 to which cells are exposed. For example, *in vitro* studies show that 1000-fold lower concentrations of IL-4 are required to induce IgG1 class-switching than that to IgE[446]. Similarly, lower concentrations of IL-4 are required to induce MHC-II expression than IgG1 class switching of mitogen-activated B cells *in vitro*[288]. In our studies, we observed MHC-II upregulation, but impaired IgG1 class-switching in HDM-immunised +/- mice (Figure 4.13). While these *in vitro* experiments were very different to our *in vivo* system, this indicates that IL-4 was available in +/- mice, but was at decreased levels compared to +/+ mice. It is possible that the amount of IL-4 required for normal expansion of the Th2 cell population also requires lower IL-4 concentrations than IgG1 switching. It may be possible to determine whether Th2 cell development is also impaired by examining mRNA transcript levels of other Th2 cytokines, like IL-5, or examining the development of Th2 cells using a reporter that identifies IL-4 producing cells without interfering with the wild-type genes. Bacterial artificial chromosomes

have been used in the past to report Th2 (IL-4) activity[383]. Similarly, using a BAC transgenic system crossed to +/+, +/- and -/- conditions could accurately determine how the loss of each allele affects Th2 cell development *in vivo*. A similar system is being explored in our lab (Ryan Kyle, personal communication).

It is possible that IL-4 by TFH cells was impaired and Th2 cells developed normally. Deficient IgG1 switching and IgE production could be caused by functional defects in the TFH compartment. TFH were shown, in this and earlier studies, to be enriched in IL-4 producing cells[275, 362, 364]. IL-4-producing cells have also been shown *in vivo* to be directly conjugated to IgE class-switched and IgG1 affinity-matured B cells[275]. Secretion of IL-4 at the immunological synapse, although not exclusively directed towards the site of interaction[451, 452], may increase by many times the concentration of IL-4 to which cognate B cells are exposed. Understanding how genetic lesions in IL-4 production impact Th2 development and B cell responses could clarify some of the most important events that lead to allergic sensitization.

4.4.8 The effect of IL-4 heterozygosity on the intestinal allergic process

Allergic mastocytosis was also impaired in IL-4+/- mice. In the model we used, allergic mastocytosis and diarrhoea require IL-4 signaling[295]. A number of studies demonstrate that local intestinal elevation of IL-4 is associated with diarrhoea development in mice[37, 38, 57, 299]. Studies also demonstrate that adoptive transfer of Th2 cells from the mesenteric lymph nodes of mice with diarrhoea can transfer diarrhoea to naive mice[38]. It is plausible that the loss of a single IL-4 allele reduced CD4+ T cell-derived IL-4 from reaching sufficient levels to promote intestinal mastocytosis. However, Th2-associated cytokines other than IL-4 are also implicated in the intestinal allergic response[37, 57, 295], or can enhance parasite-induced intestinal mast cell expansion[309, 311, 315]. Therefore, it is also possible that Th2 cell development was impaired, resulting in depressed production of other mast cell-active cytokines. A third explanation is also possible. IgE can activate mast cells and prolong their survival[30, 45, 453]. In our studies, we observed that alum-based immunisation resulted in a greater than 90% reduction in systemic IgE titres, both

prior to- and after- allergen challenge (Figure 4.14 and Figure 4.15). Therefore, it remains possible that the apparent defect in oral-allergen induced intestinal mastocytosis was indirect. Thus, we cannot definitively conclude whether the lesion was caused by impaired Th2 cell development, impaired Th2 cell function or impaired IgE production. Clarifying the level at which the impairment in mastocytosis arises has the potential to dissect specific functions of Th2 cells, and specific roles of IL-4 in the full allergic process. It will be useful to further clarify the role of IL-4 in this allergic process in future work.

While CD4+ Th2 cells are implicated in disease development, it also remains possible that the disease was dependent on IL-4 from other cellular sources. Numerous other cell types can produce IL-4[300–305, 363, 425] and, therefore, may contribute to the elevation in Th2-type cytokines that permit jejunal mastocytosis. While the parsimonious explanations remain that either local Th2 cytokine levels were too low to permit disease, or inadequate IgE was generated to sensitize mast cells, clarification of the role of CD4+ T cells in the intestinal allergic process is required.

Collectively, our investigations highlight the importance of IL-4 in the allergic process. We observed a contributory role for IL-4 in adjuvant-free Th2 cell development. We further determined that, like mice lacking both IL-4 alleles[295], missing one allele impaired IL-4-dependent antibody production, inhibited oral allergen-induced mastocytosis and ultimately prevented allergen-induced intestinal anaphylaxis. Determining which aspect causes the largest lesion could help us understand whether targeting IgE, targeting IL-4 or targeting allergen-reactive Th2 cells will provide a better long-term treatment for allergic disease.

4.5 Summary

The pTh2 assay revealed that IL-4 was able to amplify Th2 cell development in a STAT-6-dependent manner. The loss of a single allele of IL-4 impaired the B cell class-switch response to IgG1 in IL-4 heterozygous mice immunised intradermally. IL-4 heterozygosity also caused an impairment in adjuvant-based IgE production and the subsequent intestinal allergic response.

Chapter 5

Establishing an allergy-permissive environment through allergen exposure in the gut

5.1 Introduction

5.1.1 Assessing the role of CD4+ T cells in the post-sensitization phase of food allergy

In 2003, Brandt *et al.*[37], developed an IL-4-, mast cell-, and IgE-dependent model of oral allergen-induced intestinal anaphylaxis[37, 295]. In this system, mice are first sensitized to OVA by intraperitoneal injection of OVA plus alum adjuvant, and are then orally challenged with intragastric (i.g.) doses of OVA every second day, eventually resulting in acute and transient diarrhoea that resolves 120 minutes after oral challenge. One of the intriguing aspects of this system is that multiple allergen challenges are required before mice develop diarrhoea. This indicates that oral allergen challenge of sensitized mice initiates a cascade of inflammatory events that establish the appropriate inflammatory milieu to permit subsequent disease triggering. Interestingly, oral allergen challenge induces jejunal mastocytosis and increases intestinal- and systemic-IgE production, but when jejunal Th2 cytokine levels are not elevated, disease development is inhibited[299]. That is, there is a local inflammatory process invoked by oral allergen challenge that precedes, but does not guarantee, the development of the intestinal allergic response. I will refer to this process as oral potentiation, but more accurately, it is the establishment of a Th2-associated inflammatory environment that greatly exacerbates (and in the model employed, is required for) intestinally-evoked

allergic responses to orally administered allergens in previously sensitized mice.

In addition to the outlined findings, further evidence implicates intestinally-localized CD4+ T cells and Th2-type cytokines in the oral potentiation process[38, 57, 58, 295, 298, 299]. Prior to diarrhoea onset, sequential i.g. OVA challenges increase jejunal IL-4 levels in wild-type mice[37], promotes IL-4 production by intestinal CD4+ T cells[57, 58], and triggers proliferation of- and $\alpha 4\beta 7$ expression on- OVA-specific CD4+ T cells[38]. Adoptive transfer of OVA-restimulated CD4+ T cells from mesenteric lymph nodes of diarrhetic mice transfers disease susceptibility to naive mice[38]. CD4+ Th2 cell responses are also observed in the intestinal tissue of allergic individuals in the clinic[90, 454]. To more closely dissect the involvement of CD4+ T cells in local oral potentiation, we investigated how their depletion at different stages of the post-sensitization allergic process affects disease progression.

Much is still unknown about the immune molecules that govern allergic mastocytosis. This is an important issue, because in the mouse, severity of the orally-induced anaphylactic response correlates with the number of mast cells present in the intestine[36]. It is known that stem cell factor (SCF) is required for the presence of mast cells in the intestine at baseline[307], and IL-3, IL-4 and IL-9 amplify intestinal mast cell infiltration caused by parasite infection[311–313]. In agreement with this contributory role of these cytokines in the intestinal mast cell response, mice deficient in either IL-4 or IL-9 are protected from mast cell-dependent experimental allergic diarrhoea[57, 295]. Similarly, blockade of IL-4R signaling impairs disease development[295] and over-expression of IL-9 increases basal jejunal mastocytosis and promotes intestinal allergic disease[57]. However, it remains to be established whether IL-3 contributes to allergic intestinal mastocytosis and this may be a critical, previously uncharacterized component of the intestinal allergic process.

The experiments in this chapter aimed to clarify the role of CD4+ T cells and mast cell-active cytokines in the orally-induced allergic response.

5.2 Aims

In this section, we investigate the role of CD4+ T cells and non-classical Th2 cytokines in the post-sensitization allergic process.

Our specific aims were:

- 1) To establish if CD4+ T cells are required for allergic diarrhoea;
- 2) To determine whether CD4+ T cells contribute to disease maintenance;
- 3) To elucidate whether IL-3 is involved in experimental food allergy.

5.3 Results

5.3.1 The role of CD4+ T cells in oral potentiation

Our initial experiments focused on the role of CD4+ T cells in allergic diarrhoea. It is established that Th2 CD4+ T cells are increased in intestinal tissues of orally-challenged mice[57, 58] and that adoptive transfer of CD4+ T cells can render naive mice susceptible to allergic diarrhoea[38]. While IgE and mast cells are critical mediators of oral allergen-induced diarrhoea[37], numerous studies demonstrate that the presence of IgE, and of mast cells, does not automatically render mice susceptible to disease[57, 67, 299]. We were therefore interested to determine whether CD4+ T cells were influencing disease susceptibility over and above their established contribution to systemic IgE generation[275, 289, 392].

We first sensitized mice, then subsequently depleted them of CD4+ T cells with GK1.5. Mice were then i.g. challenged with 50 mg OVA and monitored for diarrhoea development. The sensitization, depletion and challenge regimen is shown (Figure 5.1A). We observed that sensitized, mock-depleted mice developed diarrhoea after 6-10 oral challenges, whereas sensitized-CD4-depleted mice were entirely protected from disease (Figure 5.1B). Sixty minutes after the final i.g. challenge, mock-depleted mice lacked colonic pellets and had voided caecae, which contrasted with the visible pellets and normal caecal content of CD4-depleted mice (Figure 5.1G). With sequential gavages we observed increases in total- and OVA specific-IgE titres in mock-depleted mice, which were prevented in CD4-depleted mice (Figure 5.1E and F). We also observed approximately 100-fold less mMCP-1 and reduced jejunal mastocytosis in CD4-depleted OVA-challenged mice (Figure 5.1C and D). This demonstrates that oral allergen-induced jejunal mastocytosis is CD4+ T cell dependent. In a second experiment, we showed that although CD4-depleted mice were protected from diarrhoea they remained susceptible to i.v.-induced systemic anaphylaxis (Figure 5.2 B and 5.2C). This was observed both with and without oral OVA challenge (Figure 5.2 B and 5.2C). While some of the mice in the PBS-fed group succumbed to fatal anaphylaxis, further experiments would be required to validate whether this reflects a protective effect of oral challenge in the absence of CD4+ T cells. It was therefore

specifically the development of an intestinal allergic response that was prevented by depletion of CD4+ T cells. These data indicate that the development an intestinal allergic response was prevented by depletion of CD4+ T cells, however, systemic sensitization was maintained in the absence of CD4+ T cells.

Figure 5.1: CD4+ T cell depletion prevented diarrhoea and oral potentiation. Mice (n=4/group) were sensitized to OVA, depleted of CD4+ T cells with GK1.5 or mock-depleted with Rat IgG and i.g. challenged with OVA as outlined in (A). (B) Diarrhoea development was monitored 45-90 minutes after i.g. OVA challenge of CD4+ T cell-depleted (white boxes) and mock-depleted (black boxes) mice. (C) Chloroacetate esterase stained cells (CAE+; mast cells) in jejunal sections collected 60 minutes after the final challenge. (D) Serum mMCP-1, (E) Total IgE and (F) OVA-specific IgE were analysed by ELISA after the stated number of i.g. OVA challenges. Data are represented as group mean±SEM. (G) Colons and caeca of mock-depleted (IgG) and CD4-depleted (GK1.5) mice were excised 60 minutes after the final challenge and photographed. Data are shown from a single experiment and were compared (C) using Student's *t*-test or (D-F) using Two-way ANOVA with Bonferroni's post-test. **p*<0.05, ***p*<0.01, *****p*<0.0001. mMCP-1 data were log-transformed prior to analysis.

Figure 5.2: CD4⁺ T cell depletion did not prevent systemic anaphylaxis. Mice (n=4/group) were sensitized and depleted of CD4⁺ T cells and subsequently fed with OVA or PBS as indicated in (A). (B) OVA-fed mice (Black triangles) or PBS-fed mice (White triangles) were monitored for diarrhoea development 45-90 minutes following each challenge. (C) 72 hours after the final gavage, mice shown in (B), or naive mice (plus symbols; n=2) were i.v.-administered 100 μ g OVA and monitored for rectal temperature. Data are shown from a single experiment and points show mean $\pm$ SEM of groups. x symbols denote one death in the PBS-fed CD4-depleted group (three total).

To determine whether short-term CD4⁺ T cell depletion conferred long-term protection from allergic diarrhoea, we depleted CD4⁺ T cells from sensitized mice and then allowed them to replenish during the oral challenge period. We observed that peripheral depletion lasted approximately two weeks (Figure 5.3A). During this time, we monitored mice for diarrhoea. Initially we observed that CD4-depleted mice were protected from allergic diarrhoea, however, diarrhoea eventually manifested in 60 percent of mice (Figure 5.3B). This correlated closely in time with the re-emergence of peripheral CD4⁺ T cells (*c.f.* Figure 5.3A and 5.3B). While we observed augmented OVA-specific antibody production in mock-depleted mice, but changes in antibody titres were negligible in the CD4-depleted group until they were CD4-replete (Figure 5.3C and 5.3D). Jejunal mastocytosis, serum mMCP-1 titres, and the amount of caecal washout were reduced in CD4-replete mice compared to mock-depleted mice (Figure 5.3E - G). Taken together, these findings indicate that CD4⁺ T cells play a central role in the development of the intestinally localized allergic response.

Figure 5.3: Diarrhoea development correlated with the re-emergence of CD4+ T cells. Mice ($n=5$ /group) were unsensitized (crosses and grey bars) or sensitized and mock-depleted (black boxes and bars) or i.p. administered 0.5 mg GK1.5 (white boxes and bars) then i.g. challenged with 50 mg OVA as depicted on the x-axis of (D). (A) Live peripheral blood CD3+ cells were examined for CD4 expression by flow cytometry before and after GK1.5 administration, mock-depletion or in unsensitized mice. CD4 depletion was significant versus both other groups at all post-depletion timepoints to $p<0.0001$. Groups were monitored for (B) diarrhoea development, (C) OVA-specific antibody production, (D) total IgE, and (E) endpoint mMCP-1 release 90-120 minutes after challenge. The horizontal dash in (D) shows the linearity limit of the assay. (F) Ninety minutes after the final challenge mice were euthanized and jejunal section were stained with CAE to quantify mast cells. (G) The picture shows caeca from two representative mock-depleted (IgG), CD4-depleted (GK1.5) or unsensitized (Unsens.) mice after final challenge. Data are shown from a single experiment and were compared (C,D) using Two-way ANOVA with Bonferroni post-test versus unsensitized mice; (E,F) using One-way ANOVA with Tukey's post-test; * $p<0.05$, ** $p<0.01$, *** $p<0.001$. OVA-specific antibody and mMCP-1 data were log-transformed prior to statistical analysis.

5.3.2 Segregating CD4+ T cell-dependent mastocytosis and IgE production

Passive sensitization with IgE enhances mast cell survival, antigen responsiveness and histamine release[30, 45, 293, 314, 453]. As well as producing effector cytokines in the tissue[57], CD4+ T cells control memory B cell activation [288, 407] and are the predominant source of IL-4 required for IgE class switching[275, 287, 289, 362]. It was therefore possible that CD4+ T cell depletion prevented antibody titres from reaching sufficient levels to permit disease, and this was the reason the treatment prevented diarrhoea. To determine if systemic antibody production was a limiting factor in intestinal disease development, we increased initial IgE titres by i.p. sensitizing mice multiple times prior to CD4+ T cell depletion (Figure 5.4A). Diarrhoea developed with similar kinetics in twice- and thrice-primed mock-depleted mice (Figure 5.4B). While it was delayed compared to mock-depleted mice, the incidence of diarrhoea also increased at similar rates in twice- and thrice-primed CD4-depleted mice (Figure 5.4B). mMCP-1 production was also impaired in CD4-depleted mice (Figure 5.4C), and only mock-depleted mice had statistically elevated jejunal mast cell numbers of PBS-fed mice (Figure 5.4D). However, there was a clear trend indicating mastocytosis increased in CD4-depleted mice by the end of the experiment (Figure 5.4D). In this experiment, we observed that CD4+ T cell depletion was maintained in both twice- and thrice-primed mice for the first five challenges (Figure 5.4E), but as they returned in thrice-primed mice, Total IgE levels began to increase (Figure 5.4F). However, critical to our results, after five oral challenges, CD4-depleted thrice-primed mice maintained similar Total IgE and OVA-specific IgE levels to mock-depleted twice-primed mice, but were protected from disease (Figure 5.4B-D). By the 5th challenge 50% of twice-primed mock-depleted mice had developed diarrhoea (Figure 5.4B). Thus, antibody titres were sufficiently high in CD4+ T cell-depleted mice to permit disease, but disease did not develop, indicating that CD4+ T cells promoted jejunal mastocytosis in a manner that was independent of their involvement in systemic antibody production.

Figure 5.4: High-titre IgE can only confer disease susceptibility in the presence of CD4+ T cells. Mice (n=5/group) received two (2P; circles) or three (3P; squares) i.p. injections of OVA plus alum adjuvant as indicated in (A), were administered GK1.5 (aCD4; white symbols) to deplete CD4+ T cells or were mock-depleted with polyclonal Rat IgG (+CD4; black symbols) and from week six were i.g. challenged with OVA or PBS (PBS). Twice-primed mice that were fed PBS served as controls (PBS; crosses). The full protocol is shown in (A). (B) Diarrhoea was monitored after each challenge. (C) mMCP-1 was determined by ELISA. (D) CAE+ (Mast) cells were examined in jejunal sections of mice after the last challenge. (E) Mice were periodically bled to monitor peripheral CD4 T cell blood content. (F) Total and (G) OVA specific-IgE were monitored during the experiment. Data are represented as group mean $\pm$ SEM. Data are shown from one experiment and were analysed (C) using Two-way ANOVA, OVA-challenged non-depleted mice versus thrice-primed CD4-depleted mice, or (D) using One-way ANOVA with Tukey's post-test. $p < 0.001$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Similar observations were made in a second experiment comparing only thrice-primed GK1.5-treated mice and twice-primed mock-depleted groups. (n=5/group).

5.3.3 Examining the role of CD4+ T cells in disease maintenance

CD4+ T cells were required for diarrhoea to be exhibited. This lead us to ask if CD4+ T cells maintained local responses after oral potentiation. Next we investigated how CD4+ T cell depletion affected established disease. We orally challenged mice until they presented with diarrhoea, then depleted them of CD4+ T cells (Figure 5.5). Administration of GK1.5 depleted CD4+ T cells to <1 per 4000 peripheral blood CD3+ T cells, whereas mock-depleted animals maintained a proportionally normal T cell pool (Figure 5.5A). This level of depletion was maintained by GK1.5 injection every four days and was also observed in all peripheral tissues, including the intestine at the experimental end-point (Figure 5.5B and 5.5C). Reciprocal increases in the CD8+ proportion were observed in all tissues (Appendices Figure 8.4). Prior to depletion, both OVA-challenged groups had similar mMCP-1 serum titres (Figure 5.5E, left), but after CD4+ T cell depletion, challenge-induced mMCP-1 release was decreased (Figure 5.5E, right). This correlated with fewer mast cells in jejunal tissues of CD4-depleted mice at the experimental end-point (Figure 5.5F), and lower levels of Total and OVA-specific IgE than mock-depleted controls following the depletion (Figure 5.5G). Despite reduction in Th2-associated parameters, diarrhoea ensued in the majority of CD4-depleted mice for at least seven further challenges (7/10 mice; Figure 5.5D). We also observed that while Total and OVA-specific IgG2a titres were unaltered (Figure 5.6A and 5.6B), CD4-depleted mice had lower Total and OVA-specific IgG1 titres than mock-depleted mice (Figure 5.6C and 5.6D), and fewer splenic and bone marrow OVA-IgG1 antibody secreting cells (ASCs) (Figure 5.6E and 5.6F). Total or OVA-specific IgG2a titres were unaltered (Figure 5.6A and 5.6D). These data suggest that while CD4+ T cells maintained underlying disease susceptibility, once instated, the response could be triggered for long periods in their absence.

Figure 5.5: Orally primed mice exhibited diarrhoea even in the absence of CD4⁺ T cells. Mice were sensitized and orally challenged with OVA ten times, at which point all challenged mice had developed diarrhoea. One group was then administered GK1.5 to deplete CD4⁺ T cells and the depletion was maintained throughout the experiment. Sensitized mice that were fed with PBS alone served as baseline controls. Black boxes and bars represent non-depleted mice, white boxes and bars represent CD4-depleted mice, and grey bars and black crosses represent baseline controls. The experimental regimen is outlined on the x-axis in (A). The dashed lines in (A) and (D) represent the point at which CD4⁺ T cells were depleted. (A) Live CD3⁺ peripheral blood cells were monitored for CD4 expression by flow cytometry. (B) After the final i.g. feed, mice were euthanized and blood, bone marrow, spleen, mesenteric lymph nodes (MLN), peters patches (PP) and the jejunal lamina propria (LPL) were examined by flow cytometry for the proportion of live CD45⁺CD3⁺ T cells that expressed CD4. (C) The intraepithelial compartment was assessed for CD4⁺ T cells as in (B). (D) Diarrhoea was monitored in PBS-fed or OVA-fed mice administered GK1.5 or Rat IgG at the dashed line. The photo shows diarrhoea in mice from the specified groups after challenge seventeen. (E) Serum mMCP-1 was measured following challenge ten (left; prior to depletion) and fourteen (right; after depletion). (F) At the experimental endpoint, jejunal sections were examined for CAE⁺ cells. (G) Serum Total IgE and (H) OVA-specific IgE was measured by ELISA. Data in (B-D) are combined from two experiments (n=8 PBS-fed; n=9 Mock-depleted; n=10 CD4-depleted) mice and (A, E-H) are shown from one of the two similar experiments with (n=4-5) mice/group, because the exact timings of the analyses were not identical. Data were compared (A, B, G, H) using Two-way ANOVA with Bonferroni's post-test, (C) using Kruskal-Wallis with Dunn's post-test, or (E, F) using One-way ANOVA with Tukey's post-test; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 5.6: CD4⁺ T cell depletion diminished the Th2-associated antibody response. OVA-sensitized mice fed with PBS (grey bars), or administered GK1.5 (white bars) or Rat IgG (black bars) after ten oral OVA feeds (shown in Figure 5.5) were bled at the experimental end-point. Sera were analysed for (A) Total IgG2a, (B) OVA-specific IgG2a, (C) Total IgG1, (D) OVA-specific IgG1. (E,F) Anti-OVA-IgG1 antibody secreting cells (ASCs) were quantitated in the bone marrow or spleens. Data represents mean $\pm$ SEM of (n=3-5) mice from one of two similar experiments. Data were analysed using One-way ANOVA with Tukey's post-test; *p<0.05, **p<0.01, ****p<0.0001.

5.3.4 The role of CD4+ T cells in systemic responses after oral potentiation

Oral potentiation has been shown to increase the severity of systemic anaphylaxis induced by i.v. allergen challenge[37]. In a final experiment using GK1.5, we examined the development of systemic anaphylaxis in mice depleted of CD4+ T cells after oral potentiation.

Similar to the previous experiment, mice were challenged until diarrhoea presented, and were depleted of CD4+ T cells for the remainder of the experiment. Again, diarrhoea ensued in the absence of CD4+ T cells (Figure 5.7A). In contrast to the previous report[37], when mice were administered 100 μ g OVA i.v. 48 hours after oral challenge, i.p.-sensitized mice demonstrated equivalent hypothermia severity, irrespective of whether they were fed OVA or not (Figure 5.7B). Unexpectedly, evidence of anaphylactic diarrhoea appeared only in mice that had been orally primed, because 10-15 minutes after i.v. challenge, only mice that had previously received oral OVA challenges had matted fur around the anus (Figure 5.7C). This occurred in both CD4-depleted and mock-depleted groups(Figure 5.7C), and was not observed in these mice when temperatures were monitored prior to i.v.-challenge. Antibody levels were similar to those reported in the previous experiment (Figure 5.7D-G). These data suggest that oral potentiation may alter the physiology of the gut, or the gut content, to permit intestinal involvement in the i.v.-induced allergic response.

Figure 5.7: Orally-primed mice developed i.v.-induced anaphylaxis with or without CD4⁺ T cells. (A) Mice were sensitized and fed OVA (boxes) or PBS (crosses). After ten challenges OVA-fed mice were administered three 0.5 mg doses of GK1.5 (white boxes) or Rat IgG (black boxes), 24 hours apart, after the tenth challenge indicated by the dashed line in (A). (B) Forty-eight hours after the final oral challenge mice were administered 100 μ g OVA i.v. and monitored for temperature changes. Completely naive mice (plus symbols) treated with 100 μ g OVA i.v. served as unsensitized controls. (C) mice were photographed 10-25 minutes after i.v. challenge. Bottoms were normal prior to i.v. challenge. Mice were bled 24 hours after the final oral challenge, prior to i.v. challenge and sera were analysed by ELISA for (D) Total IgE, (E) OVA-specific IgE, (F) OVA-specific IgG1, (G) OVA-specific IgG2a. Data represent group mean $\pm$ SEM; n=3 (naive), n=4 (PBS), or n=5 mice/group from one experiment. Data were compared using One-way ANOVA with Tukey's post-test; **p<0.01.

5.3.5 Influence of CD4+ T cells on plasma volume loss

Similar to studies of others[57], we demonstrated that diarrhoea development in orally-primed mice correlated with an increase in blood haematocrit (Figure 5.8A). This provides an additional method by which to assess the development of an intestinal allergic response. In our first experiment in this chapter, mock-depleted mice developed diarrhoea and CD4-depleted mice were protected from diarrhea (Figure 5.1). Similarly, we observed elevated blood haematocrit in mock-depleted mice with active diarrhoea (Figure 5.8B), but not in the CD4-depleted mice (Figure 5.8B and 5.8C), whereas mice that had developed diarrhoea prior to their CD4-depletion showed similar increases in their haematocrit to mock-depleted mice (figure 5.8D). While naive mice were not analysed, the PBS-fed groups and prior-to-challenge groups indicate that there was indeed a change in blood plasma volume associated with the diarrhea. Therefore, mice that presented with diarrhoea lost fluid volume in both the presence and the absence of CD4+ T cells.

Taken together, these data suggest that CD4+ T cells were required for oral allergen-induced intestinal mastocytosis that permitted allergic diarrhoea. Once established, however, intestinal allergy persisted for long periods in the absence of CD4+ T cells. Further mice remained susceptible to systemic i.v.-induced anaphylaxis in the absence of CD4+ T cells with or without prior oral potentiation.

Figure 5.8: **Haematocrit increases in intestinally anaphylactic mice with or without CD4+ T cells.** (A) OVA-sensitized mice were bled before and after the ninth oral gavage with OVA or PBS and their haematocrit was assessed. All OVA-fed mice had active diarrhoea at the time of bleeding. (B) Mice shown in (Figure 5.1) were bled prior to and following the ninth i.g. challenge for haematocrit. (C) Mice depleted of CD4+ T cells prior to being fed OVA or PBS with depletions maintained thereafter (same mice as in Figure 5.2) were bled for haematocrit prior to and following the ninth i.g. challenge. (D) After the thirteenth i.g. challenge, mice shown in Figure 5.7 were bled for haematocrit. data in each (A-D) is from a single experiment (n=3-5/group). Data were analysed using (A-C) Mann-Whitney U-test or (D) using Kruskal-wallis with Dunn's post-test; *p<0.05.

5.3.6 The cytokines that influence allergic mastocytosis

In addition to supporting orally induced IgE production, CD4+ T cells promoted mastocytosis in an additional process. In addition to IL-4 and IL-9, IL-3 is implicated in Th2-type intestinal mast cell responses. Whether oral allergen-induce mastocytosis is dependent on IL-3 is unknown.

As a start point, because IL-3 is associated with a Th2-type intestinal response[315], and allergic diarrhoea is associated with a Th2 cell profile[37, 57, 58], we were interested to determine if Th2 conditioning would trigger IL-3 production from T cells. We examined the production of IL-3 by anti-CD3 stimulated splenocytes cultured under Th0, Th1 and Th2 conditions. When total spleen cells were added to culture medium containing IL-4 (Th2 conditioning medium) and stimulated with anti-CD3 for three days, splenocyte IL-3 production reached approximately 5 ng/mL, whereas under Th0 or Th1 conditions levels were lower, reaching 1 and 0.5 ng/mL respectively (Figure 5.9A). Similarly, when total splenocytes were washed and transferred to uncoated or new anti-CD3 plates without additional IL-4, the amount of IL-3 produced in pre-conditioned Th2 spleen cell cultures was 4-5-fold higher than under Th1 conditions (Figure 5.9B). When parallel cultures were stained for intracellular cytokine, 10% of CD4+ T cells from the Th2 splenocyte cultures stained positive for IL-3 after the six hour culture period, whereas only 2% of Th1 cultured cells contained intracellular IL-3 (Figure 5.9C). Therefore, we determined that Th2 conditions promoted IL-3 production by CD4+ T cells.

Figure 5.9: Th2 cells produced more IL-3 than Th1 cells. (A) Splenocytes were cultured for three days on uncoated (Unstimulated) or anti-CD3 coated (Stimulated) plates under Th0 (grey) Th1 (white) or Th2 (black) conditions and supernatants were analysed for IL-3 by ELISA. (B) After three days stimulation on anti-CD3 under Th0 (grey) Th1 (white) or Th2 (black) conditions splenocytes cultured from individual mice were washed, transferred to restimulation medium and replated in uncoated (Rested) or anti-CD3-coated wells (Restimulated) for 6 hours and supernatants were analysed for IL-3 by ELISA. (C) Side-by-side cultures of cells in (B) had monensin added after 4 hours of culture and were stained for intracellular IL-3 two hours later. Data are represented by mean $\pm$ SEM: (A) individually cultured spleens (n=4) were collated from two experiments; data in (B and C) individually stimulated IL-3^{+/+} spleens (n=7) collated from three separate experiments. In one experiment IL-3^{-/-} mouse spleens (n=2) were included to test antibody specificity, and in (B) one spleen was not analysed under Th0 conditions due to a low cell yield. NA=Not assessed. For restimulation, 500000 cells from activated wells were replated from each condition and restimulated in medium containing IL-2 and anti-CD28. Data were analysed using Two-way ANOVA with Bonferroni's post-test; *p<0.05, **p<0.01, ****p<0.0001.

IL-3 production is known to promote the development of mast cells, but also contributes to basophil development from bone marrow cells. Recent evidence demonstrates that Thymic Stromal Lymphopoeitin (TSLP) can also promote the development of basophils from bone marrow cells[429], and TSLPR-deficient mice show reduced disease incidence in this model[132]. This led us to investigate a potential and unknown role for TSLP in mast cell generation. However, while we showed that TSLP caused a significant increase in the proportion of basophils induced to produce IL-4 (as observed previously[429]) in G4/+ mice (Figure 5.10E and 5.10F), only IL-3-containing cultures caused mast cells to develop (Figure 5.10C and 5.10F). Mast cells are the key effector cell in the ipig model of allergic diarrhoea[37], indicating that while TSLP may be involved in the allergic process[132], IL-3, rather than TSLP, promoted mast cell development from bone marrow cells.

Figure 5.10: **IL-3 but not TSLP caused mast cells to develop *in vitro*.** Bone marrow was isolated from (n=3) +/+ (black bars) or (n=3) G4/+ (white bars) mice cells were cultured from each individual mouse for five days in the presence of 10 ng/mL IL-3, 1 μ g/mL TSLP, both IL-3 and TSLP or medium alone. (A) Total cells were counted by trypan blue exclusion and numbers were used to back-calculate numbers of (B) basophils and (C) mast cells ascertained by flow cytometry. (D) The proportion of mast cells that expressed GFP and (E) the proportion of basophils that expressed GFP were assessed in each culture. (F) Left plots show the cKit^{hi}FceRI+ population used to define mast cells and the cKit^{low}FceRI+ cells that were further examined for DX5 and GFP expression (basophils; right plots). Data are shown from one experiment. Data were compared using Two-way ANOVA with Bonferroni's post-test; **p<0.01 +/+ versus G4/+.

Allergic mastocytosis and diarrhoea were impaired in IL-3-deficient mice.

To determine the potential involvement of IL-3 in CD4⁺ T cell-, IgE- and mast cell-dependent allergic intestinal anaphylaxis, we examined the development of allergic diarrhoea in IL-3^{-/-} mice. When IL-3^{-/-} mice were sensitized i.p. to OVA/Alum, Total and OVA-specific IgE were detected in serum (Figure 5.11B and C). Similar to wild-type mice, their Total and OVA-specific IgE levels increased following oral gavage challenge (Figure 5.11B and C), albeit to lower absolute levels than IL-3^{+/+} mice. IL-3^{-/-} mice were protected from allergic diarrhoea (Figure 5.11A and G) and at the experimental end-point their intestines still contained numerous pellets (Figure 5.11F). Oral OVA challenge did not increase haematocrit in IL-3^{-/-} mice, nor in PBS-fed IL-3^{+/+} mice; haematocrit in both groups remained close to 47.5%, whereas haematocrit was increased to approximately 60% of blood volume in diarrhoetic OVA-fed IL-3^{+/+} mice (Figure 5.11D). When we examined the mast cell response, mMCP-1 levels were lower in IL-3^{-/-} mice (Figure 5.11E), and this correlated with fewer jejunal mast cells in OVA-fed IL-3^{-/-} mice than IL-3^{+/+} mice (Figure 5.11F). Differences in jejunal mast cell frequencies at baseline were not statistically distinguishable, in line with no requirement for IL-3 for basal mastocytosis as reported by other groups[309]. In preliminary examinations we also observed similar Th2 cytokine production in spleen cell preparations from OVA-sensitized IL-3^{+/+} and IL-3^{-/-} mice (Appendix Figure 8.5A - D). Further, similar to an earlier study[455], we found that both IL-3^{+/+} and IL-3^{-/-} mice were susceptible to i.v. allergen-induced systemic anaphylaxis (Figure 5.12B). This occurred even when IL-3^{-/-} mice were fed with OVA (Figure 5.12B). Therefore, these data suggest that IL-3 was required for the generation of an intestinally-localised mast cell-dependent allergic response.

Figure 5.11: IL-3^{-/-} mice were protected from allergic diarrhoea. (A) OVA-sensitized IL-3^{-/-} and IL-3^{+/+} mice were i.g. fed with OVA or PBS and monitored for diarrhoea development. (B) Total IgE and (C) OVA-specific IgE were measured by ELISA. (D) Haematocrit (%PCV) was measured 50 minutes after challenge 9 and (E) mMCP-1 release was measured after challenge ten. (F) Jejunal sections from mice challenged ten times were stained for CAE⁺ (mast) cells. Photographs were taken of (G) colons 90 minutes after challenge ten or (H) bottoms 60 minutes after challenge nine. Genotypes are specified at the top of the page. (A) Diarrhoea curves are shown from one of five similar experiments each with (n=4-5) mice/group. (D) %PCV was measured once and all other data were analysed in three of the experiments with similar results in each case. Data are represented by mean $\pm$ SEM. Data were analysed (B,C) using Two-way ANOVA with Bonferroni's post-test, OVA-fed IL-3^{-/-} vs. PBS-fed IL-3^{-/-} mice or (D-F) using One-way ANOVA with Tukey's post-test; *p<0.05, **p<0.01, ***p<0.001.

Figure 5.12: **IL-3^{-/-} mice were susceptible to i.v.-induced systemic anaphylaxis.** (A) Wild-type and (B) IL-3^{-/-} mice were sensitized to ovalbumin and challenged six times with PBS or OVA and then received 100 μ g OVA i.v. and temperature change was monitored. Data are represented by mean $\pm$ SEM from a single independent experiment (n=2-3 mice/group).

5.3.7 The relative influence of IL-3 and IL-9 on allergic mastocytosis

Mice carrying a single allele of the *iFABPp*-IL-9 transgene (Tg+/+) over-express IL-9 locally in the jejunum[57]. This increases basal mast cell activity and predisposes mice to allergic diarrhoea and is also associated with higher oral allergen-induced mast cell activity compared with non-transgenic mice[57]. We next investigated whether over-expression of IL-9 could amplify jejunal mastocytosis in the absence of IL-3. In these experiments we compared Tg+/+ mice to IL-3-deficient mice carrying the transgene (Tg-/-), and sib-matched non-transgenic IL-3-deficient mice (nTg-/-). nTg-/- is used to distinguish between IL-3-/- mice and IL-3-deficient mice sib-matched to Tg-/- mice.

When sera were analysed from naive Tg+/+ mice, Tg-/- and nTg-/- mice, mice carrying the transgene had 4- to 5-fold higher serum mMCP-1 levels than nTg-/- mice (Figure 5.13A). Similarly, when jejuni were sectioned and examined for CAE+ mast cells, CAE+ cells were on average, twice as frequent in naive Tg-/- mice than naive nTg-/- mice(Figure 5.13B). CAE+ cells were present at similar levels in naive Tg-/- mice as in naive Tg+/+ mice (Figure 5.13B). Therefore, in the absence of IL-3, over-expression of IL-9 in the jejunum increased basal jejunal mast cell activity.

Figure 5.13: The *iFABP*-IL-9 transgene enhances basal jejunal mast cell activity in IL-3-deficient conditions. (A) serum mMCP-1 and (B) Jejunal CAE+ cells were determined in naive mice of the specified genotype. Pictures are representative sections of CAE staining from one high power field (HPF). Lines represent mean $\pm$ SEM of (n=17 mice/group) combined from 3 separate experiments using (n=5-7 mice/group). Data were analysed using One-way ANOVA with Tukey's post-test; *p<0.05, **p<0.01.

Next, we compared the development of allergic diarrhoea in Tg+/+, Tg-/- and nTg-/- mice. After two i.p. primes with OVA, mice of all three genotypes had approximately 2 $\mu\text{g/mL}$ Total IgE and OVA-specific IgE levels were similar (Figure 5.14D-F). With multiple i.g. OVA challenges, OVA-specific IgE levels increased to similar levels in the three OVA-fed groups (Figure 5.14D) and Total IgE levels increased to 4-6 $\mu\text{g/mL}$ in all three groups of OVA-fed mice by the experiment end-point (Figure 5.14E-G). Similar to our previous observations in IL-3-/- mice, nTg-/- mice did not develop diarrhoea (Figure 5.14A). Tg-/- mice also did not exhibit symptoms of diarrhoea (Figure 5.14A). mMCP-1 levels were low in PBS-fed nTg-/- mice and did not increase during gavage challenge (Figure 5.14B). Tg+/+ and Tg-/- mice again had elevated baseline mMCP-1 levels compared to nTg-/- mice and these remained low in PBS fed groups throughout the experiment (Figure 5.14B). OVA-fed mice of all genotypes had 100- to 200-fold increases in serum mMCP-1 levels compared to PBS-fed counterparts (Figure 5.14B) and these were highest in OVA-fed Tg+/+ mice, reaching ≈ 50000 ng/mL following the 10th i.g. OVA challenge (Figure 5.14B). While mMCP-1 levels in Tg-/- mice were elevated over nTg-/- mice by ten-fold after challenge 5, their mMCP-1 levels did not reach the levels in Tg+/+ mice at any post-challenge point (Figure 5.14B). When we examined jejunal mast cell infiltration at the experimental end-point, jejunal mast cell density was highest in OVA-fed Tg+/+ mice (Figure 5.14C) and the number of mast cells correlated approximately with serum mMCP-1 concentration (*c.f.* figure 5.14B and C). When we examined mesenteric lymph node cell expression of IFN- γ , IL-3, IL-4, IL-5 and IL-13 upon restimulation with OVA no significant differences were observed IL-3 between Tg+/+, Tg-/- and nTg-/- cultures except for the absence of IL-3 in IL-3 deficient mice (Appendix Figure 8.6A - E). These data suggest that high local IL-9 levels were not sufficient compensation to permit oral allergen-induced mastocytosis in the absence of IL-3.

Figure 5.14: **IL-3 is required for diarrhoea development in mice with high basal mast cell activity.** OVA-sensitised Tg+/+ (Squares), Tg-/- (circles), nTg-/- (triangles) mice (n=4/group) were fed with OVA (black) or PBS (white). (A) Diarrhoea development was monitored after oral gavage. (B) During the experiment, mice were bled after the stated number of challenges and serum mMCP-1 levels were determined by ELISA. (C) At the experiment end-point, jejunal sections were stained for CAE+ cells in the indicated groups. (D) OVA-specific IgE and (E-G) Total IgE were examined in each strain. Data are shown from a single experiment and represent group mean $\pm$ SEM. Data were compared using Two-way ANOVA with Bonferroni's post-test; ****p<0.0001.

With these experiments we demonstrated that IL-3^{-/-} mice had impaired allergen-induced jejunal mast cell infiltration. Next we tested whether one IL-3 allele was sufficient to permit oral allergen-induced mastocytosis, or whether both IL-3 alleles were required. *iFABP*-IL-9 mice are predisposed to oral sensitisation[57], so in this experiment, mice were orally challenged seven days after their i.p. prime. In this setting, when Tg^{+/+} mice were orally challenged with OVA, disease penetrance did not reach 100%, precluding interpretation of the impact of IL-3-heterozygosity on diarrhoea (Figure 5.15A). However, we were able to investigate the relative level of oral potentiation that had occurred by examining serum mMCP-1, IgE and jejunal mast cell infiltration. We observed that despite similar OVA-specific IgE levels at the experiment end-point (Figure 5.15C), jejunal mastocytosis was only significantly elevated in Tg^{+/+} mice, indicating that the loss of one IL-3 allele resulted in a partial impairment in intestinal mast cell infiltration (Figure 5.15B).

Numbers of CAE⁺ cells were higher in Tg^{+/-} mice compared to naive Tg^{+/+} mice (shown by the dashed line in figure 5.15B), indicating that the increase in mast cells was not due to the presence of the transgene alone (Figure 5.15B). In this modified experimental system, Total IgE did not increase after i.g. OVA challenge (Figure 5.15D). i.g. OVA challenge caused mMCP-1 levels to increase in all groups of mice. Following the 9th challenge mMCP-1 levels correlated with the number of CAE⁺ cells, and not with OVA-IgE O.D. values (*c.f.* figure 5.15B and E, versus figure 5.15B and C). These data indicate that the loss of one IL-3 allele caused a partial reduction in allergen-induced intestinal mastocytosis and further suggest that the IL-9 transgene was able to increase oral-allergen induced mast cell activity in IL-3 heterozygous conditions.

Figure 5.15: Lack of a single IL-3 allele causes a partial defect in allergen-induced intestinal mastocytosis. (A) Seven days after a single i.p. sensitisation, mice (n=5/group) were fed 50 mg OVA and monitored for diarrhoea development. (B) At the experimental end-point, jejunal CAE⁺ cells were quantified in each strain. The dashed line shows the mean number of mast cells in naive Tg^{+/+} mice sectioned in this experiment. (C) Before (0) and after (9) nine oral OVA challenges sera were analysed for OVA-specific IgE, (D) Total IgE and (E) mMCP-1 by ELISA. Data are represented by group mean±SEM (n=5 mice/group) from a single experiment. **p<0.01, ***p<0.001 by One-way ANOVA with Tukey's post-test.

5.3.8 Assessing the role of IL-3 in i.v.-induced intestinal allergy

In our studies with GK1.5 we observed that after a rest period, i.v. challenge of orally primed mice resulted in diarrhea. We sought to expand on these studies by quantifying the relative level of diarrhea by measuring the fur matting around the anus of mice following i.v. challenge. We also examined whether IL-3 was required for the i.v.-induced diarrhea. Mice were sensitized to OVA plus alum and then orally challenged 10 times with OVA, at which point 75% of OVA challenged IL-3^{+/+} mice had diarrhea (Figure 5.16A). Three days later, mice were i.v. challenged with 100

μg OVA and all sensitized groups exhibited hypothermia (Figure 5.16B). However, in contrast to the results of Figure 5.7, mice in all groups, including naive animals exhibited some fur matting (Figure 5.16C and D). While the fur matting was most pronounced in the orally primed IL-3+/+ mice, mock-primed wildtype mice also exhibited significant fur matting and diarrhea (Figure 5.16C and D). The average amount of fur matting was lower in the sensitized IL-3 deficient mice, despite at least as severe i.v.-induced anaphylaxis (Figure 5.16B - D). Taken in conjunction with the results of Figure 5.7, non-allergic parameters seem to contribute to diarrhea and fur matting in this context, such as introduction of the rectal probe and the pre-heating step. Therefore i.v.-induced fur matting may lack the sensitivity required to measure the severity of disease, but could prove useful with further optimisation or without the rectal probing step. With these preclusions in mind, the results of Figure 5.16 and Figure 5.7 indicate that IL-3-dependent oral potentiation exacerbates, rather than is necessarily required for i.v. allergen-induced diarrhea.

Taken collectively, our findings illustrate that IL-3, in addition to the roles established for IL-4 and IL-9[57, 295], is involved in oral allergen-induced jejunal mast cell infiltration.

Figure 5.16: Systemic anaphylaxis and fur matting in IL-3 sufficient and deficient mice. (A) Mice were sensitized and orally challenged with OVA. (B) Three days after the final oral challenge mice were i.v. challenged with 100 μ g OVA. Naive mice also received i.v. 100 μ g OVA. (C) Photographs were taken 50 minutes after i.v. challenge. (D) Zero, twenty and fifty minutes after i.v. challenge the area of fur matting was calculated using $\pi*((x/2)*(y/2))$; where x and y are the horizontal and vertical diameters of fur matting around the anus. Data are represented by group mean $\pm$ SEM (n=4 mice/group) from a single experiment. **p<0.01, ***p<0.001 by One-way ANOVA with Tukey's post-test. White symbols in (D) represent mice that were not measured at earlier timepoints.

5.4 Discussion

In the model we used, multiple oral feeds with OVA are required for mice to present with diarrhoea. Within the first three challenges, antigen-specific CD4+ T cells up-regulate intestinal homing molecules, jejunal IL-4 mRNA levels become elevated, intestinal antigen-specific IgE titres rise, mMCP-1 mRNA transcripts are more frequent and mastocytosis is observed[38, 299]. We refer to this process as oral potentiation and these changes are causatively or correlatively linked with the development of disease[37]. The aim of the experiments in this chapter was to clarify the immune processes involved in oral potentiation. Initially, we examined the contribution of CD4+ T cells to allergen-induced mastocytosis. We then examined the role of IL-3 in the allergen-induced intestinal mast cell response.

5.4.1 The role of CD4+ T cells in oral potentiation

Our first studies were proposed based on experimental evidence implicating local CD4+ T cells in oral potentiation. In three similar models, oral challenge of pre-sensitized mice increases Th2-associated cytokine levels in the intestinal tissues, and causes specific enhancement of Th2-type cytokines upon restimulation of CD4+ T cells. This Th2 cytokine profile is observed in mice orally challenged with OVA after: i) intraperitoneal sensitization with OVA plus alum adjuvant[37, 38, 57, 299]; ii) subcutaneous sensitization with OVA plus complete Freund's adjuvant[58]; and iii) oral sensitization with OVA plus cholera toxin[298]. This profile is also observed when milk proteins or peanut allergens are used in similar models that also result in diarrhoea, intestinal permeability and vascular leak[149, 456]. Further, in all of these oral challenge models, mast cell and eosinophil influx follow oral challenge[37, 42, 58, 149, 456]. In all three OVA-challenge models, the allergic response is critically dependent on IL-4/STAT-6 signalling, and in the model we have employed, has been shown to be dependent specifically on IgE and mast cells[57, 58, 295, 298].

In our studies, we have confirmed a central role for CD4+ T cells in the development of diarrhoea and identified two different roles for CD4+ T cells in oral potentiation. When CD4+ T cells were depleted systemically prior to OVA feeding, oral potenti-

ation did not occur (Figure 5.1). Allergen-induced mast cell activity was essentially ablated in CD4-depleted mice: mMCP-1 production was 100-fold lower and jejunal mast cell numbers averaged two per high power field, compared to approximately 35 in mock-depleted orally challenge mice. This is similar to the number we observed in unchallenged mice in other experiments. IgE production was also diminished relative to non-depleted mice, as would be expected. However, this alone was not sufficient to explain the impairment in allergen-induced mast cell recruitment, because hyper-primed, CD4-depleted mice that had IgE levels sufficient for disease exhibited delayed disease onset (Figure 5.4). These findings lead us to conclude that intestinal mastocytosis is a process independent of augmented IgE production. A previous report demonstrated that treatment of mice with FTY720, a drug that causes accumulation of lymphocytes in lymph nodes and Peyer's patches and prevents their dissemination[457] does not prevent oral potentiation[299]. Taken in this light, we consider the mast cell component of disease to be controlled by intestinally-localised Th2 mast cell-inducer (Th2m) cells, which are functionally distinct from IgE-promoting follicular T helper (TFH) cells.

5.4.2 Distinct roles for CD4+ T cells in intestinal mastocytosis and IgE generation

The requirement for CD4+ T cell function in establishing the local inflammatory milieu in the intestine in addition to supporting IgE production is a potentially important feature of the allergic process. Current evidence suggests that IgE production is controlled by IL-4-producing TFH cells found in lymph nodes[275] and lymph node- and tissue-localised CD4+ T cells are differently programmed and functionally distinct[275, 317, 368, 369]. Following helminth infection, CD4+ T cells found in the lung produce increased levels of IL-13, and significantly less IL-21 than do CD4+ T cells in the lymph nodes[275, 317]. Further, the acquisition of TFH function, or programming to egress from the lymph node to alternate sites are in part dictated by T cell receptor (TCR) antigen affinity[369]. This bifurcation of antigen-experienced cells can be observed prior to the development of germinal centres soon after T cell activation[368]. Therefore, TFH cells and Th cells that enter the tissues may arise

from separate precursor populations. In terms of the oral potentiation response, it is possible that populations of OVA-reactive CD4+ T cells that promote IgE production are distinct from those that control local mastocytosis. However, in the model we used for experimentation, mice received their first challenge four weeks after their initial sensitization. In this period, ample time existed for T cell redistribution to multiple sites. TFH cells can enter tissues, because adoptively transferred IL-21-reporter-expressing cells are found in the lungs of influenza-infected mice[367]. Furthermore, in a similar model to the one we used, both CD4+ Th2 cells and IgE producing B cells have been identified in the colonic lamina propria[58]. While IgE is not sufficient for mastocytosis on its own, interaction with B cells in the intestine may be required for Th2m cell function. However, irrespective of local B cell involvement, the data clearly indicate that CD4+ T cells control local intestinal mast cell infiltration.

5.4.3 The role of CD4+ T cells in disease maintenance

While CD4+ T cell depletion prevented oral potentiation and disease development, CD4+ T cell depletion was not sufficient to tolerise mice to allergen. In our temporary depletion studies, mice were protected from disease only while their CD4+ T cells were absent; acute and transient oral allergen-induced diarrhoea was observed in mice soon (three challenges) after CD4+ T cells emerged in the periphery (Figure 5.3). It is known that T cells are involved in oral tolerance[137, 143, 458] so to some extent, this result was to be expected. Sensitized CD4-depleted mice also remained susceptible to i.v.-induced systemic anaphylaxis (Figure 5.2), so the initial sensitization was sufficient for systemic, but not intestinally-localised allergic responses. A similar potentiating role is attributed to CD4+ T cells in the development of allergen-induced airways hyperresponsiveness. In sensitized and subsequently CD4-depleted mice, challenge-induced airways eosinophilia is ablated and subsequent airways hyperresponsiveness is prevented[459]. Intriguingly, while eosinophilia is observed in lungs of intranasally allergen-challenged mice[294] and in the intestines of orally allergen-challenged mice[37, 42, 58], neither eosinophils nor IL-5 contribute to the CD4-dependent respective airways hyperresponsiveness or diarrhoea[37, 294, 459]. The parallels in the processes indicate that the events that permit the triggering of

an allergic response in the lung or the gut mucosa are separate events from systemic sensitization.

The processes that maintain disease have also been investigated. IL-4 and IL-13, are critical in the oral potentiation period, but once disease is established systemic administration of anti-IL4 or anti-IL-13 antibodies does not prevent disease triggering[295]. Our findings are compatible with these findings. We observed that in the absence of CD4+ T cells, which are the most likely source of IL-4 and IL-13 required for oral potentiation, most mice remained susceptible to disease (Figure 5.5). Although the oral potentiation parameters remained elevated, we also observed an eventual diminution in disease incidence, and reduced mast cell activity in CD4-depleted mice, indicating that the long-term response was indeed still potentiated by CD4+ T cells (Figure 5.5). Taken together, the FTY720 studies in which oral potentiation was not inhibited[299], the partial attenuation in disease we observed following CD4-depletion (Figure 5.5), and segregation of IgE production from mastocytosis in our potentiation experiments (Figure 5.4), the data indicate that Th2m cells are required in the intestinal tissue itself. Although these findings indicate that intestinally localised Th2m cells are required for optimal oral potentiation, we also demonstrated that once established, the susceptibility conferred by oral potentiation is reasonably long-lived.

In our experiments, oral potentiation did not increase temperature loss induced by i.v. allergen administration (Figure 5.7 and 5.16). This contrasts with a previous report that indicated the severity of anaphylaxis increases in orally primed mice[37]. This may relate to the contributions of IgG-mediated processes to the anaphylactic response. While IgE-dependent systemic anaphylaxis is mast cell-dependent[43], IgG-bound basophils and macrophages can contribute to systemic anaphylaxis[40, 41, 47]. Additionally, systemic administration of antigen-specific-IgG1 can inhibit IgE-mediated anaphylactic responses induced orally in mice[67]. It is possible that significant differences in hypothermia would have been revealed if IgG-dependent processes were first neutralised in our studies. We also note that diarrhoea penetrance in the studies by Brandt *et al.* generally reached 100 percent within 6 challenges[37], whereas in our lab it can require ten or more challenges for it to be observed in all mice. Previous studies also demonstrate that low doses of allergen require more challenges to invoke diarrhoea[37]. It is possible that oral potentiation could occur faster if

we used higher challenge doses and may similarly be required for oral potentiation to heighten the systemic response. We did, however, observe liquid stools and fur matting 10 to 25 minutes after i.v. allergen challenge of orally primed mice (Figure 5.7), which indicates that the anaphylactic response was more severe in orally-primed mice, in line with the findings of Brandt *et al.*[37]. However, as noted in the results section, although fur matting measurements indicated that it remained 'less severe' than orally-primed mice, we also observed diarrhea in some i.v. challenged naive mice (Figure 5.16), indicating that additional non-allergy associated parameters of the experimental regimen could contribute to the i.v.-induced diarrhea. Determining whether i.v.-induced intestinal anaphylaxis is severe enough to be observed without introduction of the rectal probe, and further, whether this requires sensitization, or is a property for which oral OVA-feeding is sufficient needs to be addressed in future studies. The heating requirement could be removed if allergen were administered i.p. instead of i.v. in mice and permit better characterization of the i.v.-induced intestinal allergic response.

5.4.4 Clarifying the earliest events in the initiation of oral potentiation

Determining whether the first step involved in oral potentiation requires the presence of CD4+ T cells or will occur in their absence will also be important to investigate in future studies. Allergen-reactive CD4+ T cells are recruited to the allergen-exposed intestine[38, 58], which argues that the local inflammatory milieu promotes CD4+ T cell recruitment. If the earliest events in the oral potentiation process require the presence of CD4+ T cells, the implications for disease therapy will be different than if the earliest events that promote CD4+ T cell responses are initiated as a preceding independent process. CD4+ T cell activation in the mesenteric lymph node is thought to be a key step in the oral potentiation process[38], which predicates allergen and/or antigen-presenting cell transit to the mesenteric lymph node after oral challenge. OX40L-expressing dendritic cells are demonstrated as Th2-promoting cells in the intestine[434] and allergen binding to epithelial cells is associated with more severe intestinal allergic responses[139]. The way innate and non-haematopoietic cells

respond to allergen can influence the allergic process, so determining whether these changes occur in the absence of antigen-specific CD4+ T cells has the potential to determine the most appropriate targets for therapeutic intervention in atopic individuals.

There are a number of ways of further assessing the requirement for CD4+ T cells in the initiation of oral potentiation. First, if the CD4+ T cell population was depleted for a short period (two to three challenges), and then allowed to replenish, examining mastocytosis in the absence of further challenges would indicate whether or not the potentiation process is initiated by oral exposure in the absence of CD4+ T cells - Without further challenges, do mast cells infiltrate the intestine as CD4+ T cells re-emerge? If they do, this will provide evidence that oral potentiation is initiated in the absence of CD4+ T cells. As antibody treatment can be leaky, an adoptive transfer method could also be employed. Studies have demonstrated that adoptively transferring OVA-specific CD4+ T cells to naive mice, then feeding the mice OVA induces OVA-specific CD4+ T cell proliferation in mesenteric lymph nodes and causes their dissemination to the spleen[38]. In future work, mice could be depleted of CD4+ T cells, gavaged with OVA or PBS, and then receive OVA-specific CD4+ T cells once they were CD4-replete. If the transferred OVA-specific CD4+ T cells were preferentially sequestered to the jejunum, Peyer's patches or mesenteric lymph node of the OVA-fed group, this would indicate that their recruitment was initiated in their absence. Treating naive mice in a similar manner would be plausible, but may be confounded because oral exposure without sensitization inhibits Th2 polarisation[134, 143, 458]. This may affect the response of the adoptively-transferred cells. Oral potentiation has been linked to CD4+ T cell $\alpha 4\beta 7$ and CCR-6 expression[38, 299], so monitoring these molecules on emerging CD4+ T cells in CD4-replete mice could also link intestinal homing to disease. Lastly, it will be desirable to determine the frequency and activation status of OVA-specific CD4+ T cells in the gastrointestinal immune system of CD4-replete mice. These studies will have the potential to determine whether the earliest event in oral potentiation is CD4+ T cell dependent, or alternatively, if CD4+ T cells are simply a stepping-stone in an already catalysed process.

5.4.5 Requirement for oral potentiation for diarrhoea in different contexts

It is important to note that oral potentiation is not required for disease development in all allergy models. In rats, peripheral sensitization is sufficient to induce an antigen-specific IgE mediated response in the intestine[44, 54, 59]. The first intestinal exposure disrupts motility and causes diarrhoea if allergen is intubated directly into the duodenum or colon[44, 53, 59, 460]. Antigen-specific allergic intestinal responses have also been observed on first antigen challenge in subcutaneously sensitized mice[140], and those born to orally-sensitized dams[20]. In systems very close to the model we have used, diarrhoea has been observed on the first challenge. In mice with elevated intestinal mast cell numbers that are predisposed to diarrhoea development, allergic diarrhoea has been observed without an oral potentiation period, but this is again rare[67]. There is at least one other report in which diarrhoea was observed on the first challenge of a mouse[299]. This suggests that in some circumstances, the presence of IgE and mast cells are sufficient for diarrhoea to develop. However, a direct correlation between intestinal mast cell numbers and the severity of the orally-induced anaphylactic response has been demonstrated[36], and mast cell numbers increase significantly following multiple gavages[37, 38]. Further, mice transgenically modified to have high basal mast cell activity[57] and mice with naturally increased mast cell numbers[39] demonstrate heightened allergic disease susceptibility. Collectively, these data suggest that intestinal disease is influenced by the level of mastocytosis, which is increased during the CD4+ T cell dependent establishment of the diarrhea-permissive local inflammatory milieu in the intestine.

Our studies have investigated the role of CD4+ T cells in the intestinal allergic process. We find that intestinal mast cell recruitment is controlled by local CD4+ T cell activity. CD4+ T cells also promote systemic IgE production. While these processes culminate in intestinally-localised allergic responses, they can be attributed to independent functions of CD4+ T cells.

5.4.6 The role of IL-3 in mast cell-dependent Th2 immunity

Once we had determined a causal role for CD4⁺ T cells in the oral potentiation process we next investigated the potential involvement of IL-3 in intestinal allergy.

The rationale for investigating IL-3 comes from previous work identifying a role for IL-3 in Th2-associated intestinal immunity and also in some models of hypersensitivity. Parenteral allergic responses, including mast cell-dependent[43] IgE-mediated passive cutaneous anaphylaxis and OVA-induced active systemic anaphylaxis proceed unencumbered in the absence of IL-3[455]. In contrast, symptoms of oxazolone-induced contact hypersensitivity and IgE-triggered serotonin release are reduced in IL-3^{-/-} mice[396, 455]. In wild-type mice, systemic administration of IL-3 increases jejunal mast cell deposition and systemic basophil generation[301, 311, 461], whereas blockade of IL-3 signaling prevents intestinal mastocytosis in response to parasite infection[462]. Both parasite infection and oral-allergen exposure induce intestinal permeability, mast cell recruitment and mMCP-1 production in mice[37, 50, 463]. Although IL-3 has been implicated in the regulation of IgE-mediated allergic reactions, the contribution of this cytokine to oral allergen-induced intestinal allergic responses has not previously been explored.

IL-3 is known to be produced by T cells[322, 464] and T cell derived IL-3 is known to promote mast cell development[465]. When we examined the potential involvement of IL-3 in a Th2-type response, we observed that culture with IL-4 increased the amount of IL-3 produced by anti-CD3-stimulated splenocytes. At least in part, this was due to IL-3 production by CD4⁺ T cells. Confirming a wealth of previous reports (e.g., [465, 466]), we observed mast cell and basophil development from bone marrow cells cultured with IL-3. In contrast, while TSLP has recently been identified to promote IL-3-independent, IL-4-producing basophil development[429], only IL-3 gave rise to mast cells under the same culture conditions. Further, unlike the basophils that were present in cultures containing IL-3 and/or TSLP, the mast cells that developed did not express GFP, which reflects IL-4 production in the G4 mouse[273]. From these data, we conclude that IL-4 promoted IL-3 production by T cells. We also conclude that CD4⁺ T cells may contribute to intestinal mastocytosis in Th2 settings through

the production of IL-3.

5.4.7 The role of IL-3 in intestinal food allergy

IL-3 deficiency impaired the development of an intestinal allergic response in mice. When we monitored disease development in sensitized and orally challenged IL-3^{-/-} mice, they neither developed diarrhoea nor lost plasma volume; faecal pellets and caecal content were also retained. While mastocytosis was diminished in IL-3^{-/-} mice relative to wild-type mice, oral challenge increased systemic IgE titres, indicating that CD4-dependent responses still occurred. We also showed that IL-3-deficient mice remained susceptible to systemic anaphylaxis, as established previously[455]. From these data, we conclude that IL-3 was specifically required for intestinal allergic mast cell responses.

When we assessed the relative contribution of IL-3 and IL-9 to intestinal mastocytosis, our data confirmed that IL-3 was required for intestinal mast cell deposition. Over-expression of IL-9, which augments mastocytosis in IL-3-sufficient mice[57, 312], also increased jejunal mast cell numbers in IL-3-deficient mice, but was not sufficient to permit disease, or bypass the requirement of IL-3 in allergen-induced mastocytosis. Not only did this confirm that IL-3 was required for allergen-induced intestinal mast cell recruitment, but it also provided further evidence that antibody production and intestinal mast cell recruitment were independent processes. Similar observations have been made in IL-9-deficient settings[57] and support our conclusions. To confirm that the difference in IgE levels in IL-3-deficient and IL-3-sufficient mice do not underlie their protection from disease, testing whether IL-3-deficient mice develop disease when given IL-3 exogenously, as done previously in parasite infection settings[301, 311, 461], will validate the findings of our study. However, as IgE levels were similar in Tg^{+/+} and Tg^{-/-} mice, but only Tg^{+/+} mice presented with diarrhoea, we consider it unlikely that discrepancies in IgE titres underlie the differences in the intestinal mast cell response of IL-3^{+/+} and IL-3^{-/-} mice. From these data, we conclude that oral allergen-induced mastocytosis is partially dependent on IL-3.

5.4.8 The source of IL-3 that drives allergic intestinal mastocytosis

Intestinal CD4⁺ T cells are a probable source of the IL-3 that promoted the allergic intestinal mastocytosis. Intestinal helminth infection normally induces 1000-fold increases in IL-3, but if T cells are first depleted, Peyer's patch IL-3 levels are 10-fold lower than in naive mice[315]. Both IL-3 and T cells are required for helminth-induced intestinal mastocytosis, and this expedites the expulsion of some parasites[307, 309, 311, 314, 462, 467]. Thus, intestinal T cells are a potent source of mast cell-inducing IL-3. This will, however, need to be validated in a food allergy model, because committed mast cell progenitors are found in the blood and bone marrow[314, 468, 469]. It is therefore possible that IL-3 is required systemically, rather than locally, to promote intestinal allergy. Examining mast cell progenitor deposition in the intestines of IL-3^{-/-} mice, as done previously in disease-resistant IL-9^{-/-} mice[57], may indicate whether IL-3 is required locally or plays its most important role before mast cell progenitors reach the intestinal tissue.

The contribution of CD4⁺ T cell-derived IL-3 to intestinal mastocytosis will be a focus for future studies. Adoptive transfer of *ex vivo*-restimulated CD4⁺ T cells from diarrhoeic wild-type mice renders otherwise naive mice susceptible to diarrhoea[38]. Transferring IL-3-sufficient or deficient OVA-specific CD4⁺ T cells to IL-3-deficient mice would determine whether CD4⁺ T cells were a sufficient source of IL-3 to promote mastocytosis and permit disease. DO11.10 mice have an enriched pool of CD4⁺ T cells specifically recognising the OVA323-339 epitope[470] and develop allergic diarrhoea in the model we used[471]. Adoptive transfer of IL-3-sufficient or IL-3-deficient DO11.10 CD4⁺ T cells to IL-3^{-/-} mice prior to sensitization, then monitoring disease and mMCP-1 levels in the challenge model will elucidate whether or not CD4⁺ T cell IL-3-production is sufficient to promote allergic intestinal mastocytosis.

5.4.9 Additional potential effects of IL-3 on intestinal mast cell function

In addition to the reduction in jejunal mast cell numbers in IL-3^{-/-} mice that we observed, it is also possible that IL-3 alters the phenotype of intestinally-localised mast cells. While mouse mast cells differentiate in response to IL-3, exposure to additional cytokines, like IL-4 and IL-9, are required for mast cell mMCP-1 expression[472]. In humans, the process is different. IL-3 promotes the activation, rather than the differentiation of pure mast cell populations, and IL-6 and IL-10 synergise with SCF to promote human mast cell development[314]. IL-6 and IL-10 promote the development of less differentiated mast cells in mice[314], such that IL-3 may promote the maturation as well as the expansion of intestinal mast cells. Determining the mediator content of intestinal mast cells and the role of IL-3 in human intestinal allergies will be important in future studies.

5.5 Summary

We have confirmed a key role for CD4⁺ T cells in local oral potentiation. We identify that CD4⁺ T cells contribute to establishing the local inflammatory milieu in the jejunum in a manner that is additional to their role in IgE production. We determined that the allergic response is optimal in the presence of CD4⁺ T cells, but oral potentiation is sufficient for continued susceptibility to oral allergen challenge responses. IL-3 was required for diarrhoea to manifest and was required in addition to IL-9 for optimal intestinal mastocytosis.

Chapter 6

Examining milk allergenicity in Th2 immune response models

6.1 Introduction

6.1.1 Rationale for examining goat milk allergenicity

Milk allergy in the paediatric population is reasonably common. Of the five percent of children in the US that have had an allergic episode, approximately half will have been triggered by cows milk[9]. A similar incidence is reported in New Zealand infants[473]. While there are significant similarities in their composition, goat milk is less allergenic than cow milk in numerous experimental settings. In this, goat milk offers the potential to be used in a hypoallergenic milk formula that will result in fewer allergies compared with cow milk-based formulae.

Goat milk has been shown to be a weaker allergen than cow milk in a number of models. In mice sensitized orally to milks, splenocyte milk-specific IL-4 production is lower, diarrhoea less frequent and the severity of disease induced by oral feeding is lower in goat milk-fed mice[234]. In some studies, guinea pigs demonstrate more severe anaphylactic responses to cow milk than goat milk[141]. Heat-treating goat milk diminishes its allergenicity in guinea pigs to a greater extent than cow milk[238]. However, goat milk can sensitize, and induce significant allergic responses in both experimental and clinical settings[221, 238]. Therefore, to develop a hypoallergenic infant formula, clarification of the specific components of goat milk that contribute to its allergenicity is required.

In the literature, several milk components have been identified as potential sources of allergenicity. First, the casein content, and specifically the α s1-casein content,

influences the allergenicity of milk. This is observed in multiple settings. Milk sourced from goat breeds in which α s1-casein levels are low results in reduced allergic disease incidence and lower severity in guinea pig studies[249]. In another study, goat milk with 10-fold lower α s1-casein content than cow milk also resulted in lower incidences and less severe allergic responses in mice[234]. One study in mice has shown that allergenicity of cow milk can be reduced by modifying its casein:whey balance from 80:20 to 60:40[250]. Intriguingly, milks with reduced or naturally low α s1-casein content generate less BLG-specific antibody and reduce the allergic response triggered by BLG in the intestine[249, 250]. This suggests that while the casein content of milks is important, both BLG and caseins are implicated in the differential allergenicity of cow and goat milks. However, these studies may also indicate that the relative concentrations of specific proteins, rather than the intrinsic allergenicity of homologues on a protein-by-protein basis, influence milk allergenicity. Therefore, we sought to clarify whether differential allergenicity could be attributed to specific components of goat and cow milk. Finally, we considered that rather than weaker allergenicity, goat milk may have some additional allergy-suppressing properties that inhibit Th2 responses, including those to milk components. Therefore, in this section, we aimed to clarify which components and properties of goat milk influence its comparatively weak allergenicity.

6.2 Aims

In this chapter we used the models of Th2 immunity characterised in the previous chapters to evaluate the allergenicity of milk components. Specifically, our objectives were to determine:

- 1) If there are differences in the intrinsic allergenicity of homologous cow and goat milk proteins;
- 2) If goat milk can suppress Th2 responses;
- 3) If goat BLG can induce intestinal mast cell responses in sensitized mice;
- 4) If cow and goat BLG differentially affect orally-induced allergic intestinal mast cell activity.

6.3 Results

We began our studies using the pTh2 assay to compare the Th2 response induced by isolated milk proteins. As α s1-casein and BLG are major milk allergens absent or low in human milk, we compared the intrinsic allergenicity of these cow and goat homologues.

6.3.1 Examining pTh2 responses to β -lactoglobulin

In work carried out prior to this study, our lab observed that Th2 responses to food allergens were generally weak (Figure 1.3). Therefore, we examined whether Th2 responses could be increased if more allergen was administered. In this and all subsequent pTh2 assays, mice were anaesthetised, and then received the stated dose of allergen intradermally in the ear pinna. Seven days later, the draining lymph node was excised and the Th2 response was analysed.

First, we examined the Th2 response to either 1 mg or 100 μ g of cow BLG. When we examined the total number of cells infiltrating the lymph node, approximately twice as many cells were detected when 1 mg was used compared with 100 μ g (Figure 6.1A). The number of B cells and CD4+ T cells increased in mice that received 1 mg cow BLG (Figure 6.1B and C). We also observed that 1 mg BLG increased the proportion of GFP+CD4+ cells, resulting in an approximately 15-fold increase in Th2 cell numbers which was not observed at 100 μ g (Figure 6.1D and E). These data suggest that 1 mg cow BLG was immunogenic and allergenic in the pTh2 assay, while 0.1 mg was sub-immunogenic.

Next we compared the response for cow and goat BLG. In this assay, we included a 2.5 mg dose. When 1 or 2.5 mg BLG was injected, irrespective of the species source, we observed increased lymph node cellularity, which could largely be attributed to B cell and CD4+ T cell hyperplasia (Figure 6.2A-C). We also observed dose-dependent increases in the proportion and number of Th2 cells, and these increases were similar for both species ($p > 0.05$ for all parameters; Figure 6.2 A-C, E and F). These data suggest that cow and goat BLG have equivalent allergenicity in the pTh2 assay.

6.3.2 Examining pTh2 responses to casein proteins.

Caseins are also major milk allergens, with between 85 and 100 percent of milk allergic individuals exhibiting IgE binding to casein proteins[34, 183]. As significant IgE binding to one casein protein, α 1-casein, is frequently observed[182], we next examined the allergenicity of α 1-casein, and that of the whole casein fraction.

In our initial assays, we had difficulties with the solubility of the caseins, so we tested the amount of protein we were administering using standard protein quantitation assays. Solubilised HPLC-purified goat α 1-casein generated a lower O.D. reading compared with the equivalent mass of cow α 1-casein, but gave similar BCA Cu^{2+} reduction results (Figure 6.3A and B). The same mass of goat α 1-casein and whole goat caseins gave both similar absorbances and BCA Cu^{2+} reduction values (Figure 6.3A and B). These aliquots were used directly in one of the three assays used to compile Figure 6.4. An additional aliquot of goat α 1 casein and the whole casein fraction were frozen, and subsequently used for the assay shown in figure 6.5. Immunisations were performed based on the weighed mass, rather than results of either of the protein assays.

When mice were immunized with HPLC-purified α 1-casein, there was a minor increase in total cellularity in response to 1 mg cow and goat α 1-casein (Figure 6.4A), but only cow reached statistical significance. We did not observe increases in CD4+ T cell numbers in mice immunised with either cow or goat α 1-casein (Figure 6.4 B). In the mice that received 1 mg of either protein, we observed statistically **non**-significant increases in B cell number, and small, but **significant** increases in the proportion and number of Th2 cells (Figure 6.4B, D and E). At no dose did we observe differences between the two α 1-caseins (<0.05 for all parameters). These data suggest that α 1-casein isolated from cow, or from goat milk have similar and low allergenicity, in terms of their ability to initiate Th2 cell development in the absence of adjuvant.

Next we tested whether caseins are more allergenic as a whole, than the isolated α 1-casein. When we compared 333 μg masses of goat α 1 and whole caseins, we observed similar immunogenicity, as there were minor, or no difference in the total cell infiltrate, B cell infiltrate or CD4+ T cell infiltrate in draining lymph nodes (Figure 6.5A-C).

However, we observed a clear difference in the Th2 response: Equivalent masses of α s1-casein generated ≈ 2000 Th2 cells, whereas ≈ 6000 were induced by the equivalent mass of the whole casein fraction (Figure 6.5E). Similar relationships were observed for proportional Th2 responses (Figure 6.5D). These data suggest that wholegoat caseins have higher intrinsic allergenicity than goat α s1-casein alone.

We next compared the allergenicity of cow and goat whole caseins. When we immunized mice with 1 mg of whole casein fractions we observed pronounced increases in lymph node hyperplasia, B cell, CD4+ T cell and Th2 cell expansion (Figure 6.6A-E). Both cow and goat whole caseins increased the proportion and number of Th2 cells present in lymph nodes compared to non-draining lymph nodes, most significantly at the highest dose (1 mg; Figure 6.6D and E). While there may have been a small increase in Th2 proportion for cow over goat at 1 mg, no statistically significant difference was detected in the proportion or number of Th2 cells at any dose ($p > 0.05$ at all doses). Therefore, these data suggest that cow and goat whole caseins both induced Th2 cell development and did so to similar extents in the pTh2 assay.

Figure 6.1: **Dose-dependent Th2 response to cow β -lactoglobulin.** Mice (n=3/group) were injected intradermally with 600 dead *i*L3 *N. brasiliensis* larvae (G4/+ L3), 1000 μ g cow BLG, or 100 μ g cow BLG. Seven days later, draining lymph nodes were taken, and one non-draining node from each group was taken (nDLN) and analysed by trypan blue exclusion test for (A) total cell number and after flow cytometric characterization, the number of (B) B220+ cells, (C) CD4+ T cells and the (D) proportion and (E) number of CD4+ T cells that were GFP+. Bars show mean $\pm$ SEM of groups of (n=3) mice from one of two similar experiments. Data were compared using One-way ANOVA with Tukey's post-test. *p<0.05, **p<0.01.

Figure 6.2: **Similar Th2 response to cow and goat β -lactoglobulin.** G4/+ mice (n=5/group) were immunized intradermally with the stated dose of cow (squares) or goat (circles) BLG. Control +/+ (white triangles) and G4/+ (black triangles) mice were immunized with 600 dead *i*L3 *N. brasiliensis* larvae. Non-draining (nDLN) lymph nodes (crosses) were used to reflect the assay baseline. (A) Total cell numbers were calculated by trypan blue exclusion, and the number of (B) B220 and (C) CD4+ T cells were back calculated based on the (D) proportion of live cells that were B220+ (White bars) or CD4+ (Black bars). (E) The proportion and (F) number of CD4+ T cells that expressed GFP, as a surrogate of IL-4 protein induction, were quantified. Data are shown from a single experiment and the dose response was analysed using Two-way ANOVA with Bonferroni's post-test; b $p < 0.05$, bb $p < 0.01$, bbbb $p < 0.0001$ cow BLG vs. nDLN; aa $p < 0.01$, aaaa $p < 0.0001$ goat BLG vs. nDLN.

Figure 6.3: **Comparison of cow and goat casein concentrations by BCA and nanodrop.** Cow α s1-casein (cow as1), goat α s1 casein (goat as1) or goat whole casein (goat WCF) were dissolved in solution to 1 mg/90 μ L (11.111 mg/mL). (A) After diluting samples 1/20, proteins were compared for absorptive capacity by nanodrop (280 nm). (B) Serial dilutions of samples were compared to a standard 2 mg/mL BSA solution for Cu^{2+} reduction based on the BCA reduction assay. Three 1/20 dilutions were prepared from the same stock for nanodrop; BCA data were in duplicate. 600 dead *i*L3 *N. brasiliensis* larvae (L3) were also included in the assay (cross). Data are from a single experiment and were analysed by (A) Student's *t*-test, and exact *p*-values are shown or (B) Two-way ANOVA with Bonferroni's post-test; non-significant (ns) $p > 0.05$.

Figure 6.4: Similar Th2 response to cow and goat αs1 -casein. Seven days after intradermal injection of +/+ (white triangles) or G4/+ (black triangles) mice with 600 dead *i*L3 *N. brasiliensis* larvae, or cow (squares) or goat (circles) αs1 -casein at the stated dose (in μg), mice were euthanised and auricular cell infiltrates were profiled. Randomly selected non-draining lymph nodes including samples from all groups were used as baseline controls (crosses), and in one experiment PBS-immunized mice were also included (plus symbols). (A) The total number of cells was quantified by trypan blue exclusion and combined with flow cytometric analysis to determine the number of (B) B220+ cells, (C) CD4+ cells and the (D) proportion and (E) number of CD4+ T cells that expressed GFP. Symbols represent mean $\pm$ SEM of data combined from three separate experiments, each using a different titrating dose; 1000 μg Cow as1, $n=11$; 1000 μg Goat as1 $n=8$; 500 μg Cow as1 $n=3$; 500 μg goat as1 $n=5$; 333 μg cow or goat as1 $n=2$; 100 μg cow or goat as1 $n=5$; nDLN $n=20$; PBS $n=4$; +/+ L3 $n=9$; G4/+ L3 $n=11$. Dose titrations were compared against the nDLN group using Two-way ANOVA with Bonferroni's post-test; aaaa $p < 0.0001$ Goat as1 vs. non-draining, b $p < 0.05$, bbb $p < 0.001$, bbbb $p < 0.0001$ cow as1 vs. non-draining.

Figure 6.5: Th2 response to goat whole casein is stronger than to goat α s1-casein. Seven days after intradermal injection with the stated dose of whole goat caseins (WCF), goat α s1-casein (as1), PBS (as negative control), or 600 dead *N. brasiliensis* larvae (+/+ L3 and G4/+ L3 as positive controls), (A) total cell numbers, (B) B cell numbers, (C) CD4+ T cell numbers, (D) the proportion of CD4+ T cells that expressed GFP and (E) the total number of Th2 cells generated were examined. Data were generated in a single experiment and represent mean $\pm$ SEM of (n=3) 666 μ g WCF and 333 μ g as1; (n=2) 333 μ g WCF, G4/+ L3 and PBS; (n=4) +/+ L3. (A-E) For the groups indicated, data were compared by Student's *t*-test, only in the 1/3 mg groups; exact p-values are shown.

Figure 6.6: Th2 responses to cow and goat whole caseins are similar. Seven days after intradermal administration of 1 mg (1000), 0.5 mg (500) or 0.1 mg (100) cow whole casein fractions (Cow WCF) or goat whole casein fractions (goat WCF), draining lymph nodes were examined for immune responses compared to non-draining (nDLN) lymph nodes. +/+ (+/+ L3) and G4/+ (G4/+ L3) mice immunized with 600 dead *iL3 N. brasiliensis* larvae were used as positive controls. In one experiment a PBS (PBS) treated group was included as an additional negative control. (A) Total cell infiltrates, (B) B cell infiltrates, (C) CD4+ T cell infiltrates and (D) the proportion and (E) number of Th2 cells induced were recorded. Data points represent mean±SEM of (n=6) mice for *N. brasiliensis* controls, (n=14) non-draining nodes, (n=8) draining nodes at 1 mg and (n=4) draining nodes at 0.5 mg and 0.1 mg, combined from two experiments. a p<0.05, aaaa p<0.0001 goat caseins versus non-draining nodes, bb p<0.01, bbbb p<0.0001 cow caseins versus non-draining nodes by Two-way ANOVA with Bonferroni's post-test.

6.3.3 Examining the ability of goat milk to suppress Th2 responses

We used the pTh2 assay to test if goat milk can suppress Th2 responses induced by other allergens. Mice received 0.1 mg HDM antigen in the presence or absence of 25 μ L skim or whole goat milk, in a total volume of 30 μ L and seven days later lymph node cellularity was assessed. We observed increased Total, B cell and CD4+ T cell infiltration in HDM-immunized mice (Figure 6.7A-C). In terms of both proportion and number, the pTh2 response was strongest in mice immunised with HDM in the presence of whole goat milk (Figure 6.7D and E). Therefore, not only were goat milks unable to significantly suppress HDM-induced Th2 responses, the presence of whole goat milk increased the Th2 response.

Previous studies have examined the oral allergic response to milks. We next incorporated the physiological processing component of allergenicity into our investigations. Using the model developed by Brandt *et al.*[321], we examined the development of allergic diarrhoea in mice sensitised to OVA, then fed OVA dissolved in PBS, skim goat milk or whole goat milk. When mice were challenged perorally with milks alone, neither skim- nor whole-goat milk caused diarrhoea development in OVA-sensitized mice (Figure 6.7F). In contrast, when mice were challenged with 50 mg OVA, all groups began to develop diarrhoea in 3-5 challenges, and disease penetrance reached 100% in all OVA-challenged groups, even those in which the OVA was dissolved in goat milk. Therefore, allergic diarrhoea in OVA-sensitized mice was neither induced nor inhibited by the presence of skim or whole goat milk, and there was no apparent difference of the response in the presence of either skim or whole goat milk.

Figure 6.7: Neither skim nor whole goat milk prevent Th2 responses. (A-E) HDM was dissolved and then combined in a 5:25 ratio such that 30 μ L contained 100 μ g HDM in 5 μ L PBS, + 25 μ L PBS, 25 μ L skim goat milk (SGM) or 25 μ L whole goat milk (WGM). A mock-challenged group was included to set the baseline (PBS+PBS). Seven days after injection of the stated solution, (A) Total cells were counted by trypan blue exclusion and flow cytometry proportions were used to back-calculate the number of (B) B cells, (C) CD4+ T cells, (D) the proportion of CD4+ T cells that expressed GFP and (E) the number of Th2 cells. Data shown are from one of two similar experiments. (D,E) Data points represent individual mice. (A-C) Bars show mean $\pm$ SEM of these same mice. In the second experiment, 30 μ L PBS, SGM or WGM were administered to the ear 36 hours prior to HDM immunization and gave similar results. (F) OVA-sensitized mice (n=5/group) were i.g. challenged with 50 mg OVA in PBS, SGM or WGM. Control OVA-sensitized mice received 250 μ L skim goat milk (SGM; n=2) or whole goat milk (WGM; n=3) alone during i.g. challenge. Oral challenge data were generated in a single experiment. (A-E) Data were compared using One-way ANOVA with Tukey's post-test; *p<0.05, **p<0.01, ***p<0.001.

6.3.4 The oral response to milk allergens

Homologous goat and cow milk allergens induced similar Th2 responses, but the pTh2 assay does not examine the full allergic process. Using the model developed by Brandt *et al.*[37], we aimed to establish whether oral challenge with goat BLG could initiate the intestinal mastocytosis associated with oral potentiation.

Mice were sensitised twice, two weeks apart with 50 μ g goat BLG with alum, and then subsequently fed with 50 mg goat BLG, 50 mg OVA, or PBS. A control group of mice was mock-sensitized, and then fed with 50 mg goat BLG. When we examined IgE levels, total IgE increased in sensitized mice fed goat BLG (Figure 6.8A). Feeding goat BLG-sensitized mice with PBS or OVA did not increase the Total IgE above starting titres (Figure 6.8A). Similarly, mock-sensitized mice fed with goat BLG had lower IgE than the goat BLG-sensitized, goat BLG-fed group at the experimental endpoint (Figure 6.8A). We examined antibody binding to goat BLG and observed minor elevations in the BLG-fed group over other groups for both polyclonal Igs and IgG1 (Figure 6.8C and E). When we examined these mice for IgG1 binding to cow BLG, we similarly observed elevations over the other groups (Figure 6.8D and F). These data indicate that oral challenge with goat BLG increased both goat and cow BLG-specific antibody titres. Last, we examined serum mMCP-1 levels in these mice. The only group in which we detected increased mMCP-1 levels was the goat BLG-sensitized, goat BLG-fed group (Figure 6.8B) with corollary increases in jejunal mast cell frequencies (Appendix Figure 8.7). From these data, we conclude that oral potentiation was observed in goat-BLG fed mice. Therefore, goat BLG can both sensitize and initiate the oral allergic process.

To compare the allergenicity of cow and goat BLG, we used the contracted experimental model in *iFABP*-IL-9 transgenic mice. These mice were sensitised to OVA, goat BLG, cow BLG, BSA or were mock-sensitized with PBS. Seven days later, mice were orally gavaged with 50 mg of the sensitising allergen nine times. Mice were bled after the ninth challenge, and sera were analysed for parameters associated with the intestinal allergic response: mMCP-1, IgE and IgG1 production. Further, because we previously observed increases in IgG1-ASC numbers in mice sensitised and orally-primed with OVA, when we killed the mice four days after their final gavage

challenge, we also examined the number of antigen-specific ASCs in spleens of all groups of mice.

Before discussing the results of this experiment, it is important to note that in the early challenges, we observed diarrhoea development in 75 percent of cow BLG-fed mice after the third challenge (images are shown in the appendices). We attempted to bleed mice after the third challenge, but blood pressure was low in the cow BLG-fed group, and we were unable to attain sufficient blood to isolate plasma. In the preparation of this cow BLG, 2% salt (NaCl) was added to help solubilise it, so to remove any confounding this may have had, cow BLG was subsequently dialysed prior to gavage, and thereafter diarrhoea was no longer observed in cow BLG-fed mice. However, in a repeat experiment, neither sensitised nor unsensitised cow BLG-fed mice developed diarrhoea using the same mass of undialysed cow BLG; as such we were unable to reproduce the diarrhoea phenotype, nor were we able to determine whether it was IgE- and mast cell dependent. Diarrhoea developed in one of three OVA-fed mice, and in none of the BSA- or goat BLG-fed mice. Therefore, we have limited our evaluation of the results to the production of mMCP-1 and cross-reactive IgG1 detection.

When we measured mMCP-1 levels in the OVA, goat BLG, cow BLG and PBS-fed groups, we observed increases in all three groups of allergen-challenged mice over PBS-fed mice (Figure 6.9A). While OVA, goat BLG and cow BLG groups were all elevated over the mock-sensitized group, the OVA-fed group was most significantly elevated, but not significantly over the two BLG groups (Figure 6.9A). The level of Total IgE at the experimental endpoint was significantly elevated in the cow BLG group over mock-sensitized, PBS-fed mice (Figure 6.9B), and similar trends for the other groups were observed.

We also observed that ASC formation was similar for cow and goat-fed groups (Figure 6.9C-F), and also correlated approximately with the antigen-specific IgG1 titres that developed (Figure 6.10A-D). When we examined IgG1-binding to the different proteins, cow BLG- and goat BLG-specific antibodies were detected in both cow and goat BLG sensitized mice, but not OVA-sensitized mice (Figure 6.10B, C, F, G). Somewhat intriguingly, cow BLG-fed *iFABP*-IL-9 transgenic mice had higher cow BLG-specific

IgG1, and polyclonal Igs than goat BLG-fed mice at the 1/10000 dilution (Figure 6.10B and F). This same relationship was, however, not observed in the spot assay (Figure 6.9D). Such significant cross-reactivity was not observed between BLG and either OVA or BSA, in any of polyclonal, antigen-specific IgG1, or spot assays (Figure 6.9C-F and 6.10A-H). Taken together, these data may indicate that oral challenge with both cow and goat BLG causes oral potentiation; both homologues drive similar magnitude increases in mMCP-1 production but may differentially affect the B cell response. However, further investigation is required to definitively determine whether the ability of the two BLGs to drive oral potentiation differ.

Taken together, these preliminary findings suggest that the sensitising and response-evoking capacity of goat BLG and cow BLG were similar, but indicated that cow-BLG feeding increased BLG-specific IgG1 titres. These studies will benefit from replication in the future. At this stage, given the similar elevations in the mMCP-1 response, from these data we conclude that the response-evoking capacity of BLG from cow or goat milk was similar.

Figure 6.8: Goat β -lactoglobulin can induce intestinal Th2 responses. Groups of ($n=3$ /group) mice were sensitized ip to goat BLG with 50 μ g goat BLG plus 1 mg alum (goat BLG/) or mock-sensitized with PBS plus 1 mg alum (PBS/) and then orally challenged with goat BLG (/goat BPG), OVA (/OVA) or PBS (/PBS) as indicated. Mice were bled for (A) Total IgE before any and after six challenges. (B) mMCP-1 was analysed in sera after the 6th challenge. (C) goat BLG-specific polyclonal Igs, (D) cow BLG-specific polyclonal Igs, (E) goat BLG-specific IgG1 and (F) cow BLG-specific IgG1 were analysed by ELISA. (B) Data points represent individual mice or (A, C-F) mean $\pm$ SEM of these groups. Data were generated in a single experiment and were analysed using (B) One-way ANOVA with Tukey's post-test or (A, C-F) Two-way ANOVA with Bonferroni's post-test; $p<0.05$, $**p<0.01$. mMCP-1 data were log-transformed prior to analysis.

Figure 6.9: mMCP-1 levels are elevated in orally-challenged sensitized mice. *iFABPp*-IL-9 mice were i.p. sensitized, and seven days later began to receive i.g. challenges with: i) OVA (OVA/OVA), ii) cow BLG (cow BLG/cow BLG), iii) goat BLG (goat BLG/goat BLG), iv) PBS (PBS/PBS) or v) BSA (BSA). i.p. sensitizations used 50 μ g of the indicated allergen; i.g. challenges used 50 mg of that same allergen and were given every second day, three times a week for three weeks. Two hours after the 9th i.g. challenge, mice were bled and (A) serum mMCP-1 and (B) Total IgE levels were evaluated. (C-F) Antigen-specific IgG1 spot assays were performed for each allergen. FBS was used to block plates so BSA group was not analysed in the other spot assays. Data were generated in a single experiment. (A,B) Data points represent individual mice, (C-F) bars represent mean $\pm$ SEM of these same groups. Data were compared using One-way ANOVA with Tukey's post-test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. mMCP-1 data were log-transformed prior to analysis.

Figure 6.10: **Cross-reactivity observed between cow and goat β -lactoglobulin but not other allergens in *iFABPp*-IL-9 mice.** Sera from mice described in Figure 6.9 were analysed for (A) OVA-specific IgG1, (B) cow BLG-specific IgG1, (C) goat BLG-specific IgG1, (D) BSA-specific IgG1, (E) OVA-specific polyclonal Igs, (F) cow BLG-specific polyclonal Igs, (G) goat BLG-specific polyclonal Igs or (H) BSA-specific polyclonal Igs. (A, D, E, H) Represent significance against all other groups, in (B, C, F, G) statistical differences are shown for cow versus goat β -lactoglobulin groups. Data were generated in a single experiment and compared using Two-way repeated measures ANOVA with Bonferroni's post-test; ** $p<0.01$, **** $p<0.0001$.

6.4 Discussion

In this chapter we sought to establish the components of milk that influence its allergenicity. We used the pTh2 assay to compare homologous cow and goat milk proteins. We examined BLG, α s1-casein, and also the whole casein fraction. We further investigated the anti-allergic properties of skim and whole goat milks, and the oral potentiation that follows oral challenge with goat or cow BLG. In the pTh2 assays, we observed similar, dose-dependent Th2 cell development in response to primary cow and goat BLG, α s1 or whole casein fraction immunisation. These findings indicated that the intrinsic allergenicity of these goat and cow milk allergens was similar. We also found that goat milk itself did not appear to suppress Th2 responses. Our oral challenge studies identified that goat BLG can induce antigen-specific mast cell responses in the intestine, and preliminary data indicated that oral challenge with OVA, cow or goat BLG caused oral potentiation in *iFABP*-IL-9 transgenic mice. In our data we observed no significant difference in the level of potentiation invoked by either cow or goat BLG.

6.4.1 Evidence for differential allergenicity of goat and cow milk

Goat and cow milks have been compared previously. Numerous clinical studies have investigated their allergenicity. Bellioni-Businco *et al.*[192] observed that a significant proportion of cow milk allergic individuals were also allergic to goat milk[192]. Ah-Leung *et al.* presented evidence of a number of individuals sensitized to goat milk, but not cow milk[221]. Significant cross-reactivity between milk proteins from different ruminant species is usually observed[192, 247]. The IgE binding to cow α s1-casein is also frequently very strong[182]. Ballabio *et al.* recently observed that goat milk with low or no α s1-casein induces minimal skin prick positivity in some cow milk sensitized individuals[236]. There is evidence of cross-reactivity, however, there is also clinical evidence of cases in which the milks are antigenically distinct.

In experimental systems, comparisons have also been made between cow and goat milk. McLaughlan *et al.*[238] ran experiments in guinea pigs. Guinea pigs that were

given *ad libitum* access to cow milk became sensitized and demonstrated frequent fatal anaphylaxis when i.v.-challenged with cow BLG or whole caseins[238]. Guinea pigs sensitized to goat milk in the same manner also succumbed to the i.v. challenge[238]. However, it was found that if the milks were boiled prior to feeding, the incidence of fatal i.v.-induced anaphylaxis was reduced, and reduced more markedly for boiled goat milk[238]. More recently, in a contracted version of the same experiment, the incidence of fatal anaphylaxis was less frequent in goat milk fed mice[141]. Bevilacqua *et al.*[249] observed that when guinea pigs were sensitized to whole goat or whole cows milk, the antibody response was directed against BLG, and milk that had low α s1-casein content caused less intestinal anaphylaxis[249]; goat milk that had high α s1-casein caused similar disease severity to cow milk[249]. Similarly, in mice, Lara-Villoslada *et al.*[234] demonstrated that mice sensitized orally and challenged i.v. with skim cow milk released more plasma histamine, had elevated IgG1 titres, had more severe anaphylactic symptoms and a skewed Th2 cytokine response profile compared to skim goat milk treated mice[234]. Therefore, there is experimental evidence that links the allergenicity of goat milk to its α s1-casein content.

6.4.2 α s1-casein as an allergen

Given the evidence in the literature that indicates that α s1-casein is a dominant allergen, the comparatively low pTh2 response to α s1-casein was surprising. The α s1-casein content of milk has many times been associated with its allergenicity in experimental systems[141, 232, 234, 249, 250], and we expected to observe significant differences. However, not only was there no apparent difference in Th2 development between cow and goat α s1-casein, both were also poorly immunogenic. In response to a 1 mg dose, we observed that the total immune cell infiltrate increased by only two-fold, and the CD4+ T cell population did not observably expand (Figure 6.4).

These findings may indicate that α s1-casein influences the allergenicity of milk through interaction with other milk components. Caseins form micelles, and α s1-casein influences the structure and size of the micelle[230]. Johansson *et al.*[474] argue that the biasing of monoclonal antibodies that develop against caseins toward κ casein, could reflect an important role for the supramolecular structure of the casein micelle in its

immunogenicity[474]. In our studies, we observed that the whole casein fraction was more immunogenic than α s1-casein in isolation (Figure 6.5), which may support this finding. Processing of milks can alter their basic physical properties. Heat-treating milks can induce covalent linkages between BLG peptides and κ casein[475]. The combined effects of caseins and BLG may be important for the allergenicity of milks, and have been shown to be interrelated in the past[249, 250]. More recently, it has been shown that milk with high α s1-casein content has larger fat globule diameter[476]. In our studies, we observed that whole milk increased the pTh2 response to HDM to a greater extent than did skim milk (Figure 6.7); we used low α s1-casein milk in these studies, but it is possible that higher fat content, or globule size could have increased the response even more. Although we saw augmented responses to HDM in the presence of whole milk compared with skim milk in the pTh2 model, we did not see this same effect in the oral challenge model (Figure 6.7). Side-by-side comparisons of cow and goat milk fats on the pTh2 response may reveal some additional α s1-casein-associated aspects that do not relate to its intrinsic allergenicity or auto-adjuvanticity.

In the appropriate circumstances, α s1-casein can induce Th2 responses in isolation. Recently, α s1-casein from goat milk was shown to orally potentiate sensitized mice, because mMCP-1 levels increase after α s1-casein feeding[394]. In this study, α s1-casein also triggered increased IgE production, and higher IL-4 and IL-10 release by splenocytes cultured with α s1-casein[394]. This report indicates that α s1-casein can act as an allergen in mice. One difference is that this system employed a pre-sensitization, where mice were administered α s1 casein i.p. with alum adjuvant prior to i.g. challenge[394]. In our studies, we did observe some Th2 cell development in response to α s1-casein (Figure 6.4), but the addition of an exogenous adjuvant is likely to amplify the immune response. These findings may indicate that α s1-casein has low auto-adjuvanticity, but in the presence of an adjuvant can become a significant allergen. Natural adjuvants that contribute to sensitization could come in the form of staphylococcal superantigens[477], or alternatively, may not be required for sensitization if the allergen was exposed to a mucosal surface (e.g., the lung;[136]). Additionally, as there are homology differences between cow and human milk proteins, the absence of an observable effect in the mouse may relate to differences in protein

homology between cow, goat and mouse milk proteins, in contrast to cow, goat and human milk proteins. Further studies will be required to determine the comparative allergenicity of goat α s1-casein to cow α s1-casein in oral challenge models, and test alternative means by which α s1-casein could sensitize.

6.4.3 The allergenicity of BLG

We investigated BLG allergenicity using the pTh2 assay and in the oral challenge model. In our pTh2 assays, we observed equivalent allergenicity of cow and goat BLG. Both proteins induced 15-100-fold increases in Th2 cell development when injected at 1 mg, which indicated that the intact allergens were equivalently allergenic (Figure 6.2). However, evidence in the literature encouraged us to investigate the response in an oral challenge model. Goat BLG was shown in the past to induce intestinal responses in guinea pigs[249]. Cow BLG has been demonstrated to induce mast cell degranulation in oral challenge models[263]. There is also evidence in the literature that while they are both stable proteins, the digestibility of the two BLGs differ: 50-80% of goat BLG is degraded, compared to only 20% of cow BLG when subjected to sequential digestion in human gastric- and then duodenal juices[185, 478]. We considered that if goat milk was more digestible and more rapidly degraded in the murine gastrointestinal tract the amount of intact antigen available to drive the inflammatory process may be reduced and result in a similar four-fold differential in their ability to orally prime and induce mMCP-1 production.

Earlier studies undertaken at AgResearch identified that goat β -lactoglobulin can drive oral potentiation (Ali Hodgkinson, personal communication). However, mast cells can produce mMCP-1 in an IgE-independent manner[479], so it was important to confirm that the oral response only occurs in response to sensitizing antigen. In our oral challenge studies, we observed that six oral challenges with 50 mg goat BLG was sufficient to orally potentiate, because mMCP-1 titres increased from ≈ 20 ng/mL to ≈ 5000 ng/mL in orally-fed mice (Figure 6.8). We also observed elevated IgE titres, only in sensitized, allergen-fed mice. Further, feeding goat BLG-sensitized mice with OVA did not increase mMCP-1 release above mock-challenged and/or sensitized controls. Therefore, oral potentiation induced by goat BLG feeding required sensitization

and was goat BLG specific.

In the literature, the mast cell response correlates with the development and severity of oral allergic responses[36, 38]. In our studies in the previous chapter (Chapter 5), we confirmed that the intestinal mast cell response was a CD4+ T cell-dependent event. Therefore mMCP-1 production can be used to reflect the allergenicity of an orally administered protein.

In a preliminary investigation, we used the *iFABP*-IL-9 transgenic mice to compare the allergenicity of OVA, cow and goat BLG. We observed that oral potentiation occurred when any of OVA, cow or goat BLG were used, because mMCP-1 titres were elevated compared to PBS-fed mice (Figure 6.9). In terms of their comparative allergenicity, the low power of the experiment, and unexpected, but unreproducible, diarrhoea development in cow BLG-sensitized mice prevents us from making definitive conclusions. However, there were trends indicating that OVA induced the most mMCP-1 release, generated the most frequent splenic IgG1-ASCs, and had high serum IgG1 titres (Figures 6.9 and 6.10). Similarly, while mMCP-1 levels induced by cow and goat BLG were similar, the cow BLG-fed group demonstrated increases in serum cow BLG-specific antibodies that were not observed in the goat-fed group (See Appendices). Additionally, early in the response, we observed diarrhoea and severe plasmavolaemia in the cow BLG-fed group. We were unable to repeat this finding, and further were unable to link this to the allergic process because we could not bleed these mice post-challenge. However, it does indicate that oral processing may be an important feature of the differential allergenicity of cow and goat BLG that could be revealed in follow-up studies using larger experiments.

6.4.4 Future investigations that could clarify goat milk protein allergenicity

To accurately clarify the role of oral processing in milk protein allergenicity, there are several potential experiments that would be useful. One of the most informative tests will be to directly assess antibody cross-reactivity in cow and goat BLG-fed mice, which could be done using antigen inhibition tests[480]. Lung challenge models

and alternate oral challenge models have been employed using cow BLG[139, 458]. Cytokine levels produced by splenocytes, intestinal cells or mesenteric lymph node cells have been compared for other allergens[57, 394]. Examining these responses may reveal differences in the oral potentiation response, and clarify whether the trend we observe reflects a true difference, or relates to experimental variation.

The pTh2 studies could also be expanded. Examining the local Th2-mediated B cell response may indicate additional aspects of protein allergenicity that are not revealed by measuring Th2 development. While we observe IgG1 switching in the pTh2 assay, and this correlated with the Th2 response (Chapter 4), distinct mechanisms influence IgE class switching[279, 306, 445, 481]. Specifically, while both IgE and IgG1 class switching are both controlled by TFH cells and sensitive to IL-4, they are reciprocally affected by IL-21[440–443, 482]. Therefore, examining IgE switching may be additionally informative. While beyond the sensitivity of most current tools, examining IgE switching may be possible in a modified pTh2 assay using recently developed IgE-reporter mice[444, 445].

In the course of our studies, we have found no difference in the intrinsic allergenicity, as defined by primary Th2 cell induction, for BLG, α s1 or whole casein fractions isolated from cow or goat milks. Future work that analyses milk proteins in isolation, milk as a complete food and as its casein and whey fractions will have the potential to clarify the differences in cow and goat milk that render them differentially allergenic in experimental contexts.

6.5 Summary

Homologous milk allergens derived from cow and goat milk induced similar pTh2 responses to each other. α s1-casein was shown to have low allergenicity in isolation. The presence of milk fat increased the pTh2 response but did not expedite disease in the oral challenge model. Goat milk did not demonstrate strong anti-allergic properties in the models tested. Further investigation of how oral processing influences milk protein allergenicity is warranted.

Chapter 7

General Discussion

7.1 Summary of findings

During the course of this work we uncovered some novel aspects of Th2 cell development and function. In Chapter 3, we determined that biallelic IL-4 production occurs more frequently than is predicted for a monoallelic, stochastic gene expression event. In Chapter 4, we identified IL-4-independent and -dependent phases of Th2 cell development, and also found that the biallelic IL-4-producing potential is important for allergen-specific Th2 immunity. In Chapter 5, we uncovered an important role for IL-3, and established the causal role of CD4+ T cells in allergen-induced intestinal mastocytosis. We further determined that this function of CD4+ T cells was required additional to their contribution to IgE production. Our final results chapter defined the Th2-inducing capacity of goat and cow milk components. It additionally provided preliminary information about the ability of goat BLG to induce intestinal mast cell activity. The findings of this thesis reaffirm the central role of CD4+ T cells in IgE-mediated allergic disease.

7.1.1 Checkpoints along the road to allergy

The inter-related aspects of Th2 immunity investigated in this project allow us to draw a more comprehensive picture of the experimental allergic process that ultimately results in diarrhoea. We identified numerous points of potential disease intervention in mice that warrant investigation in clinical settings based on current knowledge and the findings of this thesis. These concepts are summarised in figure 7.1. Taken as a whole, we consider that there are multiple points for disease intervention, however, the further along the process, the more difficult disease will become to treat.

Figure 7.1: **Processes leading to intestinal allergy.** On first allergen exposure, naive CD4+ T cells are induced to develop into Th2 cells. These Th2 cells expand in the presence of IL-4, and in the presence of allergen, induce B cells to produce IgE, and promote mast cell deposition in the intestine through the production of IL-3, IL-4 and IL-9. Subsequent allergen exposures induce mast cell degranulation and trigger the intestinal allergic response. Potential points for disease intervention are: (1) To prophylactically administer weak or degraded allergens, administer allergens by the oral route, or use allergy-suppressing adjuvants; (2) target allergen-reactive Th2 cells; (3) Inhibit Th2m cell-dependent mast cell expansion; (4) Prevent TFH cell induction of IgE; (5) Prevent or ablate IgE synthesis; (6) Avoid allergen or apply desensitization; (7) inhibit mast cell degranulation; (8) Inhibit signaling of vasoactive products.

7.1.2 Future research directions

Evaluating Biallelic Th2 cell function *in vivo*

In this thesis, we examined Th2 cell development and function, largely using IL-4 haplo-insufficient, or IL-4-deficient reporter mice. We infer information about the regulation of IL-4 production from our data, namely that biallelic IL-4 production is more frequent than is expected based on the proposed model[401]. Determining whether this is important for specific aspects of allergic Th2 cell function, and whether this truly occurs in wild-type mice (and humans) *in vivo*, requires further investigation. The presence of biallelic IL-4 production in wild-type cells could be evaluated at the transcriptional level using single cell PCR analysis from *in vivo*-generated Th2 cells with distinguishable, but wild-type IL-4 alleles[389]. Additionally, the specific involvement of biallelic IL-4 production in Th2 immunity could be examined at a functional level. Deletion of an IL-4-silencer site results in non-specific IL-4 production[380], which could override the potential imbalance in the absolute concentration of IL-4 in IL-4 heterozygous mice. These mice would then be deficient only in biallelic IL-4 production. Examining IgG1 class-switching, the generation of IgE, and the development of allergic diarrhoea could make some headway into the role of biallelic IL-4 producing Th2 cells in the allergic immune process.

Establishing the functional or physical divergence of Th2m and TFH cells

The data in the oral allergen challenge model provides evidence for the existence of Th2m cells. However, it is unknown whether Th2m cells are a separate population from IgE-inducing cells. In addition to IgE production at systemic sites[444], antibody can be produced in high amounts in local tissues. Specifically, Kweon *et al.* identify IgE-producing B cells in the colonic intestinal lamina propria of orally-sensitized mice[58]. In this case, it is plausible that Th2m cells are also the cells involved in the amplification of systemic IgE following oral exposure, and contribute to both processes simultaneously. Determining the existence of divergent, or overlapping Th2

cell functions in the intestinal allergic process may indicate whether targeting multiple CD4+ T cell populations will be required for effective disease intervention. Additionally, establishing the importance of IL-3 in other oral allergen-challenge models, and eventually in clinical contexts will clarify the overall involvement of IL-3 in the intestinal allergic process. Simple experiments could evaluate IL-3-deficient mice in the cholera toxin model[58, 140, 483], the superantigen oral allergic disease model[456], or use IL-3R-deficient mice to validate our findings in the model we used[113]. For clinical application, initial animal experiments could also examine whether anti-IL-3 antibodies can inhibit disease development in mice.

Targeting adaptive immunity for disease intervention

The most informative experiment that will follow our oral potentiation studies in Chapter 5 arises from the CD4-depletion work. We observed that while CD4+ T cells were not required for disease maintenance, the underlying inflammatory process was diminished following their depletion. Clinically, anti-IgE therapies are effective at desensitizing, but not necessarily tolerizing allergic individuals (permitting long-term disease resolution). However, the CD4+ T cell response wanes in anti-IgE treated individuals[83]. Concomitant CD4-depletion along with administration of anti-IgE in mice (using anti-IgE hybridomas like EM-95[37]), could potentially ablate, and prevent the re-establishment of disease. This may uncover the basic processes that underlie long-term disease susceptibility and eventually lead to a combined therapy that targets allergen-reactive CD4+ T cells and IgE-producing B cells.

It would be ill-advised to use a pan-CD4-depleting antibody (like GK1.5) to treat allergic diseases, because subsets of CD4+ T cells contribute to the adaptive immune response against essentially every pathogen that causes human disease. Therefore, establishing novel ways in which allergen-reacted CD4+ T cells can be targeted will be a second required step that will precede the arrival of potential therapies in the clinic.

Addressing the "localness" of local responses

One key aspect that remains to be addressed is the extent to which oral potentiation requires local exposure. Allergen is detected in the intestines of naive allergen-fed mice, but oral challenge of sensitized mice increases intestinal permeability[37, 57] and results in 1000-fold higher (to $\mu\text{g/mL}$ concentrations) levels of allergen in the blood[68, 149]. Furthermore, in sensitized mice, systemic anaphylactic responses, in some cases, are caused by oral allergen administration[67], and response severity correlates with the administered allergen dose, and the amount that is absorbed[67, 68]. While the intestinal response requires multiple gavages to become manifest, the dissemination of allergen that follows intestinal allergen exposure argues against complete segregation of each tissue in the common mucosal immune system. In line with the concept of systemic potentiation, oral allergen challenge also increases lung mast cell numbers and exacerbates the severity of the response evoked by distinct sensitizing allergens in both the lung and the intestine[67, 149, 321]. Clarifying whether the exacerbation of the lung response requires allergen to reach the lung, or is a general feature of the Th2 response would improve our understanding of the post-sensitization CD4-dependent allergic process. Stabilizing mast cells prior to oral allergen exposure prevents the leaky gut phenotype in *iIFABP*-IL-9 mice. This could be used to distinguish between systemic potentiation via the oral route and lung potentiation induced by systemic allergen dissemination. This will be an important direction for follow up work to our studies examining oral potentiation of the intestine.

Reflecting allergenicity as a function of Th2 cell development

In our aim to clarify immunological differences between cow and goat milk, we were unable to identify quantitative differences in the Th2 response to specific proteins. However, closer analysis of the whole casein fraction may identify important aspects of milk allergenicity that our assays have not examined. Specifically, we did not analyse whole caseins in the oral challenge model; ruminant caseins demonstrate significant species-to-species cross-reactivity in humans[34] and this may be an important attribute of their ability to cause allergies. Simply comparing mMCP-1 production

in the oral challenge model using cow and goat casein fractions, or whole milks in this model, could indicate the ability of milk from each source to promote intestinal allergy. Additionally, in our suppression assays, we examined the ability of milks to suppress the allergic response to an environmental, rather than a food allergen. Future studies would also benefit from analysis of the Th2 response to food allergens in the presence- or absence of milk protein.

The cause of sensitization

The pathways that initiate sensitization after birth are unclear. Clarifying which aspects of the allergic process are modelled in the two Th2 immune response models examined in this thesis will help us understand how a protein becomes an allergen. In the G4 reporter pTh2 assay, two components should be examined in the near future. First, the influence of adjuvant on the Th2 response should be explored. While we examined the intrinsic allergenicity of homologous proteins in milks, the complex composition of milks may require multiple components to initiate the allergic process. Addition of an adjuvant may amplify Th2 responses to some proteins to a great extent than others. Similarly, cooking processes can modify the structure of isolated proteins and of whole foods[139, 160, 161, 475]; determining whether specific modifications to weak allergens can influence their allergenicity may clarify the processes that contribute to allergic sensitization in future studies. The third aspect implicated in allergic sensitization is the exposure site, and this is not necessarily reflected in the pTh2 assay. Examining allergenicity of primary allergen exposure in the lung, which models inhaled allergen exposure, has been shown to induce Th2-type responses in mice[136]. Using G4 reporter mice it may be possible to quantitate Th2 induction following the first intranasal allergen exposure, and in this reveal additional aspects of the Th2 response to allergens. While this may be below the sensitivity of the initial Th2 response, sufficient Th2 cells may develop, and thus help clarify how allergic Th2 immunity is initiated.

7.1.3 Evaluating the success of this project

In this thesis, "*Development of Th2 immune response models to evaluate allergenicity of milk proteins*", I aimed to develop assays that could be used to examine the comparative allergenicity of cow and goat milks. While I was unable to identify specific anti-allergic goat milk components, or those with intrinsically lower allergenicity than their homologous cow milk components, I was able to use the PTh2 assay and the oral allergen-induced intestinal anaphylaxis model to test the allergenicity of multiple milk proteins. The findings in chapters three, four and five provide good grounding for the investigations undertaken in these assays. In retrospect, generating empirical data for the mMCP-1 release studies could be informative in determination of the size of the experiments required to observe a predictably 'physiologically relevant' difference in 'allergenicity' in the oral challenge model. Thus, while I was unable to identify immunologic features that result in the reduced allergenicity of goat milk, the studies in this thesis provide clear evidence of how the PTh2 assay and oral challenge model can be used in synchrony as a framework in which multiple features of protein allergenicity can be evaluated.

7.1.4 Final conclusions

In this dissertation, I have used literature and experimental evidence to argue for a multi-phase inflammatory process that leads to allergic response susceptibility. The initial allergen exposure initiates IL-4-independent Th2 cell development. These cells are then expanded in the presence of IL-4, facilitate IgE production and are additionally involved in an oral potentiation phase that permits allergic response triggering in the intestine. These processes can be initiated by many individual proteins, but these vary by orders of magnitude, depending on the specific allergen involved. In this manner, allergic disease can be considered an unbalanced sum of factors: Disease is influenced by the inflammatory environment, properties of the allergen and the host that ultimately result in allergic response susceptibility.

The findings used to create this thesis can be summarised in four statements. First, primary allergen immunisation induces IL-4-independent Th2 cell development which

is followed by an IL-4-dependent phase. Second, IL-4 heterozygous mice are haploinsufficient for IL-4 in allergic disease contexts. Third, CD4⁺ T cells are critical mediators of IL-3-dependent oral allergen-induced intestinal mastocytosis. Lastly, the low allergenicity of goat milk compared to cow milk relates to its absolute composition, or changes occurring during physiological processing, rather than differences in the intrinsic allergenicity of its dominant protein components.

Despite some significant advances made in recent times, fuller understanding is required before allergies will become manageable maladies that are considered afflictions of the past. The most critical question that remains to be addressed is what permits persistent, consistently deleterious immune responses to normal, everyday proteins and foods. The aetiology of allergic disease is, and should remain at the forefront of scientific research until we have the answer.

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Chapter 8

Appendices

8.1 Supporting data

8.1.1 References for figure 1.1

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Figure 8.1: **References for figure 1.1.** These references relate specifically to figure 1.1 in the introduction section.

8.1.2 Genotyping of IL-3 and IL-9 transgene

Figure 8.2: **Genotype testing of IL-3 and IL-9.** (A) Mice were tested for the presence of the wild-type IL-9 and the IL-9 transgene. (B) Mice were tested for the presence of the wild-type IL-3 gene. Primer pairs can be found in the methods section.

8.1.3 Flow Cytometry reagents

Specificity	Fluorophore	Clone	Source
B220	Horizon V450	Ra3-6B2	BD Pharmingen
	PE-Texas Red	Ra3-6B2	BD Pharmingen
CD3	Alexafluor® 488	500A2	Invitrogen Molecular Probes
CD4	Allophycocyanin (APC)	RM4-5	BD Pharmingen
	APC/H7	GK1.5	BD Pharmingen
	Fluorescein Isothiocyanate (FITC)	GK1.5	In house
	Phycoerythrin (PE)	GK1.5	BD Pharmingen
	Peridinin Chlorophyll Protein (Percp)	RM4-5	BD Pharmingen
	Quantum dot605®	RM4-5	invitrogen Molecular Probes
CD8a	Alexafluor® 700	536.7	Biolegend
	PE-Cy7	53-6.7	BD Pharmingen
CD19	APC-H7	ID3	BD Pharmingen
CD44	PE	IM7	BD Pharmingen
CD45	Pacific Blue®	30F11	Biolegend
CD62L	Biotin	MEL-14	BD Pharmingen
	Pacific Blue®	MEL-14	Biolegend
CD117)	APC	2B8	BD Pharmingen
FceRI	PE	Mar-1	eBioscience
CD16/32	FITC	2.4G2	BD Pharmingen
human CD2	APC	RPA2.10	BD Pharmingen
I-A/I-E	PE-Cy7	M51114.15.2	Biolegend
IL-3	PE	MP2-8F8	BD Pharmingen
IL-4	APC	11B11	BD Pharmingen
	PE	11B11	BD Pharmingen
IL-5	PE	TRFK-5	BD Pharmingen
IL-10	PE	JES5-16E3	BD Pharmingen
IL-13	PE	eBio13A	eBioscience
IgD	PE	11-26c.2a	BD Pharmingen
IgE	biotin	R35-118	BD Pharmingen
IgG1	biotin	A85.1	BD Pharmingen
CD49b	PE-Cy7	DX5	eBioscience
Biotin	APC	Streptavidin	BD Pharmingen
	FITC	Streptavidin	BD Pharmingen
	PE	Streptavidin	BD Pharmingen
	PE-Texas Red®	Streptavidin	BD Pharmingen
	7-Amino Actinomycin D (7-AAD)		BD Pharmingen
	4,6-diaminidino-2-phenylindole (DAPI)		Invitrogen Molecular Probes
	Live/Dead® fixable blue		Invitrogen Molecular Probes
	GolgiStop (with Monensin)		BD Pharmingen

8.1.4 Flow cytometers

Cytometer	Laser	Filter and position	Reagents
LSRII-SORP	Ultraviolet (355 nm)	450/50B	DAPI
BD FACSCallibur	Violet (405 nm)	450/50F	Live/Dead fixable blue Brilliant Violet 421 Pacific Blue ® Horizon®V450
	Blue (488 nm)	605/40C	Qdot605
		705/70A	Percp
		515/20B	GFP FITC Alexafluor488
	Green (532 nm)	488/10C	Side Scatter (SSC)
		780/60A	PE-Cy7
		660/40C	7-AAD
		610/20D	PE-Texas Red®
	Red (633/640 nm)	575/26E	PE
		780/60A	APC-Cy7
780/60A		APC-H7	
FACSDiva	Blue (488 nm)	710/50B	Alexafluor® 700
		670/14C	APC
		530/30 (FL-1)	FITC Alexafluor®488 GFP
	Blue (488 nm)	585/42 (FL-2)	PE
		670LP (FL-3)	7-AAD
		661/16 (FL-4)	APC
		530/30 (FL-1)	PE
	585/42 (FL-2)		
	Red (633 nm)	670LP (FL-3)	APC
		661/16 (FL-4)	
Ultraviolet (355 nm)	780/60 (FL-5)	DAPI	
	450/50 (FL-6)		
		620LP (FL-8)	

8.1.5 Supplementary data

Figure 8.3: **GFP and hCD2 medFI are lower in KN2/G4 cells than G4/+, KN2/+, G4/G4 and KN2/KN2 cells.** Ten days after intradermal immunization with 100 μ g HDM, auricular lymph nodes were excised. (A) The GFP median fluorescence intensity of total GFP+ CD4+ cells was examined. (B) The hCD2 median fluorescence intensity of total hCD2+ CD4+ cells was examined. Data are shown from one of the experiments used to compile data in figures 4.2 and 4.3. AFU=Arbitrary Fluorescence Units.

Figure 8.4: *in vivo* GK1.5 treatment ablates CD4 but not CD8+ T cell populations. Representative flow plots are shown that were used to generate data in Figure 5.5B and C.

Figure 8.5: **Cytokine production by OVA or PBS-stimulated splenocytes from IL-3 sufficient and deficient mice.** Mice were sensitized to OVA plus alum twice by ip injection and then were mock-challenged with PBS 10 times. Spleens were excised and cultured with OVA (OVA) or medium alone (PBS). After four days supernatants were collected and examined for (A) IL-3, (B) IL-4, (C) IL-5 and (D) IL-13 production using a bioplex assay. Data bars show mean + SEM of individually cultured spleens (n=5/group). **p<0.01, ****p<0.0001 by Two-way ANOVA with Bonferroni's post-test.

Figure 8.6: **Cytokine production by OVA or PBS-stimulated mesenteric lymph node cells from Tg³+/+, Tg³-/- and nTg³-/- mice.** Mice were sensitized to OVA plus alum twice by ip injection and then were mock-challenged with PBS 11 times. Mesenteric lymph nodes were excised and cells were cultured with OVA. After three days supernatants were collected and examined for (A) IFN- γ , (B) IL-3, (C) IL-4 and (D) IL-5 and (E) IL-13 production by ELISA. Data bars show mean + SEM of individually cultured MLNs (n=3/group). Data were compared by One-way ANOVA with Tukey's post-test. Dashed lines represent the assay limit. ND, none detected.

Figure 8.7: **Mastocytosis in caprine BLG sensitized mice.** Jejunal mast cell frequencies were determined by CAE staining at the experimental endpoint in mice from Figure 6.8. NA not analysed.

Figure 8.8: **Liquid stool at 60 minutes post-challenge three in intestinally atopic mice fed different allergens.** 60 minutes after the third oral gavage with sensitizing antigen (day 13), *iIFABP*-IL-9 mice were photographed. Diarrhoea was not observed in any mice in a second experiment in which sensitized or naive mice were fed three times with 50 mg cow BLG.