DEVELOPMENT OF A HUMANISED MOUSE MODEL OF OVALBUMIN INDUCED ALLERGY AND INFLAMMATION AS A PLATFORM TO TEST FUNCTIONAL FOODS

By

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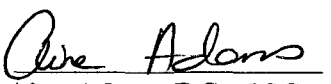
DECLARATION OF AUTHORSHIP

I certify that the work presented herin is, to the best of my knowledge, original, resulting from research performed by me, except where acknowledged otherwise This work has not been submitted in whole, or in part, for a degree at this or any other university



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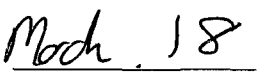

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ABSTRACT

Food allergies are most common during infancy Food allergy is a growing health problem with very limited treatment options For infants, cow's milk protein allergy (CMPA) is the predominant food allergy, occurring in 2 - 7 5 % of population Infant formula which is often cow's milk based, is the only approved alternative for breastfeeding However the proteins (casein and whey) in cow's milk result in allergic symptoms in the infants These allergic symptoms can be avoided by providing the infants with hydrolysates of cow's milk protein instead of the intact protein Whey protein hydrolysates, small bioactive peptide components of milk have been tested in mouse models for their anti-inflammatory/immunomodulatory properties however to date no studies have repeated efficacy of hydrolysates in a humanised mouse model setting In this study a large number of hydrolysates were screened *in vitro* to identify hydrolysates with anti-inflammatory properties *In vitro* we demonstrated that 147 hydrolysate significantly reduced DC maturation in a P-PARγ dependent manner To investigate the anti-inflammatory potential of hydrolysate 147 in a human relevant model of food allergy we developed a novel humanised mouse model of OVA driven food allergy We directly compared two different strains of nonobese diabetic severe combined immunodeficient mice lacking the cytokine receptor common gamma chain ($\gamma_c^{-/-}$) (NSG-SCF versus NSG-SGM3) expressing human cytokine genes for their suitability as a humanised mouse model

Using NSG-SCF mice a human immune system was developed through engraftment of $CD34^+$ hematopoietic stem cells Using an OVA sensitisation and challenge protocol the model of food allergy was developed in these humanised mice The capacity of hydrolysate 147 to mediate anti-inflammatory effects was examined through repeated administration of 147 hydrolysate after sensitisation and before challenge with OVA Both 147 hydrolysate and the wpc80 parent control equally reduced anaphylaxis. In comparison to wpc80 parent control, 147 reduced

specific antibody responses, including OVA-specific IgG1, reduced the production of the pro-inflammatory cytokines IL-6, IL-17 and induced higher levels of the anti-inflammatory cytokine IL-10 in spleens and small intestines of OVA treated mice 147 in comparison to wpc80 was more potent at promoting a Th1/Th2 balance in the spleen and most significantly suppressed the accumulation of granulocyte populations in bone marrow and liver while simultaneously reducing the T , B and myeloid cell populations in the bone marrow of OVA mice

For the first time, we have shown that a novel milk protein hydrolysate provided efficacy in comparison to its whey parent control in terms of reducing OVA driven allergy in a humanised mouse model This study demonstrated that 147 may prevent the development of other food allergies (egg-ova) in addition to CMPA (but not examined here) In addition this study, presents a framework, from which suitable conditions for testing functional foods, can be adopted in the development of novel agents for the prevention of allergic inflammation

PUBLICATIONS

Published Manuscripts

Mclean S, Healy, M, Collins C, C Carbery S, O'Shaughnessy L, Dennehy R, Adams A, Kennelly H, Corbett J, Carty F, Cahill L, Callaghan M, English K, Mahon, B, Doyle S, and Shinoy M (2016) Linocin and Omp W are involved in attachment of the cystic fibrosis associated pathogen Burkholderia cepacia complex to lung epithelial cells and protect mice against infection Infection and Immunity, 84(5) 1424-37

Manuscripts in Preparation

Adams A, Corbett J, and English K Development of a humanised mouse model of ovalbumin induced allergy and inflammation as a platform to test functional foods

Adams, A & English K Identification of hydrolysates with anti-inflammatory activity *in vitro*

Abstracts for Conference Proceedings

Anti-allergy effects of whey protein hydrolysates in human Peripheral blood mononuclear cells

Aine Adams, Bernard Mahon and Karen English

Food and allergy and Anaphylaxsis Meeting Oct 13-15 2016 Rome

Identification of immunomodulatory hydrolysates for infants diagnosed with Cow's Milk Protein Allergy

Adams, A & English K

Irish Society of Immunology Annual Meeting, 2015 Trinity Biomedicine Sciences, Institute

Comparison of the engraftment of human mast cells between NSG-SCF and NSG-SGM3 mice

Adams A & English K

Gene Editing, Transgenics & Stem cells Frontiers in Medicine and Molecular Therapeutics

Trinity Biomedicine Sciences, Institute. Sept 2015

ABBREVIATIONS

APC	Antigen presenting cell
ANOVA	Analysis of variance
BM	Bone marrow
CD	Cluster of Differentiation
CFSE	Carboxyfluorescein Succinimidyl Ester
DC	Dendritic cell
DH	Degree of hydrolysis
DH_20	Distilled water
DMSO	Dimethyl sulfoxide
DNA	Dulbecco's modified eagle's medium
DPP-IV	Dipeptidyl peptidase IV
EB/AO	Ethidium bromide/Acridine orange
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
ELISA	Enzye linked immunosorbent assay
FACS	Fluoresence-activated cell sorting
FITC	Fluorescein Isothiocyanate
FMO	Fluoresence minus one
FBS	Fetal bovine serum
Fix/Perm	Fixation/Permeabilisation
FSC	Forward scatter
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GI	Gastrointestinal
GM-CSF	Granulocyte macrophage colony stimulating factor
GVHD	Graft versus host disease
Gy	Gray

H&E	Hematoxylın and eosın
HMOS	Human mılk olıgosaccarıdes
HSC	Hematopoıetıc stem cell
HRP	Horseradısh Peroxıdase
ı v	Intravenous
IFN	Interferon
IgG	Immunoglobulın G
IgE	Immunoglobulın E
IgG1	Immunoglobulın G
IL	Interleukın
IMDM	Iscove's modıfied dulbecco's medıum
ıNKT	Invarıant natural kıller T cells
kDa	Kılodalton
KI	Knock ın
LPS	Lıpopolysaccarıde
MACS	Magnetıc-actıvatıng cell sortıng
MHC	Major hıstocompatıbılity molecule
MFI	Mean Fluorescence Intensity
MTT	3-(4, 5-dımethylthıazolyl-2)-2, 5-dıphenyltetrazolıum bromıde
NFκB	Nuclear factor kappa-lıght-chaın-enhancer of actıvated B cells
NK	Natural kıller
NOD	Non-obese dıabetıc
NSG	NOD-scıd IL-2 Rγ ^{null}
OD	Opıtical density
PB	Perıpheral blood
PBMC	Perıpheral blood mononuclear cells
PBS	Phosphate buffered salıne
PCR	Polymerase chaın reactıon
QPCR	Quantıtatıve Polymerase chaın reactıon
Pen/Strep	Penıcıllın/Streptomycın

RBC	Red blood lysis
RNA	Ribonucleic acid
mRNA	Messenger Ribonucleic acid
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute
TPO	Thromopoietin
SCF	Stem cell factor
SCID	Severe combined immunodeficiency
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SSC	Side scatter
TLR	Toll like receptor
Th1	T helper 1
Th2	T helper 2
Th3	T helper 3
Th17	T helper 17
T reg	Regulatory T cell
WPC	Whey protein control

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CHAPTER 1
INTRODUCTION

1.1 ALLERGY

"The conception that antibodies that should protect against disease are also responsible for disease, sounds at first absurd"

Clemen von Pirquet (1906)

The term "allergy" was coined by Clemen von Pirquet in 1906 to call attention to the unusual propensity of some individuals to develop signs and symptoms of reactivity or hypersensitivity reactions when exposed to certain substances Although the statement quoted above pertained to the cause of serum sickness (Silverstein 1906) allergic disorders are also associated with the production of allergen- specific IgE and the expansion of allergen specific T cell populations Both IgE and T cells are reactive with what typically or otherwise are harmless environmental substances These disorders are increasingly prevalent in the developed world and include allergic rhinitis (also known as hay fever), atopic dermatitis (also known as eczema) allergic (or atopic) asthma and some food allergies (Holgate *et al* 1999, Kay *et al* 2001) Some people develop a potentially fatal systemic allergic reaction termed anaphylaxis within seconds or minutes of exposure to allergens (Sampson *et al* 2005)

In recent years it has become clear that much of the pathology and therefore the burden of disease associated with allergic disorders reflects the long term consequences of chronic allergic inflammation at sites of persistent or repetitive exposure to allergens (Kay *et al* 2001) This realisation has led to renewed efforts to define additional therapeutic targets in allergic disease (Kraft *et al* 2007, Holgate *et al* 2008), to devise improved strategies to induce immunological tolerance to the offending allergens (Larchee *et al* 2006, Akdis *et al* 2007) and even to manipulate

the immune response to prevent the initial development of allergic disorders (Kukkonen *et al* 2007)

1.2 FOOD ALLERGY

The last decade has witnessed a significant rise in the prevalence of food allergies worldwide, as well as in the number of food proteins that can trigger an immune response (allergens) A substantial 18% increase in reported food allergies in children, under the age of 18 years, has been observed between 1997 and 2001 (Branum *et al* 2008) Food allergy is defined as "an adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food" (Boyce *et al* 2010) Within this definition are (1) IgE-mediated food allergies which occur within minutes of the ingested allergen and (2) non-IgE-mediated food allergies which are the delayed-type and occur 2-3 hours after ingestion In the majority of food allergy patients, ingestion of food allergens causes gastrointestinal (diarrhea, vomiting), cutaneous (uticaria), bronchopulmonary (dyspnea), cardiovascular (hypotension) symptoms and in severe cases, lethal anaphylactic shock (Ben-Shoshan *et al* 2011, Benhamou *et al* 2010) Recent studies indicate that up to 10% of the population in the western world now suffer from at least one type of food allergy (Prescott *et al* 2013) Food allergies are most common during infancy For infants, cow's milk protein allergy (CMPA) is the predominant food allergy, occurring in 2-3% worldwide (Host *et al* 2014) Although cow's milk avoidance is the dietary management for infants who suffer with CMPA, accidental exposure is common, which can trigger an allergic response or, in more severe cases, anaphylactic shock While it is reassuring that up to 90% of infants outgrow CMPA and develop tolerance by the age of five (Host *et al* 2014) the small percentage of

children who do not develop tolerance have an increased risk of developing conditions associated with atopy, such as asthma, dermatitis, and allergic rhinitis, or additional food allergies in later life a phenomena known as the "atopic march" (Vandenplas *et al* 2007) This prevalence of food allergy that has increased globally places a large burden on affected patients and their families The reason for this increasing prevalence remains to be elucidated, but many theories including increased hygiene, increased dietary fat, antioxidants, vitamin D insufficiency and skin sensitisation have been proposed (Lack *et al* 2008) Food allergy not only affects the patient but the whole family as well Childhood food allergy has a significant impact on general heath perception, emotional impact on the parent and limitations on family activities (Sicherer *et al* 2001) It also has been shown that the diagnosis of food allergy causes significant alterations in meal preparations, social activities, and school attendance and contributed to increased stress levels in the family (Bollinger *at al* 2006) Although quick treatment of the mild symptoms with anti-histamine or injection of epinephrine for chronic symptoms saves many lives, day to day treatment and prophylactic treatment should be established to suppress the symptoms and to improve the life of the allergic patient To understand where and how food allergy against dietary proteins can occur, we first need to briefly discuss the role of the comprehensive mucosal system

1.3. INTESTINAL IMMUNE SYSTEM

In the healthy gut, the immune system is able to create a balance between the level of protective mucosal immunity and systemic tolerance Immune homeostasis in the gut develops as a relationship that is established between the intestinal

microbiota, luminal antigens, and the epithelial barrier (Iweala *et al* 2006) Microbial colonization of the intestine begins after birth, where the sterile gut of the newborn is gradually colonized by environmental bacteria and by contact with the maternal intestinal flora and surroundings and possibly by genetic factors (Houghteling *et al* 2016) Exposure to microbial flora early in life allows for a change in the T helper 1 (T_H1)/T_H2 cytokine balance, favouring a T_H1 cell response (Qian *et al* 2017) At birth, the immune system of an infant is not fully developed and tends to be directed toward a T_H2 phenotype to prevent rejection *in utero* The T_H2 phenotype, however, leads to the stimulated production of IgE by B cells and thus increases the risk for allergic reactions through the activation of mast cells Microbial stimulation early in life will reverse the T_H2 bias and stimulate the development of T_H1 phenotype and stimulate the activity of T helper 3 cells T_H3 (T von der Weid *et al* 2001) T_H3 cells are CD4 T lymphocytes with a similar phenotype to conventional Tregs that secrete TGF-β and IL-10 and are distinctive for also expressing IL-4 (Peterson, 2012, Inobe *et al*, 1998) In this manner, their combined action will lead to the production of IgA by B cells IgA contributes to allergen exclusion and will thereby reduce exposure of the immune system to antigens Cytokines produced by the T_H1 phenotype will also reduce inflammation and stimulate tolerance toward common antigens (Kirjavainen *et al* 1999)

1.4 BASIC PATHOGENESIS OF FOOD ALLERGY

Allergic inflammation is a fundamental pathological change associated with food allergy, and type 1 hypersensitivity of the immune system is the basic mechanism of allergic inflammation (Barnes 2009) There are two phases in the basic process of

allergic inflammation the induction (sensitisation) phase and effector phase (Figure 1 1) The induction phase involves antigen presenting cells (APCs) that phagocytose food proteins and process and present them to $CD4^+$ T helper 2 cells (T_H2) The T_H2 cells in turn release pro-inflammatory cytokines such as interleukin IL-4, IL-5 and IL-13 This results in activation and class switching of B cells which produce IgE and binding of the high afficity IgE receptor on the membrane of mast cells and basophils, forming sensitised mast cells and basophils The differentiation of B-lymphocytes into IgE-expressing cells depends on three signals The first signal is delivered through the B-cell antigen receptor and is pivotal in determining the antigenic specificity of the response The second signal is primarily provided through cytokines derived from T_H2 cells, such as IL-4 and IL-13 Under tight regulation, theses cytokines stimulate transcription through Ig constant region genes The third signal is provided via the interaction between the constitutively expressed CD40 molecule on B-lymphocytes and CD154 (CD40 ligand) a molecule expressed on T-lymphocytes Thus the elevated levels of IgE observed in atopic individuals might reflect the preferential activation of T_H2 cells (Jelinek 2000) The effector phase begins when the same allergen cross-links two adjacent IgEs on sensitised mast cells or basophils activated mast cells or basophils subsequently release pro-inflammatory mediators or cytokines, thereby causing the clinical manifestations of food allergy (Figure 1 1)

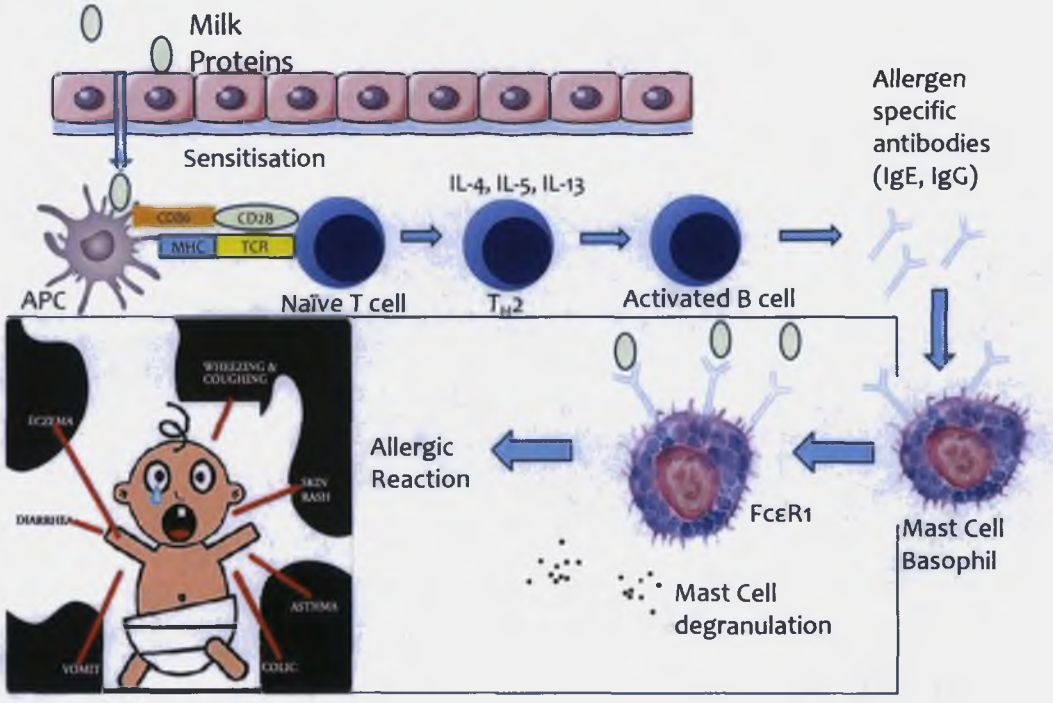


FIGURE 1.1 Schematic representation illustrating the key events during sensitisation and an allergic reaction. When antigen passes through the epithelial barrier, it is taken up and processed by APCs. These cells present the antigen in the contect of MHC class II molecule. Together with co-stimulatory molecules (CD40, CD86, CD80) and ligand binding to the CD28 receptor on antigen-specific naïve T cells, leads to T cell activation and development of T_H2 phenotype. Activated T cells stimulate B cells differentiation into plasma cells, which start to produce antigen-specific antibodies (IgE). These antibodies bind to the high-affinity receptor FcεR1 on mast cells and basophils. Upon re-exposure or challenge of the same antigen the antibodies cross-link and degranulation of the mast cell is induced. The release of a range of compounds results in allergic symptoms.

1.5. TRANSMITTERS OF ALLERGY

As shown in figure 1 1 all key cells and molecules involved in transmitting the biological message to primary effector cells are messengers of allergy, represents the most complicated process whose underlying mechanism remain unknown However, allergic inflammation reflects a complex interplay between several inflammatory cells including mast cells, basophils, T lymphocytes, dendritic cells (DC), innate lymphoid cells, eosinophils and neutrophils These cells produce multiple inflammatory mediators, including lipids, cytokines, chemokines and reactive oxygen species (Barnes 2011) In this chapter, the roles of some key cells involved in innate and adaptive immunity in the transmission from biological messages of allergen to primary effector cells will be discussed

1.6. FOOD ALLERGY AND THE INNATE IMMUNE SYSTEM

1.6.1. DENDRITIC CELLS

As a part of professional antigen-presenting cells (APCs), DCs are crucial in order to sample, process, and display antigens to naive T cells, either to initiate immune responses or to induce immune tolerance (Steinman *et al* 2003) Pertaining to food-derived antigens, roles of DCs in the intestine and associated lymphoid tissues are of particular interest, partly due to the fact that these cells can pick up antigens directly from the intestinal lumen or antigens that have been transported across the intestinal epithelial cell (IEC) (Coombes *et al* 2008) (Figure 1 2) IECs are the first line of defence against harmful pathogens and antigens These epithelial cells are highly organised and connected by tight junctions In adults the connections

are solid so the barrier is impermeable to damage by molecules but for infants the exact permeability is not known (Hill *et al* 2017) In the healthy gastrointestinal tract, however, commensal bacteria and their products modulate intestinal DCs to be tolerant via interaction with the pattern recognition receptors (PPRs) of DCs (de Kivit *et al* 2014) In addition, non-inflamed healthy IECs are also able to suppress inflammatory DCs inducing tolerogenic DCs It is the existence of tolerogenic $CD103^+$ DCs however in murine intestines and mesenteric lymph nodes that points to the important role of DCs Murine DCs were able to convert naive $CD4^+$ T into $Foxp3^+$ T Regulatory (T_{Reg}) cells via TGF-β and retinoic acid (Coombes *et al* 2007) A recent study shows a functional homology between murine $CD103^+$ DCs and human $CD141^{high}$ DCs in cross-presenting antigens to $CD8^+$ T cells (Haniffa *et al* 2012) hence eliciting a query of whether human intestinal $CD141^{high}$ DCs can also serve as tolerogenic DCs Notably in a mouse model of peanut allergy, oral sensitization with peanut extract was accompanied by a shift in intestinal DC subsets, that is, less tolerogenic $CD103^+$ DCs but more inflammatory $CD11b^+$ DCs (Smit *et al* 2011) DC-recognition of allergens can be mediated by their C-type lectin receptors, such as DC-SIGN and mannose receptor (Salazar *et al* 2013) Subsequently in the presence of IL-4 (potentially released by allergen-activated innate immune cells), allergen-presenting DCs polarize naive $CD4^+$ T cells into T_H2 cells, which in turn direct B cells to produce IgE (Galli *et al* 2008) The important role of DCs for mediating allergic reaction against food proteins is indeed supported by a finding from the adoptive transfer study of DCs from cow's milk-allergic mice into naive recipients Importantly, this DC transfer induced spontaneous production of cow's milk-specific IgE in the naive mice in the absence of antigen challenge (Chambers *et al* 2004) Omalizumab (anti-IgE antibody) reduced serum IgE and

altered the presentation by DCs to T cells (Schroeder *et al* 2010) Taken together, an interaction between IECs, and intestinal DCs under homeostatic conditions contributes to immune tolerance in the healthy gastrointestinal tract In the presence of harmful antigens however, DCs can also initiate the allergic reaction by sampling and processing allergens and then presenting allergen-derived peptides to $CD4^+$ T cells, which will be followed by the activation of pro-allergic effectors including tissue mast cells, basophils, and eosinophils These results highlight the dual role of DCs in programming the T-cell response to foods

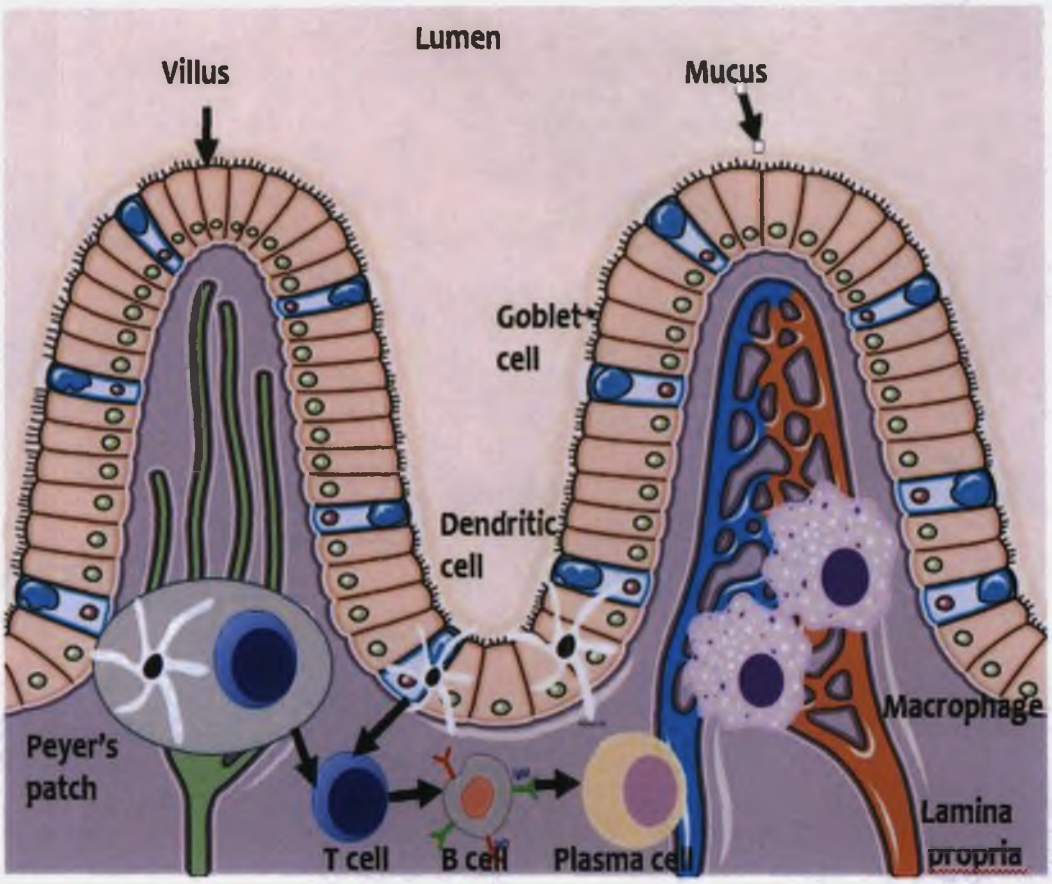


Figure 1.2 Layers of the intestinal immune barrier. The barrier consists of a protective layer of epithelial cells covered with mucus and a range of immune cells. The main cell type in the epithelial layer is the gate-keeping epithelial cells, but other specialized cells are also present. The crypts of the villi contain mucus-producing goblet cells. The lamina propria under this epithelial layer contains many immune cells including dendritic cells, macrophages, and lymphocytes. After antigen passes through the epithelial barrier, it is taken up by dendritic cells. The antigen-presenting dendritic cells migrate to the mesenteric lymph nodes and present their MHC-antigen complex to T cells inducing T-cell activation. In the Peyer's patch (organized lymphoid nodules) antigen-presenting dendritic cells also interact with T cells. T cells then drive the immune response activating other immune cells, for example, B cells.

1.6.2. MAST CELLS AND BAOPHILS: THE PRIMARY EFFECTOR CELLS OF ALLERGY

Mast cells (MCs) and basophils are the key effector cells responsible for allergic inflammation They directly respond to allergen challenge through immunoglobulin dependent or independent mechanisms (Vliagoftis *et al* 2005) In the GI tract, MCs comprise 1-5% of mononuclear cells in the lamina propria and the submucosa, and are also found intraepithelial and deep in the muscle and serosal layers Basophils are granulocytes, which mature in the bone marrow, circulate in the blood and are recruited to allergic inflammatory sites In contrast, progenitors of MCs migrate from the bone marrow to the peripheral tissues and undergo their terminal differentiation *in situ*. MCs that complete their differentiation in the skin or intestine develop into connective tissue MCs and mucosal MCs, respectively MCs and basophils express the high-affinity receptor for IgE and, upon crosslinking of FcεR1-bound IgE with antigen, rapidly produce diverse preformed mediators, cytokines (*e g* IL-4 and IL-13) and lipid mediators, leading to the induction of immediate-type hypersensitivity

Based on the anatomical location, human MCs are classified into mucosal MCs and connective tissue MCs, depending on protease content MCs are divided in two large subsets MCT, containing tryptase but little or no chymase, and MCTC, containing tryptase, chymase and carboxypeptidase MCC, which express chymase but little or no tryptase, also have been described, but they appear to be infrequent MCT prevail in the intestinal and pulmonary mucosa, near T cells, whereas MCTC are found in the skin and lymph nodes, in addition to the lung and the gut submucosa In the human small intestine, MCT represent ~98% of all MCs in the

mucosa and ~13% of MCs in the submucosa are MCT (Amin *et al* 2012) Briefly, mast-cell activity in humans can be indirectly measured by the diameter size of the induced wheal after skin prick test (SPT) with milk extract (Ford *et al* 2014) while in mice mast-cell activity can be measured by the elevation of serum levels of mouse mast-cell β-chymase/mast cell protease 1 (MCP1) a specific marker for mucosal MC degranulation (Caughey 2007) Basophils are the rarest type of granulocyte that mature in the bone marrow from $CD34^+$ myeloid stem cells and are then released into the circulation Like mast cells, basophils express the high affinity IgE receptor and synthesize histamine due to the actions of histadine decarboxylase This archetypal pro-allergic mediator is stored within intracellular granules bound to proteoglycans but is released from the cells following stimulation (Nouri-Aria *et al* 2001) The release of this biogenic amine is one of the most important pro-allergic mediators that governs the classical hallmarks of acute inflammation Although in humans MCs are more numerous than basophils, the latter are at least one order of magnitude more sensitive to IgE-mediated provocation than mast cells This along with their usual location in the blood, places basophils as potentially important contributors to systemic anaphylaxis In general, basophils are involved with late-phase reactions and are uniquely capable of rapidly releasing T_H2 cytokines IL-4 and IL-13 and both cytokines contribute to increased adhesion molecule that potentiate further inflammatory cell influx into the region (Gibbs *et al* 1996) Basophils also express CD40 ligand, which in conjunction with IL-4 and IL-13 causes class switching in B cells (Yanagihara *et al* 1998)

Human MCs in contrast produce little or no IL-4 and it seems unlikely that they can fulfil the above immunomodulatory roles as effective as basophils Upon activation MCs and basophils release three major groups of proinflammatory

mediators causing pathological damages (Kalesnikoff *et al* 2010) and clinical symptoms of allergy (Hennino *et al* 2006, Pettiper *et al* 2007) MCs release three groups of mediators, including preformed granule products, such as histamine, tryptase, chymase and heparin, newly synthesized arachidonic acid products, such as leukotriene C4 (LTC4) and prostaglandin D2 (PGD2) and cytokines, such as IL-4, IL-13, and eotaxin (Iwahiet *et al* 2005) These products greatly contribute to pathological damage in different tissues Symptoms of allergies occur after the activation of mast cells or basophils Theoretically, the degranulation of mast cells and basophils is the definitive event in allergy, whereas IgE alone serves as only one of the key messengers Although MCs and basophils play a decisive role in food allergy, the cell types involved in the sensitisation of primary effector cells are also essential for the occurrence of allergy For example, an individual allergic reaction to grass pollen or certain foods requires long process to obtain sensitisation to that specific allergen and not other allergens. This natural phenomenon suggests that MCs or basophils should be primed before activation Degranulation is a cellular process that releases antimicrobial cytotoxic or other molecules from secretory vesicles called granules found inside MCs and basophils

In humans, basophils are short-lived cells that account for less than 1% of circulating granulocytes in the blood In contrast, MCs are located in the tissue and mast cell progenitors have the potential to proliferate locally in the tissue in response to IL-3, IL-4 and IL-9 These products greatly contribute to pathological damage in different tissues (Yu *et al* 2006) These MC secretory products are activation markers for mast cells whereas histamine, LTC4 and PGD2 are activation markers for basophils A range of specific surface membrane activation markers are used to distinguish mast cells from basophils CD203c (ectonucleotide pyrophosphatase/

phosphodiesterase 3) and the high affinity IgE receptor FcεR1 are specific surface markers for MCs and basophils which are employed in flow cytometric analysis (Boumiza *et al* 2003). CD117 or c-kit is a receptor of stem cell factor which is expressed on the surface of all MCs, independent of maturation and activation status but not expressed on basophils. Thus, MCs can be distinguished by routine flow cytometry as $CD203^+$ $Fc\varepsilon R1^+$ $c\text{-}kit^+$ and basophils as $CD203^+$ $Fc\varepsilon R1^+$ $c\text{-}kit^-$. Roles of other innate immune subsets including neutrophils, monocytes, natural killer (NK), γδ T cells, innate lymphoid cells (ILCs) and natural killer T (NKT) cells during allergic reaction to food proteins remain to be elucidated. Elevated numbers of neutrophils, γδ T cells and NKT cells have been shown to accumulate in chronically inflamed digestive tissues of food allergy patients (Semeniuk *et al* 2009, Turunen *et al* 2004, Jyonouchi *et al* 2014) but the actual roles of these innate cells are unknown. It is possible that these cells are accumulated in the inflamed sites mainly because of the elevated levels of inflammatory chemokines during chronic allergic reaction and indirectly activated by the circulating inflammatory cytokines, hence contributing to the chronic inflammatory reactions (Galli *et al* 2008). Nevertheless, several murine studies suggest that these cells could play key roles during allergy. Neutrophils have been indicated to be important in both sensitization and induction of allergic skin inflammatory reactions as well as mediating alternative mechanisms of anaphylactic reaction (Mocsai 2013). On the other hand, murine tissue γδ and invariant NK T cells were suggested to exert regulatory roles to suppress food allergy (Bol-Schoenmakers *et al* 2011). Human studies are required to clarify these murine findings.

1.7 FOOD ALLERGY AND THE ADAPTIVE IMMUNE SYSTEM

1.7.1. $CD4^+$ T CELLS

$CD4^+$ T cells serve an important role as the master regulator of adaptive immune responses Through their plasticity to differentiate into at least proinflammatory $T_H1/T_H2/T_H17$ or anti-inflammatory T_{Reg} cells, $CD4^+$ T cells crucially influence the outcome of inflammatory reactions either resulting as a resolved or persistent inflammation (Zhu *et al* 2010) T_H1 and T_H2 cells are not 2 distinct $CD4^+$ T-cell subsets, but they simply represent polarized forms of the highly heterogenous $CD4^+$ T_H cell–mediated immune response T_H1 cells are characterized by the prevalent production of IL-2, IFN-γ, and TNF-α, without IL-4, IL-5, and IL-13 production In contrast, T_H2 cells are characterized by the prevalent production of IL-4, IL-5, and IL-13 in the absence of IFN-γ and TNF-α production (Romagnam 1994) The T_H1/T_H2 polarization is clear-cut in murine models on the basis of artificial immunization, whereas it is usually less restricted among human T_H cell-mediated responses Signals through contact molecules, as well as through cytokine receptors, elicit a complex series of molecular interactions that culminate in the binding of cell type-specific transcription factors and subsequent activation of cytokine genes The main transcription factor for T_H2 differentiation is GATA-3, which inhibits the production of IFN-γ, and also directly regulates IL-5 and IL-13 expression

The main transcription factor involved in T_H1 lineage commitment is the protein T-box expressed in T cells (T-bet) Transduction of T-bet into fully polarized T_H2 cells converts them into IFN-γ–producing T_H1 cells and simultaneously represses the T_H2 cytokines IL-4 and IL-5 A model for T_H1/T_H2 polarization that involves a balance between specific GATA-3 transcription factors

has been proposed (Rengarajan *et al* 2000) T_H17 cells produce a range of cytokines including IL-17A, IL-17F, IL-21 and IL-22 express the transcription factor Rorγt and are physiologically important to eliminate intracellular and extracellular pathogens and play a pivotal role in the pathogenesis of autoimmune disease T_H2 cells are known to be important for eradicating helminths However, through secretion of IL-4, IL-5, and IL-13, T_H2 cells also contribute to the pathogenesis of allergy (Galli *et al* 2008) In line with this, PBMC from patients exhibited protein-specific T_H2-polarised immune responses producing, high levels of IL-4, IL-5, and IL-13 with low production of T_H1-cytokine IFN-γ (Andre *et al* 1996, Campbell *et al* 1996, Schade *et al* 2000)

The current findings indicate that most food allergies are characterized by T_H2-polarized immune responses accompanied by the impairment of T_{Reg} cells Although it was originally proposed that each subset of $CD4^+$ T cells permanently retained a specific differentiated identity (T_H1 or T_H2 or T_H17) resulting in non-overlapping distinct subsets (O'Shea *et al* 2010) It is clear now that there is a level of plasticity in the T cell phenotype particularly during chronic inflammation *in vivo* (Hirahara *et al* 2013). This allows $CD4^+$ T cells to secrete signature cytokines that have been associated with other subsets or even to further convert into other subsets For example, it has been shown that T cells derived from chronic allergic asthma patients co-expressed and coproduced both T_H2 and T_H17 transcription factors and cytokines (Wang *et al* 2010) Thus whether T_H2-polarized immune responses in food allergic patients could also exhibit or even convert to T_H1- or T_H17-immune responses remains to be seen

1.7.2. ADAPTIVE IMMUNITY AND T-REGULATORY CELLS

Regulatory T cells (T_{Reg}) are a subset of $CD4^+$ T cells which possess potent immunomodulatory ability and are defined as $CD4^+$ $CD127^{low}CD25^{high}$ $FoxP3^+$. The expression of CD127 has been used to separate immune suppressive T_{Reg} ($CD127^{low}$) from activated effector T cell populations ($CD127^{high}$) (Hartigan-O'Connor *et al* 2007). One possible mechanism that exists to modulate allergic inflammation can be attributed to the suppressive role of T_{Reg} cells. These cells can be further classified as thymus-derived (natural T_{Reg}) or peripherally derived and whose activation is dependent on T cell receptor engagement with cytokines (induced T_{Reg}) (Abbas *et al* 2013). Their suppressive functions can occur through either secretion of anti-inflammatory cytokines (e.g., IL-10 and TGF-β and IL-35), metabolic disruption, or attenuation of DC maturation and/or functionality (Vignali *et al* 2008). It has been shown that a fine balance between Treg and pro-allergic T_H2 cells, including cell frequency and functionality, determines the development of allergy (Akdis *et al* 2004). Co-culture of Treg cells in the presence of DC reduced the antigen presentation ability and co-stimulatory function of DC through cyclic adenosine monophosphate (cAMP) production by the Treg cell and subsequently reduced the expression of the co-stimulatory molecules CD80 and CD86 on DC (Safinia *et al* 2013). Supporting evidence of a Treg suppressive role in allergy came from a clinical study of patients with IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome caused by a deletion in a noncoding region of the *FOXP3* gene, the central gene for Treg differentiation. These patients had a defect in Treg frequency as well as functionality and more importantly exhibited severe food allergic phenotype particularly against cow's milk proteins (Torgerson *et al* 2007). IPEX patients also suffer from autoimmune diabetes and/or thyroiditis

reiterating that the impairment of Treg allows many types of inflammation to occur (Cheng *et al* 2012) In addition, lower frequencies of TGF-β-producing T cells were observed in the duodenal mucosa of children with food allergy as compared to non-allergic subjects (Westerholm- Ormio *et al* 2010) To summarize, defects in Treg frequency and functionality partly contribute to food allergy pathogenesis

1.8. ALLERGY TYPICALLY BEGINS DURING INFANCY

Newborns have specific dietary needs Breast milk significantly enhances growth and development in the first months of life When breastfeeding is not possible, infant formula is the only approved infant nutrition However this is not without consequences Neonates are exposed to a large number of microorganisms, foreign proteins, and chemicals and resistance to infection relies both on the protective factors in breast milk and on the infant developing his/her own innate and adaptive (antigen-driven) immunity The neonatal immune system functions differently from that of an adult (Kelly *et al* 2000) Whether one can define the infant's immune system as classically "immunosuppressed" is somewhat debatable as little antigen exposure occurs in utero From an immunological standpoint the infant's cells require considerable "education" in the early postnatal period Deficiencies in antigen presentation (Kellt *et al* 2000) contribute to T cell immaturity The germ-free status of the intrauterine environment favors T-helper 2 (Th2) type cytokine response over a T-helper 1 (T_H1) response (Cummins *et al* 1997) Together, the naive and altered cytokine response by the infant's immune system will contribute to lower immune competence in the infant Infants are often unable to produce all of the enzymes required for complete digestion of dietary

protein such as gastric pepsin and this, coupled with their high stomach pH (up to four times higher than that of an adult), can often result in the incomplete digestion of proteins in newborns (Figure 1.3) (Henderson *et al* 2001). The allergic potential of peptides is dependent on the level of exposure and their size, with larger peptides being more allergenic and increasing risk of sensitisation to specific food proteins (Figure 1.3). As the immature gastrointestinal barrier of vulnerable infants is more permeable, it enables these larger proteins to be absorbed. Once crossed over the gastrointestinal barrier, these larger peptides can activate and overexpress Type 2 helper T-cells (T_H2), which act as key drivers of allergy. If the development of the gastrointestinal immune system and oral tolerance can be stimulated in the early stages of life, the likelihood of activating this allergic cell type and, thus, inducing sensitisation is greatly reduced (Vickery *et al* 2011).

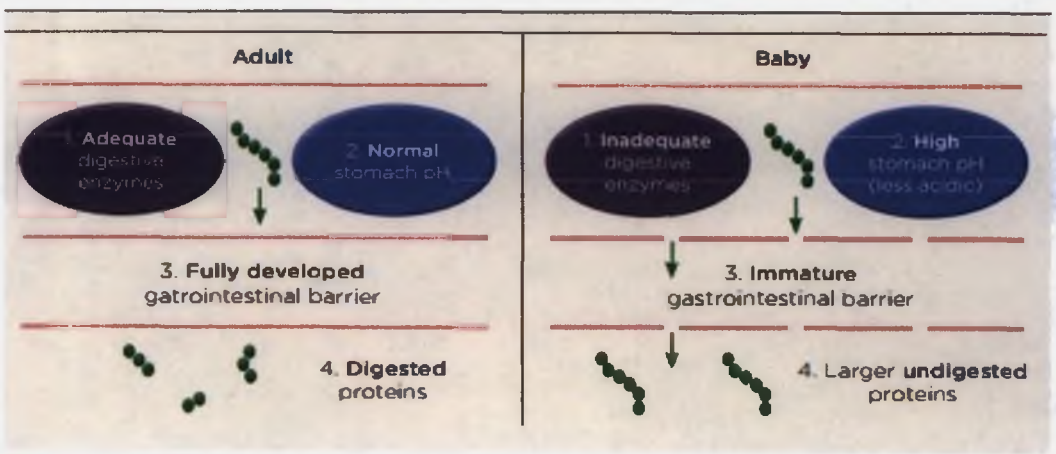


Figure 1.3. The conditions within the infants gastrointestinal tract that can potentially impact their ability to digest larger proteins and therefore increase susceptibility to allergenicity.

1.9. MECHANISM OF SENSITISATION TO FOOD-IMMUNE TOLERANCE

The gastrointestinal tract, the largest immunologic organ in the body, is exposed to a constant barrage of exogenous antigens on a daily basis (Chehade *et al* 2005) The mucosal immune system has evolved to inhibit responses to harmless antigens (e g , commensal bacteria, food protein) while maintaining the ability to mount a vigorous protective response when faced with pathogens As already discussed in section 1 3 homeostasis between tolerance and immunity is therefore an active immune process This fine balance can be modified by several factors in the intestine and when disrupted can lead to a breach in oral tolerance and inappropriate allergic sensitization to food proteins Remarkably, only a single epithelial layer separates this antigenic load from the lymphocytes, APCs, stromal cells and other immune cells in the lamina propria, which together comprise the gut associated lymphoid tissue (GALT) In the small intestine these are called the Peyer's patches (Figure 1 2) Within the Peyer's patches, unique populations of DCs interact with dietary antigens and determine the fate of the resulting adaptive response, i e , immunity versus tolerance (Chehade *et al* 2005) In this context, immune tolerance is defined as the antigen-specific suppression of cellular or humoral immune responses Normally, when the initial antigen exposure occurs through the GI tract, a robust T cell-mediated suppression develops called oral tolerance (Coombes *et al* 2008) However, in 4–6% of children, this mechanism appears to fail, leading to sensitization and elicitation (Faria *et al* 2005) Depending on the amount of antigen, three distinct immunological mechanisms can contribute to tolerance to food antigens, that is induction of regulatory T cells (T_{regs}), clonal anergy of T cells and deletion of T cells (Weiner *et al* 2011) In the mesenteric lymph nodes, T_{regs} are

mainly generated by $CD103^+CX3CR1^+$ DC Here, the tolerogenic $CD103^+CX3CR1^+$ DC present the food antigens to $CD4^+$ T cells Tolerogenic $CD103^+CX3CR1^+$ DC, but also macrophages, mostly express IL-10, and TGF-β, which induce T_{reg} formation (Manicassamy *et al* 2010) Upon interaction of DC with the T_{reg} subtype Th3, a cascade of Treg subtype induction and maintenance is initiated, while the secreted TGF-β at the same time inhibits T_H1 and T_H2 subtype T cells (Weiner *et al* 2011) This type of tolerance induction is only initiated when the food antigen is present in lower doses This mechanism is considered to be a central mechanism of tolerance induction for food antigens

At higher antigen doses, tolerance is mainly caused by clonal deletion and clonal anergy PRR-mediated activation, and especially toll like receptor (TLR) signaling, has also been found to be essential in these processes TLRs influence the T-cell response, as peptide-MHC-II-complex presentation together with a lack of co-stimulatory molecules on dendritic cells during high antigen exposure is thought to cause T-cell clonal anergy (Strobel 2003). Anergy is caused by a strong signal 1 which can be initiated through TCR activation of APC and then APC presents antigen to T cell In the absence of signal 2 (co-stimulation) anergy can also occur Clonal deletion of T cells is the other process that occurs at high antigen exposure This usually occurs in the Peyer's patches It is thought to be caused by natural killer T cells, possibly via Fas–FasL interaction with T cells that leads to apoptosis of food antigen recognizing T cells (Kim et al 2006) (Figure 1 3)

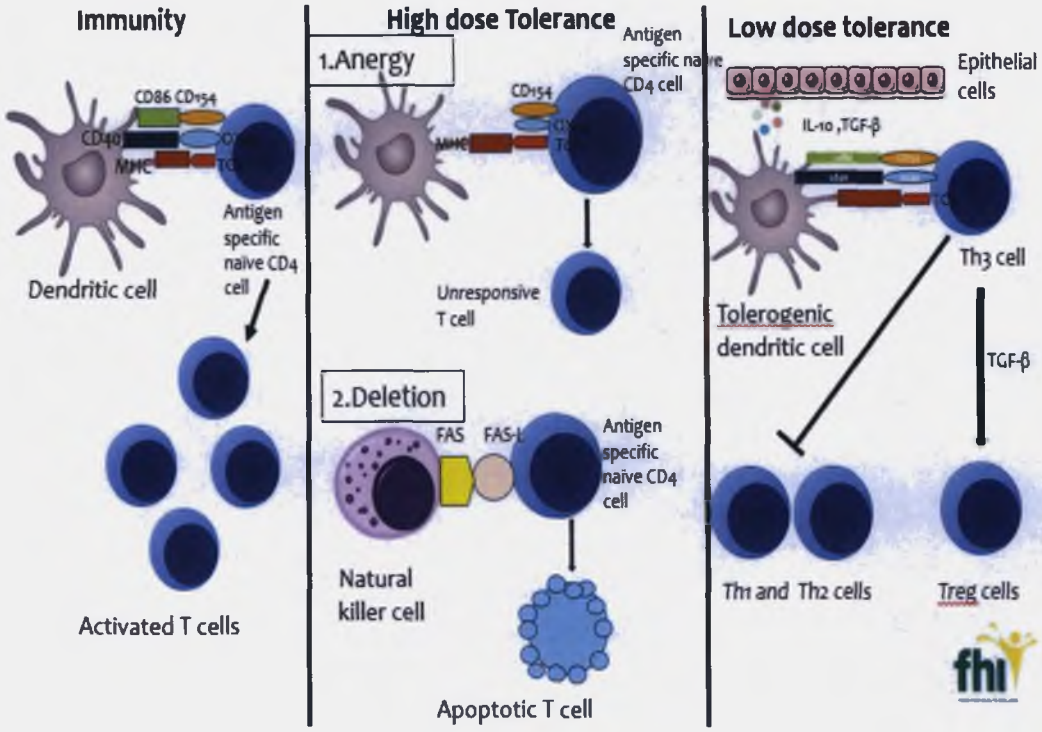


Figure 1.3. Oral tolerance induction. Normal immunity is induced by the interaction between an antigen-presenting dendritic cell and a naïve T cell. Due to the binding of the T cell receptor with the MHC-peptide complex and the co-stimulatory molecules, the T cell becomess activated. However, the default state in the gut is tolerance against dietary antigens. Different mechanisms are involved in inducing tolerance. When a high dose of antigen is present, the main mechanisms involved are anergy and deletion of T cells. Anergy occurs when co-stimulatory molecules are lacking during the interaction between dendritic cells and T cells. This leads to an unresponsive T cell. Deletion also occurs at high-antigen levels. Fas–Fas ligand-induced apoptosis is induced in antigen specific T cells after interacting with, for example, a natural killer T cell. The main mechanism inducing tolerance at a low dose of antigen is the induction of regulatory T cells. Tolerogenic factors (IL-10, TGF-β) produced by epithelial cells result in a more tolerogenic dendritic cell. When

this cell interacts with a T-helper 3 cell, the T cell produces TGF-β, which leads to differentiation towards a regulatory T cell and the inhibition of T_H1 and T_H2 cells

1.10. THERAPEUTIC INTERVENTION FOR FOOD ALLERGY

1.10.1. DIETARY PROPHYLAXIS FOR FOOD ALLERGY

The exponential increase in allergic diseases continues to remind us of the urgent need to devise strategies to curb this trend A number of approaches have been tested, including allergen avoidance, immune modulation, and dietary manipulation Supplementing the diet, rather than food allergen avoidance, is attractive because it is relatively simple and inexpensive to administer and the risk of any adverse nutritional consequences is low This strategy includes the use of probiotics and prebiotics, bioactive peptides and vitamins The innate immune system has the ability to modulate adaptive immune responses to food proteins Therefore, the type of gastrointestinal microbiota of the newborn and the preservation of intestinal permeability is crucial for preventing the development of food allergies The dietary modulation of nutritional factors through prebiotics, probiotics and bioactive preparations represent a novel approach and a challenge for dietitians and paediatric allergists The modulation of the immune system using functional foods is a promising research hypothesis in the attempt to induce a tolerogenic immune environment (Fiocchi *et al* 2010)

1.10.2. PREBIOTICS

Prebiotics have been defined as "non-digestible food components that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thereby improving host health", and recently redefined as "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits" (Cuello-Garcia *et al* 2016). In December 2016, a panel of experts convened by the International Scientific Association for Probiotics and Prebiotics (ISAPP) suggested a new definition, i.e., "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Cuello-Garcia *et al* 2016). Based on the body of available evidence, the Guidelines for Atopic Disease Prevention (GLAD-p) panel concluded that it is likely that prebiotic supplementation in infants reduces the risk of developing recurrent wheezing and possibly also the development of food allergy. However, there is very low certainty that there is an effect of prebiotics on other outcomes, other than an indirect effect due to its effect on the microbiome. In fact, their activity can be affected by many individual factors (e.g., host's microbiota or the genetic predisposition to diseases).

Environmental factors such as diet or antibiotics can also influence the use of prebiotics (Cuello-Garcia *et al* 2016). The gut microbiota has been shown to respond rapidly to dietary change (David *et al* 2014), with increased dietary fiber being associated with changes in the production of microbial metabolites including short chain fatty acids (SCFA); acetate, butyrate, and propionate. These SCFA metabolites have anti-inflammatory effects (Maslowski *et al* 2009), both locally in the gut mucosa and beyond (Thornburn *et al* 2014), inducing Treg and tolerogenic DC

(Arpaia *et al* 2013, Furusawa *et al* 2013). Lower levels of butyrate and propionate have been measured in infants with allergic disease at 1 year of age (Bottcher *et al* 2000). Prebiotic dietary sources are primarily derived from plants and commonly found in foods like chicory, asparagus, garlic, leeks, onion, and artichoke. To consider a substance a prebiotic, it must be able to resist digestion and absorption in the GI and be fermented by intestinal microbiota (Gibson *et al* 2004). Human milk contains oligosaccharides (HMOS) that also serve as prebiotics, as these non-digestible HMOS show a clear effect on the gut microbiota. HMOS are the third most abundant solid component in breast milk after fat and lactose. They play a key role in the development and well being of the baby playing a decisive role in the immature infant's immune system and promoting a healthy intestinal flora.

Clinical interventions trials of prebiotic supplementation of humans examining allergic disease outcomes have shown reduced development of atopic dermatitis /eczema in infancy; however these trails have been limited to infants fed infant formula (Arslanoglu *et al* 2008). Given the various benefits of HMOs, there has been a lot of interest in figuring out how to introduce HMOs into formula. There are several infant formulas available supplemented with functional alternatives to HMO such as galacto oligosaccarides (GOS) and/or fructo oligosaccharides (FOS) which are nondigestible carbohydrates that mimic the effect of HMOs to some extent (Roberfroid et al 2010). However, earlier this year, Nestle were the first company to replicate and produce two HMOs (2 –fucosyllactose (2-FL) and lacto-N-neotetraose (LNnT)) and add these to infant formula (Nan Optipro Supreme, Nestle, Spain) to make the composition of the infants gut more similar to breast fed babies. These ingredients were approved by the European Food Safety Authority (EFSA) and have been approved by the European Commission and the US FDA. This formula may

reduce the risk of infectious diseases in children aged up to 18 months of life, compared with babies fed with the standard formula without oligosaccharides

1.10.2 PROBIOTICS

Probiotics are living microorganisms that have been proposed as immune-modulators of the allergic response by affecting phagocytosis and production of pro-inflammatory cytokines, and thus have been advocated as therapeutic and preventive interventions for allergic diseases (Fiocchi *et al* 2015) They are present in everyday food (not only in yoghurt or fermented milk, but also in cheese, either hard or soft and also in less expected sources such as kefir, miso soup or tempe) and many people consume them daily The probiotic effects of complex oligosaccharides in human milk promote the establishment of a bifidogemc microbiota which, in turn, induces a milieu of tolerogenic immune responses to foods Earlier studies suggested a positive effect of probiotic interventions on atopic dermatitis, but meta-analyses have failed to confirm it (Rather *et al* 2016) The new World Allergy Organization (WAO) guidelines determined that it is likely that probiotic supplementation in infants reduces the risk of developing eczema and suggested that probiotics should be recommended in mothers of high-risk infants and in infants at high risk of allergic disease, where "high risk for allergy in a child" is defined as having a biological parent or sibling with an existing or history of allergic rhinitis, asthma, eczema, or food allergy The recommendations are conditional, and based on very low-quality evidence, with no specific recommendation regarding strains, dose, treatment duration etc

In terms of tolerance development in those with established food allergy, one study from Australia performed oral immunotherapy (OIT) to peanut in combination with Lactabillus rhamnosus GG, showing that 89 7% of the study participants in this

arm were desensitized to peanut. The authors speculate that this protective effect may be seen because of the possible effect of the probiotic on T regulatory cells (Tang *et al* 2015). Further scientific confirmation is required to include probiotics and prebiotics in the therapeutic plans. Practical implications and how this should be incorporated in advising food allergy sufferers are also unclear in terms of advising regular intake of foods high in short-chain fructo-oligo saccharides, fermented foods and yoghurts.

1.10.3. MILK PEPTIDES (BIOACTIVES) AS FUNCTIONAL FOODS

The last 40 years has seen a dramatic rise in allergic diseases such as asthma, eczema and hay fever, particularly in the western world. Along with these allergies has come an increase in food-related allergies. Today almost one child in every classroom worldwide has a need to avoid, milk, eggs or nuts. In Ireland, milk allergies are twice as common as egg allergies and three times as common as peanut allergies. The main treatment strategy for most allergies is based on allergen avoidance which is particularly challenging for cow's milk as it forms the basis for many milk based essential products for infant nutrition. Cow's milk is an ideal protein source as it has all nine essential amino acids (leucine, isoleucine, valine, phenylalanine, tryptophan, histidine, threonine, methionine, lysine) and so it is very important to enable more children to consume it without developing an allergy to it (Mcgregor *et al* 2014). The fact that many children are not born with milk allergy is very encouraging and suggests that there may be ways to prevent it. Children do not inherit a specific allergy but rather a genetic tendency to develop allergic disease.

Breast milk is the gold standard for the growing infant, however 0.5-1% of breast-fed infants develop cow's milk allergy after weaning to solids (Saarinen *et al*

2000) As such there has been a push for industry to produce new food products or functional foods that are capable of improving infant health within the context of food allergy These ingredients are referred to as "bioactives" as they are known to be active within the body Given Irelands position as a leading dairy producer our research funded by Food for Health Ireland (FHI) and backed by 5 industry partners (Kerry Foods, Carbery Foods, Glanbia, Dairy Gold Ingredients and Irish Dairy Board) is focused on studying bioactives in dairy products that are capable of preventing or reducing the incidence of inflammatory allergic disease in infants Milk derived bioactives have been reported to possess a variety of biological activities, including antioxidant, anti-inflammatory, anti-hypertensive and anti-cancer activities (Raikos *et al* 2014) The goal of this current research is to find bioactives with anti-inflammatory activity that can be incorporated as ingredients in functional foods There has recently been a rapid increase in consumer interest in the health-enhancing roles of specific foods or physiologically active food components, so-called functional foods (Hasler, 1998) Generally speaking all foods are functional as they have nutritional value and are fundamental for survival A functional food can be described as 'a food that can beneficially affect one or more target functions in the body beyond adequate nutritional effects in a way that is relevant to either an improved state of health and well-being and/or a reduction of risk of disease' (ISIL Europe, 2012)

There is a lack of treatment options available for food allergy While the most common treatment of food allergy is the complete avoidance, there are some pharmacological treatments available Anti-IgE (omalizamab) and anti-histamine drugs have been widely used to treat these allergic responses However, as these treatments are often costly, associated with multiple side-effects and are not

universally efficacious (Korhonen *et al* 2003) food derived milk peptides with anti-inflammatory activity could be used as a new alternative for the prevention/reduction of the incidence of inflammatory diseases such as food allergy.

1.11. MILK DERIVED HYDROLYSATES

1.11.1 POSSIBILITY TO PREVENT/TREAT ALLERGY WITH MILK PROTEIN HYDROLYSATES

While genetics play a central role in food allergy (Sicherer *et al* 2000) the environment to which we are exposed is also largely responsible, with diet being a leading component. As already discussed dietary proteins supply a pool of amino acids that form various protein molecules, which play intricate roles across the body's metabolic pathways. While dietary protein is renowned for its fundamental role in growth and tissue repair (Van Loon 2007) it also boasts a plethora of health benefits, with specific peptides playing a major role in the functioning and regulation of the immune system from birth, as well as in protecting against infection by inhibiting microbial growth (Raikoset *et al* 2014). Over the past decade, growing evidence has demonstrated the potential of dietary components in mitigating and even blockading the progression of inflammation (Giugliano *et al.*, 2006; Pan *et al.*, 2009). Peptides are an important group of bioactive substances in functional foods, which can be generated by enzymatic hydrolysis, microbial fermentation, or gastrointestinal digestion of food protein. Bioactive peptides are usually composed of 3 to 20 amino acid (AA) residues and may contain different AA composition and

sequences (Shahidi and Zhong, 2008). Recently, interest has been growing in developing anti-inflammatory peptides from food proteins for their health-promoting, desirable safety and anti-allergy effects (Dyer *et al.*, 2008; Chakrabarti *et al.*, 2014; Kumar *et al.*, 2015).

Whey protein constitutes approximately 20% of milk protein and is a natural by-product from cheese production. Whey protein is basically a mixture of globular protein, such as β-lactoglobulin (β-LG,) α-lactoferrin and BSA. As a high-quality protein source (Sousa *et al.*, 2012), whey protein can release a wide variety of bioactive peptides. However, the allergic potential of peptides is dependent on the level of exposure and their size, with larger peptides being more allergenic and increasing risk of sensitisation to specific food proteins (Figure 1.4). As the immature gastrointestinal barrier of vulnerable infants is more permeable, it enables these larger proteins to be absorbed. Following passage across the gastrointestinal barrier, these larger peptides can activate and enhance expansion of Type 2 helper T-cells (T_H2), which act as key drivers of allergy. If the development of the gastrointestinal immune system and oral tolerance can be stimulated in the early stages of life, the likelihood of activating this allergic cell type and, thus, inducing sensitisation is greatly reduced.

Proteins can be pre-digested or hydrolysed into smaller protein chains outside of the body, and are known as protein peptides or hydrolysates. The process of breaking down proteins to shorter peptide sequences is termed 'hydrolysis'. This process happens naturally in the gastrointestinal tract and can be simulated in the laboratory or on an industrial scale. During the normal transit through the gastrointestinal tract, milk proteins are exposed to proteinases such as pepsin, trypsin and chymotrypsin which break them down into smaller peptides. These peptides are

further digested by brush border peptidases present at the surface of intestinal epithelial cells where they produce amino acids, however, some oligopeptides still remain intact (Segura-Campos *et al* 2011) In laboratory or at an industrial scale, milk hydrolysates are released either by treatment of milk proteins with food grade enzymes or through fermentation with bacteria. The shorter peptide sequences often possess bioactive properties beyond their nutritional contribution along with eradicating any protein-specific allergemcity (Hartmann *et al* 2007, Kit *et al* 2003) Once the hydrolysates are released, they can potentially have bioactive properties and exert their effects in receptive cells, including those present in the gastrointestinal tract (Rico-Cabello *et al* 2012) The bioactivities of the resulting hydrolysates are variable depending on a range of factors, including the enzyme used, the processing conditions and the final size of the peptide sequence following hydrolysis (Clemente 2000) The degree of hydrolysis (DH) is defined as the percentage of cleaved peptide bonds, i e the number of hydrolysed bonds per total number of peptide bonds in the protein (Nielson *et al* 2001) This affects the size and ammo acid composition of the peptides, which subsequently determines the biological activity of the peptide Hence, DH is an important consideration from the perspective of functional food research (Jamdar *et al* 2010)

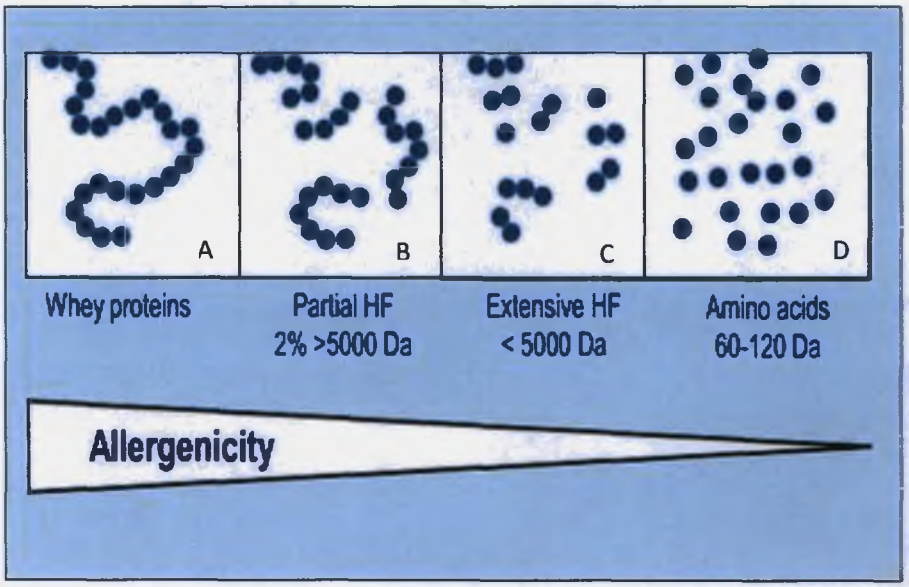


Figure 1.4 The allergenicity of proteins is dependent on their size. A) Composition of regular infant formula: proteins acceptable in tolerant infants. B) Composition of FHI proposed allergy-prevention infant formula: peptides to induce tolerance. C) Composition of specialised infant formula: smaller peptides for infants with mild allergy. D) Composition of specialised infant formula: amino acids for infants with severe allergy.

Much of the experimental data on milk hydrolysates come from model organisms such as mice and rats; however, a number of studies have been carried out in humans (Nongonierma *et al* 2016). Horiguchi *et al* (2005) examined the effects of the administration of wheat gluten hydrolysate on the immune function of healthy human volunteers and reported that NK cell activity increased significantly after intervention (6 days) without severe side effects. Soybean peptides produced by theroase or biophrase significantly increased the numbers of $CD11b^{+}$ (cell surface

antigen of macrophages and DC) and $CD56^+$ (cell surface antigen on NK cells) cells in serum of peripheral blood from healthy human volunteers after a single oral administration of 8g of soya bean peptides (Yimit *et al* 2012)

Several milk protein hydrolysates enhance immune cell function by increasing secretion of immunoglobulins. Immunoglobulins are glycoprotein molecules that specifically recognise antigens from bacteria or viruses and aid in their destruction through a highly complex and specific immune response (Calder *et al* 2002). Hydrolysates of αs1-casein, αs2-casein and β-casein stimulated the immune system through the enhancement of immunoglobulin G (IgG) and IgA concentrations (Hata *et al* 1999) Several recent studies have demonstrated that food derived peptides exert various immunomodulatory effects on both innate and adaptive immune responses including induction of or modulation of cytokine and antibody production, stimulation of lymphocytes to proliferate, augmentation of phagocytic ability of macrophages, enhancement of natural killer (NK) cell activity, improvement of the defensive ability of the body against invading pathogens and inhibition of pro-inflammatory responses to bacterial components such as lipopolysaccharide (LPS) (Zang et al 2012, Wenjia et al 2016, Yang et al 2009, Duarte et al 2010) These effects can be mediated by direct binding of food derived peptides to the receptors on the surface of immune cells The immunomodulatory capacity of peptides isolated from food protein hydrolysates is dependent on the amino acid composition, sequence length, charge, hydrophobicity and structure of the peptide molecule (Berthou *et al* 1987, Drouin & Boutin 2010) Milk hydrolysates can modulate the gastrointestinal immune system by inhibiting proliferation and maturation of localised immune cells Particular milk hydrolysates modulate the MAP kinase and NF-κβ pathways that consequently control the secretion of several

cytokines that can induce inflammatory responses and strengthen the host defence mechanisms (de Medina *et al* 2010) Immune cells, such as monocytes and macrophages, play an important role in inflammatory responses and tissue repair and remodelling by either interacting directly with microorganisms during infections and/or secretion of cytokines that mediate biological effects (Mahida 2000)

On the other hand, hydrolysates have also been shown to promote the immune response Casein peptides induced innate host immune responses in humans, by stimulating the proliferation of lymphocytes and macrophages (Meisel 1997) Similar suppression of a pro-inflammatory response by down-regulation of IL-8 in inflamed Caco-2 cells was recorded by casein hydrolysate and its size fractions Further validation of the anti-inflammatory activity was performed in porcine colonic explants and the casein hydrolysate, and its size fractions down-regulated IL-1α, IL-1β and IL-8 expression (Mukhopadhya *et al* 2015) Bovine κ-casein hydrolysate inhibited circulatory IFNγ secretion and suppressed IL-10 and FoxP3 expression in concanavalin A (ConA)-stimulated rat splenocytes (Requena *et al* 2008) This bovine κ-casein, in a human macrophage cell line U937, was associated with the suppression of circulatory pro-inflammatory cytokines IL-1β, TNFα and IL-8 production (Requena *et al* 2009) Saint-Sauveuret *et al* (2008) suggested that acidic or neutral peptide fractions from whey protein isolate better stimulated splenocyte proliferation and the cytokine secretion Javier *et al* (2014) reported that tryptic whey β-lactoglobulin digest fraction enriched in acidic and large peptides increased the secretion of IFN-γ (a T_H1 cytokine), while fractions containing short peptides enhanced TNF-α production of monocytes Vogel *et al* (2002) demonstrated that the immunomodulating and anti-inflammatory properties of lactoferricin peptide are more related to the positively charged region of the

peptide. Thus, the amino acid constituents, sequence and the length of the peptides are very important for their immunomodulatory activity.

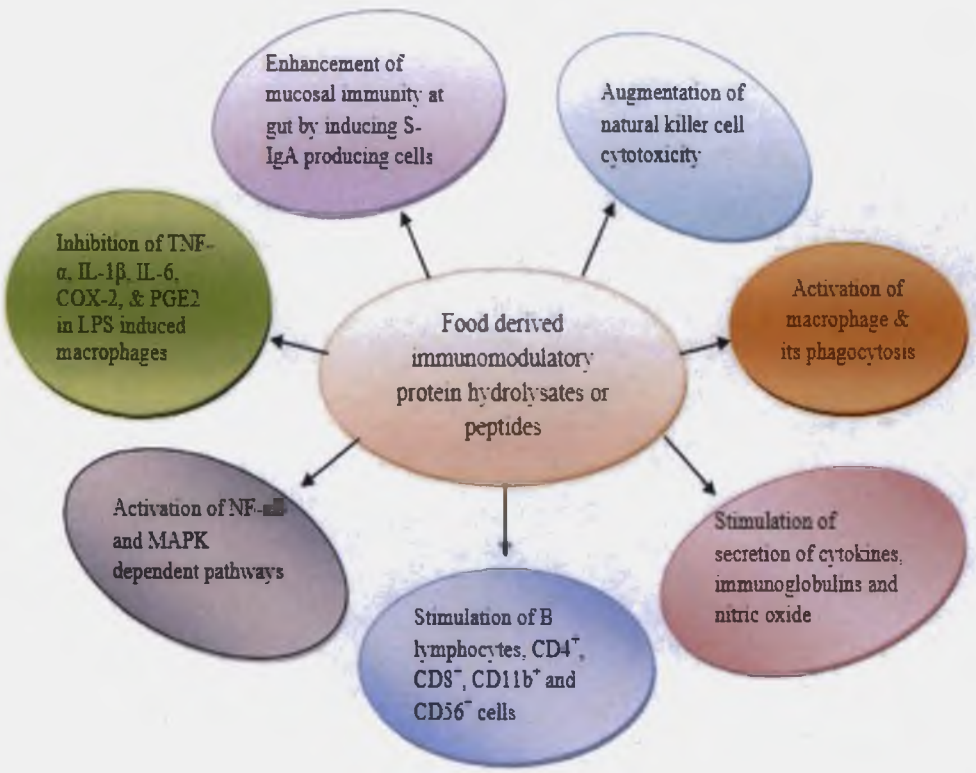


Figure 1.4 Mechanisms of action of immunomodulatory protein hydrolysates or peptides derived from food proteins

1.4 SUPPLEMENTATION OF INFANT FORMULA WITH FUNCTIONAL FOODS

Breast feeding provides the best source of nutrition for infants, however, when breast feeding is not an option or if breast milk is not available in adequate amounts, infant formula is the only alternative to meet the nutritional needs of the growing infant These include cow's milk or soy-based infant formulas, hypoallergenic infant formulas, and other formulas designed to meet the specific nutritional and dietary needs of infants Such needs include helping to lower the risk of food allergies and improve immune health These infant formulas are also developed to mimic the composition of breast milk Human breast milk is typically the sole source of nutrition required for the first six months of an infant's life It's nutrient content has not only been used to establish dietary recommendations for infants, but is also used as the model system for developing infant formulas Breast milk is unique in its physical structure and composition, with very specific types and concentrations of protein, fat, carbohydrate, vitamins and minerals, enzymes, hormones, growth factors, host resistance factors, inducers and modulators of the immune system and anti-inflammatory agents (Ballard *et al* 2014). This composition is important not only to promote infant growth and development, but also to support optimal health by protecting infants from food allergies, illnesses and to promote gut health Diet has a major impact on the child development, hence, infant and follow-on formulas are formulated to be as similar as possible to the composition of breast milk from healthy, well-nourished women

Current infant milk formulae on the market, suitable for infants with cow's milk allergy are comprised of extensively hydrolysed cow's milk proteins (Figure 1 3) These milk formulae provide the infant with the high nutritional quality of the

proteins, while also reducing their allergenic properties, due to their small size (Terheggen-Lagro 2002). However, while these extensively hydrolysed formulae are more tolerable and prevent allergic symptoms, they cannot induce tolerance to the allergen because extensive protein hydrolysis results in the loss of immunogenicity. This reduces stimulation to the immune system, inhibiting its ability to develop tolerance to these otherwise harmless milk proteins. Although strict avoidance of cow's milk is advised for the management of cow's milk allergy recent studies suggest that exposure to cow's milk allergens can, in fact, be beneficial for infants that are at risk of developing milk allergy through targeting and influencing the immune system from birth (Staden *et al* 2007).

Partially hydrolysed formulae, however, have been shown to reduce the prevalence of atopic dermatitis in infants compared to whole-protein formula (Spieldenner *et al* 2011, Vandenplas *et al* 2014). Partial hydrolysis of proteins retains the protein's immunogenicity, which provides the infant with an opportunity to develop oral tolerance to the whole protein (Hensen *et al* 2012). Among these hydrolysates, both *in vitro* and *in vivo* studies have identified cow's milk-derived whey and casein hydrolysates that have positive modulatory effects on the immune system. They have been shown to display similar beneficial effects to breastmilk on gut colonisation, through increasing *Bifidobacterium* levels, which are decreased in formula fed infants and are required to bring about immune balance, thereby promoting oral tolerance (Hernandez-Ledesma *et al* 2014, Chirico *et al* 1997). The increasing number of and health promoting effects attributed to milk-derived bioactive peptides make them potential ingredients of functional food. The main goal of the current research is to identify milk protein hydrolysates that have a capacity to

both suppress allergenic T helper cell responses and to stimulate Treg cells so they can be added to infant formula to potentially prevent allergy

1.11 ANIMAL MODELS OF ALLERGY

Unfortunately all therapeutic approaches for food allergy currently are still in clinical phase I-III and patients can only protect themselves through strict avoidance of all allergen-containing food products (Benede *et al* 2016) However many important questions about food allergy cannot be answered by *in vitro* molecular experiments alone and an appropriate *in vivo* experimental platform becomes an urgent need Mechanistic research or modifications of experimental conditions using human studies cannot be easily performed in patients Moreover the amount and types of tissue samples that can be collected from patients with food allergy are very restricted Therefore animal models become very valuable research tools of choice Animal models help us get greater insights into three major aspects of food allergy 1) the evaluation of food allergens 2) studies on immunological responses and mechanisms and 3) the development of preventative and therapeutic treatments (Oyoshi *et al* 2014) In the field of food allergy, there is insufficient information regarding the nature of food allergens and the mechanisms responsible for loss or lack of tolerance in patients It is difficult to develop a true homologous model at this time because feeding of food allergens to mice elicits oral tolerance as it does in most human subjects Instead mucosal adjuvants such as aluminium hydroxide and cholera toxin have been widely used to overcome oral tolerance to co-administered antigens to generate T_H2 responses (Ganeshan *et al* 2009) The use of these models

has already provided significant advances in our understanding of the potential mechanisms of pathogenesis of food allergy and in the development of new therapies

In recent years, the anti-inflammatory effects of several bioactive peptides and hydrolysates have been evaluated in mouse models (Chakrabarti *et al*, 2014) Two tripeptides, Valyl prolyl proline (VPP) and Isoleucyl prolyl proline (IPP) demonstrated beneficial effects in a model of intestinal enterocolitis through their mediation of anti-inflammatory activities (Chatterton *et al* 2013). They recently reported that the hydrolysate of milk casein containing both VPP and IPP improved the vascular endothelial function of subjects with stage I hypertension Feeding with peptide concentrates obtained from hydrolysis of whey proteins showed significant edema decrease in paw edema test (Mochizuki *et al* 2010) Oral intake of a corn gluten hydrolysate reduced inflammatory injury in a rat model of experimental colitis (Tavares *et al*, 2013) Furthermore Wu *et al* (2006) found that the IFN-γ/IL-4 ratio increased in spleen T cells from mice fed with chitosan hydrolysate, suggesting a change toward a more T_H1-like phenotype Since IL-10 producing Treg cells can inhibit T_H2 cells, another way to dampen down the T_H2 response is to promote the differentiation of Treg cells by administration of specific peptides (Palomares *et al* 2010) This upregulation of IL-10 production was observed in splenocytes obtained from mice treated with β-lactoglobulin trypsin hydrolysates, while intact β-lactoglobulm on the contrary downregulated IL-10 (Duan *et al* 2012) Ndiaye *et al* (2012), also found an increased amount of IL-10 producing cells in the small intestine lamina propria of mice after oral administration of yellow pea protein hydrolysate Increasing evidence suggest that protein hydrolysates could be a promising alternative anti-inflammation agent because of their high safety

Recently humanised allergy models have been developed to utilise the engraftment of human haematopoietic cells using severely immunodeficient mice. These models hold advantage over murine models of food allergy as they generate a system whereby human T and B cell compartments of the adaptive immune response capable of generating T_H2 and food-specific antibody responses against allergens can be studied *in vivo* (Shultz *et al* 2016). Such humanised mice allow for the testing of food mechanisms and specifically the role of IgE antibodies and mast cells in a humanised setting. Humanised mice are generated by the transplantation of human hematopoietic cells into recipient mice (Rongvaux *et al* 2013) and the multi-potency of these cells are discussed in the next section.

1.12. HEMATOPOIETIC STEM CELLS

The developing mammalian blood system, contains more than ten distinct mature cell types, that arise from one specific cell type, the hematopoietic stem cell (HSC). Within the system, only HSCs possess the ability of both multi-potency and self-renewal. Multi-potency is the ability to differentiate into all functional blood cells. Self-renewal is the ability to give rise to an identical daughter HSC without differentiation. Since mature blood cells are predominantly short lived, HSC continuously provide more differentiated progenitors while properly maintaining the HSC pool throughout life by precisely balancing self-renewal and differentiation (Ogawa *et al* 1993). The frequency of HSC is highest in the $CD34^+$ cell population although a lower number of HSC might be found in the $CD34^-$ fraction as well (Kondo *et al* 2003). CD34, a ligand for L-selectin is expressed by only 0.5–5% of blood cells in human fetal liver, cord blood and adult BM (Digiusto *et al* 1994, Krause *et al* 1996). These rare cells can be isolated from different sources: fetal liver, granulocyte-colony stimulating factor (G-CSF) mobilised peripheral blood

cells, adult bone marrow and umbilical cord blood (UCB) and have multi-potential, are self-renewing and/or undergo commitment to common myeloid or lymphoid progenitors (Metcalf *et al* 2007) One of the first models of HSC engraftment was described by Lapidot *et al* in which human HSCs were injected into irradiated SCID mice (Lapidot *et al* 1992) Recently, human $CD34^+$ (Burton *et al* 2017) $CD34^+ CD38^-$ (Ishikawa *et al* 2005) and $Lin^- CD34^+ CD38^-$ (Takagi *et al* 2012) HSCs have been the main cells used to repopulate immunodeficient mice, most of them isolated from UCB and enriched by magnetic separation, cell sorting or both It is well established that UCB is a better source than bone marrow of HSCs with high engraftment level (Kim *et al* 1999) $CD34^+$ cells are transplanted into adult mice by either intravenous or intrafemoral injection From these studies it is now well established that neonatal mice have more potential than adult HSCs (Ueda *et al* 2001) as they tend to have greater multilineage human hematopoietic differentiation including T and B cell development when compared to mice engrafted as adults The number of cells that needs to be injected and the engraftment that can be expected are therefore highly variable depending on the source of $CD34^+$ cells used It has been demonstrated that female NSG mice are more supportive of human engraftment than male NSG when HSC are transplanted at a limiting dose (McDermott *et al* 2010) It is therfore crucial that the appropriate transplantation protocol is chosen in order to enable the experimantal question to be answered

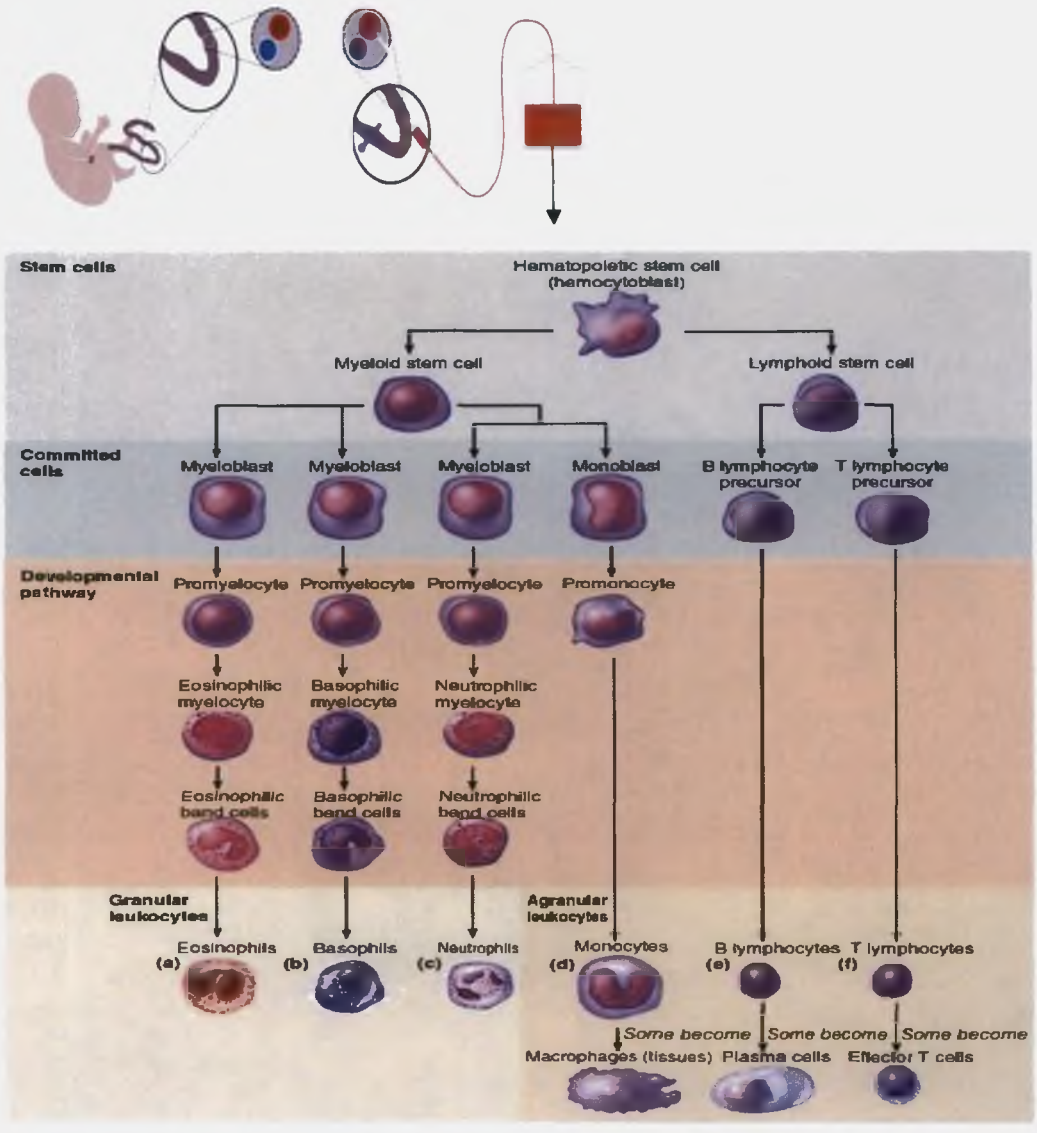


Figure 1.2 Umbilical cord blood HSC give rise to all blood cells in a process known as haematopoiesis. Hierarchical structure of haematopoiesis development is shown above.

1.12. HUMANISED MOUSE MODELS OF ALLERGY

Recently mice engrafted with human immune cells have been used to study human allergic responses in mice. These models hold advantage over murine models of allergic inflammation as they generate an adaptive immune system capable of generating food-specific antibody responses following allergen ingestion as well as fostering the development of the human innate effector cells such as mast cells and basophils. During this period such models have been improved and they have been used for an increasing array of studies (Table 1.1).

The ability to study human allergies in an *in vivo* setting in mice has opened up a wide range of possible avenues for research. Major limitations in the past have hindered the application of the approach and of these one of the most important deficiencies has been the inability of mouse factors to support human cells. Many essential factors required for human immune cell development and function are species specific and cannot be provided by the mouse. Therefore some of the studies listed in table 1.1 have adopted the strategy of replacing mouse genes with their human counterparts enabling the development and function of granulocytes (mast cells and basophils) and potentially disabling the same of the corresponding mouse cells. Human cytokines can be overexpressed as transgenes in humanised mice (Willinger *et al* 2010). This approach has been used to generate human IL-3, granulocyte macrophage colony stimulating factor (GM-CSF) and stem cell factor mice (NSG-SGM3). In these mice, human cytokine expression is driven by the cytomegalovirus promoter which leads to ubiquitous expression.

Humanised mice can also use knock in technology to replace genes in mice for e.g IL-3 and GM-CSF two cytokines crucial for myeloid cell development and

function were replaced in the mouse with the human loci for both genes to generate IL-3/GM-CSF KI mice for studying inflammatory responses in the lung In these mice there was no significant improvement in HSC engraftment and the high expression of GM-CSF in these mice ensures that any effects were evident in the lung Hense the tissue specific expression of IL-3 or GM-CSF in KI mice was sufficient for tissue-specific development and migration of human cells but not for systemically reconstituting entire human myeloid cells However the KI strategy does allow correct expression in appropriate organs (lung) where IL-3 and GM-CSF are normally expressed and at physiological concentrations (Willinger *at al* 2010) in contrast to delivery by hydrodynamic injection or overexpression as in IL-3/GM-CSF/SCF transgenic mice

To create a hematopoietic microenvironment more suitable for human myeloid development mouse strains that express the human 220-amino acid isoform of the human membrane-bound stem cell factor (mSCF) have been used These transgenic mice expressed human membrane-bound SCF from a cassette including the human SCF cDNA expressed from the human phosphoglycerate kinase (PRK) promoter and are designated NOD Cg-Prkdc ^{SCID} IL2rg ^{tmlwjl} Tg (PGK1-KITLG*220) 441Daw/J or NSG-SCF mice SCF, also known as steel factor or kit ligand critically regulates the migration and survival of mast cell precursors, promotes the proliferation of both immature and mature mast cells, enhances mast cell maturation and also plays a role in HSC maintenance (Billerbeck *et al* 2011) Takagi *et al* (2012) reported higher engraftment of $CD45^+$ (97%) in the bone marrow in comparison to the NSG control (63%) and mast cells had engrafted sufficiently in all organs Engraftment of human HSC requires preconditioning of immunodeficient recipients usually with irradiation but HSC engraftment can also occur without

irradiation in NSG-mSCF mice (Brehm *et al* 2012) In the absence of irradiation using transgenic expression of human membrane-bound SCF in non-irradiated mice, exhibited high engraftment levels of T and B cell compartments of the adaptive immune response and developed peanut-specific human IgE responses and mast cell-mediated anaphylactic sensitivity to ingested peanut following sensitisation (Burton *et al* 2017) This is the first humanised mouse model study of peanut allergy and it paves the way forward for the study of cellular and molecular interactions in the human food allergy response

To overcome the limited development of the myeloid lineage, Ito *et al* (2013) used NOG mice transgenically expressing human IL-3 and GM-CSF to show the superior engraftment of granulocytes, basophils and mast cells in comparison to mast cells that develop in NSG-SGM3 and IL-3/GM-CSF knock in mice The engraftment of human HSC in the bone marrow showed 4 and 3 fold increases and there was also an increase in myeloid DC When mice were sensitised with sera from patients that were allergic to Japanese cedar pollen and then challenged with antigen they developed a passive cutaneous anaphylaxis (PCA) reaction This was the first model to demonstrate that human cells generated in mice can mediate human allergic reactions in a reliable manner

Gut inflammation was recently assessed in a Hu-PBL-SCID-NSG- model This model was created by injection of human peripheral blood leucocytes (PBLs) which results in rapid engraftment of $CD3^{+}$T cells by the end of the first week This model is excellent for studying human T cell function *in vivo* but has only a short experimental window due to the development of lethal xenogenic GVHD, usually within 4-8 weeks PBMCs from known donors with known allergies were used for engraftment Mice injected with PBMCs form allergic donors and then challenged

with specific allergens developed intestinal wall thickening and leucocyte infiltration that were mediated by human IgE production (Eschborn *et al* 2015). Blocking IgE with the anti-IgE antibody omalizumab reduced gut inflammation. Co transfer of the allergic PBMC and the patient's own Treg blocked IgE production. This study showed the utility of this model to investigate the role of Tregs in allergic disease and the ability to assess therapeutics.

The limitation of the above study using PBMC to reconstitute NSG mice is the in ability of mast cells, granulocytes and human DC to functionally engraft. To solve this problem a triple transgenic mouse model under the control of a strong constitutive promoter provided support for hematopoietic cell development. In NSG-SGM3 mice, an immunodeficient strain that expresses transgenes for human stem cell factor (SCF), granulocyte macrophage-colony stimulating factor (GM-CSF) and IL-3 mice displayed increased human $CD4^+$ T cells in spleen, liver and bone marrow and within the $CD4^+$ population T reg cells were increased. This strain also had increased myeloid cells, $CD15^+$ granulocytes and myeloid DC in the bone marrow.

Thrombopoietin (TPO) has been demonstrated as a crucial cytokine supporting maintenance and self-renewal of HSCs. Rongvaux *et al* (2011) generated $RAG2^{-/-}\gamma_c^{-/-}$ mice in which they replaced the gene encoding mouse TPO by its human homolog. Homozygous humanization of TPO led to increased levels of human engraftment in the bone marrow of the hosts, and multilineage differentiation of hematopoietic cells was improved, with an increased ratio of myelomonocytic verus lymphoid lineages. Interestingly, the presence of human TPO improved differentiation of human granulocytes. Thus, the presence of human TPO in recipient mice favours a balance between granulocytes and lymphocytes that better reflects the human physiological condition, a finding possibly due to better maintenance and/or

differentiation of human myeloid progenitor cells Despite the clear improvement in HSC-like cell populations, as well as a better balance between the myeloid and lymphoid lineages, they did not observe any significant effect of TPO humanization on the overall engraftment levels in peripheral lymphoid tissues or in the percentages of B and T cells

Table 1.1 Humanised mouse model of Allergy

Reference	Background	Genetic modification	Cells injected	Key features
Rongvaux et al 2011	BRG **BALB/c-Rag2^{null}Il2rγ^{null}**	Human TPO knockin	Fetal liver and Cord blood	Median hCD45 increased myeloid(CD33+CD66+) IncreasedCD34+CD38+
Billerbeck 2011	NSG **NOD-scid IL2rγ^{null}**	Human IL3,GM-CSF,SCF	Fetal liver,	Median IncreasedCD33+CD15+ Increased DC Increased Treg
Ito *et al* 2013	NOG **NOD/Shi-scid IL2rg^{null}**	Human transgene IL-3,GM-CSF	Cord blood	BM 3-4X more CD34+ Increased myeloid(CD33+) Increased DC, basophil, mast
Hu-mSCF	NSG **NOD-scid IL2rγ^{null}**	Human transgene-membrane bound SCF	Cord Blood	Median human BM CD45+ significantly higher(97%) compared to NSG(63%) supports mast cell development
Willinger *et al* 2011	BRG **BALB/c-Rag2^{null}Il2rγ^{null}**	Human IL3 GM-CSF knockin	Fetal liver and Cord blood	Deficiency in mouse alveolar macrophages Development of human lung macrophages

Examining the advantages (full HSC engraftment, elevated myeloid, DC, $CD4^+$, MC) and limitations (low IgG, GVHD, no improvement in engraftment) of the humanised models and as the TPO and the NOG IL-3/GM-CSF mice were not commercially available, the NOD/SCID $IL2r\gamma^{null}$ stem cell factor (SCF) and NOD/SCID $IL2r\gamma^{null}$ triple transgenic SGM3 humanised mouse models were chosen to compare the best strain of mouse for the engraftment of mast cells and basophils. The chosen strain of NOD/SCID $IL2r\gamma^{null}$ was then used to investigate the anti-allergic potential of milk protein hydrolysates for the treatment of OVA driven allergy. Therefore, the aims of this study were to develop an experimental humanised model of ovalbumin induced allergy and inflammation that mimic the main clinical characteristics of human disease as well as to examine the allergenic and immunological properties of hydrolysed whey proteins using this model.

1.13. AIMS AND OBJECTIVES

This chapter has highlighted the current understanding of how food allergy occurs how functional foods in the form of a milk protein hydrolysate modulate cells of the immune system and explored the potential of hydrolysates as a preventative strategy for food allergy. This thesis aims to investigate two distinct areas in food allergy treatment which remain to be addressed:

(1) The direct interaction of hydrolysates with immune cells and elucidate the mechanism by which these interactions occur *in vitro*.

(2) Preclinical evaluation of the efficacy of a novel non-pharmacological treatment (in the form of a cow's milk protein hydrolysate) in an attempt to disrupt the pathogenesis of an inappropriate immune

response (allergic inflammation) in a humanised mouse model of OVA allergy

Despite the advances made in our understanding of how hydrolysates modulate the immune system the direct action of hydrolysates with cells of the innate (dendritic cells, MCs and basophils) and adaptive (T cells and B cells) system remain unclear The elucidation of these actions will be beneficial as it may pave the way for the use of hydrolysates as a functional food ingredient in formula to help induce tolerance in allergy prone infants, thereby reducing the risk of allergy and associated atopic conditions later in life The goal of chapter 3 is to determine if and how hydrolysates modulate the activation of the immune system *in vitro* and to show the mechanism by which such effect is mediated.

Chapter 4 of this thesis will compare the engraftment kinetics of two strains of NSG mice in order to choose the best strain of mouse for the engraftment of mast cells and basophils, the main effector cells of allergy for the establishment of an experimental platform to confidently test functional foods *in vivo* The chosen mouse strain was used in Chapter 5 to ascertain whether a novel cow's milk protein hydrolysate can alter Th2 driven allergy sufficient to suppress allergic inflammation in a humanised mouse model of OVA allergy

Overall this study is designed to further the current knowledge of the bioactive properties of milk protein hydrolysates in terms of their anti-inflammatory activity It is hoped that this new platform will allow direct assessment of how these potential anti-inflammatory functional foods act on human cells without having to do tests on human volunteers These food derived milk peptides could be used as a new alternative in the form of a functional food for the treatment and management of inflammatory diseases This thesis will advance our understanding of how a novel

milk protein hydrolysate may suppress allergic inflammation *in vivo* and will be of real value to nutritional companies, including infant formula manufacturers as this new experimental approach (testing functional foods in a human relevant system) will strongly increase the predictability of success in advance of investment in costly intervention trials

CHAPTER 2

MATERIALS AND METHODS

2.1 METHODS

2.2 REGULATORY ISSUES

2.2.1 ETHICAL APPROVAL AND HPRA COMPLIANCE

All procedures involving the use of animals or human materials were carried out by licensed personnel Ethical approval for all work was granted by the ethics committee of Maynooth University (BRESC-2015-007) Project Authorisation was received from the HPRA (AE19124/P005) whereby the terms of the animal experiments within this project were outlined and adhered to

2.2.2 COMPLIANCE WITH GMO AND SAFETY GUIDELINES

All GMO/GMM work was performed according to approved standard operation procedures and recording protocols approved by the Environmental Protection Agency (Ireland) Safe working practices were employed throughout this study as documented in the Biology Department, Maynooth University Safety manual.

2.2.3 ANIMAL STRAINS

The following mouse strain was used Nonobese diabetic (NOD) severe combined immunodeficiency (SCID) common gamma deficient Cg-Prkdc ^{scid} IL2rg ^{tm1Wjl}/Tg (PGK1-KITLG*220) 441 Daw/J, mice abbreviated as NSG-mSCF mice expressing human stem factor (SCF) and NOD Cg-Prkdc^{scid} IL2rg^{tm1Wjl} /Tg (CMVIL3,CSF2,KITLG)1Eav/ MloySzJ mice abbreviated as NSG SGM3 mice expressing human SCF, hGM –CSF and hIL-3 (Jackson Labs, Bar Harbour, Maine,

USA). All mice were housed according to Heath Products Regulatory Agency (HPRA) guidelines and used with ethical approval under the terms of AE19124/P005 project authorisation from HPRA. Sample sizes for animal experiments were determined by statistical power calculation using SISA. SISA software is online at http://home.clara.net/sisa/power.htm

2.3 CELL CULTURE

2.3.1 HUMAN PERIPHERAL BLOOD MONONUCLEAR CELL (PBMC) ISOLATION

Whole blood buffy coat packs, which contained red blood cells, white blood cells and platelets, were supplied by the Irish Blood Transfusion Service (IBTS) at St. James's Hospital, Dublin. PBMC were isolated from whole blood by density gradient centrifugation. The contents of buffy coat packs were diluted 1 in 2 with phosphate buffered saline (PBS) (Oxoid Ltd., Basingstoke, Hampshire, England). 25 ml diluted blood was carefully layered on top of 15 ml lymphoprep (Axis-Shield PoC AS, Oslo, Norway) in a 50 ml centrifugation tube (Sarstedt). The samples were centrifuged at 2400 rpm for 25 min at room temperature with no brake and low acceleration. After centrifugation, the white buffy coat layer containing PBMC was removed into a sterile 50 ml tube, leaving red blood cells and remaining plasma behind. PBMC were centrifuged at 1800 rpm for 10 min at 4 °C with the brake at normal settings. Supernatant was removed and the PBMC pellet was washed in 20 ml of PBS and centrifuged at 1500 rpm for 5 min at 4 °C for a total of two times. Remaining red blood cells were lysed using 5ml 1x red blood cell lysis buffer (Biolegend, London, UK) for 5 min. 25 ml of complete RPMI (cRPMI) (RPMI 1640 (Sigma-Aldrich) supplemented with 10 % (v/v) heat inactivated FBS, 50 U/ml penicillin (Sigma-Aldrich), 50 µg/ml streptomycin (Sigma-

Aldrich), 2mM L-glutamine (Sigma-Aldich) and 0.1% (v/v) 2-mercaptoethanol (Gibco)) (Table 2.1) was added to stop lysis. PBMC were centrifuged at 1000 rpm for 10 min at 4 °C to remove platelets. The PBMC pellet was resuspended in 25 ml of cRPMI and counted.

2.3.2 DENDRITIC CELL ISOLATION AND CULTURE

Human $CD14^+$ monocytes were isolated for *in vitro* generation of dendritic cells using human CD14 microbeads (Miltenyi 130-050-201) according to manufacturer's instructions (Auburn, CA, USA).MN). Briefly, PBMC were isolated (see section 2.2.1) and resuspended in 80 µl of MACS buffer to a cell density of 10×10^6 cells. 20 µl of CD14 microbeads were added and cells incubated for 15 min at 4 °C. 2 ml of MACS buffer was then added per 1×10^7 total cells and the cells were centrifuged at 300 x g for 10 minutes. All the supernatant was aspirated and up to 10^8 cells were resuspended in 500µl of MACS buffer. LS columns were placed into a MidiMACS separator and rinsed with 3 ml of buffer. The cell suspension was applied to the column and the flow-through containing unlabelled cells was collected. The column was washed with a further 3 x 3 ml of buffer and the column was removed from the separator and placed on a 15 ml collection tube. 5 ml of MACS buffer was pipetted onto the column and the magnetically labelled $CD14^+$ cells were flushed out by firmly pushing the plunger into the column. The $CD14^+$ cell suspension was centrifuged at 300 g for 5 min then resuspended in cRPMI and counted. On day 3, 1.5 ml of cell suspension was removed from each dish and discarded. 1.5 ml of fresh cRPMI supplemented with 50 ng/ml GM-CSF (R&D Systems, Abingdon, UK) and 70ng/ml IL-4 (R&D) was added. After 6 days, cells were harvested by gentle aspiration and centrifuged at 300 g for 5 min. Cells were resuspended in cRPMI (Table 2.1) and counted. Cells were seeded at appropriate concentrations relevant to experimental assays.

2.3.3 $CD4^+$ T CELL ISOLATION

Human $CD4^+$ T cells were isolated using Human Miltenyi Naive $CD4^+$ T cell isolation kitII (130-094-131) according to the manufacturer's instructions (Auburn, CA, USA) Briefly, PBMC were resuspended in 40 µl 1X MACS buffer to a cell density of 10×10^6 cells and 10 µl of Naive $CD4^+$ T cell biotin- antibody cocktail II was added and cells incubated for 5 mm at 4 °C 30µl of MACS buffer was then added per 1×10^7 total cells After addition of buffer, 20 µl of Naive $CD4^+$ T cell Microbead Cocktail II per 1×10^7 cells was added and further incubated for 10 min at 4 °C LS columns were placed into a MidiMACS separator and rinsed with 3 ml of buffer The cell suspension was applied to the column and the flow-through containing unlabelled cells, representing the enriched naive $CD4^+$ T cells was collected The column was washed with a further 3 ml of buffer and the unlabelled cells were collected and combined with the flow–through from the first wash The $CD4^+$T cell suspension was centrifuged at 300 g for 5 min then resuspended in cRPMI and counted

2.3.4 HUMAN T CELL POLARISATION

Isolated naive CD4 T cells (see "Human CD4 T cell isolation protocol") were stimulated using agonist antibodies directed to plate bound CD3 1mg/ml (BD Pharmmgen) and plate bound CD28 1mg/ml (BD Pharmingen) Plates were coated with 1µg/ml anti-hCD3 in PBS for 2 hours at 37°C Cells were stimulated in the presence or absence of polarising cytokines (human) with or without 1 mg/ml hydrolysates added on Day 0 or on Day 3 The cocktails used to drive specific T–cell subsets and their concentrations are represented in the table below

Phenotype	Cocktail	Conc	Supplier	Cat no
Th1	rIL-2	10ng/ml	R&D	202-1L-010
	anti-hIL-4	5μg/ml	R&D	MAB304
	rIL-12	10ng/ml	R&D	219-1L
Th2	rIL-2	10ng/ml	R&D	202-1L-010
	rIL-4	12 5ng/ml	R&D	204-1L-010
	anti-IFNγ	5μg/ml	R&D	MAB2852
T Reg	rIL-2	5μg/ml	R&D	202-1L-010
	rTGF-β	2ng/ml	R&D	204-B-002
Th-17	rIL-1β	10ug/ml	R&D	240-1B-002
	r-6	20ng/ml	R&D	206-1L-010
	rIL-23	10ng/ml	R&D	1290-IL-010
	rTGF-β	2ng/ml	R&D	204-B-002
	anti-hIFNγ	10μg/ml	R&D	MAB2852
	anti-hIL-4	10μg/ml	R&D	MAB304

Cells were transferred to coated anti-CD3 96 round bottom well plates (Fisher, Ballycoolin, Ireland) at 1 0 x 10^6 cells/ml 2μg/ml anti-CD28 was added to cells Cells were incubated for three days at 37°C On day 3 cells were refed with 10ng/ml IL-2 and hydrolysates (1mg/ml) were added and incubated for a further 3 days at 37°C On day 6 cells were harvested

2.3.5 MEASUREMENT OF CELL VIABILITY

Cells were resuspended in their specific growth media and diluted 1/2 in 2 % (w/v) ethidium bromide/acridine orange (EB/AO) (Sigma-Aldrich) 10 µl was pipetted on to a haemocytometer, live cells (green) and dead cells (orange) were counted using a fluorescent light microscope

2.3.5.1 MTT ASSAY

Cell viability was also assessed using the Methylthiazoldiphenyltetrazolium bromide (MTT) assay based on the reduction of MTT reagent into purple formazan crystals by viable metabolically active cells Briefly, CFSE stained PBMC were harvested on Day 4, washed with PBS and incubated with 20µl of MTT solution (0.5mg/ml) for 3h at 37°C The media was removed and the formazan crystals were then dissolved in 200µl of DMSO and centrifuged for 5 minutes at 300 x g and incubated at 37°C for 5 minutes The absorbance (optical density (O D)) of the samples were measured at 570nm using a microplate reader (BioTek EL800,Swindon, UK) with Gen5 Data Analysis Sotware Cell viability was determined as a % of control cells

2.3.6 CRYOPRESERVATION AND RECOVERY OF CELLS FROM LIQUID NITROGEN

For short term storage, $CD34^+$ haematopoietic stem cells were resuspended at 1-2 x 10^6/ml ($CD34^+$) in 500 µl freezing medium (DMEM containing 10% (v/v) Hyclone FBS (LabTech) supplemented with 10 % (v/v) Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich)) For PBMC cryopreservation, cells were resuspended at 5 x 10^7/ml m heat

inactivated FBS. 50 µl of DMSO was added to a 1.5 ml cryo-tube (Thermo Fisher Scientific, Massachusetts, USA) before the resuspended PBMC were added to the cryo-tube. Cells were gradually cooled at 1°C per minute overnight and then transferred to liquid nitrogen for storage. To recover cells, vials were quickly thawed at 37 °C. Just as the vial contents thawed, cells were transferred to a 15 ml tube (Sarstedt) where 5 ml of warmed medium was added (drop by drop) before cells were centrifuged at 300 g for 5 min. The pellet was resuspended in 1 ml of complete cell specific culture media, counted and used for experiments as required.

2.4 CELL CHARACTERISATION BY FLOW CYTOMETRY

2.4.1 GENERAL FLOW CYTOMETRY AND CHARACTERISATION OF CELLS

For analysis by flow cytometry, cells (PBMC, DC or $CD4^+$ T_H1, T_H2, T-Reg, T_H17 cells)) were harvested, washed in sterile PBS and resuspended in FACS Buffer (PBS supplemented with 2 % heat inactivated FBS) to yield approximately 1 x 10^5 cells/FACS tube (4 ml polypropylene tubes) (Falcon, BD Biosciences, Oxford, UK) or 1 x 10^5 cells/well in 96 well V bottom Plate (Lennox, Dublin, Ireland). Fluorochrome conjugated antibodies (Table 2.3) or isotype controls were incubated with cells for 15 min at 4 °C. After 15 min, cells were washed in 2 ml of FACS Buffer, vortexed and centrifuged at 300 g for 5 min. The supernatant was removed and cells resuspended in 50 µl counting beads (3 x 10^5/ml) (Calibrite™ Beads, BD Biosciences) or 100 µl of cell fixative (PBS supplemented with 2 % (v/v) formaldehyde solution (Sigma-Aldrich)). Cells were then analysed by flow cytometry (Accuri C6 flow cytometer, BD Biosciences) using CFlowPlus software (BD Biosciences).

2.4.2 CFSE LABELLING OF PBMC AND MEASUREMENT OF T CELL PROLIFERATION *IN VITRO*

Human PBMC were resuspended at 5 x 10^7/ml in warm PBS PBMC (5 x 10^7) were labelled with 10 µM carboxyfluorescein succimmidyl ester (CFSE) Cells were incubated for 10 minutes at room temperature in the dark After 10 minutes, 2 ml of cold PBS was added and PBMC were centrifuged at 600 g for 5 minutes then washed twice in PBS Labelled PBMC (5 x 10^4/well) were seeded into a 96 well round bottom tissue culture plate (Fisher, Ballycoolin, Ireland) CD3/CD28 Dynabeads® beads (Invitrogen) were added (1 x 10^4/well) to activate PBMC proliferation Hydrolysates (0 5 mg/ml, 1mg/ml or 2 mg/ml) were added one hour later After 4 days, PBMC with or without hydrolysates (1 mg/ml) were harvested and the level of proliferation by $CD3^+$ cells was analysed by flowcytometry (section 2 3 1) and enumerated using counting beads (3 x 10^5/ml) (Calibrite ^{TM} beads, BD Biosciences)

2.4.3 INTRACELLULAR STAINING FOR TRANSCRIPTION FACTOR EXPRESSION IN HUMAN T CELLS

FoxP3, T-bet, Gata-3 and Rorγt was analysed intracellularly using a FoxP3 staining kit (eBioscience) Briefly, $CD4^+$ T cells were washed in 150µl FACS buffer and centrifuged at 950rpm for 5 mins For FoxP3 assays, $CD4^+$ T cells were labelled with CD4 FITC and CD25 APC or corresponding isotype control antibodies for 15 min at 4 °C For T_H1 assays (T-bet), CD4+ T cells were labelled with CD3 FITC and CD4 APC or corresponding isotype control antibodies for 15 min at 4 °C For T_H2 assays (Gata-3), CD4+ T cells were labelled with CD3 FITC and CD4 PE or corresponding isotype control antibodies for 15 min at 4 °C For T_H17 assays (Ror-γt), $CD4^+$ T cells were labelled with CD3 FITC and CD4 PERCP or corresponding isotype control antibodies for 15 min at 4

°C The cells were washed twice in 150µl of FACS buffer, centrifuged at 950rpm for 5 min and fixed with 100µl fix/permeabilisation buffer (eBioscience) for 1 hour The cells were then permeabilised with 200µl permeabilisation buffer , washed with 150µl FACS buffer and blocked using 3 µl 2% rat serum for 15 min The cells were labelled with T-bet, Gata-3, FoxP3, Ror-γt or isotype control antibodies and left at 4° C overnight Samples were washed twice with 150µl FACS buffer, resuspended in counting beads (3 x 10^5/ml) and analysed by flow cytometry (Accuri C6 flow cytometer, BD Biosciences) using CFlowPlus software (BD Biosciences)

2.5 MILK PROTEIN HYDROLYSATES

2.5.1 WHEY AND CASEIN HYDROLYSATES

A number of Whey and Casein hydrolysates (and their regenerates / upscaled samples) were supplied by our collaborators at Food for Health Ireland (FHI), University of Limerick (Alice Nongonierma) Due to Intellectual Property Rights method of generation could not be released by FHI Lead functional compounds 56 (LFC56) and its regenerate 56R were included as controls These two hydrolysates were included as they proved to be functionally bioactive in a bioassay screening platform in FH1 phase 1 Control milk protein substrates were supplied by the industry partners The whey protein concentrate (wpc80 81% (w/v) protein) was obtained from Carbery Milk products Group (Ballineen, Cork) wpc80 is a food grade powder containing high quality whey protein It is manufactured from dairy whey using ultrafiltration and spray drying technology Sodium caseinate (NACN-90 4% (w/v) protein was obtained from Kerry Ingredients (Listowel, Ireland) NACN is a food grade powder containing high quality casein protein

2.5.2 PREPARATION OF HYDROLYSATE (FREEZE DRIED MILK POWDERS)

Before dissolving the powders, the hydrolysates were allowed to equilibrate at room temperature in order to avoid water condensation on the powder Carefully 0 015g of powder was weighed out on a precision scales The powder was dispersed in 1 5ml sterile water (stock concentration – 10mg/ml) using gentle agitation The hydrolysates were stirred gently for 15 –30 min at room temperature to ensure complete dispersion When the powders were fully dispersed in the water the hydrolysates were centrifuged at room temperature at 5000 g for 5 min and the supernatant was retained The supernatant was filtered through 0 2 mm cellulose acetate filters (Whatman GD/X) and the hydrolysates were added at a working concentration of 0 5 mg, 1mg/ml or 2 mg/ml to cells Soluble peptides were present in 5% (w/v) aqueous dispersions of these freeze-dried powders

2.5.3 STORAGE OF HYDROLYSATES

On receipt of samples, all hydrolysates were stored at -20°C in the presence of desiccant (e g , silica gel)

2.6 BIOCHEMICAL METHODS

2.6.1 PROTEIN EXTRACTION

Intracellular protein was extracted from human dendritic cells 24 well plates were placed on ice and media removed Cells were washed with 1 ml of ice cold PBS Cells were scraped in 1 ml ice cold PBS and added to a 1 5 ml tube Samples were centrifuged at 15,000 rpm for 5 mm and PBS removed The pellets were then resuspended in 90 µl cell lysis buffer (Table 2 2) and left on ice for 10 min. Samples were resuspended

by gently pipetting to aid thee lysis process an left on ice for a further 10 min Protein lysates were then subjected to centrifugation at 12,000 g for 10 min at 4 °C 90 µl of the supernatant which constitutes the intracellular protein was added to a new 1 5 ml tube and stored at – 20°C Prior to loading protein lysates, samples were mixed with 4X sample buffer(Table 2 2) and boiled for 5 min

2.6.2 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

SDS-PAGE was carried out in accordance with the Laemmli method as modied by Studier (Laemmli 1970, Studier 1973). Samples and appropriate prestained (10-180 kDa) protein markers were loaded into separate 0 75 mm wells Electrophoresis was performed at 70 V through a 5% polyacrylamide stacking gel and then through a 10% polyacrylamide resolving gel at 90 V for up to 2 hours

2.6.3 IMMUNOBLOTTING

Following separation by electrophoresis, the proteins were transferred electrophoretically to nitrocellulose membranes (GE Heathcare, Buckinghamshire, England) in a BioRad Trans –Blot Turbo Blotting System (BioRad,) at 2 5 A for 30 mins 1 layer of extra thick blotting paper (BioRad) was placed on the bottom surface of the Trans-blot System followed by one layer of nitrocellulose membrane The resolving gel was then placed on top with care to avoid air bubbles Finally 1 more layer of extra-thick blotting paper was added and the unit closed Following transfer , non-specific binding was blocked by incubating the nitrocellulose membranes at room temperature for 1 h with blocking buffer(tris buffered saline (TBS)), 0 1% Tween-20 with 5% (w/v) non-fat dry milk (Table 2 2) under gentle agitation The membranes were then incubated under agitation at 4 ^{0} C overnight with the primary antibody diluted in TBS containing 0 1%

(v/v) Tween-20 (TBST) with 5% BSA as indicated in Table 2 4 The membranes were subsequently subjected to 3 x 5 min washes in TBST (Table 2 2) Membranes were then incubated in a secondary antibody (Table 2 5) specific for the primary antibody in TBST containing 5% (w/v) skimmed milk powder for 1 h in the dark at room temperature The membranes were then washed a further 3 times for 5 min each in TBST in the dark The immunoreactive bands were detected using enhanced chemiluminescence development (WesternBright ECL HRP Substrate, Advansta, Labtech)

2.6.4 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) OF CYTOKINES

All ELISA were carried out according to manufacturer's instructions (R & D Systems, Abingdon, UK or eBioscience, Paisley, Scotland) Specific capture antibodies (human IFNγ, IL-2, IL-4, IL-5, IL-6, 1L-10 , IL-12/IL-23 p40, IL-13 & IL-17) in PBS were added to 96 well Nunc-Immuno^{TM} plates (Thermo Fisher Scientific) and incubated overnight at room temperature Plates were then washed 3 times in wash buffer (PBS supplemented with 0 05 % v/v Tween 20) and then incubated in blocking solution (PBS supplemented with 1 % w/v BSA) for a minimum of 1 h Plates were then washed and incubated with 100 µl/well of sample supernatant or corresponding cytokine standard for 2 hr at room temperature After washing, plates were incubated with specific detection antibodies for a further 2 h at room temperature Plates were washed again and incubated with 100 µl/well of streptavidin horseradish peroxidase (HRP) (R & D Systems) conjugate diluted 1/40 in specific reagent diluent (Tris Buffered solution (TBS) (Sigma-Aldrich) supplemented with BSA for 20 min After washing, plates were incubated with 100 µl/well of tetramethylbenzidine (TMB) substrate (Sigma-Aldrich) for 20 min at room temperature out of direct light The reaction was stopped after 20 min by adding 50

µl/well of 1 M H_2SO_4. The absorbance (optical density (O.D)) of the samples and standards were measured at 450 nm for all ELISA using a microplate reader (BioTek EL800,Swindon,UK) with Gen5 Data analysis software. The cytokine concentration of each sample was determined by comparison to the standard curve of known cytokine concentrations using GraphPad Prism5 software.

2.6.5 MEASUREMENT OF TOTAL IgE AND IgG1

Total IgE and IgG1 were carried out according to manufacturer's instructions (eBioscience, Paisley, Scotland). Specific capture antibodies (human IgE ,IgG1) in PBS were added to 96 well Nunc-Immuno^{TM} plates (Thermo Fisher Scientific) and incubated overnight at room temperature. Plates were then washed 3 times in wash buffer (PBS supplemented with 0.05 % v/v Tween 20) and then incubated in blocking solution (PBS supplemented with 1 % w/v BSA) for a minimum of 2 h. Plates were then washed and incubated with 50 µl/well of sample sera or corresponding standard for 2 hr at room temperature. After washing, plates were incubated with specific detection antibodies for a further 1 h at room temperature. After washing, plates were incubated with 100 µl/well of tetramethylbenzidine (TMB) substrate (Sigma-Aldrich) for 20 min at room temperature out of direct light. The reaction was stopped after 20 min by adding 50 µl/well of 1 M H_2SO_4. The absorbance (optical density (O.D)) of the samples and standards were measured at 450 nm for all ELISA using a microplate reader (BioTek EL800,Swindon, UK) with Gen5 Data analysis software. The antibody concentration of each sample was determined by comparison to the standard curve of known concentrations of IgE, IgG1 using GraphPad Prism5 software.

2.6.6 MEASUREMENT OF OVA SPECIFIC IgG1

Ova specific IgG1 was assayed by enzyme-linked immunosorbant assay (ELISA) 96 well Nunc-Immuno^{TM} plates (Thermo Fisher Scientific) were coated with 5µg/ml OVA extract diluted in carbonate buffer (pH 9 6) and left overnight at 4°C Plates were then washed 3 times with 200 µl washing buffer and blocked with 250µl 1% bovine serum albumin (BSA) in PBS with 1% Tween 20 for two hours at room temperature After washing, 50 µl diluted serum samples were added to the plates and incubated overnight at 4°C Following incubation, plates were washed, and 100 µl of 1 250 diluted HRP-conjugated anti-human IgG antibody (eBioscience, Paisley, Scotland) were added to each well The plates were further incubated for one hour at 37°C After washing, plates were incubated with 100 µl/well of tetramethylbenzidine (TMB) substrate (Sigma-Aldrich) for 20 min at room temperature out of direct light The reaction was stopped after 20 min by adding 50 µl/well of 1 M H_2SO_4. The absorbance (optical density (O D)) of the samples were measured at 450 nm for all ELISA using a microplate reader (BioTek EL800, Swindon, UK) with Gen5 Data analysis software

2.7 MOLECULAR TECHNIQUES

2.7.1 RNA ISOLATION

Total RNA was extracted using trizol® reagent (Invitrogen) according to the manufacturers's instructions Briefly, 1 x 10^6 cells were lysed in 1ml trizol at room temperature for 5 mm 100 µl of RNA-grade 1-Bromo-3-chloropropane (SigmaAldrich) was added to the cells, mixed vigorously and incubated at room temperature for 5 min Samples were centrifuged at 12,000 g for 15 min at 4°C Two distinct layers resulted

with RNA remaining in the clear, aqueous upper layer. 350-400 µl of RNA was carefully removed, ensuring the lower white DNA layer was not disturbed, and precipitated with 500 µl isopropanol (Sigma-Aldrich). The samples were incubated at room temperature for 10 min and followed by centrifugation for 10 min at 4 °C. The resulting RNA pellet was washed with 1 ml 75 % (v/v) ethanol and centrifuged at 7,500 g for 5 min at 4 °C. The ethanol was aspirated and the RNA pellet was allowed to briefly air dry prior to resuspension in 30 µl RNase-free water (Promega, Southhampton, UK). The purity and concentration of RNA was determined using a spectrophotometer (Nanodrop 2000, ThermoScientific, Wilmington, DE, USA) which calculated the ratio of absorbance at 260 nm to280 nm. A ratio between 1.8 and 2.0 indicated sufficient purity of the RNA. Samples outside this range were discarded.

2.7.2 DNASE TREATMENT OF RNA

Genomic DNA was removed from RNA samples by treatment with DNase I (Invitrogen, Paisley, UK). 1 µl of DNase (Amplification grade) was added to 500 ng of RNA and incubated for 15 min at room temperature. 1 µl of 25mM EDTA (Invitrogen), to inactivate the DNase, was added to the mixture and incubated at 65^0C for 10 min.

2.7.3 cDNA SYNTHESIS

Following DNase treatment of RNA, total RNA was reverse transcribed using 5X All-In-One Mastermix (ABM-NBS Biologicals, Cambridgeshire, UK). This ready to use mastermix formulation , containing EasyScript Reverse Transcriptase, RNaseOFF ribonuclease inhibitor, dNTP mix ,Oligo (dT)s and random primers ,was diluted to a 1X

concentration in nuclease-free water (ABM-NBS Biologicals) The conditions for cDNA synthesis were as follows 42 ^{0}C for 50 min, 85°C for 5 min, and 4 ^{0}C for 10 min Quantification of cDNA was performed by measuring the absorbance value of the sample 260nm Samples were stored -20°C until required

2.7.4 REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

PCR was used to determine the presence of specific DNA sequences (or mRNA following reverse transcription) using primers summarised in Table 2 6 Expression of the housekeeping gene, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a positive control PCR reactions contained 2 5 mM MgCl2 (Promega), 25 mM dNTP (Promega), 1 x GoTaq reaction buffer (Promega), 40 U/ml Taq polymerase (Promega) and 0 4 pM of the appropriate primer (NCBI BLAST) The reaction mastermix was adjustedto a final volume of 24 µl with nuclease-free water The PCR conditions wereas follows denaturation at 95 °C for 45 sec (2 min for first cycle), annealing at 58^{0}C for 45 sec (optimal annealing temperatures are summarised in Table 2 7) and extension for 45 sec at 72°C DNA products were resolved on a 1 3 % w/v agarose gel and detected by binding of gel red (Biotium, Hayward, CA)

2.7.5 AGAROSE GEL ELECTROPHORESIS

Agarose gels were prepared by adding 1 3% (w/v) agarose in TAE buffer and heating until completely dissolved The solution was then cooled before 5 µl GelRed nucleic acid stain (Biotium) was added and the solution waspoured into a gel tray Following solidification, agarose gels were submerged in TAE buffer and subjected to electrophoresis at 110 V Samples were run simultaneously with a 1 Kb molecular weight

ladder Nucleic acid products were visualised under ultraviolet (UV) light (254nm) and images acquired using a Gel Logic 212 Pro gel documentation system (Carestream Health,Connecticut,USA)

2.7.6 REAL TIME-POLYMERASE CHAIN REACTION (qPCR)

cDNA was analysed for the quantification of mRNA expression Briefly, cDNA (500 ng) were amplified in the presence of SYBR® Green Jumpstart Taq ReadtyMix PCR mastermix (Qiagen) Accumulation of gene-specific products were measured continuously by means of fluorescence detection over 40cycles Each cycle consisted of denaturation at 95 °C for 15 sec, annealing at 95 ^{0}C for 30 sec, (Table 2 6) and extension at 72 ^{0}C for 45 sec followed by a melt curve cycle of 95 °C for 15 sec, 55 °C for 15 sec and 95 °C for a final 15 sec Quantification of target gene expression was obtained using an Eco Real-Time PCR System (Illumina, San Diego, CA USA) Expression was quantified in relation to the housekeeping gene GAPDH using the delta CT method The ΔCT method was determined by subtracting the GAPDH value form the target CT value for each sample The fold change in relative gene expression was determined by calculating the $2^{-\Delta ct}$ values (Schmittgen & Livak 2008)

2.8 HUMANISED MOUSE MODEL

2.8.1 INTRAVENOUS ADMINISTRATION OF HUMAN CD34$^+$ HSCs

A humanised mouse model was developed from a protocol described by Brehm *et al* (Brehm *et al* 2012) and Billerbeck *et al.* (Billerbeck *et al* 2012). NSG-mSCF mice (Prkdc ^{scid} IL2rg ^{tmlWjl}/Tg PGK1-KITLG*220) 441 Daw/J and NSG-SGM3 mice (NOD CgPrkdc^{scid}IL2rg^{tmlWjl}/TgCMVIL3,CSF2,KITLG)1Eav/MloySzJ were two strains chosen to study engraftment of human CD34$^+$ hematopoietic stem cells (HSCs) in NSG mice. NSG-SGM3 mice were irradiated with a conditioning dose of 1.0 Gray (Gy) of whole body gamma irradiation. NSG-mSCF mice were non-irradiated. Cord blood-derived flow-sorted human CD34$^+$ (HSCs) purchased from Lonza (Basel, Switzerland) or freshly isolated human CD34$^+$ HSCs (AnthonyNolan/Holles St) were administered into 3-6 week old NSG-SCF and NSG-SGM3 mice by intravenous injection into the tail vein using a 27 gauge needle and a 1 ml syringe between 4 h but no longer than 24 h following irradiation. Before infusion CD34$^+$ HSCs were washed three times with sterile PBS. CD34$^+$ HSCs (5×10^4- 1×10^5) cells/mouse were administered on day 0. Each mouse received a total of 0.3 ml. Following i.v injection, animals were returned to their cages where they were monitored closely for the first hour and at regular intervals thereafter for any signs of distress or ill health. Animals were weighed 3 times per week and weight loss was documented accordingly. Any animals which displayed greater than 15 % total bodyweight loss were sacrificed humanely. Engraftment of CD34$^+$ HSCs was examined in peripheral blood, spleen, bone marrow, liver and thymus using flow cytometry at 12, 16 and 20 weeks post transplantation.

2.8.2 $CD3^+$ CELL DEPLETION OF CORD BLOOD

Fresh human cord blood (80 -170ml) was purchased from Anthony Nolan Trust UK (Anthony Nolan Cell Therapy Centre, Nottingham, UK) and shipped overnight at room temperature (15-25^{0}C) The next morning the $CD3^+$ cells were depleted using the RosetteSep system (Stemcell Technologies, Grenoble,France) 1 25ml RosetteSep Human CD3 Depletion cocktail was added for every 25ml of cord blood This was mixed and incubated for 20 minutes at room temperature The cord blood was then diluted 1 4 in RoboSep buffer (PBS with 2% foetal calf serum (FCS) and 1mM EDTA) This was carefully layered over Lymphoprep (Axis Shield PoC AS, Oslo, Norway), with 35ml of blood for 15ml of Lymphoprep and spun at 1200 g for 20 minutes at 20°C and with the brake off The enriched cells were transferred to a clean Falcon tube and diluted in an equal volume of RobSep buffer (Stemcell Technologies, Grenoble, France) and spun at 300 g for 10 minutes $CD3^+$ cell depleted cord blood cells were then resuspended in RoboSep buffer for further processing using the EasySep separation (described below) system

2.8.3 $CD34^+$ CELL PURIFICATION USING EASYSEP

EasySep (Stemcell Technologies, Grenoble, France) human cord blood CD34 positive selection kit II (Cat no 17896) was used for labelling between 80-170ml of Anthony Nolan cord blood or 60-140 ml of Holles St cord blood 75µl of RosetteSep cord blood CD34 pre-enrichment cocktail II was added for every 15ml of cord blood. This was mixed and incubated for 20 minutes at room temperature The cord blood was then diluted

1:2 in RoboSep buffer (PBS with 2% foetal calf serum (FCS) and 1mM EDTA Stemcell Technologies). This was carefully layered over Lymphoprep (Asis-Shield PoC AS, Oslo, Norway) with 30ml of blood for 15ml of Lymphoprep and spun at 1200 x g for 20 minutes at 20°C with the brake off. The enriched cells were transferred to a clean Falcon tube and diluted in an equal volume of RoboSep buffer (Stemcell Technologies, Grenoble, France) and spun at 300 x g for 10 minutes with the brake low. The pre-enriched cells were then resuspended in 0.75 ml- 1.0 ml in EasySep buffer and transferred to a 14 ml polystyrene round-bottom tube (Corning 17 x 100 mm).100µl of human CD34 positive selection cocktail was added for every ml of sample. This was mixed and incubated for 10 minutes. The EasySep magnetic nanoparticles were vortexed for 30 seconds to give a uniform suspension and then added to the cell suspension at 50µl/ml of cells. This was mixed and incubated at room temperature for 5 minutes. The cell suspension was brought to a total volume of 2.5ml with EasySep buffer. The cells were mixed by gentle pipetting and the tube containing the cell suspension was placed, without a lid, into the big easy magnet and set aside for 5 minutes. In one continuous movement the magnet containing the tube with the cell suspension was inverted thus pouring off the supernatant. Once upright the tube was removed from the magnet and the remaining cells were resuspended in EasySep buffer. The cells were once again mixed by gentle pipetting and the tube containing the cell suspension was placed, without a lid, into the magnet and set aside for 5 minutes. In one continuous movement the magnet containing the tube with the cell suspension was inverted thus pouring off the supernatant. This was repeated 3 more times and the final cell suspension was topped up with EasySep buffer centrifuged at 300 x g for 10 minutes with the brake low. All the supernatant was aspirated and finally the yield and purity of the labelled fraction was determined using flow cytometry before the cells were either injected into mice or cryopreserved in 90% FCS and 10% DMSO.

2.8.4 ISOLATION OF HUMAN CELLS FROM MOUSE SPLEEN AND THYMUS

12, 16 and 20 week old male and female NSG-SCF and NSG-SGM3 mice were sacrificed by cervical dislocation Spleens and thymus were removed aseptically from mice into a 50 ml tube containing cRPMI (Table 2 1).Splenocytes and thymocytes were isolated by homogenising spleens and thymus through a 70 μm filter into a fresh 50 ml tube using a sterile plunger The homogenates were centrifuged at 300 g for 5 min and resuspended in 5 ml of 1 x red blood cell lysis buffer solution (BioLegend, San Diego, CA,) for 5 mm at room temperature 5 ml of cRPMI was added to the suspension to neutralise the lysis solution which was then centrifuged at 300g for 5 min Supernatant was removed and the cells were then resuspended for FACS analysis (Section 2 9 1)

2.8.5 ISOLATION OF HUMAN CELLS FROM MOUSE BONE MARROW

12, 16 and 20 week old male and female NSG-SCF and NSG-SGM3 mice were sacrificed by cervical dislocation Femurs and tibias were removed aseptically from mice into a sterile petri-dish and the surrounding muscle removed The ends of each bone were removed and the bone marrow was flushed into a sterile Petri dish using a 27 gauge needle and into cRPMI (Table 2 1) Cell aggregates were disrupted using a 19 gauge needle and 5 ml syringe Bone marrow aggregates were homogenised through a 70μm filter into a fresh 50 ml tube using a sterile plunger and the isolated cells were then suspended in 25 ml cRPMI This homogenate was layered over 15 ml lymphoprep density gradient (Axis-Shield) and centrifuged at 2400 rpm for 25 min with no brake and low acceleration The

interface was collected by aspiration into a fresh labelled 50ml tube The interface was washed twice with 25 ml PBS and centrifuged at 300 g for 5 min Supernatant was removed and the cells were resuspended for FACS analysis (Section 2 9 1)

2.8.6 ISOLATION OF HUMAN CELLS FROM MOUSE LIVER

Livers were removed aseptically from mice into a 50 ml tube containing cRPMI (Table 2 1) Tissues were homogenised through a 70µm filter into a fresh 50 ml tube using a sterile plunger and the isolated cells were then suspended in 25 ml cRPMI This homogenate was layered over 15 ml lymphoprep density gradient (Axis-Shield) and centrifuged at 2400 rpm for 25 min with no brake and low acceleration The interface was collected by aspiration into a fresh labelled50 ml tube The interface was washed twice with 25 ml PBS and centrifuged at 300 g for 5 mm Supernatant was removed and the cells were resuspended for FACS analysis (Section 2 9 1)

2.8.7 ISOLATION OF HUMAN CELLS FROM MOUSE PERIPHERAL BLOOD

At 12, 16 and 20 weeks after HSC transplantation, peripheral blood was collected from the facial vein of humanised NSG-SCF and NSG-SGM3 mice Briefly 100-150 µl of blood was collected into an eppendorf tube containing 200µl of 2mM EDTA and mixed thoroughly 1 0 ml of 1X RBC Lysis Buffer was added and each tube was gently vortexed for 10 sec and incubated at room temperature, protected from light, for 10-15 minutes All samples were centrifuged at 350 x g for 5 minutes The supernatant was removed

without disturbing pellet and the pellet was resuspended for FACS analysis (Section 2 9 1)

2.9 HUMANISED MOUSE MODEL OF OVA ALLERGY

2.9.1 INTRAVENOUS ADMINISTRATION OF FROZEN HUMAN CD34$^+$ HSCs

A humanised mouse model was developed from a protocol described by Brehm *et al* (Brehm *et al* 2011) Female NSG-mSCF mice (Prkdc ^{scid} IL2rg ^{tm1Wjl}/Tg PGK1-KITLG*220) 441 Daw/J were chosen to study engraftment of human CD34$^+$ hematopoietic stem cells (HSCs) in NSG mice Cord blood-derived flow-sorted human CD34$^+$ (HSCs) purchased from Lonza (Basel, Switzerland) were administered by intravenous injection into the tail vein using a 27 gauge needle and a 1 ml syringe Before infusion CD34$^+$ HSCs were washed three times with sterile PBS CD34$^+$ HSCs (5 x 10^4) cells/mouse were administered on day 0 Each mouse received a total of 0 3 ml Following i v injection, animals were returned to their cages where they were monitored closely for the first hour and at regular intervals thereafter for any signs of distress or ill health Animals were weighed 3 times per week and weight loss was documented accordingly Any animals which displayed greater than 15 % total bodyweight loss were sacrificed humanely In addition an animal welfare score sheet was utilized throughout the study Engraftment was monitored in samples of peripheral blood using flow

cytometry on Day 46 and in samples of spleen, liver, bone marrow and thymus on Day 84 preceding allergen sensitization and on Day 130 at the end of the study

2.9.2 SENSITISATION AND CHALLENGE OF OVA MICE

After 3 months of stem cell engraftment, mice were sensitized by intra-peritoneal (i p) injection with Ovalbumin (OVA) (grade V, Sigma-Aldrich, UK) plus alum (Imject Alum, ThermoScientific, Rockford,USA) at a 1 2 ratio Each mouse received a total of 100ug of the solution (OVA+Alum) in 0 3ml once a week for two weeks Ova was delivered i p using a 27 gauge needle and a 1 ml syringe Control groups were sham sensitized with sterile PBS plus alum Allergen challenge was performed once one week after the last gavage (147 or wpc80) by gavage feeding with 50mg of OVA in 0 3 ml PBS Following each procedure the animals were returned to their cages where they were monitored for any signs of distress or ill health. In addition, an animal welfare score sheet was utilized throughout the study

2.9.3 PREPARATION AND ADMISTRATION OF HYDROLYSATE 147 AND WPC80

One day after the last sensitization, Hydrolysate 147 (FHI, UL, Limerick) and the parent compound whey protein concentrate 80 (WPC80) (Carbery Milk products Group Ballineen, Cork) were prepared as in section 2 5 2 Each mouse received a total of 50mg of the solution (147 or WPC80) in 0.3ml once a week for 5 weeks. Administration was carried out by oral gavage Briefly the solutions were loaded into a 1 ml syringe

attached to a feeding needle (Vet Tech,Cheshire, UK) Mice were carefully scruffed and the feeding needle was inserted into the mouth of the mouse The feeding needle was carefully guided down the oesaphagus, where the 0 3ml solution of hydrolysate 147 or WPC80 was released from the syringe Following each procedure the animals were returned to their cages where they were monitored for any signs of distress or ill health

2.10. ANAPHYLACTIC SCORING SYSTEM

Anaphylactic symptoms were evaluated for 30 to 40 minutes after the first challenge dose by using a scoring system as follows 0, no symptoms, 1, scratching and rubbing around the nose and head, 2, puffiness around the eyes and mouth, diarrhea, pilar erecti, reduced activity, and/or decreased activity with increased respiratory rate, 3, wheezing, labored respiration, and cyanosis around the mouth and the tail, 4, no activity after prodding or tremor and convulsion, 5, death

2.11. CELLULAR AND CYTOKINE ANALYSIS FROM OVA MICE

2.11.1. ISOLATION OF HUMAN CELLS FROM THE SPLEEN, BONE MARROW, LIVER , PERIPERAL BLOOD AND THYMUS

All tissues were prepared as before (Section 2 8 4-2 8 7) The cells were resuspended for FACS analysis (Section 2 10 5)

2.11.2 ISOLATION OF HUMAN CELLS FROM THE MESENTERIC LYMPH NODE

The small intestine together with mesentery was surgically removed The intestine was arranged in a fan-shape to visualize the mesenteric lymph nodes Mesenteric

lymph nodes (MLN) were removed into a 24 well plate containing cRPMI (Table 2 1) MLNs were isolated by homogenising through a 70 μm filter into a fresh 50 ml tube using a sterile plunger The homogenate was centrifuged at 300 g for 5 min, supernatant was removed and the cells were then resuspended in fresh cRPMI and counted for an ex *vivo* culture (Section 2 10 3)

2.11.3 CYTOKINE ANALYSIS FROM MLN CELL CULTURES

Spleens and mesenteric lymph nodes were removed from mice as described above and single cell suspensions were prepared Cells were seeded at 2 x 10^5 per well in a 96 well round bottom plate and cultured in cRPMI Cells were unstimulated (media alone) or stimulated with 100ug/ml OVA (Sigma-Aldrich) Supernatants were harvested after 72 h for detection of IL-4, IL-5 and IL-13 Cytokines in supernatants were detected by ELISA as described in Section 2 6 1

2.11.4 CYTOKINE ANALYSIS FROM THE TISSUES OF OVA MICE

The small intestine, spleen and liver were removed from mice as described above and were immediately snap frozen and stored at – 80 ^{0} C Tissues were thawed and gut contents removed The tissues were chopped finely and homogenized using an Ultra-Turrax homogemzer (IKA, Staufen, Germany) in 1 ml of chilled homogenization buffer (PBS 2 % heat inactivated FBS supplemented with protease inhibitor cocktail (Roche, Dublin ,Ireland)) The homogenate was microcentrifuged at 13,000 rpm for 15 min at 4 ^{0}C The supernatant was removed and stored at -20 ^{0}C Protein extracts were analysed for IL-4, IL-6, IL-10, IL-12, IL-13, IL-17 and IFN-γ Cytokines in supernatants were detected by ELISA as described in Section 2 6 1

2.12 ANALYSIS OF HUMAN CELL ENGRAFTMENT *IN VIVO* BY FLOW CYTOMETRY

2.12.1 DECTECTION OF LEUCOCYTES, MYELOID CELLS, B CELLS, T CELLS, MAST CELLS/BASOPHILS AND DENDRITIC CELLS IN MOUSE TISSUES

For analysis by flow cytometry, single cell suspensions from spleen, bone marrow liver, thymus and peripheral blood were harvested, washed in sterile PBS and resuspended in FACS Buffer (PBS supplemented with 2 % heat inactivated FBS) to yield approximately 1 x 10^5 cells/well in 96 well V bottom Plate (Lennox, Dublin, Ireland) Fluorochrome conjugated antibodies (Table 2 4) or FMO controls were incubated with cells for 15 min at 4 °C After 15 min, cells were washed in 2 ml of FACS Buffer, vortexed and centrifuged at 300 g for 5 min The supernatant was removed and cells resuspended in 150 µl facs buffer Cells were then analysed by flow cytometry (Attune NXT Acoustic Focusing Cytometer) using attune NXT software (Thermo Fisher Scientific)

2.12.2 DETECTION OF CYTOKINE PRODUCTION BY HUMAN CELLS

IFN-γ and IL4 were analysed intracellularly by flow cytometry. Briefly, splenocytes recovered from *in vivo* studies following stimulation with 100ng/ml Phorbal 12-myriate 13-acetate (PMA) (Sigma-Aldrich) and 1ug/ml ionomycin (Sigma-Aldrich) and 1X Brefeldin A for 4 h, were washed in 150µl FACS buffer and centrifuged at 950rpm for 5 min in 96 well v bottomed plates. Splenocytes were labelled with CD45 Pacific Orange, mouse CD45 Pecy7, CD3 Alexa Flour , CD4 FITC, or corresponding FMO control antibodies for 15 min at 4 ^{0}C.The cells were washed twice in 150 µl FACS buffer, centrifuged at 950 rpm for 5 min and fixed with 100 µl fix/permeabilisation buffer (eBiocsience) for 1 h. The cells were then permeabilised with 200 µl permeabilisation buffer (eBioscience), washed with 150 µl FACS buffer and blocked using 3 µl 2% rat serum for 15 min. The cells were labelled with IFNγ APC, IL-4 PE dazzle or FoxP3 brillant violet 421 or fmo control antibodies and left at 4 ^{0}C for 1 h. Samples were washed twice with 150 µl FACS buffer and analysed by flow cytometry (Attune NXT Acoustic Focusing Cytometer) using attune NXT software (Thermo Fisher Scientific).

2.12.3 INTRACELLULAR STAINING OF CELLS TO DETECT FOXP3 EXPRESSION

FoxP3 expression was analysed intracellularly using a FoxP3 staining kit (eBioscience). Briefly , splenocytes recovered form in *vivo* studies were washed in 150 µl FACS buffer and centrifuged at 950 rpm for 5 min. Splenocytes were labelled with CD45 Pacific Orange, mouse CD45 PECY7, CD3 Alexa Flour 700 , CD4 FITC, CD127 PERCP 5.5 and CD25 PECY7 or corresponding FMO control antibodies for 15 min at

4 ^{0}C The cells were washed twice in 150 µl FACS buffer, centrifuged at 950 rpm for 5 min and fixed with 100 µl fix/permeabilisation buffer (eBiocsience) for 1 h The cells were then permeabilised with 200 µl permeabihsation buffer (eBioscience), washed with 150 µl FACS buffer and blocked using 3 µl 2% rat serum for 15 min The cells were labelled with FoxP3 brillant violet 421 or fmo control antibodies and left at 4 ^{0}C for 1 h Samples were washed twice with 150 µl FACS buffer and analysed by flow cytometry (Attune NXT Acoustic Focusing Cytometer) using attune NXT software (ThermoFisherScientific)

2.13 HISTOLOGY

2.13.1 TISSUE PREPARATION

Small Intestine was harvested from experimental mice on day 130 and fixed in 10% (v/v) neutral buffered formalin (Sigma-Aldrich) for at least 24 h Samples were transferred to 70% ethanol for a further 24 h Samples were processed for histology using an automated processor (Shandon Pathcentre, Runcorn, UK), which immersed the tissues in fixatives and sequential dehydration solutions, including ethanol (70%, 80%, 95% x 2, 100% x 3) and xylene (x 2) (Sigma-Alrich) After processing, tissues were embedded in paraffin wax (Sigma-Aldrich) using the Shandon Histocenter 2 (Shandon) and left set overnight at 4 °C 4µm sections were cut using a microtome (Shandon Finesse 325, Thermo-Shandon, Waltham, MA, USA) Sections were placed in cold water before being transferred to a hot water bath (42 °C) to remove any folding of the sections Tissue sections were placed onto microscope slides (VWR, Ballycoolin, Ireland), and left to air-dry overnight at RT Samples were then stained with H &E (Section 2 12 2) or Toluidine Blue (Section 2 12 3) and blindly scored using the system outlined in section 2 12 4

2.13.2 HAEMATOXYLIN/EOSIN STAINING

Before commencement with H&E staining slides were heated to 56 °C for a minimum of 1 h to aid wax clearance Slides were then transferred to xylene (Sigma-Aldrich) for 10 min each This was repeated with fresh xylene for a further 10 mm Samples were then re-hydrated following immersion in 3 decreasing concentrations of ethanol (100% x 2, 95% (v/v) and 80%) for 5 min each Sections were then transferred to dH_20 for 5 min before being immersed in Haematoxylin (Sigma-Aldrich) for 3 min Samples were then washed under dH_20 for 2 min before being placed in 1 % acid alcohol for 20 seconds Samples were washed again under dH_20 before being immersed in Eosin Y (Sigma-Aldrich) for 3 min and back to washing again under dH_20 Slides were dehydrated through immersion in a series of increasing ethanol concentrations (80%, 95% and 100%) for 5 min each Samples were air dried, mounted with DPX mountmg media (BDH) and examined under a light microscope

2.13.3 TOULIDINE BLUE STAINING

Before commencement with Toluidine staining slides were heated to 56 °C for a minimum of 1 h to aid wax clearance Slides were then transferred to xylene (Sigma-Aldrich) for 10 min each This was repeated with fresh xylene for a further 10 min Samples were then re-hydrated following immersion in 3 decreasing concentrations of ethanol (100% x 2, 95% (v/v) and 80%) for 5 min each Sections were then transferred to dH_20 for 5 min before being immersed in Toluidine blue (Sigma-Aldrich) solution for 40 sec Slides were dehydrated through immersion in a series of increasing ethanol concentrations (80%, 95% and 100%) for 5 min each Samples were then immersed in

Xylene for 3 mins Samples were air dried, mounted with DPX mounting media (BDH) and examined under a light microscope

2.13.4 HISTOLOGICAL SCORING

Following H&E staining, slides were coded without reference to prior treatment and examined in a blind manner A semi-quantitative scoring chart was used to assess the degree of inflammation and related histological changes of the Small Intestine Pathological scoring was carried out as follows

SCORE	Small Intestine /Jejunum
0	Normal
1	Mild hyperplasia with minor mononuclear cell infiltration
2	Dispersed but mild villous blunting, necrosis and increased cell infiltration
3	Dispersed and moderate villous blunting, necrosis with further increased cell infiltration and colonic crypt ulceration
4	Dispersed and severe villous blunting, necrotic cells with pervasive mononuclear cell infiltration and colonic crypt ulceration

2.14 STATISTICAL METHODS

The students paired t test was used when statistical analysis was required between two experimental groups One way ANOVA was used to test for statistical significance of differences when multiple experimental groups were compared Power analysis was carried out to determine the number of animals that would yield a significant difference

in the in vivo studies. Statistical methods (Power analysis (SISA)) were used to determine the minimum number of animals per treatment group to obtain a power in the study. SISA software is online at http://home.clara.net/sisa/power.htm.

Table 2.1 Media for Cultured Cells

Media	Composition	Supplier
Complete media for Human PBMC Culture	RPMI 1640	Sigma-Aldrich
	10% (v/v) heat inactivated FBS	Labtech
	50U/ml penicillin	Sigma-Aldrich
	50µg/ml streptomycin	Sigma-Aldrich
	2mM L-glutamine	Sigma-Aldrich
	0.1% (v/v) 2-mecaptothanol	Gibco
Complete media for Human T cell polarisation Culture	TexMacs Media	Stem Cell
	10% (v/v) Hyclone FBS	Labtech
	50U/ml penicillin	Sigma-Aldrich
	50µg/ml streptomycin	Sigma-Aldrich
	2mM L-glutamine	Sigma-Aldrich
	0.1% (v/v) 2-mecaptothanol	Gibco
Complete media for For Human Dendritic cell culture	IMDM	Sigma-Aldrich
	10%(v/v) Hyclone FBS	Labtech
	50U/ml penicillin	Sigma-Aldrich
	50µg/ml streptomycin	Sigma-Aldrich
	2mM L-glutamine	Sigma-Aldrich
	0.1% (v/v) 2-mecaptothanol	Gibco

Table 2.2 Antibodies for Flow Cytometry

Antibody	Fluorochrome	Clone	Isotype	Supplier
CD3	APC/FITC	UCHT1	Mouse IgG1k	eBioscience
CD4	APC/FITC	SK3	Mouse IgG1k	eBioscience
CD8	FITC/PE	RPA-T8	Mouse IgG1k	eBioscience
CD11C	FITC	3.9	Mouse IgG1k	eBioscience
CD14	PE	61D3	Mouse IgG1k	eBioscience
CD1A	FITC	H1149	Mouse IgG1k	eBioscience
CD19	FITC/APC	H1B19	Mouse IgG1k	eBioscience
CD20	FITC	2H7	Mouse IgG1k	eBioscience
CD25	APC	BC96	Mouse IgG1k	eBioscience
CD34	PE	4H11	Mouse IgG1k	eBioscience

CD45	FITC/PERCP	2D1	Mouse IgG1k	eBioscience
CD86	PE	IT2.2	Mouse IgG1k	eBioscience
CD123	APC	6H6	Mouse IgG1k	eBioscience
CD117	FITC/APC	104D2	Mouse IgG1k	eBioscience
CD203C	PE	NP4D6	Mouse IgG1k	eBioscience
CD33	FITC/APC	HIM3-4	Mouse IgG1k	eBioscience
CD294	PE	BM16	Mouse IgG1k	eBioscience
FoxP3	PE	E7	Mouse IgG1k	eBioscience
T-bet	PE	4B10	Mouse IgG1k	eBioscience
Gata-3	APC	TWAJ	Mouse IgG1k	eBioscience
Ror-γt	APC	AFKJS-9	Mouse IgG1k	eBioscience
HLA-DR	PE	L243	Mouse IgG2a k	eBioscience

Table 2.3 Antibodies for Flow Cytometry Attune NxT

Antibody	Fluorochrome	Clone	Isotype	Supplier
CD3	Alexa Fluor 700	HIT3a	Mouse IgG2ak	Biolegend
CD4	FITC	OKT4	Mouse IgG1k	eBioscience
CD8	Pacific Blue	RPA-T8	Mouse IgG1k	Invitrogen
CD19	Brillant Violet 510	H1B19	Mouse IgG1k	eBioscience
HLA-DR	PerCp- Cy5.5	LN3	Mouse IgG1k	eBioscience
HLA-DR	PE/CY7	L243	Mouse IgG2ak	Biolegend
CD25	PE/CY7	BC96	Mouse IgG1k	Biolegend
FCεR1	PE	AER-37	Mouse IgG1k	eBioscience
mCD45	PE/CY7	30-F11	Rat IgG2bk	Biolegend
CD45	Pacific Orange	2D1	Mouse IgG1k	Invitrogen
CD45	APC-H7	2D1	Mouse IgG1k	BD Pharmingen
CD117	Brilliant Violet 510	104D2	Mouse IgG1k	eBioscience
CD117	PE/CY7	104D2	Mouse IgG1k	eBioscience
CD127	PerCp/Cy5.5	A019D5	Mouse IgG1k	eBioscience
IFN-γ	PerCp/Cy5.5	45.B3	Mouse IgG1k	eBioscience

FoxP3	Brıllant Vıolet 421	206D	Mouse IgG1k	eBıoscıence
IL-4	PEDazzle 594	MP4-25D2	Rat IgG1k	Bıolegend

Table 2.4 Buffers

Buffer	Composition
Blocking Buffer for Immunoblotting	TBS, 0 1% (v/v) Tween-20 (TBST) wıth 5%(w/v) non-fat dry Mılk
Laemmli sample buffer	62 5mM Trıs-HCL, pH6 8, 10 % (w/v) glycerol, 2% (w/v) SDS, 0 7 M β-mercaptoethanol and 0 001% (w/v) bromophenal blue
Phosphate Buffered Saline (PBS)	2 7mM KCl, 1.5mM KH_2P0_4, 137mM (PBS) NaCL and 8 mM Na_2HPO_4, pH 7 4
RIPA Lysis Buffer	50mM Trıs-HCL pH 7 4, 1% (v/v) Igepal, 150mM NaCL, 0 5% (w/v) Sodıum Deoxycholate, 1mM EDTA, 0 1% (w/v) SDS 1mM Na_3VO_4, 1 mM PMSF and protease ınhıbıtor cocktaıl
SDS Running Buffer	25mM Trıs, 192 mM glycıne, 01% SDS
TAE(Tris-acetate-EDTA) Buffer	40mM Trıs Base, 0 1%(v/v) glacıal acetıc Buffer acıd, 1Mm EDTA
TBS (Tris buffered Saline)	25Mm Trıs, pH 7 4, contaınıng 0 14M NaCL
TBST(Tris buffered Saline with Tween)	25Mm Trıs, pH 7 4, contaınıng 0 14M NaCL 0 1% (v/v) Tween -20
Transfer Buffer	25mM Trıs,192 mM glycıne, 20% (v/v)methanol

Table 2.5 Primer Sequences

Primer	Forward 5 – 3	Reverse 3 - 5	Product Size(bp)	Anneal temp (°C)
GAPDH	ACAGTTGCCATG TAATGTAGACC	TTTTTGGTTGA GCACAG	150	58
PPAR-γ	AGTGTTGTACAGA	GCATCTGTCTT	200	58

Table 2.6 Primary Antibodies for Immunoblotting

Primary Antibodies For Immunoblotting	Dilution Factor	Diluent	Supplier
Phospho-p65	1:1000	5%BSA TBST	Cell Signalling
β-actin	1:200	5% Milk TBST	Sigma

Table 2.7 Secondary Antibodies for Immunoblotting

Secondary ECL Antibodies	Dilution Factor	Diluent	Supplier
Anti-Mouse HRP	1:1000	5% Milk TBST	Cell Signalling
Anti-Rabbit HRP	1:1500	5% Milk TBST	Cell Signalling

CHAPTER 3

WHEY HYDROLYSATE 147 EXERTS ANTI-INFLAMMATORY EFFECTS BY SUPPRESSING NF-κB PATHWAY ACTIVATION THROUGH A PPAR-γ DEPENDANT MECHANISM

3.1 INTRODUCTION

While milk contains components that can modulate the immune system. An increasing number of studies suggest that milk proteins, including whey and casein may contain biologically active peptides with immunomodulatory properties in their sequences (Wichers 2009). These bioactive peptide sequences can be released during digestion in the gut and can also be produced by *in vitro* enzyme hydrolysis from parent milk proteins (Phelan and Kerins 2011). Hydrolyzation of cow's milk proteins, both whey and casein fractions, results in a mixture of different peptide-sequences which is called a hydrolysate. Hydrolysates are already being produced on a large scale, mainly for the use of managing cow's milk allergy in infants (e.g include Pepti, Aptamil, and Nutricia,). Protein-derived peptides formed during hydrolysis have been shown to modulate the gastrointestinal tract at different levels, besides preventing IgE induced allergic responses. Immunoregulatory peptides found in cow's milk hydrolysates were shown to induce mucus production (Matinez *et al* 2013, Trompette *et al* 2003) and to promote anti-inflammatory responses in intestinal epithelial cells (Mukhopadhya *et al* 2015, Piccolomini *et al* 2012). Once taken up into the lamina propria, hydrolysates may also directly influence both innate and adaptive immune cells (Cheng *et al* 2015, Wong *et al* 1998). Following exposure to casein hydrolysates *in vitro* macrophages showed both inhibition of inflammatory responses (Malinowski *et al* 2014, Chen *et al* 2015) and an increased *in vitro* phagocytic activity (Sandre *et al* 2001,Mao *et al* 2005, Lebrun *et al* 2004) *in vivo* (Pan *et al* 2013,Kazlauskaite *et al* 2005). Several studies have focused on the *in vitro* modulation of lymphocyte proliferation by individual whey proteins (β-LG, α-LA,) and mixtures of whey

protein concentrates (WPCs) (Mercier *et al* 2004, Miyauchi *et al* 1998, Orsi 2004) However, few studies have evaluated the effect of peptides naturally present in whey or those produced by enzyme hydrolysis of whey Miyauchi *et al* (1997) showed that bovine lactoferrin (LF) inhibited the proliferation of murine splenocytes while a peptic hydrolysate of LF promoted cell proliferation especially in a B-cell enriched fraction .The authors concluded that the peptic hydrolysate may in fact contain both immunostimulatory and immunoinhibitory peptides Furthermore, cow's milk hydrolysates induced differentiation of specific T cell subsets, especially the formation of interleukin-10 (IL-10) producing regulatory T cells, in cultured human (Lahart *et al* 2011), and in murine (Duan *et al* 2012) lymphocytes Therefore, several groups, including ours, propose to help develop a nutritional product enriched with immunomodulatory peptides to help prevent food allergies including cow's milk allergy in infants Milk bioactives generated from either sodium caseinate (NaCaS) or whey protein concentrate (WPC) may possess anti-inflammatory or anti-allergic properties The identification of such bioactives may be useful in promoting tolerance to cow's milk proteins and thus may be beneficial in infant formula to alleviate the symptoms of CMPA or perhaps to induce a tolerogenic response to CMP We hypothesize that these hydrolysates may exert significant anti-inflammatory/ anti-allergic effects, by modulating the production of pro-inflammatory Th1, Th2, or Th17 derived cytokines or through the modulation of dendritic cells or T cell functions Therefore the objectives of this chapter are·

- To screen a range of whey and casein hydrolysates for anti-inflammatory activity
- To identify hydrolysates that have immunosuppressive effects on T cell proliferation and cytokine production *in vitro*
- To determine the effects of hydrolysates on Th1, Th2, Th17 and T Reg differentiation
- To determine the effects of hydrolysates on DC maturation and cytokine production
- To identify a mechanism by which hydrolysates can influence key signal transduction events involved in inflammatory processes

3.2. HYDROLYSATES INHIBIT T CELL PROLIFERATION *IN VITRO*

In the past, studies have demonstrated differential findings with regard to the capacity for hydrolysates (whey or casein derived) to modulate the immune response via suppression of T cell proliferation For example, hydrolysates obtained from soy and whey (or more specifically the proteins β-lactoglobulin or lactoferrin) were shown to enhance proliferation in murine spleen lymphocytes (Wong *et al* 1998) Two synthetic peptides corresponding to the sequences f50-51(Tyr-Gly) and f18-20 (Tyr-Gly-gly) of the whey protein α- lactalbumin enhanced both the *in vitro* proliferation and protein synthesis of PHA-stimulated human peripheral blood lymphocytes (Kayser and Meisel, 1996) However, Cross and colleagues (1999) showed that a modified WPC rich in glycomacropeptide (GMP), a κ-casein-derived peptide, present in appreciable amounts in some whey protein concentrate (WPCs) suppressed T and B lymphocyte proliferative responses to mitogens in a dose dependent manner (Cross *et al* 1999) Therefore, in collaboration with FHI, this study sought to identify hydrolysates with immunosuppressive properties, starting with an investigation of the effects of hydrolysates on T cell proliferation These effects were assessed using a human relevant system in the form of human peripheral blood mononuclear cells (PBMC) Human biology-based *in vitro* screening systems are valuable as they better reflect the human condition with better predictive outcomes than nonhuman biology-based test methods

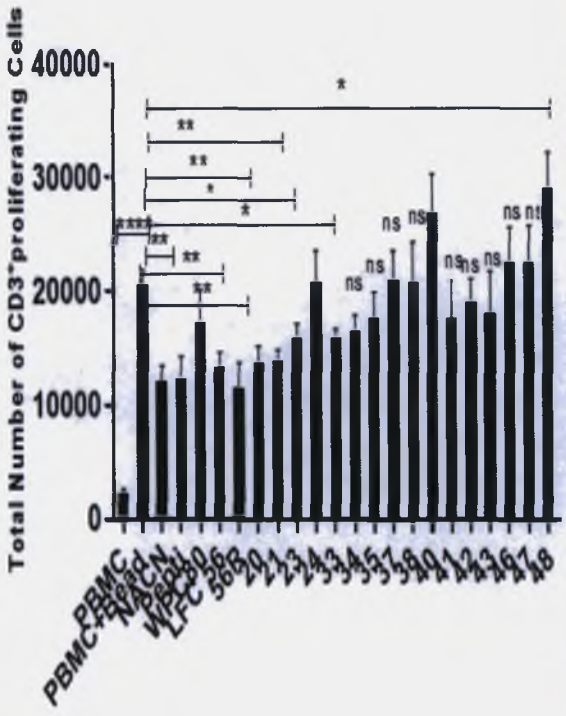
At the outset of this experiment it was important to include some control compounds that are currently used in infant formula Whey protein concentrate 80 (WPC80,) and sodium caseinate (NaCn) are food grade intact protein powders used as standard ingredients in infant formula Pepti (Aptamil, Nutricia, Dublin) is an extensively hydrolysed powder formula,

currently used for the treatment of cow's milk allergy in infants. Lead functional compound 56 (LFC56) and its upscaled sample LFC56R were included as they have been previously screened in an extensive bioassay screening platform in FHI 1 and were shown to have potential health benefits on the immune system

In this study, the influence of hydrolysates on T cell proliferation was assessed by measuring the proliferation of activated and CFSE labelled PBMC, as detailed in section 2 3 3 Briefly, PBMC were labelled with a fluorescent proliferation dye, activated with CD3/CD28 beads and cultured for four days in the presence or absence of hydrolysates (1mg/ml) Cells were harvested on day four and stained with fluorescent antibody CD3 and fluorescent dye-7-aminoactinomycin D (7-AAD) to examine viability in $CD3^+$ proliferating T cells PBMC proliferation (proliferation dye dilution) was analysed by flow cytometry

In comparison to the PBMC alone group, $CD3^+$ T cells present in the PBMC activated with anti- CD3/CD28 beads proliferated significantly In the presence of intact sodium caseinate (NaCn), Pepti (extensively hydrolysed formula), lead functional compound 56 (LFC56) and its regenerate (LFC56R) the proliferation of PBMC was significantly reduced ($P< 0\ 001$) In the presence of whey protein hydrolysates (20, 21) and sodium caseinate hydrolysates (23, 33) the proliferation of PBMC was significantly reduced ($P < 0\ 05$ $P < 0\ 001$) respectively (Figure 3 1) In contrast casein hydrolysate 48 significantly increased the proliferation of PBMC ($P < 0\ 05$) PBMC cultured in the presence of the remaining casein hydrolysates (24, 34, 35, 37, 38) and whey hydrolysates (40, 41, 42, 43, 46, 47) or the intact whey protein control sample (WPC80) did not significantly differ in terms of proliferation kinetics in comparison to the positive control sample (PBMC plus CD3/CD28 activation beads) This data suggests that specific hydrolysates have either immunosuppressive or

immunostimulatory capacity in the context of modulating T cell proliferation *in vitro* However, more detailed characterization is required to better understand the effects hydrolysates have on other aspects of the immune system



LFC	SOURCE	Significance between PBMC and Hydrolysate
NACN	Carbery Milk Products	**
WPC80	Kerry Ingredients	NS
Pepti	Nutricia	**
LFC56	UL	**
56R	UL	**
20	UL	**
21	UL	**
23	UL	*
24	UL	NS
33	UL	*
34	UL	NS
35	UL	NS
37	UL	NS
38	UL	NS
40	UL	NS
41	UL	NS
42	UL	NS
43	UL	NS
46	UL	NS
47	UL	NS
48	UL	*

Figure 3.1. Hydrolysates have differential effects on T cell proliferation. PBMC (5 x 10^4 per well) were labelled with CFSE in a 96 well round bottom plate and co-cultured with anti-CD3/CD28 beads in the presence or absence of hydrolysates (1mg/ml). On Day four, cells were harvested and stained with fluorescent antibody CD3 and fluorescent 7AAD viability dye to analyse $CD3^+$ proliferation using flow cytometry. Proliferation of gated CD3+CFSE diluted live cells was analysed. The assay was performed on three PBMC donors (n=3). Statistical analysis was carried out using one way ANOVA Tukey Multiple Comparison Test where * <0.05,** < 0.01 , **** < 0.0001.UL= University of Limerick

3.3. ANTI-INFLAMMATORY EFFECTS OF SODIUM CASINATE AND WHEY PROTEIN HYDROLYSATES IN PBMC ACTIVATED CELLS

One of the main features of food proteins that play a role in their capacity to induce an allergic response, is their ability to promote Th2 effector pathways, favoring Th2 rather than Th1 immunity. IFN-γ and IL-2 are key pro-inflammatory cytokines produced by Th1 cells to mediate inflammatory events during food allergy. Th2 cells secrete IL-4 which is considered to drive the allergic response. T regulatory cells secrete IL-10, which plays a pivotal role in the maintenance of immune tolerance and suppression of allergic inflammation. IL-6 is a cytokine with well-defined pro- and anti-inflammatory properties and is involved in the regulation of the Th17 immune response and inflammation. As outlined before, hydrolysates may help drive T-helper cells (Th1, Th2, T-Reg and Th17) to produce increasing or decreasing amounts of various cytokines. Therefore this study explored further the potential anti-allergic potential of milk peptide hydrolysates in terms of the secreted cytokine profile. Therefore, hydrolysates were assessed in a proliferation assay as described in section 3.2, in order to identify which hydrolysates were effective at modulating cytokines in supernatant, determined by ELISA.

In the supernatants, a number of whey (41, 46, 47) and casein (48) hydrolysates reduced the levels of IFN-γ (Figure 3.2) while other whey (21) and casein hydrolysates (23, 34, 38) significantly increased the levels of IFN-γ ($P<0.05$, $P<0.01$, $P<0.001$, $P<0.001$). A number of whey hydrolysates (21, 40, 41, 42, 43) and casein hydrolysates (23, 24, 35) significantly inhibited the Th2 response by decreasing the levels of IL-4 ($P< 0.0001$) and some hydrolysates (34, 37, 42, 46) significantly decreased the pro-inflammatory cytokine IL-6 ($P<0.05$, 0.01). Only two casein hydrolysates (35, 37) were found to significantly increase the level of IL-10 ($P < 0.0001$). Table 3.1 shows a detailed summary of all the hydrolysates tested.

Immunomodulating peptides were selected for further studies on the basis of their either their anti- proliferative activity, IFN-γ stimulating properties, IL-10 induction and absence of IL-4 inducing capacity So based on the results of this initial screen a number of whey hydrolysates (40,42) and casein hydrolysates (23,34,35) and their optimised regenerate (77,79,80,84,85) were selected for further analysis

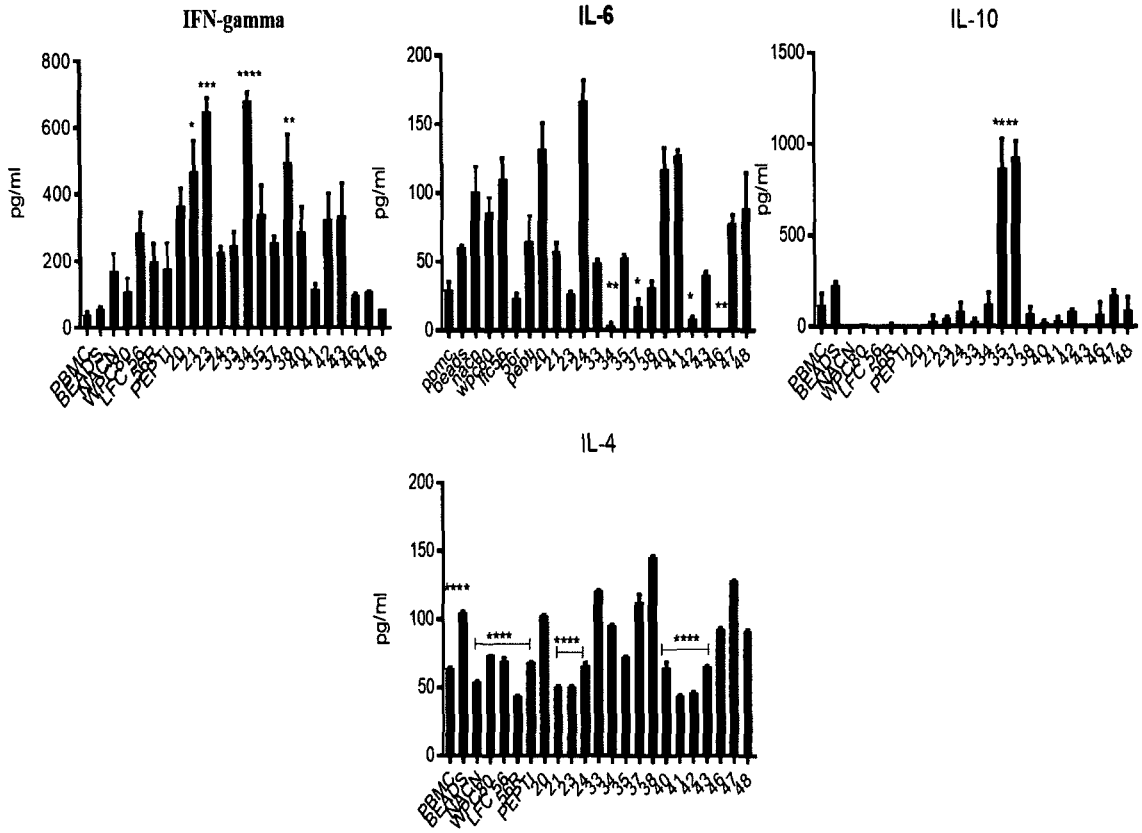


Figure 3.2. Hydrolysates alter the production of pro and anti-inflammatory cytokines *in vitro*. T cell proliferation assays were carried out as described before in figure 3 1 On day four, supernatant was collected for detection of IL-4, IL-6, IFN-γ and IL-10 Some hydrolysates significantly reduced the pro-inflammatory cytokines IL-6 and IFN-γ Some hydrolysates significantly reduced the T_H2 cytokine IL-4 while other proteins provided an anti-inflammatory response (35, 37) The assay was performed on three PBMC donors (n=3) Statistical analysis was carried out using one-way ANOVA Tukey Multiple Comparison Test where *<0 05,**< 0 01 and *** <0 001, **** <0 0001 Stars are in comparison to the PBMC+ beads group

Table 3.1. Proliferation and Cytokine Screen of Milk Protein Hydrolysates

LFC	PROLIF	IFNγ	IL-6	IL-10	IL-4
NACN	↓★★	ND	↑	↓	↓★★★★
PEPTI	↓★★	ND	↑	↓	↓★★★★
WPC80	↓	↓	↑	↓	↓★★★★
LFC56	↓	↑	↑	↓	↓★★★★
LFC56R	↓	ND	↓	↓	↓★★★★
20	↓★★	↑	↑	↓	↑
21	↓★★	↑	ND	↓	↓★★★★
23	↓★	↑★★★	ND	↓	↓★★★★
24	ND	ND	↑★	↓	↓★★★★
33	↓★	↑	ND	↓	↑
34	↓	↑★★★	↓★	↓	↓
35	↓	↑	ND	↑★★★★	↓
37	ND	↑	↓★	↑★★★★	↑
38	ND	↑	ND	↓	↑
40	↑	↑	↑	↓	↓★★★★
41	ND	↓★	↑	↓	↓★★★★
42	ND	↑	↓★	↓	↓★★★★
43	ND	↑	ND	↓	↓★★★★
46	ND	↓★★	↓★	↓	↓
47	ND	↓★★	↑	↑★★	↑
48	↑★	↓★★	↑	↑	↓

↑ Indicates increased proliferation/production above PBMC +beads

↓Indicates decrease proliferation/production compared PBMC +beads

★★ Indicates level of significance when compared to PBMC +beads

ND= no difference compared to PBMC+ beads

3.4 DEGREE OF HYDROLYSIS MAY IMPACT ALLERGENICITY OF HYDROLYSATES

In order to obtain desirable functional properties of milk protein hydrolysates the chemical breakdown of the compound, a process termed hydrolysis must be carried out under strictly controlled conditions to a generally low degree of hydrolysis (DH) (Alder-Nissen *et al* 1979) DH is defined as the percentage of peptide bonds cleaved (Alder-Nissen 1976) The degree of hydrolysis has been shown to influence the biological activities of the hydrolysates, as the size and amino acid composition of the hydrolysate is altered (Chen *et al* 2012) In all experiments whey protein concentrate 80 (WPC80) and sodium caseinate (NaCn) served as controls Pepti, (Aptamil, Danone Nutricia,) an extensively hydrolysed formula for the dietary management of Cows Milk Allergy was also included as a control Two lead functional compounds LFC56 and its optimized sample LFC56R are also included as controls as they have previously been screened to contain bioactive compounds Both whey and casein hydrolysates with a degree of hydrolysis (DH) ranging from 3 1% to 45% were tested (Table 3 1) In terms of allergenicity the smaller the DH% the less risk for an allergic reaction The highest degree of hydrolysis was found in sodium casein hydrolysate 34 followed by whey protein hydrolysate 20 and the lowest was found in whey protein hydrolysate 46 Only a few studies have addressed the issue of DH% One in vitro study indicated that the degree of hydrolysis had no effect on both the cytotoxic and immunomodulatory properties of NaCas in human Jurkat cells (Lahart *et al* 2011) However, a recent study found that whey samples induced TNFα and IL-10 in a DH dependent manner in human PBMCs (Kiewiet *et al* 2017) They found lower DH, was associated with enhanced immunoregulatory effects Hence, the

thorough evaluation of the bioactivity of individual hydrolysates generated under different physico-chemical conditions is essential, as the findings cannot be generalised. The samples highlighted in yellow below were the five hydrolysates chosen for further evaluation in T_H1, T_H2, T_H17 and $T\text{-}_{Reg}$ assays, to specifically examine immunomodulatory effects.

Table 3.2 Characteristics of milk protein hydrolysates tested

Sample Description	DH%	Starting Substrate	Enzyme Source
20	39.4	WPC	Aspergillus
21	12.9	WPC	Bacillus
23	15.9	NaCas	Bacillus
24	19.8	NaCas	Bacillus
33	29.3	NaCas	Bacillus
34	48.2	NaCas	Aspergillus
35	18.8	NaCas	Aspergillus
37	33.5	NaCas	Aspergillus
38	14.5	NaCas	Aspergillus
40	2.3	WPC	Aspergillus
41	2.0	WPC	Aspergillus
42	15.3	WPC	Bacillus
43	1.9	WPC	Aspergillus
46	0.0	WPC	Aspergillus
47	12.8	NaCas	Bacillus
48	0.6	NaCas	Aspergillus
WPC80	0.0	WPC	.....
NACN	0.0	NaCas	.....

Table 3.3 List of Whey protein and Sodium Caseinate hydrolysates and their regenerated samples

Sample Description	DH%	Regen	Starting Substrate	Enzyme Source
42	15.3	79	WPC	Bacillus
40	15.3	77	WPC	Bacillus
23	15.9	80	NaCas	Aspergillus
34	48.2	84	NaCas	Aspergillus
35	18.8	85	NaCas	Aspergillus

3.5. SPECIFIC HYDROLYSATES ARE PROTOLOEROGENIC/ANTI-INFLAMMATORY WITHOUT ANY EFFECTS ON T CELL PROLIFERATION

The results available thus far suggest that milk peptide hydrolysates may have immunomodulatory properties, with the potential to dampen pro-inflammatory responses (inflammation or allergy) and promote regulatory responses (anti-inflammatory) in a certain direction. In order to assess the potential of milk protein hydrolysates to alter/modulate an unbalanced immune status, such as that representative of CMPA five of the hydrolysates (42,40, 23, 34,35) and their regenerates (79, 77,80, 84, 85) (Table 3.3) were re-screened for their proliferative/suppressive potential as described previously (see section 3.2). In the presence of whey and casein hydrolysates the proliferation of CD3+T cells with a whole PBMC population

PBMC did not change significantly (Figure 3 3) Overall the five hydrolysates and their optimized regenerate sample did not negatively or positively effect T cell proliferation driven by CD3/CD28 beads

Although there was no influence on T cell proliferation, hydrolysates may have influenced the cytokine production by the PBMC population Levels of IFN-γ, IL-2, IL-6, IL-10, IL-17 and IL-4 were examined In this assay no IL-4 was detected In the $PBMC^{+}$ bead population there was a significant increase in the levels of IL-6, IL-10 and IL-17 (Figure 3 4) None of the hydrolysates or controls had any significant effect (either increase or decrease) on IFN-γ or IL-2 All hydrolysates and controls (except 42 and 84) significantly reduced the levels of pro-inflammatory cytokine IL-6 Only pepti and NaCN (controls) and hydrolysate 35 and its regenerate 85 significantly reduced the levels of IL-17 Importantly only hydrolysate 35 and its regenerate 85 significantly increased the levels of the anti-inflammatory cytokine IL-10

In summary, hydrolysate 35 and its regenerate 85 significantly reduced the production of the pro-inflammatory cytokine IL-6 and IL-17 and significantly increased the anti-inflammatory cytokine IL-10 The purpose of this study is to find a hydrolysate that can outperform the current treatment formula options (Pepti) with regards to immunomodulatory ability The data supported a potential enhanced immunomodulatory ability for hydrolysate 35 over and above pepti High levels of anti-inflammatory (IL-10) and low levels of Th17 cytokine have been identified and found to be important in gut homeostasis (Langrish *et al* 2005) In healthy individuals, low numbers of Th17 cells are present, mainly in the lamina propria (Huber *et al* 2012) Several studies have also demonstrated a role for hydrolysates in increasing the IL-10 production in T cells (Lahart *et al* 2011, Cian *et al* 2012) The overall suppression of IL-17 and upregulation of IL-10 with hydrolysate 35 and its regenerate 85 could not be explored

further as the enzyme preparation needed to make the hydrolysate could no longer be supplied by the manufacturer Unfortunately, this excluded hydrolysate 35 from the rest of the study Based on this screen summarized in Table 3 3 and on a similar screen completed with mouse cells within FHI by our collaborators at DCU, whey hydrolysate 42 and it's regenerate 79, and sodium caseinate hydrolysate 34 an it's regenerates 84 and 132 and were chosen for further testing in Th1, Th2, Th17 and Treg polarization assays

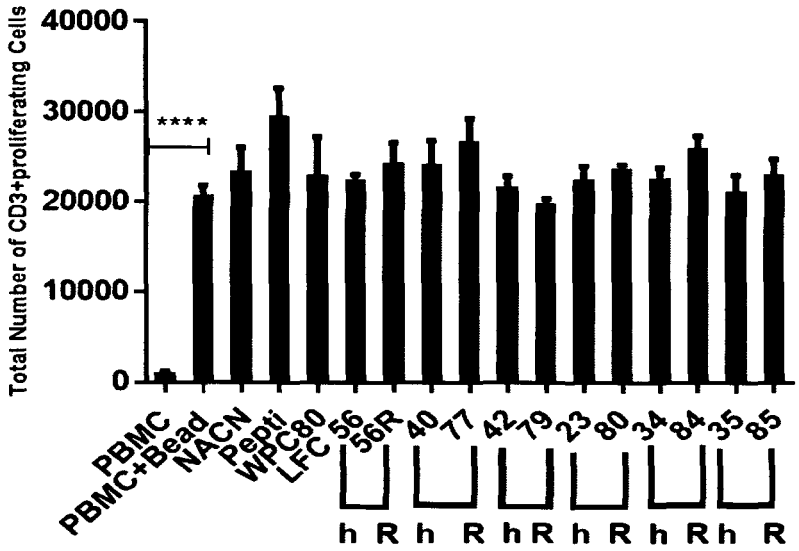


Figure 3.3 Hydrolysates and their regenerates do not impair T cell proliferation. PBMC (5 x 10^4 per well) were labelled with CFSE in a 96 well round bottom plate and co-cultured with anti-CD3/CD28 beads in the presence or absence of hydrolysates (1mg/ml) On Day four, cells were harvested and stained with fluorescent antibody CD3 and fluorescent 7AAD viability dye to analyse $CD3^+$ proliferation using flow cytometry The assay was performed on three PBMC donors (n=3) Statistical analysis was carried out using one-way ANOVA Tukey Multiple Comparison Test where **** p<0 0001 Stars are in comparison to the PBMC+ beads group

h=hydrolysate R=Regenerate

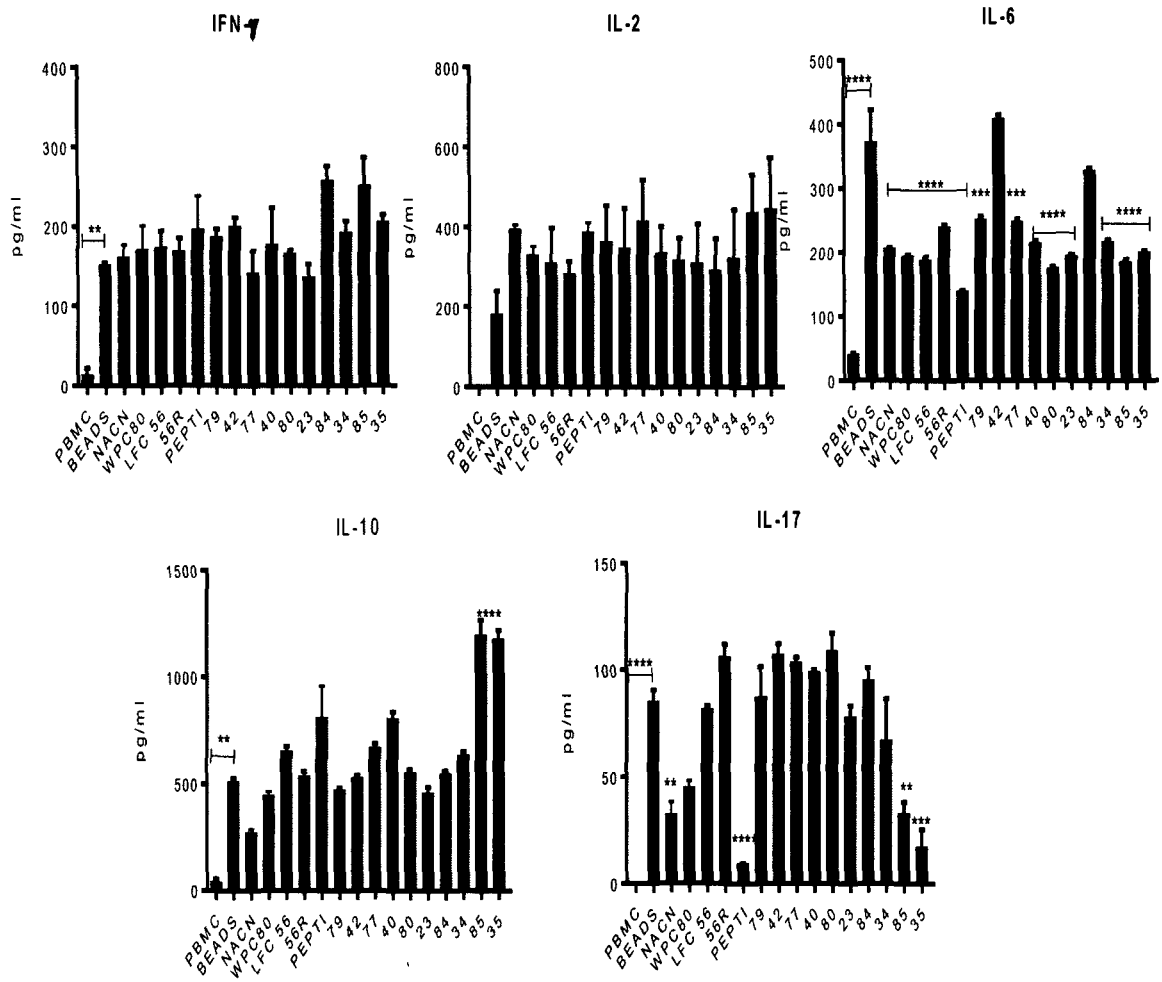


Figure 3.4 Hydrolysate 35 increased the anti-inflammatory cytokine IL-10 and suppressed the levels of IL-17. T cell proliferation assays were carried out exactly as described before in figure 3 1 On day four, supernatant was collected for detection of IL-2, IL-6, IFN-γ, IL-10 and IL-17 There was no change detected in the Th1 cytokines but a number of the hydrolysates and their regenerates decreased pro-inflammatory IL-6 and IL-17 levels and increased the anti-inflammatory IL-10 level The assay was performed on three PBMC donors (N=3) Statistical analysis was carried out using one-way ANOVA Tukey Multiple Comparison Test where *<0 05, **< 0 01 and ***<0 001 ****< 0 0001 Stars are in comparison to the PBMC+ beads group.

<0 001 Stars are in comparison to the PBMC+ beads group

Table 3.4 Proliferation and Cytokine Screen 2

LFC	Prolif	IFN-γ	IL-6	IL-10	IL-17	IL-2
NACN	UC	UC	↓ ★★★★	↓	↓ ★★	↑
PEPTI	↑	↑	↓ ★★★★	↑	↓ ★★★★	↑
WPC80	↑	↑	↓ ★★★★	↓	↓	↑
56	UC	↑	↓ ★★★★	↑	UC	↑
56R	↑	↑	↓ ★★★★	↑	↑	↑
42	UC	↑	↑	UC	↑	↑
79	↓	↑	↓ ★★★★	↓	UC	↑
40	↑	↑	↓ ★★★★	↑	↑	↑
77	↑	↓	↓ ★★★★	↑	↑	↑
23	UC	↓	↓ ★★★★	↓	↓	↑
80	UC	UC	↓ ★★★★	↓	↑	↑
34	UC	↑	↓ ★★★★	↑	↓	↑
84	↑	↑	↓	↑	↑	↑
35	UC	↑	↓ ★★★★	↑ ★★★★	↓★★★	↑
85	↑	↑	↓ ★★★	↑ ★★★★	↓★★	↑

↑ Indicates increased proliferation/production above PBMC +beads

↓Indicates decrease proliferation/production compared PBMC +beads

★★ Indicates level of significance when compared to PBMC +beads

UC/ND= unchanged/ no difference compared to PBMC+ beads

3.6. HYDROLYSATE DO NOT NEGATIVELY IMPACT CELL VIABILITY

As a quality control procedure, the possible effect of hydrolysates on T cell viability in a T cell proliferation assay was assessed This is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product The cells are then solubilised with an organic solvent (eg DMSO) and the released, solubilised formazan reagent is measured spectrophotometrically Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells Cytotoxicity of the hydrolysates was determined on CFSE labelled PBMC using 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazoliumbromide (MTT) Notably, the PBMC only group that did not receive activation (survival) signals had significantly reduced viability The level of MTT activity in the culture medium was similar in the presence and absence of the hydrolysate, indicating that it was not toxic to the cells (Figure 3 7)

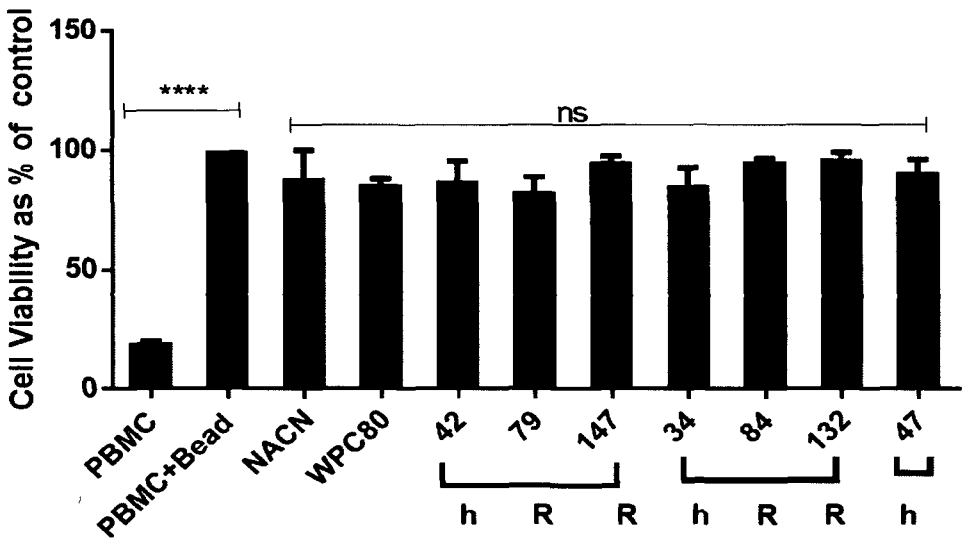


Figure 3.5. Hydrolysates have no negative impact on cell viability. PBMC (5 x 10^4 per well) were labelled with CFSE in a 96 well round bottom plate and co-cultured with anti-CD3/CD28 beads in the presence or absence of hydrolyates (h) or the regenerates (R) (1mg/ml) On Day four, CFSE stained PBMC were harvested into a 96 well round bottom plate , washed with PBS and incubated with 20µl of MTT solution (0 5mg/ml) for 3 h at 37 ^{O}C The media was removed and the formazan crystals were dissolved in 200µl of DMSO, incubated at 37 ^{O} C for 5 minutes and centrifuged for 5 minutes at 300 x g The absorbance (optical density (OD)) of the samples was measured at 570nm using a microplate reader The assay was performed on three PBMC donors (n=3) Cell viability was determined as a % of control cells (PBMC + beads) Statistical analysis was carried out using one way ANOVA Tukey Multiple Comparison Test where ****<0 0001

3.7 HYDROLYSATES IMPAIR TH1 AND TH2 DIFFERENTIATION

Although the above results show no effect of hydrolysates on number of proliferating $CD3^+$ T cells response, hydrolysates may prevent or inhibit an allergic response by skewing Th2 responses towards a Th1 phenotype (Tang *et al* 2001). Th1 immune activation is defined by a distinct pattern of cytokine expression characterized by the secretion of IFN-γ and expression of the transcription factor T-bet. The differentiation and expansion of Th2 cells is characterized by the secretion of IL-4, IL-5 and IL-13 and expression of the master transcription factor Gata-3. Despite the obvious potential benefit for peptide hydrolysates to promote a Th1/Th2 skew, few studies have examined this effect. Therefore this study assessed the potential of milk protein hydrolysates to drive immune responses in a certain direction. Sodium caseinate hydrolysate, 47 displayed an anti-inflammatory profile in murine T cell subsets (Th1, Th2, Th17 and Treg) within the infant nutrition group in FHI (data not shown. Hense hydrolysate 47 and the regenerate of 34 (132) were examined in T helper polarisation assays.

For the Th1 and Th2 polarisation assays, isolated human naïve $CD4^+$ T cells (purity> 95%) were transferred to 96 round bottom plates and stimulated with plate bound anti-human CD3/anti-CD28 antibody and incubated for 3 days in the presence or absence of polarising cytokines Th1(IL-2 (10 ng/ml), IL-12 (10ng/ml) and neutralising antibody anti-IL-4 (5μg/ml)) and for Th2 . ((IL-2) (10 ng/ml) IL-4 (12.5 ng/ml)) and neutralising antibody (anti-IFN-γ (5μg/ml)). Hydrolysates (1mg/ml) were added on Day 3 and cells were re-stimulated with IL-2 (10ng/ml). On day 6 cells were harvested and intracellular cytokine staining for T-bet (Th1) and Gata-3 (Th2) and cytokine production of IFN-γ, IL-2 (Th1) and IL-4 (Th2) was examined. Figure 3.8 outlines the gating strategy for the identification of $CD4^+$ T-bet^+ Th1 cells. Figure 3.9 shows that hydrolysates 132 and 47 significantly decreased T-bet transcription factor

expression (MFI & number) in polarized Th1 cells after addition of hydrolysates on Day 3 (P <0 01) however regenerate 79 significantly increased the number of $CD4^+$ cells producing T-bet (P< 0 05) Unexpectedly, there was no change detected in IFN-γ or IL-2 in the Th1 culture supernatants after 6 days of culture

All the hydrolysates including the whey protein control (wpc80) significantly decreased Gata-3 transcription factor expression in polarized Th2 cells after the addition of hydrolysates on Day 3 (Figure 3 8)(P< 0 001) However addition of hydrolysates had no effect on the levels of IL-4 in the supernatants after 6 days of culture (Figure 3 7)

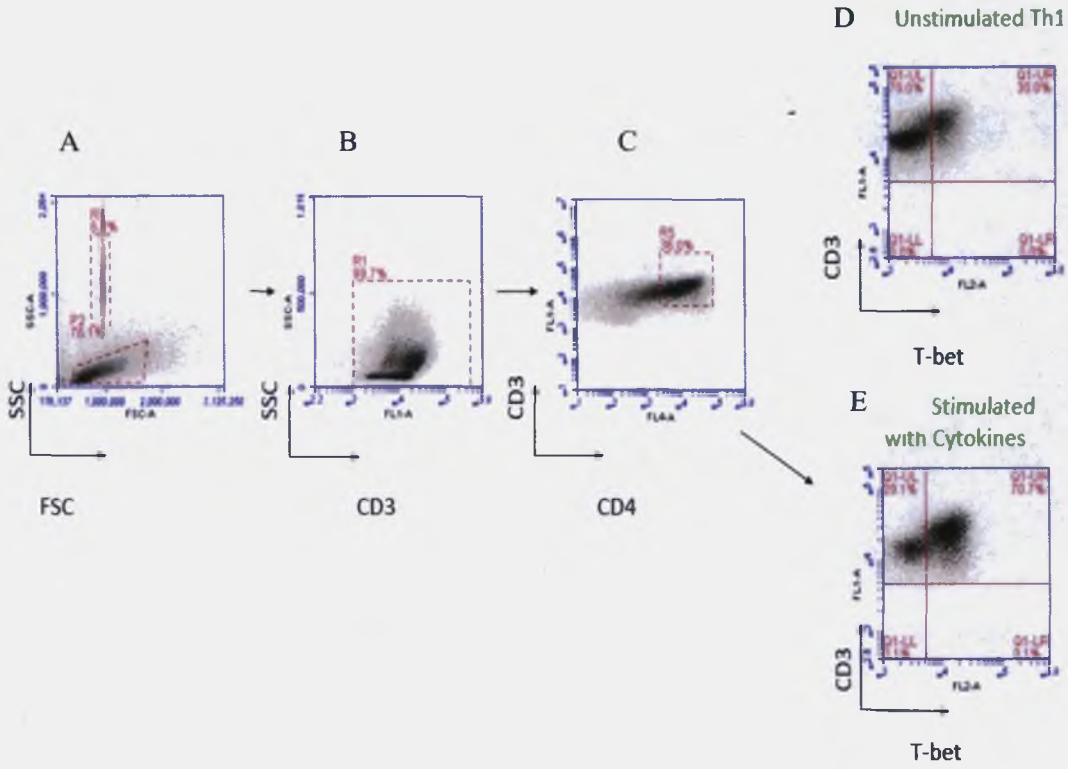


Figure 3.6 Representative example of gating strategy used to identify human CD3$^+$T-bet$^+$ Th1 cells. (A) Cells were gated first on FSC against SSC to select the live lymphocyte population (B) Illustrates the gating position for human CD3$^+$ (FITC) expression (C) represents the gating position for human CD3$^+$ (FITC) CD4$^+$ (APC) T cells within the lymphocyte population, (D) and (E) illustrates the gating position for CD3$^+$ (FITC) and T-bet (PE) expression in unstimulated (CD3/CD28) and stimulated (CD3/CD28 +IL-2 (10 ng/ml), IL-12 (10ng/ml) and anti-IL-4 (5µg/ml) cells within the CD3$^+$ (FITC) CD4$^+$ (APC) T cell population. All other gating positions were determined using matched isotype controls.

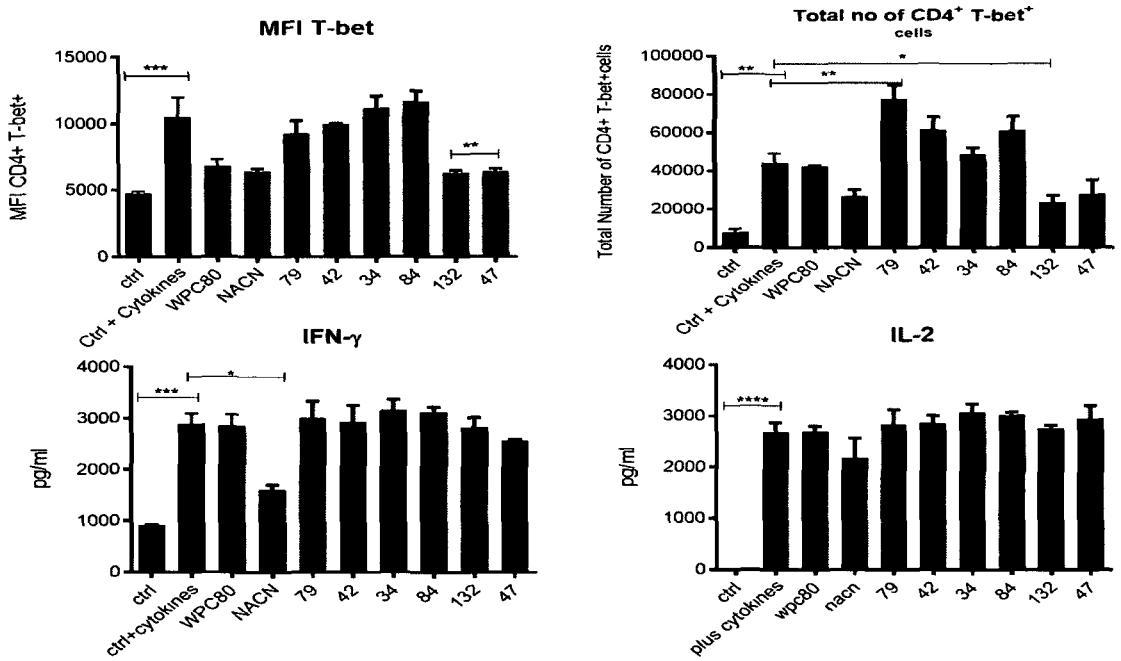


Figure 3.7. Hydrolysates 132 and 47 significantly decrease T-bet transcription factor expression in polarised Th1 cells. Isolated human naive $CD4^+$ T cells (purity> 95%) at 1 0 x 10^6 cells/ml were transferred to 96 round bottom plates coated with 100µl of plate bound anti-human CD3 (1µg/ml) antibody 20µl of anti-CD28 (2µg/ml) was then added to cells $CD4^+$ T cells were incubated for 3 days in the presence or absence of polarising cytokines IL-2 (10 ng/ml), IL-12 (10ng/ml) and neutralising antibody anti-IL-4 (5µg/ml)) and hydrolysates (1mg/ml) were added on Day 3 Cells were re-stimulated with IL-2 (10ng/ml) on Day 3 On Day 6, cells were harvested and stained with CD4 (APC) CD3 (FITC) and fixed, permeabilised and stained intracellularly with fluorescent antibody for T-bet (PE) to analyse (A) MFI of T-bet and (B) No of $CD4^+$T-bet^+ cells (PE) using flow cytometry Supernatant was collected and analysed by ELISA for the detection of (C) IFN-γ and (D) IL-2 The assay was performed on three different PBMC donors (n=3) Statistical analysis was carried out with one way ANOVA Tukey Multiple Comparison Test where**** significant to control (1µg/ml plate bound CD3/CD28) plus cytokines** p<0 01 and ***p<0 001

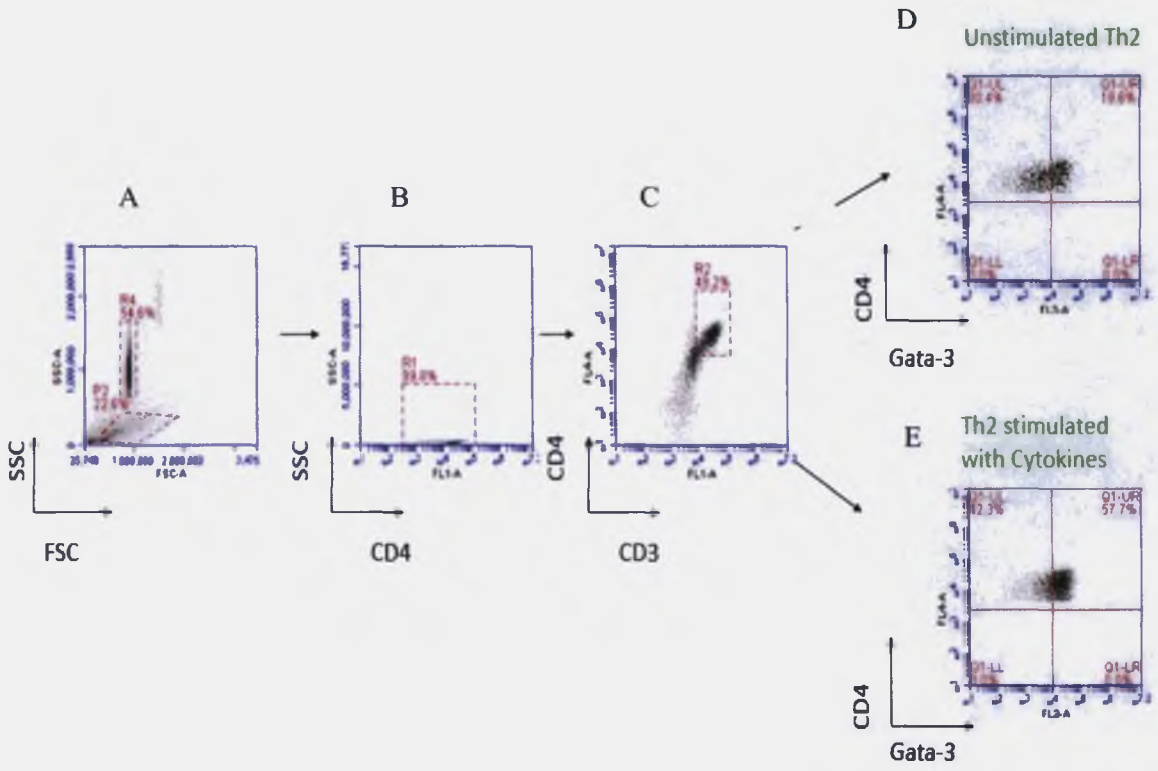


Figure 3.8. Representative example of gating strategy used to identify human CD4$^+$Gata-3$^+$ Th2 cells. (A) Cells were gated first on FSC against SSC to select the live lymphocyte population (B) Illustrates the gating position for human CD4$^+$ (APC) expression (C) represents the gating position for human CD3$^+$ (FITC) CD4$^+$ (APC) T cells within the lymphocyte population, (D) and (E) illustrates the gating position for CD4$^+$ (APC) and Gata-3 (PerCP) expression in unstimulated and stimulated (CD3/CD28 + IL-2 (10 ng/ml) IL-4 (12.5 ng/ml) and IFN-γ (5μg/ml) cells within the CD3$^+$ (FITC) CD4$^+$ (APC) T cell population. All other gating positions were determined using matched isotype controls.

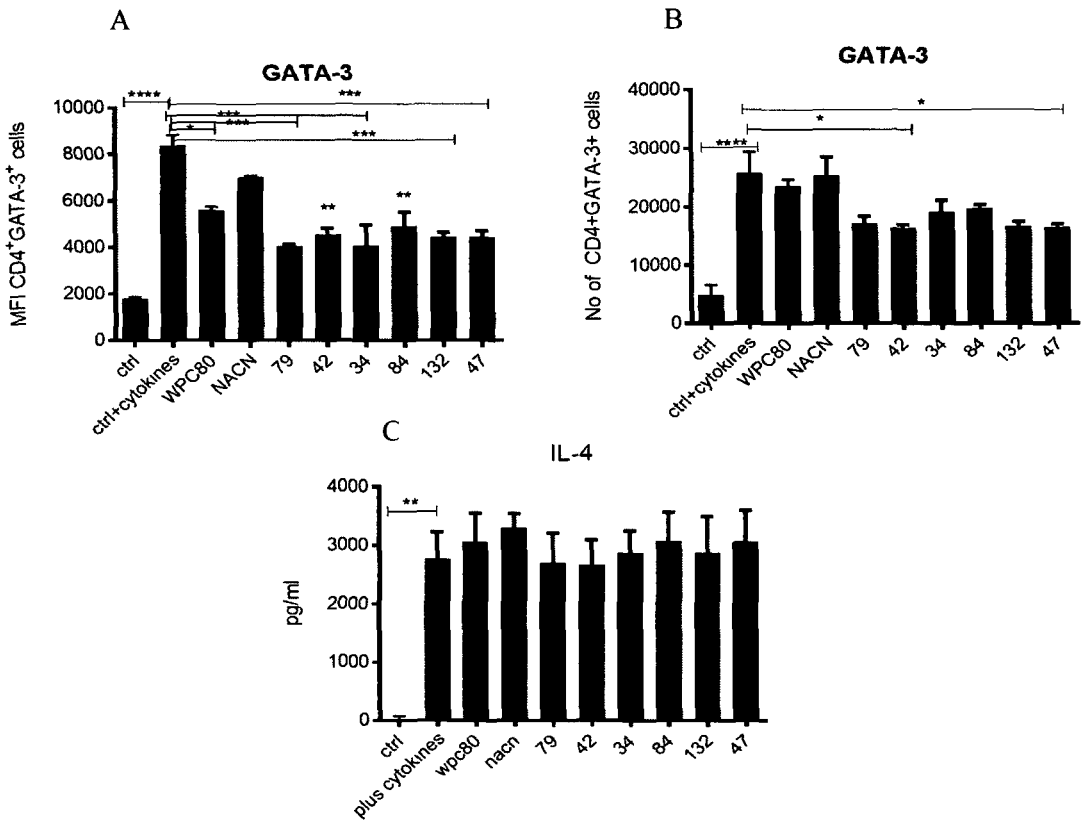


Figure 3.9. All hydrolysates decreased Gata-3 expression in polarised Th2 cells after addition of hydrolysates on Day 3 Isolated human naive $CD4^+$ T cells (purity> 95%) at 1 0 x 10^6 cells/ml were transferred to 96 round bottom plates coated with 100µl of plate bound anti-human CD3 (1µg/ml) antibody 20µl of anti-CD28 (2µg/ml) was added to cells $CD4^+$ T cells were incubated for 3 days in the presence or absence of polarising cytokines, IL-2 (10 ng/ml) IL-4 (12 5 ng/ml) and neutralising antibody (anti-IFN-γ (5µg/ml) Hydrolysates (1mg/ml) were added on Day 3 Cells were re-stimulated with IL-2 (10ng/ml) on Day 3 On Day 6, cells were fixed, permeabilised and stained intracellularly with fluorescent antibody for Gata-3 (PerCp) to analyse Th2 polarisation using flow cytometry (A) MFI and (B) no of $CD4^+$ Gata-3^+ cells Supernatant was collected and analysed by ELISA for the detection of IL-4 The assays were performed on three different PBMC donors (n=3) Statistical analysis was carried out with one way ANOVA where **** *p<0 0001 ***p<0 001 **p < 0 01

3.8. HYDROLYSATES IMPAIR TH17 DIFFERENTIATION BUT HAVE NO IMPACT ON T-REGULATORY EXPRESSION

Although food allergy was classically thought to be a Th2-type response, recent studies have shown that the Th1/Th2 paradigm is an oversimplified view of the real situation Besides Th1 and Th2 cells, other T-cell subtypes, such as T-Reg and Th17 have been identified and found to be important in maintaining balance in the immune system (Dang *et al* 2016, Stelmaszczyk-Emmel *et al* 2013, Dhubanehal *et al* 2013) Because IL-10 producing T-reg cells can inhibit Th2 cells, another way to dampen the Th2 response is to promote the differentiation of Treg cells by administration of peptides (Palomares *et al* 2010) Various studies have shown an effect of hydrolysates on Treg formation by showing an IL-10 upregulation after treatment of lymphocytes with a hydrolysate obtained from the seaweed *Porphyra columbina* (Cian *et al* 2012) or casein hydrolysate (Lahart *et al* 2011) Other studies suggest that Th17 cells are important in maintaining homeostasis in the intestine and thus protect the state of tolerance (Huber *et al* 2012) Differentiation of Th17 cells is controlled by a "master regulator" of transcription the orphan nuclear receptor Ror gamma t (Rorγt) whereas differentiation of T-Reg is controlled by the transcription factor FoxP3 Despite the apparent opposite effects of T-Reg and Th17 immunity, there are very few studies that have addressed the issue of T helper cell differentiation skewed by peptides Therefore this study sought to assess the potential of milk protein hydrolysates to promote Treg differentiation or modulate Th17 differentiation Human $CD4^+$ naive T cells were polarized under T-reg (IL-2 (5μg/ml) and TGF-β (2ng/ml)) or Th17 (IL-1β(10μg/ml), IL-6 (20ng/ml), IL-23 (10ng/ml), TGF-β (2ng/ml) anti-IL-4 (10μg/ml),anti-IFN-γ (10μg/ml), conditions for 3 days before addition of hydrolysates on day 3 On day 6 cells

were harvested and intracellular cytokine staining for Foxp3 and Rorγt was performed The mean fluorescence intensity (MFI) of Ror-γt (Th17) and FoxP3 (T-reg) and cytokine production of IL-10 (T-reg) and IL-17 recovered from the supernatants were examined after 6 days in culture in presence or absence of hydrolysates Figure 3 10 outlines the gating strategy for the identification of $CD4^+$ $Ror\gamma t^+$ Th17 cells All hydrolysates and the control (wpc80) significantly decreased Rorγt expression ($P< 0.0001$) in comparison to the positive control (1μg/ml plate bound CD3/CD28 plus cytokines) in polarized Th17 cells after addition of hydrolysates on Day 3 In terms of IL-17 cytokine production, all hydrolysates significantly hampered the production of Th17 in the supernatants after 6 days of culture (Figure 3 9)

In the case of T-regulatory Foxp3 expression, as can be seen in figure 3 12 only the whey control sample (wpc80) significantly ($P<0.05$) reduced the percentage of $CD4^+$ $CD25^+$ $Foxp3^+$ cells in comparison to the positive control (1μg/ml plate bound CD3/CD28 plus cytokines) This effect was mirrored in the amount of IL-10 secreted in the supernatants on day 6 Both hydrolysate 47 and wpc80 control significantly down-regulated IL-10 cytokine production after addition of the hydrolysates on day three of culture ($P<0.001$) in comparison to the positive control (1μg/ml plate bound CD3/CD28 plus cytokines) Whereas hydrolysate 42 and its regenerate 79, 34 and its regenerates 84 and 132 did not enhance or reduce the levels of IL-10 secreted in the supernatants on day 6

In summary, all hydrolysates significantly decreased the percentage of the master regulator transcription factor Rorγt with a corresponding downregulation of IL-17 detected in the supernatants However, the presence of hydrolysate 47 and intact protein wpc80 control in the Treg polarisation assays hampered production of IL-10 The remaining hydrolysates do not negatively impact Treg Foxp3 expression or IL-10 cytokine production This data suggest that

hydrolysates may use different mechanisms to regulate these T cell subsets and depending on peptides present, hydrolysates can exert positive or negative effects

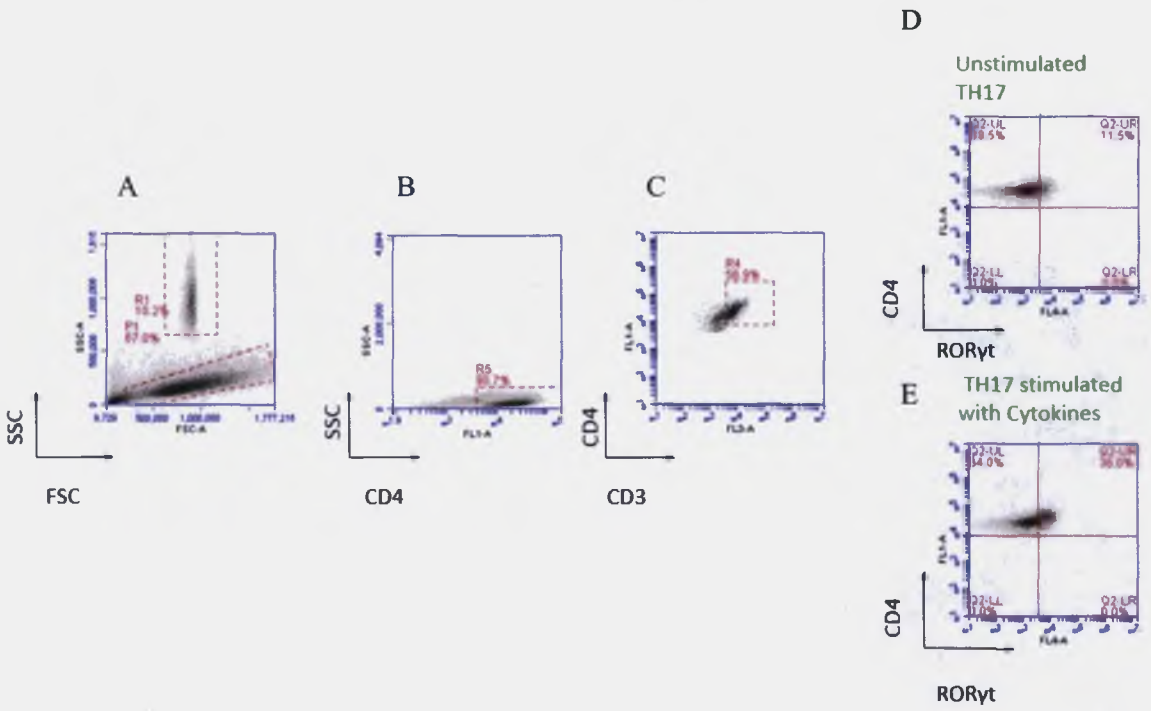


Figure 3.10. Representative example of gating strategy used to identify human CD3$^+$Rorγt$^+$ Th17 cells (A) Cells were gated first on FSC against SSC to select the live lymphocyte population (B) Illustrates the gating position for human CD4$^+$ (FITC) expression (C) represents the gating position for human CD3$^+$ (PerCp) CD4$^+$ (FITC) T cells within the lymphocyte population, (D) and (E) illustrates the gating position for CD4$^+$ (FITC) and Rorγt$^+$ (APC) expression in unstimulated and stimulated (IL-1β(10μg/ml), IL-6 (20ng/ml), IL-23 (10ng/ml), TGF-β (2ng/ml) anti-IL-4 (10μg/ml),anti-IFN-γ (10μg/ml) cells within the CD3$^+$ (PerCp) CD4$^+$ (FITC) population. All other gating positions were determined using matched isotype controls.

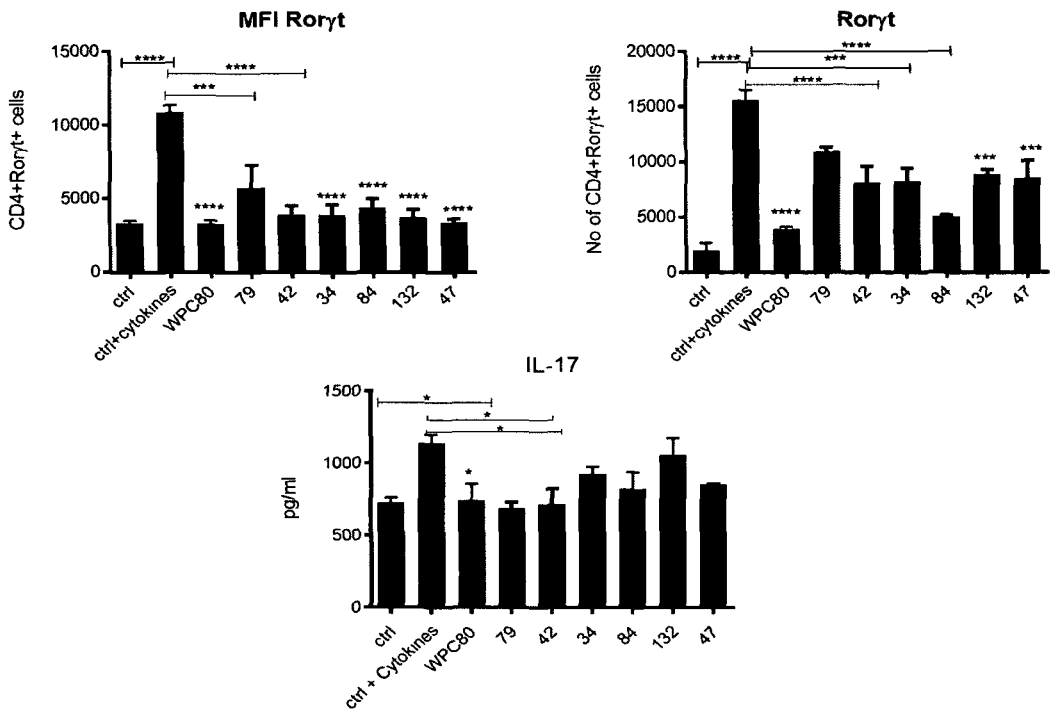


Figure 3.11. Hydrolysates reduce the MFI and number of CD4$^+$Rorγt$^+$ cells and suppress the levels of IL-17 production in polarised Th 17 cells. Human naive CD4$^+$ T cells were activated with anti-CD3/CD28 exactly as described in figure 3 10 CD4$^+$ T cells were incubated for 3 days in the presence or absence of polarising cytokines, IL-1β (10 μg/ml), IL-6 (20 ng/ml), IL-23 (10ng/ml), TGF-β (2ng/ml) and neutralising antibodies, anti-IFN-γ (10μg/ml), anti-IL-4(10μg/ml) Hydrolysates (1mg/ml) were added on Day 3 and re-fed with cytokines On Day 6, cells were harvested and stained with CD4 (APC) CD3 (FITC) and these cells were fixed, permeabilised, and stained intracellularly with fluorescent antibody for Rorγt (PerCp) and analysed by flow cytometry Supernatant was collected and analysed by ELISA for the detection of IL-17 The assay was performed on three different PBMC donors (n=3) Statistical analysis was carried out with one way ANOVA Tukey Multiple Comparison Test where **** <0 0001 ***<0 001 and * <0 05

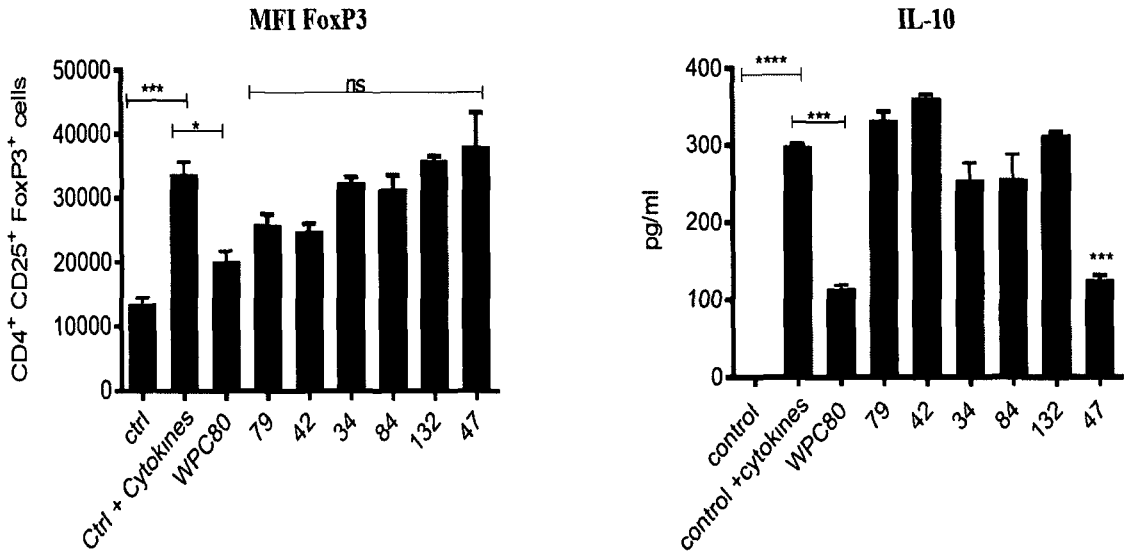


Figure 3.12. Intact whey protein (wpc80) significantly decreased the Foxp3 expression and IL-10 production in a Treg polarisation assay. Purified human naive $CD4^+$ T cells (purity> 95%) at 1 0 x 10^6 cells/ml were transferred to 96 round bottom plates coated with 100µl of plate bound anti-human CD3 (1µg/ml) antibody 20µl of anti-CD28 (2µg/ml) was added to cells $CD4^+$ T cells were incubated for 3 days in the presence or absence of polarising cytokines (IL-2 (5µg/ml) TGF-β (2ng/ml) and hydrolysates (1mg/ml) were added on Day 3 On Day 6, cells were harvested and stained with CD4 (FITC) CD25 (APC) and these cells were fixed, permeabilised, and stained intracellularly with fluorescent antibody for Foxp3 (Pe) to analyse T-Reg cytokine production using flow cytometry and supernatant was collected and analysed by ELISA for the detection of IL-10 The assay was performed on three different PBMC donors (n=3) Statistical analysis was carried out with one way ANOVA Tukey Multiple Comparison Test where **** <0 0001 ***p<0 001 and* <0 05

3.9. HYDROLYSATES IMPAIR DC MATURATION AND CYTOKINE PRODUCTION

Dendritic cells (DC) initiate primary and drive secondary T cell- mediated immune reactions against a wide range of antigens (Banchereau *et al* 1998, Lanzavecchia *et al* 2000) This property is due to their ability to capture antigens at the immature stage and to present processed antigens to naive T lymphocytes at the mature stage in secondary lymphoid organs (Cella *et al* 1997) The transition from immature to mature DC is characterised by a number of changes, including the increased expression of MHC class II and costimulatory molecules (CD80, CD83, CD86), increased secretion of IL-12 which are necessary to deliver accessory signals required for the activation and the differentiation of naive T cells into type 1 (IL-2, IFN-γ) or type 2 (IL-4,IL-5, IL-13) cytokine-producing cells (Th1 and Th2) (Narala *et al* 2007) The level of IL-12 secretion by DC induced by some microbial constituents such as lipopolysaccharide (LPS) is a key factor in the outcome of immune responses Hydrolysates have been shown to inhibit inflammatory responses and even stimulate tolerogenic responses in other antigen presenting cells (macrophages) (Oseguera-Toledo *et al* 2011) Very few studies have examined the effect of hydrolysates on human monocyte- derived dendritic cells Thus the ability of hydrolysates to interfere with DC maturation, was examined Human monocyte derived DC were generated using human CD14 microbeads (Miltenyi) and MACS columns $CD14^+$ cells were supplemented with 50ng/ml GM-SCF and 70ng/ml IL-4 and grown for three days On day 3 they were re-fed with cytokines and on day 6 harvested and stimulated with LPS in the presence or absence of hydrolysates for 24 h After 24 h, the expression of costimulatory molecule CD86 was analysed on DC by flow cytometry (Fig 3 13) Supernatants

from these cultures were collected at 24 h and cytokine secretion was analysed by ELISA (Fig 3 13) Maturation of DC with LPS for 24 h resulted in a significant increase increase in maturation marker expression, CD86 (96%) and (173606 MFI) by flow cytometry (Fig 3 13 B) However addition of hydrolysates and control samples (WPC80, NaCN) significantly reduced the expression of CD86 The most significant decrease in CD86 was observed in DC cultures in presence of 132 and 47 (Fig 3 13) P <0 0001 Following addition of hydrolysates and control samples, DC secreted significantly less pro-inflammatory IL-12p40 (p<0 01) These data suggested that hydrolysates disrupt the maturation of DC significantly reducing the upregulation of CD86 expression, in conjunction with a significant decrease in IL12-p40 production These data are consistent with a hypothesis suggesting that hydrolysates maintain DC in a semi-mature or tolerogenic state

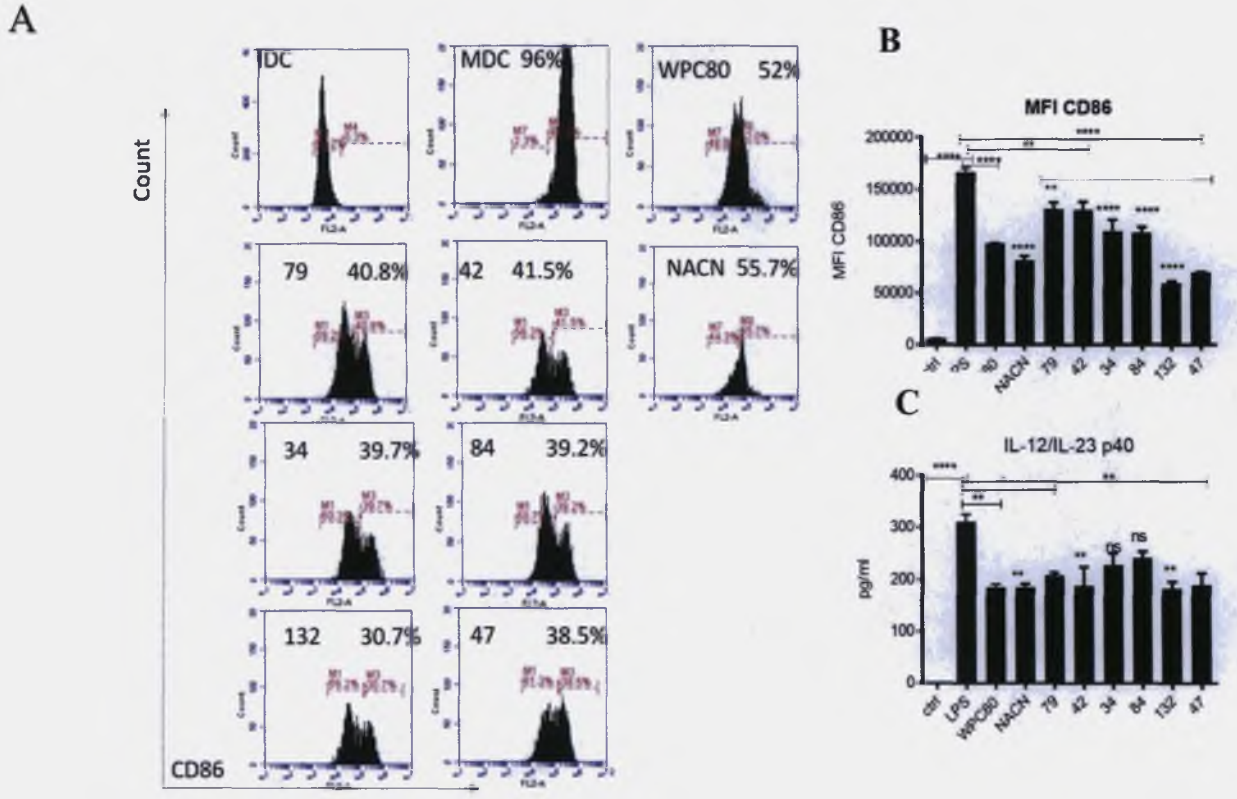


Figure 3.13. Hydrolysates reduce LPS driven dendritic cell maturation and cytokine production in human monocyte derived dendritic cells. On day 6, immature DC (iDC) were stimulated with LPS (200 ng/ml) (mDC) and cultured in the presence or absence of hydrolysates for 24 h. On day 7, DC were harvested and stained with CD1A (FITC) and CD86 (PE) for the expression of maturation marker by flow cytometry. (A) Percentage positive CD86 presented as representative histograms, (B) protein expression levels were expressed as mean fluorescent intensity (MFI) and (C) supernatants were collected after 24 h for cytokine analysis by ELISA of four independent experiments (n=4) Statistical analysis was carried out using the one way ANOVA Tukey where **** <0.0001 **p<0.01.

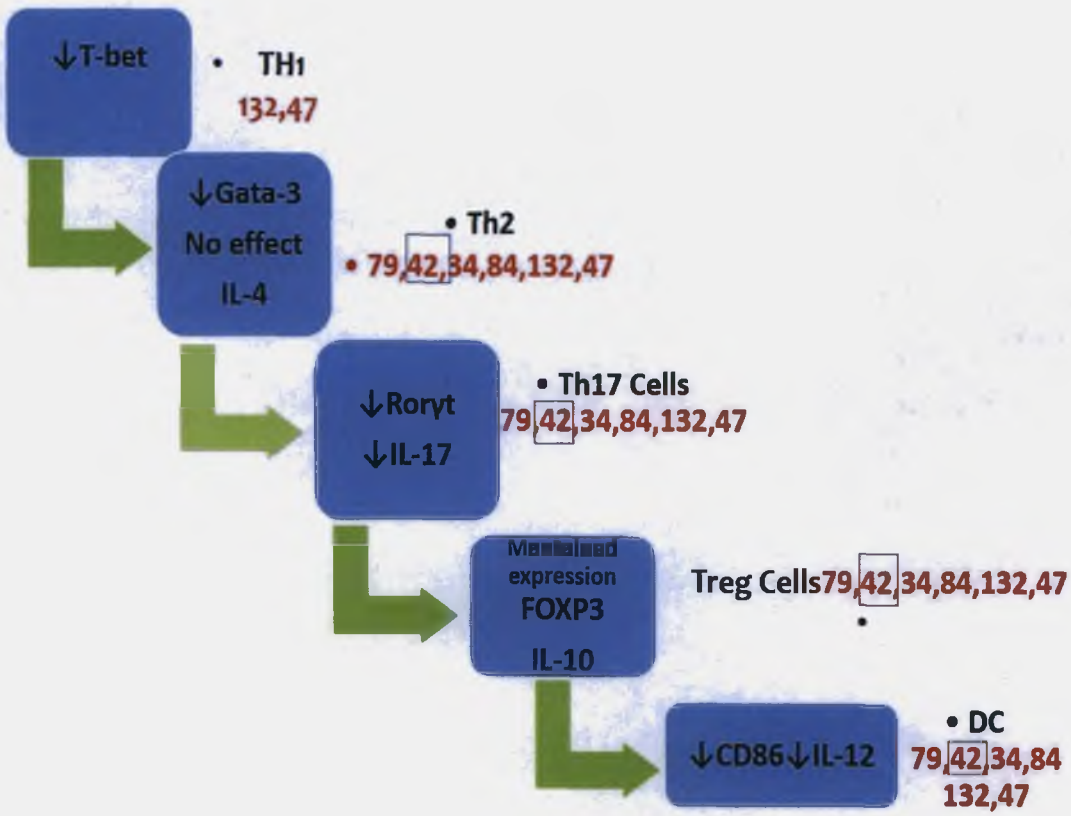


Figure 3.14.Summary of findings on the effect of hydrolysates on Th1, Th2, Th17, Treg and DC cultures.

Table 3.5 List of whey protein samples and their optimised samples

Sample Description	DH%	Comments	Conditions	Starting Substrate	Enzyme Source	Activity/Reason brought forward
42	15.3	Original sample	pH 7	WPC	Bacillus	Anti-inflammatory ↓DC ↓Th2 ↓TH17
79	15.3	Regen sample	pH 7	WPC	Bacillus	Anti-inflammatory ↓DC ↓Th2 ↓TH17
147-3hr 147 6 hrs	15.3	Upscaled samples	Starting ph of 7.0 3hrs&6hrs	WPC	Bacillus	

3.10 OPTIMISED HYDROLYSATE 147 HAS ALLERGY SUPPRESSING PROPERTIES

Thus far we have identified hydrolysates which promote an anti- inflammatory /anti-allergy T cell and DC phenotype summarised in Fig 3.14. There was no clear choice of hydrolysate for further evaluation in an *in vivo* setting as many of the hydrolysates exerted anti-inflammatory effects but based on other bioassays within FHI, hydrolysate 42 was chosen. For the purpose of hydrolysates going forward for *in vivo* testing , 2 upscaled samples of whey hydrolysate 42 (147 3 hr and 147 6 hr) (Table 3.5) were screened to confirm their

ımmunomodulatory role *ın vıtro* before they were tested *ın vıvo* 147 3 hrs and 147 6 hrs refers to the amount of tıme the hydrolysate was treated wıth the dıgestıve enzyme preparatıon durıng hydrolysıs as longer hydrolysıs tımes can change the ımmunoreactıvıty of protein allergens (Nongonıerma & Fıtzgerald 2014, Wróblewska B *et al* 2004)

The prolıferatıve abılıty of ıncreasıng doses (0 5 mg/ml 1 0 mg/ml, 2 0 mg/ml) of hydrolysates (147 3 hr and 147 6hr) was assessed by measurıng the prolıferatıon of actıvated and labelled perıpheral blood mononuclear cells (PBMC) ın the presence or absence of hydrolysates, as detaıled ın sectıon 2 3 3 Addıng ıncreasıng doses of hydrolysates had no effect on the prolıferatıon of $CD3^+$ T cells (wıth a whole PBMC populatıon stımulated wıth antıCD3/CD28 (Fıgure 3 15)

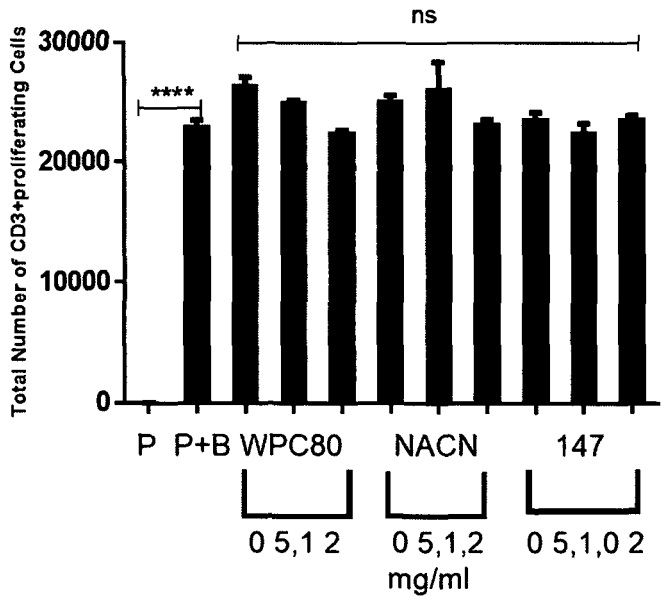


Figure 3.15. Hydrolysate 147 had no impact on T cell proliferation. PBMC (5 x 10^4 per well) were labelled with CFSE in a 96 well round bottom plate and co-cultured with anti-CD3/CD28 beads in the presence or absence of hydrolysates (0 5, 1mg or 2mg/ml) On Day four, cells were harvested and stained with fluorescent antibody CD3 and fluorescent 7AAD viability dye to analyse $CD3^+$ proliferation using flow cytometry The assay was performed on three PBMC donors (n=3) Statistical analysis was carried out using one-way ANOVA Tukey Multiple Comparison Test where **** <0 0001 Stars are in comparison to the PBMC+ beads group

3.11. OPTMISED HYDROLYSATE 147 ENHANCES TH1 DIFFERENTIATION

The results so far suggest that specific hydrolysates impair or enhance Th1 differentiation However when the two new fractions of optimized hydrolysates 147 (3 hr) 147 (6 hr) were tested in a polarized Th1 assay a more enhancement effect was observed (Figure 3 16 A) Hydrolysates 147 3 hr and 6 hr and whey control protein (WPC80) significantly increased the T-bet transcription factor expression ($P < 0.001$, $P < 0.01$) Previously there was no change found with the levels of IFN-γ in the supernatants after 6 days of culture under Th1 polarising conditions with ELISA (Figure 3 7) A more sensitive read-out utilizing intracellular flow cytometry was carried out to detect the percentage of $CD4^+$ cells producing IFN-γ On day 6, Th1 cells were harvested and stimulated with PMA and ionomycin for 4 hrs, and stained intracellularly for IFN-γ Hydrolysate 147 (6hrs) significantly increased the mean fluorescent intensity of $CD4^+$T-bet$^+$cells and the percentage of $CD4^+$ cells producing IFN-γ in Th1 polarised cells ($P < 0.05$) (Figure 3 16 B)

As whey hydrolysate 42 was the original hydrolysate this data suggests that hydrolysate 146 (6 hr) which was hydrolysed for a longer time resulted in a more potent Th1 inducer than hydrolysate 42

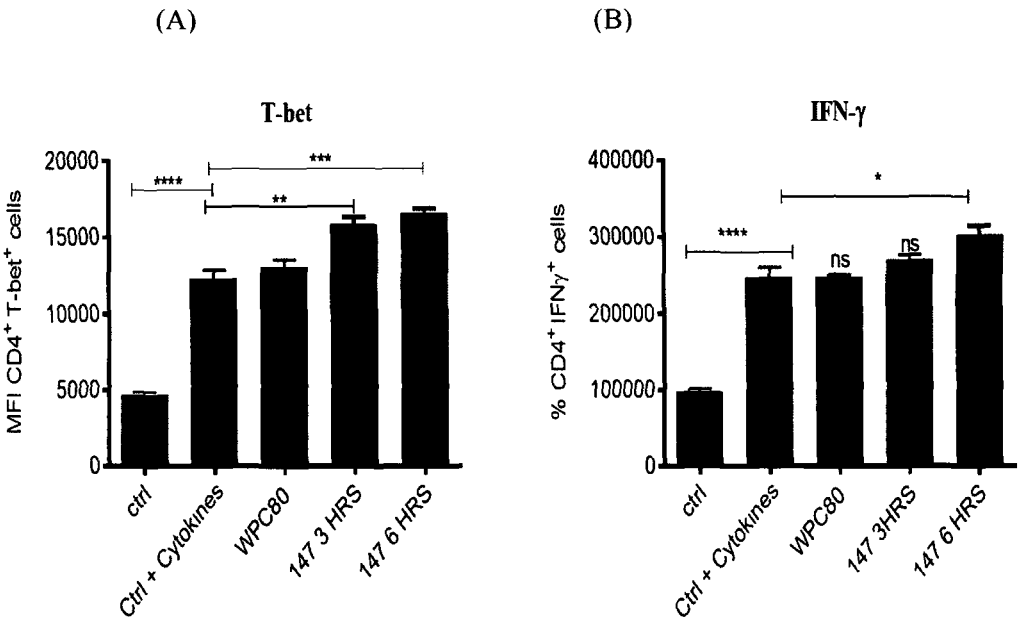


Figure 3.16. Hydrolysate 147 (6 hrs) significantly increases T-bet transcription factor expression and percentage CD4$^+$ IFN-γ$^+$ cells in polarised Th1 cells after addition of hydrolysates on Day 3. Isolated human naive CD4$^+$ T cells (purity> 95%) were set up exactly as described in figure 3 9 Cells were recovered on Day 6 and stained with CD4 (APC) CD3 (FITC) and fixed, permeabilised and stained intracellularly with fluorescent antibody for T-bet (Pe) or stimulated with 100ng/ml PMA, 1 μg/ml ionomycin and 1X Brefeldin A for 4 h and stained intracellularly with fluorescent antibody for IFN-γ (pe) (A) Graphical representation of protein levels of transcription factor t-bet (MFI) and percentage of human CD4$^+$ IFN-γ$^+$ cells is represented in (B) respectively The assay was performed on three different PBMC donors (n=3) Statistical analysis was carried out with one way ANOVA Tukey Multiple Comparison Test where**** significant to control (1μg/ml plate bound CD3/CD28) plus cytokines***p < 0 001** p <0 01* p <0 05

3.12. BOTH FRACTIONS OF HYDROLYSATE 147 IMPAIRS TH2 DIFFERNTIATION.

Thus far the results suggest that specific hydrolysates impair Th2 differentiation The two new fractions of optimized hydrolysates (147 3 hr, 147 6 hr) were tested in a Th2 polarised assay and the same effect was seen (Figure 3 17 A) Hydrolysates 147 3 hr and 6 hr significantly decreased the Gata-3 transcription factor expression ($P < 0.05$, $P < 0.01$) Previously there was no change found with the levels of IL-4 in the supernatants after 6 days of culture under Th2 polarising conditions with ELISA (Figure 3 9) A more sensitive assay of intracellular flow cytometry was carried out to detect the percentage of $CD4^+$ cells producing IL-4 On day 6 Th2 cells were harvested and stimulated with PMA and ionomycin for 4 hrs, and stained intracellularly for IL-4 The capacity of the new fractions (147 3 hr, 147 6 hr) to impair Th2 differentiation were examined using a Th2 polarisation assay There was a significant decrease in the percentage of $CD4^+$ cells producing IL-4 when the Th2 polarised cells were cultured in the presence of hydrolysate 147 3 hr, 147 6 hr and WPC80 ($P < 0.001$) (Figure 3 17 B)

(A) (B)

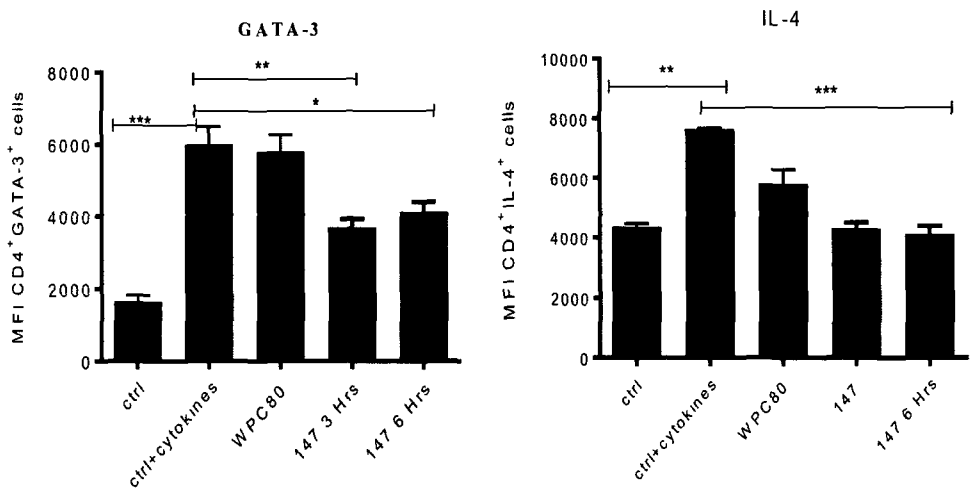


Figure 3.17. Hydrolysate 147 significantly decrease Gata-3 expression and IL-4 cytokine production in polarised Th2 cells. Isolated human naive $CD4^+$ T cells (purity> 95%) were set up as described in figure 3 11 On Day 6, cells were fixed, permeabilised and stained intracellularly with fluorescent antibody for Gata-3 (PerCp) or IL-4 (PE) to analyse Th2 cytokine production using flow cytometry or cells were stimulated with 100ng/ml PMA, 1μg/ml ionomycin and 1x Brefeldin A for 4 h and analysed by intercellular flow cytometry (A) Graphical representation of protein levels of transcription factor Gata-3 (MFI) and percentage of human $CD4^+$ $IL\text{-}4^+$ cells is represented in (B) respectively The assay was performed on three different PBMC donors (n=3) Statistical analysis was carried out with one way ANOVA Tukey Multiple Comparison Test where *<0 005 **p<0 01***p < 0 001

3.13. HYDROLYSATES 147 AND WHEY PROTEIN CONTROL INHIBIT TH17 DIFFERENTIATION WITHOUT ANY CHANGE IN T-REGULATORY EXPRESSION

Human $CD4^+$ naïve T cells were polarized under T-reg and Th17 conditions for 3 days before addition of hydrolysates on day 3. The mean fluorescence intensity (MFI) of Rorγt (Th17) and Foxp3 (T-reg) and cytokine production of IL-17 recovered from the supernatants after 6 days in culture in presence or absence of hydrolysates was examined. All hydrolysates and control (wpc80) sample significantly decreased Rorγt transcription factor expression ($P< 0.0001$) in comparison to the positive control (1μg/ml plate bound CD3/CD28 plus cytokines) in polarized Th17 cells after addition of hydrolysates on Day 3 (Figure 3.18A). In terms of IL-17 cytokine production, all hydrolysates and whey control sample slightly reduced the production of Th17 in the supernatants after 6 days of culture ($P<0.05$) (Figure 3.18B).

In the case of T-regulatory Foxp3 expression, there was a slight increase in the mean fluorescent intensity of Foxp3 with hydrolysate 147 6 hr but this increase was not significant. (Figure 3.19). Overall hydrolysate 147 and wpc80 did not positively or negatively impact on Treg expression or on expression of Foxp3.

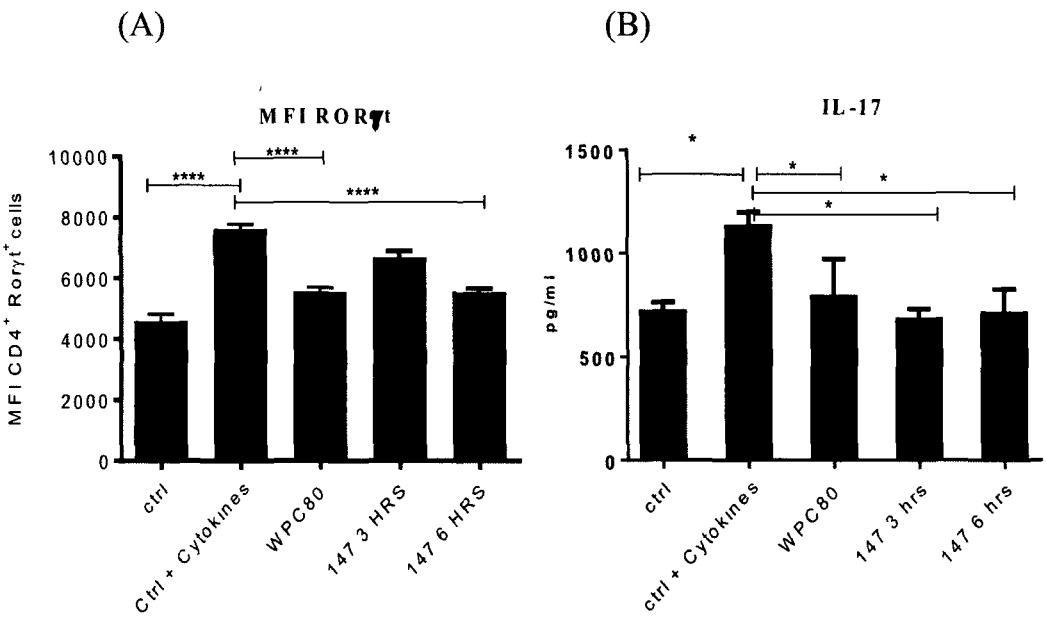


Figure 3.18. Hydrolysate 147 and whey protein control reduce the MFI of CD4$^+$Rorγt$^+$ cells and suppress the levels of IL-17 in polarised Th 17 cells. Isolated human naive CD4$^+$ T cells (purity> 95%) at 1 0 x 10^6 cells/ml were isolated as described before in figure 3 13 On Day 6, cells were harvested and surfaced stained with CD3 (APC) CD4 (FITC) and then cells were fixed, permeabilised and stained intracellularly with fluorescent antibody for Rorγt (PerCp) and analysed by flow cytometry (A) Graphical representation of protein levels of transcription factor Rorγt (MFI) and (B) Supernatant was collected and analysed by ELISA for the detection of IL-17 The assay was performed on three different PBMC donors (n=3) Statistical analysis was carried out with one way ANOVA Tukey Multiple Comparison Test where *<0 05 **<0 01 ****<0 0001

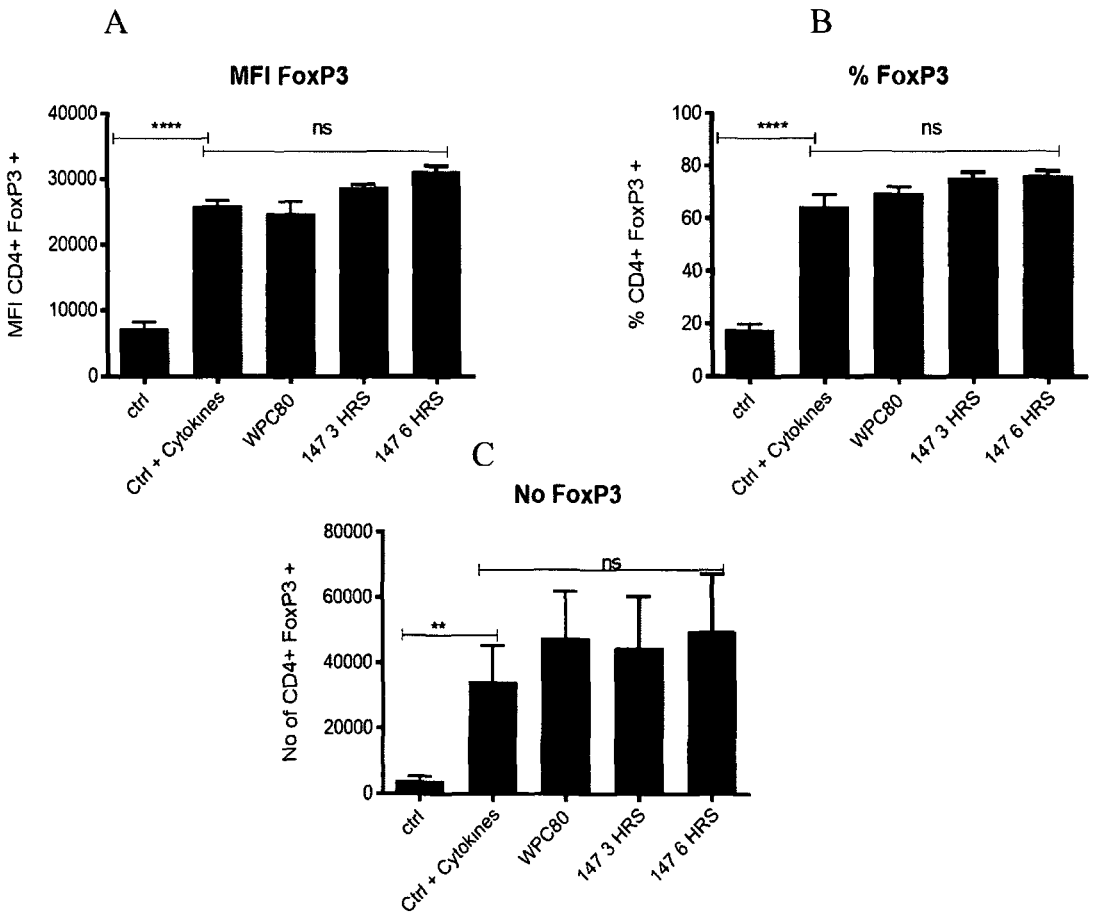


Figure 3.19. No change detected in Foxp3 with hydrolysate 147. Purified naive CD4+ T cells were prepared as described in Fig 3 14 On Day 6, cells were harvested and stained with CD4 (FITC) CD25 (APC) and these cells were fixed, permeabilised, and stained intracellularly with fluorescent antibody for Foxp3 (Pe) to analyse (A) MFI (B) percentage and (C) total no of $CD4^{+}Foxp3^{+}$ cells using flow cytometry The assay was performed on three different PBMC donors (n=3) Statistical analysis was carried out with one way ANOVA Tukey Multiple Comparison Test where **** <0 0001

3.14. NEW OPTIMISED HYDROLYSATES INHIBIT DC MATURATION AND CYTOKINE PRODUCTION

Th2 cells and its associated cytokines (IL-4, IL-5, IL-13) produce many of the pathological features of allergic disease however they depend on the instruction from antigen-presenting DCs to differentiate and act. DCs provide the first step of the immune recognition of allergens and thus represents the decision maker of the immunological outcome. Hense in the absence of activation signals, allergen challenged DC induce the proper T cell unresponsiveness and tolerance. In contrast in the presence of these signals the outcome may result in the induction of T cell activation, division and Th2 differentiation. The maintenance of balance between tolerance and immunity is a complex process and hydrolysates have been shown to maintain the balance towards tolerance (Oseguera-Toledo *et al* 2011). Previously, hydrolysate 42 and it's regenerate 79 significantly reduced the LPS driven dendritic cell maturation and cytokine production in human monocyte derived DC. To assess whether the new fraction 147 6hr has an impact on DC maturation, human monocyte derived DC were stimulated with LPS in the presence or absence of hydrolysate 147 6hr for 24 h. After 24 h, the expression of co-stimulatory molecule CD86 was analysed on DC by flow cytometry and the cytokine secretion in the supernatants was analysed by ELISA. Following addition of 147 hydrolysate, there was a significant decrease in the expression of the co-stimulatory molecule CD86 (Figure 3.20A) and DC secreted significantly less pro-inflammatory IL-12 in presence of hydrolysate 147 and wpc80 (Figure 3.20B). This data provides further evidence that hydrolysates 42 and its regenerate have significant immunomodulatory potential (Figure 3.21).

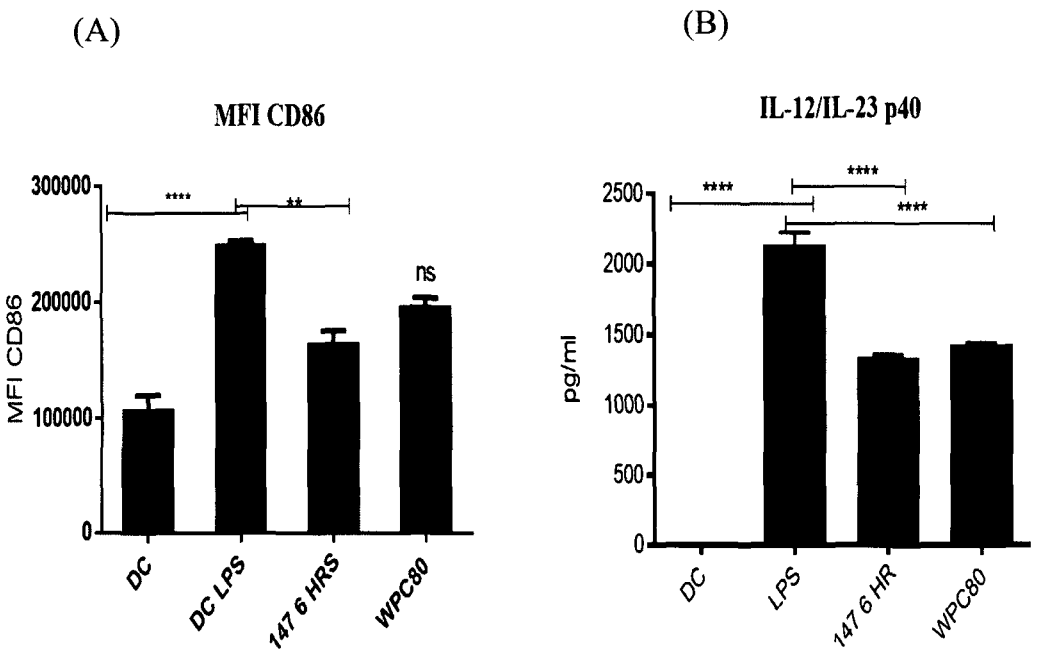


Figure 3.20. Optimised 147 hydrolysate inhibits the LPS driven dendritic cell maturation and reduces the cytokine production in human monocyte derived DC. On day 6, immature DC (iDC) were stimulated with LPS (200 ng/ml) (mDC) and cultured in the presence or absence of hydrolysates for 24 h On day 7, DC were harvested and stained with CD1A (FITC) and CD86 (PE) for the expression of maturation marker by flow cytometry (A) protein expression levels were expressed as mean fluorescent intensity (MFI) and (B) supernatants were collected after 24 h for cytokine analysis by ELISA of four independent experiments (n=2) Statistical analysis was carried out using the one way ANOVA Tukey where **** <0 0001 **p<0 01

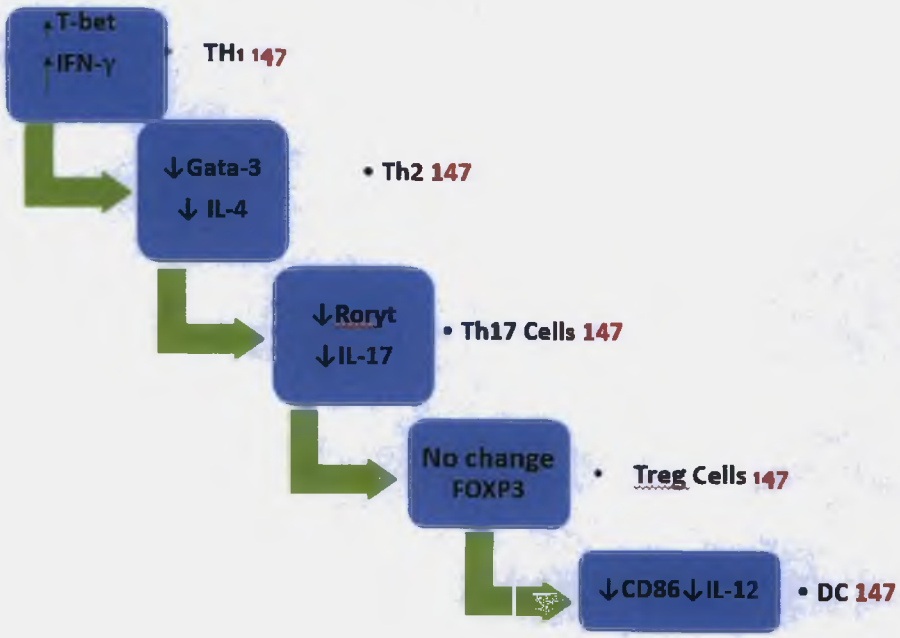


Figure 3.21. Summary of findings on the effect of hydrolysate 147 on Th1, Th2, Th17, Treg and DC cultures.

3.15. HYDROLYSATE 147 INCREASED THE EXPRESSION OF PEROXISOME PROLIFERATOR—ACTIVATED RECEPTOR GAMMA IN HUMAN MONOCYTE- DERIVED DENDRITIC CELLS

Although many immunoregulatory effects of hydrolysates have been described, the underlying mechanisms to explain the aforementioned effects are still unknown Peroxisome proliferator-activated receptors (P-PAR) are ligand-activated transcription factors belonging to the nuclear receptor superfamily They exercise homeostatic functions in the intestine at the interface between nutrient metabolism and immunity, and their main functions are related with the regulation of genes involved in glucose and lipid metabolism and in cellular differentiation (Tenenbaum *et al* 2003, Desverhgne *et al* 2004, Feige *et al* 2006) P-PARγ is activated by both natural ligands, such as polyunsaturated fatty acids (PUFAs) or by synthetic ligands, such as thiazolidinediones—a class of antidiabetic drug and both have been investigated for their anti-inflammatory effects (Tyagi *et al* 2011) Although P-PARγ is expressed in various tissues and cell types, including pancreas, liver, kidney, immune cells (eg, lymphocytes, monocytes, and macrophages), it is also expressed in dendritic cells (Gosset *et al* 2001, Letellier *et al* 2008) Synthetic P-PARγ agonists including troglitazone and rosiglitazone were shown to suppress production of inflammatory cytokines by these cells (Letellier *et al* 2008) However some agonists in clinical use have series side effects such as weight gain, increased bone fracture and heart failure (Rizos *et al* 2009) Conversely, natural products like milk derived peptides which so far display anti-inflammatory effects may also activate P-PAR

P-PARγ is of particular interest to the food industry beacuse P-PAR-γ activators and their precursors e g linolenic and linoleic acid are abundant in several foods

To assess whether P-PARγ has a role in the immunomodulatory effect of hydrolysates, on DC, human monocyte derived dendritic cells were stimulated with LPS (in the presence and absence of hydrolysate 147) and the expression of P-PARγ mRNA was examined P-PARγ mRNA expression was significantly higher in the presence of hydrolysate 147 ($P<0.01$) and less so with wpc80 ($P < 0.05$) in comparison to negative control (immature dendritic cell) (Figure 3 21)

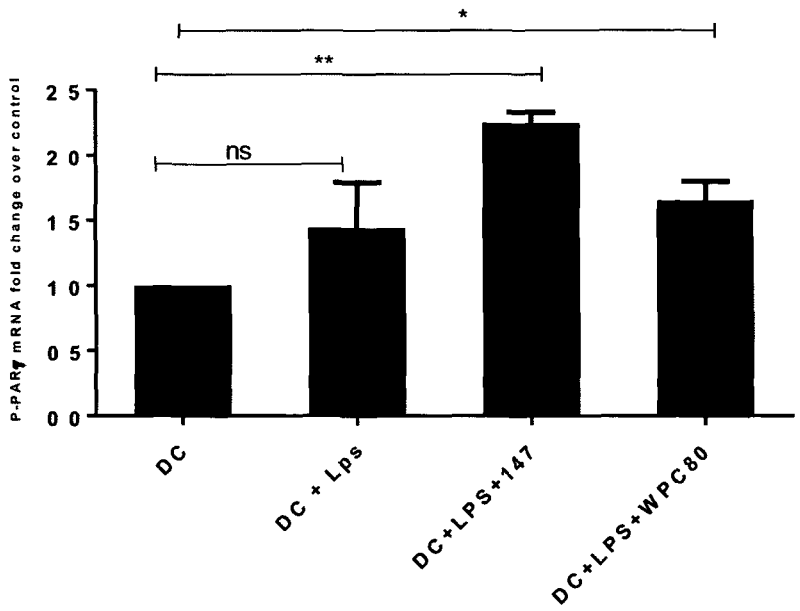


Figure 3.21. Expression of P-PARγ *in vitro* in human monocyte derived dendritic cells Monocytes were cultured in the presence of GM-CSF (50ng/ml) and IL-4 (70ng/ml) to generate DC On Day 6, immature DC (iDC) were seeded at 2 x 10^5 per well in a 24 well plate and left alone or stimulated with LPS (200 ng/ml) and cultured in the presence or absence of 147 and WPC80 (1mg/ml) for 24 h After 24 hours the cells were collected for quantitative PCR (qPCR) analysis RNA from each sample was extracted, cDNA was synthesised and used as a template for qPCR P-PARγ mRNA expression was relative to the housekeeping gene GAPDH Hydrolysate 147 and whey control significantly increased the mRNA expression of P-PARγ after 24 hours These experiments were repeated using 3 PBMC donors to generate human monocyte derived dendritic cells (n=3) and the results shown are representative of this Statistical analysis was carried out using unpaired student t-test where *<0 05 and**< 0 01

3.16. WHEY PROTEIN-DERIVED PEPTIDES MODULATE THE EXPRESSION OF PRO-INFLAMMATORY PHENOTYPE OF MDDC

The demonstration that P-PARγ activators like milk protein hydrolysates may regulate the synthesis of cytokines and /or surface molecules by various immunocompetent cells (Fitzgerald *et al* 2015, Clark *et al* 2000) raised the possibility that P-PARγ could play a role in hydrolysate modulation of DC activation and cytokine production

P-PARγ is a nuclear receptor that supresses inflammatory gene expression when activated in the inflammatory response in DC (Gosset *et al* 2001) P-PARγ also acts as a transcription factor to suppress the signal transduction and consequent activation of pro-inflammatory transcription factors such as NFκB (Letellier *et al* 2008) Therefore, it is postulated that as P-PARγ is expressed on DC and hydrolysates activate P-PARγ, addition of 147 hydrolysate to activated DC may result in inhibition of NFκB signaling Specifically in order to test whether the effects of whey protein hydrolysate in suppressing the inflammatory phenotype of activated DC was blocked, a synthetic P-PARγ inhibitor BADGE was used DC were treated for 24 h with the hydrolysate or with a synthetic P-PARγ agonist troglitazone (25 μM) in the presence or absence of BADGE (10μM) and then activated with LPS (200ng/ml) P-PAR-γ agonist (p-parγ+) significantly reduced the MFI of the maturation marker CD86 (P<0 001) and simultaneously inhibited the production of IL-12 (P< 0 0001)(Figure 3 23) Similarly, the whey hydrolysate 147 significantly reduced the MFI of the maturation marker CD86 (P<0 01) and simultaneously inhibited the production of IL-12 (P< 0 0001) In the presence of antagonist BADGE (P-PARγ-), the effects of both troglitazone and the whey hydrolysate 147 were prevented This data suggest that the modulation of LPS monocyte

derived dendritic cells response by whey derived bioactive peptides is mediated by the activation of P-PARγ (Figure 3 23)

In summary, the activation of P-PARγ in MDDC decreased secretion of IL-12, a pivotal cytokine in Th1 polarisation The P-PARγ agonist , troglitazone decreased CD86 expression The net effect of these changes appeared to be an inhibition of DC immunogenicity marked by diminished T-cell activation capacity Fitzgerald *et al* (2014) found a similar effect with a casein hydrolysate in TNF-α activated endothelial cells where the modulation of TNF-α induced adhesion molecules response by casein derived bioactive peptides was mediated by activation of PPAR-γ

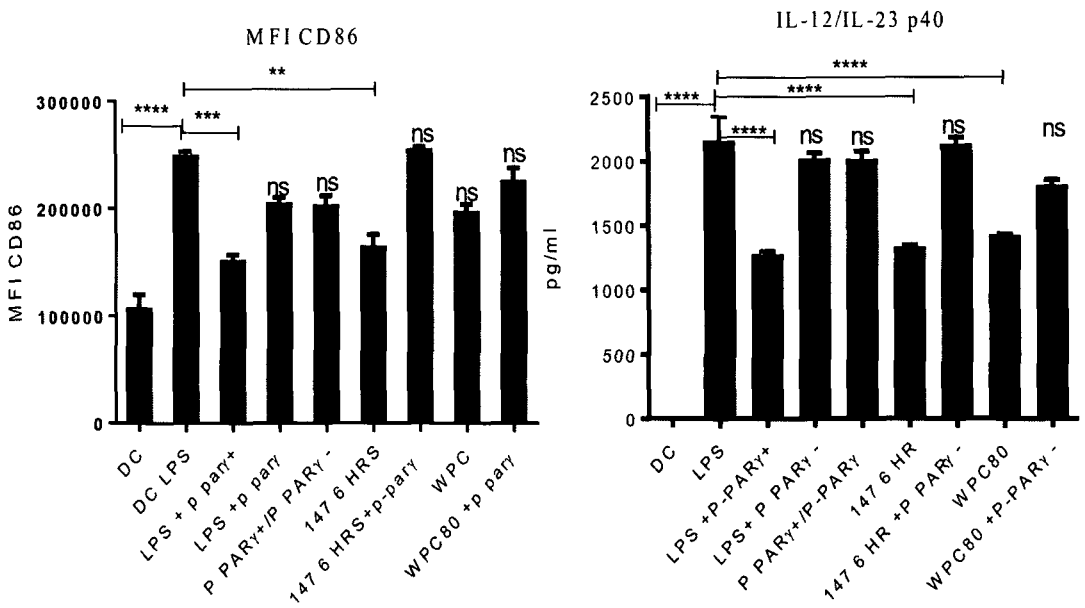


Figure 3.23. The anti-inflammatory effects of whey hydrolysate 147 can be mimicked in the presence of a P-PARγ agonist troglitazone or reversed in the presence of a P-PARγ antagonist, BADGE On Day 6, immature MDDC (5 X 10^6 cells/ml) were harvested The P-PARγ antagonist Badge (10μM) was added 1 h before the stimulation of MDDC with LPS (200 ng/ml) (mDC) and troglitazone (25μM) agonist added 10 min before stimulation MDDC were cultured in the presence or absence of agonist /antagonist or with hydrolysates +/- antagonist for 24 h On Day 7, DC were harvested and stained with CD86 (PE) for the expression of maturation marker by flow cytometry (B) Supernatants were collected after 24 h for cytokine analysis by ELISA These experiments were repeated using three PBMC donors (n=3) Hydrolysates and P-PAR γ agonist troglitazone significantly decreased the maturation marker CD86 and inhibited the production of IL-12p40 by DC while the P-PARγ antagonist restored the maturation marker CD86 and cytokine level of IL-12p40 back to a mature MDDC phenotype Statistical analysis was carried out with one way ANOVA Tukey Multiple Comparison Test where **** < 0 0001, **<0 01

3.17. WHEY HYDROLYSATE 147 EXERTS ITS ANTI-INFLAMMATORY EFFECTS BY SUPPRESSING NF-κB PATHWAY ACTIVATION THROUGH P-PARγ DEPENDENT MECHANISMS

NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a protein complex that controls transcription of DNA, cytokine production and cell survival The DC maturation process is known to depend on the activation of NF-κB following LPS stimulation To explore the hypothesis that the hydrolysate exerts it effects in DC via NF-κB through activation of P-PARγ, DC were treated for 24 h with the hydrolysate (147) or whey protein control (wpc80) or troglitazone (25μM) in the presence or absence of BADGE (10μM) and then activated with LPS (200ng/ml for 10 mins) and the resulting DC lysates were analysed by western blotting Phosphorylation of the p65 subunit of NF-κB was used as an index of NF-κB activation As shown in Figure 3 24, LPS activates the NF-κB pathway inducing the phosphorylation of NF-κB p65 (P <0 0001, Figure 3 24 A, B) Phosphorylation of the NF-κB p65 was significantly reduced by hydrolysate 147 (P<0 01), by whey protein control wpc80 (P <0 01) and by the P-PARγ ligand (p-parγ+) troglitazone (P<0 01) In the presence of BADGE (p-parγ-), the effects of both the whey hydrolysate, whey control and troglitazone on NF-κB p65 phosphorylation were completely suppressed, demonstrating that the modulation of NF-κB activation in DC by the whey hydrolysate 147 was mediated by a P-PARγ dependent mechanism

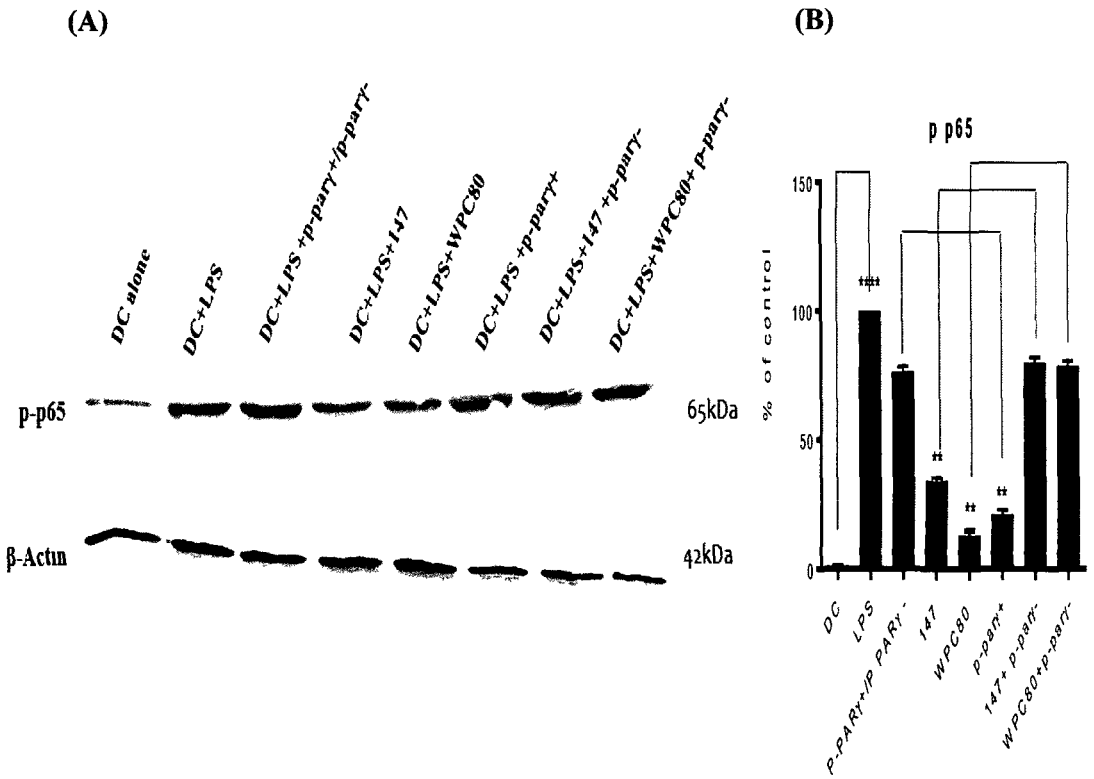


Figure 3.24. Whey hydrolysate 147 and wpc80 exert there anti-inflammatory effects by suppressing NF-κB pathway activation through PPAR-γ dependent mechanism. Phosphorylation of the p65 subunit of NF-κB was used as an index of NF-κB activation (A) DC were treated with whey hydrolysate 147, WPC80 or troglitazone (p-parγ+ (25 μM)) for 24 h and with or without BADGE (p-parγ- (10 μM)) followed by 10 min stimulation with LPS (200ng/ml) Phosphorylation of p65 was estimated by Western Blotting experiments (10 μg of total proteins) and β-actin was used as a loading control (B) Protein levels (assessed as ratio of protein OD normalized to the OD of β-actin) were shown as a percentage of control (LPS activated cell) (n=2) Statistical analysis was carried out using unpaired student *t* test where **** <0 0001 DC vs LPS and ** <0 01 treatment vs BADGE

3.18. SUMMARY

The main objectives of this chapter were to 1) to identify hydrolysates that have immunosuppressive effects on T cell proliferation and cytokine production *in* vitro 2) to determine the effects of hydrolysates on Th1, Th2, Th17 and T Reg differentiation 3) to determine the effects of hydrolysates on DC maturation and cytokine production and 4) to identify a mechanism by which hydrolysates can influence key signal transduction events involved in inflammatory processes The addition of hydrolysates to PBMC cultures had differential effects on T cell proliferation however for the most part, hydrolysates did not significantly interact with T cell proliferation In terms of immunomodulatory ability, a number of whey and casein hydrolysates reduced the levels of the Th1 cytokine (IFN-γ), while other hydrolysates significantly increased the levels of IFN-γ after 4 days in PBMC activated cells Several hydrolysates inhibited the Th2 response by decreasing the signature cytokine of allergy IL-4 and some hydrolysates significantly decreased the pro-inflammatory cytokine IL-6 Two casein hydrolysates increased the level of IL-10 Based on these results two whey hydrolysates and three casein hydrolysates and their optimised regenerates were selected for further analysis to build the body of evidence that hydrolysates may have immunomodulatory properties with the potential to dampen pro-inflammatory responses and promote regulatory responses These selected hydrolysates and their regenerates had no effect on the number of proliferating $CD3^+$ T cells Differential modulation was again evident in this second screen where several hydrolysates and their regenerates decreased both pro-inflammatory IL-6 and IL-17 with little or no effect on Th1 cytokines IFN-γ, or IL-2

Unfortunately, the most promising hydrolysate 35 and it's regenerate 85 with an enhanced immunomodulatory ability over and above Pepti had to be excluded from the study Hydrolysates were then chosen for further screening based on the results of this screen and a screen performed with mouse cells within FHI

In terms of suppression in polarized Th1 cells, upscaled hydrolysate 132 and 47 significantly decreased T-bet transcription factor expression, however addition of upscaled hydrolysate 79 to Th1 polarised cells, significantly increased the number of $CD4^+$ T-bet^+ cells All hydrolysates significantly decreased the master transcription factor of Th2 cells, Gata-3 in polarized Th2 cells while both the original hydrolysates 42 and 47 significantly reduced the number of Gata-3^+ cells, but there was no change detected in the Th2 culture supernatants (Figure 3 9) When hydrolysates were added to Th17 polarised cells, all hydrolysates significantly reduced the protein expression of the transcription factor Rorγt, number of $CD4^+$ Rorγt^+ cells and suppressed the levels of IL-17 (Figure 3.11) Adding WPC80 control to Treg polarised cells, hampered the production of IL-10 and protein expression of Foxp3 (Figure 3 12)

The third part of this study was to determine the effects of hydrolysates on DC maturation and cytokine production Human monocyte derived DC stimulated with LPS in the presence or absence of hydrolysates (42, 79, 132 and 47) disrupted the maturation of DC and significantly prevented the upregulation of CD86 co-stimulatory molecule expression in conjunction with a significant decrease in IL-12p40 cytokine production (Figure 3 13)

The last part of this study was to identify a mechanism by which hydrolysates can influence key signal transduction events involved in inflammatory processes P-PAR-γ a member of the nuclear receptor superfamily, has recently been described as a modulator of macrophage and

dendritic functions, are activated by natural ligands (PUFAs) or by synthetic ligands (Troglitazone) and both have been investigated for their anti-inflammatory effects P-PAR-γ are expressed in spleen and in Peyer's patches (Braissant *et al* 1996, Greene *et al* 1995) and are also expressed in DC (Gosset *et al* 2001) Many anti-inflammatory natural products activate P-PAR therefore natural immunomodulatory milk protein hydrolysates may activate or modulate P-PAR-γ to exert there anti-inflammatory effects This was first investigated by examining the P-PAR-γ mRNA expression in MDDC in the presence or absence of hydrolysate 147 P-PAR-γ mRNA expression was significantly higher in the presence of hydrolysate 147 (Figure 3 22) Probing this further, lead us to test whether the effects of whey hydrolysate 147 in suppressing activated DC was blocked, by treating DC with hydrolysate or with a synthetic P-PAR-γ agonist troglitazone in the presence or absence of a synthetic P-PAR-γ inhibitor BADGE In the presence of the antagonist BADGE the anti-inflammatory effect of hydrolysate 147 and the synthetic agonist troglitazone was prevented (Figure 3 2) In the DC lysates obtained from activated DC treated with hydrolysate or agonist in the presence or absence of BADGE phosphorylation of NF-κB p65 was significantly reduced by hydrolysate147 and by P-PAR-γ agonist However in presence of BADGE the NF-κB p65 protein levels were significantly reduced This suggests that the inhibition is direct and is dependent on activation of NF-κB by hydrolysate 147 mediated through a P-PARγ dependent mechanism This mechanism could provide information for a more efficient application of specific immunomodulating milk peptides to induce anti-allergic /anti-inflammatory effects in infants with food allergy

CHAPTER 4

COMPARISON OF THE ENGRAFTMENT OF HUMAN IMMUNE POPULATIONS IN NSG-SCF VERSUS NSG-SGM3 MICE

4.1 Introduction

Development of 'humanized' mice, which involves transplantation of human hematopoietic stem cells in immunodeficient animals, has provided a promising platform to study human immune responses *in vivo* (Legrand *et al* 2009). The humanised mouse system, a xenogenic transplantation and engraftment model for hematopoietic stem cells (HSCs) and peripheral blood mononuclear cells (PBMCs) facilitates the investigation of human immune systems *in vivo* (Manz 2007, Shultz *et al* 2007). Mice with functional human immune systems have potential to serve as a novel platform to test functional foods. Evaluation of functional foods i.e. anti-inflammatory milk protein hydrolysates in a humanised mouse models brings us one step closer to validate their biological activity in a human relevant system. Mast cells and basophils play a critical role in allergy and this makes them attractive candidates for targeting allergic diseases. To this end, mice need to fully support the development of human myeloid immune cells especially granulocytes like mast cells and basophils in order to be capable of mounting effective human allergic responses.

As explained in Chapter 1, there are numerous issues with regards the current animal mouse models of food allergy that make the prospect of a humanised mouse model attractive. Humans and mice display numerous discrepancies in both innate and adaptive immunity. The immunological limitations of these models were increasingly being realized which prompted the development of mice that fully support human hematopoiesis *in vivo.* NSG or NOD-*scid* IL2Rg ^{null} mice are the most common strain of immunodeficient mice currently being used for the generation of humanised mice but the differentiation of human HSC into functional myeloid cells requires the delivery of human cytokines *in vivo.* Thus, to facilitate this a number of strains have been engineered

to allow the expression of human genes that are relevant in human allergy for example SCF, IL-3, GM-SCF, and TPO.

Despite, or perhaps because of the continuous efforts to improve the currently available models, the development of humanised mice remains complicated by the fact that there is no consensus on how it should be approached. Although some investigators use peripheral blood leukocytes to reconstitute mice, human hematopoietic stem cells (HSCs) have been shown to provide better engraftment, and present less risk of the mice developing graft vs host disease. However factors that must be considered when developing these models include the following: 1) a number of different human cell types can be used to engraft mice, 2) NSG mice are available on a number of background strains, 3) mice can be irradiated or not to achieve higher levels of engraftment, 4) mice can be engrafted both as adults and neonates and 5) both male and female mice can be used (Shultz *et al* 2015). To date there have been few studies on the engraftment of humanised mice that have directly compared these factors.

The development of animal models that mimic human allergic responses is crucial to study the pathophysiology of disease and to generate new therapeutic methodologies. In Chapter 1, the rational for choosing NSG-SCF and NSG-SGM3 was discussed. Based on this discussion, $CD34^+$ stem cells derived from UCB were chosen to repopulate immunodeficient mice with human immune cells in this study. The engraftment levels of two strains of immunodeficient mice, both with the IL2rγ -/- mutation, were compared after adoptive transfer of human $CD34^+$ cells derived from umbilical cord blood (UCB). The two strains used were as follows: NOD.Cg-Prkdc ^{scid} IL-2rg ^{tmlWJl} Tg (PGK1-KITLG*220) 441Daw/J; human membrane bound stem cell factor (NSG-SCF) and triple transgenic NOD.Cg-Prkdc ^{scid} Tg; human stem cell factor (hSCF), granulocyte-

macrophage colony-stimulating factor (GM-CSF) and interleukin -3 (IL-3) mice (NSG-SGM3) We chose to compare the engraftment levels of hHSCs in irradiated NSG-SGM3 versus non-irradiated NSG-SCF mice to study the development of human myeloid, T, B cells, basophils and mast cells in these two strains of mice as no study has directly compared these models In this chapter the engraftment levels of human immune cells in NSG-SCF and NSG-SGM3 mice were compared, various methods of $CD34^+$ cell isolation from UCB were tested, purified cells were transferred into conditioned NSG-SGM3 and non-conditioned NSG-SCF and the engraftment of these mice was characterised In summary the objectives of this chapter were,

1) To identify which strain of mouse provided the best host for engraftment of human immune cells involved in the allergy response (T, B , myeloid mast cells and basophils)
2) To identify the conditions facilitating the optimal development of humanised mice,
    - Engraftment in male versus female mice
    - Engraftment with fresh versus frozen $CD34^+$ HSC
    - The dose of $CD34^+$ required for optimal engraftment levels of T, B, myeloid mast cells and basophils in NSG-SCF and NSG-SGM3 mice

4.2. THREE HSC SOURCES FOR HUMANISATION OF MICE

There are several factors to address before commencing with the generation of humanised mice but none are more important than the source of HSC We choose three sources of HSC 1) fresh umbilical cord blood (UCB) provided by the cord blood bank Anthony Nolan Trust Nottingham, UK, 2) UCB donated from consented women who underwent planned caesarean sections at the national maternity hospital (NMH), Holles

St, Dublin and 3) frozen cord blood - derived flow sorted human $CD34^+$ cells purchased from Lonza (Basel, Switzerland).

4.3 ISOLATION OF $CD34^+$ CELLS FROM FRESH UMBILICAL CORD BLOOD.

Clinical standard UCB was obtained from the Anthony Nolan Trust, Nottingham, UK. It was shipped overnight (by air) and the next morning the $CD3^+$ T cell were depleted from the UCB using the RosetteSep system (Stem cell Technologies , Grenoble France)(Section 2.8.2) before enrichment for $CD34^+$ cells was performed with the positive selection kit II (Stem cell Technologies) as detailed in section 2.8.3. Flow cytometry staining for $CD34^+$ was used to determine the purity of the cell preparations. After using the Easy Sep human cord blood kit, which involves two separate isolation steps, the cell suspension contained only 4.2 % $CD34^+$ cells (Figure 4.1). The flow through containing the cells that were not magnetically labelled and thus were poured off was also examined to ensure no $CD34^+$ cells that could be collected by an extra purification step remained. No $CD34^+$ cells were present in the flow through highlighting a problem with either the kit used to isolate the cells or the blood obtained from Anthony Nolan Trust. However, it was suspected that the UCB was X-rayed at customs in transit despite having an X-ray exempt form on the packaged cells (private communication Kam Varma, PdP couriers, UK) which killed the cells (Figure 4.1). Thus new fresh cord was obtained again from the Anthony Nolan Trust shipped overnight (non-irradiated) and enrichment for $CD34^+$ cells was performed again with the positive selection kit II (Stem cell Technologies). The new Easy Sep kit produced a cell suspension with 90.9% $CD34^+$ cells (Figure 4.2). Furthermore the yield was acceptable (3.8×10^6 cells total,

enough to engraft approximately 36 mice) (Table 4.1). Table 4.1 summarises the various attempts to purify $CD34^+$ cells from fresh cord blood from Anthony Nolan. Of the 3 attempts to isolate $CD34^+$ cells from the Anthony Nolan Trust sufficient purity (> 90% $CD34^+$ cells) was only achieved once (Table 4.1).

The next source of UCB was obtained from consented women volunteers at the NMH Holles St, Dublin. Human UCB samples were obtained according to institutional guidelines approved by the NMH ethics committee. The major challenge here was to get a yield of UCB sufficient to permit injection into a number of mice. Based on our experience of 11 UCB samples obtained from consented donors from the NMH (Table 4.1) 4 out of 11 times we received volumes of 80-140ml which resulted in sufficient yields (1.2×10^6- 2.78×10^6) and purity (> 90%) to permit injection into mice. However 2 out of 11 times UCB blood volume were very low (6-9ml) which resulted in very low yields and purity (Table 4.1). There were several times when we got sufficient volumes of UCB (65-85ml) but for some unforeseen reason, also resulted in low yields and purity. Several factors may affect the $CD34^+$ cell yield. First, one needs to find a density gradient matrix that works well for UCB, which is not necessarily the same as is efficacious for adult peripheral blood. Moreover, during the purification of UCB cells away from red blood cells, performed on lymphoprep gradient medium (Axis Shield PoS, AS, Oslo, Norway) retention of red blood cells occurred at the interface or just below when there was a delay of several hours between withdrawal of blood from the cord (due to emergency caesarean sections) and its collection which could not be controlled which resulted in the low yields and purity detailed in Table 4.1.

The third source of HSC; frozen cord blood - derived flow sorted human $CD34^+$ cells purchased from Lonza (Basel, Switzerland) allowed for greater flexibility in terms of planning experiments and therefore were the most stable source as they were already

purified and contained sufficient cell numbers and purity of $CD34^+$ cells to perform experiments (Figure 4 3) In this chapter, some experiments were completed with UCB obtained from Anthony Nolan, some with NMH but most were completed with purified HSC from Lonza (Basel, Switzerland)

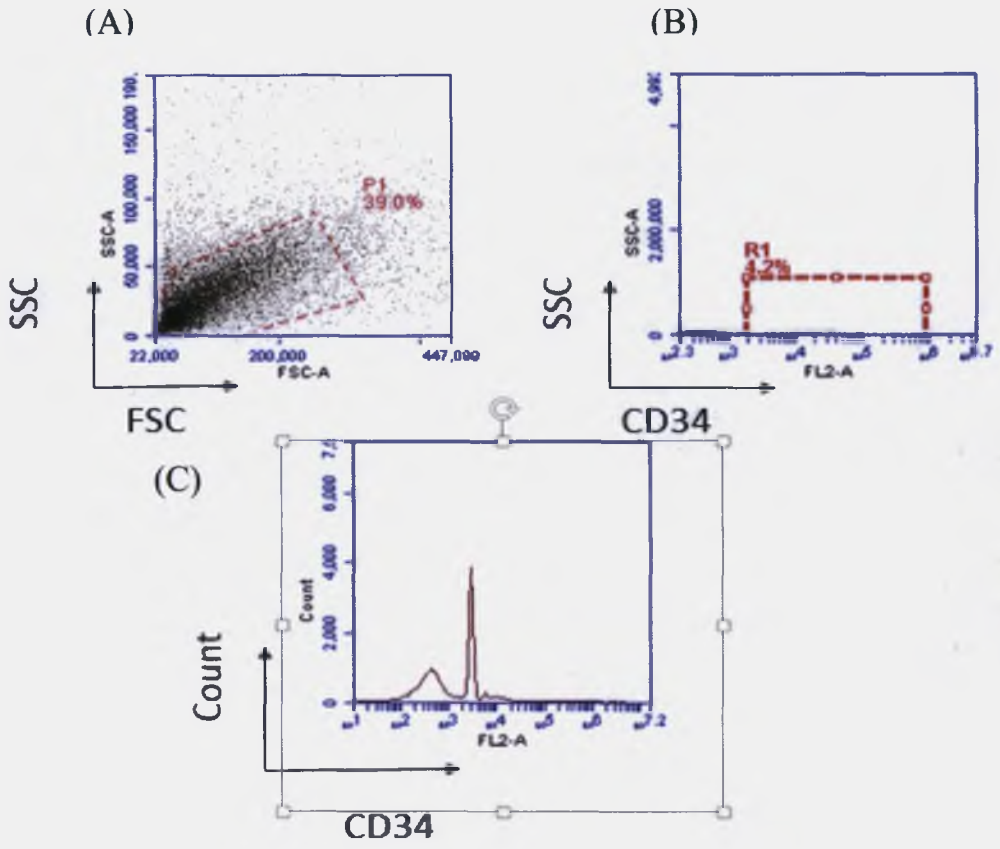


Figure 4.1. CD34$^+$ cell purification from fresh UCB. Using the Easy Sep system CD34$^+$ cells were isolated from 24-36 hr old cord blood obtained from the Anthony Nolan Trust. Analysis for surface expression of CD34$^+$ (PE) by flow cytometry was used to determine the purity of the cell preparations. (A) Illustrates the gated HSC population from SCC against FSC plot, (B) represents the gating position for human CD34$^+$ (PE) and (C) purity (red open histogram)

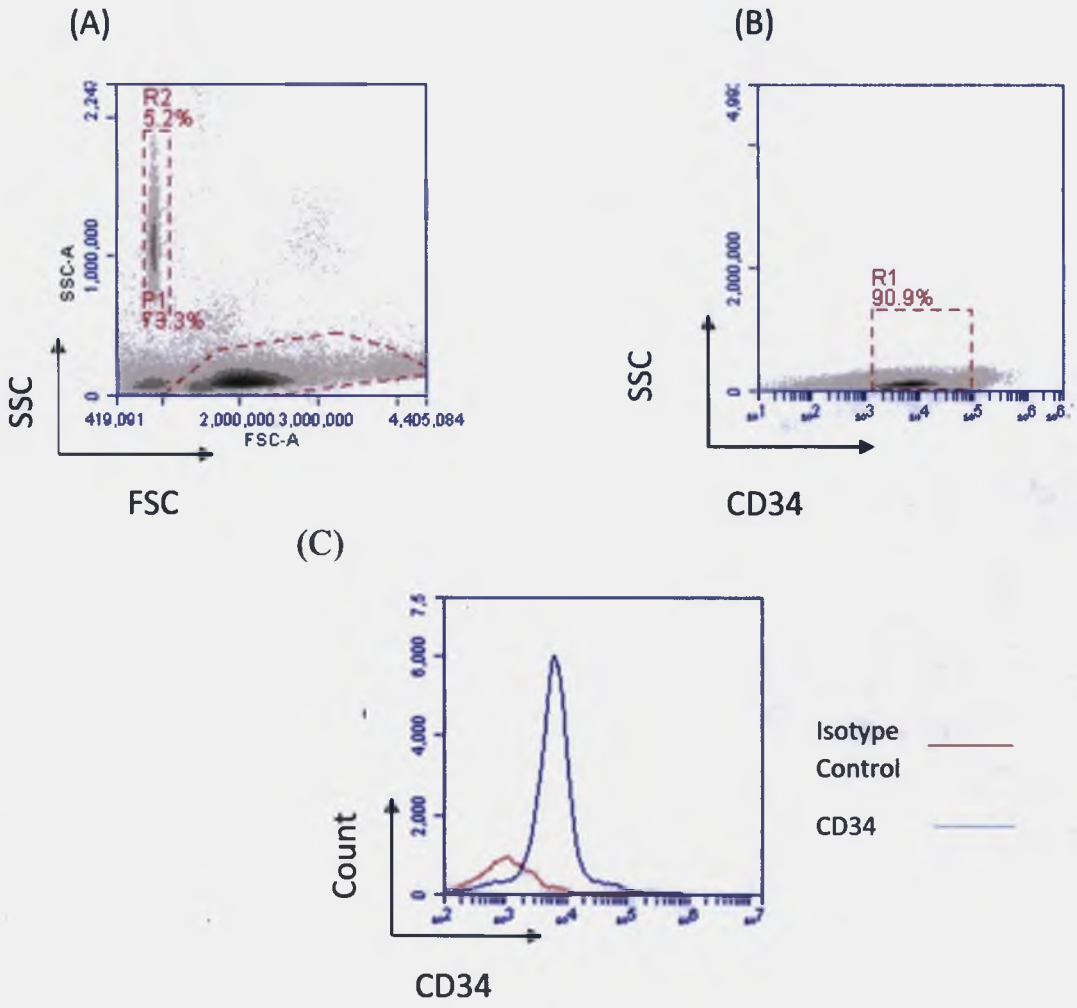


Figure 4.2. CD34$^+$ cell purification from fresh UCB. Using the Easy Sep system CD34$^+$ cells were isolated from 24-36 hr old cord blood obtained from the Anthony Nolan Trust. Analysis for surface expression of CD34$^+$ (PE) by flow cytometry was used to determine the purity of the cell preparations. (A) Illustrates the gated HSC population from SCC against FSC plot, (B) represents the gating position for human CD34$^+$ (PE) and (C) purity (blue open histogram) and red peak represents isotype control. Data is from Donor 100544 (170ml) and is representative of one experiment.

Table 4.1 List of CD34⁺cell purifIcations

Cord Blood Sample vol	Source of Cells	Total cell yield CD34+	Purity of CD34+ cells (%)
114ml	Anthony Nolan	2×10^5	4.2
127 ml	Anthony Nolan	6.4×10^5	82.0
170ml	Anthony Nolan	3.8×10^6	90.9
80ml	NMH	6.6×10^5	68.0
120ml	NMH	1.2×10^6	93.0
85ml	NMH	6×10^5	78.0
80 ml	NMH	1.6×10^6	95.6
80ml	NMH	4.4×10^5	74.0
65 ml	NMH	2.19×10^5	76.0
9ml	NMH	5.8×10^5	81.0
6ml	NMH	2×10^5	86
140 ml	NMH	2.78×10^6	94.0
100ml	NMH	7×10^5	83.0
100 ml	NMH	1.3×10^6	91.0

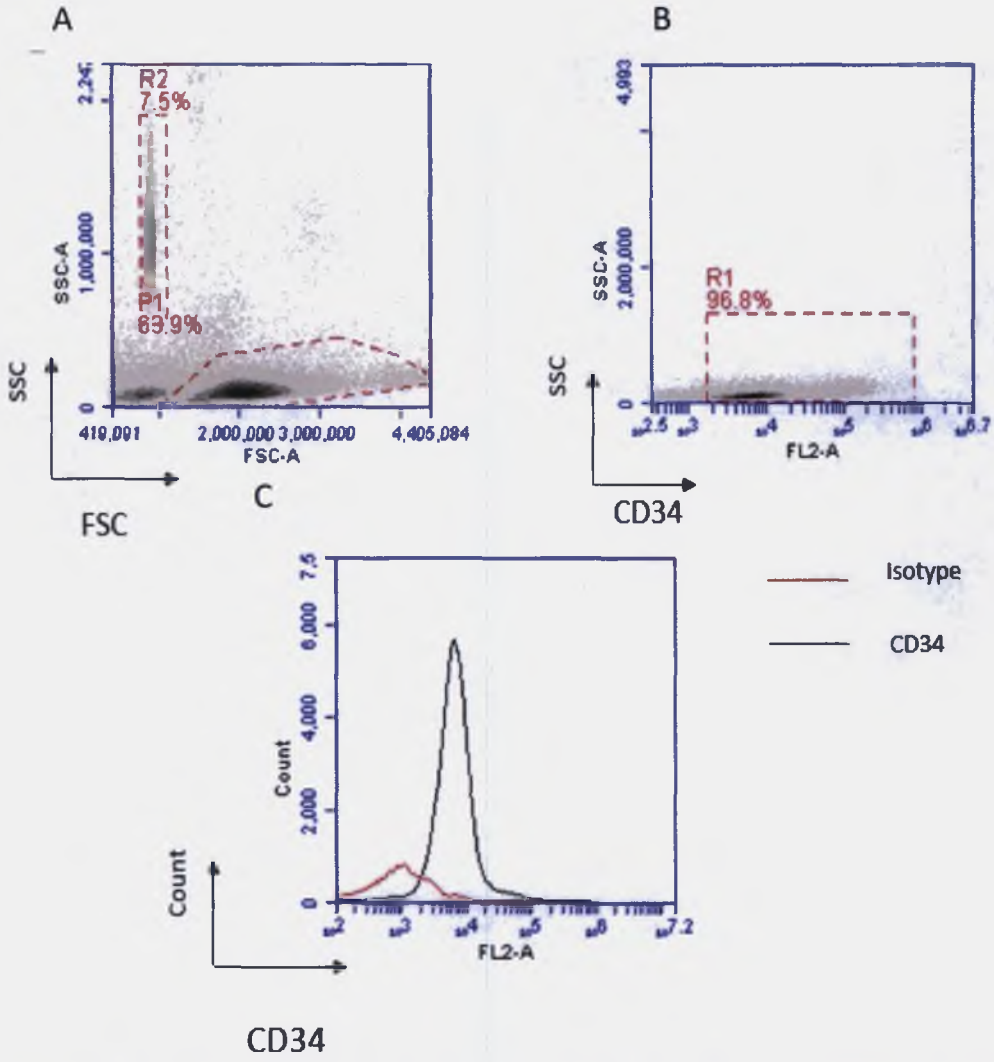


Figure 4.3. Purity analysis of CD34$^+$ stem cells by flow cytometry. Isolated hematopoietic CD34$^+$ cells obtained from Lonza were thawed as in section 2.1 and were analysed for surface expression of CD34$^+$ (PE) by flow cytometry to determine the purity of the cell preparations. (A) Illustrates the gated HSC population from SCC against FSC plot, (B) represents the gating position for human CD34$^+$ (PE) and (C) purity (black open histogram) and red peak represents isotype control.

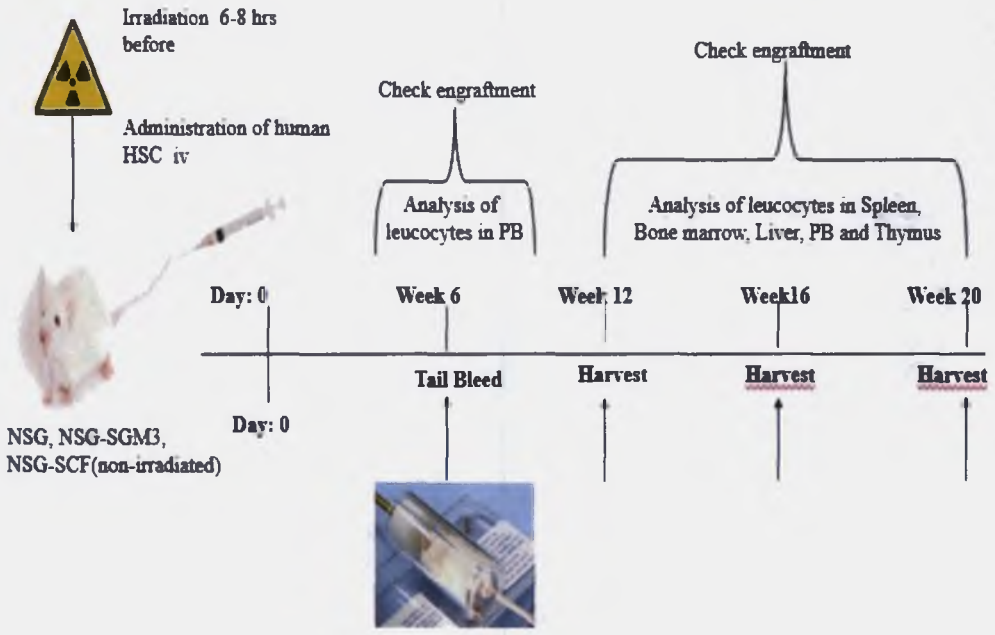


Fig 4.4. Timeline of the CD34$^+$ stem cell reconstitution in NSG, NSG-IL-3/GM-SCF/SCF (SGM3) and NSG- membrane bound SCF mice (mSCF). NOD-SCID IL-2ry^{null}(NSG) and human stem cell factor, granulocyte-macrophage colony-stimulating factor and interleukin -3 expressing NOD-SCID IL-2ry^{null}(NSG-SGM3) mice were exposed to a sub-lethal dose (2.4Gy) of gamma irradiation. 1 x 10^5 CD34$^+$ HSCs was then administered intravenously (300 µl) via the tail vein on day 0 into NSG, NSG-SGM3 and NOD-SCID IL-2ry ^{null} human membrane bound stem cell factor (NSG-SCF). Engraftment was monitored in samples of peripheral blood using flow cytometry at 6 weeks and engraftment was monitored in bone marrow, liver, spleen thymus and peripheral blood at 12, 16 and 20 weeks post reconstitution with human CD34$^+$ HSCs.

4.4. 3-WEEK OLD NSG AND NSG-SGM3 MICE WERE RADIOSENSITIVE AT SUB-LETHAL DOSES

Neonatal mice classically show better engraftment compared to adults (Ishikawa *et al* 2005) but injecting them with HSCs is delicate work In contrast, weaning and i v tail injection of 3-4 week old mice is an easier procedure and they have been shown to display better engraftment than adult mice (Shultz *et al* 2016) To determine the effect of using 3 week old mice instead of adult mice (6 week +), NSG and NSG-SGM3 mice were conditioned with low dose whole body irradiation (2 4 Gy) whereas NSG-SCF mice were not irradiated 4 hrs later human HSC (1 x 10^5 $CD3^+$ cell depleted $CD34^+$ cells) obtained from the Anthony Nolan Trust were injected into the tails veins of 3 strains of NSG mice (Figure 4 4) After HSC administration, weight loss and survival of each mouse was monitored 3 times a week The administration of HSC to non-irradiated NSG-SCF mice had no effect on survival throughout the study (Figure 4 5) However, unexpectedly at approximately week 8 post HSC transplantation NSG and NSG-SGM3 mice that were generated displayed an increased rate of mortality compared to NSG-SCF mice that were not irradiated (Figure 4 5) Hense, all mice were subsequently monitored every day until the end of the experiment Autopsy results revealed gross abnormalities in the thymus of these mice which resembled radiation damage It is known that sublethal irradiation causes damage to neurons in the developing brain (Kameyama *et al* 1994) For e g radiation damage of newborn mice increases numbers of binucleated neuron heterokaryons consisting of Purkinje cells fused to bone marrow-derived cells (Espejel *et al* 2009) On closer examination the deaths in the NSG and NSG-SGM3 mice may have been as a result of their young age (3 weeks) in conjunction with too high a irradiation dose (240 cGy) because non-irradiated NSG-SCF mice did not become moribund after 20 weeks after HSC transplantation (Figure 4 6) After

consultation with a senior staff member from Jackson laboratories a lower dose of radiation of 140cGy for NSG and 100 cGy for NSG-SGM3 was recommended for these radiation sensitive strains instead of the 240 cGy dose administered previously (personal communication, Dr Ralph Gareus, Jackson Laboratories, Maine, USA). All subsequent experiments were performed with 3 week old mice and reduced radiation doses for NSG-SGM3 mice.

All remaining mice (NSG, NSG-SGM3, NSG-SCF) were compared for their engraftment levels of immune cells in the three strains of immunodeficient mice, all with the IL2Rγ ^{null} mutation. 20 weeks after adoptive transfer of human CD34$^+$ cells, spleens, bone, liver, peripheral blood and thymus were harvested and stained with human antibodies to examine the overall engraftment in these organs. The percentage of human CD45$^+$ cells was first examined in all three strains of mice. Comparable percentages of human CD45$^+$ cells were present in the spleen, bone marrow, livers, thymus and in the circulation of all mice tested (Figure 4.6). Reconstitution was considered to be successful if the percentage of human CD45$^+$ cells in organs tested was more than 25% (Shultz *et al* 2016). Furthermore, there were considerably more human leucocytes present in the bone marrow of NSG-SCF mice than in the bone marrow of NSG-SGM3 and NSG mice (Mean 73.5% vs 55.9% vs 41.5%) (Figure 4.6). This increase in engraftment kinetics between strains was also observed with the myeloid cells. Notably there were significantly more CD33$^+$ myeloid cells found in the spleen in NSG-SCF mice than in the spleen of NSG-SGM3 mice (Mean 59.5% vs 33.2% $P< 0.05$). This trend also continued in the B cell lineage where there were significantly more CD20$^+$ B cells found in the liver and bone marrow of NSG-SCF mice than in the other two strains ($P<0.001$, $P<0.05$) (Figure 4.6). There were little or no CD3$^+$ T cells present in the spleen, bone marrow or liver of NSG mice at 20 weeks post transplantation of HSCs but this lack of

T cell development may be a direct result of the damage to the thymus in these mice There were more $CD3^+$ T cells found in the spleen and bone marrow of NSG-SCF mice than the spleen and bone marrow of NSG-SGM3 and NSG mice but the difference was not significant The highest level was again found in the liver (Mean 12 17) (Figure 4 6) The engraftment of the effector cells of allergy (mast cells and basophils) were analysed in the spleen, bone marrow and livers Human cells expressing the phenotypic markers of mast cells and basophils (FcεRI and CD203c) were present at slightly higher levels in spleen and bone marrow of NSG-SCF (Figure 4 7) No mast cells or basophils were detected in any of the tissues of NSG mice over the course of this experiment (Data not shown) Mast cells and basophils were further distinguished by assessing human c-kit expression As shown in figure 4 7B there was an increase in the total number of mast cells and basophils recovered in the spleen, bone marrow and liver of NSG-SCF in comparison to NSG-SGM3 tissues but this difference was not significant In addition the numbers of basophils and mast cells in the various tissues was investigated ~3770 in the spleen, 2290 in the bone marrow and 9112 of $FcεRI^+$ $CD203c^+$ cells in the liver were mast cells in NSG-SCF mice In comparison, fewer mast cells were recovered in the spleen and bone marrow of NSG-SGM3 mice but higher numbers were recovered in the liver of these mice (Figure 4 7)

In summary there was higher engraftment of immune cells in the tissues of non-irradiated NSG-SCF mice in comparison to the reduced hematopoiesis found in irradiated humanised NSG and NSG-SGM3 mice which was possibly caused by the radiation dose which resulted in a decreased ability of transplanted and irradiated human HSC to repopulate following radiation injury

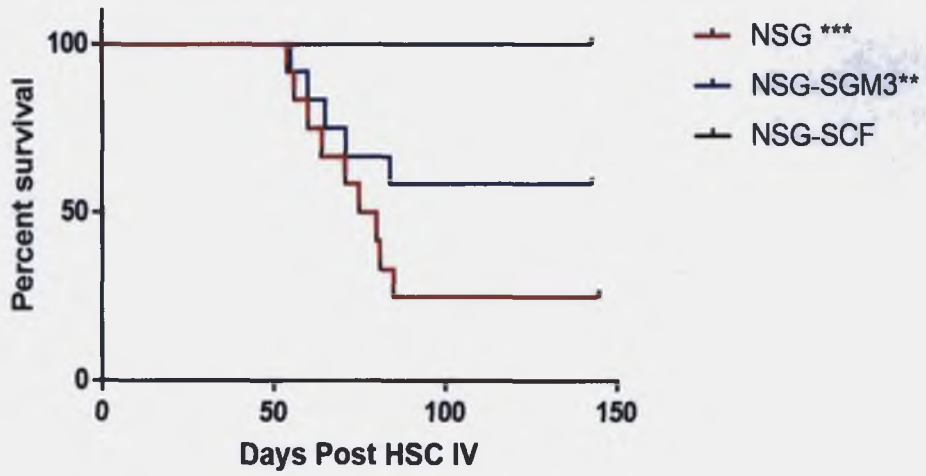


Figure 4.5. NSG and NSG-SGM3 3 week old male and female mice were susceptible to sub lethal irradiation. 1 x 10^5 human CD34+ HSCs were isolated from 24-36 hr old cord blood from Antony Nolan Trust UK and administered to irradiated NSG and NSG-SGM3 (2.4 Gy) and non-irradiated NSG-SCF mice on day 0. Transplanted mice were monitored twice a week until day 55 and then every day for the duration of the experiment. n=12 for each group. Statistical analysis was carried out using a Mantel-Cox test where ***<0.001, **<0.01.

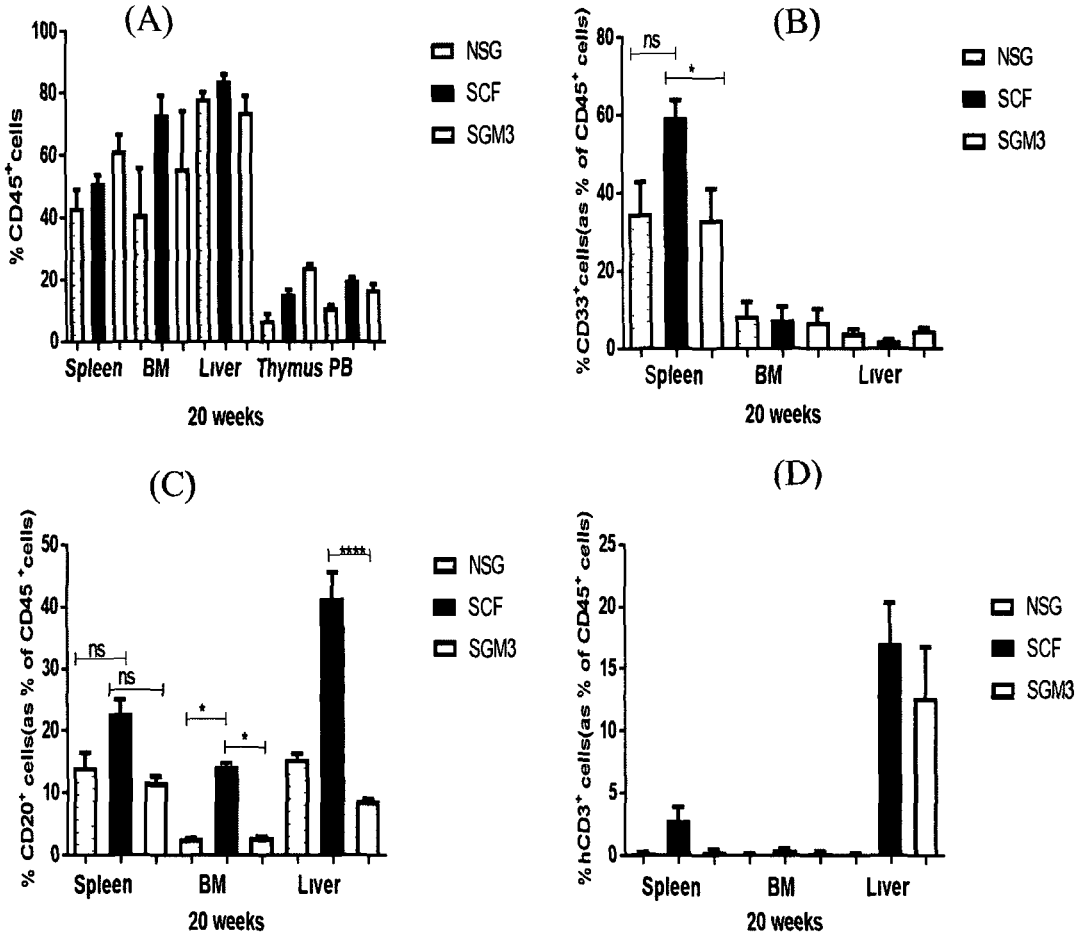


Figure 4.6. Human CD45, CD33, CD20 and CD3 cells in spleen, bone marrow, liver and peripheral blood and thymus at 20 weeks post engraftment with HSC from Anthony Nolan Trust cord blood a) human $CD45^+$,b) human $C33^+$ and c) human $CD20^+$ cells and d) human $CD3^+$T cells as a % of human $CD45^+$ cells m NSG versus NSG-SCF versus NSG-SGM3 mice (n=3 at week 20) NSG mice were set up exactly as described in figure 4 1 Tissues were harvested on week 20 and cells were recovered and stained with CD45 (PerCp), CD33 (FITC), CD20 (APC) and CD14 (PE) and analysed by flow cytometry Graphical representation of the percentage of (A) human $CD45^+$ cells (B) myeloid (C) B cells and (D) T cells recovered in the spleen, bone marrow, liver and peripheral blood n=3 per group (1 HSC donor) at week 20 Statistical analysis was carried out using one-way ANOVA Tukey with multiple comparison test where****< 0 0001 and * <0 05

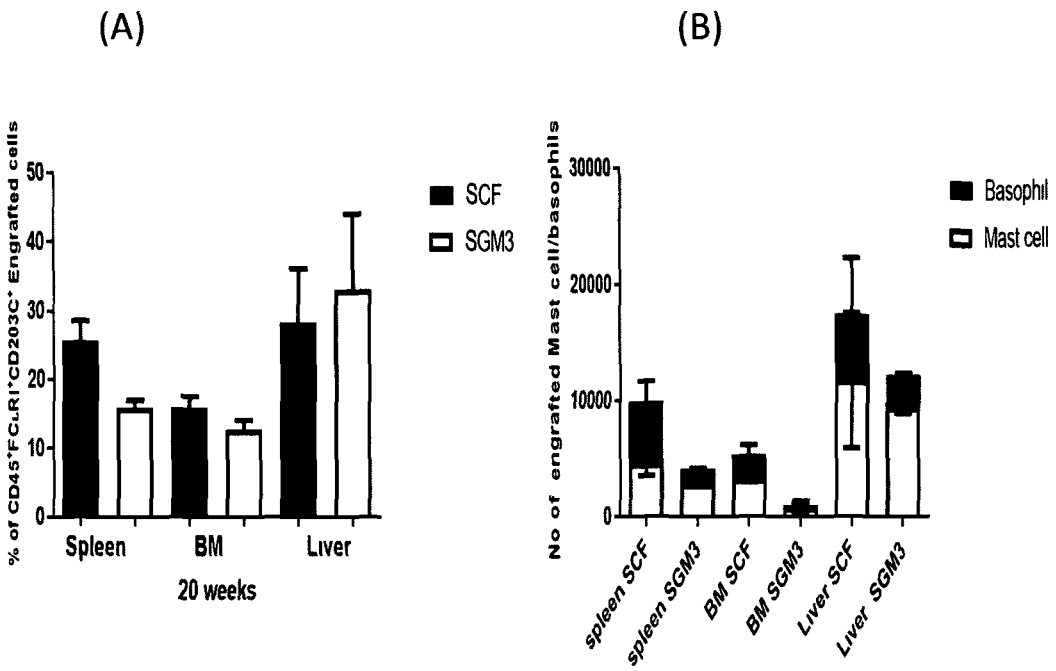


Figure 4.7. Higher engraftment of mast cells and basophils in NSG-SCF mice compared to NSG-SGM3 mice after adoptive transfer of HSCs from Anthony Nolan Trust. The mice were set up as described in figure 4 4 Cells were recovered at week 20 from spleen, bone marrow and liver and surface stained with hCD45$^+$ (fitc) hFCεR1$^+$ (apc), hCD203C$^+$ (pe) cells and anti-human c-kit Ab (PerCp) (A) Graphical representation of the percentage of human mast cells (hFCεR1$^+$hCD203C$^+$hCD45$^+$ cells) recovered from spleen, bone marrow and liver of NSG-SCF and NSG-SGM3 mice (B) Total number of basophils (hFCεR1$^+$hCD203C$^+$hCD45$^+$c-kit$^-$) and mast cells (hFCεR1$^+$hCD203C$^+$hCD45$^+$ c-kit$^+$) recovered in the indicated tissues The total number of cells was assessed using counting beads during flow cytometry n=4 per group, 1 HSC donor

4.5. HUMANISED MICE POSSESS SIMILAR LEVELS OF HUMAN CELLS AT 12, 16 AND 20 WEEKS POST ENGRAFTMENT WITH HSCs

Using protocols created by Billerbeck *et al.* (2012) and Brehm *at al.* (2012) as a guideline and starting point, a humanised mouse model was developed. The majority of studies using humanised mice assess engraftment 12 weeks post transfer of HSCs (Shultz *et al* 2007, Ishikawa *et al* 2017, Billerbeck *et al* 2011) whereas 16 to 22 weeks are required for functional development of mast cells and basophils (Ito *et al* 2013, Takagi *et al* 2011). NSG-SGM3 mice were conditioned by low dose total body irradiation of 1.0 (Gy). 1 x 10^5 $CD34^+$ stem cells obtained from Lonza (characterisation , Figure 4.3) were injected intraveneously into three week old NSG-SGM3 and non-irradiated NSG-SCF mice and the engraftment of leucocytes was examined using flow cytometry at 6, 12, 16 and 20 weeks (Figure 4.1). At the outset it is important to note that any mouse with more than 25% human $CD45^+$ cells is considered successfully humanised (Shultz *et al* 2016). NSG-SGM3 mice engrafted with isolated $CD34^+$ stem cells had lower levels of human $CD45^+$ cells in their circulation at 6 weeks (16.3% +/- 1.8 versus 30.8 % +/- 1.3) than NSG-SCF mice (Figure 4.8). Notably, NSG-SCF mice achieved the level of engraftment (25%) associated with successful humanisation at 6 weeks. Importantly in NSG-SGM3 the average percentage of human $CD45^+$ cells more than doubled between 6 and 12 weeks (16.2% vs 42%) and the average percentage of human $CD45^+$ cells in NSG-SCF also increased to 56% (Figure 4.9). NSG-SCF and NSG-SGM3 mice engrafted with $CD34^+$ stem cells from Lonza on average had high levels of human $CD45^+$ cells in the bone marrow, liver, thymus and peripheral blood at 12 weeks. Low levels of $CD45^+$ cells were found in the spleen of NSG-SCF at 12 weeks in comparison to the significantly higher levels found in the spleen of NSG-SGM3 mice

(15% +/- vs 41% +/- P<0 05) However by 20 weeks comparable levels of human $CD45^+$ cells were found in both strains of mice (Figure 4 9) Over the 12 week engraftment period, human $CD45^+$ cells levels remained high in the liver and bone marrow of NSG-SCF mice Whereas over time the levels of $CD45^+$ cells decreased in the thymus and in the circulation of NSG-SCF and NSG-SGM3 mice (Figure 4 9)

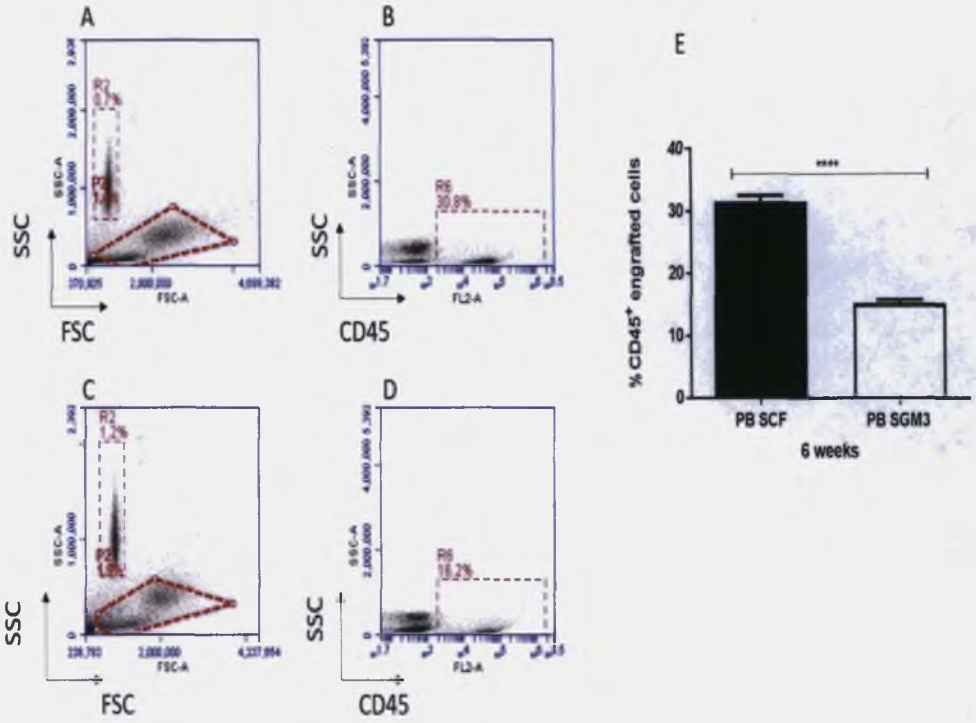


Figure 4.8 NSG-SCF mice have higher leucocyte engraftment in the circulation at 6 weeks post reconstitution with HSC. Comparison of NSG-SCF and NSG-SGM3 PB engrafted with CD34+ Lonza cells. Tail bleeds were performed 6 weeks after HSC injection into the tail vein. The blood was lysed and stained with CD45 (PerCp) antibody and analysed by flow cytometry. (A) Illustrates the viable gated lymphocyte population of NSG-SCF from SSC against FSC plot (B) Percentage of human $CD45^+$ cells in the peripheral blood of one NSG-SCF mouse (C) Illustrates the viable gated lymphocyte population of NSG-SGM3 from SSC against FSC plot (D) Percentage of human $CD45^+$ cells in the peripheral blood of one NSG-SGM3 mouse (E) Graphical representation of the percentage of $CD45^+$ cells in the peripheral blood at 6 weeks. Images A-D are representative of one tail bleed from each mouse. n= 11 per group using one HSC Lonza donor. Statistical analysis was carried out using unpaired t-test where **** <0.0001.

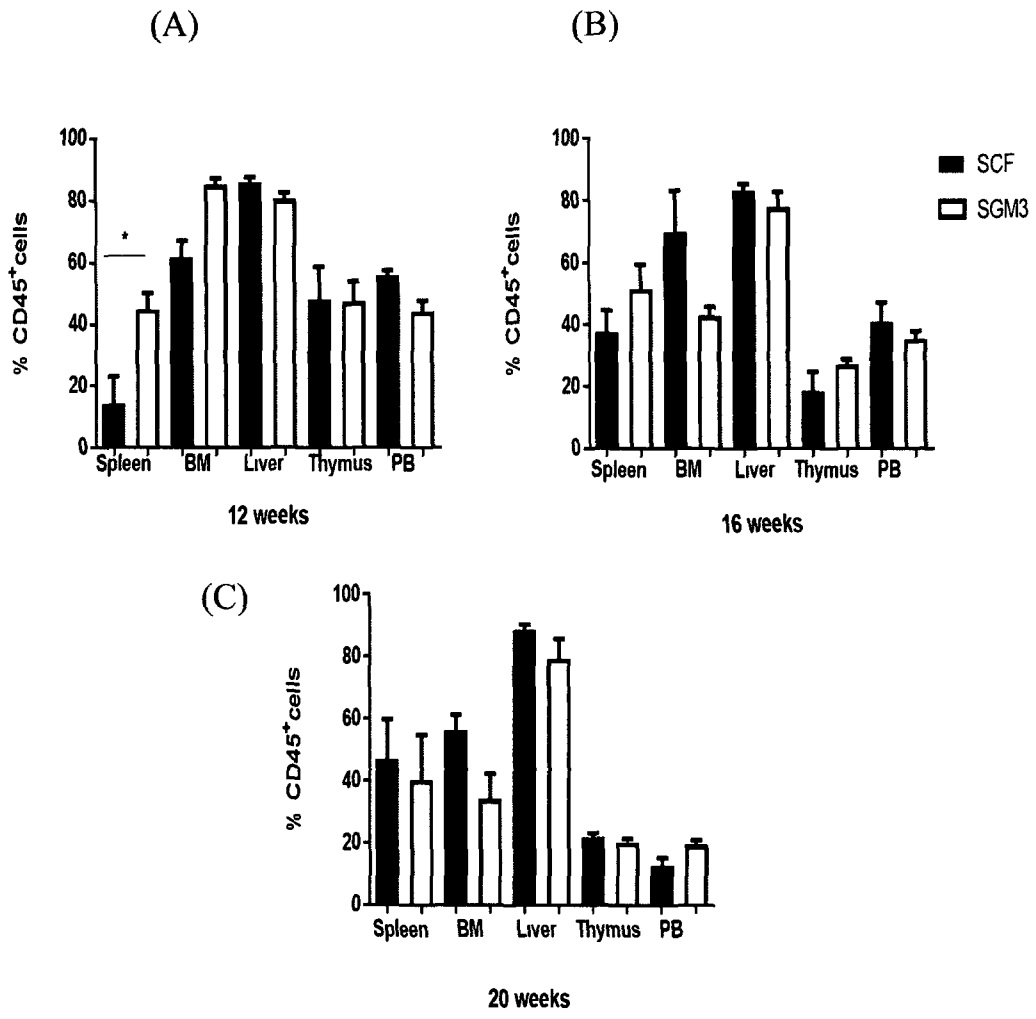


Figure 4.9. Time course of engraftment of human CD45$^+$ using CD34$^+$ cells obtained from Lonza in NSG-SCF versus NSG-SGM3 mice. NSG mice were set up exactly as described in figure 4 4 Tissues were harvested on week 12, week 16 and week 20 and cells were recovered and stained with CD45 (PerCp) antibody and analysed by flow cytometry Graphical representation of the percentage of human CD45$^+$ cells recovered in the spleen, bone marrow, liver, thymus and peripheral blood n=4 per group (1 HSC donor) at weeks 12 (A) and (B) week 16, n=3 at (C) week 20 Statistical analysis was carried out using one-way ANOVA Tukey with multiple comparison test where * <0 05

4.6. NSG-SCF/NSG-SGM3 MICE RECONSTITUTED WITH CD34$^+$ STEM CELLS HAD ENHANCED HUMAN T CELLS IN THE BONE MARROW AND LIVER.

Having demonstrated that human CD45$^+$ cells repopulate conditioned NSG-SGM3 and non-conditioned NSG-SCF mice that received human HSCs, the human lymphoid and myeloid cells present were further characterised By 20 weeks, the frequency of human myeloid cells within the total CD45$^+$ population was higher in spleen and livers of NSG-SCF and NSG-SGM3 mice (Figure 4 10) than in bone marrow or PB There was no difference observed between the strains in the B cell population across all tissues tested at 20 weeks, however there were significantly more T cells in the bone marrow of NSG-SCF than NSG-SGM3 and significantly more T cells in PB in NSG-SGM3 than NSG-SCF by 20 weeks (Figure 4 10) B cell frequencies decreased over time in spleen, bone marrow and liver in NSG-SCF mice from 27% at week 12 to approximately 19% at Week 20 in spleen, (figure 4 11) from 23% at week 12 to 16% at week 20 in bone marrow (Figure 4 12) and from 19% at week 12 to 9% at week 20 in liver (Figure 4 13)

Similar decreases in B-cell frequencies were observed in spleen, bone marrow and liver of NSG-SGM3 mice This overall reduction in B cells was paralleled by a proportional increase in CD3$^+$ T cell frequencies in bone marrow in NSG-SCF mice Notably, there were significantly more CD3$^+$ T cells found in the bone marrow of NSG-SCF mice than in the bone marrow of NSG-SGM3 mice at 16 and 20 weeks ($P < 0\ 01$, Figure 4 12) This significant change in the lymphocyte population has been described previously (Billerbeck *et al* 2013) To further characterise the increased T-cell population in NSG-SCF mice, CD4$^+$ to CD8$^+$ T cell ratios were compared in both mouse strains In the spleens of NSG-SCF mice, the ratio of CD4$^+$ to CD8$^+$ T cells was

approximately 5 1 (P<0 01) and 4 1 in the spleens of NSG-SGM3 mice (Figure 4 14) In the bone marrow of NSG-SCF mice the ratio of $CD4^+$ to $CD8^+$ T cells was slightly higher at 6 1 (P<0 01) in comparison to the ratio of 3 1 in the bone marrow of NSG-SGM3 mice Lower ratios of 4 1 (P < 0 01, NSG-SCF) and 3 1 (P < 0 05, NSG-SGM3) were found in the livers of these mice Together the results show that the transgenic expression of human SCF, IL-3 and GM-CSF significantly promotes the expansion of human $CD4^+$ T helper cells in these mice which are vital cells in the generation of immune responses

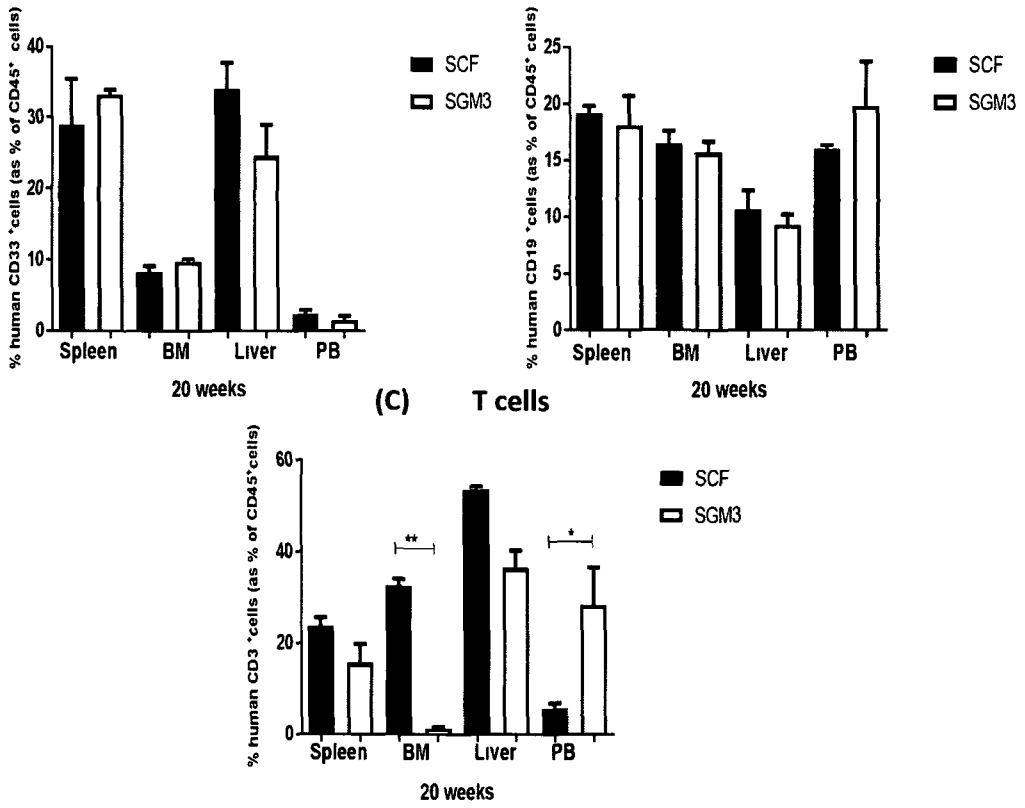


Figure 4.10 Human Myeloid, B and T cells present in spleen, bone marrow, liver and peripheral blood at 20 weeks post engraftment a) human $CD33^+$,b) human $CD19^+$ and c) human $CD3^+$ cells as a % of human $CD45^+$ cells in NSG-SCF versus NSG-SGM3 mice reconstituted with $CD34^+$ Lonza cord blood cells NSG mice were set up exactly as described in figure 4 1 Tissues were harvested on week 20 and cells were recovered and stained with CD33 (FITC), CD19 (PE) and CD3 (APC) and analysed by flow cytometry Graphical representation of the percentage of (A) human $CD33^+$ (B) human $CD19^+$ (C) $CD3^+$ recovered in the spleen, bone marrow, liver and peripheral blood n=3 per group (1 HSC donor) at week 20 Statistical analysis was carried out using one-way ANOVA Tukey with multiple comparison test where * < 0 05 < **0 01

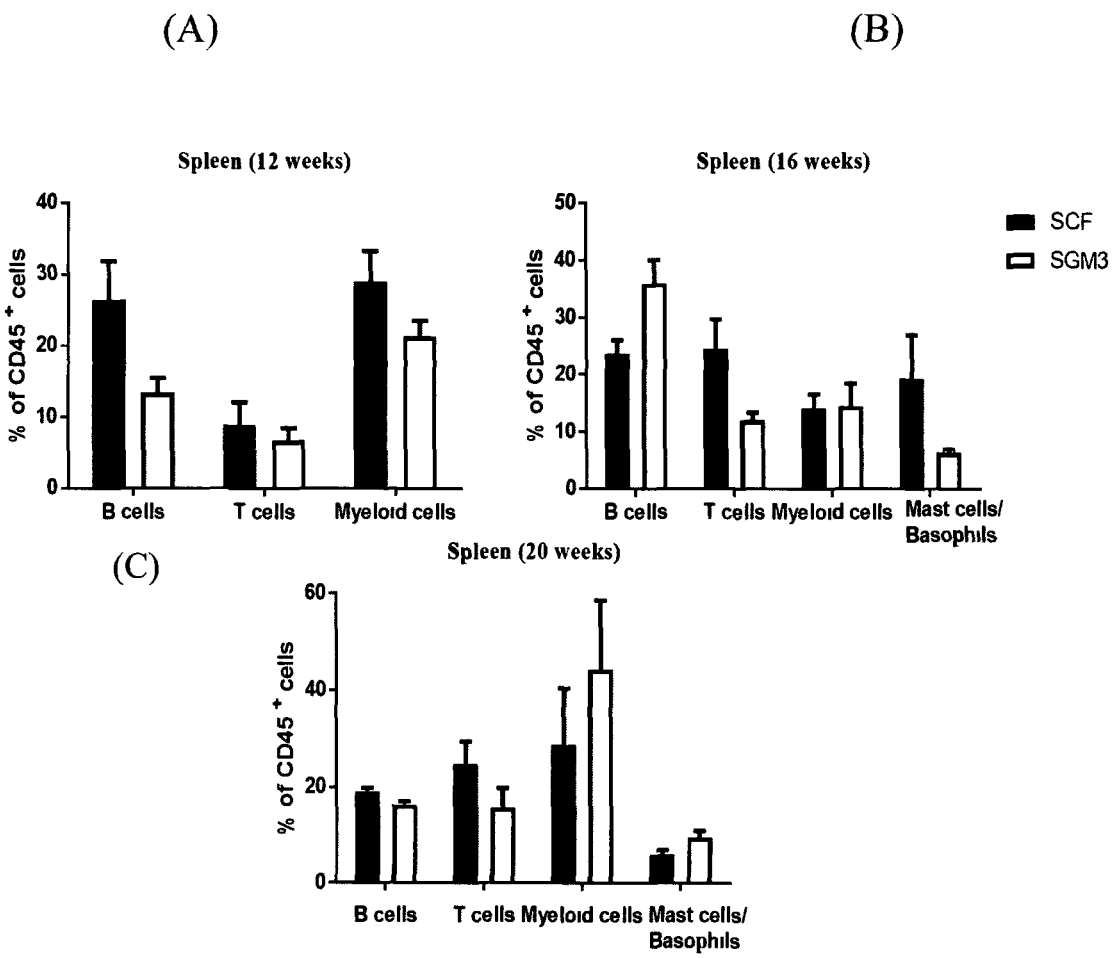


Figure 4.11 Time course of spleen engraftment of human B cells ($CD19^+$), T cells ($CD3^+$), myeloid cells ($CD33^+$) and mast cells/basophil cells ($CD203C^+FC\varepsilon R1^+$) as a % of human $CD45^+$ cells in NSG-SCF versus NSG-SGM3 mice using Lonza cord blood cells

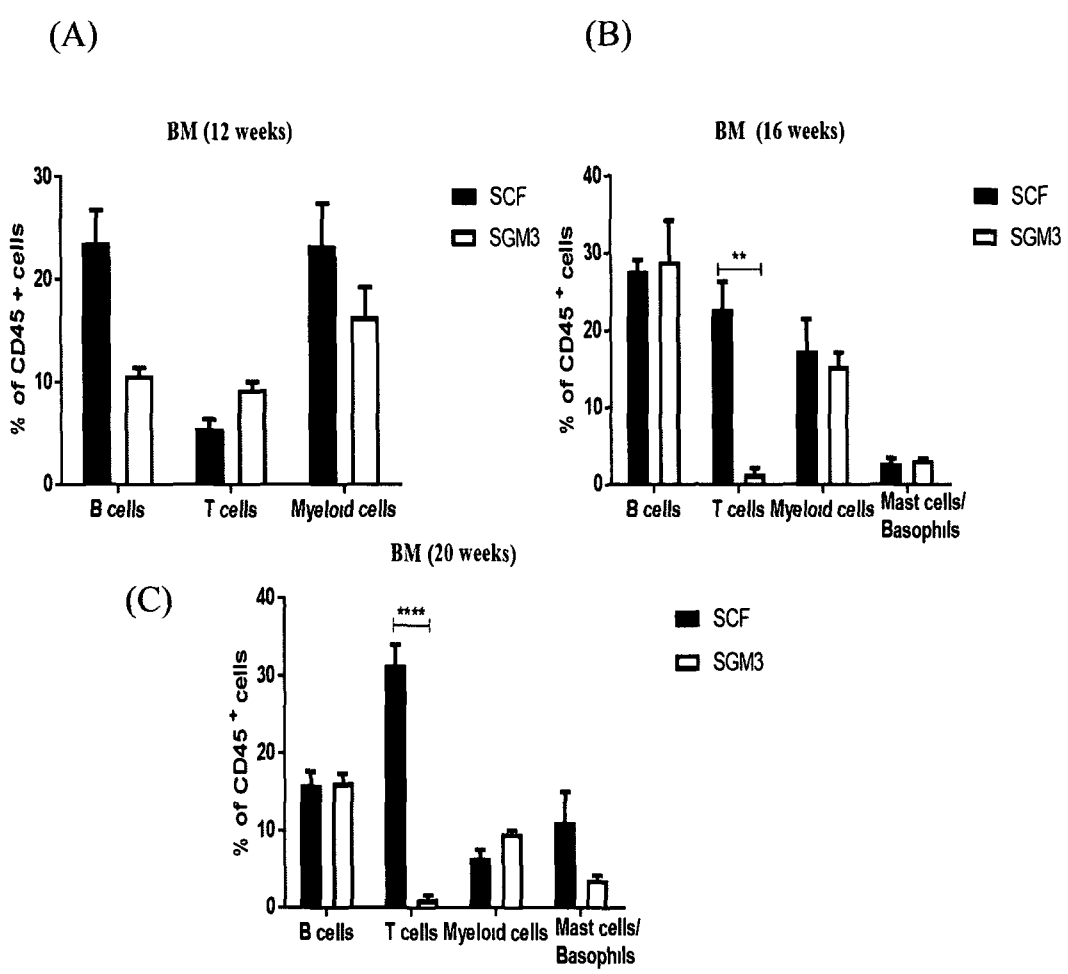


Figure 4.12. Time course of bone marrow engraftment of human B cells ($CD19^+$), T cells ($CD3^+$), myeloid cells ($CD33^+$) and mast cells/basophil cells($CD203C^+FC\varepsilon R1^+$)as a % of human $CD45^+$ cells in NSG-SCF versus NSG-SGM3 mice using Lonza $CD34^+$ cord blood cells Statistical analysis was carried out by one way ANOVA Tukey with multiple comparison test where **** <0 001 **, 0 01 (n=4 at weeks 12 (A) and week 16 (B) n=3 at week 20 (C))

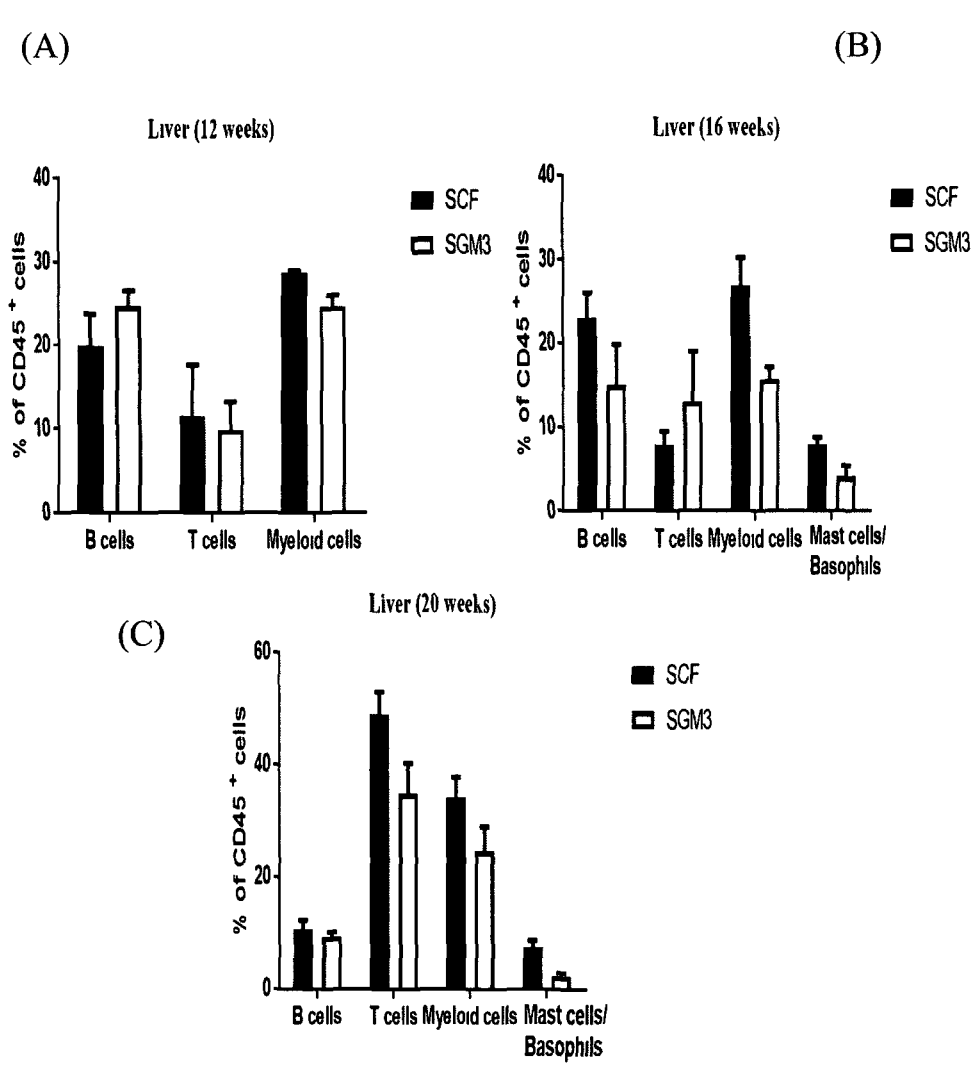


Figure 4.13. Time course of engraftment in the liver Human B cells ($CD19^+$), T cells ($CD3^+$), myeloid cells ($CD33^+$) and mast cells/basophil cells($CD203C^+FC\varepsilon R1^+$)as a % of human $CD45^+$ cells in NSG-SCF versus NSG-SGM3 mice using Lonza cord blood cells (n=4 at weeks 12 (A) and week 16(B) n=3 at week 20 (C)

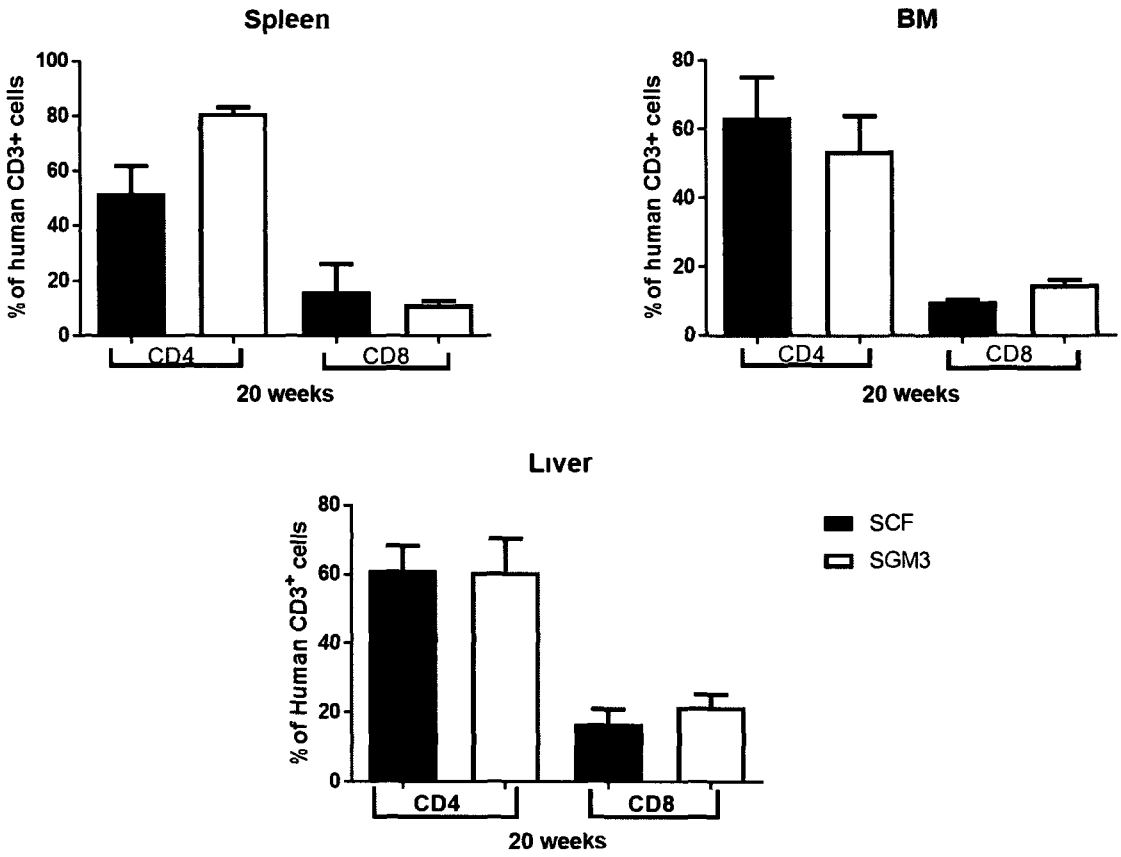


Figure 4.14. Increased frequencies of $CD4^+$ lymphocyte in SCF and SGM3 mouse tissues at 20 weeks. Analysis of human $CD4^+$ to $CD8^+$ T-cell ratios in spleen, bone marrow and liver The mice were set up as described in figure 4 1 Cells were recovered from the spleen (A), bone marrow (B) and liver (C) and surface stained with CD45 (fitc), CD3 (APC), CD4 (PerCP) and CD8(PE) and analysed by flow cytometry Graphical representation of the percentage of cells that were $CD4^+CD8^+$ (gated on human $CD45^+$ $CD3^+$ T cells), n=11 per strain Statistical analysis was carried out using one way ANOVA Tukey with multiple comparison test where * <0 5, **<0 01

4.7. DEVELOPMENT OF HUMAN MAST CELLS/BASOPHILS IN HSCF VERSUS SGM3 NSG RECIPIENTS

We next investigated the development of human mast cells and basophils in the membrane-bound hSCF expressing NSG mice versus the triple transgenic hSCF, hGM-CSF and hIL-3 expressing mice. Using the gating strategy as described in figure 4.15, the engraftment of mast cell and basophils was analysed by flow cytometry. The cell surface marker, CD203c, has been identified as specific for basophils and mast cells among cells of hematopoietic lineage. CD203c is an ecto-nucleotide pyrophosphatase/phosphodiesterase expressed on resting cells at low levels and its expression is rapidly up-regulated following activation (Buhring *et al* 1999). The high affinity IgE receptor Fc epsilon receptor 1 (FcεR1) is expressed on mast cells and basophils and plays a central role in the IgE mediated allergic response. The stem cell factor receptor c-kit (CD117), a tyrosine-protein kinase that acts as cell-surface receptor for the cytokine KITLG/SCF and is expressed on mast cells independent of tissue site maturation or activation status (Metcalfe *et al* 2016) and it's presence distinguishes mast cells from basophils.

As we have seen previously in figure 4.11, 4.12, 4.13 comparable percentages of mast cells had engrafted in the spleen, bone marrow and liver in both strains of mice by week 20 (Figure 4.16 A). Overall similar numbers of mast cells ($CD45^+CD203C^+FC\varepsilon RI^+$) were recovered in spleen and bone marrow in both strains of mice however there was a significant increase in mast cells in the livers of NSG-SGM3 in comparison to NSG-SGM3 (Figure 4.16B). When these cells were further stained with human c-kit antibody (CD117), the majority of cells recovered in the spleen ,bone marrow and livers of NSG-SCF mice were mast cells. However in the bone morrow and livers of NSG-SGM3 mice significantly more basophils ($CD45^+CD203C^+FC\varepsilon RI^+$ c-

kit^{-}) had engrafted than mast cells (Figure 4 16C) Strikingly, there were over ~4000 basophils recovered in the livers of NSG-SGM3 mice ($P<0.0001$) Mast cell progenitors and mature mast cells reside in high frequencies in the spleen and bone marrow of normal immunocompetent mice (Arinobu *et al* 2005) so transgenic expression of human membrane bound SCF influenced the mast cell development in the hematopoietic organs of NSG-SCF mice In comparison, the expression of SCF, IL-3 and GM-CSF resulted in significantly increased number of basophils found in the liver of these immuno-deficient mice

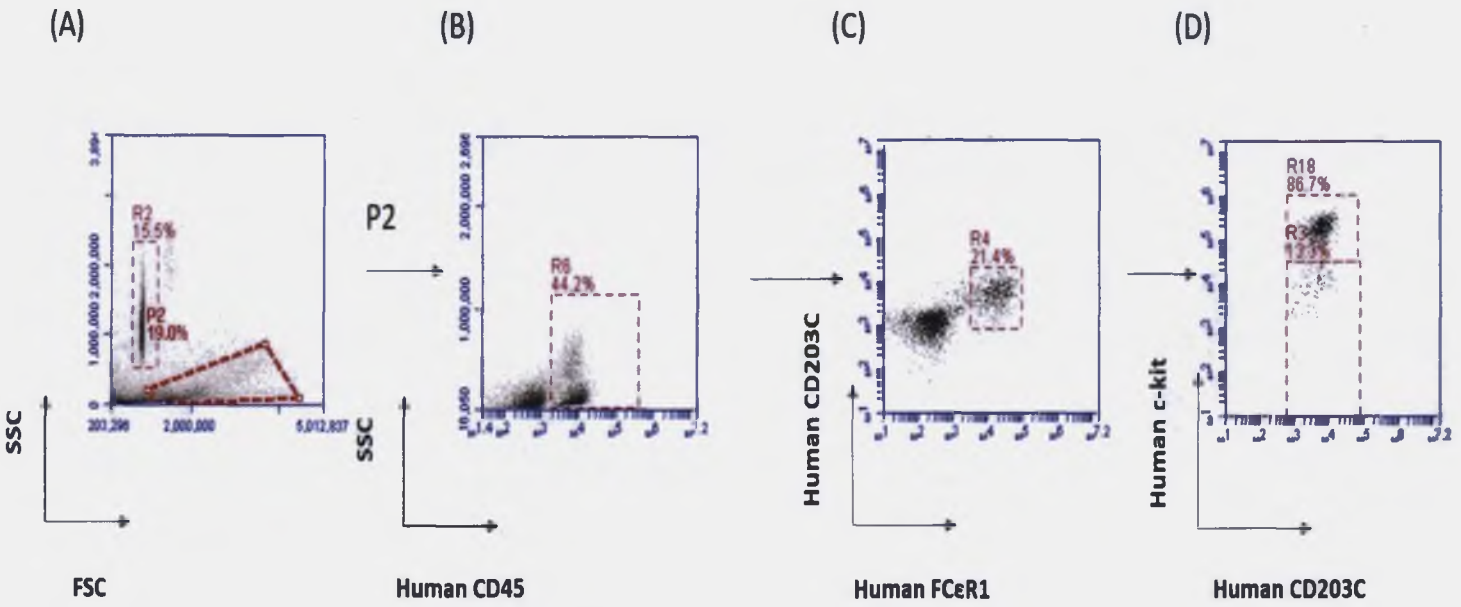


Figure 4.15.Representative example of gating strategy used to identify human mast cells and basophils. (A) Illustrates the gated lymphocyte population from SSC against FSC plot,(B) represents the gating position for human $CD45^+$ (FITC) expression within the lymphocyte population, (C) illustrates the gating position for $CD203C^+$ (PE) and $FC\varepsilon RI^+$ (APC) expression within the $CD45^+$ population and (D) represents the gating position for mast cells ($CD45^+$ $CD203C^+$ $c\text{-}kit^+$) and basophils ($CD45^+$ $CD203C^+Ckit^-$). All gating positions were determined using matching isotype controls.

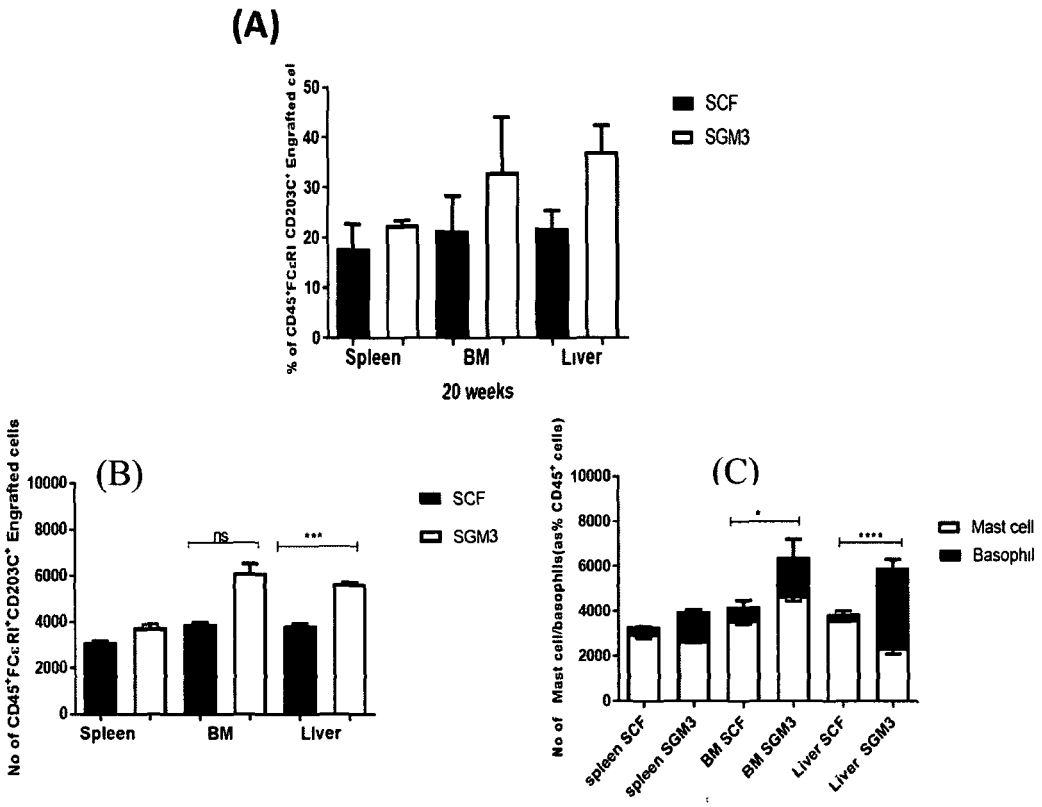


Figure 4.16. Differential distribution of mast cells and basophils in tissues of humanised mice. The mice were set up as described in figure 4 4. Cells were recovered at week 20 from spleen, bone marrow and liver and surface stained with hCD45$^+$ (fitc) hFCεR1$^+$ (apc), hCD203C$^+$ (pe) cells and anti-human c-kit Ab (PerCp) (A) Graphical representation of the percentage of human mast cells (hFCεR1$^+$hCD203C$^+$hCD45$^+$ cells) recovered from spleen , bone marrow and liver (B) the number of mast cells (hFCεR1$^+$hCD203C$^+$hCD45$^+$) and (C) the number of mast cells (hFCεR1$^+$hCD203C$^+$hCD45$^+$c-kit$^+$) and basophils (hFCεR1$^+$hCD203C$^+$hCD45$^+$ c-kit$^-$) recovered in the indicated tissues from hSCF and hSGM3 mice The total number of cells was assessed using counting beads during flow cytometry Statistical analysis was carried out using one way ANOVA and 2 way ANOVA Tukey with multiple comparison test where **** < 0 0001 n=4 per group

4.8. FEMALE NSG-SCF ENGRAFTED WITH CD34+ STEM CELLS HAD ENHANCED LEUCOCYTE, MYELOID AND T AND B CELL RECONSITUTION WHEN COMPARED WITH MALE NSG-SCF MICE.

In order to explore the use of humanized mice (transplanted with human hematopoietic stem cells as *in vivo* models) to evaluate the effects of functional foods and immune therapies on adaptive immune responses, the question of possible gender aspects need to be considered Notta *et al* (2010) reported that female non-obese diabetic/severe combined immunodeficient (NOD/SCID)/IL2Rγc−/− (NSG) mice transplanted with limiting numbers of human $CD34^+$ CD38− cord blood hematopoietic stem cells (CB-HSCs) showed better engraftment of human $CD45^+$ hematopoietic cells than males Subsequent work reported faster growth of human tumor lines in NSG male mice, further supporting differences of xenograft cell engraftment and growth in female and male hosts (Padura *et al* 2010) Here, we evaluated if engraftment of $CD34^+$ CB-HSC into 6-week-old NSG-SCF mice was affected by gender

Umbilical cord blood was obtained from volunteers who were undergoing elective c-sections from the national maternity hospital (NMH) Dublin in accordance with the ethical committee guidelines for heath related research studies Cord blood (2 hrs old) samples were first processed for isolation of mononuclear cells (MNC) using lymphocyte separation medium (Lymphoprep) Cord blood MNCs were then enriched for human $CD34^+$ cells using Easy Sep Human Cord blood CD34 positive selection KIT II (section 2 8 3) Purity of the $CD34^+$ separated cells was > 90% (Table 4 1) NSG-SCF male and female mice were injected with a moderate dose ($1x10^5$ HSCs) via the

tail vein on day 0 (Figure 4.4). 20 weeks later, flow cytometry was used to determine the levels of $CD45^+$ cell chimerism in the spleen, bone marrow, liver, peripheral blood and thymus. When $1x10^5$ HSCs were transplanted, the percentages of human $CD45^+$ were comparable across all tissues but the absolute numbers of human $CD45^+$cells in the spleen of non-irradiated NSG-SCF mice were significantly higher (~ 6- fold higher) than that observed in male mice ($P< 0.0001$, Figure 4.18). Female NSG mice displayed a trend toward higher numbers of human $CD45^+$ cells compared with males in the Liver (female vs male: 235143 vs 104387, $P<0.05$). This trend continued with the myeloid compartment. Females displayed a 10 fold increase (85645 vs 8603.5) in the number of $CD33^+$ myeloid cells in the spleen compared with males ($P< 0.0001$, 4.18(C)). There were also differences in lineage distribution of human T and B cells engrafted in male or female recipients (Figure 4.15). The percentages and absolute numbers of $CD3^+$ T cells in the spleen and liver were significantly increased when compared with males. Females displayed 3-fold (24.5 vs 6.95) and 6-fold (32.6 vs 5.1) percentage increase in the spleen and liver and 11- fold (66001 vs 5827) and 15- fold (90,317 vs 6002) increase in the absolute numbers ($P< 0.0001$, Figure 4.15). The absolute numbers of $CD20^+$ B cells in the spleen of female mice also increased 6 – fold (301,207 vs 45,879) compared with males. These results are consistent with Notta *et al* (2010), who found that the recipient gender played a critical role in the engraftment and proliferation of human HSCs. Therefore, $CD34^+$ cord blood cells repopulate at higher numbers in spleens and livers of females compared with age-matched male NSG-SCF mice.

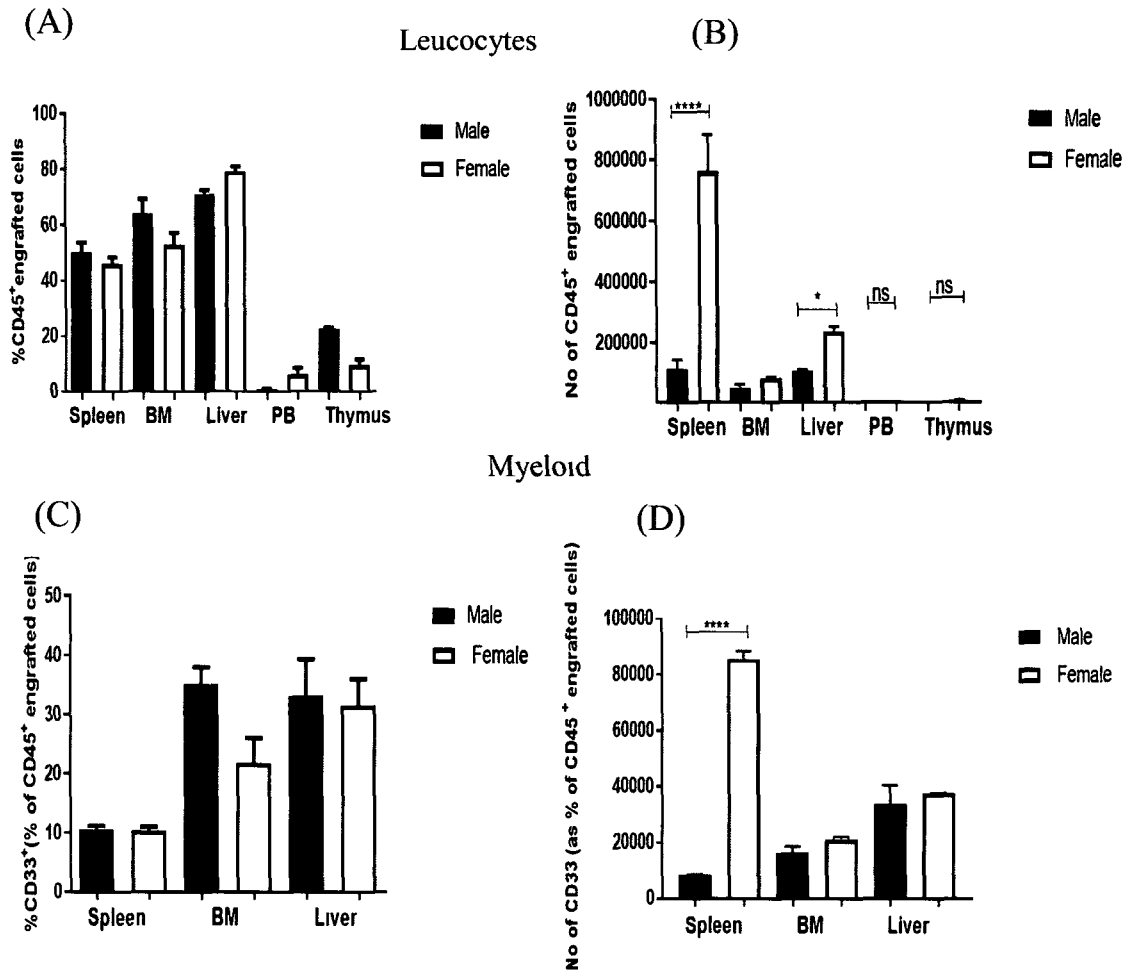


Figure 4.18 The total number of leucocyte and myeloid cells engrafted in the spleen of NSG-SCF mice were significantly increased in female mice NSG mice were set up exactly as described in figure 4 4 Tissues were harvested on week 20 and cells were recovered and stained with CD45 (PerCp) and CD33 (FITC) antibody and analysed by flow cytometry Graphical representation of the percentage(A) and number of human $CD45^+$ cells (B) percentage (C) and number (D) of myeloid cells recovered in the spleen, bone marrow, liver, thymus and peripheral blood The total number of human cells was assessed using counting beads during flow cytometry n=6 per group (1 HSC donor) at week 20 Statistical analysis was carried out using one-way ANOVA Tukey with multiple comparison test where **** <0 0001* <0 05

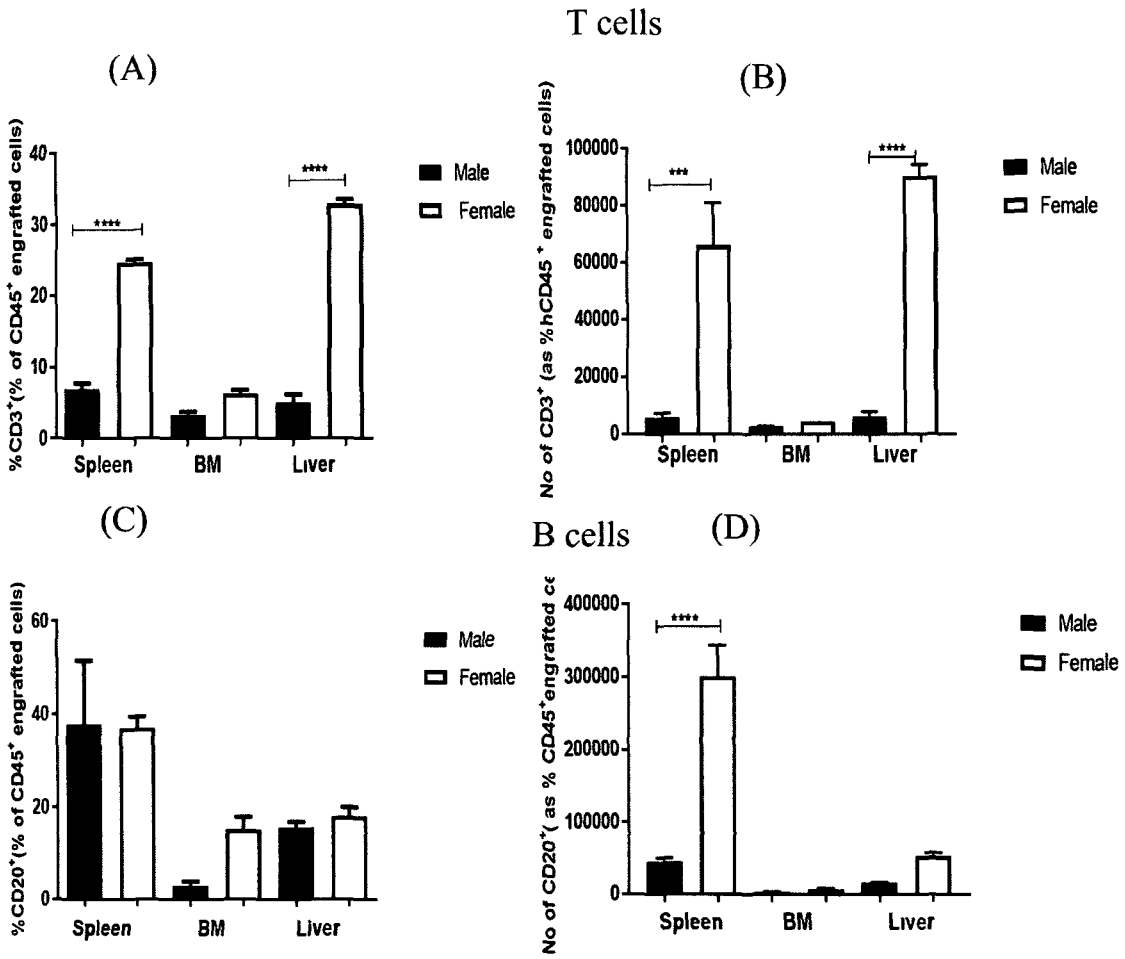


Figure 4.19. Engraftment of T and B cells in the spleen was significantly increased in female NSG-SCF mice NSG mice were set up exactly as described in figure 4 4 Tissues were harvested on week 20 and cells were recovered and stained with CD3 (APC) and CD20 (FITC) antibody and analysed by flow cytometry Graphical representation of the percentage (A) and number of human T cells (B) cells and percentage (C) and number (D) of B cells recovered in the spleen, bone marrow, and liver The total number of human cells was assessed using counting beads during flow cytometry n=6 per group (1 HSC donor) at week 20 Statistical analysis was carried out using one-way ANOVA Tukey with multiple comparison test where **** <0 0001* <0 05

4.9. EVEN AT LIMITING DOSES OF HSC FEMALE NSG-SCF MICE CONTINUE TO DISPLAY HIGHER NUMBERS OF HUMAN CHIMERISM COMPARED TO MALE MICE

As we have already shown in section 4. 8, female NSG-SCF mice are far superior hosts for the engraftment of higher numbers of leucocytes, myeloid T cells and B cells, in the spleen and livers of NSG-SCF mice. To explore this further, engraftment levels with a lower dose ($5x10^4$) of HSCs was examined in female versus male NSG-SCF mice.

Cord blood (6 hrs old) was obtained from donors at the NMH and $CD34^+$ cells were obtained as detailed in section 2.6.3. $CD34^+$ cell purity was > 95% (Table 4.1). Non-irradiated NSG-SCF male and female mice were injected with $5x10^4$ HSCs via the tail vein on day 0. After 12 and 20 weeks, flow cytometry was used to determine the levels of $CD45^+$ cell engraftment in peripheral blood. High levels of $CD45^+$ cells at 12 weeks (37.1%) and 20 weeks (45.7%) were observed in the peripheral blood of female NSG-SCF mice (Figure 4.20). In a side by side comparison experiment, male NSG-SCF mice had lower levels of $CD45^+$ in the circulation at 12 and 20 weeks (7.7% and 17.5 %) (Figure 4.20). Comparable percentages of $CD33^+$ myeloid cells were detected across all tissues at week 20 (Figure 4.21). In the bone marrow and livers of female NSG-SCF mice significantly increased percentages of $CD3^+$ T cells had engrafted compared to male mice ($P<0.001$,$P<0.05$, Figure 4.20). In addition there was a significant increase in the percentages of $CD20^+$ B cells in the livers of female mice compared to male mice ($P<0.05$) (Figure 4.21). This gender differential increase in lineage cells continued through to the mast cells where there was a dramatic increase in the number of $CD45^+Fc\varepsilon R1^+$ $CD203c^+$ cells in the spleens of female mice ($P< 0.0001$, Figure 4.22).

In summary, engraftment levels of high (1×10^5) and low (5×10^4) HSC doses was superior in female NSG-SCF mice providing better multilineage engraftment than their male equivalents Importantly this gender bias must be taken into account when designing humanised mouse model studies

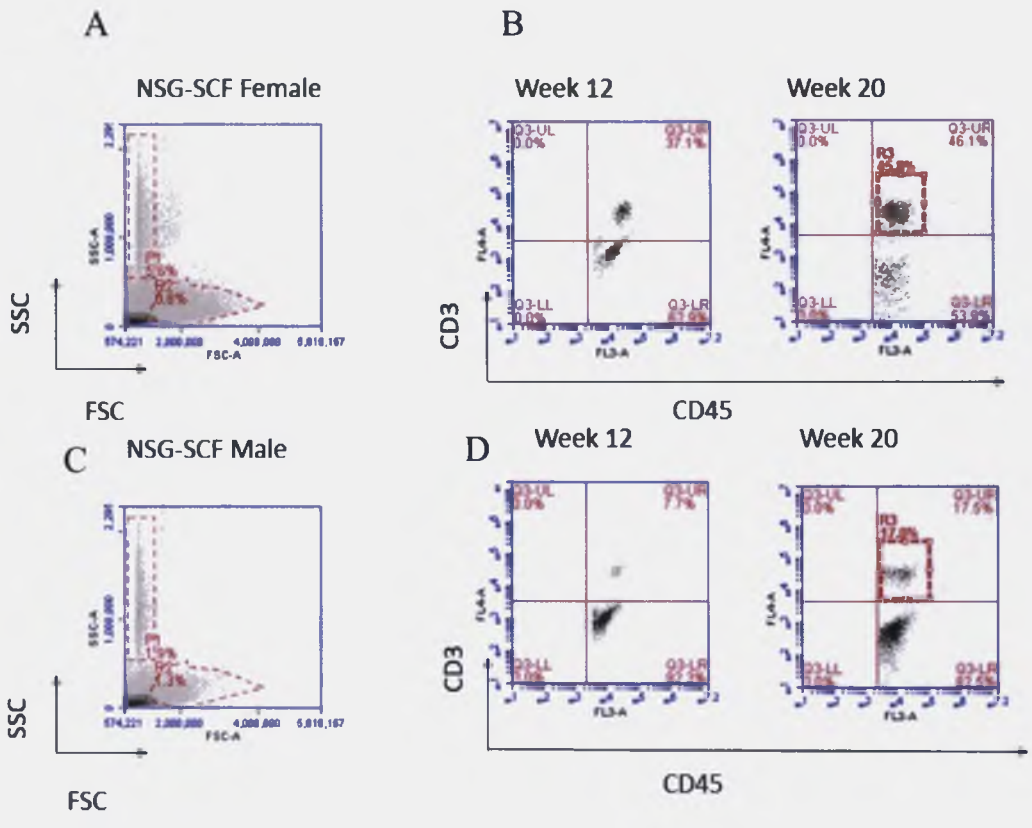


Figure 4.20. Enhanced T cell engraftment in peripheral blood of female mice at lower dose of HSC compared to male mice. Comparison of Female and male NSG – SCF mice engrafted with $CD34^+$ HSCs isolated from UCB from NMH. Tail bleeds were performed and the blood was lysed and stained with CD45 (PerCp) antibody and analysed by flow cytometry. (A) Illustrates the viable gated lymphocyte population of NSG-SCF from SSC against FSC plot (B) Percentage of human $CD45^+$ cell in the peripheral blood of one female NSG-SCF mouse. (C) Illustrates the gated lymphocyte population of male NSG-SCF from SSC against FSC plot and (D) Percentage of human $CD45^+$ in the peripheral blood of one male NSG-SCF mouse. Image representative of one tail bleed form each mouse n= 4 using one HSC NMH donor.

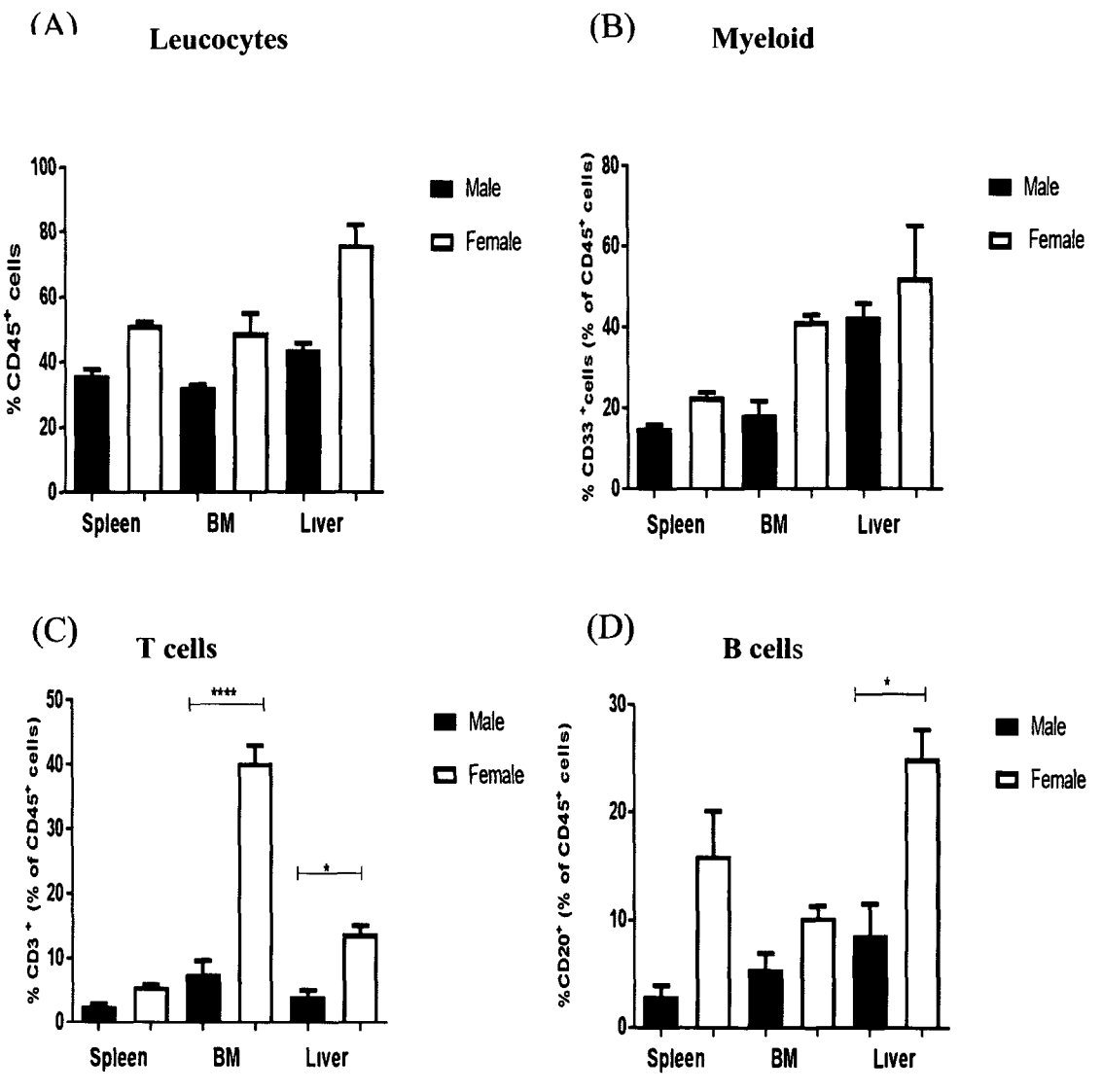


Figure 4.21 Female NSG-SCF mice display increase in engraftment compared to male mice. 20 weeks after adoptive transfer of 5 x 10^4 $CD34^+$ HSC from UCB from NMH Dublin, male versus female SCF mice were analysed for human $CD45^+$ (Leucocyte)(A), $CD33^+$ (Myeloid) (B), $CD3^+$ (T cells) (C) and $CD20^+$ (B cells) (D) engraftment Graphical representation of the percentage and numbers of leucocytes myeloid T cells and B cells in each organ The flow cytometry data are representative of 4 animals/group Statistical analysis was carried out by one way ANOVA Tukey with multiple comparison test where **** < 0 001

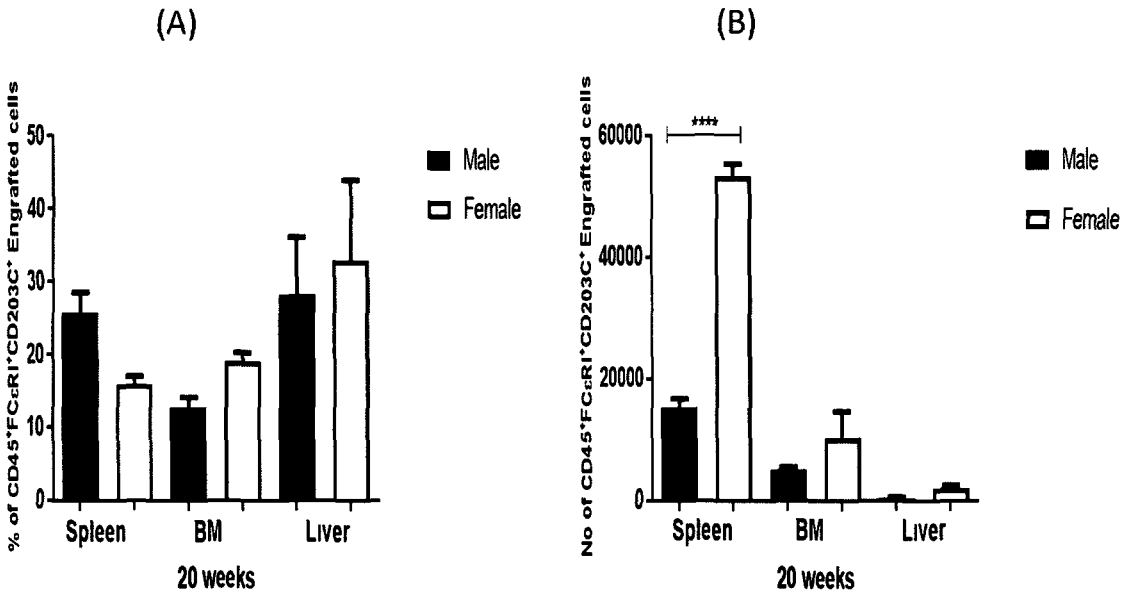


Figure 4.22. Increased number of mast cells engrafted in the spleens of female NSG-SCF mice injected with lower dose of HSCs compared to male mice. Cells were isolated from spleen, bone marrow and liver from hSCF mice at 20 weeks after HSC transplantation and were stained with anti-hCD45 (fitc), anti-hCD203C (pe) and anti-hFCεRI (apc) antibodies Graphical representation of the percentage (A) and numbers (B) of hCD203C$^+$FCεR1$^+$ CD45$^+$ mast cells in each organ The flow cytometry data are representative of 4 animals/group Statistical analysis was carried out by one way ANOVA Tukey with multiple comparison test where **** < 0 001

Table 4.2 Humanised mice generated with Anthony Nolan HSC

Mouse Group no	Strain	No of mice engrafted/No of mice surviving to end point	CD34 positive cells used	Mean Engraftment 20 weeks (% human CD45 $^{+}$ cells)				
				Spleen	BM	Liver	PB	Thymus
3	NSG	12/3	Anthony Nolan	43 25	41 15	78 35	11	6 8
4	NSG-SCF	12/12	Anthony Nolan	51 2	73 2	83 97	19 95	15 5
5	NSG-SGM3	12/7	Anthony Nolan	61 5	56	74 05	16 98	24 2

Table 4.3 Humanised mice generated with Lonza HSC

Mouse Group no	Strain	No of mice engrafted/No of mice surviving to end point	CD34 positive cells used	Mean Engraftment 20 weeks (% human CD45 $^{+}$ cells)				
				Spleen	BM	Liver	PB	Thymus
1	NSG-SCF	11/11	Lonza	46 9	56 4	88 2	12 75	21 7
2	NSG-SGM3	11/9	Lonza	40 4	34 15	79 36	19 42	20 3

Table 4.4 Humanised mice generated with NMH HSC

Mouse Group no	Strain	No of mice engrafted/No of mice surviving to end point	CD34 positive cells used	Mean Engraftment 20 weeks (% human CD45 $^{+}$ cells)				
				Spleen	BM	Liver	PB	Thymus
6	NSG-SCF Male	6/6	NMH	50 26	64 17	79 25	8 0	9 5
7	NSG-SCF Female	6/6	NMH	48 43	53 05	81 6	15 0	22 5
8	NSG-SCF Male $5x10^4$	8/8	NMH	36	35 4	43 8	5 4	4 7
9	NSG-SCF Female $5x\ 10^4$	8/8	NMH	51 45	49	76	21 65	7 1

4.10 SUMMARY

The mam aims of this chapter were to (1) isolate viable haematopoietic stem cells from umbilical cord blood cells and (2) to repopulate conditioned and non-conditioned NSG-SGM3/NSG-SCF mice with human immune cells using haematopoietic stem cells and to (3) identify the optimal strain of mice that best supported the development of human myeloid cells especially mast cells and basophils

As discussed in Section 4 1 human umbilical cord blood was chosen to repopulate NSG-SCF and NSG-SGM3 mice with human cells Thus establishing a source of human HSCs was the first step in the generation of humanised mice While awaiting ethics approval for procuring cord blood samples directly from women from NMH, umbilical cord blood was sourced from the Anthony Nolan non-profit cord blood bank, Nottingham, UK Of the three attempts to obtain $CD34^+$ HSC from Anthony Nolan cord blood sufficient purity and yield (enough cells to engraft mice) was only achieved once In the initial experiment $CD34^+$ HSCs were injected into 3 week old irradiated NSG, NSG-SGM3 and non-irradiated (NSG-SCF) mice to compare the engraftment levels of human cells However by week 20 only 25% of NSG and 58% of NSG-SGM3 mice had survived (Table 4 2) The deaths in mice was related to the high dose irradiation and engraftment of the human HSCs because 100% of un-irradiated transplanted NSG-SCF mice survived till the end of the study As a result there were lower levels of engraftment of human cells in the circulation and tissues of these two strains of mice than that seen in the tissues of NSG-SCF mice In terms of mast cell and basophil cell numbers, NSG-SCF mice had higher levels of mast cells in the spleen, bone marrow and liver in comparison to levels found in NSG-SGM3 mouse tissues

Procuring UMB from consented volunteers in NMH proved to be very unreliable both in terms of volumes and quality of UCB However sufficient volumes and yields of UCB were achieved on 4 occasions to allow investigations into if dose of HSC or gender have any effect on engraftment levels in NSG-SCF mice Overall, $CD34^+$ cord blood cells repopulated at higher numbers in spleens and livers of females compared with age-matched male NSG-SCF mice There was also increased engraftment of T cells and B cells in the bone marrow and liver at the lower dose of HSC Importantly, there was increased numbers of mast cells in the spleens of female NSG-SCF mice at this limiting dose

Due to the unreliability of both Anthony Nolan and NMH UCB, it was decided to purchase frozen vials of flow sorted $CD34^+$ HSCs were purchased from Lonza which allowed greater control over the timing of sample and processing and there were no issues with yield as each vial had a defined number of cells In this side by side comparison experiment with irradiated (1 0 cGy) NSG-SGM3 and non-irradiated NSG-SCF mice, the leucocyte engraftment levels in blood was significantly increased at 6 weeks in NSG-SCF mice compared with NSG-SCF mice (Figures 4 8) Both strains of mice had comparable myeloid engraftment across all tissues tested (Table 4 10A) but NSG-SCF had significantly increased $CD3^+$ T cells in the bone marrow and liver while NSG-SGM3 mice had significantly higher T cells in the circulation (4 10C) Similar percentages of mast cells and basophils were detected in spleen, bone marrow and liver in both mouse strains but there were differences in the distribution of these cells in the tissues of humanised mice In the spleen, bone marrow and livers of NSG-SCF and NSG-SGM3 mice, there were comparable % and number of mast cells across the tissues but when the c-kit expression was analysed there were more basophils detected in the bone marrow and livers of NSG-SGM3 mice

Isolating viable hematopoietic stem cells from umbilical cord blood was an arduous and unpredictable process SCF, also known as steel factor or kit ligand may serve as a guidance cue that directs HSCs to their stem cell niche and it plays an important role in HSC maintenance GM-CSF and IL-3 stimulate the differentiation of HSCs in myeloid progenitor cells and IL-3 stimulates proliferation of all cells in the myeloid lineage (erythrocytes, thrombocytes, granulocytes and monocytes) All of these cytokines are species specific and expression of human SCF, IL-3 and GM-CSF in NSG-SGM3 resulted in moderate levels of human $CD45^+$ engraftment in the spleen and bone marrow with higher engraftment in the livers of NSG-SCF and NSG-SGM3 mice Comparable total numbers of human leukocytes were detected in liver, spleen and bone marrow in both mouse strains 20 weeks after transplantation with lower levels detected in the PB and thymus in both strains (Table 4 2, 4 3)

There were no remarkable differences in the engraftment kinetics between NSG-SCF and NSG-SGM3 (summarised in table 4 2, 4 3) with the exception of higher engraftment of T cells in the bone marrow of NSG-SCF versus higher engraftment in the circulation of NSG-SGM3 and higher numbers of basophils in the bone marrow and livers of NSG-SGM3 Ideally more screening with multiple donors would have re-confirmed our findings but due to limited time and budget and to avoid the unnecessary complications with irradiation, the NSG-SCF strain was chosen as the host for the engraftment of human immune cells involved in the allergy response for future experiments By comparing male and female engraftment kinetics we found that gender does play a role at low and high doses of HSC in NSG-SCF mice Female NSG-SCF transplanted with lover dose of HSC had significantly higher numbers of T and B cells and mast cells than their male equivalents and at 20 weeks had higher levels of $CD45^+$

across all tissue tested (Table 4 4) and this was taken into consideration going forward to *in vivo* testing

CHAPTER 5

DEVELOPMENT OF A HUMANISED MOUSE MODEL OF OVALBUMIN INDUCED ALLERGY AND INFLAMMATION AS A PLATFORM TO TEST FUNCTIONAL FOODS

5.1 Introduction

The ability of milk protein hydrolysate 147 to suppress immune responses *in vitro* and some of the associated mechanisms through which this occurs were identified in chapter 3 However the findings that hydrolysate 147 can modulate the immune response (Chapter 3) needs to be proven in an *in vivo* setting In Chapter 4 mice were developed with a humanised adaptive immune system complete with tissue engraftment by human mast cells In this chapter, the next step was to utilise our humanised mouse system in a relevant food allergy (ovalbumin) model with functional human immune systems capable of mounting T helper specific responses against the common food allergen ovalbumin This study sought to test the capacity of the functional food component hydrolysate 147 as an anti-inflammatory agent in a food (OVA) driven allergic inflammatory humanised mouse model

Mouse models for human allergies including atopic dermatitis (Kotani *et al* 2000), asthma (Iwata *et al* 2003) and food allergies (Li *et al* 2001, Dearmann *et al* 2005, Bowmann *et al* 2009, Knippels *et al* 2009) have allowed researchers to elucidate important fundamental principles of the cellular and molecular mechanisms of these diseases These studies, together with the development of methods for the creation of transgenic, knockout, and knockin mice, have provided added impetus and powerful tools for mouse research, and have led to a dramatic increase in the use of mice as model organisms Studies on mice have contributed immeasurably to our understanding of human biology All too often, however, mice respond to experimental interventions in ways that differ markedly from humans Endostatin, the anticancer drug, is but one of many treatments that cure cancer in mice but have

limited effectiveness in humans (Perlman *et al* 2016) Interestingly, allergy is one field in which the transcriptional analysis approach has shown remarkable consistency between murine and human samples For example, a recent study using a murine model of atopic dermatitis (AD) included comparisons with data from affected human skin and showed a high degree of homology in the gene expression profile (Ando *et al* 2013) Using genetically modified mice the authors definitively showed key roles for T cells and mast cells in disease pathogenesis Similarly, in a murine model of severe asthma, Yu *et al* (2013) performed transcriptional comparison analysis between the murine lung and patient lung biopsy specimens Their data elegantly showed a highly significant association in gene expression patterns that was lost in mast cell deficient mice but restored if mast cells were reconstituted by means of adoptive transfer There is no doubt that such validation approaches will be an important aspect of mechanistic studies moving forward, especially given that researchers in the field of allergy possess a strong collection of tools to study the diseases

One such tool that can be exploited are humanised mouse models that bring us one step closer to the human situation Mice that are administered human stem cells develop a human immune response, as opposed to the immune response which a mouse would normally display Humanised mouse models can be adapted to provide a humanised model that would better mirror human immune responsiveness, and therefore potentially provide more meaningful information in terms of treating allergic disease in humans rather than the information provided by the current mouse model Humanised mouse models of inflammation and food allergy provide a platform from which novel therapies can be assessed and their performance of alleviating allergic conditions can be investigated in a clinically relevant manner Recently in a humanised mouse model of peanut allergy, Burton *et al* (2016) used NSG-SCF mice

to successfully predict the allergenic potential of peanut proteins and demonstrated the human food allergic sensitisation/ allergic response could be abolished by anti-IgE (omalizumab) treatment They elegantly showed that in animals receiving omalizumab treatment before sensitisation were completely protected from loss of core body temperature (symptom of anaphylaxis) in response to peanut challenge which resulted in a significant increase in serum levels of total IgE, an effect that is commonly observed in human subjects treated with the antibody that is attributed to the persistence of stable circulating omalizumab IgE complexes (Burton *et al* 2016) In a similar manner we propose to generate a humanised mouse model of OVA allergy to successfully predict the allergenic potential of ovalbumin (egg) proteins and demonstrate that the inflammatory/allergic response can be elevated by a milk peptide hydrolysate treatment

Therefore the objectives of this chapter were,

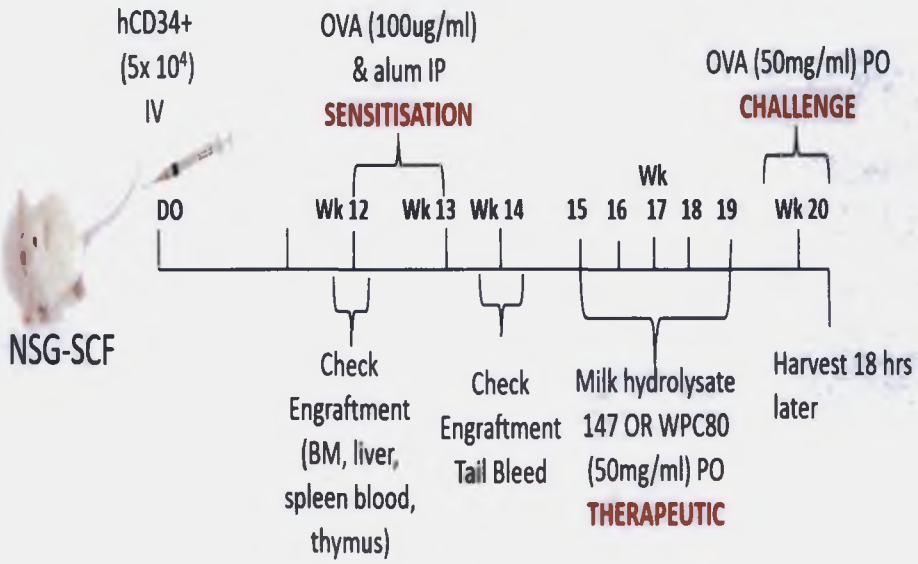
- To assess the immunomodulatory capacity of novel milk whey derived protein hydrolysate 147 in a humanised mouse model of OVA inflammation against whey protein concentrate control
- To determine if milk protein hydrolysate 147 has anti-inflammatory/anti-allergic effects in the humanised model of OVA inflammation

5.2. HUMANISED MICE POSSESS SUFFICIENT LEVELS OF HUMAN CELLS AT WEEK 12 BEFORE ALLERGEN SENSITISATION

The first step in the development of the model was to ensure sufficient engraftment of human $CD34^+$ cells as variability can exist across hCB $CD34^+$ stem cells donors Human umbilical cord blood–derived $CD34^+$ cells were purchased from Lonza (Basel, Switzerland) After thawing the frozen vials of cells according to the manufacturer's protocol, all the cells (over 90% viability) were used for transplantation 5×10^4 cord blood $CD34^+$ hHSC were transplanted i v via the tail vein into 84 female NSG-SCF on day 0 (Figure 5 1) 12 weeks later a small group of NSG-SCF mice were analysed to determine levels of engraftment of human hematopoiesis in the spleen, bone marrow, liver, thymus and peripheral blood to check that there was sufficient levels of engraftment of human cells present before a sensitisation regimen could commence Flow cytometry was performed to evaluate the engraftment levels of human $CD45^+$ cells Engraftment levels of human $CD45^+$ leucocytes in the spleen, liver, bone marrow, thymus and peripheral blood were high (mean 72 4%, 75 37%, 79 63%, 32 17% and 53 3% respectively) (Figure 5 2A) The development of human lymphoid and myeloid cells in the engrafted human CD45 hematopoietic cell populations by flow cytometry using antibodies against hCD3, hCD19 and hCD33 was next examined The percentage of human $CD33^+$ myeloid cells within the total human $CD45^+$ population was high in the spleen, bone marrow and liver of NSG-SCF mice (Figure 5 2 B, C, D) Comparable percentages of $CD19^+$ B cells were also found in all three organs (19 6%, 22 85%, and 28 24% respectively) (Figure 5 2 B,C,D) In the spleen, bone marrow and liver a similarly moderate human $CD3^+$ T cell chimerism of approximately 22% was achieved (Figure 5 2 B, C, D) Mast cells and basophils are the primary effector cells in allergic responses thus so it was important to examine the

development of human mast cells and basophils in each CB $CD34^+$ before commencement of an immunisation regime Overall the percentages of $CD45^+$ $CD203c^+$ $ckit^+$ (mast cell) or $CD45^+$ $CD203C^+$ $ckit^-$ (basophils) within the spleen and bone marrow were similar (Figure 5 2 B, C, D) However, a higher percentage of human mast cells was achieved in the liver of NSG-SCF mice (Mean 24%) Previously in chapter 4, higher percentage and numbers of human mast cells had engrafted in the spleen, bone marrow and livers of female NSG-SCF mice by 20 weeks (Figure 4 6) It is very promising for the success of the model that even at 12 weeks post transplantation of $CD34^+$ cells mast cells and basophils had engrafted and at sufficient levels

In summary, NSG-SCF mice had sufficient levels of immune cell engraftment of leucocytes, myeloid, T, B, mast cells and basophils in the spleen, bone marrow and liver, 12 weeks post transplantation of $CD34^+$ HSCs This equates to engraftment of a functional human immune system for use in investigating the therapeutic effect of hydrolysates in a model of OVA induced allergy and inflammation



Readouts

Engraftment data (wk 12) – Flow cytometry
Small Intestine– Histology
Serum IgE, IGg1, Ova-IgG1
Mesenteric lymph node –Ova stimulation and Cytokine analysis
Cytokine Analysis: Small Intestine, Spleen, Liver – (ELISA)
Analysis of Th1 (IFN-g), Th2 (IL-4), Treg, CD33+ Myeloid cells, DC in Spleen, BM and liver

Figure 5.1. Development of humanised mouse model of OVA allergy. 6-8 week old female NSG-SCF mice were injected by tail vein injection with 5 x 10^4 HSC on day 0. Tissues were harvested on week 12 to check engraftment in spleen, bone marrow, liver, peripheral blood and thymus. Sensitisation: Mice were sensitised by intraperitoneal injection of either OVA (100ug/ml) with alum or saline with alum once a week for two weeks (week 12 and week 13). Tail bleeds were performed to check the levels of IgE antibody production by ELISA. Treatment: 147 hydrolysate or whey protein control was delivered by oral administration for 5 doses (50mg/ml per dose) once a week for 5 weeks from week 15 to week 19. Challenge: One week later (week 20) all mice were challenged with OVA antigen (50mg/ml) via oral delivery. The development of OVA allergy was monitored 30 minutes after challenge. 18 hrs later all mice were humanely sacrificed and read outs performed.

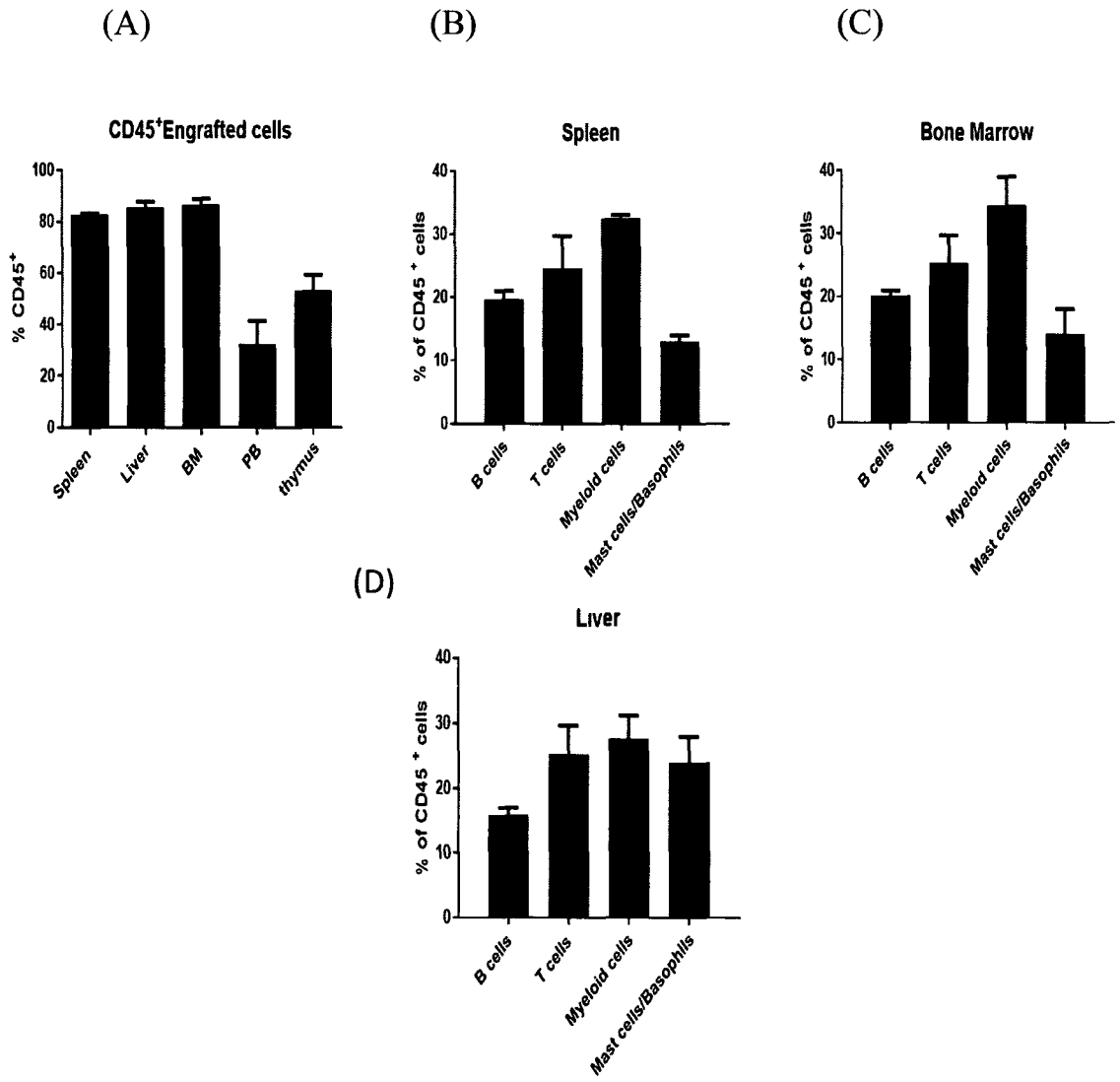


Figure 5.2. Engraftment of human cells in female NSG-SCF mice 12 weeks after transplantation of CD34$^+$ cells. Mice were set up exactly as described in figure 5 1 Tissues were harvested on week 12 and cells were recovered and stained with antibodies and analysed by flow cytometry Graphical representation of the (A) percentage of human CD45$^+$, cells recovered in the spleen, bone marrow, liver, thymus and peripheral blood of mouse (B) percentage of B cells (CD19$^+$), T cells (CD3$^+$), myeloid cells (CD33$^+$) and mast cells/basophils (CD45$^+$CD203$^+$FcεR1$^+$) recovered in the spleen (C) percentage of B (CD19$^+$), T (CD3$^+$), myeloid (CD33$^+$) and mast cells/basophils (CD45$^+$CD203$^+$FcεR1$^+$) recovered in the bone marrow (D) percentage of B (CD19$^+$), T (CD3$^+$), myeloid (CD33$^+$) and mast cells/basophils (CD45$^+$CD203$^+$FcεR1$^+$) recovered in the liver of OVA sensitised mice n=2 per group (4 HSC donors) at week 12

5.3 MICE SENSITISED TO OVA IN THE PRESENCE OHYDROLYSATE 147 AND WHEY PROTEIN CONTROL ARE PROTECTED FROM SYSTEMIC ANAPHYLAXIS-LIKE SYMPTOMS AFTER SUBSEQUENT ORAL CHALLENGE WITH OVA.

Depending on the route of exposure, dose of allergen and the presence of a suitable adjuvant, the immune response can result in either sensitisation or mucosal tolerance induction (Perrier *et al* 2010, Mine *et al* 2007 and Repa *et al* 2008) An effective approach to overcome the oral tolerance induction is pretreatment of mice by systemic intraperitoneal (i p) administration of allergen with aluminium hydroxide (alum) a potent T_H2 adjuvant followed by repeated intra-gastric treatments (Dearman *et al* 2007) Brandt *et al* (2009) used a modification of the widely used murine model of asthma to induce small intestinal allergic inflammation After two systemic administrations of OVA in alum, exposure of mice to OVA by the oral route resulted in the generation of an acute antigen-specific diarrhea and allergic inflammation of the small intestine This study utilised a modification of the latter food allergy model by including a treatment regimen after sensitisation and before challenge Specifically this study sought to determine if allergy specific immunotherapy in the form of milk peptide hydrolysate 147 could prevent the allergic response NSG-SCF mice were divided into four groups, OVA sensitised, PBS (control), OVA sensitised plus hydrolysate (147) and OVA sensitised plus whey protein control (wpc80) (Table 5 1) All groups of NSG-SCF mice (6-11 per group) were transplanted with 5 x 10^4 cord blood $CD34^+$ hHSC i v via the tail vein on day 0 After checking for engraftment of human immune cells at 12 weeks mice were sensitised by intraperitoneal injection of either OVA (100ug/ml) with alum or saline with alum once a week for two weeks (week 12 and week 13) 147 hydrolysate or whey protein control was delivered by oral administration for 5 doses (50mg/ml per dose) once a week for 5 weeks from

week 15 to week 19 (Figure 5.1). One week later (week 20) all mice were challenged with OVA antigen (50mg/ml) via oral delivery and the systemic anaphylactic symptoms were evident within 15 to 30 minutes. The severity of anaphylaxis was scored as indicated in Table 5.2. The most severe reactions were observed in mice sensitized with OVA plus alum and challenged with OVA antigen (50mg/ml). In the OVA treated group systemic anaphylaxis-like symptoms developed within 30 mins in four of seven mice. Mice responded with laboured respiration (marked change in breathing) and lethargy and were huddled. Control mice (PBS) challenged with OVA antigen (50mg/ml) did not respond with any of the symptoms. In contrast, the symptoms were significantly reduced in mice of the hydrolysate 147 and wpc80 control group. 5 of 12 (147 treated) and 6 of 11 (wpc80) mice responded with scratching around the nose and head and the remainder of mice displayed no symptoms (Figure 5.3). Our experimental regime (one oral challenge) of OVA however, did not induce any acute responses such as allergen-induced diarrhea as all mice had normal faeces.

Table 5.1 Experimental groups

Experimental groups	Sensitisation	Treatment	Challenge
Sensitised OVA/Alum	OVA	-	OVA
Non-sensitised PBS/Alum	PBS	-	OVA
Sensitised OVA/Alum	OVA	147	OVA
Sensitised OVA/Alum	OVA	WPC80	OVA

Table 5.2 Anaphylaxis scoring system

Score	Symptoms
0	No Symptoms
1	Scrathing around nose and mouth
2	Swelling around the eyes and mouth; pillar erection; reduced activity; higher breathing rate
3	Shortness of breath; blue rash around the mouth and tail; higher breathing rate
4	No activity after stimulation, shivering and muscle contractions
5	Death by shock

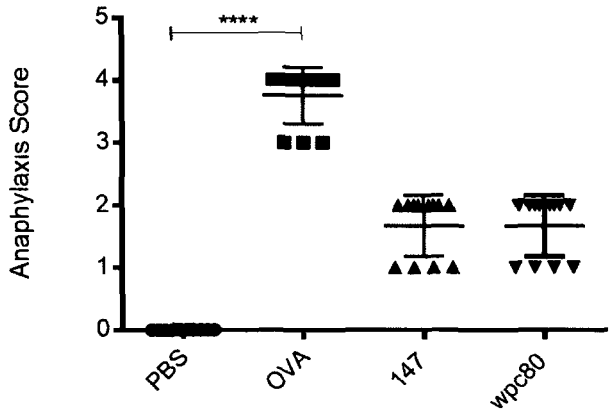


Figure 5.3 Hydrolysate 147 and wpc80 control significantly reduced the systemic anaphylactic (symptom) scores in OVA sensitised mice. 6-8 week old female NSG-SCF mice were injected by tail vein injection with 5 x 10^4 HSC (Lonza) on day 0 Tissues were harvested on week 12 to check engraftment in spleen, bone marrow, liver, peripheral blood and thymus Mice were sensitised by intraperitoneal injection of either OVA (100ug/ml) with alum or saline with alum once a week for two weeks (week 12 and week 13) and on week 14 tail bleeds were performed to check the levels of IgE antibody production by ELISA 147 hydrolysate or whey protein control was delivered by oral administration for 5 doses (50mg/ml per dose) once a week for 5 weeks from week 15 to week 19 Mice (n = 6 to 11) were challenged intragastrically with OVA (50mg/ml) Thirty mins later, the symptoms of anaphylaxis were scored on a scale from 0 (no symptoms) to 5 (death), closed circles, closed square, closed triangles indicate individual mice Statistical analysis was carried out by one way Anova Tukey with Multiple Comparison Test where **** < 0 0001

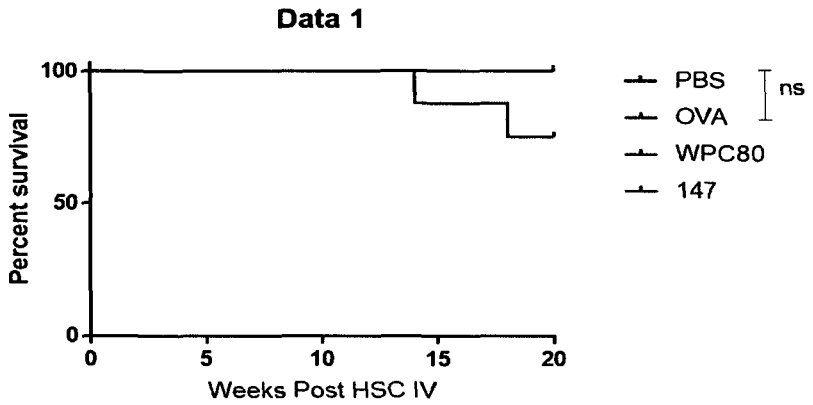
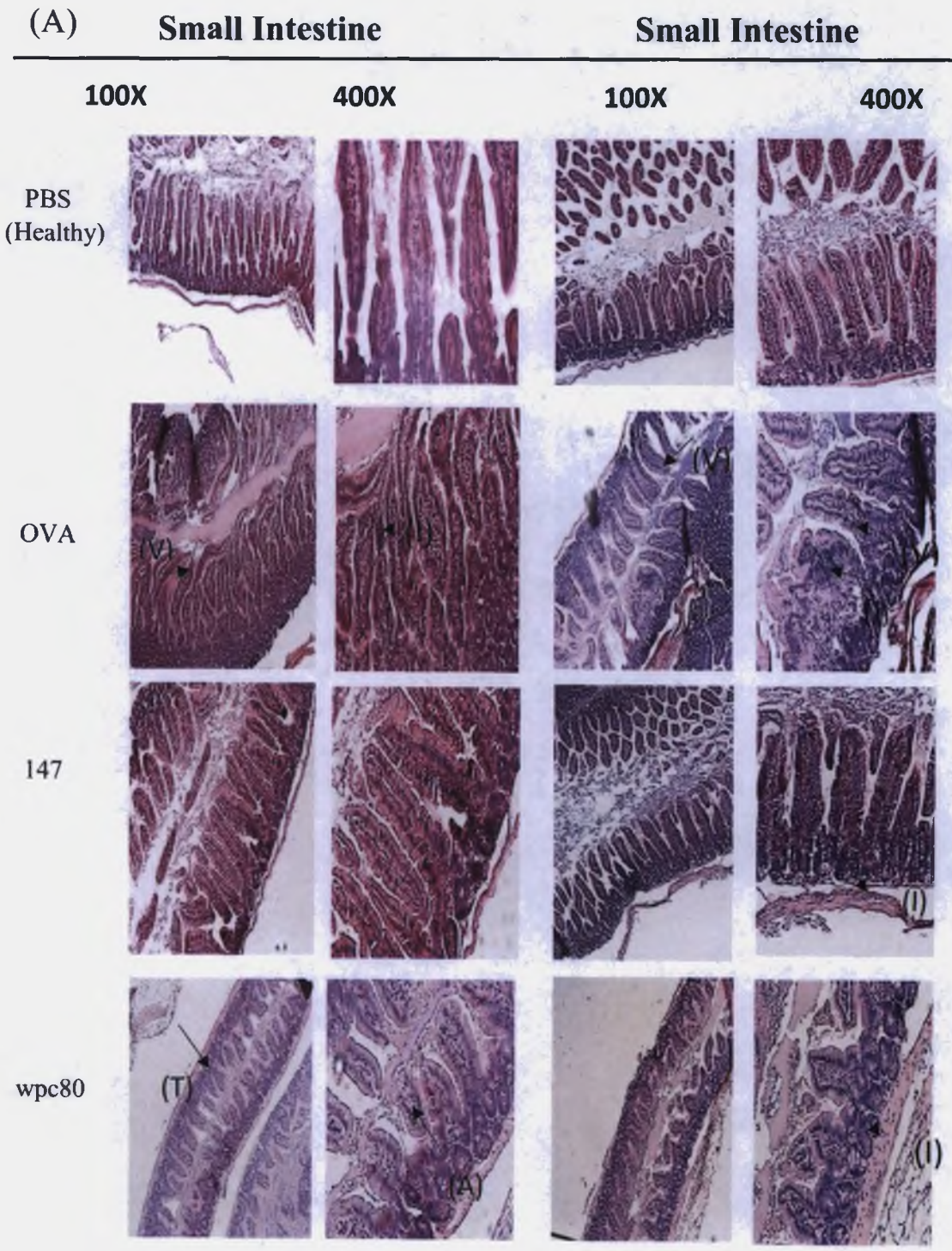


Figure 5.4.Ova sensitised mice had reduced survival in comparison to 147 and wpc80 treated mice. 5 x 10^4 human HSC were administered i v to female NSG-SCF mice on day 0 Mice were sensitised by i p injection of either (100µg/ml) with alum or saline with alum once a week for two weeks (week 12 and week 13) 147 hydrolysate or whey protein control was delivered by oral administration for 5 doses (50mg/ml per dose) once a week for 5 weeks from week 15 to week 19 One week later (week 20) all mice were challenged with OVA antigen (50mg/ml) via oral delivery Mice were monitored three times a week for the duration of the experiment n=6 for PBS and OVA groups, n= 11 for 147 and wpc80 treated groups

5.4. HYDROLYSATE 147 AND WPC80 SIGNIFICANTLY IMPROVED OVA-DRIVEN ALLERGIC GI PATHOLOGY

The primary target organs for allergic reactions are the skin and the respiratory system but the main immune inflammatory events occur in the gastrointestinal tract and these events are central to the pathophysiology of food allergy (Knight *et al* 2007) OVA sensitisation hampered the survival of NSG-SCF mice (75% survived) whereas 100% of the mice survived in 147 and wpc80 treated groups (Figure 5 4) It is hypothesised that feeding hydrolysates between the sensitisation and challenge phase may alter the "inflammatory" status of the gastrointestinal tract Therefore histological analysis was carried out to compare hydrolysate 147 efficacy against the efficacy of it's parent control wpc80 in alleviating OVA-driven pathology in the small intestine NSG-SCF mice received HSC (5×10^4) on day 0 After two systemic administrations of OVA in alum (Week 12 & 13), 147 hydrolysate and whey protein control 80 (wpc80) were administered once a week for 5 weeks (50mg/ml) via oral gavage in the humanised OVA model (Figure 5 2) One week after treatment with 147 hydrolysate or wpc80, oral challenge with 50mg/ml of OVA antigen was performed 18 hrs later the OVA target organ (small intestine) was harvested and placed in formalin for histological analysis Tissue sections were stained with H&E and the histological score was evaluated for each treatment group according to the criteria described in section 2 10 4 (Erben *et al,* 2014) Histological analysis of the jejunum in the small intestine showed that OVA sensitised mice exhibited characteristics of allergic inflammation which included lymphocyte infiltration (I) or blunting (v) (Figure 5 5 A&B) PBS control mice exhibited normal small intestinal tissue morphology with no accumulation of infiltrating cells However OVA sensitised mice that received no therapy displayed frequent villi destruction and blunting accompanied by infiltrating lymphocytes

(Figure 5 5 A &B) OVA sensitised mice that received 147 hydrolysate and whey protein control (wpc80) were shown to have similar effects in improving small intestine pathology (Figure 5 5B) 147 significantly reduced the level of villi destruction and lymphocyte infiltration into the lamina propria (Figure 5 5 A&B) However both 147 and wpc80 had infiltrating lymphocytes in the connective tissue wpc80 displayed more inflammatory changes such as villous atrophy, (A) crypt elongation (E) and thickened basal layers (T) in comparison to 147 hydrolysate treatment The histology findings suggest that hydrolysate 147 and whey protein control mediate significant protection in the jejunum of the small intestine as illustrated by less inflammatory pathology in this organ


(A)
Small Intestine
Small Intestine
100X
400X
100X
400X
PBS
(Healthy)
OVA
147
wpc80
(V)
(V)
(V)
(I)
(T)
(A)
(I)

(B)

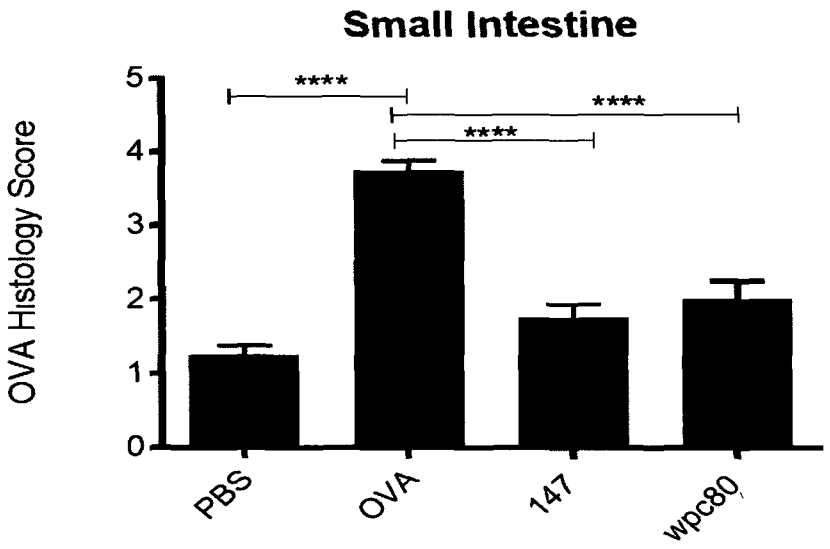


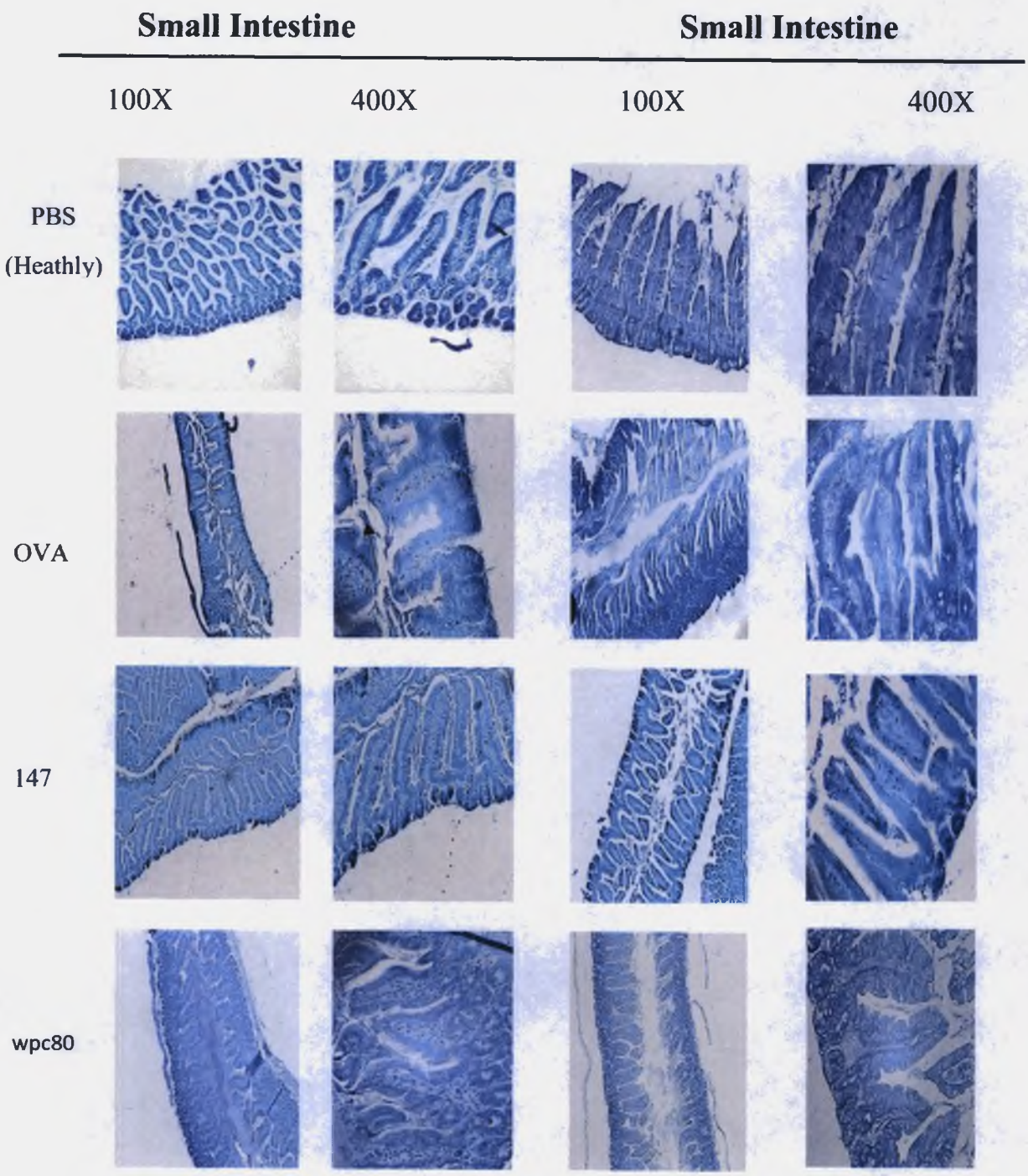
Figure 5.5: Milk peptide whey hydrolysate 147 and whey protein concentrate control (WPC80) treatment significantly reduced villi destruction in the jejunum of OVA mice. The OVA model was set up exactly as described in figure 5 2 Tissue samples were harvested 18 hr after the OVA challenge on week 20, formalin fixed, paraffin embedded and stained with H&E (A) Represented images were analysed for lymphocyte infiltration (I) and villi destruction and displayed for each group Images were captured at 100X and 400X A well defined histology scoring system as described in section 2 10 4 was carried out blinded and used to determine the level of allergic inflammation between the groups in the small intestine (B) n=6-11 per group (4 HSC donors) Statistical analysis was carried out using one way ANOVA Tukey where ****< 0 0001

5.5. MAST CELLS WERE RESIDENT IN THE CONNECTIVE TISSUE OF OVA SENSITISED NSG-SCF MICE.

Animal and clinical studies have revealed that sensitisation to allergens and subsequent mast cell activation by cross-linking of FcεRI with IgE and food antigen are critical for the induction of food allergy (Brandt *et al* 2003, Lin *et al* 2002) As we have already observed, the engraftment of human mast cells in the spleen, bone marrow and liver at 12 weeks (Figure 5 2) it was important to investigate the presence of mast cells in the gastrointestinal tissues Many of the symptoms are mediated by mast cell activation in the GI leading to release of inflammatory mediators that drive much of the allergic reaction with pathological consequences (Karra *et al* 2009) As in the previous experiment on day 141, 18 hrs after OVA challenge small intestine was harvested and placed in formalin for histological analysis Tissue sections were stained with toluidine blue to identify the presence of mast cells Mast cells are often classified according to their location or protease subtype and typically are grouped as mucosal or connective tissue-type subpopulations of mast cells (Galli *et al* 1984) No mast cells were identified in the jejunum of PBS, 147 hydrolysate treated or wpc80 treated mice (Figure 5 6) However on closer examination of the connective tissue in the submucosa of OVA sensitised mice, small numbers of mast cells were present (Figure 5 6) Toluidine blue stained mast cells red- purple or violet (metachromatic staining) and the background blue (orthochromatic staining) Connective tissue mast cells contain numerous granules rich in histamine and serotonin (Friend *et al* 1996) Mast cells appeared to be intact and elongated in form and were purple in colour but appeared to have weak purple granules which indicates an intermediate level of sulfation of heparin monomers It is known that the level of sulfation of heparin determines its functional activity (Faarberg *et al* 2001) Overall there were limited numbers of mast cells present and no mast cells revealed features of de-granulation in

the connective tissue of OVA sensitised mice The enzyme histochemical detection of chloroacetyl esterase was also used to try and detect mast cells in the jejunum of these mice but unfortunately this method revealed no mast cells (data not shown)

(C)


Small Intestine
Small Intestine
100X
400X
100X
400X
PBS
(Heathly)
OVA
147
wpc80

(A)

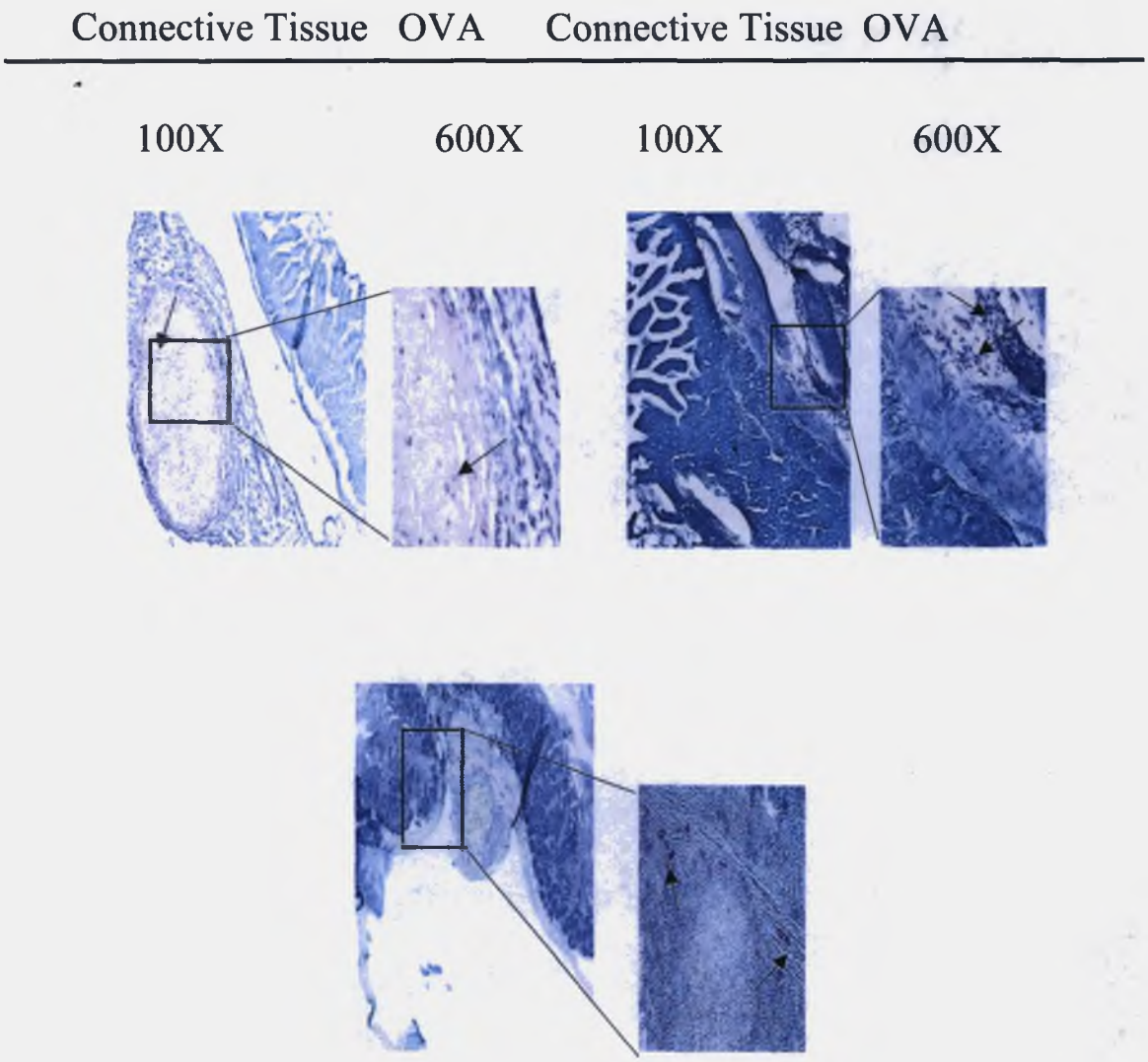


Figure 5.6. Mast cells were only visible in connective tissue of OVA sensitised NSG-SCF mice that received no therapy. The OVA model was set up exactly as described in figure 5.2. Tissue samples were harvested on day 141 (18h after the OVA challenge) formalin fixed, paraffin embedded and stained with toluidine blue.

(A) Representative images of β-metachromasia mast cells in the connective tissue in the submucosa of the small intestine. Images were captured at 100X and 600X. n= 6 per group (4 HSC donors).

(C) Representative images of Toluidine blue stained jejunum with no mast cells present in PBS, OVA, 147 and WPC80 treated tissue.

5.6 HYDROLYSATE 147 TREATMENT ALTERS THE OVA-SPECIFIC IgG1 ANTIBODY LEVELS IN A HUMASISED MOUSE MODEL OF OVA ALLERGY

IgE antibodies can play an important role in conferring immunologic specificity to effector cell activation in patients with allergic disease (Galli *et al* 2012) IgE is the isotype found at by far the lowest concentrations in the circulation (50-200 ng/ml total circulating IgE in healthy subjects vs approx 10mg/ml for IgG) (Dullaers *et al* 2012) However IgE can be found at much higher levels in patients with allergic diseases (Platts *et al* 2016) Ova sensitisation in mice is known to induce IgE and a powerful T_H2 response Therefore it was important to explore whether oral treatment with whey hydrolysate 147 could decrease total IgE antibody concentrations in OVA sensitised mice After transplantation of human $CD34^+$ HSCs on day 0 and checking for engrafted human immune cells at week 12, mice were sensitised by intraperitoneal injection of either OVA (100ug/ml) with alum or saline with alum once a week for two weeks (week 12 and week 13) One week later, tail bleeds were performed and serum IgE antibody levels were determined by ELISA Low levels of total IgE antibody were detected in the serum of control mice whereas there was a significant increase in IgE antibody produced in the serum of OVA sensitised mice (Mean 79 85 vs 651 15 P<0 0001) one week after the last sensitisation (Figure 5 7A)

In contrast, levels of IgE detected in the sera of all groups at week 20 (after treatment and challenge) were similar (Figure 5 7B) It is important to note that PBS mice were challenged with OVA and this is likely to result in the increase from 100ng/ml to 400ng/ml seen between figure 5 7 A and B Clearly IgE levels alone do not explain susceptibility to allergic disease Many patients can experience near-fatal anaphylaxis despite having low or undetectable levels of circulating IgE (Simons *et al*

2007) Thus, in addition to IgE there is an important humoral response generated during primary sensitization that leads to production of allergen-specific IgG1 Qualitative changes in the OVA-specific IgG antibody response may possibly be an important mechanism underlying the clinical efficacy of 147 hydrolysate immunotherapy As in the previous experiment, blood samples were collected at sacrifice on day 141 Total serum IgG1 and OVA specific serum IgG1 levels were determined by ELISA There were low levels of total IgG1 detected in control mice compared to significantly high levels in OVA sensitised and wpc80 treated mice, ($P<0.01$, $P<0.05$) (Figure 5 8 A) Significantly higher levels of OVA-specific IgG1were induced in the OVA and OVA and wpc80 treated groups ($P<0.001$, $P<0.05$) However there was a significant reduction in OVA specific IgG1 produced in the sera of OVA and 147 hydrolysate treated mice ($P < 0.01$) (Figure 5 8B)

In summary, treatment with hydrolysate 147 and its control wpc80 had little or no effect in changing the levels of total IgE levels However the ability of 147 to dampen T_H2 associated OVA-specific IgG1 may give an indication of the potential success of this treatment

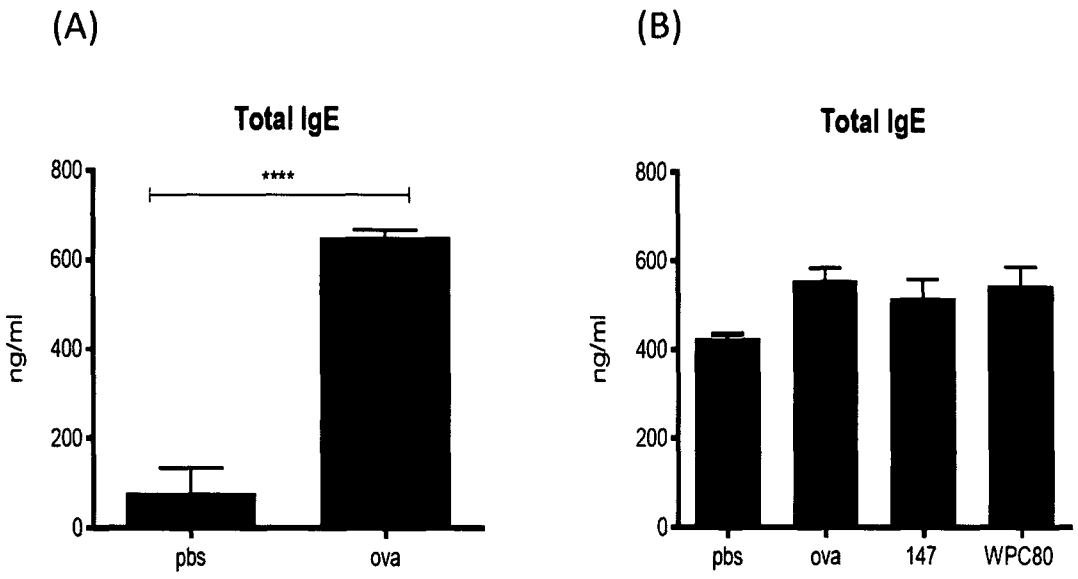


Figure 5.7 Oral treatment with milk protein hydrolysate 147 or WPC80 does not change total IgE antibody responses to sensitising allergen. Tail bleeds were performed 1 week after the final sensitisation (week 14), serum was processed and frozen at -80^{0}C until analysis of total IgE by ELISA (Ready Set-GO kit, eBioscience San Diego ,USA) In the same way at end of study on day 141, facial bleeds were performed, processed and frozen at -80^{0}C until analysis of total IgE by ELISA (A) Levels of IgE detected in the serum in PBS and OVA sensitised mice at 14 weeks and (B) Levels of IgE detected in serum of PBS, OVA sensitised, OVA sensitised and 147 treated and OVA sensitised and wpc80 treated mice at 20 weeks IgE levels were calculated by reference to a human IgE standard curve Statistical analysis was carried out using unpaired student *t*-test where **** <0 0001

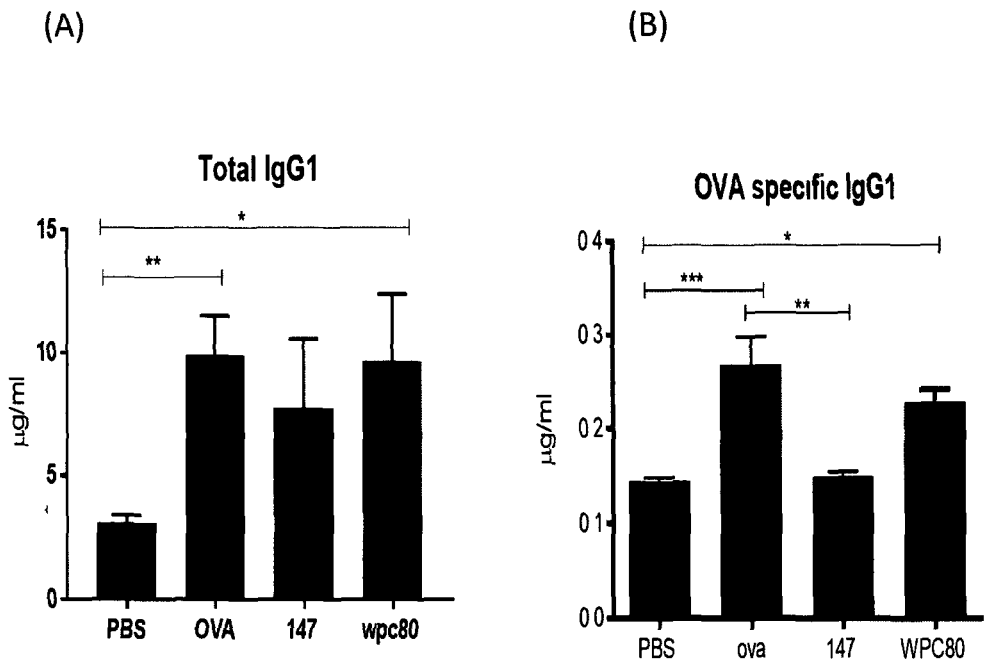


Figure 5.8. Hydrolysate 147 treated mice exhibit a significant suppression of anti-ova IgG1 antibody response. The OVA model was set up exactly as described in Figure 5 2 On day 141, mice were bled through the facial vein and 200µl of blood was collected Serum was processed and frozen at -80°C until analysis Levels of total IgG1 and OVA-specific IgG1 in serum were determined by ELISA (Ready-SET-GO, eBioscience, San Diego, USA) Briefly, 96-well plates were coated either with OVA (5µg/ml) or anti-human IgG1 (10µg/ml) overnight Serum samples were diluted 1/100 for IgG1 and 1/10 for OVA specific IgG1 Anti-human IgG1 were applied followed by HRP-conjugated anti-human IgG antibody (1/250) for detection (A) Total IgG1 antibody level and (B) OVA specific IgG1 detected in sera of PBS, OVA, 147 treated and wpc80 control treated mice IgG1 levels were calculated by reference to a human IgG1 standard curve Statistical analysis was carried out with one way ANOVA Tukey with multiple comparison test where ***<0 001, **<0 01, *<0 05

5.7 EX VIVO CYTOKINE PRODUCTION BY MLN REDUCED BY HYDROLYSATE 147 TREATMENT.

Mesenteric lymph nodes (MLNs) are an important site of T cell activation in the small intestine and activation within the MLNs is likely a key event in the development of T_H2-biased inflammation within the gastrointestinal tract (Knight *et al* 2007) As there were differences detected in antibody responses between the groups of mice the question was posed if the differences in antibody responses were also associated with cytokine milieu Mesenteric lymph nodes (MLN) were removed at sacrifice (day 141) Single cell suspensions were prepared in RPMI-1640 containing 10% fetal bovine serum and 1% penicillin-streptomycin Cells (2 x 10^5/well) were cultured in flat- bottom 96-well plates without any stimuli or in presence of OVA (100µg/well) for 72 hrs (37^0C, 5% CO_2) Supernatants were collected and stored at -20^0C until analysis IL-4, IL-5 and IL-13 were determined by ELISA (R&D) Local and systemic cell responses to PBS, OVA, OVA plus 147 treated and OVA plus wpc80 were evaluated in all 4 groups of mice as *in vitro* cytokine production by MLN after exposure to corresponding allergen IL-4 production from controls (PBS group) was low in response to OVA stimulation, however the levels of IL-4 were significantly increased in MLN cells exposed to OVA ($P<0.001$) Importantly, IL-4 cytokine production was significantly reduced in the OVA plus wpc80 and OVA plus 147 groups (Figure 5 9)

In summary, on the local level in MLNs, a significantly higher production of the Th2 cytokine IL-4 was detected in the OVA treated mice after an *in vitro* exposure to OVA

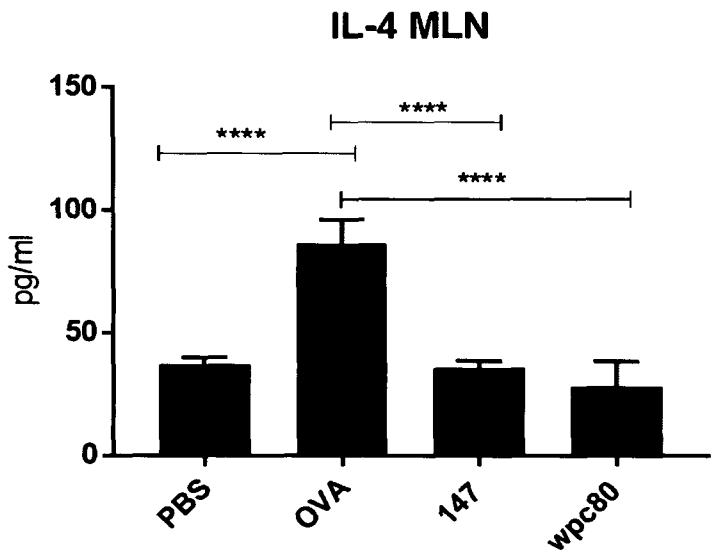


Figure 5.9. Oral administration of milk peptide hydrolysate 147 and parent control wpc80 inhibits the ex vivo cytokine production of IL-4 by Mesenteric Lymph nodes (MLN) in OVA treated mice. Mice were set up exactly as described in Figure 5 2 Mesenteric lymph node cells were recovered on Day 141 and prepared from all four groups of mice (PBS, OVA sensitised, 147 and WPC80 fed mice) using a cell strainer MLN cells (2 x 10^5/well) were cultured with or without 100ug/ml OVA for 72 h in culture medium in triplicate Supernatants were frozen at -20^{0}C until determination of IL-4 concentration by ELISA n= 6 per group and plated in duplicate Statistical analysis was carried out using one way ANOVA Tukey with multiple comparison test where ****< 0 0001

5.8. 147 HYDROLYSATE TREATMENT SIGNIFICANTLY REDUCED THE TH2 AND PROINFLAMMATORY CYTOKINES IN THE GI AND SPLEEN OF OVA MICE

The main driver of OVA induced inflammation are T_H2 cells Given the capacity of hydrolysates to potentially alter the $T_H1/T_H2/T_H17$ balance or enhance T_{Reg} function, it was important to investigate whether hydrolysate 147 anti-inflammatory/ immunomodulatory activity would impact the systemic and GI organs of OVA sensitised mice Therefore cytokines profiles for the small intestine, spleen and liver were determined Using the same model set up as described in figure 5 2, small intestine, spleen and liver were harvested, snap frozen and homogenates were used to detect the of levels of T_H2 (IL-4, IL-13) T_H1 (IFN-γ, IL-12) pro-inflammatory (IL-6, IL-17) and anti-inflammatory (IL-10) cytokines by ELISA

As expected, in the small intestine there was a significant increase in the levels of the T_H2 cytokine IL-4 in the mice that were sensitised with OVA antigen (Figure 5 10) However, both 147 and wpc80 treatment significantly decreased the levels of the T_H2 cytokine IL-4 There was a trend for reduction in IL-13 in the small intestine of mice treated with 147 in comparison to OVA sensitised mice (Figure 5 10) 147 treatment significantly reduced the levels of the T_H1 cytokine IL-12 in comparison to high levels of the cytokine detected in both OVA sensitised mice and wpc80 treated mice There was a significant reduction in the levels of pro-inflammatory cytokines IL-6 and IL-17 in the small intestine of 147 treated mice in comparison to OVA sensitised mice and wpc80 treated mice (Figure 5 10) However the most significant finding was the increase in the levels of the anti-inflammatory cytokine IL-10 in the small intestine of 147 treated mice in

comparison to OVA mice In the small intestine of wpc80 treated mice there was a significant increase in the levels of IL-10 in comparison to OVA mice but not as high as 147 treated mice The cytokine profile is broken down in Table 5 3

The cytokine profile in the spleen was very similar to the cytokine profile found in the small intestine 147 treatment significantly decreased the levels of the T_H2 cytokine IL-4 in comparison to OVA sensitised mice (Figure 5 11) However the levels of the T_H2 cytokine IL-13 after treatment with wpc80 were similar to the levels detected in the spleens of OVA sensitised mice compared to 147 treatment (Figure 5 11) There was a significant ~5 fold decrease in the T_H1 cytokine IL-12 in both spleens of 147 treated and wpc80 treated mice in comparison to OVA sensitised mice (Figure 5 11) 147 treatment again significantly decreased the levels of pro-inflammatory IL-6 and IL-17 in comparison to OVA sensitised mice with less of a decrease found in the spleens of wpc80 treated mice However similar to effect in the small intestine, treatment with 147 resulted in a ~2 fold increase in IL-10 levels in comparison to OVA sensitised mice The data is summarised in Table 5 4

IL-4 changes in the liver did not reach statistical significance, however both 147 and wpc80 treatment significantly reduced the T_H2 cytokine IL-13 in comparison to OVA sensitised mice (Figure 5 12) The level of the T_H1 cytokine IL-12 was reduced with 147 treatment however there were no changes detected in the levels of the pro-inflammatory cytokines (IL-6, IL-17) with either 147 hydrolysate or wpc80 control However, in keeping with the anti-inflammatory trend seen in the small intestine and spleen there was a significant increase in the levels of IL-10 in the livers of 147 and wpc80 treated mice (Figure 5 12) Table 5 5 summarises this data

Collectively these results demonstrate further that 147 hydrolysate is anti-inflammatory/ anti-allergic as it provides protection in both systemic and GI tissues above that provided by it's parent control (wpc80) This was characterised by the overall reduction in T_H2 cytokines, Th1 cytokines and pro-inflammatory cytokines and increase in anti-inflammatory cytokines in systemic and G1 tissues in the humanised OVA model

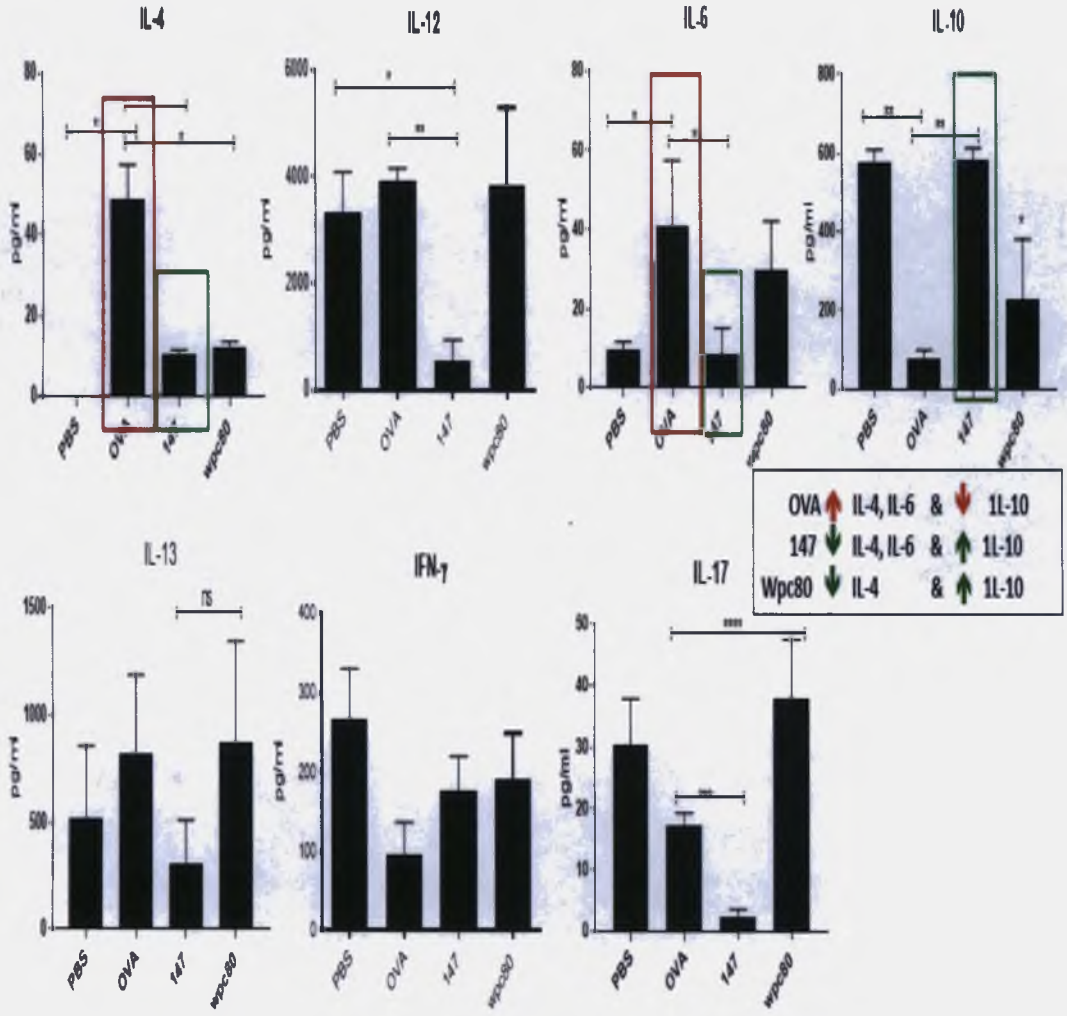


Figure 5.10 Hydrolysate 147 significantly reduced TH1, TH2, pro-inflammatory (IL-4, IL-6, IL-17) and increased the anti-inflammatory cytokines (IL-10) detected in the small intestine of OVA mice. The OVA model was set up exactly as described in figure 5.2. Tissue samples were harvested on Day 130, immediately snap frozen and stored at -80 ^{0}C. Homogenates were prepared and ELISA was used to detect TH1 (IFN-γ, IL-12), TH2 (IL-4, IL-13) pro-inflammatory (IL-6, IL-17) and anti-inflammatory (IL-10) cytokines. Concentration of cytokine is expressed as pg cytokine per ml tissue n=11 per group. Statistical analysis was carried out using one way ANOVA Tukey with multiple comparison test where * <0.05, **< 0.01 ***<0.001.

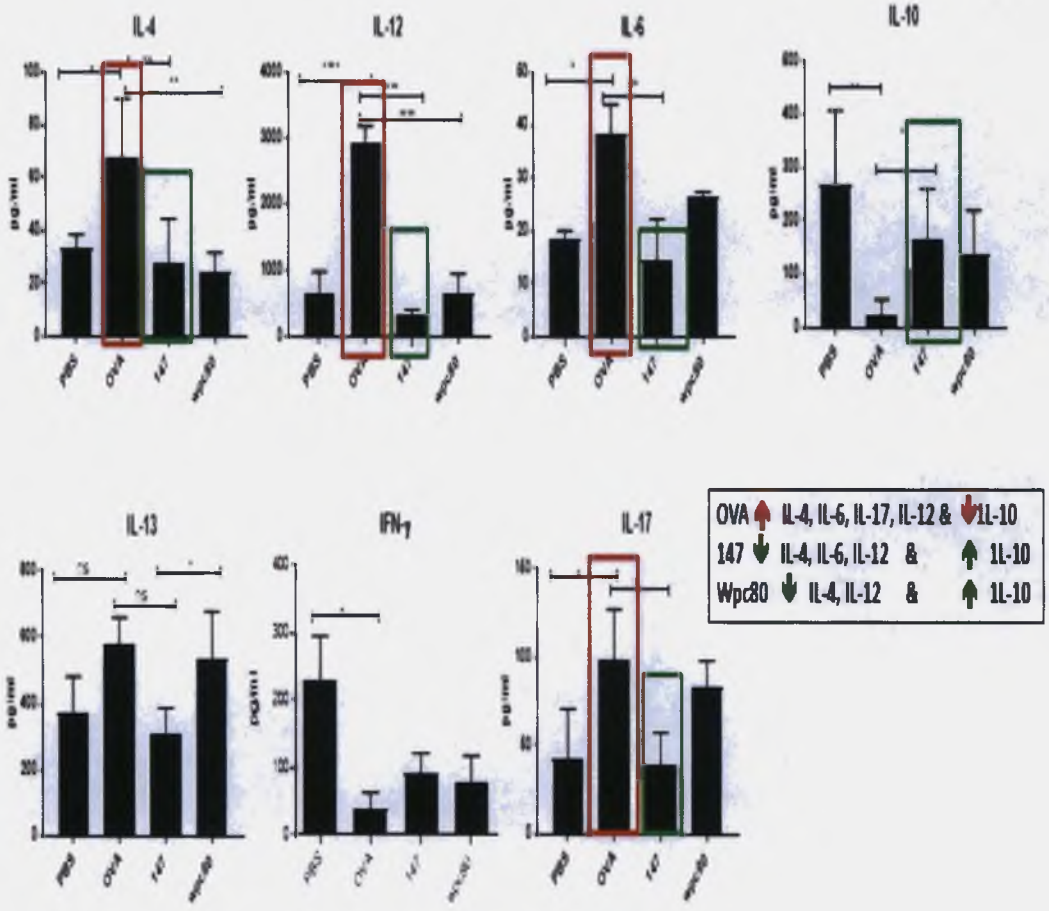


Figure 5.11: Hydrolysate 147 significantly reduced pro-inflammatory (IL-4, IL-6, IL-17 and increased anti-inflammatory IL-10 cytokines detected in the spleen of OVA mice. The OVA model was set up exactly as described in figure 5.2. Tissue samples were harvested on day 141, immediately snap frozen and stored at -80 ^{0} C. Homogenates were prepared and ELISA was used to detect TH1 (IFN-γ, IL-12), TH2 (IL-4, IL-13) pro-inflammatory (IL-6, IL-17) and anti-inflammatory (IL-10) cytokines. Concentration of cytokine is expressed as pg cytokine per ml tissue n=11 per group. Statistical analysis was carried out using one way ANOVA Tukey with multiple comparison test where * <0.05, **< 0.01 ***<0.001.

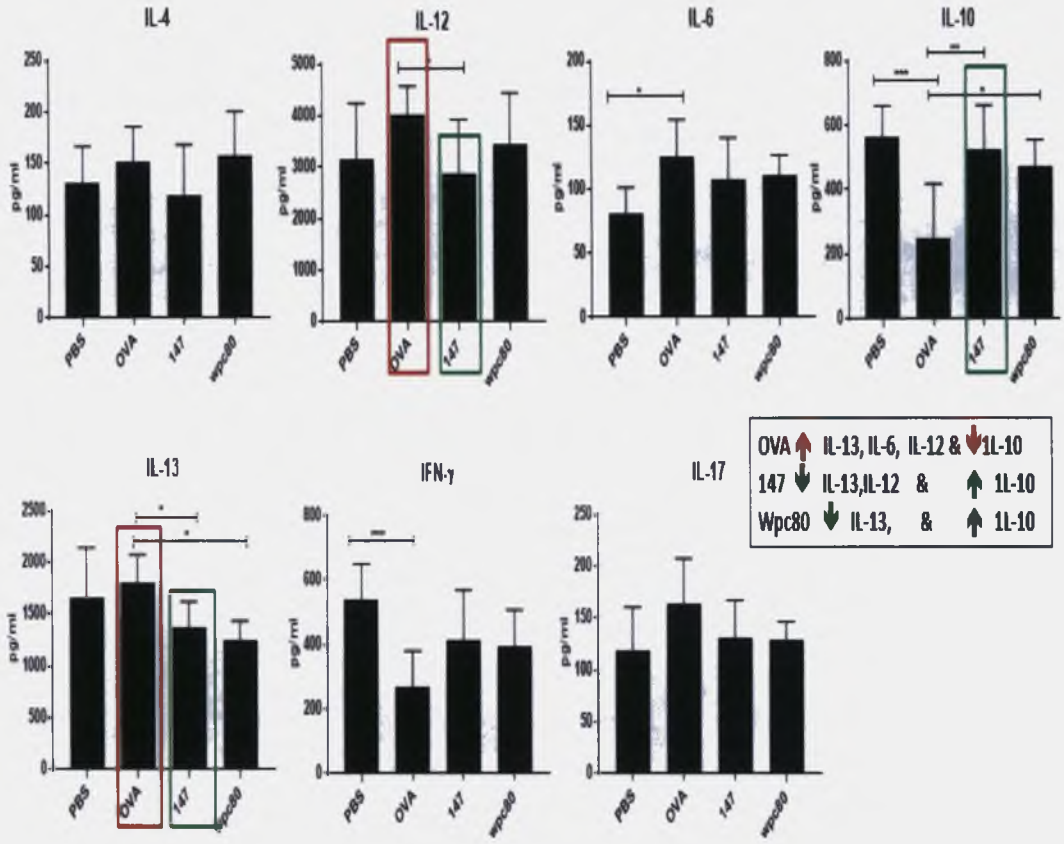


Figure 5.12. Hydrolysate 147 significantly reduced pro-inflammatory (IL-13, IL-12) and increased anti-inflammatory cytokine IL-10 detected in the liver of OVA mice. The OVA model was set up exactly as described in figure 5.2. Tissue samples were harvested on day 141, immediately snap frozen and stored at -80 ^{0} C. Homogenates were prepared and ELISA was used to detect TH1 (IFN-γ, IL-12), TH2 (IL-4, IL-13) pro-inflammatory (IL-6, IL-17) and anti-inflammatory (IL-10) cytokines. Concentration of cytokine is expressed as pg cytokine per ml tissue n=11 per group. Statistical analysis was carried out using one way ANOVA Tukey with multiple comparison test where * <0.05, **< 0.01 ***<0.001.

Table 5.3 Cytokine profile in the Small intestine

	PBS	OVA	147	WPC80
IL-4	ND	↑*	↓*	↓*
IL-13	↑	↑	↓	↑
IL-6	↑	↑*	↓*	↑
IL-12	↑	↑	↓**	↑
IL-17	↑	↑	↓****	↑****
IL-10	↑	↓	↑**	↑
IFN-γ	↑	↓	↑	↑

Table 5.4 Cytokine profile in the Spleen

	PBS	OVA	147	WPC80
IL-4	↑	↑*	↓**	↓**
IL-13	↑	↑	↓	↑
IL-6	↑	↑*	↓**	↑
IL-12	↑	↑****	↓****	↓****
IL-17	↑	↑*	↓*	↓
IL-10	↑	↓**	↑*	↑
IFN-γ	↑	↓*	↑	↑

Table 5.5 Cytokine profile in the liver

	PBS	OVA	147	WPC80
IL-4	↑	↑	↑	↑
IL-13	↑	↑	↓*	↓*
IL-6	↑	↑*	↑	↑
IL-12	↑	↑	↓*	↑
IL-17	↑	↑	↑	↑
IL-10	↑	↓***	↑**	↑*
IFN-γ	↑	↓***	↑	↑

5.9. REGULATORY T CELLS IN OVA MICE WERE NOT SIGNIFICANTLY ALTERED BY 147 HYDROLYSATE OR WPC80 TREATMENT.

Food allergy is a type 2 helper T cell (T_H2) dependent disease whose prevalence is increasing in industrialized countries as a direct consequence of reduced tolerance to food antigens The generation of regulatory T cells (T_{Reg}) is a key component of oral tolerance, and compelling experimental evidence has demonstrated that functional allergen-specific T_{Reg} cells play a major role in healthy immune responses to allergens and clinically successful allergen-specific immunotherapy (Palomares *et al* 2013) Since IL-10 producing T_{Reg} cells can inhibit Th2 cells, another way to dampen the T_H2 response is to promote the differentiation of Treg cells by administration of specific peptides (Palomares *et al* 2010) To date few studies have shown an effect of hydrolysates on T_{Reg} formation *in vivo* Duan *et al* (2012) showed an upregulation of IL-10 production in splenocytes obtained from mice treated with β-lactoglobulin trypsin hydrolysates, while intact β-lactoglobulin on the contrary downregulated IL-10 Based on these findings, the effect that 147 hydrolysate treatment has on T_{Reg} cells during OVA driven allergy was investigated in the humanised mouse model

All four groups of mice (PBS, OVA, OVA plus 147, OVA plus wpc80) were sacrificed on day 141 and the spleens were harvested for analysis Human T_{Reg} cells were defined as $CD4^+CD25^+CD127^{low}FoxP3^+$ after gating on the human $CD45^+$ cells recovered from the spleens and determined by intracellular flow cytometry Figure 5 13 outlines the 8- colour gating strategy to identify these cells on the Attune NxT flow cytometer

Very small populations of T_{Reg} cells were present in the spleens harvested The highest percentage and numbers of T_{Reg} cells were found in the PBS control group

(Figure 5 14) The total numbers of human T_{Reg} cells were significantly decreased in the spleens treated with 147 or wpc80 in comparison to PBS control mice These results suggest that 147 or wpc80 has no effect on the number and percentage of T_{Regs} ($CD127^{low}$ $CD25^{+}$ $Foxp3^{+}T_{Reg}$ found in the spleen it may suggest that T_{Reg} may have been found in the small intestine and this warrants further investigaton

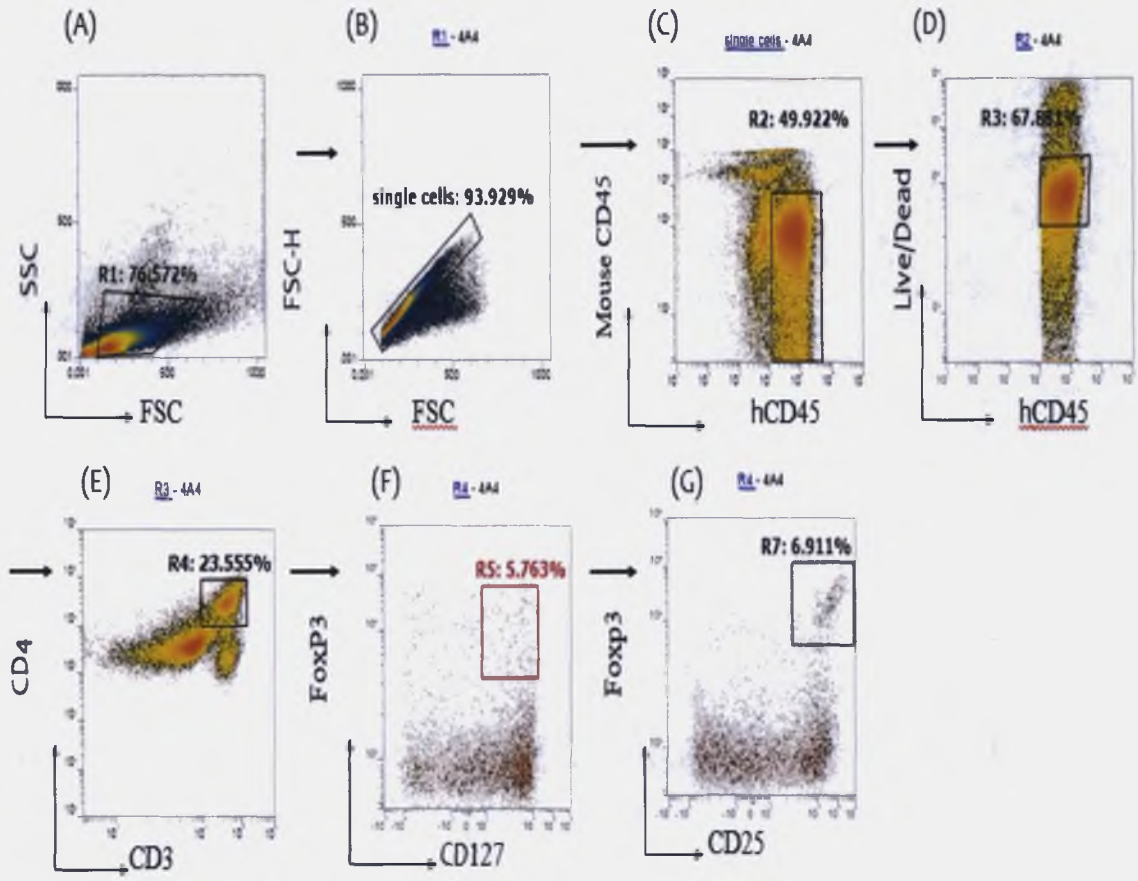


Figure 5.13. Representative example of gating strategy used to identify human $CD45^+CD3^+CD4^+$ T cells producing $Foxp3^+$ (A) Illustrates the gated population from SSC against FSC plot, (B) represents the gating position for single cells (C) illustrates the gating position for human $CD45^+$ (Pacific orange) cells and mouse $CD45^+$ (Pe) cells within the single cells (D) represents the gating position for Live /Dead stain (E-Flour 780) versus $hCD45^+$ expression. (E) represents the $CD3^+$ (Alexa Flour) $CD4^+$ (FITC) T cells within the $CD45^+$ population. (F) Illustrates the gating position for $Foxp3^+$ (BV421) $CD127^{low}$ (PerCP) within the $CD3^+CD4^+$ population (G) represents the gating position for the total $Foxp3^+$ $CD25^+$ (PE/CY7) cells within the $hCD45^+$ population. All gating positions were determined using FMO controls.

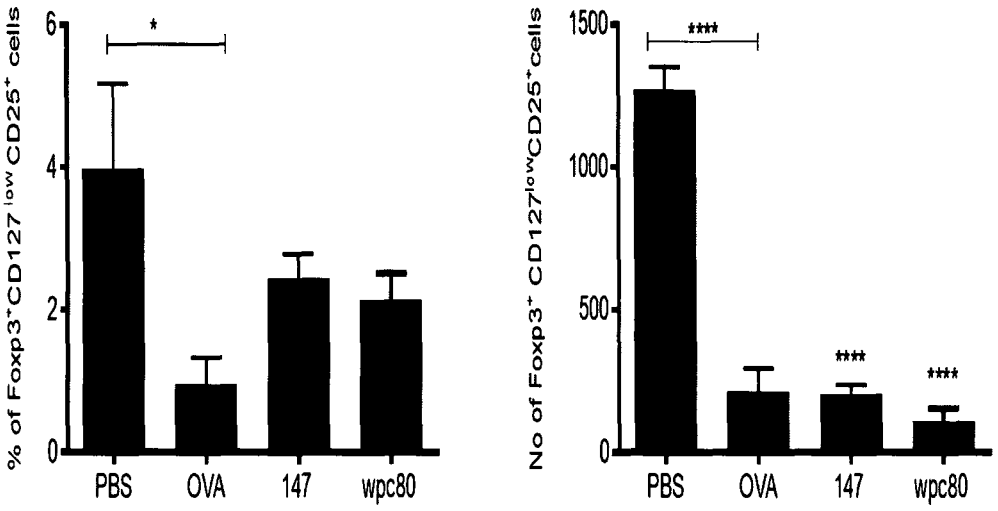


Figure 5.14. Hydrolysate 147 and WPC80 treatment had no significant effect on the number and percentage of Treg cells in the spleens of OVA driven mice The OVA model was set up exactly as decribed in figure 5 2 Cells were recovered on day 141 Human Treg cells were defined as CD127 ^{low} $CD25^{+}Foxp3^{+}$ after gating on the human $CD45^{+}$ $CD3^{+}$ $CD4^{+}$ T cells and analysed by intracellular flow cytometry Graphical representation of the total number and percentage of human Treg cells recovered from the spleen N=6 per group (2 HSC donors) Statistical analysis was carried out in comparison to the PBS group using one way ANOVA Tukey Multiple Comparison Test where *<0 05 ****<0 0001

5.10. HYDROLYSATE 147 SKEWS THE T-CELL DIFFERENTIATION FROM A TH2 SUBTYPE TOWARD TH1 WHICH IS BENEFICIAL IN FOOD ALLERGY

T_H1 and T_H2 cells are not 2 distinct $CD4^+$ T-cell subsets, but they simply represent polarized forms of the highly heterogenous $CD4^+$ T_H cell–mediated immune response (Romagnani 1999) T_H1 cells are characterized by the prevalent production of IL-2 and IFN-γ, without IL-4, IL-5, and IL-13 production By contrast, T_H2 cells are characterized by the prevalent production of IL- 4, IL-5, and IL-13 in the absence of IFN-γ production Some bioactive peptides from hydrolysates have the ability to modulate the immune response in a hypoallergenic way by balancing the T_H1/T_H2 response (Moller *et al* 2008) However few studies have addressed the issue of T-cell differentiation skewed by hydrolysates *in vivo* Wu *et al* (2006) demonstrated that the IFN-γ/IL-4 ratio increased in spleen T cells from mice fed with chitosan hydrolysate, suggesting a change toward a more T_H1-like phenotype We have previously shown *in vitro* that hydrolysate 147 decreases IL-4 production in a T_H2 polarisation assay (Figure 3 18b) Therefore to build on this finding, the effect that 147 hydrolysate has on the development of human $CD4^+$ T cells producing either IFN-γ (T_H1) or IL-4 (T_H2) during OVA driven inflammation was analysed in the spleen using intracellular flow cytometry

On day 141, human $CD4^+$ T cells were examined in the spleen from all groups of mice Using the gating strategy as described in figure 5 15, the T_H1 and T_H2 cytokine production was analysed in these cells by intracellular flow cytometry (Figure 5 16) In OVA mice, there was increased percentage and number of $CD45^+CD4^+$ T cells producing IL-4 and decreased percentage and number of

$CD45^+CD4^+$ T cells producing IFN-γ compared to PBS control mice (Figure 5 16) In contrast, treatment with 147 (but not wpc80) in OVA mice reduced the percentage and number of $CD45^+CD4^+$ T cells producing IL-4 and increased percentage and number of $CD45^+CD4^+$ T cells producing IFN-γ (Figure 5 16) Table 5 3 summaries the data in figure 5 16 and displays the ratio of IFN-γ/IL-4 (T_H1/T_H2) between the groups of mice There was a lower IFN-γ/IL-4 ratio (both in the percentage and number) in OVA mice compared to PBS mice Treatment with 147 (but not wpc80) increased the ratio (both in percentage and number) of IFN-γ/IL-4 in the spleen compared to OVA mice This data demonstrates further that 147 has immunomodulatory ability as it is capable of skewing the T-cell differentiation from a T_H2 subtype toward T_H1, which is beneficial in allergic inflammation

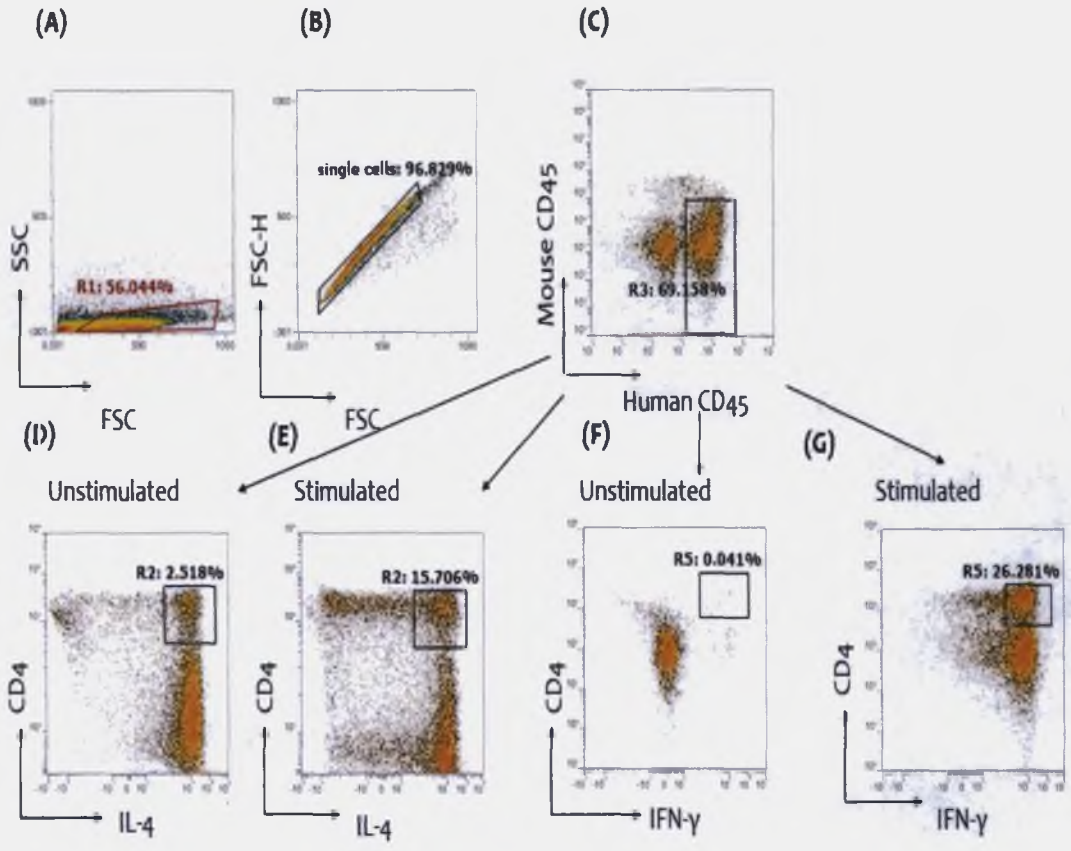


Figure 5.15. Representative example of gating strategy used to identify human splenic $CD45^+CD4^+$ T cells producing IL-4 (Th2) or IFN-γ (Th1) (A) Illustrates the gated splenic population from SSC against FSC plot (B) represents the gating position for single cells (C) illustrates the gating position for human $CD45^+$ (Pacific Orange) and mouse $CD45^+$ (PE) population (D) represents the gating position for the unstimulated $CD45^+$ $CD4^+$ (FITC) IL-4 $^+$(PE Dazzle) population (E) Illustrates the stimulated $CD45^+$ $CD4^+$ (FITC) IL-4 $^+$(PE Dazzle) population (F) represents the unstimulated $CD45^+CD4^+$ IFN-γ^+ (APC) and (G) Illustrates the stimulated $CD45^+CD4^+$ IFN-γ^+ (APC). All gating positions were determined by FMO controls.

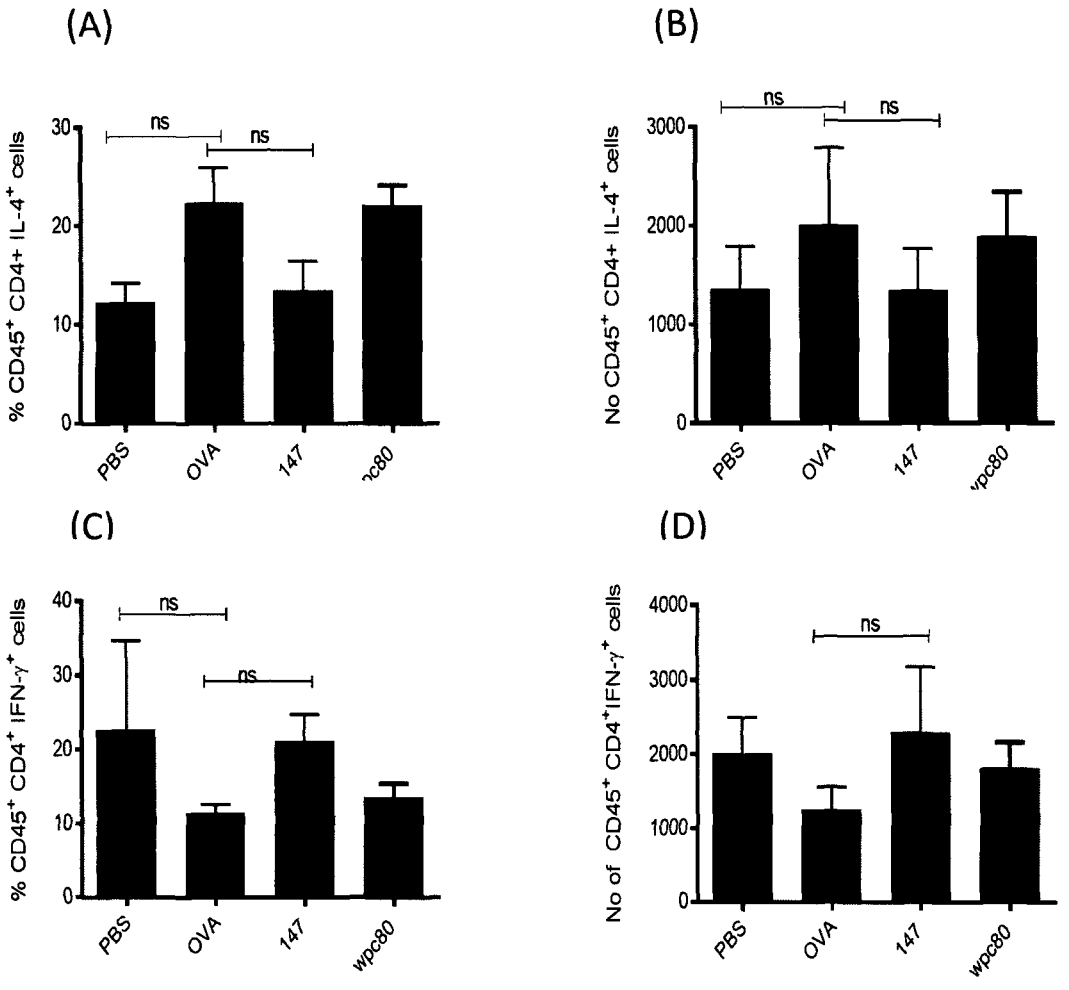


Figure 5.16 Increased IFN-γ/IL-4 ratio in splenocytes of 147 treated mice The OVA model was set up exactly as described in figure 5 2 Spleens were recovered on day 141 and single cell suspensions of splenocytes were stimulated with 100ng/ml Phorbal 12-myristate 13-acetate, 1 μg/ml ionomycin and 1μg/ml brefeldin A for 4 hrs and analysed by intracellular flow cytometry Graphical representation of the percentage (A) and total number of human $CD4^+$ IFN-γ cells (B) recovered in the spleen and percentage (C) and total number of human $CD4^+$ IL-4 cells (D) recovered in the spleens N=6 per group (2 HSC donors) Statistical analysis was carried out using one way ANOVA Tukey with Multiple Comparison test

Table 5.3 Comparison of ratio of Th1/Th2 cells in spleen of OVA mice

Group	Mean% IFN-γ cells	Mean No IFN-γ cells	Mean % IL-4 cells	Mean No IL-4 cells	Ratio IFN-γ/IL-4 %	Ratio IFN-γ/IL-4 No
PBS	**22.72**	**2021**	**12.3**	**1356**	**1.84**	**1.49**
OVA	**11.51**	**12.51**	**22.47**	**2010**	**0.51**	**.62**
147	**20.93**	**2305**	**13.56**	**1349**	**1.54**	**1.70**
WPC80	**13.66**	**1821**	**24.3**	**1893**	**0.56**	**0.96**

5.11 HYDROLYSATE 147 REDUCED THE MAST CELL LOAD IN THE BONE MARROW OF HUMANISED MICE

Mast cells are viewed as key players in IgE-dependent allergies and anaphylaxis (Voehringer 2013) and several lines of evidence suggest that basophils participate in allergic disease (Schroeder 2011) However, allergic inflammation reflects a complex interplay between several inflammatory cells, including mast cells, basophils, lymphocytes, dendritic cells, eosinophils and neutrophils (Barnes 2011) In a recent humanised mouse model study of anaphylactic peanut allergy, anaphylaxis in peanut fed humanised NSG-SCF mice was mediated by engrafted human mast cells through production of human IgE antibody produced by engrafted B cells and omalizumab (anti-IgE) treatment completely protected the mice from anaphylaxis (Burton *et al* 2017) However this study failed to show what effect the treatment had on the development of mast cells in these mice As we have previously found that mast cells and basophils engraft in the bone marrow of NSG-SCF mice (Figure 5 2) the next step was to investigate if 147 hydrolysate treatment would affect the development of the main effector cells in allergy, mast cells and basophils

Using the same model set up as described in figure 5 1, on day 141, femurs were removed from all groups of mice and bone marrow was harvested Human mast cells were defined as $CD203c^{+}FC\varepsilon RI^{+}$ $c\text{-}kit^{+}$ after gating on the human $CD45^{+}$ cells recovered from the bone marrow and determined by flow cytometry Figure 5 17 outlines the 6- colour gating strategy to identify these cells on the Attune Nxt flow cytometer As expected, the highest percentage of mast cells ($CD45^{+}$ $CD203c^{+}$ $c\text{-}kit^{+}$ cells) was found in the bone marrow of OVA sensitised mice that received no treatment compared to PBS mice (Figure 5 18A) Treatment with 147 and wpc80 significantly decreased the percentage and number of mast cells present (Figure 5 18

B and C) When these mast cells were further analysed for their expression of c-kit there was significantly lower numbers of mast cells ($CD45^+$ $CD203c^+$ $Fc\varepsilon R1^+$ c-kit^+) and basophils ($CD45^+$ $CD203c^+$ $Fc\varepsilon R1^+$ c-kit^-) present in the bone marrow of 147 and wpc80 mice (Figure 5 18 E) Overall 147 and wpc80 had a similar profile to PBS mice (Figure 5 18, A,B,D) however there were significantly less numbers of mast cells and basophils present with 147 and wpc80 treatment

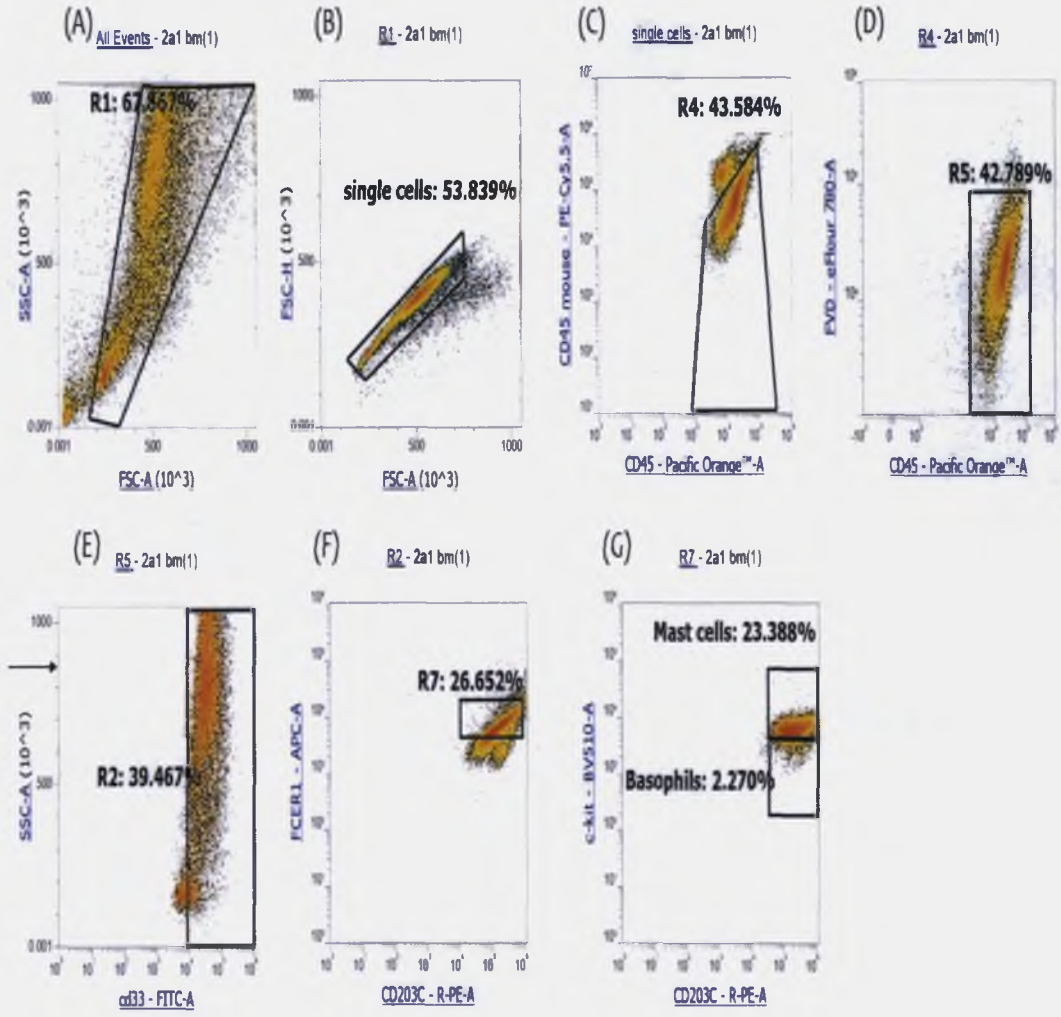


Figure 5.17. Representative example of gating strategy used to identify human mast cells and basophils. (A) Illustrates the gated population from SSC against FSC plot, (B) represents the gating position for single cells (C) illustrates the gating position for human $CD45^+$ (Pacific orange) cells and mouse $CD45^+$ (Pe) cells within the single cells (D) represents the gating position for Live /Dead stain (E-Flour 780) versus $hCD45^+$ expression. (E) Illustrates the $CD33^+$cells within the $CD45^+$ population (F) represents the $CD203C^+$ (PE) $FC\varepsilon R1^+$ (APC) cells within the $CD33^+$ population. (G) Illustrates the gating position for $CD203C^+$ $c\text{-}kit^+$ (mast cell) and $CD203C^+$ $c\text{-}kit^-$(basophils) within the $FC\varepsilon R1^+CD203C^+$ population. All gating positions were determined using FMO controls.

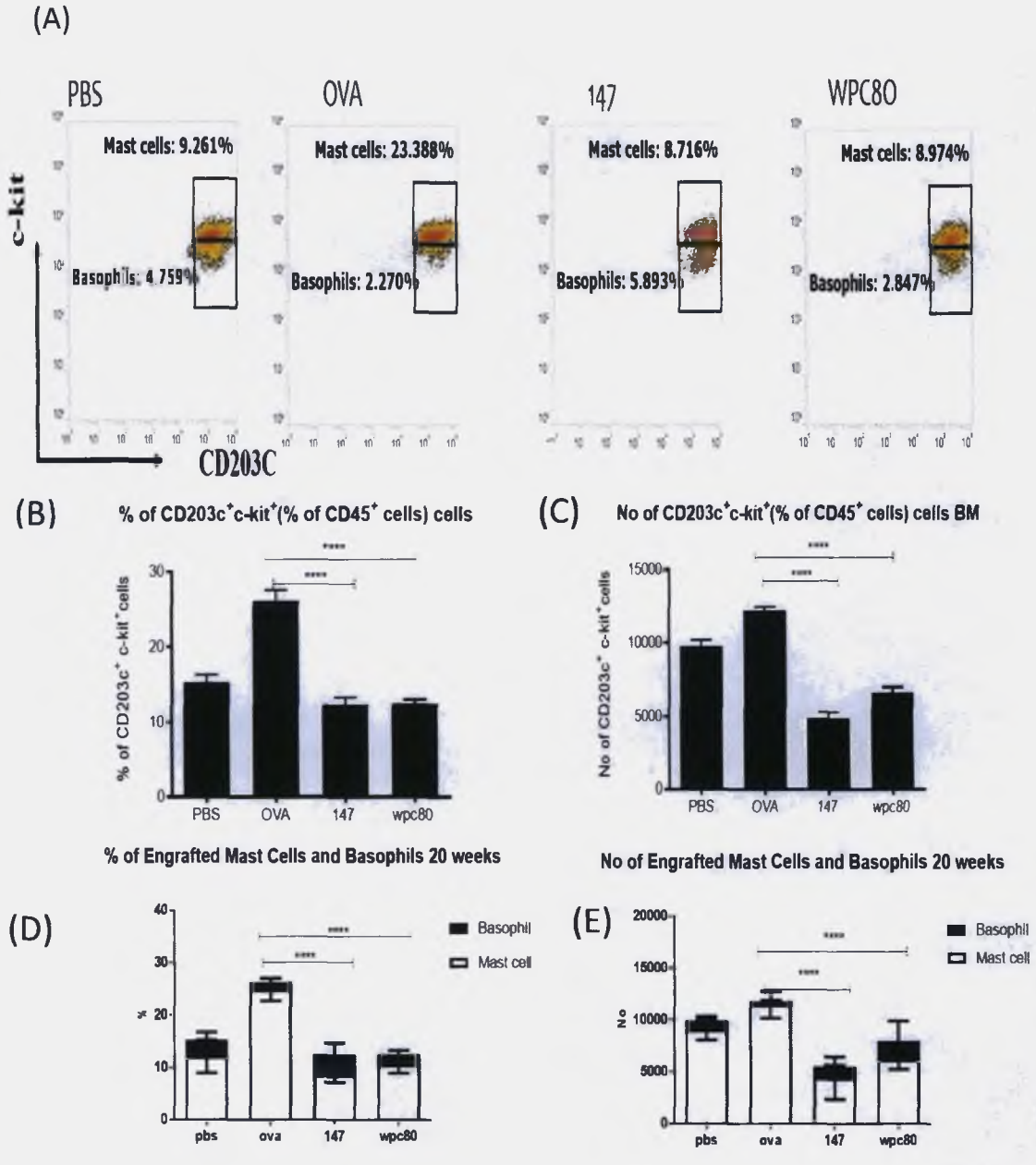


Figure 5.18. Percentage and number of mast cells were significantly reduced in the bone marrow of 147 hydrolysate & wpc80 mice. The OVA model was set up as described in Figure 5.1. Cells were recovered on day 141 and analysed by flow cytometry. (A) Flow cytometry plots of $CD45^+$ (Pacific Orange) $CD203c^+$ (PE) $c\text{-}kit^+$ cells (BV521) in bone marrow of PBS, OVA, OVA $^+$147 and OVA$^+$WPC80 mice. (B) percentage and (C) number of engrafted $CD45^+$ $CD203c^+$ $c\text{-}kit^+$ in bone marrow and (D) percentage and (E) number of mast cell ($CD45^+$ $CD203c^+$ $Fc\varepsilon R1^+$ $c\text{-}kit^+$) and basophils ($CD45^+$ $CD203c^+$ $Fc\varepsilon R1^+$ $c\text{-}kit^-$) in bone marrow of engrafted mice. N=6 per group for PBS and OVA and N=11 for 147 and WPC80 treated groups. Statistical analysis was carried out with one way ANOVA Tukey with Multiple Comparison Test where * < 0.05, **< 0.01***< 0.001

5.12 DIFFERENTIAL DISTRIBUTION OF LYMPHOCYTES, MONOCYTES AND GRANULOCYTES IN BONE MARROW OF 147 TREATED MICE ARE ACCOMPANIED BY REDUCTIONS IN T-LYMPOCYTES

The coordinated production of leukocytes in bone marrow is crucial for innate and adaptive immunity Inflammation alters normal leukocyte production by promoting granulopoiesis over lymphopoiesis (Ueda *et al* 2005) Developing granulocytes and lymphocytes compete for a bone marrow niche where reductions in one compartment result in expansion by the other (Ueda *et al* 2005) As outlined before, milk hydrolysates consist of mixtures of smaller peptides with different properties that can influence the immune response to be more suppressive/tolerogenic (Korhonen *et al* 2003) Herein, we used a model of OVA driven inflammation to investigate if ingesting a novel hydrolysate after sensitisation and before challenge would result in differential expression of lymphocyte, granulocyte or monocyte populations in OVA mice

As before, all groups of NSG-SCF mice were set up as described in figure 5 1 On day 141, bone marrow and liver were harvested and differences in lymphocyte subtypes were analysed by flow cytometry Representative dot plots showing the distribution of leucocytes by size (forward light scatter) versus granularity (side angle scatter) are illustrated in figure 5 19 A There was an increased frequency and number of granulocytes in the bone marrow of OVA mice compared to PBS mice (Figure 5 19 A, B, C) Treatment with 147 (but not wpc80) in OVA mice significantly reduced the frequency and number of granulocytes and concurrently increased the frequency and number of lymphocytes (Figure 5 19 A) There was an increased frequency and number of granulocytes in the liver of OVA mice compared to PBS mice A similar trend of significantly increased lymphocytes in comparison to low levels of

granulocytes was also seen in the livers of 147 treated mice however there was no change in cell number between the groups (Figure 5 20 B,C) To evaluate the distinct phenotypic switch in the bone marrow further, absolute cell counts were performed in the bone marrow of all groups of mice Human myeloid, T and B cells recovered from the bone marrow were determined by 7 colour flow cytometry Treatment with 147 (but not wpc80) significantly decreased the number of $CD45^+$ leucocytes found in the bone marrow of these mice compared to OVA mice (Figure 5 21 A) Notably there was lower absolute number of $CD3^+$ $CD4^+$ lymphocytes (but not wpc80) in the 147 treatment group in comparison to OVA mice (Figure 5 21C) There was lower absolute number of $CD3^+$ $CD8^+$ lymphocytes (as well as wpc80) in the 147 treatment group in comparison to OVA mice (Figure 5 21 D) This potential for reducing the number of lymphocyte phenotypes continued through to the myeloid and B cells 147 significantly decreased the number of human myeloid $CD33^+$ cells (as well as wpc80) and B ($CD19^+$) cells (but not wpc80) in OVA mice (Figure 5 21 B, E)

In summary, the data suggests that 147 has a big impact on the granulocyte populations as it significantly altered the ratio of immune cell populations in the bone marrow and liver In the bone marrow and liver there was a relative increase in the ratio of lymphocytes compared to granulocytes However in looking at cell number across the populations there was no actual increase in the cell number but there was a significant decrease in the granulocyte cell number It is possible that 147 promoted the accumulation of another cell type found in the lymphocyte gate for e g natural killer (NK) or invariant natural killer cells (iKT) cells or innate lymphoid cells (ILCs) that we did not analyse here iKT are a subgroup of T cells with a immunoregulatory function which have recently been shown to play a role in CMPA in infants (Jyounouchi *et al* 2011) Here the authors for the first time have demonstrated that

iNKT from children with IgE mediated food allergy especially those with milk allergy were reduced in number and exhibited a Th2 response to milk sphingomyelin, a type of lipid found commonly in milk and eggs.

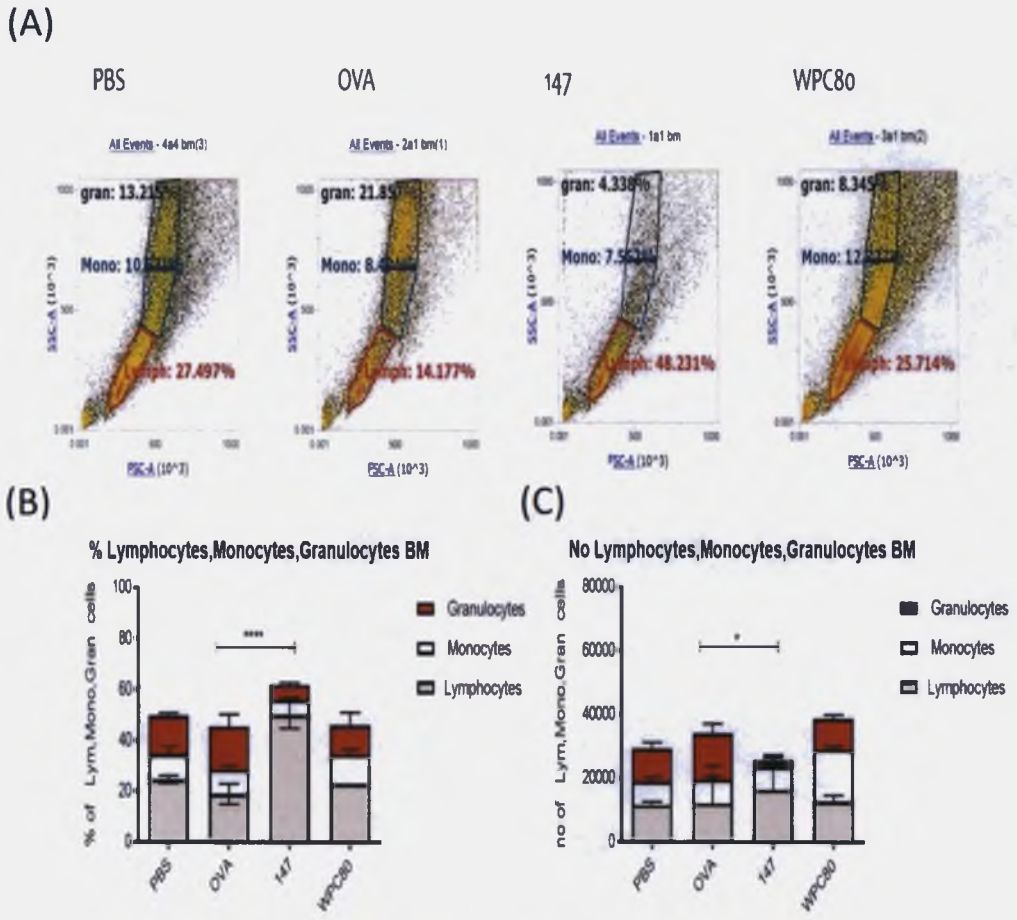


Figure 5.19. 147 hydrolysate decreases the percentage and number of granulocytes in the bone marrow of OVA challenged mice. The OVA model was set up as described in Figure 5.1. Cells were recovered on day 141 and analysed by flow cytometry. (A) Representative flow cytometry plots of lymphocyte, monocyte and granulocyte distribution in bone marrow of PBS, OVA, OVA plus 147 and OVA plus WPC80 mice. (B) percentage and (C) number of lymphocytes , monocytes and granulocytes in bone marrow of PBS, OVA, OVA plus 147 and OVA plus WPC80 mice. N=6 per group for PBS and OVA and N=11 for 147 and WPC80 treated groups. Statistical analysis was carried out with two way ANOVA Tukey with Multiple Comparison Test where * < 0.05, ****0.001

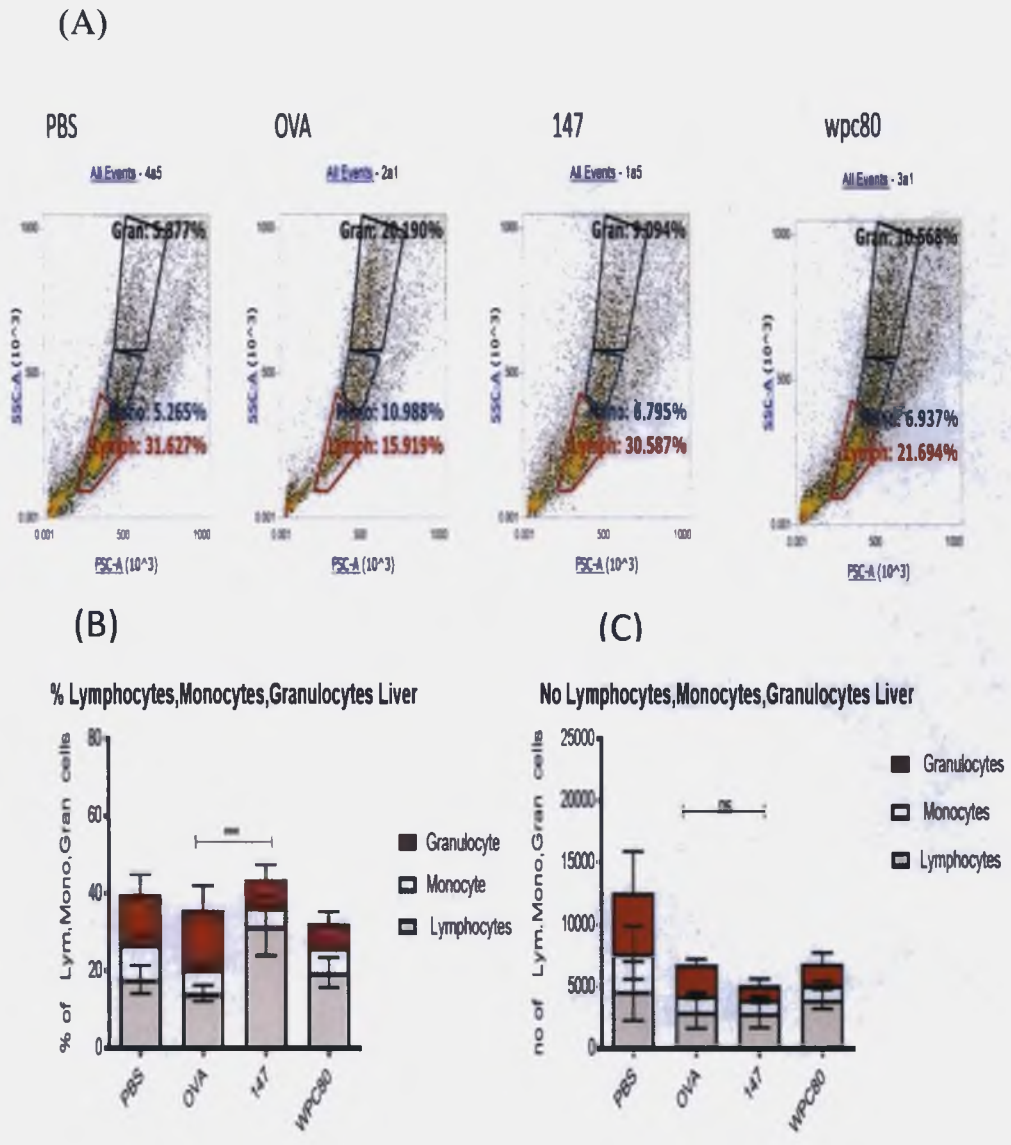


Figure 5.20. 147 hydrolysate decreases the percentage of granulocytes in the liver of OVA challenged mice. The OVA model was set up as described in figure 5.1. Cells were recovered on day 141 and analysed by flow cytometry. (A) Representative flow cytometry plots of lymphocyte, monocyte and granulocyte distribution in liver of PBS, OVA, OVA plus 147 and OVA plus wpc80 mice. (B) percentage and (C) number of lymphocytes, monocytes and granulocytes in bone marrow of PBS, OVA, OVA plus 147 and OVA plus wpc80 mice. N=6 per group for PBS and OVA and N=11 for 147 and wpc80 treated groups. Statistical analysis was carried out with two way ANOVA Tukey with Multiple Comparison Test where ***0.001.

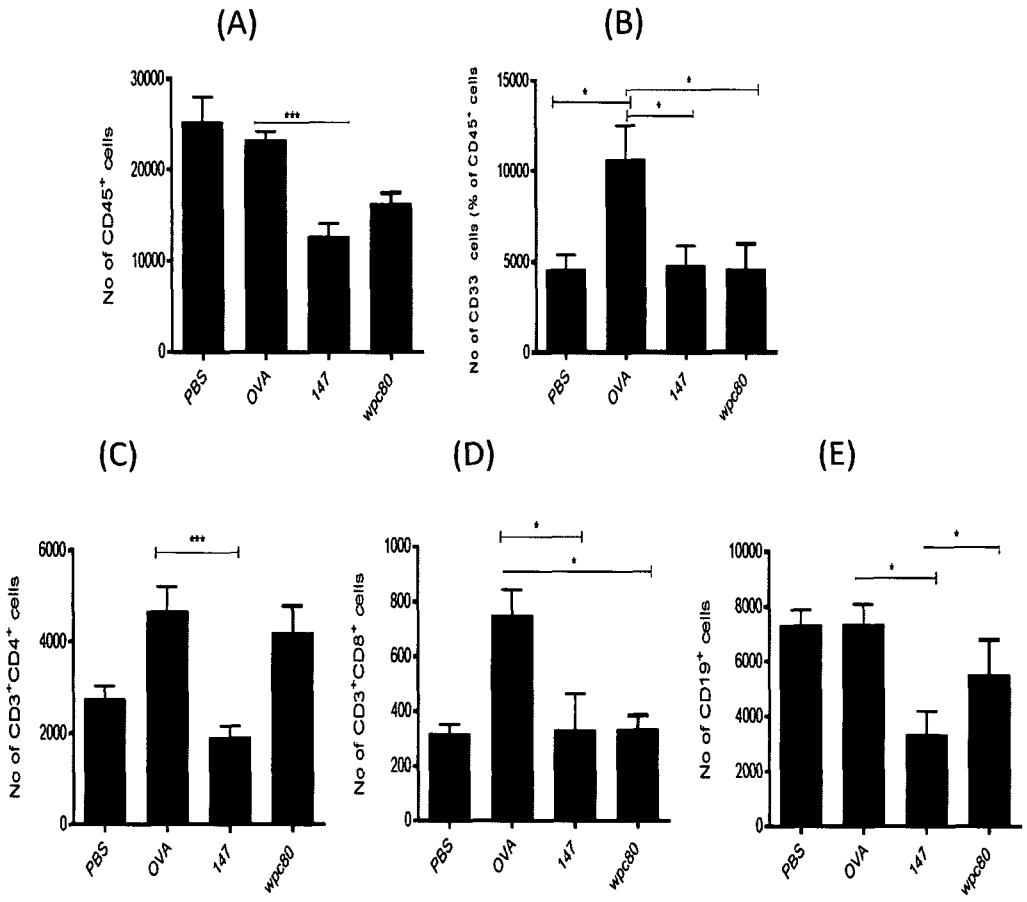


Figure 5.21. Significant reductions in T lymphocyte populations in bone marrow of OVA challenged mice treated with hydrolysate 147. The OVA model was set up as described in Figure 5 1 Cells were recovered on day 141 and analysed by flow cytometry (A) absolute number of Leucocytes ($CD45^+$) cells in the bone marrow (B) absolute number of myeloid ($CD33^+$) cells in bone marrow and (C) absolute number of $CD3^+CD4^+$ (D) absolute number of $CD3^+CD8^+$ (E) absolute number of B (CD19+) cells N=6 per group for PBS and OVA and N=11 for 147 and wpc80 treated groups Statistical analysis was carried out with one way ANOVA Tukey with Multiple Comparison Test where * < 0 05

5.13. HYDROLYSATE 147 SIGNIFICANTLY REDUCES THE ACTIVATION OF DENDRITIC CELLS IN THE BONE MARROW OF HUMANISED MICE

The prevalence of allergic diseases has increased rapidly in recent years It is well established that the deleterious allergic response is initiated by T-cell recognition of major histocompatibility class II-peptide complexes at the surface of antigen-presenting cells While this first signal gives antigen specificity to the adaptive immune response, a second nonspecific costimulatory signal is required by T cells to become fully activated This signal is provided by interactions between antigen-presenting cells and T cells through molecules borne at the surfaces of the two cell types Depending on the type of molecules involved, this secondary signal can promote the development of an inflammatory allergic reaction or may favor immune regulation Several molecules of the B7 family (CD80, CD86, PD-1, ICOS, CTLA-4) and tumor necrosis factor receptor family (OX40, CD30, 4-1BB, Fas, CD27, CD40) play an important role in delivering costimulatory signals in early and late phases of allergic response (Lombardi *et al* 2010) In Chapter 3, the demonstration that addition of milk hydrolysate 147 to activated monocyte derived dendritic cells resulted in down regulation of co-stimulatory molecule CD86 expression raised the possibility that hydrolysate 147 could also down regulate the expression of costimulatory molecules *in vivo*

Using the same model as described in figure 5 1, on day 141 bone marrow cells were isolated from the femurs of mice, single cells suspensions were analysed for the expression of dendritic cell markers $CD11c^+$ and co-stimulatory marker of activation $CD86^+$ by flow cytometry In the bone marrow there was significant reduction in the absolute number and percentage of cells in mice

challenged with OVA and treated with hydrolysate 147 in comparison to OVA challenged mice ($P<0.05$, $P<0.001$) This finding is consistent with the finding from Chapter 3 Treating mice with wpc80 after sensitisation and before challenge also significantly reduced the absolute numbers of $CD45^+CD11C^+CD86^+$ in the bone marrow of these mice (Figure 5.22) Downregulation of the dendritic activation marker CD86 in 147 treated mice adds to the growing body of evidence that hydrolysate 147 has anti-inflammatory capabilities in a humanised mouse mode of OVA allergy

Next the development of human myeloid subsets was examined in the bone marrow of humanised mouse model In mice sensitised and challenged with OVA, percentage and number $CD45^+CD33^+$ HLA-DR^- granulocytes were increased compared to PBS mice Importantly, this subset of cells (% and number) was significantly reduced in mice that were treated with 147 ($P<0.001$, Fig 5.23 A, B) However a similar effect was seen in the bone marrow of mice that were treated with wpc80 ($P<0.001$)

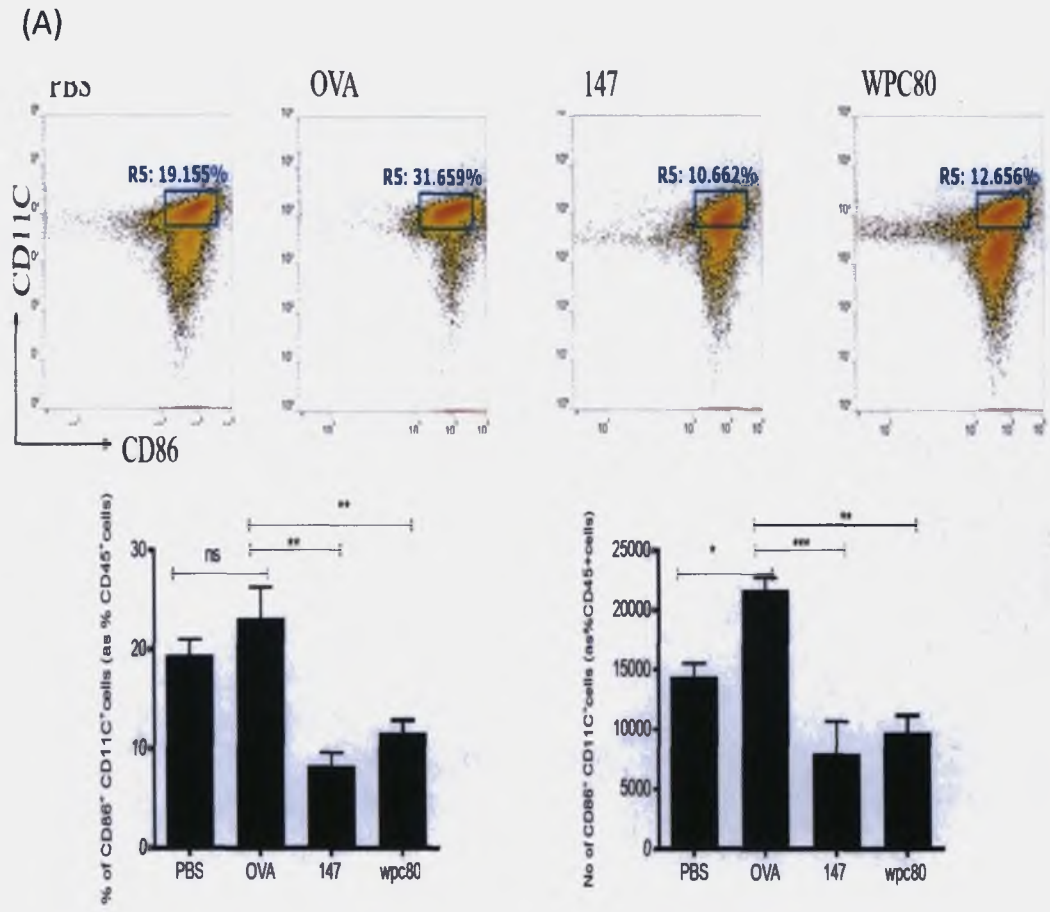


Figure 5.22. Reduction of co-stimulatory molecule CD86$^+$ cells in the bone marrow of OVA mice is mediated by 147 hydrolysate treatment. The OVA model was set up exactly as described in figure 5.2. On day 141, bone marrow was harvested, mechanically digested and single cell suspensions were analysed for the expression of CD45$^+$, CD86$^+$ CD11c$^+$ by flow cytometry. (A) Representative flow cytometry graphs of percentage CD45$^+$ CD86$^+$ CD11c$^+$ (B) percentage and (C) number of CD45$^+$ CD86$^+$ CD11c$^+$ cells in bone marrow. n=6 per group for PBS and OVA and N=11 for 147 and wpc80 groups. Statistical analysis was carried out using one way ANOVA Tukey Multiple Comparison Test where * <0.05 **< 0.01 and

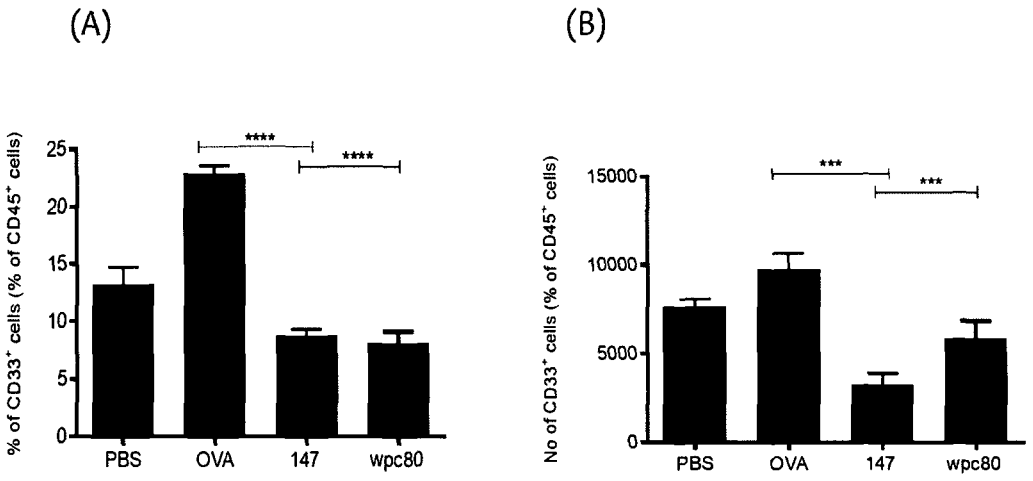


Figure 5.23. 147 treatment significantly reduced the percentage and total number of CD33$^+$ HLA-DR$^-$ granulocytes in the BM of OVA sensitised mice. The OVA model was set up exactly as described in figure 5 2 On day 141, bone marrow was harvested, mechanically digested and single cell suspensions were analysed for the expression of CD45$^+$, CD33$^+$HLA-DR$^+$ by flow cytometry (A) percentage and (B) number of CD45$^+$ CD33$^+$HLA-DR$^+$ in bone marrow n=6 per group for PBS and OVA and N=11 for 147 and wpc80 groups Statistical analysis was carried out using one way ANOVA Tukey Multiple Comparison Test where * <0 05 and ***< 0 001

5.14 SUMMARY

Throughout this chapter studies were designed to 1) assess the immunomodulatory capacity of novel milk protein hydrolysate 147 in a humanised mouse model of OVA inflammation against whey protein control and 2) to determine if milk protein hydrolysate 147 has anti-inflammatory/anti-allergic effects in the humanised model Whey 147 hydrolysate and its parent control wpc80 were tested after sensitisation and before the challenge/ effector phase of the allergic immune response to investigate the capacity for these treatments to alter the pre-disposed T_H2 environment that already exists in these mice 30 mins after challenge with OVA antigen, 147 and wpc80 treated mice had reduced anaphylactic symptoms in comparison to OVA sensitised mice that received no intervention (Figure 5 3) Investigating the GI protection by hydrolysates, histological analysis revealed that damage in the target organ of allergic mice was greatly diminished in mice exposed to 147 hydrolysate and wpc80 treatment (Figure 5 6) There were limited number of mast cells present in the connective tissue of OVA mice and no mast cells were present in the GI tissues of 147, wpc80 or PBS mice

For further investigation, cytokines profiles in OVA mice following these treatments were analysed Figures 5 6-5 8 confirmed that 147 treatment significantly reduced the Th2 and pro-inflammatory cytokine profile in the small intestine, spleen and in the liver of OVA driven mice There was a significant increase in the anti-inflammatory cytokine IL-10 in all three tissues The source of the IL-10 remains unidentified but may have been produced by telorogenic dendritic cells In terms of changing T cell differentiation, from an allergic T_H2 to a more tolerant subtype, spleens that were treated with hydrolysate 147 had higher T_H1/T_H2 ratios This skewing of the immune response is very beneficial attribute in a food allergy model

Looking more closely at the effector cells of allergy, mast cells were significantly decreased in the bone marrow of NSG-SCF mice treated with hydrolysate 147 (and wpc80) and they mirrored the effect seen in the PBS control mice However the most striking phenotype was that 147 treatment blocked the accumulation of granulocytes in the bone marrow and liver This suppression of granulopoiesis resulted in over production of lymphocytes However, phenotypic analysis by flow cytometry revealed a significant reduction in the leucocytes ($CD45^+$), T-lymphocyte subsets ($CD3^+$ $CD4^+$, $CD3^+CD8^+$) myeloid ($CD33^+$) and B cells ($CD19^+$) in the bone marrow of 147 treated mice, a phenomena which may be mediated by either a regulatory or suppressor type of cell

Oral immunotherapy for food allergy alters the expression of the co-stimulatory molecules on DC (Gorelik *et al* 2015) There was significant reduction of co-stimulatory molecule CD86 in the bone marrow of mice receiving 147 hydrolysate treatment which again points to a tolerogenic type of immune regulation There were significantly lower numbers of $CD33^+HLA\text{-}DR^-$ granulocytes in the bone marrow of 147 and wpc80 treated mice in comparison to OVA mice (Figure 5 23) Importantly 147 hydrolysate was more anti-inflammatory than its parent control wpc80 as shown in summary (Figure 5 24)

Collectively, this data suggests that hydrolysate 147 has no implications for the risk of inducing allergic symptoms in already sensitised mice and represents a potent anti-inflammatory compound that could be added as a functional food ingredient as an immune balancing compound Following these investigations, this chapter adds significant knowledge on our understanding of the anti-inflammatory effects a novel milk peptide hydrolysate 147 has in reducing OVA driven allergy in a humanised mouse model and presents a framework from which suitable conditions for testing

functional foods can be adopted in the development of novel agents for the prevention of allergic inflammation.

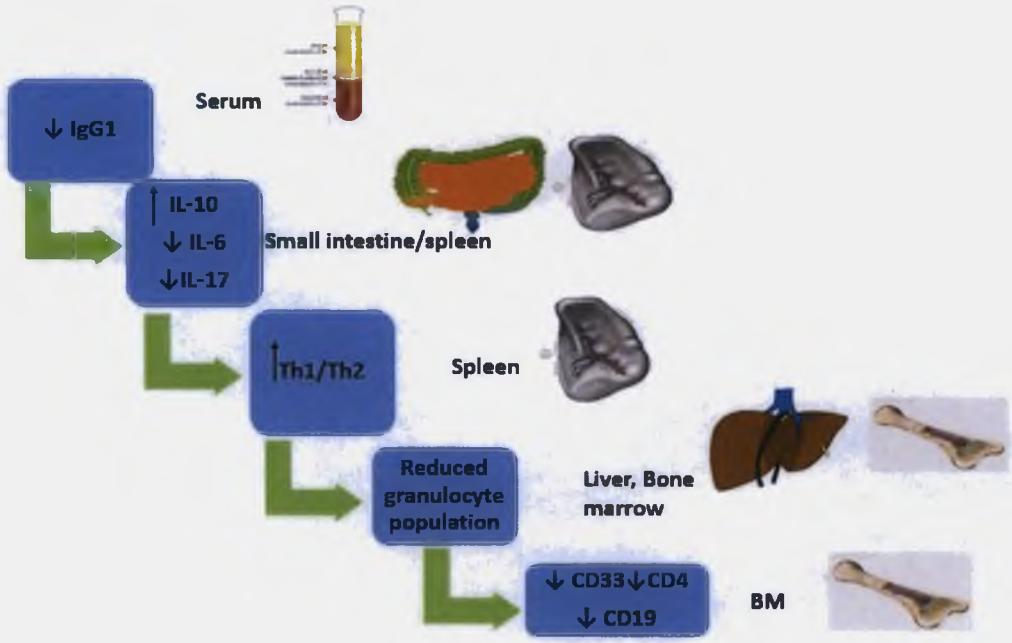


Figure 5.24: Whey milk peptide hydrolysate 147 provided additional anti-inflammatory/pro- tolerogenic effects over it's intact whey protein control in a humanised mouse model of ovalbumin induced allergy and inflammation. Addition of 147 to OVA mice resulted in lower production of T_H2 antibody IgG1 in serum, lower expression of pro-inflammatory cytokines IL-6, IL-17 and higher expression of the anti-inflammatory cytokine IL-10 in both spleen and small intestines of OVA mice than wpc80. 147 was more potent than wpc80 at providing a T_H1/T_H2 balance in the spleen and had a significant impact on reducing the granulocyte populations in bone marrow and liver of OVA mice in comparison to wpc80. 147 reduced the $CD4^+$T and $CD19^+$B lymphocytes and $CD33^+$ myeloid cells in the bone marrow of OVA mice.

CHAPTER 6
DISCUSSION

In the last two decades many studies have demonstrated that bovine milk is a source of bioactive compounds These bioactive molecules are naturally contained in milk Milk-derived bioactive peptides are usually encrypted and kept inactive within the primary structure of milk protein and they are generated by proteolysis of casein (α-, β-, γ- and κ-casein) and whey proteins (β-lactoglobulin, α-lactalbumin, serum albumin, immunoglobulins, lactoferrin and protease-peptone fractions) Both whey and casein contain a number of immunomodulatory peptides that are naturally present or that are part of the primary sequence of whey and casein proteins These peptide sequences can be released during digestion in the gut and can also be produced by *in vitro* enzymatic hydrolysis. Proteins that are pre-digested or hydrolysed into smaller protein chains outside of the body are known as hydrolysates These milk hydrolysates are known to possess different immunological properties to the whole protein and have been proven to beneficially modulate the immune system The increasing number of and health promoting effects attributed to milk-derived bioactive peptides make them potential functional ingredients These hydrolysates can be used to prevent or mitigate allergic reactions via the development of targeted food products or functional food ingredients Although human milk is generally accepted as the feeding choice, more than 70% of the infants in Western Europe are formula fed from 12 weeks on (Bosscher *et al* 2009) Infant formula, which is often cow's milk based, is the only approved alternative for breastfeeding However, cow's milk proteins cause allergic symptoms in 2-3% of the infants (Sackwsen *et al* 2011) Cow's milk is largely considered the most important ingredient source for infant formula, as it is made up of many up of many of the constituents found in breastmilk, such as essential amino acids that are required to support the developmental needs of the infant Current milk formula on the market suitable for infants with CMPA, are comprised of extensively hydrolysed cow's milk proteins These milk formulae provide the infant with the high nutritional quality of the proteins, while also

reducing the allergenic properties, due to their small size (Terheggen-Lagro *et al* 2002) However, while these extensively hydrolysed formula are more tolerable and prevent allergic symptoms, they cannot induce tolerance to the allergen because extensive protein hydrolysis results in the loss of immunogenicity This reduces stimulation to the immune system, inhibiting its ability to develop tolerance to these otherwise harmless milk proteins Although strict avoidance of cow's milk is advised for the management of CMPA, recent studies suggest that exposure to cow's milk allergens can, in fact be beneficial for infants that are at risk of developing CMPA through targeting and influencing the immune system from birth (Staden 2007) Partially hydrolysed formulae however have been shown to reduce the prevalence of atopic dermatitis in infants compared to whole-protein formula (Spieldenner *et al* 2011, Vandenplas *et al* 2014, Hernandez-Ledesma *et al* 2014) Partial hydrolysis of proteins retains the protein's immunogenicity which provides the infant with an opportunity to develop oral tolerance to the whole protein (Henson *et al* 2012) Milk hydrolysates generated from either casein or whey protein may possess anti-inflammatory or anti-allergic properties and thus may be beneficial in infant formula to alleviate the symptoms of CMPA while promoting immune balance Based on the recommendations that infant formula should mimic the effects of human milk in the infant as much as possible, several groups, including ours proposed to develop a nutritional product enriched with immunomodulatory peptides to prevent food allergy in infants

The first chapter (chapter 3), sought to identify hydrolysate's that had immunomodulatory effects using *in vitro* screening assays Firstly, the ability of 20 hydrolysates to suppress T cell proliferation was investigated in human PBMCs We chose to use human PBMCs as they are a relevant source of human immune cells and have been used previously to study immunoregulatory effects of dietary molecules (Vogt *et al* 2013) including cow's milk proteins (Zaczynska *et al* 2014) Milk peptide hydrolysates isolated

from whey and casein proteins have been shown to have inhibitory effects on T cells by suppressing the proliferation of PBMCs isolated from healthy donors (Sutas *et al* 1996) In this study Sutas *et al*, (1996) hypothesised that the inhibitory effect of the whey and casein hydrolysates was strengthened by the additional hydrolysis with the use of bacterial enzymes from the *Lactobacillus Casei* strain Similar studies using the BrdU test to assess the effect of milk casein peptide on the proliferation of PBMCs found a concentration-dependent stimulation of cells after 10 hrs of exposure of the cells to milk peptides but longer incubations resulted in T cell suppression (Fiedorowic *et al* 2011) Thus in collaboration with FHI, this first screening study sought to identify hydrolysates with immunomodulatory properties *in vitro* as this likely has implications in determining the outcome of an immune response Therefore the influence of hydrolysates on T cell proliferation and cytokine production *in vitro* was assessed Pepti (Aptamil) a current infant milk formula on the market suitable for infants with CMPA (extensively hydrolysed) was included as a bench mark control in these assays Using a proliferation dye dilution assay both whey (20, 21) and sodium caseinate hydrolysates (23, 33) and their parent control (NaCn) were shown to suppress T cell proliferation, however there was no evidence of enhanced suppression over that of pepti control The majority of the hydrolysates (10 out of 16) had no effect on T cell proliferation but some were effective at modulating the cytokines in the supernatant The capacity of T cells to produce an inflammatory reaction is largely controlled by their repertoire of cytokines and food allergies have traditionally been considered to be Th2 cell-derived immunopathologies Therefore the inhibition of the Th2 cytokines (such as IL-4) and the induction of Th1 (IFN-γ) and anti-inflammatory cytokines (such as IL-10) is a relevant approach to shift the balance in allergy A number of whey and casein hydrolysates inhibited the T_H2 response by decreasing the levels of IL-4 and increasing the levels of IFN-γ and some casein hydrolysates reduced the pro-inflammatory cytokine IL-6 while increasing the

anti-inflammatory cytokine IL-10 These initial results indicated the ability of certain milk peptide hydrolysates to favourably balance pro-inflammatory and anti-inflammatory/pro-tolerogenic responses in stimulated PBMC To further confirm these immunomodulatory effects, a cohort of whey (40,42) and casein hydrolysates (23,34,35) and their optimised regenerates (79,77,80 84,85) were re-screened for their suppressive potential and ability to dampen pro-inflammatory responses and promote regulatory responses These hydrolysates and their corresponding regenerates had no effect on T cell proliferation but they differentially modulated the production of pro (IL-6, IL-17) and anti-inflammatory (IL-10) cytokines *in vitro* Notably, hydrolysate 35 and it's regenerate significantly upregulated the production of IL-10 and suppressed the levels of IL-17 over and above pepti formula Whey hydrolysate have been shown previously to induce TNFα or IL-10 production in resting PBMCs (Rodriguez-Carrio *et al* 2014) High IL-10 levels induced by intact whey or mildly hydrolyzed whey indicate an overall anti-inflammatory effect of whey and whey hydrolysates Anti-inflammatory effects of whey hydrolysates have indeed been described in animals using standard tests including paw edema models (Taveras *et al* 2013) A number of hydrolysates and their regenerates were anti-proliferative, increased the levels of Th1 cytokine (34) or significantly decreased the pro-inflammatory cytokines IL-6 (34,35,79), or decreased IL-17 (34,35,85) or increased anti-inflammatory IL-10 (35,85) Hydrolysate 35 and its regenerate 85 had to be excluded from further studies due to cessation of enzyme preparation by manufacturer Based on this second screen and a screen completed with mouse cells within FHI, whey hydrolysate 42 and it's regenerate 79, and sodium caseinate hydrolysate 34 an it's regenerates 84 and 132 were chosen for further testing in Th1, Th2, Th17 and Treg polarisation assays

Although, measuring cytokine levels in supernatants provides an overall view of cytokine concentration, it does not provide any detail of cytokines being produced at a cellular level by

particular cells Therefore the next approach was to assess the effect hydrolysates have on Th1, Th2, Th17 and Treg to draw further conclusions about the effect of hydrolysates on T helper cell differentiation It has long been recognized that antigen presenting cells (APCs) guide (if not dictate) the sensitization of allergy by directing differentiation of uncommitted (naive) $CD4^+$ T helper (Th) cells towards Th1, Th2, Th17 and Treg phenotypes For example, the presence of IFN-γ and IL-12 in the local milieu skews towards Th1 (expression of T box expressed in T cells (T-bet)), IL-4 towards Th2 (expression of GATA-3), transforming growth factor (TGF)-β and IL-10 towards Treg (expression of forkhead box P3 (Foxp3)) and IL-6 and IL-23 towards Th17 (expression of RORgammat) in human $CD4^+$ T cells (Zang *et al* 2014) No study to date has addressed the topic of T cell differentiation skewed by peptides using human T helper polarised cells One study investigated the effect of a yak milk hydrolysate on the Th1/Th2 balance my measuring mRNA levels of Th1 cytokines (IL-2 and IFN-γ) and the Th2 cytokine IL-4 in murine spleen lymphocytes (Mao *et al* 2007) They found that yak milk casein hydrolysate increased the Th1 cytokines but it did not alter the Th2 cytokines (Mao *et al* 2007) Furthermore Wu *et al* (2006), found that the IFN-γ/IL-4 ratio increased in spleen T cells from mice fed chitosan (shrimp) hydrolysate, suggesting a change toward a more Th1-like phenotype Therefore, the effect of hydrolysates on driving immune responses in a certain direction was explored in Th1 and Th2 polarisation assays Addition of whey hydrolysate 79 on day 3 increased the number of $CD4^+$ T-bet cells while addition of casein hydrolysates 132 and 47 decreased the MFI and number of $CD4^+$T-bet cells in a polarised Th1 assay However no hydrolysate altered the production of the Th1 cytokines (IL-2, IFN-γ) in this Th1 assay In a Th2 polarisation assay, all hydrolysates including wpc80 decreased the MFI Gata-3 expression however when we analysed the number of $CD4^+$ Gata-3 cells by intracellular flow cytometry only hydrolysate 42 and 47

significantly decreased the number of Th2 cells producing Gata-3 and no hydrolysate altered the levels of the Th2 cytokine (IL-4) measured by ELISA

Th17 have been identified as a unique $CD4^+$ T helper subset characterised by IL-17 production that promotes tissue inflammation (Harrington *et al* 2005, Park *et al* 2005) Understanding their function during homeostatic and inflammatory conditions is continuously evolving, however, it is increasingly clear that Th17 cells are critical in protecting and maintaining the homeostasis particularly in the lamina propria of the small intestine (SI), where they are abundantly present (Ivanov *et al* 2006) In healthy individuals, low numbers of Th17 cells are present, mainly in the lamina propria (Huber *et al* 2012) During inflammatory disease, IL-17 induces the recruitment of neutrophils and the chemokine CCL20 attracts more Th17 cells (Esplugues *et al* 2011) $CD4^+$ T cells from children with food allergies have been shown to produce lower levels of IL-17 ex vivo *In vitro* $CD4^+$ T cells from these allergic children showed impaired IL-17 production after antigen administration (Bin *et al* 2013) In a recent cow's milk allergy mouse model study, whey sensitized animals showed an increased percentage of total Th1, activated Th17 and activated Treg cells, however, these increases were not observed in animals sensitized with the whey hydrolysate (Kiewiet *et al* 2017) Therefore the effect of hydrolysates (developed in FHI) on Th17 cytokine production and transcription factor expression (Rorγt) was investigated All hydrolysates significantly decreased the Rorγt (MFI) expression and number of $CD4^+$ $Ror\gamma t^+$ cells Importantly, hydrolysate 42 and it's regenerate 79 significantly suppressed the levels of IL-17 in polarised Th17 cells after 6 days of culture in a similar manner to the parent control wpc80

Treg cells are a heterogeneous population of T cells with suppressive and immunoregulatory properties that are essential for maintaining tolerance to innocuous substances and preventing excessive or misguided immune responses to pathogens In

humans, several studies show that the balance between allergen-specific Th2 and Treg cells recognizing the same T-cell epitopes determines whether an individual develops allergy (Th2) or has a healthy response (Treg) (Akdis 2012) This balance can potentially be maintained by administration of hydrolysates Treg cells are one of the main producers of IL-10, an anti-inflammatory cytokine that induces the down-regulation of Th1 cells and the enhancement of B cell survival, proliferation and antibody production, among other actions Casein hydrolysates were found to stimulate IL-10 production in treated ConA stimulated human Jurkat T cells (Lahart *et al* 2011) Upregulation of IL-10 production was also observed in splenocytes from mice treated with the whey protein β-lactoglobulin trypsin hydrolysates, while intact β-lactoglobulin on the contrary, downregulated IL-10 (Duan *et al* 2012) In a similar manner in our Treg polarisation assays, intact wpc80 and casein hydrolysate 47 decreased Foxp3 expression and IL-10 production however the majority of the hydrolysates neither enhanced or reduced the expression of Foxp3 and IL-10 cytokine levels These findings are not entirely similar to the published studies above where hydrolysates were shown to increase levels of IL-10 but differences in cell types (murine splenocytes, human Jurkat T cells) may account for these variances Our study used pure populations of polarised $CD4^+$ T helper cells whereas the Lahart & O'Callaghan study used immortalised T lymphocyte cells

Dendritic cells (DCs) are recognized as the most potent antigen-presenting cells, which are solely capable of inducing a primary immune response Hydrolysates delivered orally might interact with immune cells, including DCs, in the gut-associated lymphoid tissue (GALT) As hydrolysates had been found to possess immune-modulating properties, it was hypothesized that the hydrolysates in bovine milk may stimulate tolerogenic responses in antigen presenting cells by interfering with DC maturation Very few studies have examined the effect of hydrolysates on human monocyte derived dendritic cells therefore the capacity

for milk-derived hydrolysates to modulate DC maturation was examined Both whey (79 42) and casein (34, 84) hydrolysates suppressed the effector functions of DCs as they significantly reduced the expression of the co-stimulatory molecule CD86 and levels of IL-12, with casein 132 and 47 being more potent at reducing production of IL-12 Similar to our findings, Li *et al* (2017) showed that avian- derived pepsin hydrolysate effectively inhibited hpopolysaccharide (LPS)-induced bone marrow derived dendritic cells (BMDCs) maturation through reduced expression levels of CD86 and production of TNF-α, IL-12p70, and RANTES, and enhanced production of IL-10 Tolerogenic DC can present antigen to antigen-specific T cells, but fail to deliver adequate costimulation for effector T cell proliferation (Morelli, 2007) These combined studies suggest that peptides in hydrolysates may contribute to preventing allergy by inducing a more tolerogenic response in dendritic cells

Maturation of DCs by LPS is initiated by binding to Toll-like receptor 4 (TLR4) Differences in immunomodulatory effects of hydrolysates might be explained by differences in TLR receptor activating properties, since some studies suggested an interaction between TLRs, mainly TLR2 and 4, and varying hydrolysates (Iskander *et al* 2013, Ndiaye *et al* 2012) The interaction of peptides with TLRs can induce immune effects, since these receptors are known to sample many dietary molecules in the intestine in order to regulate immune effects (Kiewiet *et al* 2017) Limited findings suggest that TLR activation not only induces tolerogenic dendritic cells, but activation of TLRs on antigen –presenting cells also prevents a Th2 driven allergic response by skewing a more Th1 response (Gangloff *et al* 2004) This raises questions surrounding the observed effects on DCs described above The effects mediated by hydrolysates on DC maturation could also be induced via TLRs expressed on dendritic cells While this was not investigated here this is an area which calls for future investigation to show the direct interaction of hydrolysate peptides and TLRs on dendritic cells

Ideally we wanted a hydrolysate that reduced Th2, enhanced or maintained Foxp3 and produced IL-10 and reduced DC maturation Whey hydrolysates 42 and its regenerate 79 and casein hydrolysates 34, and its regenerates 84,132 and casein hydrolysate 47 all reduced Th2 and DC maturation As 47 reduced IL-10 production from in a Foxp3 Treg polarisation assay it was excluded from further analysis Hydrolysates 79, 42, 34, 84, and 132 did not expand or reduce Treg expression but instead maintained Foxp3 expression Having identified hydrolysates which promoted an anti- inflammatory /anti-allergy T cell and DC phenotype, there was no clear choice of hydrolysate to choose for further evaluation in a humanised mouse model Then based on the results of other screening bio-assays like (Dipeptidyl-peptidase IV (DPP-IV) assay which is a screening assay for glucose metabolism and murine T cell polarisation assays within FHI, hydrolysate 42 was chosen 2 upscaled samples of whey hydrolysate 42 (147 3 hr and 147 6 hr) were generated and screened to confirm their immunomodulatory role *in vitro* before they were tested *in vivo* Firstly the effect of increasing doses (0 5 mg/ml 1 0 mg/ml, 2 0 mg/ml) of hydrolysates (147 3 hr and 147 6hr) on T cell proliferation in a whole PBMC population was examined in vitro Neither hydrolysate had any impact on the proliferation of $CD3^+$ T cells in a CFSE labelled PBMC assay However, the data demonstrated that the hydrolysates are not toxic at these high concentrations in vitro In hindsight we questioned the value of using T cell proliferation assays to screen hydrolysates given that there was no effect on T cell proliferation at any of the doses examined Moving forward, the DC assay would be more useful to screen hydrolysates

Utilizing intra cellular flow cytometry proved to be more effective that ELISA in detecting the ability of hydrolysate 147 (6hrs) to significantly increase the percentage of $CD4^+$T-bet^+ cells producing IFN-γ in Th1 polarised cells Utilizing intracellular flow cytometry also proved to be instrumental in detecting the ability of 147 3 hrs, 147 6 hr and

wpc80 to significantly decrease the percentage of $CD4^+$ cells producing IL-4 in Th2 polarised cells were previously no Th2 cytokine effect was seen Consistent with earlier studies, both upscaled samples of hydrolysate 42 (147 3hr and 6hr) and wpc80 significantly decreased Ror-γt transcription factor and IL-17 cytokine expression in a Th17 polarisation assay In addition, there was no change detected in the MFI, percentage or number of $Foxp3^+CD4^+$ T cells when upscaled hydrolysates 147 (3 or 6hr) or wpc80 were added in a Treg polarisation assay This data showed 147's ability to alter the Th1/Th2 balance by increased production of T-bet and Th1 cells producing IFN-γ, inhibited production of GATA-3 and Th2 cells producing IL-4, inhibited Ror-γt Th17 polarisation but does not interfere with Treg balance

All of the findings thus far have suggested that hydrolysate 147 has anti-inflammatory/anti-allergic properties however the underlying mechanism to explain the aforementioned effects are still unknown Therefore the next approach was to identify a mechanism by which this anti-inflammatory effect was being achieved *in vitro* Following a comprehensive review of existing literature to identify potential targets, peroxisome prohferator-activated receptor gamma (PPAR-γ) was identified as an interesting candidate PPAR-γ belongs to the nuclear receptor class II superfamily, showing pleiotropic effects in inflammatory processes in their capacity as ligand-dependent transcription factor (Zingarelh and Cook, 2005) P-PARγ was found to be expressed in different cell types, involving B and T lymphocytes (Greene *et al*, 2000), monocytes/macrophages (Tontonoz et al , 1998) and DC (Nencioni *et al*, 2002) It has been shown that P-PARγ was active in DCs and it was likely to be a regulator of many aspects of DC biology (Gosset *et al* 20001, Nencioni *et al* 2002) Tissue-resident DCs are exposed to various dietary substances raising the question of whether there are natural mediators (like milk derived bioactive peptides) that are capable of P-PARγ activation Indeed, several compounds have been shown to activate P-PARγ in DCs, such as lysophosphatidic (LPA) which is a G protein receptor antagonist and oxidized low density lipoproteins (oxLDL)

which are proteins associated with atherosclerosis initiation and progression (Hourton *et al* 2001, Smyth *et al* 2008) However, most of our current knowledge on P-PARγ's function in DCs is derived from synthetic agonist treatment *in vitro* Data provided by these studies suggest that P-PARγ activation negatively affects functional maturation of DCs in response to environmental stimuli It has recently been shown that a casein hydrolysate inhibited the pro-inflammatory NF-κB pathway through activation of PPAR-γ (Fitzgerald *et al* 2015)

Therefore as P-PARγ ligands have been described as negative regulators of DC maturation, it was imperative to investigate if 147 hydrolysate expressed P-PARγ n DC LPS stimulated human monocyte derived dendritic cells (MDDC) expressed higher levels of P-PARγ mRNA than immature DC P-PARγ mRNA expression was significantly higher in the presence of hydrolysate 147 (less so with wpc80) in LPS matured DC This increase in P-PARγ mRNA expression likely corresponds to activation of P-PARγ Given that 147 hydrolysate activates P-PARγ we further examined whether the effects were suppressed by the specific P-PARγ antagonist BADGE Importantly, the inhibitory effects of 147 hydrolysate on co-stimulatory molecule and IL-12 cytokine expression were completely prevented when DC (matured in the presence LPS) were treated with BADGE Similarly BADGE prevented the anti-inflammatory effects of P-PARγ agonist troglitazone in LPS stimulated DC The inhibitory effect of P-PARγ on DC maturation was mediated in part through nuclear factor of activated T cells, nuclear factor kappa-light chain enhancer of activated B cells NF-κB A growing number of studies have highlighted the interaction of P-PARγ and NF-κB P-PARγ could inhibit activation of NF-κB through several mechanisms and then repress NF-κB-mediated transcription of proinflammatory cytokines (Genolet *et al* 2004, Kelly *et al* 2004) It is well established that the transcription factor NF-κB has a key role in mediating inflammatory responses including the regulation of DC development, maturation and APC function (Rescigno *et al* 1998) NF-κB activation controls both the

expression of costimulatory molecules and MHC, as well as the production of cytokines like IL-12 (Ouaaz *et al* 2002) In resting conditions, NF-κB is maintained inactive in the cytosol of cells forming a complex with its inhibitor subunit The phosphorylation of the p65 subunit of NF-κB on serine -536 is an important step in the activation of the NF-κB pathway via activation of several kinases including IκB kinases (IKKs)

Therefore the effects of whey hydrolysate and troglitazone on the phosphorylation of p65 subunit were examined as an index of NF-κB pathway activation Our results showed that the PPAR-γ ligand troglitazone suppressed the activation of NF-κB by LPS in DC, decreasing the phosphorylation of p65 147 hydrolysate and wpc80 induced similar responses Moreover, BADGE suppressed the effects of both troglitazone and the hydrolysate suggesting a role for P-PARγ in the NF-κB pathway modulation by 147 hydrolysate (Figure 6 1) Given that NF-κB plays a key role in regulating the expression of co-stimulatory molecules in DC our data on the suppression of NF-κB suggests that whey hydrolysate 147 may exert its anti-inflammatory effects on cytokine production and co-stimulatory expression through NF-κB Similar findings were found with a casein hydrolysate that exerted anti-inflammatory effects on human endothelial cells by suppressing NF-κB pathway activation through P-PARγ dependent mechanisms (Fitzgerald *et al* 2015) It has been reported that endogenous ligands of PPAR-γ such as cyclopentenone prostaglandins, a metabolite of prostaglandin D2 might also exert anti-inflammatory effects through suppression of NF-κB activation in a P-PARγ independent manner via direct inhibition of other signaling components of the NF-κB pathway, such as IκB kinase (Rossi *et al* 2000) There are also studies that reported that P-PARγ ligands may have anti-inflammatory effects in a P-PARγ and NF-κB independent manner interfering with other pathways such as activation of ERK (Wilmer *et al* 2001) or inhibition of AP-1 pathways (Perez-Sala *et al* 2003)

As the hydrolysate used in this study is a complex mixture of peptides, we cannot exclude the possibility that together with the direct effect on NF-κB through P-PARγ, the hydrolysate might exert its anti-inflammatory effect by acting at other levels of NF-κB activation or even by modulating other pathways However, further study is required to specifically identify the functional components (peptides) of hydrolysate 147 Many of the known bioactive peptides are multifunctional and can exhibit different bioactivities, such as immunomodulation or antimicrobial, antioxidant, antithrombotic, and antihypertensive properties (Hartmann *et al* 2007, Meisel 2004) Interestingly, a recent study showed potential anti-inflammatory property of casein hydrolysates mediated by the modulation of NF-κB (Fitzgerald *et al* 2014) In addition, in the present study, we reported an anti-inflammatory bioactivity of the whey hydrolysate, and we demonstrated that the hydrolysate acts by inhibiting transcription factors (NF-κB and PPAR-γ) that are involved in the regulation of several inflammatory pathways, which in turn, are common to many diseases Therefore, our results may offer a useful basis to understand the molecular mechanisms which may regulate many bioactivities attributed to milk derived bioactive peptides

Chapter 3 extensively investigated the anti-inflammatory effects of whey and casein hydrolysates *in vitro* and provided comprehensive findings on the anti-allergic/anti-inflammatory ability of whey hydrolysate 147 While many have reported on the immunomodulatory effects of hydrolysates there have been few reports or in-depth studies providing a mechanism to support such interactions Notably this chapter has defined the direct effects of hydrolysates on the key cells involved in allergy and proposed an anti-inflammatory mechanism of action by which hydrolysates can modulate their effects These findings enhance our knowledge of the bioactive properties of hydrolysates *in vitro* and provide a basis from which these investigations can be expanded upon *in vivo*

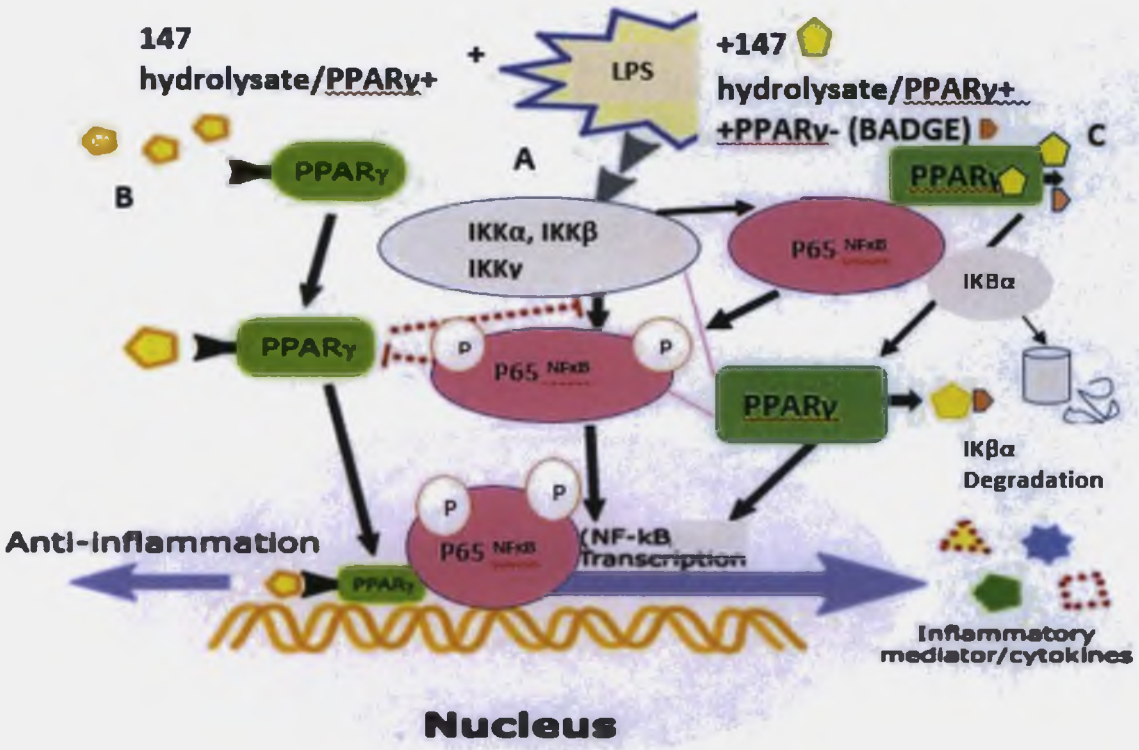


Figure 6.1.Whey hydrolysate 147 exerts its anti-inflammatory effects by suppressing NFkB pathway activation through PPARγ dependent mechanism (A) Inactive NFkB dimers are sequestered in the cytoplasm by interaction with the IKB alpha (IKB).Upon stimulation with LPS the IKB proteins are phosphorylated by one or a number of IKB kinases (IKK), ubiquitinylated and degraded thereby allowing the NFkB complex to translocate into the nucleus. The active NFkB complex translocates into the nucleus where it mediates gene transcriptions and leads to the inflammatory response. (B)147 hydrolysate and PPARγ+ agonist (Troglitazone) suppressed the activation of NFkB by LPS in DC by inhibiting the phosphorylation of p65 and translocation to the nucleus. (C) PPARγ – antagonist (BADGE) suppressed the effects of both 147 hydrolysate and PPARγ+ and allowed the NFkB complex to translocate to the nucleus and exert an inflammatory response.

Chapter 3 identified *in vitro* evidence that hydrolysate 147 contains specific immunomodulating peptides which increase Th1 cytokines and decrease Th2 cytokines and impair TH17 cells and contribute to preventing allergy by inducing a more tolerogenic response in dendritic cells This suggested that hydrolysate 147 could act therapeutically to prevent allergic or inflammatory conditions where the induction of a more balanced immune system could be beneficial Therefore a model system to examine the potential of hydrolysate 147 to be used therapeutically as a anti-allergy/anti-inflammatory functional food in infant formula needed to be developed Although it may seem obvious, the goal for using mouse models is to gain insight into the value of specific agents (147 hydrolysate) for use against food allergy As a result of this, the starting point for choosing a mouse model has to be how it relates to the human disease Mouse models, despite their individual limitations, have been useful tools for the study of allergic diseases *in vivo* (Helm *et al* 2002, Hogan *et al* 2001) They have been instrumental in advancing our understanding of the underlying pathophysiologic mechanisms, assessing the allergenicity of food products, and evaluating new therapeutic strategies (Niggleman *et al* 2001, Knippels *et al* 2002) Mouse models offer the ability to study sensitization, studies that are not possible in humans for obvious ethical reasons Mouse models vary in terms of sensitization protocols (type of food allergen, dose, route of administration, use of adjuvants), and the methods used to assay the allergic response after antigen challenge (measurement of inflammatory mediators, functional assays of gut function, morphologic studies) It is also important to consider the genetic background of the animal in eliciting an immune response

For example, Balb/c mice are good IgE responders, whereas other mouse strains such as C3H/HeJ mice vary in IgE production although they have been used in establishing good models of food allergy (Li *et al* 1999, Li *et al* 2000) This may be related to the recent

observation that this mouse strain has a point mutation in Toll-like receptors preventing lipopolysaccharide responses by peritoneal macrophages that might be necessary to elicit a full IgE response (Helm *et al* 2002) Smaller laboratory animals also offer the potential for genetic manipulation in which specific effector cells of inflammation (mast cells, eosinophils) or mechanisms of antigen recognition (IgE, antigen-specific T-cell receptors) are manipulated Examples include mast cell-deficient mice (Wershil *et al* 2000) and ovalbumin-specific T-cell receptor transgenic mice (Shida *et al* 2002) As discussed in chapter 1, despite the benefits of mouse models for food allergy, there are limitations which make the prospect of using humanised mouse models more attractive To date, there is no animal model that can identify known food allergens, predict the allergic potential of novel food proteins, or mimic the human food-allergic sensitization and allergic responses (Helm *et al* 2002) However, humanised mice with components of the human immune system can bridge this gap as they have been used successfully for studying the human immune system *in vivo* and for predicting the allergic potential of food proteins (Burton *et al* 2016) Severely immunodeficient mouse strains, such as NOD/Shi-scid IL2ry ^{null}, NOD/LtSz-scid IL2ry ^{null} (NSG) and BALB/c Rag2 ^{null} IL2ry ^{null} enabled long-term engraftment of human tissues because of the total lack of the endogenous mouse immune system, enormously improving the generation of humanised mice (Ito *et al* 2002,Yahata *et al* 2002, Shultz *et al* 2005,Traggiai *et al* 2004) These strains are highly immunodeficient, lacking B, T, and NK cells, and their genetic background is permissive for human hematopoietic engraftment and differentiation Upon human CD34^{+} hematopoietic stem and progenitor cell transplantation, most human hematopoietic populations (including B cells, T cells, monocytes, dendritic cells, erythrocytes, and platelets) can develop and are detectable in these models (Shultz *et al* 2007, Legrand *et al* 2006, Ito *et al* 2002, Traggiai *et al* 2004, Ishikawa *et al* 2005) However the differentiation of human myeloid lineage cells, especially granulocytes and mast cells, in

conventional human HSC NOG mice had been inefficient (Shultz *et al* 2012) until several groups endeavoured to improve the efficiency of human granulopoiesis in humanised mice by engineering mice to express human genes encoding these cytokines Mice that express the human myeloid cytokines IL-3 and GM-CSF showed increased support of myeloid differentiation (Ito *et al* 2013, Willinger *et al* 2011)

In particular the NOG IL-3/GM-CSF transgenic mouse strain established by Ito resulted in the stable presence of mast cells and basophils and these cells were functional as mice developed PCA reactions when exposed to serum from patients with cedar pollinosis (Ito *et al* 2013) The development of such strains of mice that mimic human allergic responses allow for the study of novel therapeutics for food allergy for e g a milk protein hydrolysate with anti-inflammatory properties to help combat inflammatory disease Therefore it was important to identify which strain of mouse would provide the best host for the engraftment of human immune cells involved in the allergic response (T, B, myeloid, eosinophils, mast cells, basophils) As the NOG IL-3/GM-CSF was not commercially available the next option was to use commercially available strains of NOD/Shi-scid-IL-2Rγ ^{null} mice that express human cytokines to support the differentiation of human granulocytes and mast cells The first mouse strain chosen was a triple transgenic mouse model expressing human stem cell factor (SCF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukin-3 (IL-3) NOD/Shi-scid-IL-2Rγ ^{null} mice termed NSG-SGM3 These mice were chosen as previously transplantation of CD34^{+} HSCs into NSG-SGM3 mice lead to 1) robust human hematopoietic engraftment of immune cells in blood, spleen, bone marrow and liver 2) slightly enhanced development of CD33^{+} myeloid cells and CD15^{+} granulocytes 3) elevated myeloid dendritic cell (mDCs) frequencies in the bone marrow of humanised NSG-SGM3 mice and 4) elevated human CD4^{+} Foxp3^{+} Treg cell populations m the bone marrow,

spleen , liver and blood of humanised NSG-SGM3 mice compared with nontransgenic NSG mice (Billerbeck *et al* 2011)

The second strain chosen was a single transgenic mouse model that expressed membrane-bound human stem cell factor termed NSG-SCF mice Previously Takagi *et al* 2012 demonstrated that after transplantation of $CD34^+$ HSCs into NSG-SCF mice human granulocytes accounted for the majority of engrafted human cells in the bone marrow of these mice and c-kit^+ human mast cells differentiated efficiently in the bone marrow, spleen and mucosal tissues thereby supporting human myeloid development in these mice In addition, NSG-SCF mice were chosen as transgenic expression of human mSCF enabled heightened levels of hematopoietic cell chimerism in the absence of irradiation thereby eliminating the complications associated with irradiation (Brehm *et al* 2012) To date there is no study that compares these two strains of mice for the development of mast cells and so this comparison may prove to be useful in future investigations of human mast cells

As most studies compare the engraftment of humanised mice to a non-transgenic strain, NSG were included in this comparison study Haematopoietic engraftment in the spleen, bone marrow, liver, peripheral blood and thymus was evaluated by flow cytometry 20 weeks after $CD34^+$ cells were transplanted into all three strains of NSG mice Comparable levels of human leucocyte ($CD45^+$) engraftment were observed in the spleen, bone marrow and liver across all three strains of mice whereas lower levels of leucocytes were found in the peripheral blood and thymus When comparing myeloid levels in the tissues of NSG, NSG-SCF and NSG-SGM3 engrafted humanised mice, we observed a significant increase in myelopoiesis in the spleen of NSG-SCF mice, an increase which was comparable to the increase seen by Takagi *et al* 2012 with myeloid cells B cells engrafted at higher rates in the spleen and bone marrow of NSG-SCF compared to smaller proportions in NSG and NSG-SGM3 Little is known about human immune cell reconstitution in the livers of humanized

mice Upon further examination of the immune subset composition of the human $CD45^+$ population, we found that there were significant $CD3^+$ T-cell and $CD20^+$ B-cells populations residing in the livers of NSG-SCF humanized mice which is similar to the findings of Dykstra *et al* (2016) Similar T cell increases were found in the livers of NSG-SGM3 Current understanding concerning the education of developing T cells holds that selection occurs in the thymus on MHC molecules expressed by stromal cells of non-hematopoietic origin (Bix *et al* 1992) Although human hematopoietic cells bearing human MHC molecules might enter the mouse thymus in NSG mice, some published studies have predicted that these cells cannot effectively select developing human T cells (Shultz *et al* 2012, Bix *et al* 1992) Although we see low levels of $CD3^+$ T cells engraftment in the spleen and bone marrow moderate levels (~ 12 to 17%), of engraftment were observed in the livers of NSG-SCF and NSG-SGM3 mice We cannot presume that human MHC restricted T cell development may be functional in these mice as no functional assay was performed

The main aim of this study was to find the most appropriate host for the engraftment of mast cells and basophils (in addition to T and B cells) so our focus next turned to analysing the populations of effector cells in these mice No mast cells or basophils could be detected in the tissues of NSG mice, however there was an increase in the total number of mast cells and basophils recovered in the spleen, bone marrow and liver of NSG-SCF mice in comparison to those found in tissues of NSG-SGM3 but the difference was not significant Overall there was higher engraftment of immune cells in the tissues of non-irradiated mice however this statement comes with one caveat Three week old NSG and NSG-SGM3 mice engrafted poorly as a result of the radiation dose (2 4Gy) given to these mice and was not a result of the HSC donor As already discussed in Chapter 4, radiation damage in NSG and NSG-SGM3 mice resulted in significant damage to the thymus of these mice Thus, the ability of transplanted human HSC to repopulate following radiation injury was significantly reduced

in these mice NSG mice have been previously shown to survive up to 125 days with this sub-lethal dose of irradiation (> 10 weeks old) (Brehm *et al* 2012) however as these mice were irradiated at weaning age (3 weeks old) they were much more sensitive to the dose of radiation All subsequent experiments with the NSG-SGM3 mice were carried out with a dose 1 0Gy

Using the lower dose of irradiation, the engraftment kinetics of immune cells was assessed at 12, 16 and 20 weeks post transfer of flow sorted $CD34^+$ HSC (Lonza) into irradiated NSG-SGM3 and non-irradiated NSG-SCF mice At the time of harvest, no observation of any gross macroscopic abnormalities were visible in these mice Flow cytometry was performed to evaluate the engraftment levels of human $CD45^+$ cells The engraftment levels of leucocytes was higher in the peripheral blood (PB) of NSG-SCF compared to the PB of NSG-SCF at 6 weeks post HSC transfer However by 12 weeks, comparable levels of leucocytes had engrafted in the PB of NSG-SGM3 and NSG-SCF mice By 20 weeks similar levels of leucocytes had engrafted in PB in both strains of mice Engraftment in the thymus mirrored that was seen in the PB Significantly more leucocytes had engrafted in the spleens of NSG-SGM3 at 12 weeks but by 16 and 20 weeks similar levels of $CD45^+$ had engrafted in the spleens of both strains of mice At 16 and 20 weeks engraftment levels of $CD45^+$ cells in the bone marrow were slightly higher in NSG-SCF mice compared to NSG-SGM3 mice but by far the highest levels of leucocytes were found in the livers in both strains of mice and they remained high over the course of the experiment Spleens were markedly enlarged in both transgenic strains

Consistent with this finding, high levels of myeloid cells had engrafted in the spleen and livers of NSG-SCF and NSG-SGM3 mice Moderate levels of B cells were found in the spleen, bone marrow, liver and PB at 12 and 16 weeks but levels decreased between 16 and 20 weeks in both strains of mice Notably, the only difference between the two strains of

mice was seen in the engraftment of T cells There were significantly more T cells in the bone marrow of NSG-SCF than NSG-SGM3 at 16 and 20 weeks and significantly more T cells in the circulation of NSG-SGM3 mice than NSG-SCF at the end of the study The increase in the $CD3^+$ cell engraftment in the PB of NSG-SGM3 is in agreement with the Billerbeck study that found an overall reduction in B cells was paralleled by a proportional increase in $CD3^+$ T cell frequencies (Billerbeck *et al* 2012) This increase was further confirmed on examination of the $CD4^+$ to $CD8^+$ T-cell ratios in the tissues of the mice Higher $CD4^+$ to $CD8^+$ T-cell ratios were found in the spleen, bone marrow and livers in NSG-SCF and NSG-SGM3 mice This data suggests that the transgenic expression of SCF, GM-CSF and IL-3 promotes the expansion of human $CD4^+$ T cells in these humanised mice

In this study the development of mast cells and basophils in humanised mice was compared in order to find which strain of mouse was the better host for the engraftment of the effector cells of allergy Recently, Takagi *et al* (2012) demonstrated that the development of human mast cells was significantly improved in their hSCF transgenic NSG strain compared with normal NSG mice However the expression of the high affinity IgE receptor (hFCεR1) on mast cells was not evaluated In this study, we examined whether human cells expressing hFCεRI or CD203c, (phenotypic markers) were present There was a clear subpopulation of $hFC\varepsilon R1^+$ $CD203c^+$ cells present in the spleen, bone marrow and liver from NSG-SCF and NSG-SGM3 mice There were no measurable differences in the frequencies and numbers of $hFC\varepsilon R1^+$ $CD203c^+$ in the spleen and bone-marrow of these mice however there were significantly more mast cells detected in the livers of NSG-SGM3 mice in comparison to NSG-SCF When these cells were further distinguished by assessing their human c-kit expression there were more mast cells that were $c\text{-}kit^+$ in spleen, bone marrow and livers of NSG-SCF mice while more basophils ($c\text{-}kit^-$) were present in the spleen, bone marrow and liver of NSG-SGM3 mice Notably significantly more basophils (~60%) developed in the

livers of NSG-SGM3 than in NSG-SCF mice This suggests that mast cells and basophils may have been differentially distributed in various tissues One other immunodeficient mouse strain carrying the human IL-3 and GM-CSF genes have been generated IL-3/GM-CSF-Tg (transgenic) (Ito *et al* 2013) It is noteworthy that there were similarities in phenotypes between our study and the Ito study For example, there was high engraftment of HSCs in IL-3/GM-CSF, with an increase in the $CD3^+$ T cell population at 12 week post HSC transplantation and $FC\varepsilon RI^+CD203c^+$ mast cells and basophils were significantly higher in NOG IL-3/GM-CSF than in non-Tg mice and were differentially distributed ~ 70 and 50% of $FC\varepsilon RI^+CD203c^+$ cells of the whole $CD45^+$ population in the spleen and bone marrow were mast cells (Ito *et al* 2013) The overall objective of this study was to identify which strain of mouse provided the best host for engraftment of human immune cells involved in the allergic response The data suggested that both strains would be useful for engraftment of and study of immune cells involved in allergy NSG-SCF mice had the highest potential from the point of view of HSC maintenance and myeloid and mast cell engraftment from the first study but there was no real difference between the two strains in terms of engraftment of human immune cells in the second stud Ideally more HSC donors and studies comparing the two strains would have re-affirmed these findings but due to limited time and budget no further studies were completed comparing the two strains Based on these findings and to avoid the complications of irradiation, NSG-SCF mice were chosen as the best host for the humanised mouse model of food allergy

After choosing the best host, conditions such as fresh versus frozen $CD34^+$ HSC, male versus female mice and dose of $CD34^+$ were optimised to facilitate the optimal development of this humanised mouse model An equally important component, after identification of the best host in the development of humanised mice, is the choice of engrafting cells $CD34^+$ cells can be isolated from adult bone marrow, fetal liver, UCB and G-CSF–mobilised

peripheral blood cells (Alexandre *et al* 2016) Although human PBMC have been used to study IgE-mediated allergic responses (Weigmann *et al* 2012) several investigators have used UCB to study IgE-mediated allergic responses (Takagi *et al* 2012, Ito *et al* 2013, Burton *et al* 2016) For this reason and since UCB have multi-potency and self-renewal capabilities higher than adult bone marrow (Metcalf 2007) UCB was chosen as the optimal type of HSC 3 sources of UCB were tested for the humanisation of mice Fresh UCB from the cord blood bank Anthony Nolan Trust, Nottingham, UK, fresh UCB donated from consented women undergoing planned CS at NMH, Dublin and frozen cord blood derived flow sorted $CD34^+$ cells purchased from Lonza In our experience, the frozen purified $CD34^+$ cells from Lonza, were the most reliable source for the generation of humanised mice as they allowed for greater flexibility in terms of planning experiments and they contained sufficient numbers of $CD34^+$ cells to perform experiments However their high cost must be taken into consideration when planning experiments

The dynamic interplay between donor hematopoietic stem cells (HSCs) and the recipient microenvironment governs the survival and proliferation of HSCs in a transplantation setting (Notta *et al* 2010) A complex network of cells within the microenvironment regulates HSC function via direct interaction or secretion of cytokines that act in an autocrine or paracrine manner (Scadden 2006) During the course of our functional analyses of HSCs using NSG-SCF mice, we observed that the recipient gender played a critical role in the engraftment and proliferation of human HSCs Specifically, female NSG mice were far superior to their male counterparts in facilitating the engraftment of human HSCs When 1 x 10^5 HSCs were transplanted into male and female NSG-SCF mice, striking differences in engraftment were observed Absolute numbers of human $CD45^+$ cells in the spleen and liver were 6 fold and 2 fold higher than male mice, myeloid cells in the spleen were 10 fold higher, T cells in the spleen and liver were 11 fold and 15 fold higher and B cells in the spleen increased 6 fold

compared with male NSG-SCF mice Engraftment levels with a lower dose of HSCs (5×10^4) also resulted in differences in engraftment between male and female mice In the PB the percentage of $CD3^+$ T cells at 12 and 20 weeks increased 4 fold and 2 fold higher than male mice, T cells in the bone marrow and liver were 5 fold and 3 fold higher and B cells in the liver increased 3 fold compared to their male equivalent Lastly there was a 3 fold increase in the absolute number of mast cells in the spleens of female mice compared to male mice These findings are in agreement with Notta *et al* who reported that female NSG mice transplanted with limiting numbers of human $CD34^+$ cord blood HSCs showed better engraftment of human $CD45^+$ cells than males (Notta *et al* 2010) They proposed 2 reasons for the existence of this gender difference (1) female NSG mice might be more immunodeficient than males, or (2) gender- associated factors, such as steroid hormones, can positively or negatively regulate human HSCs Although further experiments are required to identify gender-specific mechanisms of HSC engraftment, our study revealed their key role and supported the concept of recipient gender as a critical variable in the context of stem cell transplantation studies

Food allergy is an important public health issue in industrial countries due to the increasing prevalence and the potential life-threatening consequence, effecting up to 8% of children and 4 % of adults (Platts-Mills 2015, Ashley et al 2015) Often patients experience a reduction in physical and psychologic well-being and suffer from a decreased quality of life due to disease activity The continuous rise in the number of people affected by an allergic condition indicates an urgent need for better diagnosis and more efficient treatment/prevention options Food allergies are often observed in early childhood The finding that children can mount allergic responses to food which they have never been exposed to before, raised the question whether certain allergens might be transferred by breast-milk feeding Indeed, it has been demonstrated that major food allergens are present in

breastmilk (Pastor-Vargas *et al* 2015) However, it is still a matter of debate whether these allergens may induce tolerance or if they might promote sensitisation in children (Bion *et al* 2016) Although breastmilk is the optimal feed for infants, it is important that research continues to improve the composition of formula available to infants for whom breastmilk is not an option In collaboration with FHI, we embarked on innovative research to find specific hydrolysed cow's milk peptides that were anti-inflammatory/anti-allergic that could help to induce tolerance in allergy prone infants, thereby reducing the risk of allergies and associated atopic conditions later in life In chapter 3, the ability of milk protein hydrolysate 147 to suppress immune responses *in vitro* and the associated mechanism through which this occurs were identified In chapter 4, the transgenic NSG-SCF mouse was identified as the best host for the engraftment of immune cells involved in the allergic response

Therefore the next step in Chapter 5 was to develop an experimental humanised mouse model of ovalbumin induced allergy and inflammation that mimic the main clinical characteristics of human disease as well to examine the anti-inflammatory/ tolerogenic properties of a novel milk protein hydrolysate in this model The advancement of humanised models to study food allergy and inflammation has provided a clinically relevant setting by which the pathophysiology of the disease can be studied What makes them more advantageous over mouse models of food allergy is that they are designed to facilitate the engraftment of adult human immune cells within the mouse Thus the cells driving the allergic disease are human and are capable of mounting an immune response to sensitised and challenged antigen thus making this model clinically relevant and useful for assessing novel therapeutic interventions including milk protein whey hydrolysates While there are a limited number of humanised mouse models available to study food allergy we and others have found the NSG-SCF mouse strain to be superior in terms of myeloid engraftment compared to other models (Takagi *et al* 2012, Coughlan *et al* 2016) While this humanised mouse model was originally developed by

Takagi *et al*, several experiments were carried out (Chapter 4) to find the best host for the engraftment of human immune cells involved in the allergic response and NSG-SCF was found to be the more appropriate host Many approaches were undertaken to enhance this model, in particular the use of flow sorted CD34+ HSCs, instead of freshly isolated $CD34^{+}$HSCs, female mice instead of male mice and lower dose of HSC Both sexes are afflicted with food allergies in humans, although there are relative differences based on age, among adults, most afflicted are males, among children are females (DunnGavin *et al* 2006) Most mouse models have used female mice as females tend to exhibit more robust IgE antibody responses in general (Venugopal *et al* 1995, Melgert *et al* 2005) A recent study using hazelnut allergy mouse model, reported that female mice exhibit higher IgE responses compared to males (Parvataneni *et al* 2009) Thus the findings in Chapter 4, facilitated the performance of a larger scale *in vivo* study with 4 HSC donors $CD34^{+}$ flow sorted cells purchased from Lonza, combined with the lower dose of HSC required, allowed the inclusion of more mice per study On this basis, and building on the findings in Chapter 3, this humanised mouse model of ovalbumin induced allergy and inflammation was used as a platform to test a novel functional compound, hydrolysate 147 against food allergy

To address how hydrolysate 147 affects the severity of allergic responses *in vivo*, a modified protocol of a well characterised food antigen ovalbumin was used (Dearman *et al* 2007) This model consisted of sensitisation, treatment and challenge phases of experimental food allergy that used Th2-type sensitisation with OVA/alum i p injections followed by repeated gavages of the treatment (147) and challenge of the OVA antigen to mimic physiological allergen exposure The question may be asked why ovalbumin antigen which is the main protein found in egg white was used instead of whey or casein which are the main proteins found in milk The choice of OVA as a model allergen was also driven by the clinical observation that allergy to egg in early life is one of the strongest predictors of

progression of the allergic march, the process by which children gradually develop severe allergic diseases like atopic dermatitis, rhinitis and asthma (Lambrecht 2017) In order to assess the relative allergemcity of novel proteins, it is essential to use known allergens in humans as reference proteins OVA is a T cell-dependent antigen commonly used as a model protein for studying antigen-specific immune responses in mice The murine immune response to OVA has been well characterized, to the extent that the immunodominant peptides for eliciting T cell responses have been identified (Weaver *et al* 2008) Anti-OVA antibodies are detectable 8-10 days after immunization using enzyme-linked immunosorbent assay so it was foreseeable that these responses would also be elicited in a humanised mouse model Normally allergen ingestion by humans or mice results in tolerance so consequently adjuvants such as aluminium hydroxide (alum) are used to overcome oral tolerance It is not well understood to what extent adjuvants are needed to promote an allergic response, but adjuvant signals appear crucial at least in a range of animal studies Adjuvants influence both the activation and subsequent migration of dendritic cells (DCs) to a draining lymph node, which reside in the vicinity of the first exposure site to potential allergens In several animal models of food allergy, sensitization by the i p route with the use of aluminium hydroxide have been used successfully to decipher the inherent allergenic potential of proteins and the clinical utility of alum in humans has been approved (Lambrecht *et al* 2009) Therefore alum was used with OVA in our model to stimulate an IL-4-driven Th2 response

Previously, we used an *in vitro* platform to screen immunomodulatory effects of a range of whey and casein hydrolysates in a systematic way Our data showed that a specific whey hydrolysate induced a greater anti-inflammatory profile than intact wpc80 Since higher levels of anti-inflammatory cytokines have a regulatory function in allergy (Chung *et al* 2004, Akdis *et al* 2006) it was hypothesised that this whey protein hydrolysate may also have *in vivo* immunomodulatory effects in a OVA allergy setting Therefore, we investigated the

immunomodulatory capacity of the hydrolysate 147 in a humanised mouse model of ovalbumin induced allergy and inflammation

The first step in the development of the model was to ensure that the mice were sufficiently humanised i e that human immune cells involved in the allergic response had engrafted Engraftment levels of lymphoid ($CD45^+$), myeloid ($CD33^+$), T ($CD3^+$) and B ($CD19^+$) cells as well as the effector cells of allergy (mast cells and basophils) were monitored in the spleen, bone marrow, liver, PB and thymus after 12 weeks before an immunisation regimen began Sufficiently high levels of all immune cells including mast cells and basophils engrafted after 12 weeks post transplantation with HSCs which was important for the success of the model going forward

Most animal studies showing beneficial effects of hydrolysates in allergy mainly focus on immunoglobulin levels as a measure for sensitisation (Von Esh *et al* 2011, Fritsche *et al* 1997, Adel-Patient *et al* 2012, Peng *et al* 2004) while only a few studies investigated effects of whey hydrolysates on immune populations involved in the allergic response (Van Esh *et al* 2011, Adel-Patient *et al* 2011) A wide range of in vivo and ex vivo readouts were performed to assess or support the anti-allergic capacity of whey hydrolysate 147 The first common readout to assess the clinical symptoms of allergy was adopted from Li *et al* (2001) Mice were sensitised, treated with 147 and wpc80 and challenged with OVA and anaphylaxis symptoms were monitored 30 mins after intragastric challenge A high anaphylactic score was recorded for the OVA mice and no score for the PBS, with reduced symptom scores recorded for the 147 and wpc80 treatment mice In a humanised mouse model of peanut allergy, peanut anaphylaxis was blocked by treatment with ant-IgE antibody In this study they went one step further to test whether human mast cells were responsible for the response By using Balb IgE deficient mice, which are immunocompetent and have an intact mast cell compartment, they elegantly demonstrated that no anaphylaxis occurred in mice

that received human IgE This confirmed that human IgE cannot elicit responses by murine mast cells and these findings strongly implicated that engraftment by human mast cells triggered by human IgE antibodies, produced by engrafted B cells were the sole inducers of anaphylactic responses to peanut exhibited by the humanised mice (Burton *et al* 2016) In a similar murine model of OVA allergy, a novel immunomodulatory protein isolated from the edible mushroom *Flammulina veluties* was tested for its anti-inflammatory properties, mice sensitised to OVA in the presence of the mushroom protein (FIP-*fve*) were protected from systemic anaphylaxis after oral challenge with OVA (Hiesh *et al* 2003)

The GI tract is central to the pathophysiology of food allergy It was hypothesised that feeding hydrolysates would alter the inflammatory status of the GI and this proved to be the case 147 and intact protein wpc80 displayed a similar protective profile in the jejunum of OVA mice compared to OVA mice OVA mice had all the hallmarks of allergic inflammation which included lymphocyte infiltration, villi destruction and blunting, which were mostly absent in 147 and wpc80 treated mice 147 and its intact control wpc80 were significantly efficacious in protecting the target organ from allergic inflammation No other humanised mouse model has examined the inflammatory status of the GI tract However, in the mouse model study, Hiesh *et al* (2003) also examined the histological changes of intestine (jejunum) after oral challenge with OVA and found in the FIP-*fve* treatment group, the intestine was nearly normal except for very mild mucosal oedema

In the intestinal tract, mast cells are mainly located in the lamina propria and they contribute both to tolerance for food as well as to sustain the immune response against pathogens (Mekori *et al* 2015) In allergic individuals, ingestions of harmless food proteins leads to the stimulation of allergen-specific T-helper 2 cells and the production of cytokines which are responsible for the production of allergen specific IgE IgE binds to the high-affinity receptor for IgE (FcεRI) on mast cells Allergen crosslinking of cell-surface-bound

IgE leads to mast cell degranulation resulting in allergic symptoms therefore it was important to investigate the presence of mast cells in the GI tract No mast cells were identified in the jejunum of PBS, 147 or wpc80 by toluidine blue staining but limited numbers were found in the connective tissue in OVA mice 18 hours after the intragsatric challenge These mast cells appeared to be intact and revealed no features of de-granulation It is important to note that our experimental regimen only included one intragastric challenge Previously Brandt *et al* (2003) demonstrated that elevated levels of mast cell numbers following OVA challenge only increased between challenge 3 and 5 in a mouse model of OVA induced intestinal inflammation Thus, we propose that more mtragastic challenges may have enhanced the mast cell numbers in the GI tract of these mice which was recently demonstrated by Burton *et al* (2016) where a significant mast cell expansion was evident in the jejunum and spleen of peanut fed mice after intragastric feeding once a week for 8 weeks

A humanised mouse model of food allergy represents an important tool for studying the mechanisms of induction and repression of an allergic reaction, as well as for the development of an immunotherapy to prevent or minimize such an adverse reaction Total IgE and IgG1 (Th2 response) and OVA specific IgG1 are good markers for the induction of an allergic response in mice Therefore it was important to explore whether oral treatment with whey hydrolysate 147 could decrease these markers One week after the final sensitisation low levels of total IgE antibody were detected in the serum of PBS mice whereas there was a significant increase in IgE antibody produced in the serum of OVA sensitised mice However at the end of the study, levels of IgE antibody were similar across all groups (PBS, OVA, 147, wpc80) Unfortunately, OVA specific IgE could not be detected in our ELISA screening system In the recent humanised mouse model study of peanut allergy, Burton *et al* (2016) observed variable effects on the production of peanut-specific IgE with an anti-IgE (omalizumab) treatment and they concluded that the reductions in

detection might be related to interference with the anti-IgE capture antibody in their peanut specific IgE ELISA , which may have been the case in our assay Both total IgG1 and OVA specific IgG1 antibody levels were significantly reduced with 147 hydrolysate and not with intact protein wpc80 These results may point to the possibility that the production of the so called blocking IgG may be involved in the immunodulatory mechanism induced by 147 hydrolysate Only peanut specific IgG1 was significantly reduced with omalizumab treatment but total IgG1 remained high in the humanised mouse model of peanut allergy (Burton *et al* 2016) OVA sensitised mice showed increased levels of total and OVA specific IgG1 compared to the non-sensitised PBS control group, whereas in peanut sensitised mice increased levels of peanut specific IgG1 and lower levels of total IgG1 were found in the serum of NSG-SCF mice In a mouse model of OVA allergy mice receiving oral FIP-*fve* had an impaired OVA-specific IgE response while the IgG2a response was markedly elevated indicating a Th1 enhanced response (Hsieh *et al* 2003) Inclusion of IgG2a and IgG4 which is associated with the development of tolerance in children in future studies would add further weight to the anti-inflammatory/pro-telorogenic evidence of 147 hydrolysate To study the immunomodulatory effects of whey hydrolysates, we measured the ex vivo cytokine production by mesenteric lymph nodes (MLN) During sensitization and challenge, the intestinal immune cells are the first to encounter the administered antigen In this challenge model, antigens will first encounter the MLN, which may affect the peripheral immune response Peyer's patches and the MLN play important roles in allergic responses to food allergens as removal of these lymphoid organs results in a decrease in allergic responses (Nakajima-Adachi *et al* 2014) The role of the MLN in oral tolerance and regulating the allergic reaction has been studied most The results showed that IL-4 local production by MLN from the mice that were treated with 147 hydrolysate and wpc80 were significantly reduced in comparison to MLN from OVA sensitised mice In a similar mouse model of

OVA allergy where treatment with heated OVA was compared with native OVA, the levels of the Th2 cytokines (IL-4, IL-5 and IL-13) were higher in culture media obtained from MLN exposed to native OVA in comparison to low or non-detectable levels with the heated OVA Golias *et al* 2013)

Given the capacity of hydrolysates to alter the Th1/Th2/Th17 balance or enhance Treg function, it was important to explore whether hydrolysate 147 anti-inflammatory/anti-allergic activity would impact the systemic and GI organs of OVA sensitised mice Our humanised mouse model was used to examine the effect of 147 hydrolysate on the production of these cytokines (IL-4, IL-13, IL-6, IL-17, IL-12, IFN-γ and IL-10) Notably 147 hydrolysate provided greater protection in both systemic and GI tissues over and above that provided by it's parent control (wpc80) This was exemplified by the overall reduction in the Th2 cytokines and pro-inflammatory cytokines and increase in the anti-inflammatory cytokine IL-10 in these tissues Importantly, 147 and not wpc80 decreased the levels of IL-13, IL-6 and IL-17 and increased IL-10 in the small intestine, similar difference was seen in the spleen where 147 and not wpc80 decreased the levels of IL-13, IL-6 and IL-17 and 147 and not wpc80 increased the level of the anti-inflammatory IL-10 in the liver

Both Treg deficiency and Treg dysfunction have been found to be associated with the development of allergic diseases However only a limited number of studies have assessed the role of Tregs in IgE food allergy The generation of Treg is a key component of oral tolerance and to date few studies have shown an effect of hydrolysates on Treg formation in vivo Our study found no difference between the Treg frequencies in the spleens of OVA, 147 and wpc80 mice however the levels were significantly reduced in comparison to the PBS control group This reduced Treg expression in splenocytes of OVA, 147 and wpc80 may point to a suppressive state or it may suggest that Tregs may be elsewhere for example in the small intestine Assessing the balance of Treg to activated T cells may provide a more

sensitive measure of Treg expression than enumeration of Treg populations Healthy subjects showed a clear and robust increase in this ratio following allergen exposure (Akis *et al* 2004) which fits with the convincing argument that the dominant response in healthy subjects is a Treg response compared to a transient increased ratio for sensitised infants and delayed response in allergic infants In the recent humanised peanut allergy study (Burton *et al* 2016) there was a higher percentage of Foxp3CD127 ^{low} CD25^{+} cells in the spleens of unsensitised mice than that seen in our model which again may be due to the differences in sensitisation regimens but the authors failed to show the effect that the anti-IgE treatment had on development of Treg response

Many researchers regard allergy as a Th2 weighted imbalance, and immunologists have been investigating ways to redirect allergic Th2 responses in favour of Th1 responses to try to reduce the incidence of atopy Some groups have been looking at using high dose exposure to allergen to drive up the Th1 response in established disease and other groups have been studying the use of mycobacterial vaccines in an attempt to drive a stronger Th1 response in early life Few studies have addressed the issue of T-cell differentiation skewed by hydrolysates *in vivo* Here, we demonstrated that there was an increased Th1/Th2 (IFN-γ/IL-4) ratio in splenocytes of 147 treated mice but not in mice treated with intact wpc80 Significantly there was a lower Th1/Th2 ratio in OVA mice compared with PBS control Babies tend to be born with a Th2 biased immune responses over Th1 response These can be switched off rapidly postnatally under the influence of microbiological exposure or can be enhanced by early exposure to allergens or potentially by exposure to immune balancing infant formula This data demonstrates further that 147 has immunomodulatory capacity to skew the T cell differentiation from a Th2 subtype toward Th1 which would be highly beneficial in infant formula

The use of animals transgenic for the obligatory mast cell human growth factor SCF in this model ensured the differentiation and localisation of human mast cells in the tissues of the humanised mice engrafted with human $CD34^+$ cells NSG that were not injected with HSC had no tissue mast cells, whereas engrafted animals had mast cells in the bone marrow, spleen and liver As mast cells had engrafted sufficiently at 12 weeks the next step was to investigate if sensitisation, treatment and challenge would alter the effector cells of allergy in bone marrow of PBS, OVA, 147 treated and wpc80 treated mice Percentage and number of mast cells were significantly reduced in the bone marrow of 147 and wpc80 control mice Mast cell numbers in 147 and wpc80 mice were more equivalent to that seen in the bone marrow of the PBS control group OVA sensitised mice had increased percentages (~24%) and number of mast cells in the bone marrow which was likely a result of their activation by antigen specific IgE antibodies as lower mast cell percentages of ~15 % were observed before sensitisation occurred at 12 weeks post transfer of HSC cells In a recent NSG-SCF peanut mouse model study a significant percentage and number of mast cell engrafted in spleen, and small intestine, 6 months after transplantation with $CD34^+$ HSCs (Burton et al 2016) but again no data was presented on the development of mast cells post treatment with anti-IgE therapy

Dendritic cells (DCs) are the most important antigen presenting cells to activate naive T cells, and they are therefore the central players of the immune system crossing the bridge between innate and adaptive immunity To play such an important role and keep the balance between health and disease, they have a unique set of features that enables them to operate at the interface of host defense and tolerance One such feature involves the process of DC maturation following encounter with antigen The upregulation of co-stimulatory molecules such as CD86 usually results in development of a functional immune response and in the context of allergen an allergic reaction Alternatively, differential kinetics of co-stimulatory

molecule expression may favour immune regulation Addition of milk hydrolysates to activated dendritic cells *in vitro* in Chapter 3 resulted in a significant decrease in CD86 co-stimulatory molecule expression In this humanised mouse model there was significant reduction in absolute number and percentage of $CD86^{+}CD11c^{+}$ DC in the bone marrow of 147 treated mice and wpc80 mice in comparison to OVA mice which points again to a more tolerogenic DC population Analysis of human dendritic cell populations in spleens of peanut sensitised NSG-SCF mice after IgE treatment revealed a reduction in the MFI of $CD11c^{+}$ $HLA\text{-}DR^{+}$ cells in the spleens of treated mice in comparison to peanut sensitised mice Thus treatment did have an effect of the activation of dendritic cells in this model

The majority of myeloid cells in the bone marrow were $CD33^{+}HLA\text{-}DR^{-}$ granulocytes However treatment with 147 hydrolysate and its intact control wpc80 significantly reduced the percentage and number of granulocytes in the bone marrow of NSG-SCF mice Probing this further resulted in unveiling the most striking result with hydrolysate 147 Investigation of the proportions of lymphocytes, monocytes and granulocytes (through basic lymphocyte gating on FSC and SSC) in the bone marrow revealed a remarkable finding Treatment with 147 and not wpc80 selectively blocked granulocytes accumulation in the bone marrow of 147 treated mice with a corresponding increase in the lymphocyte populations Closer phenotypic analysis using cell number data however revealed a significant reduction in $CD45^{+}$cells, T helper cells, cytotoxic T cells, B cells and myeloid cells in the bone marrow of these mice This somewhat contradictory finding may be explained in the following manner, 147 promoted the accumulation of another cell type (for e g NK, iNKT, ILCs, none of which were analysed here) which may explain the increased percentage of cells in the lymphocyte gate and that this unidentified lymphocyte population resulted in the suppression of the granulocyte population Natural killer cells are a subset of lymphocytes that principally participate in innate immunity but may also have important roles in determining the outcome

of the adaptive immune responses Recent developments in the discovery of NK cell subsets have supported their potential role in allergic diseases via their contribution to allergen-specific immune suppression, allergen-specific TH1 cell generation and IgE production

Little is known about the contribution of the invariant natural killer T (iNKT) cells in the onset of food allergy However they have been shown to have a role in CMPA INKT cells suppressed cow's milk allergic symptoms in mice and differentially regulated oral sensitization when mice were sensitised with casein and whey (Schoutenet *et al* 2012) This dramatic reduction in the granulocyte population remains unexplained but warrants further investigation

In Chapter 5, we evaluated the immunomodulatory capacity of the 147 hydrolysate, demonstrating the anti-inflammatory and tolerogenic potential of 147, using a humanised mouse model of OVA allergy However it is important to highlight the similarities and differences between the two treatments Results revealed that both hydrolysate 147 and wpc80 offered preventive and therapeutic protection against allergy to OVA in the GI tract, by reduced anaphylaxis score in GI of treated mice, both inhibited the local *ex vivo* production of IL-4 by MLNs In the small intestine, both 147 and wpc80 decreased IL-4 and increased IL-10, both reduced the production of the Th2 cytokine IL-4 and pro-inflammatory cytokine IL-12 in the spleen and they both reduced the cytokine production of the Th2 cytokine IL-13 and of IL-12 in the liver Both 147 and wpc80 decreased the mast cell load in the bone marrow and both decreased the percentage of $CD8^{+}$T lymphocytes and reduced the co-stimulatory molecule expression in the bone marrow Importantly 147 alone exerted a marked reduction in humoral IgG1 antibody response, decreased the Th2 response while promoting anti-inflammatory and balancing Th1/Th2 response in the spleen 147 alone reduced the production of pro-inflammatory cytokines IL-6, IL-12 and IL-17 in the small intestine, decreased IL-6 and IL-17 and increased production of anti-inflammatory IL-10 in

the spleen Treating OVA mice with 147 hydrolysate resulted in an overall suppression of the granulocyte populations in the bone marrow and livers of 147 treated mice which was accompanied by reduction of $CD3^{+}CD4^{+}$T lymphocytes and B cells in the bone marrow This hydrolysate was more effective than intact wpc80 in desensitising mice by virtue of its lower allergenicity and its immunomodulating capacity

Future studies using this humanised model should include dose–sensitization data Ideally, an appropriate animal model should be (a) validated by dose–response curves with different sensitizations and (b) be sensitive for distinguishing a threshold beyond which significant sensitization would be predicted Thus, one can conclude that both forms of this protein possess sensitizing capacity and the doses relevant for human exposure should be taken into consideration when qualifying the potential risks for humans Also to enhance its clinical relevance more gastric challenges need to be performed to generate a more clinically relevant model The contribution made from host murine mast cells was not explored in this model To completely rule out the possibility that murine mast cells might be mediating the human IgE effect, examination of murine mast cell protease 1 in these mice would be required Measuring the contribution that TSLP, IL-25 and IL-33, along with ILC2s, may have in the intestine during food allergy sensitization may provide additional predictive markers of sensitizing capabilities Measuring NK activity in the bone marrow during food allergy may provide a regulatory role for these important innate cells Another interesting concept is to explore the role of hydrolysates and the microbiome in food allergy prevention in these mice The altered dysbiosis in the GI tract has a huge effect on the development of food allergy This presents an interesting concept whereby the interplay between immune cells of allergy and the microbiome can be explored

This thesis showed that DC maturation was the best screening assay for the identification of potential anti-inflammatory hydrolysates Th1/Th2/Th17/Treg polarisation

assays were also useful in screening hydrolysates Importantly in chapter 3 we identified the capacity for 147 to inhibit DC maturation in a PPAR-γ NFκB dependent pathway In chapter 4, the best strain of mouse was identified for the engraftment of immune cells involved in allergy In Chapter 5 a novel humanised mouse model of OVA induced allergy and inflammation was developed which verified the *in vitro* findings in chapter 3 that 147 reduced Th2 and promoted Th1/Th2 balance in the spleen Also the reduction in DC maturation in the bone marrow confirmed the earlier findings *in vitro* with monocyte derived dendritic cells Importantly this thesis provides the proof of concept that a whey protein hydrolysate is anti-allergic/anti-inflammatory in a humanised mouse model of OVA allergy This was demonstrated by presenting the capacity for 147 to increase the IgG1 response, promote a Th1/Th2 ratio in the spleen, increase the systemic production of the anti-inflammatory IL-10 while reducing the pro-inflammatory cytokines (IL-12,IL-6, IL-17) in small intestine and (IL-6, IL-17) spleen, by altering the ratio of granulocyte and lymphocyte populations while reducing T and B cell populations in the bone marrow, effects which outnumbered wpc80 effects This thesis has furthered our knowledge of humanised mouse models of allergy as a platform to test functional foods

CHAPTER 7

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