Mining marine materials for novel functional ingredients that modulate the immune response for benefit in chronic inflammation

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Thesis Submitted for the Award of Ph.D.

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September 2018

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Acknowledgements

There have been many people who have helped me through the past few years, without whom I would not have made it this far. These are just some of those I'd like to thank:

My supervisor Professor Christine Loscher, for giving me this opportunity and her never ending support throughout. Her belief in me and ongoing mentorship means so much. I would never have had the courage to pursue a PhD without her.

My lab, past and present, who provided endless cans of coke and laughs over the many ELISA plates. To Conor who I'm sure I drove mad by being super untidy. To the best lab mate and friend anyone could ask for, Niamh, the best gift my PhD brought me was you and the many laughs, naps and funny noises. The BRS, from the early days with Claire and Gill, to taking the helm with Niamh and Garvan and saying yes to a 3rd year with Jonathan and Dan when I said I never would. A society that gave me with the best friends I could have ever asked for, countless trips and nights out and even a broken finger. To all the BRS members who are now firm friends, you've given me a life time of memories and too many hangovers. Especially Jonathan, who has been a best mate throughout, providing guidance, support and a passion for the League of Ireland.

Ruth Bone, Louise Keogh and Dr. Niamh Quinn (Kearney) for being there from Mr Moriarty's Junior Cert science class to now.

Ronan, I couldn't have gotten here without the love, support and pizza you have given in the past year. I have never laughed as much and I can't wait for the next chapter with you.

My sister Jess who has been the best big sister anyone could ask for. You have forever provided guidance, advice and lifts. I will get a driving licence soon. My nephew Rhys, 10 years ago you came into my life and changed it forever. You are wise beyond your years and it is a pleasure to watch you grow into the young man you are becoming. Sam, for the never ending cuddles and licks and all the distractions (Sam is a dog). My Aunts, Uncles, Cousins and lifelong friends, thank you for being the best family. Ann, for always believing and delivering cans of coke throughout my PhD. Toni for basically being a second sister. Lily, for loving and always bragging about her Grandchildren.

My Mam, Janice Connick, for everything. The never ending love, support and guidance you have selflessly given my entire life. I will be eternally grateful. You have been and always will be my rock. There isn't a big enough way to say thank you.

Ballymun, for instilling in me the fight to achieve everything I wanted and not believing those who said I couldn't just because I was from Ballymun. Where you grow up does not, and should not, decide your worth. Hup the flats!

This thesis is dedicated to the memory of my father

David Connick
(1962-2011)

You said I could do anything, so I did.

Table of Contents

List of Figures

List of Tables

Abbreviations

APC	Antigen presenting cell
BMDC	Bone marrow derived dendritic cell
BSA	Bovine serum albumin
CD	Cluster of differentiation
CD	Crohn's disease
cDNA	Complementary DNA
CO_2	Carbon dioxide
DC	Dendritic cell
dH_2O	Distilled water
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
ECACC	European Collection of Cell Cultures
ECFP	European Common Fisheries Policy
EDTA	Ethylenediaminetetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
EU	European Union
FBS	Fetal bovine serum
GMCSF	Granulocyte-macrophage colony stimulating factor
HCl	Hydrochloric acid
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
IC	Indeterminate colitis
IFN	Interferon

IL	Interleukin
IRAK	Interleukin 1 receptor associated kinases
JAK	Janus kinases
KO	Knockout
LC	Liquid chromatography
LOX	Loxoribine
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MCP	Macrophage chemoattractant protein
MG	Molecular grade
$MgCl_2$	Magnesium chloride
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
mRNA	messenger RNA
MS	Mass spectrometry
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NaCl	Sodium chloride
NFκB	Nuclear factor-κB
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween 20
PCR	Polymerase chain reaction
pH	Log of the hydrogen ion concentration
RA	Rheumatoid arthritis
RPM	Revolutions per minute
RT	Room temperature
TAE	Tris-acetate-EDTA

Th	T helper cell
TLR	‘Toll-like’ receptor
TMB	3, 3', 5, 5' tetramethylbenzidine
TNF	Tumor necrosis factor
Treg	T regulatory cell
UC	Ulcerative colitis
β	Beta

Units

%	Percent
ANOVA	Analysis of variance
^{o}C	Degrees Celsius
g	Gram
kg	Kilogram
L	Litre
mg	Milligram
mL	Millilitre
ng	Nanogram
pH	Negative logarithm of the hydrogen ion concentration
SD	Standard deviation
SE	Standard error
SEM	Standard error of the mean
µg	Microgram
µL	Microlitre

Presentations and Posters

Connick, K.M., Loscher, C.E. (30th January 2015). Look and Sea. The Annual School of Biotechnology Research Day, Dublin, Ireland. Oral presentation.

Connick, K.M., Loscher, C.E. (17th - 18th September 2015). Investing marine material as source of immunomodulatory functional peptides. Irish Society of Immunology Annual Conference, TBSI, Trinity College Dublin, Ireland. Poster presentation.

Connick, K.M., Loscher, C.E. (29th January 2016). Discover Ireland's oceans, the science starts here. The Annual School of Biotechnology Research Day, Dublin, Ireland. Oral presentation.

Connick, K.M., Loscher, C.E. (30th June – 1 st July 2016). Mining marine material for novel functional ingredients. Researchfest @ Inspirefest, Bord Gais Energy Theatre, Dublin, Ireland. Oral Presentation.

Connick, K.M., Loscher, C.E. (3rd - 4th November 2016). Mining marine sources for functional ingredients with immunomodulatory activities. Inflammatory Disease, Biochemical Society Conference, NUI Maynooth, Ireland. Oral and poster presentations.

Connick, K.M., Loscher, C.E. (7th November 2016). Flow cytometry as a screening tool in the search for functional ingredients. Cytometry Society Ireland (CSI 2016), TBSI, TCD, Dublin Ireland. Poster presentation.

Connick, K.M., Loscher, C.E. (18th - 19th May 2017). Mining marine materials for novel functional peptides which modulate the immune response. 10th International Food and Nutrition Conference, Las Vegas, Nevada, USA. Poster presentation.

Connick, K.M., Loscher, C.E. (26th January 2018). How super is your superfood? 10th Annual School of Biotechnology Research Day, Dublin, Ireland. Video presentation.

Connick, K.M., Loscher, C.E. (8th March 2018). Under the sea. DCU Tell It Straight Runner up. DCU, Ireland.

Abstract

Mining marine materials for novel functional ingredients that modulate the immune response for benefit in chronic inflammation.

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Marine life is rich in biodiversity and a wealthy source of novel bioactive compounds. Ongoing research in marine bio-actives has identified immunomodulating ingredients, which have potential to support the immune system. Irish waters have been used a source of food for thousands of years and there is a long tradition of fishing and seaweed harvesting along the west coast. This project aimed to mine new anti-inflammatory compounds from Irish marine sources. Three species were identified as potential sources of these compounds and investigated in this project. 1. *Palarmia palmata*, a red seaweed, 2. Boarfish, a small mesopelagic fish found in the Atlantic shelf and 3. Blue Whiting, a north east Atlantic pelagic fish. These species, all found off the west coast of Ireland, are significant sources of protein and this project assessed these proteins for the presence of bioactive protein hydrolysates which displayed anti-inflammatory effects. Inflammation plays a major role in a large number of chronic diseases such as inflammatory bowel disease and rheumatoid arthritis. Low grade chronic inflammation is also heavily implicated in aging and many side effects associated with aging. This phenomenon known as inflammaging is considered to be at the core of most age-related diseases including Alzheimers' disease, atherosclerosis and diabetes. Therefore, protein hydrolysates with anti-inflammatory activity could have potential in the treatment of these conditions.

This project aimed to identify anti-inflammatory protein hydrolysates and/or peptides from a marine food source for use as functional food ingredients which reduce chronic inflammation in disease and aging. Using *in vitro* studies novel marine protein hydrolysates were identified that modulated the immune response through their effects on cytokine secretion by dendritic cells, naïve undifferentiated $CD4^+$ T cells and Th1, Th2 and Th17 cells. Candidate hydrolysates were then fractionated using HPLC in the search for the individual bioactive peptides that had these properties. Candidate bioactive peptides were then examined in an *in vivo* murine model of dextran sodium sulphate-induced colitis to assess their therapeutic application in a disease setting.

A number of marine protein hydrolysates with the ability to modulate inflammation were identified from all species screened *in vitro*. A hydrolysate fraction from Blue Whiting displayed potent anti-inflammatory effects *in vitro*, this anti-inflammatory effect was then shown to translate into an *in vivo* murine model of chronic inflammation, reducing disease-associated symptoms to that of healthy control levels. The protein hydrolysates examined are sourced from marine foods, and hence could be advantageous over other sources of potential functional ingredients as they are already fit for human consumption. They are also from low value sources and their potential as sources of novel functional food ingredients could add much needed value to these marine organisms. Marine organisms such as *Palmaria palmata*, boarfish and blue whiting offer real potential as sources of novel bioactive protein hydrolysates for incorporation into functional foods to modulate immune function and improve health.

Chapter 1:
Introduction

1.1 Introduction

The link between diet and human health is well known, advances in research into understanding the association between health and food has led to the development of functional foods and ingredients. Functional foods not only provide nutrition and satisfy appetite, they also offer added benefits such as prevention or reduction of disease (Siro *et al*., 2008). Foods marketed as a functional food, or nutraceutical, typically have added ingredients with specific health benefits (Coppens *et al.,* 2006). As of December 2006, functional foods and functional ingredients are regulated by the European Union (EU) (Regulation (CE) 1924/2006). This regulation states that nutritional and health claims of products must be regulated by the European Food and Safety Authority (EFSA), including their presentation, labelling and promotion (EFSA, 2006). The functional food market has experienced rapid growth over the past two decades as the general population has become more health conscious (Moons *et al*., 2018). A report published in 2004 showed the global functional food market to be estimated at $33 billion (Menrad, 2003), with Europe having the third largest market in the world (Bech-Larsen and Scholderer, 2000). This figure has undoubtedly risen as the demand for new novel functional ingredients continues to grow.

1.2 Functional food and ingredients

Functional foods have been around for many years, with the term first being used in the 1980's (Roberfroid, 2000). This group of food includes commonly used products, like orange juice and breakfast cereal, which have been fortified with vitamins and minerals, such as vitamin C and folic acid (Malinow *et al*., 1998). This fortification of food with nutrients which provide added benefits such as vitamin C boosting the immune system (Gleeson *et al.*, 2004), was then expanded into micronutrients such as omega-3 polyunsaturated fatty acids (PUFA) which have been shown to restore immunodeficiency (Gogos *et al*., 1998). Other types of functional foods include enriched food, which are foods with added ingredients which would not normally be found in those foods (Patch *et al*., 2005). Examples of enriched foods are those with added probiotics, probiotics contain live cultures of bacteria such as *Lactobacillus sp*. These provide proven health benefits in diseases such as inflammatory bowel disease (IBD) by balancing the microbiota and restoring healthy populations of bacteria (Schultz *et al*., 1998). Prebiotics, an emerging

group of functional ingredients, provide stimulation of so called good bacteria in the gut and help to support a healthy immune system. These have been shown to be beneficial to gut health and in the treatment of gastrointestinal related disorders (Tuohy *et al*., 2003). Naturally occurring plant based phytosterols are also being used to enrich food such as margarine (Flora ProActiv®) as they have lower absorption than cholesterol in humans, hence help lower cholesterol and improve cardiovascular disease and overall health (Jones and AbuMweis, 2009). Another group of functional foods, are enhanced food, where specific healthy ingredients of the food have been increased through genetic manipulation or special feed. One example of this is the use of special chicken feed to increase the level of omega-3 PUFA in the eggs that chicken lay (Mesias, 2011).

The potential uses and benefits of functional foods are far reaching and have the potential to impact the daily lives of many people. Vitamin and mineral deficiency have traditionally been a major target of the market, with foods such as cereal, including All Bran®, fortified with folic acid in order to reduce the risk of infants born with neural tube birth defect (NTD) (Crider *et al.*, 2011). Another common use of functional ingredients is in improving cardiovascular health. A common cardiovascular protective functional food is stanol and sterol ester enhanced margarine with many types currently on the Irish market, including Flora ProActiv®, Benecol® and I Can't Believe It's Not Butter® (Hasler *et al*., 2000). Anti-inflammatory diets which include functional ingredients that have been shown to reduce inflammation include ginger (Grzanna *et al.*, 2005), curcumin (Chainani, 2003) and salmon peptides (Ahn *et al*., 2012).

1.2.1 Sources of functional food and ingredients

Sources of functional ingredients are greatly varied and include plants, animals, animal products, fish and other marine organisms to name but a few. Dairy products have been at the forefront so far in developments in functional foods with products such as bifidobacterium, a probiotic dairy product, being very successful in the market (Saarela, 2007). An emerging focus in dairy functional ingredient research is the potential use of bioactive protein hydrolysates in infant milk formula. Some infants who can not be breastfed and are instead bottle fed using infant milk formula. This can result

in sensitisation and an immunological response to cow's milk proteins, known as cow's milk protein allergy (CMPA) (Scaillon and Cadranel, 2006). Current treatments include the use of hydrolysed infant milk formula such as Aptamil Pepti1®, which breaks the cow's milk proteins into smaller peptide strands, this can result in the immune system not mounting the same immune response, however the infant does not become tolerised to the proteins and can suffer allergic reactions in the future (Fritsché, 2003). Research is ongoing into dairy-based hypoallergenic protein hydrolysates which can both prevent CMPA but also help tolerise the infant to cow's milk protein (Kiewiet *et al*., 2018).

1.2.2 Marine sources of functional food and ingredients

Marine organisms are a largely unused source of functional ingredients (Lordan *et al.*, 2011). The marine environment is home to vast numbers of extremely diverse organisms which offer real potential for bioactive compounds. This potential has already been shown by the numerous marine-based compounds with varied bioactivity that have been identified so far (D'Orazio *et al.*, 2012). Fish oils and marine bacteria such as *Photobacterium profundum* are rich sources of omega-3 PUFA's (Allen and Bartlet, 2002) which have been shown to reduce obesity in mice (Ruzickova *et al*., 2004) and prevent cognitive decline and onset of dementia (Sydenham *et al,* 2012). Carotenoids, which are powerful antioxidants, have been isolated from crustaceans (Linán-Cabello *et al*., 2002), marine algae such as *Turbinaria ornata* (Kelman *et al*., 2012) and the marine microorganism, the diatom *Odontella aurita* (Xia *et al.,* 2013). Phenolic compounds have also displayed antioxidant activity and have been found in many marine organisms, this includes the macroalgae *Symphyocladia latiuscula* (Zhang *et al*., 2007), the marine sponge *Aplysina aerophoba* (Ahn *et al.*, 2003) and the Atlantic sea cucumber *Cucumaria frondosa* (Mamelona *et al.*, 2007). Phenolic compounds from the marine algae *Ascophyllum nodosum* have also been reported to display anti-proliferative effects (Zhang *et al.,* 2007, Nwosu *et al.*, 2011). Anti-tuberculosis compounds such as the peptide neamphamide, isolated from the marine sponge *Neamphius sp.,* have also been isolated from marine sources (Yamano *et al*., 2012).

Table 1.1 Marine sources of bioactive compounds, the bioactive compounds and activity.

Source species	Compound	Bioactivity	Reference
Photobacterium profundum **(Bacteria)**	Omega-3 PUFA	Reduce obesity Prevent cognitive decline	Ruzickova *et al*., 2004 Sydenham *et al,* 2012
Odontella aurita **(Microorganism Diatom)**	Carotenoid	Anti-oxidant	Xia *et al.,* 2013
Aplysina aerophoba (**Sea sponge)**	Phenol	Anti-oxidant	Ahn *et al.*, 2003
Cucumaria frondosa **(Sea cucumber)**	Phenol	Anti-oxidant	Mamelona *et al.*, 2007
Neamphius sp. **(Sea sponge)**	Peptide	Anti-tuberculosis	Yamano *et al*., 2012
Lobophytum crassum **(Soft coral)**	Diterpenoid	Anti-inflammatory	Lin *et al.*, 2013

Marine algae, both macroalgae and microalgae, have already provided many novel antioxidant compounds, includingthe microalgae *Spirulina* and *Chlorella* (Ötleş and Pire, 2001) and the macroalgae *Avrainvillea longicaulis* (Zubia *et al.,* 2007). Anti-bacterial activity has been demonstrated in compounds isolated from macroalgae *Falkenbergia billebrandii* (Lima-Filho *et al.*, 2002) and *Caulerpa racemosa* (Kandhasamy and Arunachalam, 2008). Anti-viral activity has been displayed by a glycolipid isolated from the brown macroalgae *Sargassum vulgare* with the ability to inhibit the Human herpes simplex virus-1 and –2 (HSV-1 and HSV-2) (Plouguerné *et al*., 2013).

Many anti-inflammatory molecules have also been found in algae such as the polyketide from *Ecklonia cava* which has been shown to inhibit tumour necrosis factor α (TNFα) and interleukin-6 (IL-6) in macrophage *in vitro* (Yang *et al*., 2012). Another TNFα inhibitor found in algae is the meroterpenoid from the brown seaweed *Cystoseira usneoides* (de los Reyes *et al.*, 2013). The ability to modulate the immune system is not limited to compounds found in algae, numerous marine organisms have demonstrated their ability to alter the immune response. For example, the compounds called diterpenoids from the soft coral *Lobophytum crassum* inhibit TNFα and IL-12 secretion from bone marrow derived dendritic cells (BMDCs) (Lin *et al.*, 2013).

Table 1.2 Algae (macro and micro) sources of bioactive compounds, the bioactive compounds and activity.

Sources species	Compound	Bioactivity	Reference
Turbinaria ornata **(Macroalgae)**	Carotenoid	Anti-oxidant	Kelman *et al*., 2012
Symphyocladia latiuscula **(Macroalgae)**	Phenol	Anti-oxidant	Zhang *et al*., 2007
Spirulina **(Microalgae)**	Fatty Acid	Anti-oxidant	Ötleş and Pire, 2001
Chlorella **(Microalgae)**	Fatty acid	Anti-oxidant	Ötleş and Pire, 2001
Avrainvillea longicaulis **(Macroalgae)**	Unknown	Anti-oxidant	Zubia *et al.,* 2007
Falkenbergia billebrandii **(Macroalgae)**	Unknown	Anti-bacterial	Lima-Filho *et al.*, 2002
Caulerpa racemosa **(Macroalgae)**	Unknown	Anti-bacterial	Kandhasamy and Arunachalam, 2008
Sargassum vulgare **(Macroalgae)**	Glycolipid	Anti-viral	Plouguerné *et al*., 2013
Ecklonia cava **(Macroalgae)**	Polyketida	Anti-inflammatory	Yang *et al*., 2012
Cystoseira usneoides **(Macroalgae)**	Meroterpenoid	Anti-inflammatory	de los Reyes *et al.*, 2013)

Fish oil has been traditionally used as an anti-inflammatory for hundreds of years and has the ability to suppress the pro-inflammatory cytokines IL-1β, TNFα and IL-6 in humans (Blok *et al*., 1997). However, fish protein hydrolysates have taken front row in research into marine bioactive compounds with numerous fish hydrolysate-based products now on the market. These include the fish hydrolysate based product PeptAce®, a hydrolysate from Bonito found in the Atlantic Ocean, which has been shown to be cardioprotective as an angiotensin-converting-enzyme (ACE) inhibitor (Carrasco-Castilla *et al*., 2012). Another fish hydrolysate product currently on the market is Seacure®, a hydrolysate from deep sea white fish from the Pacific Ocean, which claims to help protect the cells of the gastrointestinal tract (Marchbank *et al*., 2009) has been shown to display immunomodulatory activity by boosting the immune system through the increase of cytokines such as IL-10, IL-4 and IL-6 *in vivo* (Duarte *et al.*, 2006).

Functional foods have been claimed to help prevent disease onset, but recent focus has been on developing bioactive compounds as functional ingredients which help to treat disease and disease symptoms. Bioactive peptides from a range of sources including casein from milk, proteins from sardines and ovalbumin from eggs display ACE inhibitory activity and are useful in the treatment of cardiovascular disease (Erdmann *et al*., 2008). Probiotics and prebiotics are common therapies in the treatment of mild to moderate IBD and other gastrointestinal tract disorders, they work through enhancing the growth of commensal bacteria and promoting balance in the natural microbiota of the gut which is often imbalanced in IBD (Nell *et al*., 2010). Peptides isolated from egg whites demonstrated the ability to reduce symptoms of IBD and decrease pro-inflammatory cytokines such as TNFα, IL-6, IL-1β and interferon gamma (IFNγ) in an *in vivo* model of IBD (Lee *et al*., 2009). Gamma-linoleic acid extracted from the oil of plant seeds such as sunflower seed oil has been shown to effectively reduce symptoms of rheumatoid arthritis (RA) in patients (Zurier *et al*., 1996). Numerous functional food ingredients have also displayed anti-diabetic effects with compounds isolated from mushrooms (Perera and Li, 2011), flaxseed (Oomah, 2001), seaweed (Holdt and Kraan, 2011), cereal (Brennan and Cleary, 2005) and fish (Ktari *et al*., 2013), to name but a few, all reducing the symptoms of diabetes. Compounds isolated from marine organisms, such as fish proteins and protein hydrolysates, have proven anti-inflammatory effects with real potential in the treatment

of inflammatory diseases such as IBD and RA. Functional ingredients, like probiotics, are already used as complementary treatments in patients with IBD or in IBD remission (Sartor, 2004). Dietary therapies for IBD and gastrointestinal disorders are also currently in use, through specific diets such as the Mediterranean diet which is considered anti-inflammatory (De Filippis *et al*., 2016) with functional ingredients playing a major role. Functional food and the use of dietary intervention in the treatment of disease is advancing at a rapid pace and is considered the future of both disease prevention and treatment (Witkowski *et al*., 2018).

Table 1.3 Sources of functional ingredients which treat disease, their bioactive compounds and activity.

Source	Compound	Bioactivity	Reference
Milk	Casein	Cardiovascular disease (ACE Inhibitory)	Erdmann *et al*., 2008
Sardines	Protein	Cardiovascular disease (ACE Inhibitory)	Erdmann *et al*., 2008
Eggs	Ovalbumin	Cardiovascular disease (ACE Inhibitory)	Erdmann *et al*., 2008
Eggs	Peptides	IBD (Reduction in pro-inflammatory cytokines)	Lee *et al*., 2009
Sunflower seed oil	Gamma linoleic acid	RA (reduced symptoms)	Zurier *et al*., 1996
Mushrooms	Protein	Diabetes	Perera and Li, 2011
Flaxseed	Omega-3 PUFA	Diabetes	Oomah, 2001
Seaweed	Extracts	Diabetes	Holdt and Kraan, 2011
Cereal	Betaglucans	Diabetes	Brennan and Cleary, 2005
Zebra Fish	Protein hydrolysates	Diabetes	Ktari *et al*., 2013

1.3 The immune response and inflammation

The immune response provides protection from invading pathogens and helps in the repair of tissue following injury. It can be divided into two categories; innate or adaptive immunity (Vivier *et al*., 2011). Innate immunity serves as the body's first line of defence and is present from birth. Defences of the innate immune system include

physiological aspects such as the ability of lysosymes in mucus to cleave bacterial cell walls (Shilhavy *et al*., 2010), anatomic such as the skin as a barrier to invading pathogens (Elias, 2007) and phagocytic such as the ability of macrophages to engulf and destroy bacteria (Greenberg and Grinstein, 2002). The adaptive immune system, in contrast, is considered learned immunity, it is built up over time and involves the recognition of antigens following its destruction or removal by the innate immune system. This occurs when macrophage phagocytose antigens and displays them on their surface membrane at major histocompatibility complex (MHC) sites and these are recognised by a small subset of specialised T cells, known as T helper cells (Flajnik and Kasahara, 2000). Another process which begins upon phagocytosis of antigens is the secretion of pro-inflammatory cytokines by macrophage cells. This release of cytokines then helps to orchestrate the adaptive immune response through attraction and activation of T cells and dendritic cells (DCs) among other immune cells (Banchereau and Steinman, 1998).

Both the innate and adaptive immune responses can drive acute and chronic inflammation. Acute inflammation limits tissue damage caused by injury or infection through limiting the spread of phathogens and attracting macrophage and neutrophil cells to the site in order to destroy and remove the invading antigens (Savill *et al*., 1989). Acute inflammation also initiates tissue repair through proliferation of connective tissues and various other processes (Sporn and Roberts, 1986). Chronic inflammation is classed as persistent and systemic inflammation and can last for weeks, months and even years. It may develop following acute inflammation or independently and is the underlying cause of many inflammatory related disorders (Xu *et al*., 2003). During chronic inflammation cytokines interact resulting in the movement of monocytes to the site of inflammation where activation factors such as in IFN-g and granulocyte-macrophage colony stimulating factor (GM-CSF) differentiate the monocytes into either macrophage or dendritic cells (Palucka *et al*., 1998) Cytokines known to mediate chronic inflammatory processes include IL-1β, IL-6, IL-17, IFNγ, and TNFα (Stumpo *et al.*, 2010).

1.3.1 Cytokines

Cytokines are small signaling proteins which play a fundamental role in the immune system through allowing communication between the immune response and host tissue cells (Budd *et al*., 2016). They act via binding to a receptor which then sends a signal to a recipient cell resulting in a change in function or phenotype of the cell. They exist in broad families, such as the IL-12 super family, that are structurally related but display diverse functions (Vignali and Kuchroo, 2012). They are secreted by many cells and function can vary greatly. The major classes of cytokines include pro- and anti-inflammatory cytokines, cytokines of neutrophil and eosinophil recruitment and cytokines of T cell recruitment and growth factors (Hanada and Yoshimura, 2002). In chronic inflammation and inflammatory diseases there are large increases in pro-inflammatory cytokines and decreases in anti-inflammatory cytokines. Pro-inflammatory cytokines such as TNFα and IL-1β have become targets of therapeutics to treat these diseases (Butler *et al.*, 1995). Anti-inflammatory cytokines such as IL-10 are currently in use as therapeutic agents to regulate inflammation and treat inflammatory disorders (Asadullah *et al*., 1998). Many more cytokines are currently being researched as potential therapeutic targets or therapeutic agents.

1.3.1.1 IL-1β

IL-1β was first purified from rabbits in 1977 and the human IL-1β gene was then cloned in 1984 (Henderson and Pettipher, 1989). This led the way for research using recombinant IL-1β and the many functions of this cytokine were uncovered. Although this research indicated IL-1β's role in inflammation, it was only in more recent times through the study of anti-IL-1β therapies, did its crucial role in inflammation become clear (Spohn *et al.*, 2017). Nearly all microorganisms induce IL-1β secretion via toll-like receptor (TLR) ligands, however IL-1β also induces itself in monocytes such as macrophage and dendritic cells (Monteleone *et al*., 2015). This self-stimulation has been shown to result in sustained levels of IL-1β mRNA levels over 24 hours compared to the decline in levels seen following a peak at 4 hours in TLR ligand stimulation, this self-stimulation is considered to play a possible role in chronic inflammation (Netea *et al*., 2015). This cytokine has many functions, its main one being co-stimulation of T cells (Bevilacqua *et al*., 1984), enhancement of natural killer (NK) cells (Hunter *et al.*, 1995) and to act as a chemoattractant to other inflammatory molecules (Matsushima and Oppenheim, 1989). It

has been shown to play pivotal roles in a number of inflammatory disorders including RA (Ruscitti *et al*., 2015), diabetes (Herder *et al.*, 2015) and IBD (Spohn *et al*., 2017). IL-1β has been identified as a target for anti-inflammatory therapies (Williams *et al*., 2000).

1.3.1.2 IL-6

IL-6 is a pro-inflammatory cytokine secreted by T cells and monocytes. It has numerous functions including stimulation of acute phase protein synthesis by the liver, inducing B cells to mature into antibody producing plasma cells and activation and differentiation of T cells (Samoilova *et al*., 1998). Alongside these functions, IL-6 plays a major role in chronic inflammation and is thought to be crucial to the transition from neutrophil to monocyte recruitment seen as an acute inflammatory response develops into chronic inflammation (Kaplanski et al., 2003). Upregulation of this cytokine has been observed in a variety of inflammatory diseases and it has been linked to the pathogenesis of inflammatory diseases such as RA, type I diabetes, psoriasis, and IBD (Feghali and Wright, 1997). IL-6 inhibition is currently used in the treatment of RA through the use of an antibody called Tocilizumab which received FDA approval in 2010 (Bijlsma *et al*., 2016).

1.3.1.3 IL-10

IL-10 is an anti-inflammatory cytokine and a crucial immunoregulator during pathogenic infection, suppressing excessive T cells responses (Ruffell *et al*., 2014). It modulates the expression of pro-inflammatory cytokines such as IL-1β, IL-6, and TNFα, but also directly inhibits the proliferation of Th1 cells and secretion of IFNγ and IL-2 by Th1 cells. IL-10 also reduces the chemokine production thus preventing DC migration to lymph nodes and the differentiation of Th1 cells from naïve undifferentiated $CD4^+$ T cells (Castillo and Kolls, 2016). IL-10 knockout (KO) mice have displayed excessive secretion of pro-inflammatory cytokines and the development of inflammatory diseases including IBD and RA (O'Mahony *et al*., 2001). IL-10 has been identified as a potential therapeutic agent in the treatment of inflammatory disease and has been trialed using recombinant IL-10 in the treatment of IBD (Fedorak *et al*., 2000).

1.3.1.4 IL-17

IL-17, considered a major inflammatory cytokine, was first discovered in 1993 and named IL-17 in 1995 (Anthonysamy *et al.*, 1999). IL-17 has many roles but its main function is in the activation of the tissue response and in initiating the neutrophil-led immune defence. It is mainly secreted by Th17 cells but it has also been shown to be secreted by NK cells and neutrophils (Cua and Tato, 2010). It has been shown to have synergistic effects with a number of other pro-inflammatory cytokines such as TNFα and IL-1β. IL-17 levels are elevated in numerous inflammatory diseases such as RA and psoriasis (Miossec, 2009) and it has been identified as a target for therapeutics for inflammatory disease with anti-IL-17 therapy is currently used in clinic for the treatment of psoriasis (Campa *et al.*, 2016).

1.3.1.5 IFNγ

IFNγ is a pro-inflammatory cytokine which is secreted primarily by $CD4^+$ T cells, mainly Th1 cells but also antigen presenting cells (APCs) such as macrophage and dendritic cells. Its main function is in the activation of macrophage and DCs (Nathan *et al*., 1983). This activation results in increased MHC expression and increased secretion of IL-12, inducing the production of more IFNγ and driving a Th1 cell phenotype in naïve undifferentiated $CD4^+$ T cells (Yoshimoto *et al*., 1998). It has been implicated in chronic inflammatory diseases with significantly increased levels of IFNγ seen in RA (Cañete *et al.*, 2000), IBD (Bisping *et al.*, 2001) and diabetes (Suk *et al.*, 2001). Anti-IFNγ therapy is currently in clinical use in the treatment of psoriasis with antibodies such as HuZAF commonly used (Harden *et al*., 2015).

1.3.1.6 TNFα

TNFα is a multifunctional cytokine produced by many cells including macrophage, dendritic cells, NK cells and lymphocytes. It has a variety of functions such as mediating the expression of genes for cytokines, activation of a variety of cells including macrophage and the inducing the production of IL-1β and IL-6 (Dick *et al.,* 1998). TNFα can also influence the function of APCs by altering their ability to present antigens and hence upregulate the expression of co-stimulatory factors upon presentation of antigens (Cella *et al*., 1997). TNFα has been extensively studied and its role in chronic inflammation

and inflammatory disease well documented (Bradley, 2008). Development of TNFα inhibitors has been one of the most active areas in drug development in recent times and various inhibitors such as the antibodies infliximab, etanercept and adalimumab are commonly used in clinic for the treatment of a broad range of inflammatory disease including RA and IBD (Thalayasingam and Isaacs, 2011).

Table 1.4 Cytokines produced by immune cells and their primary functions

Cytokine	Secreted by	Function	Inflammatory Status
IL-1β	Macrophage Dendritic cells B cells	Co-stimulates T cells Induces NK cell activity Chemoattractant	Pro-inflammatory
IL-4	Th2 cells	Th2 polarisation Macrophage activation	Anti-inflammatory
IL-6	Macrophage Dendritic Cells Th17 cells	Activates T cells Th17 differentiation	Pro-inflammatory
IL-10	T regulatory cells Dendritic cells Macrophage	Immunosuppressant Inhibits Th1 cell response	Anti-inflammatory
IL-12	Macrophage Dendritic Cells	Th1 cell development Stimulates APCs	Pro-inflammatory
IL-13	Th2 cells	Macrophage activation	Anti-inflammatory
IL-17	Th17 cells	DC maturation	Pro-inflammatory
IL-23	Macrophage Dendritic cells	Expansion and survival of Th17 cells	Pro-inflammatory
IFNγ	Th2 cells Macrophage	Th1 expansion	Pro-inflammatory
TNFα	Macrophage Dendritic cells T cells	Amplifies inflammation Induces cytokine release	Pro-inflammatory

1.3.2 Immune cells

The immune system is a complex network of cells, known collectively as leukocytes, which function both independently and as part of wider systems with other cells and tissues, to provide protection against invading pathogens. The innate immune system is made up of phagocytes, such as neutrophils, eosinophils, basophils, dendritic cells and macrophage. These cells provide the body's first line of defence against invading pathogens by engulfing the pathogen, secreting cytokines and displaying the antigen on their surface which initiates the adaptive immune response (Delneste *et al.*, 2007). The adaptive immune response is made up of cells known as lymphocytes which are considered memory cells. These cells remember invading pathogens from previous attacks and help coordinate the immune response based on this memory. They are classed as either B-lymphocytes (B cells) that produce antibodies and activate T cells or T-lymphocytes (T cells) which destroy compromised cells and help initiate the immune response (Bluestone and Abbas, 2003).

1.3.2.1 T Cells

T cells are the dominant lymphocytes. They arise in the bone marrow but undergo differentiation and proliferation in the thymus. They can be found throughout the body however, they are found in large numbers in sites of activation such as the lymph nodes and spleen (Swirski *et al*., 2009). T cell activation is critical for the initiation and regulation of the immune response. They are divided into two major subsets; T helper cells (Th) which express CD4 on their cell surface and recognise antigens complexed to MHCII molecules on the surface of APCs, and cytotoxic T cells (CTs) which express CD8 on their cell surface and recognise antigens complexed to MHCI molecules on the surface of APCs (Parkin and Cohen, 2001). $CD8^+$ T cells are essential in the downregulation of the immune response and also exhibit cytotoxic activity in the killing of specific tumour cells, cells infected with virus particles and any cells displaying foreign antigen coupled to MHCI (Murali-Krishna *et al*., 1998). $CD4^+$ T cells direct the immune response and differentiate into distinct cell subsets such as Th1, Th2 and Th17 cells. These are classified by their functions and unique cytokine secretion profiles (Zhu and Paul, 2008). Dysregulation of $CD4^+$ T cell function has been implicated in the pathogenesis of inflammatory diseases including RA, IBD and psoriasis (Langrish *et al*., 2005).

1.3.2.1.1 Th1 cells

Naïve undifferentiated $CD4^+$ T cells are polarised into a Th1 phenotype through the cytokines IL-12, IL-27, IFNγ and the transcription factors signal transducer and activator of transcription (STAT)-1 and STAT-4 and the intracellular adhesion molecule-1 (ICAM-1) alongside T-bet. Th1 cells secrete IL-2, IL-2, IL-15, TNFβ and IFNγ which activate macrophage, NK cells and CTs. Their primary role is regulation of the immune response and they are principal effectors of cell mediated immunity. Dysregulation of Th1 function has been implicated in a number of inflammatory disorders including RA where Th1 cells dominate synovial fluid (Yamada *et al*., 2008). They also play a major role in IBD through interactions with Th17 cells (Olsen *et al*., 2011).

1.3.2.1.2 Th2 cells

The differentiation of Th2 cells is largely dependent on the cytokine IL-4 alongside transcription factors STAT-6 and GATA3 (White *et al*., 2010). Their function is to promote humoral immunity, allergic responses and constrain cell mediated immunity and inflammation and they secrete IL-4, IL-5 and IL-13. During a Th2 cell response, IL-4 and IL-13 stimulate B cells to produce IgE whilst IL-5 activates eosinophils (Straumann *et al*., 2001). Th2 cells act in a paradigm with Th1 cells, known as the Th1/Th2 paradigm. They can antagonise each other and inhibit differentiation of the other cells with IL-4 secretion inhibiting Th1 cell differentiation and IFNγ inhibiting Th2 differentiation (Romagnani, 1997). Th2 cells contribute to atopic diseases, rather than inflammatory, driving allergy and other such disorders such as asthma (Del Prete *et al.,* 1993).

1.3.2.1.3 Th17 cells

The Th17 cell subset was discovered more recently than Th1 and Th2 cells and similarly to these cells, it requires specific cytokines and transcription factors for their differentiation. IL-6 and TGFβ are required from the polarisation of a naïve undifferentiated $CD4^+$ T cell to a Th17 cell (Verma *et al*., 2013). Th17 cells are characterised by the secretion of IL-17. This cytokine is a major player in inflammation and induces the secretion of IL-1β, TNFα and GMCSF. These cytokines induce the expression of the orphan nuclear receptor (RORγt) and upregulation of the production of IL-17 and IL-23 (Weaver and Hatton, 2009). Th17

cells have been implicated in many inflammatory disorders including RA and IBD (Miossec, 2009).

Table 1.5 $CD4^+$ T cell subsets

T helper cell subset	Differentiated by	Cytokines secreted	Inhibited by
Th1	IL-12 IL-27 ICAM-1	IFNγ IL-2	IL-4 IL-17
Th2	IL-10 OX40L IL-4	IL-4 IL-5 IL-13	IFNγ IL-12
Th17	IL-23 IL-1β IL-6 TNFα TGFβ	IL-17 IL-22 IL-6	IFNγ IL-4

1.3.2.2 Dendritic cells

DCs are professional APCs, which are abundant at body surfaces and within tissues. They are considered professional APCs due to their ability to activate and differentiate naïve T cells (Banchereau *et al*., 2000). There are three major subsets; plasmacytoid, conventional and monocyte-derived, characterised by origin and function. Following interaction with antigens, DCs undergo maturation, during which they process the antigen and induce production of MHC molecules and cytokines. They then migrate to the lymphatic system, to present antigens to undifferentiated naïve T cells and induce immunity (Cella *et al*., 1997). DCs interact with pathogens through TLRs found on the surface of the cell. TLRs bind to specific pattern association molecule patterns (PAMPs) on the pathogen and this ligation leads to phagocytosis and the secretion of a number pro-inflammatory cytokines such as IL-12, TNFα and IL-1β. These cytokines then facilitate the activation of both the innate and adaptive immune responses. Due to this ability to activate the adaptive immune response, DCs are known as the link between the innate and adaptive immune systems (Palucka and Banchereau, 1999). DCs have been linked to inflammatory disease. In IBD, they are considered key initiators of the inflammatory response and they secrete large amounts of inflammatory cytokines (Hart *et al*., 2005).

1.3.2.3 Macrophage

Macrophages are key innate immune cells that play a key role in tissue homeostasis, wound healing, pathogen clearance and regulation of inflammation (MacMicking *et al*., 1997). They are primary mediators of inflammation that perform various functions in both the innate and adaptive immune responses. Their primary role is to recognise and phagocytose invading pathogens. They express pattern recognition receptors (PRRs) on their surface such as TLRs that allow them to recognise PAMPs. This results in the phagocytosis of pathogens and the initiation of immune pathways that result in the secretion of inflammatory cytokines (Mosser and Edwards, 2008). Macrophage also initiate the adaptive immune system through the presentation of antigens through MHC II molecules to $CD4^+$ T cells (Iwasaki and Medzhitov, 2010). Macrophage can be activated into one of three distinct subsets, classically activated (M1), alternatively activated (M2) and regulatory macrophage. This activation is dependent on the pathogen encountered or the environment of the macrophage (Martinez *et al*., 2008). M1 cells are generated

following stimulation with pro-inflammatory cytokines such as IFNγ and TNFα or LPS. M1 macrophage secrete cytokines such as TNFα, IL-1β and IL-12 and are associated with increased expression of MHCII and CD86 (Sica and Mantovani, 2012). Overproduction of M1 macrophage can lead to a range of inflammatory diseases such as RA and IBD (Mills, 2012). M2 macrophage are induced by IL-4 and IL-13, cytokines secreted by a Th2 subset response. They can also be detrimental when overproduced and are increased in RA (Mulherin *et al*., 1996).

1.4 Inflammation and inflammatory disease

Acute inflammation is the immune system's normal response to injury or invasion by pathogens, it helps in the repair and adaptation of many tissues and is a vital part of the immune response which is usually tightly regulated. It is driven by key immune cells such as dendritic cells, macrophage and T cells (Moilanen and Vapaatalo, 1995). Chronic inflammation has many of the same features of acute inflammation, such as also being driven by key immune cells, however unlike acute, chronic is usually unregulated, low grade, persistent and harmful (Doring, 1994). The underlying cause of chronic inflammation is largely unknown with many different mechanisms hypothesized, including activated immune cells leading to the production of pro-inflammatory cytokines which drive an inflammatory response (Dinarello, 2000). This increase in cytokines can also alter the phenotypes of nearby cells such as T helper cells which can be detrimental to the normal function of surrounding tissue (Fantini *et al.*, 2004).

This persistent sub-clinical inflammation is a major risk factor in numerous diseases such as RA, psoriasis, IBD, atherosclerosis and diabetes (Cohen *et al.*, 2012). These diseases are often classed as inflammatory disease due to the crucial role inflammation plays in their onset and pathogenesis. RA is a chronic inflammatory and destructive disease which affects the joints of patients (Machold *et al*., 2006). Long term prognosis of patients is poor with 80% of patients being disabled within 20 years (Versteeg *et al*., 2018). The synovial membrance in the joints of patients with RA is characterised by increased vascularity, hyperplasia and the infiltration of inflammatory cells such as $CD4^+$ T cells (Baeten *et al*., 2000). $CD4^+$ T cells are the main orchestrator of cell mediated immune

responses and have been strongly associated with RA (van Amelsfort *et al*., 2004). Through this mediation $CD4^+$ T cells stimulate other immune cells such as macrophage and DCs to produce pro-inflammatory cytokines such as TNFα, IL-1β and IL-6 (Buchan *et al*., 1988). These cytokines have been implicated as having primary roles in the pathogenesis of RA and inhibition of these has been shown to treat the disease (Arend and Dayer, 1995). Current treatment of RA includes cytokine targeting therapeutics such as anti-TNFα antibodies like adalimumab (Weinblatt *et al*., 2003) and the immunosuppressive agent methotrexate (Maini *et al*., 1999). Anti-cytokine therapies are often used in conjunction with immunosuppressive agents such as methotrexate in the treatment of moderate to severe RA (Lipsky *et al.*, 2000). Treatment with these therapies alone or in combination often results in immunosuppression of the patient and infection by opportunistic pathogens is common (Doran *et al.*, 2002). Therapies which target multiple cytokines, such as Tofacitinib, are becoming increasingly popular with research into the complex cytokine pathway involved in RA ongoing (van Vollenhoven *et al*., 2012). However, the same immunosuppressive effects as seen with single cytokine inhibition are still being reported (Zerbini and Lomonte, 2012). Dietary intervention, specifically the use of functional ingredients such as fish oil and omega-3 PUFAs, has been shown to help reduce symptoms, but not to the same degree as commonly used therapies. They are recommended however, for use as complementary therapies alongside traditional approaches (Tedeschi and Costenbader, 2016).

Psoriasis is another inflammatory disorder which is driven by immune cells such as $CD4^+$ T cells and cytokines such as TNFα, IFNγ, IL-6, IL-12 and IL-17 (Arican *et al*., 2005). It is characterised by inflammation of the skin but can also manifest in joint inflammation in a disorder known as psoriatic arthritis. Psoriasis, like RA and most inflammatory disorders, is an idiopathic disorder and disease symptoms, rather than the cause of the disease, are treated (Al-Suwaidan and Feldman, 2000). Current therapies include corticosteroids such as Halobetasol propionate (Pariser *et al*., 2016), a vitamin D derivative Calcipotriol ointment (Koo *et al*., 2016), the anthracene derivative Dithranol (Schaefer *et al.*, 1980), phytotheraphy known as UVB (Nordal and Christensen, 2004) and phytochemotherapy known as PUVA which involves the use of an oral or topical therapeutic known as Psoralen which causes increased sensitivity to UVA light (Lozinski *et*

al., 2016). Cyclosporine, a steroid sparing agent, is also used in the treatment of psoriasis, as it is safer in long term use than steroids however it also suppresses the immune system and increases the individual's risk of infection (Ellis *et al.*, 1986). In severe psoriasis, the immunosuppresant methotrexate is also used, however similar side effects as in RA are seen (Menting *et al*., 2016). The use of specialised diets, such as the anti-inflammatory Mediterranean diet, in reducing severity of psoriasis have been trialed and have received success in reducing symptoms of the disease (Barrea *et al*., 2015).

Atherosclerosis is also thought to be immune cell mediated with pro-inflammatory cytokines playing a major role. Atherosclerosis is a disease in which arteries become chronically inflamed and plaque from cholesterol and lipids builds up as a result (Packard and Libby, 2008). $CD4^+$ T cells and macrophage cells are thought to be key drivers of inflammation in the disease (Portugal *et al*., 2009). Treatment of atherosclerosis includes dietary and lifestyle changes with a lipid-lowering diet being a key change in the treatment of most patients (Watts *et al*., 1992). Dietary treatment is not always enough, and the use of cholesterol lowering medication known as statins (Okazaki *et al*., 2004). Stents are also used to treat moderate to severe atherosclerosis in order to clear the blockage and reduce the risk of heart attack and stroke (Van de Ven *et al.*, 1999). Advancements in stent technology have produced drug eluting stents which can open the artery back up and deliver anti-inflammatory drugs to the area in order to reduce the level of inflammation in the artery wall (Schofer *et al.*, 2003).

Diabetes mellitus is a metabolic disorder which has recently been also classified as an inflammatory disorder due to the critical role inflammation plays in the disease. The activation of the innate immune system and the resulting chronic inflammation are considered relevant factors in the pathogenesis of the disease (Pickup, 2004). It is caused overall by a lack of (or deficiency in) insulin, a hormone required for regulation of blood sugar, which is normally produced in the pancreas (American Diabetes Association, 2014). Pro-inflammatory cytokines have been named critical factors in the development of microvascular diabetic complications, including nephropathy (Navarro *et al.*, 2006). Inflammation in general has been implicated in both type I and type II diabetes. In type I diabetes, a T cell mediated autoimmune response against pancreatic β-cells is thought to

play a role in this assault with the release of inflammatory cytokines possibly inhibiting the regeneration of β-cells (Eizirik *et al*., 2009). There is increasing evidence of the role chronic inflammation plays in the onset and pathogenesis of type II diabetes with major risk factors such as age, inactivity and poor diet all thought to be possible causes of chronic inflammation (Duncan *et al.*, 2003). Recombinant insulin therapy through subcutaneous injection is the main treatment of diabetes, specifically type I (Dane *et al*., 2014). Insulin pumps are used as a form of artificial pancreas to deliver small amounts of insulin at regular intervals (Haidar *et al*., 2015). Diet and exercise are the first port of call in the treatment of type II diabetes with weight loss crucial in treating the disease (Inzucchi *et al*., 2015). Alongside lifestyle change, metformin is commonly used in the treatment of type II diabetes, as it lowers blood sugar and can reduce the cardiovascular risks associated with the disease (Gong *et al*., 2016).

IBD encompasses several inflammatory diseases of the gastrointestinal tract including Crohn's Disease (CD), ulcerative colitis (UC) and indeterminate colitis (IC) (Podolsky, 1991). Whilst many other diseases affect the gastrointestinal tract and may cause inflammation, IBD is characterised as being idiopathic, and the disorders which fall under IBD are classified by their clinical and pathological symptoms rather than their causes (Benchimol *et al*., 2010). CD and UC are the two most recognisable forms of IBD, although they are often indistinguishable from each other. UC is characterised by chronic inflammation of the colon whilst CD can affect the entire gastrointestinal tract (Boughton-Smith *et al*., 1993). In both diseases however, the inflammatory response begins with a mass infiltration of neutrophil and macrophage cells, which release proinflammatory cytokines such as TNFα (Kojouharoff *et al.*, 1997). This activates either a Th1 or Th2 cell phenotype in the gut mucosa and initiates a cytokine cascade which drives the disease (Hanauer, 2006). Symptoms of IBD include severe diarrhea often with the presence of blood, fever, fatigue, abdominal pain, abdominal cramping, loss of appetite and weight loss (Carter *et al*., 2004). IBD is typically diagnosed before the age of 30, with pediatric IBD incidences increasing (Bernstein *et al.*, 2010). Common therapies for IBD include the use of corticosteroids for mild to moderate disease (Bernstein, 2015), antibiotics such as metronidazole (Isaacs and Sartor, 2004), anti-inflammatories such as the 5-aminosalicylic Mesalazine (Travis and Jewell, 1994), immunosuppressants like

methotrexate (Willot *et al*., 2011) and cytokine inhibition therapies such as anti-TNFα antibodies like adalimumab (Humira®). Due to the complexities of IBD, the lack of understanding in the mechanisms and differences of CD and UC and patients responding differently to treatments, no current therapy is considered the gold standard. Many therapies such as antibiotics and corticosteroids are not suitable for long term use (Sartor, 2004) and others such as immunosuppresants and anti-cytokine therapies often leave the patient at risk of opportunistic infections (Irving and Gibson, 2008).

Inflammaging whilst not necessarily considered an inflammatory disease, is an inflammatory condition which effects a vast majority of the elderly population and is an underlying cause of many age-related inflammatory diseases (Franceschi and Campisi, 2014). Thought to be a side effect of the dramatic increase in life expectancy seen over the last century, inflammaging is caused by the overstimulation of the immune system by antigenic stress over time causing immune inefficiency with age (Franceschi *et al*., 2000). This inefficiency results in low grade inflammation which contributes to the frailty seen in older populations and eventual mortality (Wu *et al.*, 2015). Theories behind the driving forces of inflammaging are deeply rooted in evolutionary theory and it is thought to be a consequence of the remodelling of the innate and acquired immune system which results in the production of chronic inflammatory cytokines with significant increases of IL-6, IL-1β, TNFα and other pro-inflammatory cytokines seen in elderly people (Baylis *et al*., 2013). This increase in pro-inflammatory cytokines activates the inflammasome and is caused by the upregulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and STAT proteins (Franceschi and Capisi, 2014). The majority of age related diseases share an underlying inflammatory pathogenesis caused by the activation of the inflammasome by proinflammatory cytokines. Inflammaging has been implicated as a possible cause or risk factor in many age-related diseases such as Alzheimer's disease, cardiovascular disease, macular degeneration, degenerative arthritis and diabetes (Vasto *et al*., 2007). Popular therapies for inflammaging include low dose aspirin or statins, however dietary intervention and the use of nutrition as a tool in treating inflammaging has been identified as having real potential in the treatment of this phenomenon (Sun *et al*., 2016).

Inflammatory disease and inflammaging are characterised by an infiltration of immune cells such as $CD4^+$ T cells, DCs and macrophage and an increase in the secretion of pro-inflammatory cytokines such as TNFα, IL-1β, IL-12, IL-6 and IFNγ. This cascade is dysregulated in both disorders resulting in chronic inflammation (Elenkob *et al.*, 2005). Treatment for inflammatory diseases relies heavily on suppression of the overall immune response, often resulting in side effects such as infection. Whilst research into therapies which combat inflammaging is in its infancy, a need for therapies which reduce levels of pro-inflammatory cytokines but maintain the immune response of patients has been identified and is considered the future of inflammatory disease and inflammaging treatment (Weisshof *et al*., 2018).

1.5 Thesis objectives

Chronic inflammation is a global epidemic affecting millions of people. As the average life expectancy in developed countries grows, so too does the phenomenon of inflammaging, and the increased risk of inflammatory disease. Dietary intervention and the use of functional ingredients which demonstrate anti-inflammatory activity and the ability to both prevent and treat inflammatory disease is emerging as a potential therapeutic alternative or as a complementary therapy to existing therapeutics. Due to this emerging market of functional foods with immunomodulatory activity, the demand for novel sources of these ingredients is expanding. Several functional ingredients, such as omega-3, are already sourced from the marine world. But due to its vast ecology and the presence of extremely diverse organisms, the marine world offers real potential for new novel functional ingredients with the ability to modulate the immune response.

- This thesis aims to investigate three marine organisms found in Irish waters as sources of potential anti-inflammatory protein hydrolysates. It will examine protein hydrolysates generated from the seaweed *Palmaria palmata* and the fish boarfish and blue whiting in order to assess their ability to modulate the cytokine response of key immune cells.
- Protein hydrolysates ability to alter the immune response of DCs and naïve undifferentiated $CD4^+$ T cells will first be analysed. Potential candidate hydrolysates which display potent anti-inflammatory activity in the suppression of pro-inflammatory cytokines and promotion of anti-inflammatory cytokines will then be brought forward and their ability to modulate the activity of T helper cell subsets investigated.
- The impact of variables in the hydrolysates such as the origin of the starting material, time of harvest and hydrolysis method used will be studied and its effect on commercial viability considered.
- Candidates which display specific potent anti-inflammatory activity and are considered commercially viable options will then be studied *in vivo* in a murine model of inflammatory disease.

Chapter 2:
Materials and Methods

2.1 Materials

2.1.1 Stock buffers and reagents

Table 2.1 Stock buffers. All buffers prepared in laboratory for use in experiments. All reagents use in buffers obtained from Sigma Aldrich, Ireland.

Buffer	Supplier
Phosphate-buffered saline (PBS) (1X) 150 mM	NaCl (5.84 g/L) Na_2HPO_4 (4.72 g/L) NaH_2PO_4 (2.64 g/L)
PBS tween 20 (PBST)	NaCl (5.84 g/L) Na_2HPO_4 (4.72 g/L) NaH_2PO_4 (2.64 g/L) Tween 20 (0.05% v/v)
Tris-buffered saline (TBS) (1X) 150 mM	Tris base (6.05 g/L) NaCl (8.76 g/L)
Tris-acetate-EDTA (TAE) (pH 8.0)	Tris base (10.8 g/L) Boric acid (5.5 g/L) 0.5 M EDTA (40 mL)
Reagent diluent	BSA (1 % w/v); in 1X PBS
TBS Reagent diluent	BSA (0.1 % w/v) Tween 20 (0.05 % v/v); in 1X TBS
Phosphate-buffered saline (PBS) (1X) 150 mM	NaCl (5.84 g/L) Na_2HPO_4 (4.72 g/L) NaH_2PO_4 (2.64 g/L)

The constituents of each buffer are dissolved in 990 mL distilled and deionised water and adjusted to a final pH of 7.4 (unless otherwise stated). The solutions are made up to a final volume of 1 L. All components were analytical grade.

2.1.2 Cell culture materials

Table 2.2 Cell Culture Materials. All cell culture materials and reagents and corresponding sources

Material	Supplier
Tissue culture flasks (T-25 cm^2/T-75 cm^2)	Nunc™
Sterile Petri Dishes	Nunc™
6, 24 and 96-well tissue culture plates	Nunc™
Dimethyl sulphoxide (DMSO)	Sigma Aldrich, Ireland
rGM-CSF	Sigma Aldrich, Ireland
Trypan blue (0.4% v/v)	Sigma Aldrich, Ireland
Sterile Water	Sigma Aldrich, Ireland
Beta-mercaptoethanol	Sigma Aldrich, Ireland
Red Blood Cell Lysis Buffer	Sigma Aldrich, Ireland
EDTA	Sigma Aldrich, Ireland
0.2 µm Filter	Sigma Aldrich, Ireland
CellTiter 96® Aqueous One Solution	Pierce, UK
Foetal Bovine Serum (FBS)	Gibco, UK
RPMI-1640	Gibco, UK
DMEM	Gibco, UK
Penicillin Streptomycin	Invitrogen™, UK
Balb/C Mice	Charles River, UK
Easysep© mouse $CD4^+$ T-cell isolation kit	Stemcell Technologies, UK
5 mL polystyrene falcon tube	BD Biosciences, UK
96 well round bottom plate	Sarstedt, Ireland

2.1.3 ELISA materials

Table 2.3 ELISA Reagents. All ELISA reagents used and corresponding sources.

Reagent	Supplier
96-well Microtitre Plate	Nunc™
3,3',5,5'-tetramethyl-benzidine (TMB)	BD, UK
Tween® 20	Sigma Aldrich, Ireland
Bovine Serum Albumin (BSA)	Sigma Aldrich, Ireland
DuoSet ELISA Kits	R&D Systems®
1X DPBS	Invitrogen™, UK

2.1.4 T cell differentiation reagents

Table 2.4 Differentiating cytokines and antibodies. All cytokines and antibodies used in T cell differentiation and corresponding sources.

Reagent	Supplier
rmIL-2	R&D Systems®, UK
rmIL-4	R&D Systems®, UK
rmIL-6	R&D Systems®, UK
rmIL-12	R&D Systems®, UK
rmIL-23	R&D Systems®, UK
rhTGFβ	R&D Systems®, UK
Anti-mouse IL-4 Neutralising Antibody	R&D Systems®, UK
Anti-mouse IFNγ Neutralizing Antibody	R&D Systems®, UK
Anti-mouse CD3 Monoclonal Antibody	BD, UK
Anti-mouse CD28 Monoclonal Antibody	BD, UK

2.1.5 RNA isolation and cDNA synthesis reagents

Table 2.5 RNA isolation and cDNA synthesis reagents. All reagents used for RNA isolation and cDNA synthesis and corresponding sources.

Reagent	Supplier
Nucleospin RNA II Columns	Macherey-Nagel, Germany
DEPC Treated Water	Invitrogen, UK
High Capacity cDNA Reverse Transcription Kit	Applied Biosystems, USA
Molecular Grade Ethanol	Sigma Aldrich, Ireland
RNaseZAP	Sigma Aldrich, Ireland
RNA Later	Sigma Aldrich, Ireland

2.1.6 qPCR reagents

Table 2.6 Quantitative PCR reagents. All reagents used for qPCR and corresponding sources.

Reagent	Supplier
Taqman Universal Mastermix	Applied Biosystems, USA
SYBR® Green Mastermix	Roche, Ireland
Microamp® Optical Adhesive Film	Applied Biosystems, USA
Microamp® Optical 96 Well Plate	Applied Biosystems, USA
GusB PrimeTime qPCR Primers	IDT, UK
IL-1β PrimeTime qPCR Primers	IDT, UK
TNFα PrimeTime qPCR Primers	IDT, UK
IL-6 PrimeTime qPCR Primers	IDT, UK
IFNγ PrimeTime qPCR Primers	IDT, UK
IL-17 PrimeTime qPCR Primers	IDT, UK
IL-10 PrimeTime qPCR Primers	IDT, UK

2.1.7 Immunohistochemistry reagents

Table 2.7 Immunohistochemistry reagents. All reagents used for H&E staining, tissue processing and paraffin embedding and corresponding sources.

Reagent	Supplier
Paraffin Wax	VWR, Ireland
Formalin	Sigma Aldrich, Ireland
Xylene	Sigma Aldrich, Ireland
Hydrochloric Acid	Sigma Aldrich, Ireland
Sodium Bicarbonate	Sigma Aldrich, Ireland
Ethanol	Sigma Aldrich, Ireland
Harris Haemotoxylin	Sigma Aldrich, Ireland
Eosin	Sigma Aldrich, Ireland
Histoclear	National Diagnostics, Ireland
Histobond Microscope Slides	RA Lab, Ireland

2.1.8 *in vivo* dextran sodium sulphate induced murine model of colitis materials and reagents

Table 2.8 Materials and reagents for *in vivo* murine model of DSS induced colitis. All reagents used for DSS-induced murine model of colitis and corresponding sources.

Reagent	Supplier
Oral Dosage Needles	Vet-tech, UK
Dextran Sodium Sulphate	Sigma Aldrich, Ireland
DPBS	Invitrogen, UK

2.1.9 Equipment

Table 2.9 Equipment used. All equipment used and corresponding sources.

Reagent/Material	Supplier
Class II Laminar Air Flow Unit - Holten 2010	ThermoElectron Corporation, USA
Incubator 37°C with 5% CO^2 and 95% dehumidified air - Model 381	ThermoElectron Corporation, USA
Inverted Microscope Olympus CKX31	Olympus Corporation, Toyko, Japan
Olympus IX81 motorised inverted microscope with Hamamatsu ORCA – ER digital camera C4742-80	Mason Technologies, Ireland
Heraeus Fresco 17 Centrifuge	Thermo Scientific, Ireland
Universal 32R Centrifuge	Hettich Zentrifugen, Germany
Mini Table Top Centrifuge	Technico, UK
Specific Pathogen Free Environment	Biological Research Unit**,** DCU
JB Series Waterbath	Grant, UK
LightCycler 96 qPCR	Roche, Ireland
PTC-200 Peltier Thermo Cycler	MJ Research, Ireland
Nanodrop™ ND-1000	Nanodrop Technologies, USA
Qiagen Tissue Lyser LT	Qiagen, Germany

2.2 Methods

2.2.1 Hydrolysates

Hydrolysates were prepared by the Fitzgerald group in the University of Limerick. *Palmaria palmata* starting material was sourced wild from Mweenish Island (County Galway) and Black Head (County Clare) and from longlines in Ard Bay (County Galway). Boarfish and blue whiting were sourced from the Killybegs Fishermans Association as facilitated by Board Iascaigh Mhara.

Hydrolysate generation

Proteins were isolated from starting material using the pH isolation method (Harnedy *et al*., 2014), protein was then incubated with various enzymes such as corolase, flavourzyme and alcalase, before being cooled in ice and centrifuged. Supernatant from this process was then freeze dried and sent to DCU for screening.

Hydrolysate preparation methods

Hydrolysates were first weighed into an Eppendorf 2 mL sterile tube (Starstedt, Ireland) and re-suspended in 1 mL sterile water in a Class II laminar hood (Holten 2010 – ThermoElectron Corporation, USA). These were then rotated for 30 mins on a circular rotator and centrifuged using a table top centrifuge at 2000 g (Mini, Technico UK) for 5 mins. Supernatant was then passed through a 0.2 µm filter (Sigma Aldrich, Ireland) and collected in a sterile Eppendorf tubes. Hydrolysates were added to the cells from this working stock at a concentration of 0-2 mg/mL.

2.2.2 Isolation and culture of bone marrow derived dendritic cells

Day 1 – Bone marrow Isolation

Isolation of bone marrow-derived dendritic cells (BMDCs) was an established laboratory protocol adapted from Lutz *et al.* (1999). Bone marrow was isolated aseptically, in a Class II Laminar hood, from the tibia and femurs of Balb/C mice aged 12+ weeks, which were housed in a specific pathogen free (SPF) environment and sourced from Charles River. This was achieved by culling the mouse, carefully removing the legs from the body with a surgical scissors and tweezers and removing all fur and flesh. When all that remains is two

clean bones, these were cut at either end and flushed with RPMI using a 27.5 g needle and syringe into a sterile 50 mL falcon. Bone marrow still intact was then gently broken up using a 19.5 g needle and syringe. Cells were then centrifuged for 5 mins at 1200 rpm, supernatant removed, pellet re-suspended in RPMI (supplemented with 20% heat inactivated foetal bovine serum (FBS), 100 µg/ml/100 µg/ml penicillin/streptomycin 50 ng/mL rGM-CSF) to allow for 10 mL of cells per bone isolated. 10 mL of cells were then added to each petri dish and incubated at 37°C.

Day 4 – Feeding cells

The petri dish was tipped forward at a 45° angle to ensure cells were not disturbed. Using a transfer pipette ~ 6 mL of media was gently removed. Ten mL of pre-warmed supplemented RPMI was added to each petri dish and returned to the 37°C incubator.

Day 7 – Counting and plating BMDCs

Adherent cells were dislodged from the surface using a cell scraper (Sarstedt, Ireland) before being collected in a 50 mL falcon. Cells were centrifuged at 1200 rpm for 5 mins, resuspended and counted using the trypan blue exclusion method (described in section 3.3.2). The cell concentration was adjusted with RPMI and cells were plated at 1×10^6 cells / mL at a volume of 250 µL on 96 well cell culture plates.

2.2.3 Isolation and culture of $CD4^+$ T-cells

2.2.3.1 Splenocyte isolation

T-cells were isolated aseptically in a class II laminar hood (Holten 2010 – ThermoElectron Corporation, USA) from female Balb/c mice aged 6-12 weeks. Spleens were removed and collected in RPMI-1640/10% (v/v) FBS on ice. A single cell suspension was achieved by pushing each spleen through a cell strainer (40 µm, BD falcon). Cells were then washed with RPMI-1640/10% (v/v) FBS and counted.

2.2.3.2 $CD4^+$ T-cell magnetic particle isolation

(Stemcell technologies- Easysep© Mouse $CD4^+$ T-cell isolation kit Catalog#19852)

Cells were prepared in RPMI-1640 at a concentration of 1 x 10^6 cells/mL. The cell solution was then transferred to a 5 mL polystyrene falcon tube (BD Biosciences). The Easysep© mouse $CD4^+$ T-cell enrichment cocktail was then added at 50 µL/mL of cells, mixed well and left to incubate at room temperature for 10 mins. This cocktail contains a selection of antibodies targeting cell surface markers of all cells excluding T-cells (CD8, CD11b, CD11c, CD19, CD24, CD24, CD45R, CD49b and TER119), negatively selecting for $CD4^+$ T cells. The Easysep© streptavidin rapidspheres™ were vortexed and added to cells at a concentration of 75 µL/mL of cells, this was then mixed well and incubated at room temperature for 2.5 mins. The suspension was then made up to a total volume of 2.5 mL and mixed gently before being placed into the Easysep© magnet at room temperature for 2.5 mins. The supernatant that was poured off contained the $CD4^+$ T-cells. These cells were centrifuged at 300 g for 5 mins, counted and resuspended in cRPMI media. Cells were then stimulated with 5 µg/mL plate bound anti-CD3 and 2.5 µg/mL anti-CD28 agonists for three days and supernatants collected for ELISA cytokine analysis.

2.2.3.3 Polarisation of Th1, Th2 and Th17 T cells

Cells were stimulated using agonist antibodies directed to CD3 (clone: 145-2C11) (BD Biosciences, Ireland) and CD28 (clone: 37.51) (BD Biosciences, Ireland). Plates were coated with 5 µg/mL anti-CD3 for 2 hours at 37°C, 5% CO_2. Anti-CD28 (5 µg/mL) was then added to cells. Cells were stimulated in the presence of polarizing cytokines. The cocktails used to drive specific T cell subsets and their concentrations are presented in Table 2.10. After 2 hours, cells were transferred to coated anti-CD3 plates and incubated at 37°C, 5% CO_2. All media was removed from cells and cells were rested with the addition of new media. New anti-CD3 plates were coated (2.5 µg/mL) for 2 hours at 37°C, 5% CO_2. Cells were transferred to new anti-CD3 plates and anti-CD28 (2.5 µg/mL) added in order to re-stimulate the cells. Cells were then incubated for a further 24 hours at 37°C, 5% CO_2. Supernatants were then harvested.

Table 2.10 List of T cell subset polarising cocktails. List of polarising reagents and concentrations used to drive T cell subset phenotypes.

Reagent	Supplier	Concentration	Source
Th1	rmIL-12	10 ng/mL	R&D Systems®,
	anti-IL-4	10 µg/mL	UK
Th2	rmIL-2	10 ng/mL	R&D Systems®,
	rmIL-4	10 ng/mL	UK
	anti-IFNγ	10 µg/mL	
Th17	rmIL-6	20 ng/mL	R&D Systems®,
	rmIL-23	10 ng/mL	UK
	rhTGFβ	2 ng/mL	
	anti-IFNγ	10 µg/mL	
	anti-IL-4	10 µg/mL	

2.2.4 Culture of cell line

The murine macrophage cell line J774A.1 was obtained from the European Collection of Cell Cultures (ECACC; Salisbury, UK). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, UK) supplemented with 10 % (v/v) foetal bovine serum (FBS) (Invitrogen, UK), 50 U penicillin and 50 µg streptomycin (Invitrogen, UK). The semi-adherent cell line, J774A.1, was subcultured by dislodging the cells from the surface of the flask using a cell scraper (Sarstedt, Ireland). Cells were recovered by centrifugation in a Sigma 2K15 centrifuge (Sigma Laborzentrifugen, Germany) at 400 g for 5 minutes, diluted in fresh medium and then transferred to a sterile 75 cm^2 tissue culture flasks for further growth. All cultures were maintained in a 37^{o}C, in a 5 % CO_2 humidified atmosphere. All work was carried out in a MSC-Advantage laminar flow cabinet (Thermo, USA) to ensure safety and sterility.

2.2.5 Cell Enumeration and Viability

Cells were counted and their viability assessed using trypan blue exclusion. Trypan blue is negatively charged, so it is excluded from viable cells. Therefore, only the cells with damaged cell membranes (i.e. dead cells) will stain blue. Cell suspension (100 µL) was mixed with 150 µL PBS and 250 µL trypan blue solution (0.4% v/v) (Sigma-Aldrich, Ireland). After ~5 mins cells were applied to a haemocytometer and examined using an inverted

microscope. Cells inside the central grid were counted. A viable cell count was determined using the following formula: Cell/mL= N x 5 x 10^4 where, N= average cell number counted, 5 = dilution factor and 10^4 = constant.

2.2.6 MTS Assay

To measure cytotoxicity a CellTiter 96® Aqueous One Solution (Pierce, UK) was employed. This is a colorimetric method for determining the number of viable cells in a sample. It contains an MTS tetrazolium compound (Owen's reagent) which is bioreduced by cells into a soluble coloured formazan product. The quantity of formazan product is measured at an absorbance reading of 450nm and is directly proportional to the number of living cells in the culture medium. BMDCs were plated in a 96-well plate with 100 µL per well at a concentration of 1x10^6 cells/mL. Hydrolysates were then added at increasing concentrations (0.25 – 2 mg/mL) 1 hour prior to stimulation with LPS (100ng/ml). Cells alone and 10% (v/v) DMSO controls were also used. CellTiter 96® Aqueous One Solution (20 µL) was added to each well of the 96-well plate. The plates were incubated for 4 hours at 37°C in 5 % CO_2 and absorbance read at 490 nm. The cell viability of each sample was calculated by treating the absorbance of the cells alone control as 100% and comparing the remaining samples to this and expressing results as percentage viability. DMSO (10% v/v) is cytotoxic and was used a negative control.

2.2.7 Enzyme linked immuno-sorbent assay (ELISA)

Cell suspension at a concentration of 2.5 x 10^5 cells/250 µL was added to each well of a Nunc™ 96 well tissue culture plate. Protein hydrolysates were added in various concentrations (0.25 mg/mL – 2 mg/mL) (with or without 100 ng/mL LPS in BMDCs to induce an inflammatory response). The plates were placed in a 37°C incubator, 5% CO_2, for 24 hours. After 24 hour incubations, the cell supernatants were taken and aliquoted into sterile 96 well tissue culture plates and stored at -20°C until required. All experiments were performed in triplicate. DuoSet ELISAs were performed according to the manufacturer's instructions (R&D Systems Ltd). Each sample and standard was assayed in triplicate for each of the cytokines indicated.

2.2.8 Dextran sulfate sodium induced model of colitis

Dextran sulfate sodium (DSS) was used to induce colitis in Balb/c mice. Female Balb/c mice (n=24) were housed in the SPF unit of the BRU at DCU. DSS was administered in the drinking water at a final concentration of 5% (w/v) for 7 days. Water with DSS was prepared fresh every day. Following 7 days of DSS, mice were administered daily intra-gastric (IG) gavage with PBS or hydrolysed blue whiting protein in PBS for 7 days. Mice were sacrificed on day 14 and organs harvested. Mice were split into four groups for this study as described in Table 2.11.

Table 2.11 DSS experimental groups

Days	Control	DSS Alone	DSS + H26	DSS + F1
Days 1 – 7	No DSS.	5% DSS	5% DSS	5% DSS
Days 8 - 14	PBS IG	PBS IG	H26 (50 mg in 0.2mL) in PBS IG	F1 (50 mg in 0.2mL) in PBS IG

2.2.9 RNA isolation from colonic tissue

Colonic tissue was homogenised in lysis buffer RA1 (Macherey-Nagel, Germany) containing beta-mercaptoethanol using the Qiagen Tissue Lyser LT (Qiagen, Germany) for 5 mins at 50 Hz. Total RNA was then isolated using a Nucleospin® RNA II Kit (Macherey-Nagel, Germany). The viscosity of the lysate was cleared by filtration through a Nucleospin® Filter and centrifuging (11,000 g) for 1 min. 70% ethanol was then added to homogenised lysate to adjust the RNA binding conditions. A Nucleospin RNA II column was used to bind RNA with the addition of Membrane Desalting Buffer to remove salt and improve the efficiency of rDNAse digestion. DNA was digested using DNAse reaction mixture at room temperature for 15 mins. Buffer RA2 and RA3 were added after 15 mins in order to inactivate rDNAse and wash the column. The Nucleospin® RNA II column was placed into a nuclease free collection tube and RNA eluted in RNAse free water.

2.2.10 RNA quantitation

The concentration of RNA was measured using the Nanodrop® ND-100 (Nanodrop Technologies, USA). The purity of RNA was determined using 260 nm and 280 nm absorption wavelengths. All RNA used had an A280/A260 ratio between 1.8 and 2.1.

2.2.11 cDNA synthesis

Complementary DNA (cDNA) was generated from RNA using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, USA). Reverse transcriptases included in the kit were used to synthesise single strand cDNA using the RNA strands as template. cDNA was then used as a template in subsequent qPCR experiments. RNA (2 µg) was used in each cDNA reaction on the PTC-200 PCR thermal cycler (MJ Research, Ireland). Table 2.12 displays settings used.

Table 2.12 Thermal cycling conditions on PTC-200 PCR thermal cycler

Steps	1	2	3	4
Temperature (°C)	25	37	85	4
Time (mins)	10	120	5	∞

2.2.12 Quantitative polymerase chain reaction (qPCR)

PCR was prepared in triplicate for each sample. cDNA, Taqman® Gene Expression Mastermix (Applied Biosystems, USA) and RNAse free water (Invitrogen, UK) were added to each well of a 96 well reaction plate (Applied Biosystems, USA). Plates were then sealed with optically clear film (Applied Biosystems, USA). Plates were centrifuged (Heraeus Fresco 17 Centrifuge, Germany) at 1000 g for 1 min and ran on a Lightcycle 96® system (Roche, Ireland) under the conditions in Table 2.13.

Table 2.13 Thermal cycling conditions on Lightcycler96® thermal cycler. All conditions were continued for 40 cycles.

Steps	1	2	3	4
Temperature (°C)	50	95	95	60
Time (mins)	2	1	15 (secs)	15 (secs)

2.2.13 PCR data analysis

PCR results were analysed using the Lightcycler 96® software (Roche, Ireland) and Microsoft Office Excel (Microsoft, USA). For gene expression analysis a relative quantitation method in which gene levels are expressed as a fold difference between a sample and a calibrator such as untreated tissue from healthy control mice was used. Glucuronidase-β (GusB) was used as a endogenous control in order to normalise samples. Normalised samples were then compared to the calibrator using ΔΔCt method:

$$\Delta\Delta Ct = \Delta Ct\ sample * - \Delta Ct\ calibrator **$$

$$* \Delta Ct\ sample = Ct\ sample - Ct\ endogenous\ control$$

$$** \Delta Ct\ calibrator = \frac{Ct\ calibrator}{Ct\ control} - Ct\ endogenous\ control$$

2.2.14 Colonic tissue sectioning

Tissue sections of 0.5 cm were removed from the distal colon and arranged into swiss rolls. These were then submerged in formalin and incubated at room temperature overnight in order to fix the rolls. Following this tissue was then put into a TP1020 processor (Leica Biosystems, Germany) for paraffin tissue processing. Table 2.14 outlines steps of this processor in order to ensure adequate dehydration of the tissue.

Table 2.14 Dehydration of colonic tissue

Steps	1	2	3	4
Reagent	70% EtOH	96% EtOH	100% EtOH	100% EtOH
Time (hours)	1	1	1	1

The program continued with two 1 hour Xylene (Sigma Aldrich, Ireland) steps to remove traces of ethanol before two 1 hour paraffin embedding steps. The tissue was then fixed in small paraffin blocks and left to set overnight. Sections (0.5 µm) were then cut from the paraffin blocks and placed onto microscope slides. Slides were baked at 40°C for 40 mins.

2.2.15 Haemotoxylin and eosin staining

Paraffin embedded tissue was immersed in Histoclear (National Diagnostics, Ireland) to de-paraffinise it for 10 mins, twice. Tissue was then rehydrated by immersion using the protocol outlined in Table 2.15

Table 2.15 Rehydration of colonic tissue

Steps	1	2	3
Reagent	100% EtOH	95% EtOH	70% EtOH
Time (mins) x number of repeats	5 mins x 2	2 mins x 1	2 mins x 1

Slides were then stained with Harris haemotoxylin (Sigma Aldrich, Ireland) for 10 mins and washed under running tap water for 5 mins. The tissue was then differentiated in 1% acid/alcohol for 30 secs (3 times) and again washed under running tap water for 1 min. Slides were then placed in 0.1% sodium bicarbonate (Sigma Aldrich, Ireland) for 1 min and washed again under a running tap for 5 mins. Slides were then rinsed in 95% EtOH (Sigma Aldrich, Ireland) for 10 dips before being immersed (using dipping method) in Eosin (Sigma Aldrich, Ireland) for 1 min. Slides were then dehydrated again using the method described in Table 2.16

Table 2.16 Dehydration of H&E stained slides

Steps	1	2	3	4
Reagent	70% EtOH	95% EtOH	100% EtOH	Histoclear
Time (mins) x number of repeats	3 mins x 1	3 mins x 2	3 mins x 1	3 mins x 1

Slides were then mounted with coverslips using mouting medium (DPX) and cover slips pushed firmly to remove bubbles.

2.2.16 Statistical Analysis

One-way analysis of variance (ANOVA) was used to determine significant differences between more than two samples. When this indicated significance ($p \leq 0.05$), post-hoc Student-Newmann-Keul test was used to determine which conditions were significantly different from each other. The level of statistical significance was indicated by $*p \leq 0.05$, $**p \leq 0.01$$***p \leq 0.001$. Parametric statistical tests were used throughout this study. Sample size was not large enough to test distribution and thus it was assumed the data was normally distributed. Data is presented as a mean + SEM unless otherwise indicated.

Chapter 3:

Investigating the potential of *Palmaria palmata* as a source of anti-inflammatory protein hydrolysates

3.1 Introduction

Macroalgae refers to a diverse group of multicellular marine organisms that are often characterised into red, brown or green macroalgae. Seaweed falls under the category of macroalgae, as it is macroscopic marine algae. Macroalgae, due to their unique metabolic pathways, have been identified as a rich source of potential bioactive compounds. Many bioactive compounds have already been identified from macroalgae, including anti-tumour and anti-oxidant activity from *Cladophora surera* (Lezcano *et al.,* 2018), anti-microbial and anti-cancer activity from *Cystosphaera jacquinotii* (Martins *et al.,* 2018) and neuroprotective properties from *Gelidiella acerose* (Syad *et al.,* 2016).

Palmaria palmata (PP) is a red macroalgae which has been identified as a potential source of novel bioactive compounds. It is found on northern coasts of both the Pacific and Atlantic Oceans, including along the North and West coast of Ireland. PP has been studied both here in Ireland and abroad. It is rich in protein, having the second highest protein content of any common macroalgae, with protein making up roughly 35% of its dried weight (Galland-Irmoul *et al.,* 1990). Due to this high protein content it is being investigated as a potential alternative protein source (Bjarnadóttir *et al.,* 2018) as the world looks for more sustainable sources of protein as its population grows. It has been found to contain bioactive compounds which exhibit anti-oxidant and anti-proliferation capabilities (Yuan *et al.,* 2005), as well as inhibition of renin (Fitzgerald *et al.,* 2012) and dipeptidyl peptidase IV (DPP IV) (Harnedy *et al.,* 2015). A common theme in bioactive compounds found in PP is that the majority are peptide chains, which is unsurprising considering its high protein content.

One of the more widely consumed seaweeds, PP has been eaten for centuries, possibly even millenia, in countries such as Ireland, Canada, Iceland and France. Also known as Dulse or Dillisk, it is commonly eaten as a snack food, when dried, but its use as a functional food, commerically and domestically has begun to increase in recent years. As the general population has become more health focused, interest in seaweeds such as PP has risen. It is high in protein, as previously mentioned, with alanine, aspartic acid and

glutamic acid being the most abundant amino acids present (Channing and Young, 1953). PP also contains essential fatty acids and vitamin K (Mouritsen *et al.,* 2013). Vitamin K plays a crucial role in bone health and in blood clotting (Price and Nishimito, 1980). Current research on PP however has focused mainly on its proteins, due mainly to the high yield achieved from the plants.

Protein hydrolysation is the process of hydrolysing or breaking down protein chains into smaller peptide chains. This can occur in a number of different ways including changing the pH, causing denaturation of the protein, or through the use of enzymes. In this project, enzymatic hydrolysation was utilised in the preparation of all hydrolysates. Through the hydrolysation process, many new peptide chains are unlocked and these may possess bioactivity which wasn't present before hydrolysation. PP protein hydrolysates have been widely investigated for potential beneficial properties. PP proteins enzymatically hydrolysed have been shown to possess potent anti-oxidant and ACE inhibitory effects (Beaulieu *et al.,* 2016). Wang *et al.* (2010) and Harnedy and Fitzgerald (2013) also found protein hydrolysates enzymatically isolated from PP to have anti-oxidant acivity. Hydrolysates have also been identified with anti-hypertensive and anti-diabetic activity (Admassu *et al.,* 2018). Similarly, our collaborators on this project Harnedy and Fitzgerald (2013) found the hydrolysates used during the course of this project to have cardioprotective, anti-diabetic and anti-oxidant potential. Following this, Harnedy *et al.* (2015) purified and identified DPP-IV inhibitory peptides from these hydrolysates.

PP can be grown in the wild or cultivated in pools. It is often dried before use and if harvested from the wild it is usually dried on the rocks beside the sea (Le Gall *et al.,* 2004). There are many small businesses across Europe, Iceland and North America which harvest wild PP, dry it and package it for sale. In Ireland, several such companies operate along the west coast. The majority of successful PP farming projects have taken place in in-land pools, however, in northern Spain the largest cultivation of red macroalgae in open sea outside of Asia has been very successful (Martinez *et al.,* 2006). PP is harvested in Ireland

all along the west coast, both in the wild and farmed. PP for this project was taken from both wild and farmed sites. Wild seaweed was harvested from Mweenish Island, Co. Galway and Black Head, Co. Clare. Farmed seaweed was harvested from longline cultivation in Ard Bay, Co. Galway.

Macroalgae proteins have been shown to differ in bioactivity depending on the harvest time and place of harvest of the macroalgae. PP hydrolysates screened in this study were found by Harnedy *et al.* (2014) to differ in their DPP-IV inhibition and anti-oxidant activity depending on the time of year the harvest occurred. Another red macroalgae, *Porphyra dioica,* was also found to have seasonal variation in its bioactivity (Stack *et al.,* 2018).

PP whole protein has been used in a human trial by collaborators Allsopp *et al.* (2016) in the University of Ulster. In this study, 40 participants were fed with either a placebo bread or bread enriched with whole protein PP. They found that inflammatory markers such as serum levels of C-reactive protein (CRP) and thyroid stimulating hormone (TSH) increased in participants fed with the enriched bread, although not to clinically significant levels with serum CRP and TSH. In a parallel, an *in vitro* study on Caco-2 cells they found similar results with increases in the pro-inflammatory cytokine IL-8. This stimulation of inflammation is one of the few reported cases where PP has been found to be immuno-stimulatory. Hydrolysation of the PP could reduce this effect and make it possible to identify anti-inflammatory peptides.

Immunomodulatory peptides are a current focus of research, particularly those isolated from food sources. Peptides from casein and whey proteins have gained recognition as potential immunomodulators that could be incorporated into food for use in a functional food or nutraceutical market (Agyei and Danquah, 2012). Protein use in therapeutics has increased dramatically over the last two decades, with insulin being the first recombinant protein therapeutic. This has paved the way for the introduction of a whole class of

therapies (Leader *et al.,* 2008). One such therapy is etanercept, Enbrel®, a fusion protein used in the treatment of many inflammatory diseases. Etanercept inhibits TNFα from acting and has been used to treat a number of diseases including RA, psoriasis and IBD (Nash *et al.,* 2001). However, the inhibition of such a vital cytokine in the immune system often leaves patients immunocompromised and as such it is reserved for those with life-limiting inflammatory disease.

The majority of sufferers of inflammatory disease do not experience life-limiting symptoms, they are people such as elderly people suffering the phenomenon known as 'inflammaging' and drastic therapies such as etanercept are not viable options. This so-called low grade chronic inflammation is often overlooked in therapeutics development and left to non-steroidal anti-inflammatory drugs (NSAIDs). The long-term use of NSAIDs is not recommended and so there is a demand for new anti-inflammatory treatments which can be used over an extended period of time has emerged. This project aims to identify and develop bioactive peptides from marine based sources which have the ability to decrease this low grade systemic inflammatory response and can be used long-term.

Inflammaging is the low grade chronic inflammation people develop as they age. Immunosenescence is the term used to describe the overall effect of aging on the immune system, which includes inflammaging, the increased risk of infectious disease and the severity of the disease when it occurs (Fülöp *et al.,* 2018). Inflammaging has been implicated as being the underlying cause of a number of age-related diseases including Parkinsons' disease (Calabrese *et al.,* 2018), Alzheimers' disease (Giunta *et al.,* 2008.), chronic obstructive pulmonary disease (COPD) (Rahman *et al.,* 2012.) and cardiovascular disease (Shayganni *et al.,* 2016) amongst others. This phenomenon is described as an unavoidable aspect of aging (Flynn *et al.,* 2018) but whilst unavoidable it may be possible to decrease its effects through the modulation of the immune system to a more anti-inflammatory state. It has been shown that high levels of circulating pro-inflammatory cytokines are present in inflammaging including IL-1β, IL-6 and TNFα (Wood, 2018). NF-κB signalling has also been shown to play a role in this chronic inflammation.

NF-κB is a signalling protein of the innate immune system which, alongside maturation of DCs and signalling Tcells, also causes a pro-inflammatory cascade of cytokines such as IL-1, IL-2, IL-12 and TNFα when activated. These pro-inflammatory cytokines have been identified as a possible target for therapeutics to combat this phenomenon.

It is hypothesised that functional foods, and functional ingredients, which target the chronic inflammatory response experienced in inflammaging could be a possible alternative to therapeutics (vel Szic *et al.,* 2015). Functional foods are foods which provide added benefits of more than just nutrition to the consumer. Whilst the term has been heavily promoted in recent years, functional foods have been around for a long time. They are very much already part of the daily diet of Irish people and include food such as milk fortified with vitamin D (Avonmore Super Milk) and probiotic drinks such as Yakult. Proteins isolated from food such as seaweed are ideal functional ingredients as they are fit for human consumption and commonly eaten. This project aims to identify a protein hydrolysate from PP which could be used as a potential functional ingredient to combat inflammaging.

3.1.1 Chapter Aims

The purpose of this chapter is to investigate the potential of PP hydrolysates as bioactive peptides with anti-inflammatory properties for use in inflammaging. It aims to demonstrate the potential immunomodulatory capabilities of PP hydrolysates on key immune cells, BMDCs and T-cells. This gives insight into the potential of PP hydrolysates as functional ingredients which benefit the immune system.

Firstly, a dose response of PP selected hydrolysates was carried out in order to determine the optimal dose of hydrolysate for this project. A dose of range of 0.25 - 2.0 mg/mL was chosen as this has been previously used by our collaborators on this project. Following a consultation, it was discovered that several sample preparation methods were being used across the project by our collaborators hence these were compared and the optimum chosen for this work.

The cytotoxicity of PP hydrolysates on both BMDCs and T-cells was first examined to ensure samples were not negatively impacting cell viability. A full screen of PP hydrolysates on BMDCs was then carried out in order to study the hydrolysates ability to modulate cytokine secretion. In conjunction with this, a full screen of PP hydrolysates was also carried out on $CD4^+$ T cells in order to assess their effect on the adaptive immune response. Data was then analysed and potential candidates for further screening chose to be brought forward into T cell subset screening in order to gain insight on their mechanism of action.

PP hydrolysates were harvested and their proteins isolated and hydrolysed by collaborators in the University of Limerick. Hydrolysates were generated through the use of enzymatic hydrolysation as described in Harnedy and Fitzgerald (2013). Enzymes used to hydrolyse the whole protein include corolase, alcalase and flavourzyme. Whole protein controls were made through the same process but no enzyme was added to these samples. In total twenty-seven (27) samples of PP were provided by the University of Limerick for screening including nine (9) unhydrolysed whole protein controls and

eighteen (18) protein hydrolysates. **Table 3.1** outlines the controls for each hydrolysate. For screening purposes, hydrolysates are grouped according to their control.

Table 3.1 Whole protein control and corresponding hydrolysates as grouped for analysis

Whole Protein Control (WPC)	Hydrolysates
WPC 2	4
	5
	6
WPC 7	9
	10
WPC 12	14
	15
	16
WPC 17	18
	19
WPC 20	21
	22
WPC 23	24
	25
WPC 26	27
	28
WPC 31	32
	33
	34
WPC 35	36
	37
	38
	39

Several PP whole protein controls, also known as starting material, differed in harvest location and harvest time. PP hydrolysates from the same starting material differed in the enzyme used to hydrolyse the whole protein. Table 3.2 describes these WPCs and their hydrolysates.

Table 3.2 Harvest time, location and enzyme used for hydrolysis.

WPC	Hydrolysate	Enzyme	Location	Time of Year
17	18	Alcalase	Mweenish Island Galway (wild)	April
	19	Corolase	Mweenish Island Galway (wild)	April
20	21	Alcalase	Ard Bay Galway (farmed)	April
	22	Corolase	Ard Bay Galway (farmed)	April
23	24	Alcalase	Black Head Clare (wild)	July
	25	Corolase	Black Head Clare (wild)	July
2	4	Alcalase	Black Head Clare (wild)	October
	5	Flavourzyme	Black Head Clare (wild)	October
	6	Corolase	Black Head Clare (wild)	October

3.2 Results

3.2.1 Dose response of *Palmaria palmata* hydrolysates on BMDCs

It was first necessary to investigate the optimal dose at which PP hydrolysates had the greatest immunomodulatory effects on BMDCs. This was achieved by analysing the cytokine secretion of BMDCs treated with a dose response of PP hydrolysates using a range from 0.25 mg/mL to 2.0 mg/mL. Hydrolysates were chosen at random for these experiments.

These are displayed in **Figure 3.1**. All doses successfully modulated cytokine secretion. The doses 0.25 mg/mL and 0.5 mg/mL had the least anti-inflammatory effects. 2.0 mg/mL had the most potent anti-inflammatory effects, but reduced levels of all cytokines below basal levels. 1.0 mg/mL reduced levels of pro-inflammatory cytokines whilst still maintaining a basal level response and as a result this was chosen as the optimal dose for future investigations.

One-way ANOVA with a post Newman-Keuls was used to assess differences between groups. Cells treated with hydrolysate only were compared to cells alone, cells treated with hydrolysates + LPS were compared to cells alone + LPS. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to untreated cells (no hydrolysate).

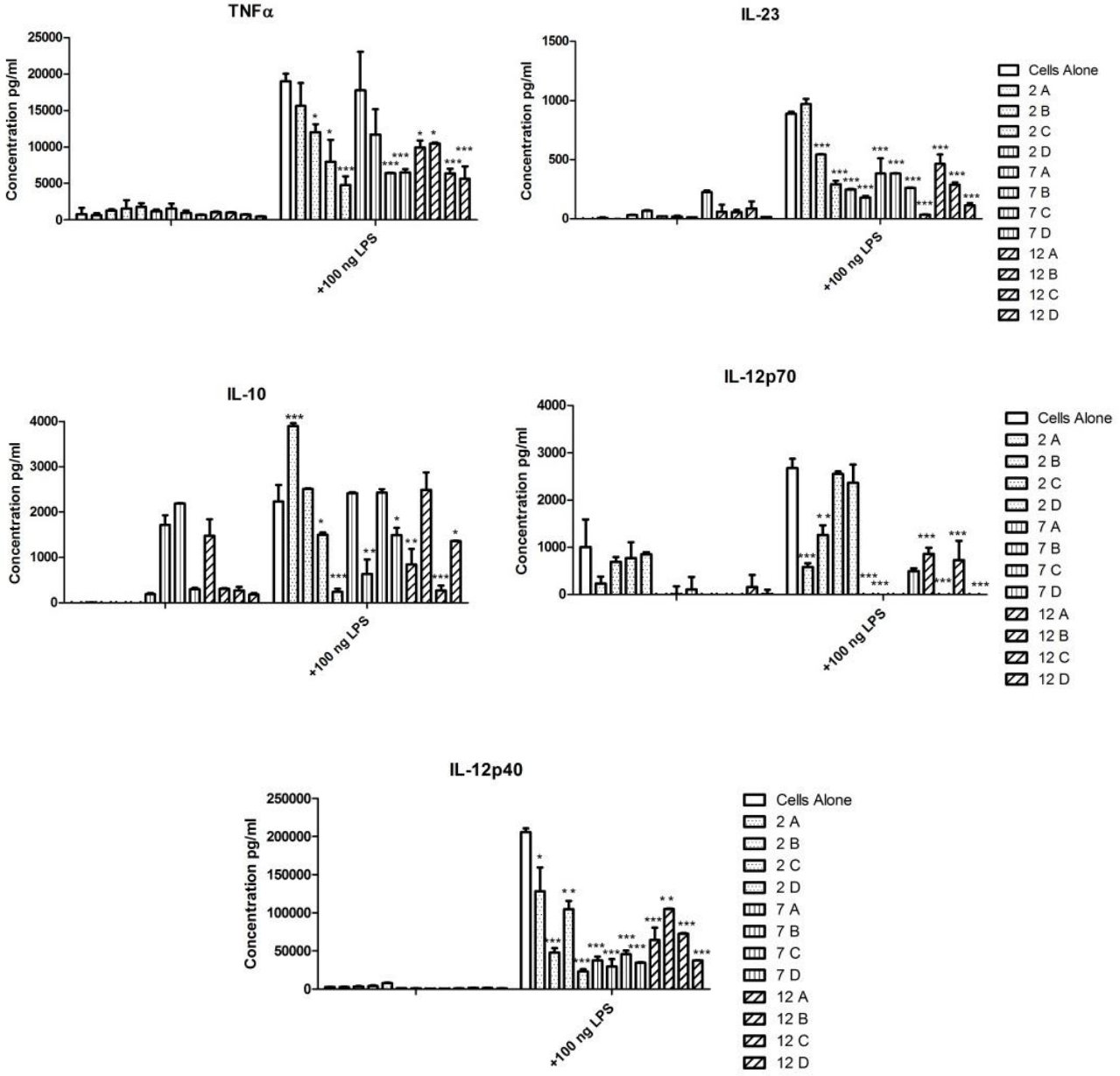

Figure 3.1 Secretion of TNFα, IL-23, IL-10, IL-12p70 and IL-12p40 by BMDCs when treated with *Palmaria palmata* hydrolysates (0.25 – 2.00 mg/mL). BMDCs were treated with hydrolysates 2, 7 or 12 in a dose range as follows: A - 0.25 mg/mL, B - 0.50 mg/mL, C - 1.00 mg/mL and D - 2.00 mg/mL. Cells marked +LPS were then stimulated with 100 ng/mL LPS. Secretion of TNFα, IL-23, IL-10, IL-12p70 and IL-12p40 was measured using ELISA (R&D Duoset).

3.2.2 Sample preparation method impacts *Palmaria palmata* hydrolysates ability to modulate cytokine secretion of BMDCs

A standard operating procedure for *in vitro* sample preparation was not in place across collaborators when this project began. This was essential to the project to ensure work from all collaborators was comparable. Three preparation methods (A, B and C) were investigated. Method A involved hydrolysates being weighed directly into an eppendorf 2 mL tube, suspended in sterile water, rotated on a circular rotator for 30 mins, centrifuged on a table top centrifuge at 5000 g for 5 mins and sterile filtered using a 0.2 µm filter and the filtrate used for analysis. Method B involved hydrolysates also being weighed directly into a 2.0 mL eppendorf tube and suspended in sterile water before being rotated on a circular rotator for 30 mins and sterile filtered using a 0.2 µm filter and the filtrate used for analysis. Finally, method C involved weighing of the hydrolysates directly into a 2.0 mL eppendorf tube, suspended in RPMI media, rotated on a circular rotator for 30 mins, centrifuged on a table top centrifuge at 5000 g for 5 mins and sterile filtered using a 0.2 µm filter and the filtrate used for analysis. BMDCs were plated in triplicate at a concentration of $1x10^6$ cells/mL and rested for 2 hours. Cells were then treated with hydrolysates at a dose of 1 mg/mL as had been previously optimised in **Section 3.2.1** and then those marked + LPS were stimulated with LPS (100 ng/mL) marked +100 ng LPS and incubated for 24 hours. Secretion of cytokines (IL-10, IL-1β, IL-21, IL-23, TNFα, and IL-12p70) was then quantified using ELISA (R&D Duoset).

Figure 3.2 shows the cytokine secretion of IL-10, IL-1β, IL-21, IL-23, TNFα, and IL-12p70 from BMDCs when treated with whole protein controls (WPCs) WPC2, WPC7 and WPC12 prepared using three different methods. Method preparation significantly altered modulation of cytokines by hydrolysates. Method A and B resulted in the modulation of cytokines by hydrolysates in a similar manner to each other. They both suppressed secretion of IL-12p70. Method C's effect on IL-12p70 did not display the same potency. Method A and C reduced levels of IL-1β, with all methods reducing levels of IL-23. Method A (sterile H_2O and centrifugation) was chosen as the optimal sample preparation method due to its anti-inflammatory profile and its ease of use.

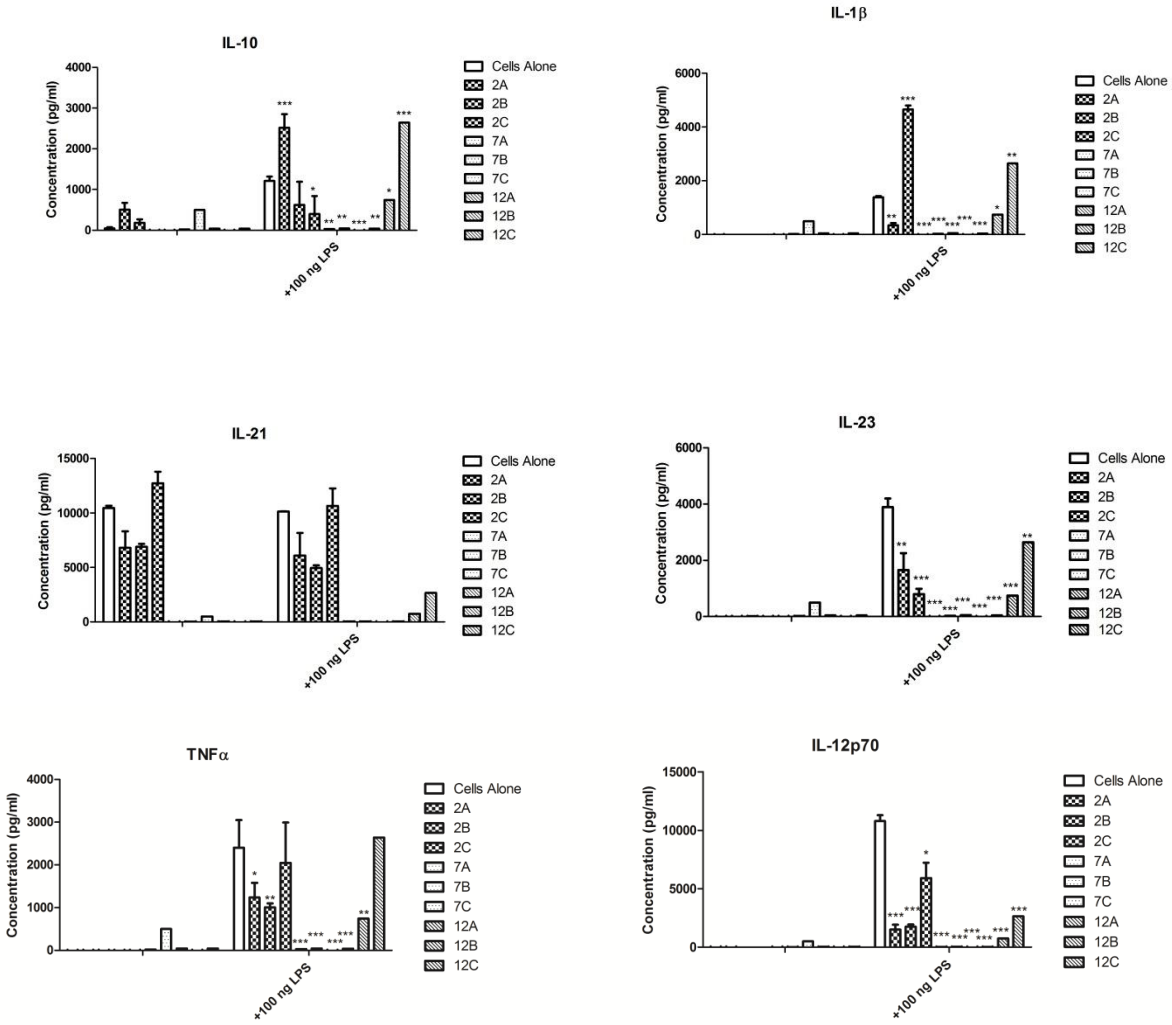

Figure 3.2 Secretion of IL-10, IL-1β, IL-21, IL-23, TNFα, and IL-12p70 by BMDCs when treated with *Palmaria palmata* hydrolysates from different preparation methods. BMDCs were isolated from Balb/c mice and differentiated in rGMCSF for seven days. On day 7 cells were treated with hydrolysates (1 mg/mL). Hydrolysates were prepared using three different methods (A, B and C). Cells marked +LPS were then stimulated with 100 ng/mL LPS. Secretion of IL-10, IL-1β, IL-21, IL-23, TNFα, and IL-12p70 was measured using ELISA (R&D Duoset). One way ANOVA with a Post test Newman Keuls was used to assess differences between groups. Cells treated with hydrolysate only were compared to cells alone, cells treated with hydrolysates + LPS were compared to cells alone + LPS. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to untreated cells (no hydrolysate).

3.2.3 *Palmaria palmata* hydrolysates do not impact dendritic cell viability.

After optimisation of sample preparation and the determination of the optimal dose of PP (1 mg/mL), it was necessary to confirm that PP hydrolysates had no negative effect on the viability of BMDCs. Cells were isolated from the bone marrow of Balb/c mice and incubated at 37°C 5% CO_2 for seven days with rGMCSF (Sigma Aldrich, Ireland). BMDCs were plated in triplicate (100 µL/well) at a concentration of $1x10^6$ cells/mL on a 96 well plate and rested for 2 hours. Cells were then treated with hydrolysates (1 mg/mL) or 10% DMSO (positive control) before being stimulated with LPS (100 ng/mL) and incubated for 24 hours. Viability was assessed using an MTS assay (Cell Titer 96 Aqueous One Solution – Promega) according to the manufacturer's instructions. Results were expressed as a percentage of cells alone. At 1 mg/mL PP hydrolysates did not have a significant negative effect on cell viability on BMDCs when compared to untreated cells (**Figure 3.3**). Results are expressed as a percentage of cells alone (untreated cells). Error bars represent mean ± 3SEM (standard error of mean).

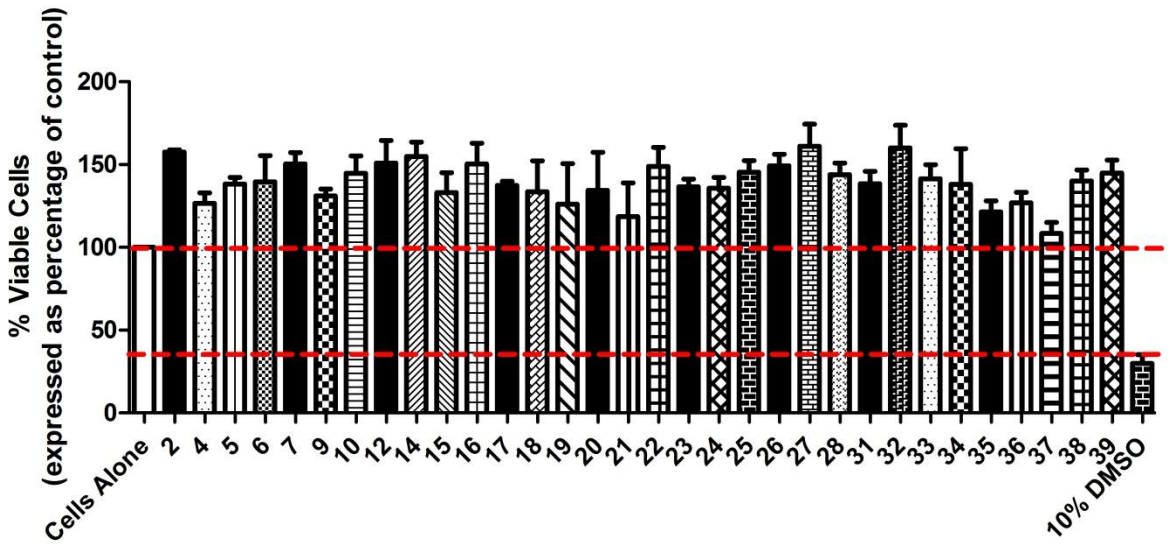

Figure 3.3 BMDC viability following treatment with *Palmaria palmata* hydrolysates and LPS stimulation. BMDCs were isolated from Balb/c mice and differentiated in rGMCSF for seven days. On day 7 cells were treated with hydrolysates and stimulated with 100 ng/mL LPS for 24 hours. DMSO (10% v/v) was included as a positive control of cytotoxicity. MTS assay (Cell Titer 96 Aqueous One Solution) was used to assess cell viability. Results are expressed as a percentage of cells alone (untreated cells).

3.2.4 The dose of *Palmaria palmata* hydrolysates used does not impact undifferentiated $CD4^+$ undifferentiated T cell viability.

In order to investigate the effects of PP hydrolysates on undifferentiated CD^+ T cells, the effects of the PP hydrolysates on the viability of undifferentiated $CD4^+$ T cells was next assessed. Cells were isolated from the spleens of Balb/c mice using the EasySep Mouse $CD4^+$ T cell Enrichment Kit (Stemcell Technologies, UK). $CD4^+$ T cells were plated in triplicate at a concentration of $1x10^6$ cells/mL on a 96 well plate, 100 µL/well. The cells were then stimulated with plate bound anti-CD3 (5 µg/mL) and anti-CD28 (5 µg/mL). They were then treated with hydrolysates (1 mg/mL) or 10% DMSO (negative control). Cells were incubated for 72 hours. Viability was assessed using an MTS assay (Cell Titer 96 Aqueous Solution – Promega) according to the manufacturer's instructions. Results were expressed as a percentage of cells alone. At 1 mg/mL PP hydrolysates did not have any significant effect on cell viability on naïve undifferentiated T cells when compared to untreated cells (**Figure 3.4**).

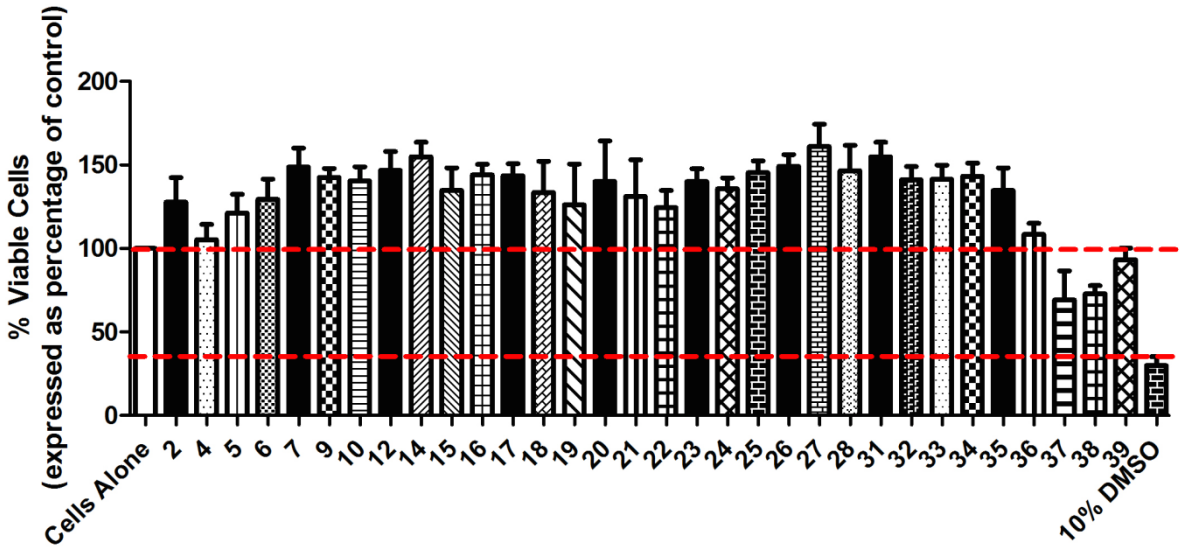

Figure 3.4 Undifferentiated CD4$^+$ T cell viability expressed as a percentage of cells alone following treatment with *Palmaria palmata* hydrolysates and whole protein controls. CD4$^+$ T-cells were isolated from the spleens of Balb/c mice, stimulated as described and treated with hydrolysates or whole protein controls. DMSO (10% v/v) was included as a positive control of cytotoxicity. MTS assay (Cell Titer 96 Aqueous One Solution) was used to assess cell viability.

3.2.5 Cytokine secretion of BMDCs treated with *Palmaria palmata* protein hydrolysates

In order to identify whether PP hydrolysates possessed anti-inflammatory properties, their ability to modulate pro-inflammatory cytokines secreted from BMDCs was examined. BMDCs were isolated as before and treated with hydrolysates at a dose of 1 mg/mL, some of these (marked + 100 ng LPS) were stimulated with 100 ng/mL LPS. Secretion of cytokines (TNFα, IL-10, IL-6 and IL-12p40) was then assessed using ELISA (R&D Duoset). Whole protein controls (WPCs) are shown as black bars and paired with their hydrolysed proteins as outlined in **Table 3.1**. Cells alone are shown as a white bar.

Figure 3.5 and **Figure 3.6** show the effects of WPC 2 and its hydrolysates H4, H5 and H6 and WPC7 and its hydrolysates H9 and H10 on secretion of TNFα, IL-12p70, IL-23 and IL-10 (**Figure 3.5**) and IL-1β, IL-6 and IL-12p40 (**Figure 3.6)** from BMDCs. WPC2 reduced levels of TNFα ($p<0.001$), IL-23 ($p<0.001$) and IL-1β ($p<0.001$) and promotes levels of IL-6 ($p<0.01$). H4 significantly reduced levels of TNFα ($p<0.001$), IL-23 ($p<0.001$) and IL-12p40 ($p<0.05$) and increased levels of IL-10 ($p<0.01$), IL-1β ($p<0.001$) and IL-6 ($p<0.01$). H5 decreased levels of TNFα ($p<0.001$) and IL-1β ($p<0.001$). H6 reduced levels of TNFα ($p<0.001$) and IL-23 ($p<0.001$) and increased production of IL-12p70 ($p<0.01$) and IL-10 ($p<0.01$). WPC7 significantly decreased levels of TNFα ($p<0.001$), IL-23 ($p<0.001$) and IL-1β ($p<0.001$) and increased IL-10 ($p<0.01$). H9 significantly reduced TNFα ($p<0.001$) and IL-23 ($p<0.001$) whilst increasing levels of IL-12p70 ($p<0.001$). H10 displayed selective anti-inflammatory effects differing to that of its control (WPC 7). H10 selectively decreased levels of IL-12p70 ($p<0.01$), TNFα ($p<0.001$), IL-12p40 ($p<0.05$) and IL-6 ($p<0.05$) whilst maintaining levels of IL-23. This hydrolysate also increased IL-10 production ($p<0.001$). As a result of this, hydrolysate H10 is considered a potential candidate for further screening.

Figure 3.7 and **Figure 3.8** show the effects of WPC12 and its hydrolysates H14, H15 and H16 and WPC17 and its hydrolysates H18 and H19 on the secretion of TNFα, IL-12p70, IL-23 and IL-10 (**Figure 3.7**) and IL-1β, IL-6 and IL-12p40 (**Figure 3.8)** from BMDCs. WPC12 significantly reduced levels of TNFα ($p<0.001$) and IL-23 ($p<0.001$), and increased levels of IL-10 ($p<0.001$), IL-6 ($p<0.01$) and IL-12p40 ($p<0.01$). H14 reduced levels of TNFα ($p<0.001$), IL-23 ($p<0.001$), IL-10 ($p<0.01$) and IL-1β ($p<0.05$) whilst promoting IL-

12p70 production ($p<0.01$). H15 decreased TNFα ($p<0.001$) and IL-23 ($p<0.001$) and increased levels of IL-12p70 ($p<0.001$), IL-10 ($p<0.001$) and IL-12p40 ($p<0.01$). H16 decreased TNFα ($p<0.001$), IL-23 ($p<0.001$), IL-10 ($p<0.01$), IL-1β ($p<0.01$) and increased IL-12p70 ($p<0.05$). WPC17 decreased levels of TNFα ($p<0.001$) and IL-23 ($p<0.001$) and increased levels of IL-12p70 ($p<0.001$). H18 reduced levels of TNFα ($p<0.001$), IL-23 ($p<0.001$) and IL-1β ($p<0.01$) and increased levels of IL-12p70 ($p<0.05$). H19 decreased levels of TNFα ($p<0.001$) and IL-23 ($p<0.001$) whilst increasing production of IL-12p70 ($p<0.01$) and IL-1β ($p<0.001$). All hydrolysates displayed anti-inflammatory properties, however, no PP hydrolysates in Figure 3.7 and Figure 3.8 were chosen as potential candidates at this time as no significant difference was seen between the activity of the hydrolysates and their whole protein controls.

Figure 3.9 and **Figure 3.10** show the effects of WPC20 and its hydrolysates H21 and H22 and WPC23 and its hydrolysates H24 and H25 on secretion of TNFα, IL-12p70, IL-23 and IL-10 (**Figure 3.9**) and IL-1β, IL-6 and IL-12p40 (**Figure 3.10**) from BMDCs. WPC20 significantly decreased levels of TNFα ($p<0.001$), IL-23 ($p<0.001$) and IL-10 ($p<0.01$). H21 reduced TNFα ($p<0.01$), IL-23 ($p<0.001$) and IL-10 ($p<0.001$) and increased levels of IL-12p70 ($p<0.001$) and IL-1β ($p<0.01$). H22 decreased levels of TNFα ($p<0.01$), IL-23 ($p<0.001$) and IL-10 ($p<0.001$). WPC23 decreased TNFα ($p<0.01$), IL-23 ($p<0.001$), IL-10 ($p<0.05$) and IL-1β ($p<0.01$) and increased levels of IL-12p70 ($p<0.001$). H24 decreased TNFα ($p<0.01$), IL-23 ($p<0.001$), IL-10 ($p<0.001$) and IL-1β ($p<0.01$). H25 reduced levels of TNFα ($p<0.01$), IL-23 ($p<0.001$), IL-10 ($p<0.05$) and IL-1β ($p<0.01$) whilst promoting production of IL-12p40 ($p<0.001$). All hydrolysates displayed anti-inflammatory properties, however, no PP hydrolysates in **Figure 3.9** and **Figure 3.10** were chosen as potential candidates at this time as no hydrolysates displayed significant anti-inflammatory activity differently to that of its whole protein control.

Figure 3.11 and **Figure 3.12** show the effects of WPC 26 and its hydrolysates 27 and 28 and WPC 31 and its hydrolysates 32, 33 and 34 on secretion of TNFα, IL-12p70, IL-23 and IL-10 (**Figure 3.11**) and IL-1β, IL-6 and IL-12p40 (**Figure 3.12**) from BMDCs. WPC26 significantly decreased pro-inflammatory cytokines IL-12p70 ($p<0.001$) and IL-23 ($p<0.01$) whilst increasing fellow pro-inflammatory cytokine subunit IL-12p40 ($p<0.01$). H27 also

decreased IL-12p70 ($p<0.001$) and IL-23 ($p<0.01$) and increased IL-12p40 ($p<0.05$). H28 has similar effects on IL-12p70 ($p<0.001$), IL-23 ($p<0.01$) decreasing both and increasing IL-12p40 ($p<0.05$) but also decreased levels of anti-inflammatory cytokine IL-10 ($p<0.05$) and pro-inflammatory IL-1β ($p<0.05$). WPC31 decreased IL-12p70 ($p<0.001$) and IL-23 ($p<0.05$) production and increased levels of IL-10 ($p<0.001$), IL-1β ($p<0.01$) and IL-12p40 ($p<0.001$). H32 decreased levels of IL-12p70 ($p<0.05$), IL-23 ($p<0.01$) and IL-1β ($p<0.05$) and increased levels of IL-12p40 ($p<0.01$). H33 reduced levels of IL-12p70 ($p<0.001$), IL-23 ($p<0.05$), IL-1β ($p<0.01$) and increased levels of IL-12p40 ($p<0.01$). H34 decreased TNFα ($p<0.05$), IL-23 ($p<0.001$), IL-1β ($p<0.05$) and IL-6 ($p<0.05$) and increased levels of IL-12p40 ($p<0.01$). All hydrolysates displayed anti-inflammatory properties, however, no PP hydrolysates in Figure 3.11 and Figure 3.12 were chosen as potential candidates at this time as no hydrolysates displayed activity differently to that of their whole protein controls, indicating that activity was not as a result of hydrolysis.

Figure 3.13 and **Figure 3.14** show the effects of WPC35 and its hydrolysates H36, H37, H38 and H39 on secretion of TNFα, IL-12p70, IL-23 and IL-10 (**Figure 3.13**) and IL-1β, IL-6 and IL-12p40 (**Figure 3.14**) from BMDCs. WPC35 reduced levels of IL-12p70 ($p<0.05$) IL-10 ($p<0.001$) and IL-1β ($p<0.01$) and increased IL-12p40 ($p<0.001$). H36 decreased TNFα ($p<0.05$), IL-12p70 ($p<0.001$), IL-10 ($p<0.001$) and IL-1β ($p<0.001$) and increased levels of IL-12p40 ($p<0.05$). H37 decreased levels of IL-10 ($p<0.001$) and IL-1β ($p<0.001$) and increased IL-12p40 ($p<0.001$). H38 reduced IL-12p70 ($p<0.001$), IL-23 ($p<0.05$), IL-10 ($p<0.001$) and IL-1β ($p<0.001$) and promoted production of IL-12p40 ($p<0.001$). H39 decreased production of IL-12p70 ($p<0.001$), IL-1β ($p<0.01$) and IL-10 ($p<0.001$) and increased IL-12p40 ($p<0.001$). All hydrolysates displayed anti-inflammatory properties and hydrolysates H36, H38 and H39 were considered potential candidates for further screening as they displayed activity significantly different to that of their whole protein control WPC35.

These results were then further considered when next examined the PP hydrolysates effects on the cytokine secretion of naïve undifferentiated T cells, before potential candidates were chosen for further screening.

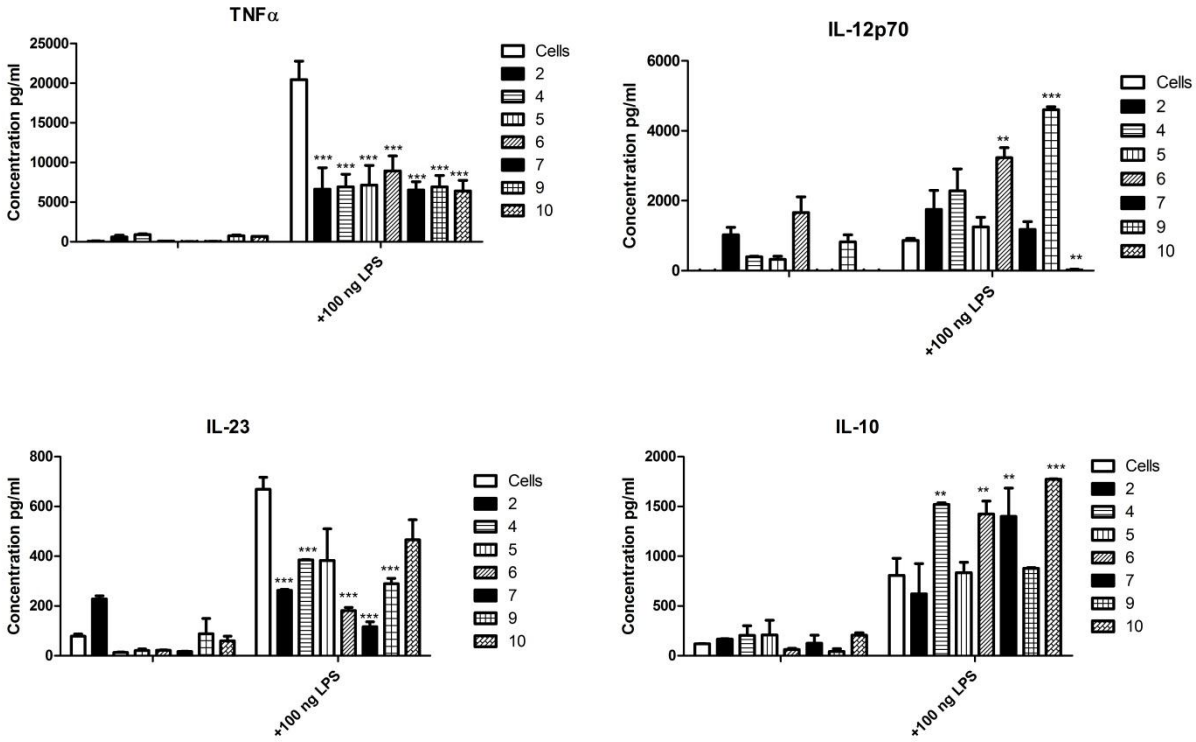

Figure 3.5 Secretion of TNFα, IL-12p70, IL-23 and IL-10 by BMDCs treated with *Palmaria palmata* hydrolysates 2 – 10. BMDCs were isolated from Balb/c mice and differentiated in rGMCSF for seven days. On day 7 cells were treated with hydrolysates and stimulated with 100 ng/mL LPS. Secretion of TNFα, IL-12p70, IL-23 and IL-10 was measured using ELISA (R&D Duoset). One way ANOVA with a Post test Newman Keuls was used to assess differences between groups. Cells treated with hydrolysate only were compared to cells alone, cells treated with hydrolysates + LPS were compared to cells alone + LPS. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to untreated cells (no hydrolysate).

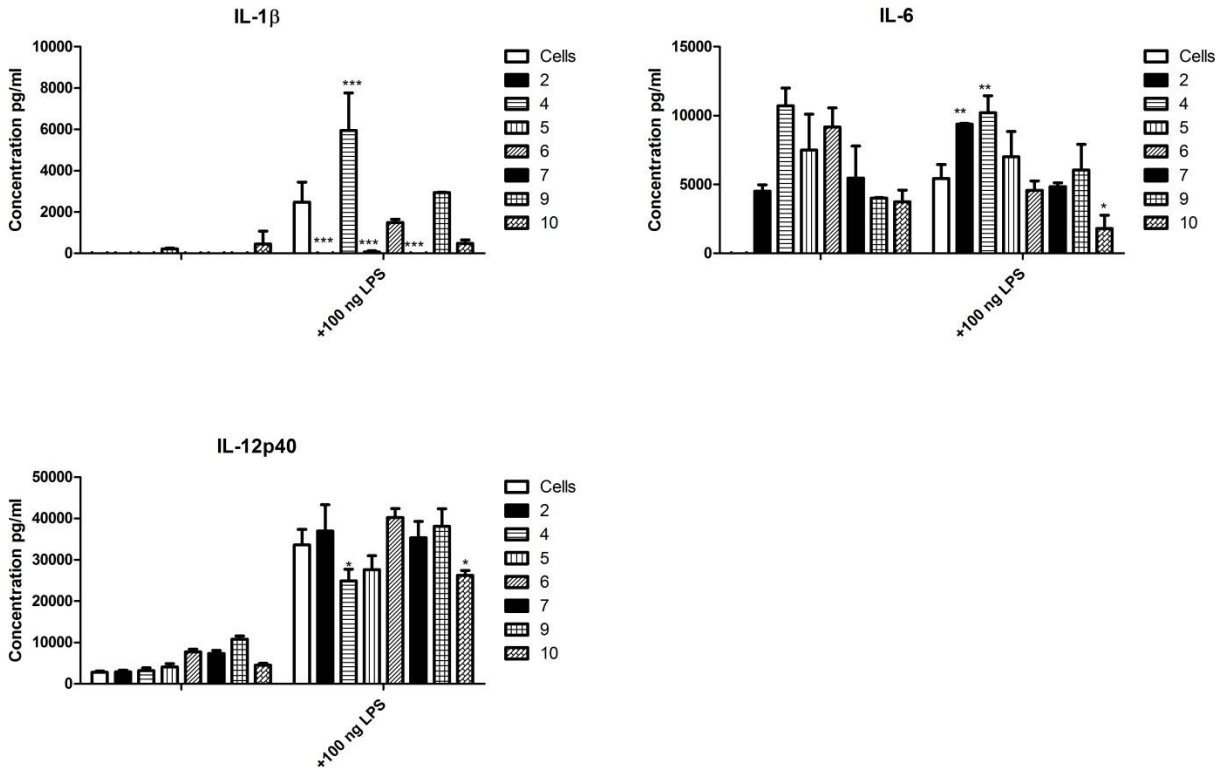

Figure 3.6 Secretion of IL-1β, IL-6 and IL-12p40 by BMDCs treated with *Palmaria palmata* hydrolysates 2 – 10. BMDCs were isolated from Balb/c mice and differentiated in rGMCSF for seven days. On day 7 cells were treated with hydrolysates and stimulated with 100 ng/mL LPS. Secretion of IL-1β, IL-6 and IL-12p40 was measured using ELISA (R&D Duoset). One way ANOVA with a Post test Newman Keuls was used to assess differences between groups. Cells treated with hydrolysate only were compared to cells alone, cells treated with hydrolysates + LPS were compared to cells alone + LPS. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to untreated cells (no hydrolysate).

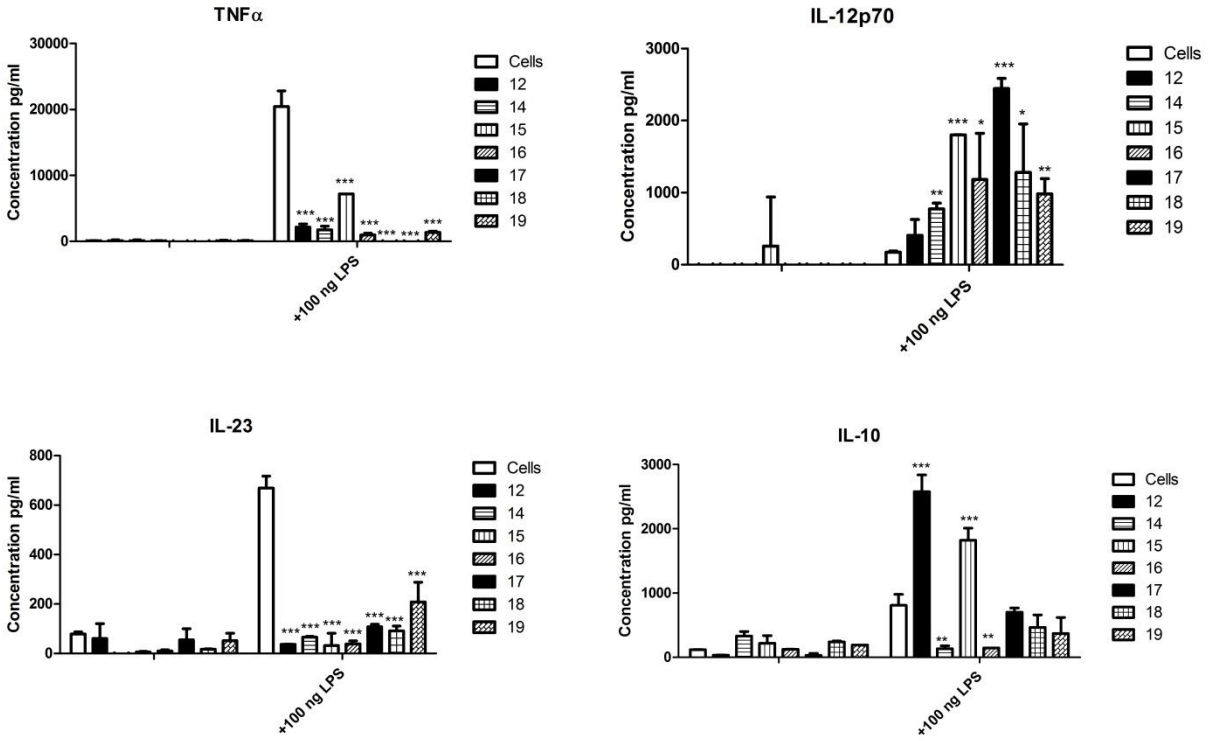

Figure 3.7 Secretion of TNFα, IL-12p70, IL-23 and IL-10 by BMDCs treated with *Palmaria palmata* hydrolysates 12 -19. BMDCs were isolated from Balb/c mice and differentiated in rGMCSF for seven days. On day 7 cells were treated with hydrolysates and stimulated with 100 ng/mL LPS. Secretion of TNFα, IL-12p70, IL-23 and IL-10 was measured using ELISA (R&D Duoset). One way ANOVA with a Post test Newman Keuls was used to assess differences between groups. Cells treated with hydrolysate only were compared to cells alone, cells treated with hydrolysates + LPS were compared to cells alone + LPS. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to untreated cells (no hydrolysate).

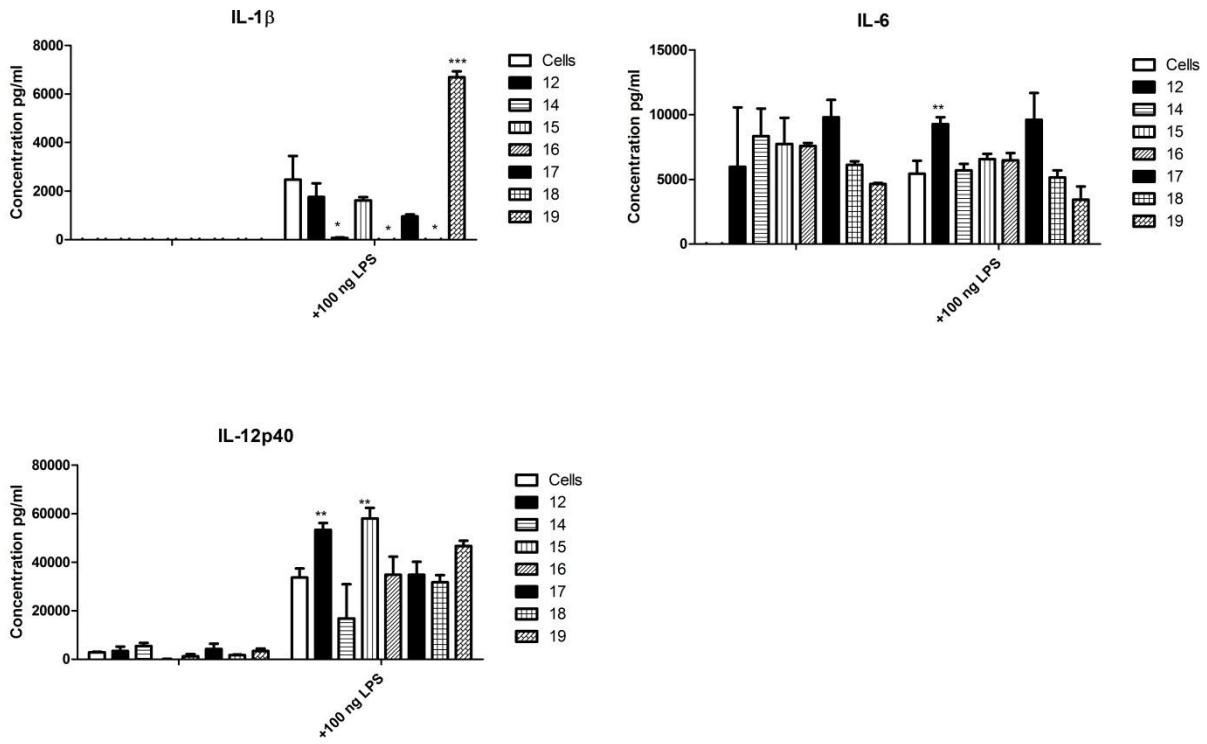

Figure 3.8 Secretion of IL-1β, IL-6 and IL-12p40 by BMDCs treated with *Palmaria palmata* hydrolysates 12 - 14. BMDCs were isolated from Balb/c mice and differentiated in rGMCSF for seven days. On day 7 cells were treated with hydrolysates and stimulated with 100 ng/mL LPS. Secretion of IL-1β, IL-6 and IL-12p40 was measured using ELISA (R&D Duoset). One way ANOVA with a Post test Newman Keuls was used to assess differences between groups. Cells treated with hydrolysate only were compared to cells alone, cells treated with hydrolysates + LPS were compared to cells alone + LPS. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to untreated cells (no hydrolysate).

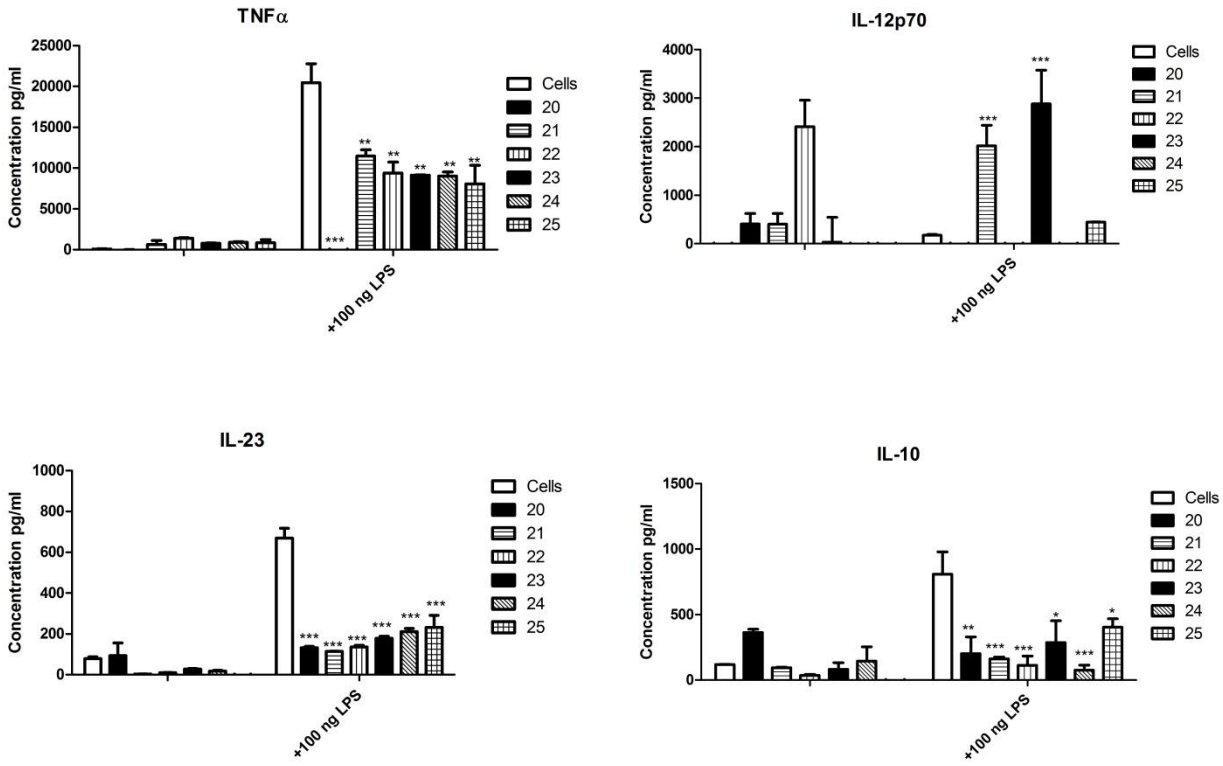

Figure 3.9 Secretion of TNFα, IL-12p70, IL-23 and IL-10 by BMDCs treated with *Palmaria palmata* hydrolysates 20 - 25. BMDCs were isolated from Balb/c mice and differentiated in rGMCSF for seven days. On day 7 cells were treated with hydrolysates and stimulated with 100 ng/mL LPS. Secretion of TNFα, IL-12p70, IL-23 and IL-10 was measured using ELISA (R&D Duoset). One way ANOVA with a Post test Newman Keuls was used to assess differences between groups. Cells treated with hydrolysate only were compared to cells alone, cells treated with hydrolysates + LPS were compared to cells alone + LPS. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to untreated cells (no hydrolysate).

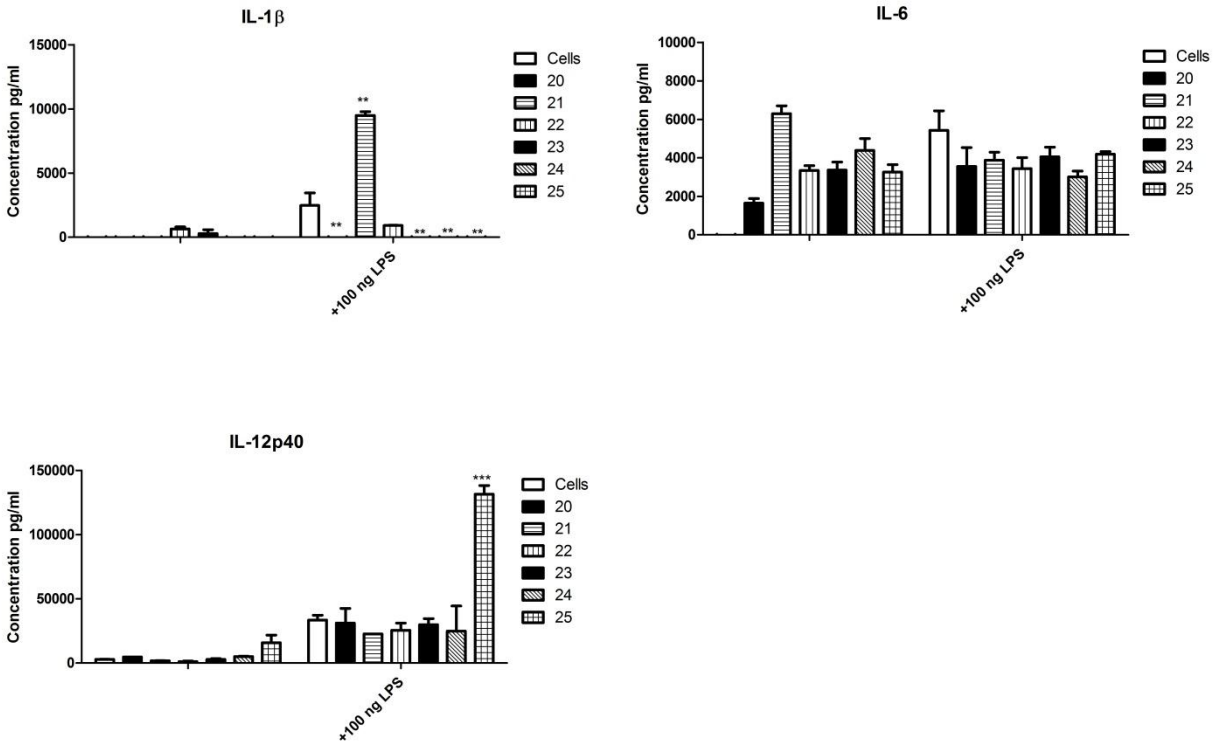

Figure 3.10 Secretion of IL-1β, IL-6 and IL-12p40 by BMDCs treated with *Palmaria palmata* hydrolysates 20 - 25. BMDCs were isolated from Balb/c mice and differentiated in rGMCSF for seven days. On day 7 cells were treated with hydrolysates and stimulated with 100 ng/mL LPS. Secretion of IL-1β, IL-6 and IL-12p40 was measured using ELISA (R&D Duoset). One way ANOVA with a Post test Newman Keuls was used to assess differences between groups. Cells treated with hydrolysate only were compared to cells alone, cells treated with hydrolysates + LPS were compared to cells alone + LPS. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to untreated cells (no hydrolysate).

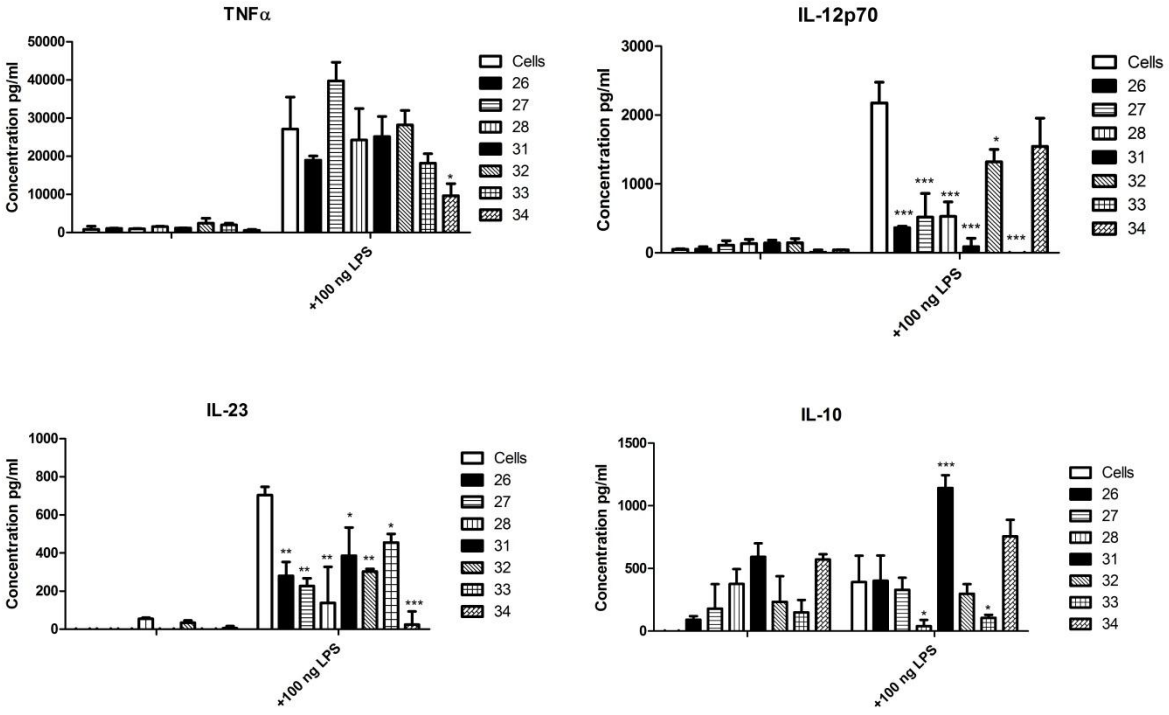

Figure 3.11 Secretion of TNFα, IL-12p70, IL-23 and IL-10 by BMDCs treated with *Palmaria palmata* hydrolysates 26 - 34. BMDCs were isolated from Balb/c mice and differentiated in rGMCSF for seven days. On day 7 cells were treated with hydrolysates and stimulated with 100 ng/mL LPS. Secretion of TNFα, IL-12p70, IL-23 and IL-10 was measured using ELISA (R&D Duoset). One way ANOVA with a Post test Newman Keuls was used to assess differences between groups. Cells treated with hydrolysate only were compared to cells alone, cells treated with hydrolysates + LPS were compared to cells alone + LPS. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to untreated cells (no hydrolysate).

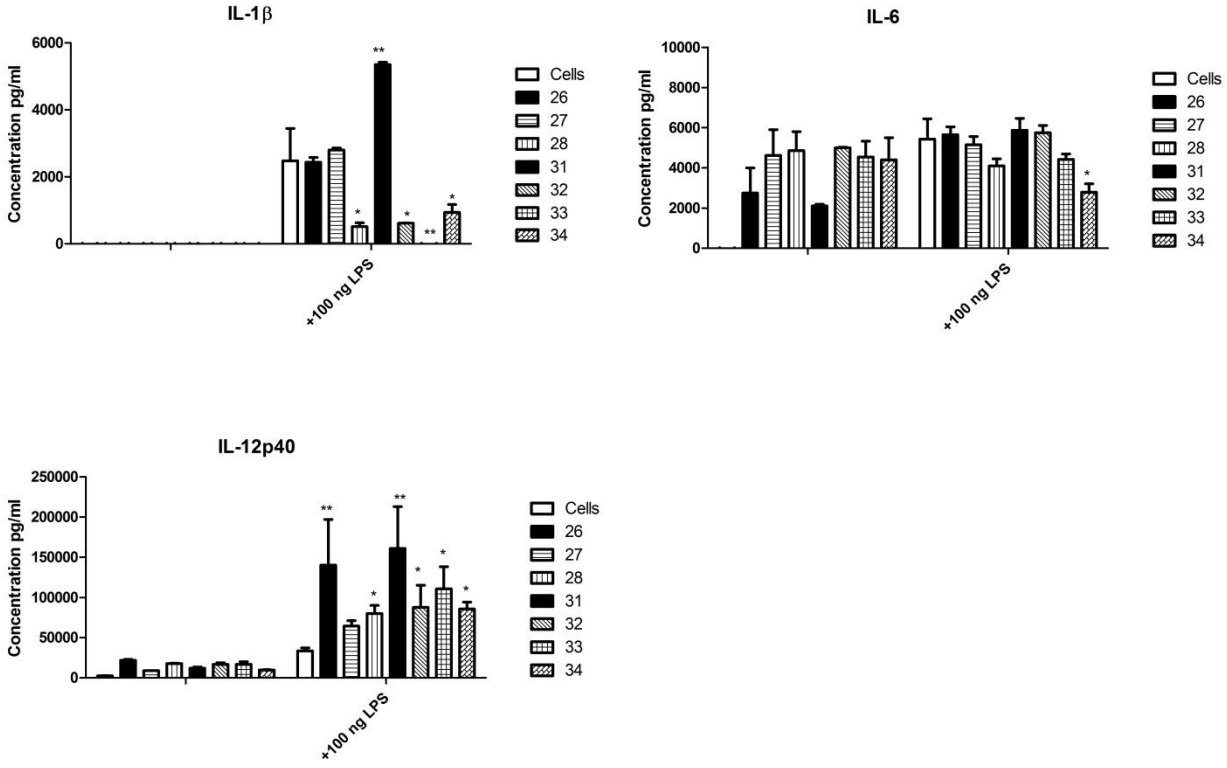

Figure 3.12 Secretion of IL-1β, IL-6 and IL-12p40 by BMDCs treated with *Palmaria palmata* hydrolysates 26 - 34. BMDCs were isolated from Balb/c mice and differentiated in rGMCSF for seven days. On day 7 cells were treated with hydrolysates and stimulated with 100 ng/mL LPS. Secretion of IL-1β, IL-6 and IL-12p40 was measured using ELISA (R&D Duoset). One way ANOVA with a Post test Newman Keuls was used to assess differences between groups. Cells treated with hydrolysate only were compared to cells alone, cells treated with hydrolysates + LPS were compared to cells alone + LPS. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to untreated cells (no hydrolysate).

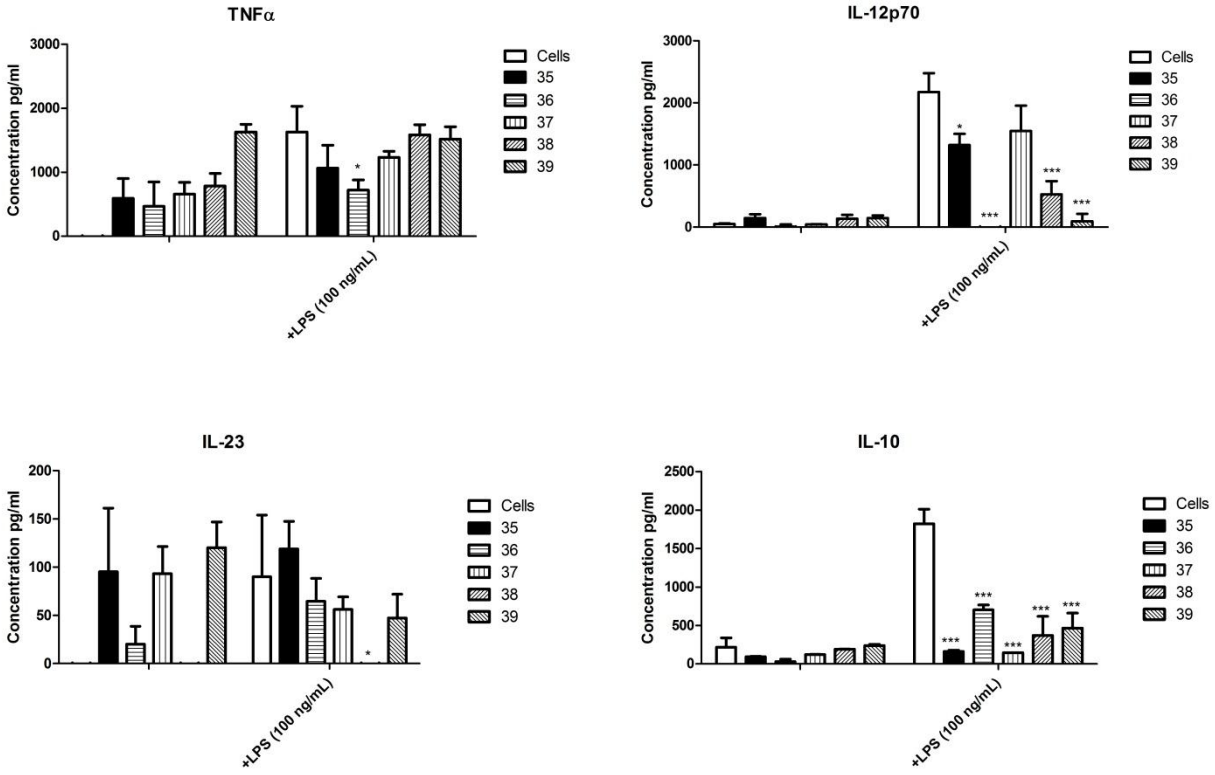

Figure 3.13 Secretion of TNFα, IL-12p70, IL-23 and IL-10 by BMDCs treated with *Palmaria palmata* hydrolysates 35 - 39. BMDCs were isolated from Balb/c mice and differentiated in rGMCSF for seven days. On day 7 cells were treated with hydrolysates and stimulated with 100 ng/mL LPS. Secretion of TNFα, IL-12p70, IL-23 and IL-10 was measured using ELISA (R&D Duoset). One way ANOVA with a Post test Newman Keuls was used to assess differences between groups. Cells treated with hydrolysate only were compared to cells alone, cells treated with hydrolysates + LPS were compared to cells alone + LPS. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to untreated cells (no hydrolysate).

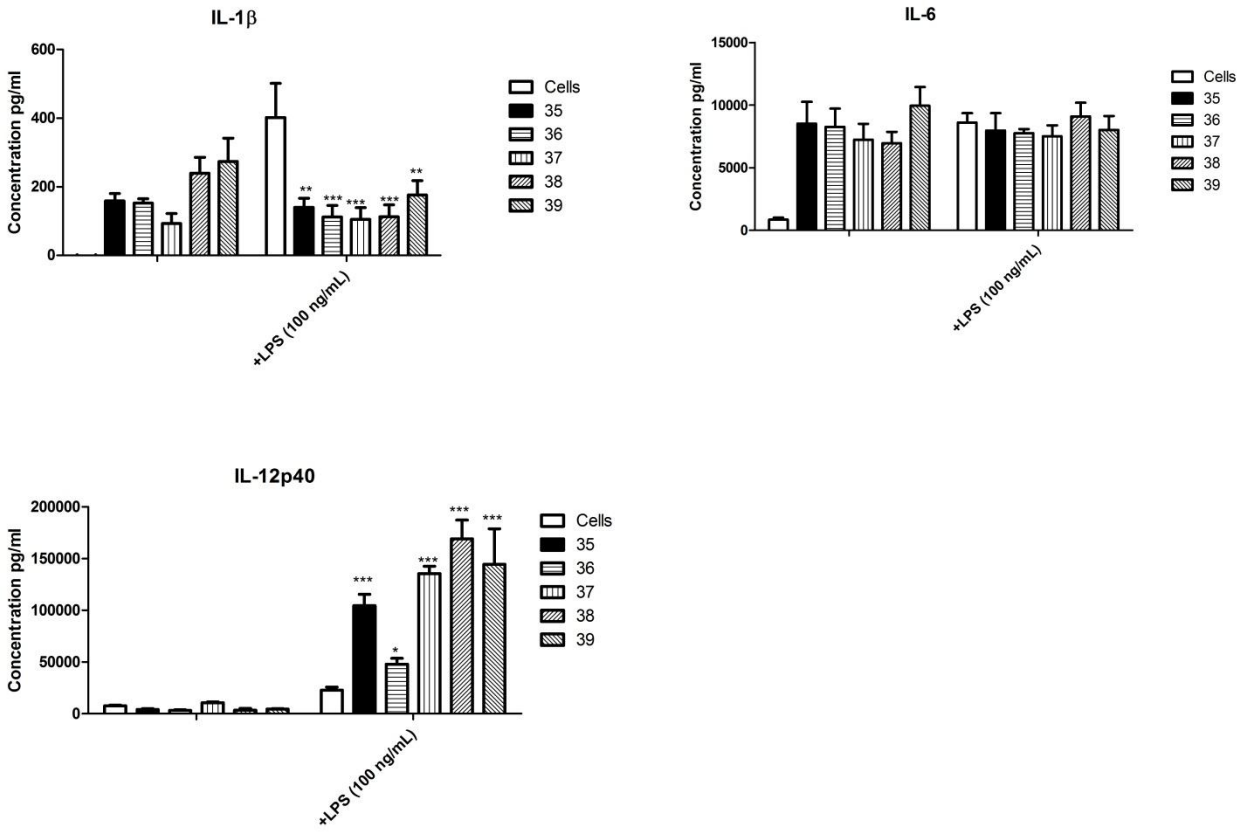

Figure 3.14 Secretion of IL-1β, IL-6 and IL-12p40 by BMDCs treated with *Palmaria palmata* hydrolysates 35 - 39. BMDCs were isolated from Balb/c mice and differentiated in rGMCSF for seven days. On day 7 cells were treated with hydrolysates and stimulated with 100 ng/mL LPS. Secretion of IL-1β, IL-6 and IL-12p40 was measured using ELISA (R&D Duoset). One way ANOVA with a Post test Newman Keuls was used to assess differences between groups. Cells treated with hydrolysate only were compared to cells alone, cells treated with hydrolysates + LPS were compared to cells alone + LPS. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to untreated cells (no hydrolysate).

3.2.6 Cytokine secretion of CD4+ T cells treated with *Palmaria palmata* protein hydrolysates.

PP protein hydrolysates were screened on undifferentiated $CD4^+$ T cells and their effects on cytokine secretion analysed. $CD4^+$ T cells were isolated as before using an EasySep Mouse $CD4^+$ T cell Enrichment Kit (Stemcell Technologies, UK) and treated with hydrolysates at a dose of 1 mg/mL. Secretion of cytokines (IL-17, IL-2, IL-4, IL-6, IL-13, IFNγ and IL-10) was then assessed using ELISA (R&D Duoset, UK).

Figure 3.15 shows secretion of IL-13, IL-6, IL-10, IL-17, IL-4 and IFNγ from undifferentiated $CD4^+$ T cells treated with WPC2, hydrolysates H4, H5, H6, WPC7, hydrolysates H9 and H10. WPC2 decreased IL-6 ($p<0.001$). H4 reduced levels of IL-6 ($p<0.001$) and IL-2 ($p<0.05$). H5 decreased IL-6 ($p<0.001$), IL-4 ($p<0.01$) and IFNγ ($p<0.001$) and increased IL-17 ($p<0.001$) and IL-2 ($p<0.001$). H6 increased IL-13 ($p<0.05$) production and reduced levels of IL-6 ($p<0.001$) and IL-2 ($p<0.05$). WPC7 decreased IL-13 ($p<0.01$), IL-6 ($p<0.001$), IL-4 ($p<0.01$) and IFNγ ($p<0.001$) whilst increasing IL-17 ($p<0.001$). H9 reduced levels of IL-6 ($p<0.001$), IL-10 ($p<0.01$), IL-4 ($p<0.05$) and IL-2 ($p<0.05$). H10 increased IL-17 ($p<0.001$) and decreased IL-13 ($p<0.01$), IL-6 ($p<0.001$), IL-4 ($p<0.01$) and IFNγ ($p<0.001$). Hydrolysate H10 displayed potential anti-inflammatory properties both in BMDCs, by decreasing TNFα and IL-12p70, and in $CD4^+$ T cells, by decreasing IFNγ. Therefore, H10 was chosen as a candidate to be brought forward into T cell subsets for further investigation.

Figure 3.16 shows secretion of IL-13, IL-6, IL-10, IL-17, IL-4 and IFNγ from undifferentiated $CD4^+$ T cells treated with WPC12, hydrolysates H14, H15, H16, WPC17, hydrolysates H18 and H19. WPC12 decreased production of IL-13 ($p<0.05$), IL-6 ($p<0.001$), IL-10 ($p<0.05$), IL-4 ($p<0.001$) and IFNγ ($p<0.001$) and increased levels of IL-17 ($p<0.001$). H14 increased IL-17 ($p<0.001$) but also decreased IL-6 ($p<0.001$), IL-10 ($p<0.05$), IL-4 ($p<0.001$) and IFNγ ($p<0.001$). H15 decreased IL-10 ($p<0.01$), IFNγ ($p<0.01$) and IL-2 ($p<0.05$). H16 reduced levels of IL-6 ($p<0.001$), IFNγ ($p<0.001$) and IL-2 ($p<0.05$). H17 decreased IL-6 ($p<0.001$), IL-10 ($p<0.001$), IL-4 ($p<0.001$), IFNγ ($p<0.01$) and IL-2 ($p<0.05$). H18 reduced IL-6 ($p<0.01$), IFNγ ($p<0.05$) and IL-2 ($p<0.05$) and increased IL-17 ($p<0.05$). H19 decreased IL-6 ($p<0.05$), IL-4 ($p<0.001$) and IL-2 ($p<0.05$) whilst increasing IL-10 ($p<0.001$) and IL-17 ($p<0.05$).

Hydrolysate H19 displayed the ability to selectively alter cytokine secretion of both BMDCs, by reducing pro-inflammatory cytokines TNFα and IL-23 and increasing anti-inflammatory cytokine IL-10, and in $CD4^+$ T cells, differing to that of the abilities of its control WPC17. Therefore, H19 was chosen as a candidate to be brought forward for further screening in T cell subsets.

Figure 3.17 shows secretion of IL-13, IL-6, IL-10, IL-17, IL-4 and IFNγ from $CD4^+$ T cells treated with WPC20, hydrolysates H21 and H22, WPC23, hydrolysates H24 and H25. WPC20 reduced levels of IL-13 ($p<0.001$), IL-6 ($p<0.05$), and IL-2 ($p<0.001$). H21 decreased IL-2 ($p<0.001$) and increased IL-13 ($P<0.05$). H22 reduced IL-2 ($p<0.01$) production. WPC23 and hydrolysates H24 and H25 all reduced levels of IL-2 ($p<0.001$) also. H21 was chosen to be brought forward for further screening as it displayed selective modulation of pro-inflammatory cytokines in BMDCs and the ability to induce a Th2 cell type in T cells through increasing production of IL-13. H22 was also chosen to be brought forward to investigate its effects on the cytokine secretion in T cell subsets.

Figure 3.18 shows secretion of IL-13, IL-6, IL-10, IL-17, IL-4 and IFNγ from $CD4^+$ T cells treated with WPC26 and its hydrolysates H27 and H28 and also WPC31 and its hydrolysates H32, H33 and H34. WPC26 decreased IL-13 ($p<0.01$) and increased IL-17 ($p<0.05$) and IL-2 ($p<0.001$) production. H27 increased levels of IL-13 ($p<0.001$) whilst decreasing IL-4 ($p<0.05$). H28 decreased levels of IL-4 ($p<0.01$) and increased IL-2 ($p<0.001$). WPC31 decreased IL-13 ($p<0.05$). H32 increased IL-13 ($p<0.001$) and decreased IL-4 ($p<0.01$). H33 increased IL-17 ($p<0.05$) and decreased IL-4 ($p<0.01$). H34 also increased IL-17 ($p<0.05$) and decreased IL-4 ($p<0.01$). No hydrolysates displayed significant differences in their ability to modulate cytokine secretion to that of their WPC and none were brought forward into future investigations.

Figure 3.19 shows the secretion of IL-13, IL-6, IL-10, IL-17, IL-4 and IFNγ from $CD4^+$ T cells treated with WPC35, hydrolysates H36, H37, H38 and H39. WPC35 decreased IL-13 ($p<0.05$), IL-10 ($p<0.05$) and IFNγ ($p<0.001$) in undifferentiated T cells. Hydrolysate H36 also reduced levels of IL-13 ($p<0.01$), IL-10 ($p<0.01$) and IFNγ ($p<0.001$). H37 decreased IL-13 ($p<0.05$) and IL-10 ($p<0.01$). H38 reduced IL-13 ($p<0.01$), IL-17 ($p<0.05$)

and IFNγ ($p<0.001$). H39 reduced IL-13 ($p<0.001$), IL-10 ($p<0.05$) and IFNγ ($p<0.001$) whilst increasing levels of IL-4 ($p<0.05$) and IL-2 ($p<0.01$). Due to their ability to decrease levels of pro-inflammatory cytokines TNFα, IL-12p70 and IL-23 secreted from BMDCs and their ability to reduce IFNγ from $CD4^+$ T cells, hydrolysates H36, H38 and H39 were brought forward for further screening in T cell subsets.

Hydrolysates H10, H19, H21, H22, H25, H36, H38 and H39 were next assessed on T cell subsets to examine their specific effects on T cell responses.

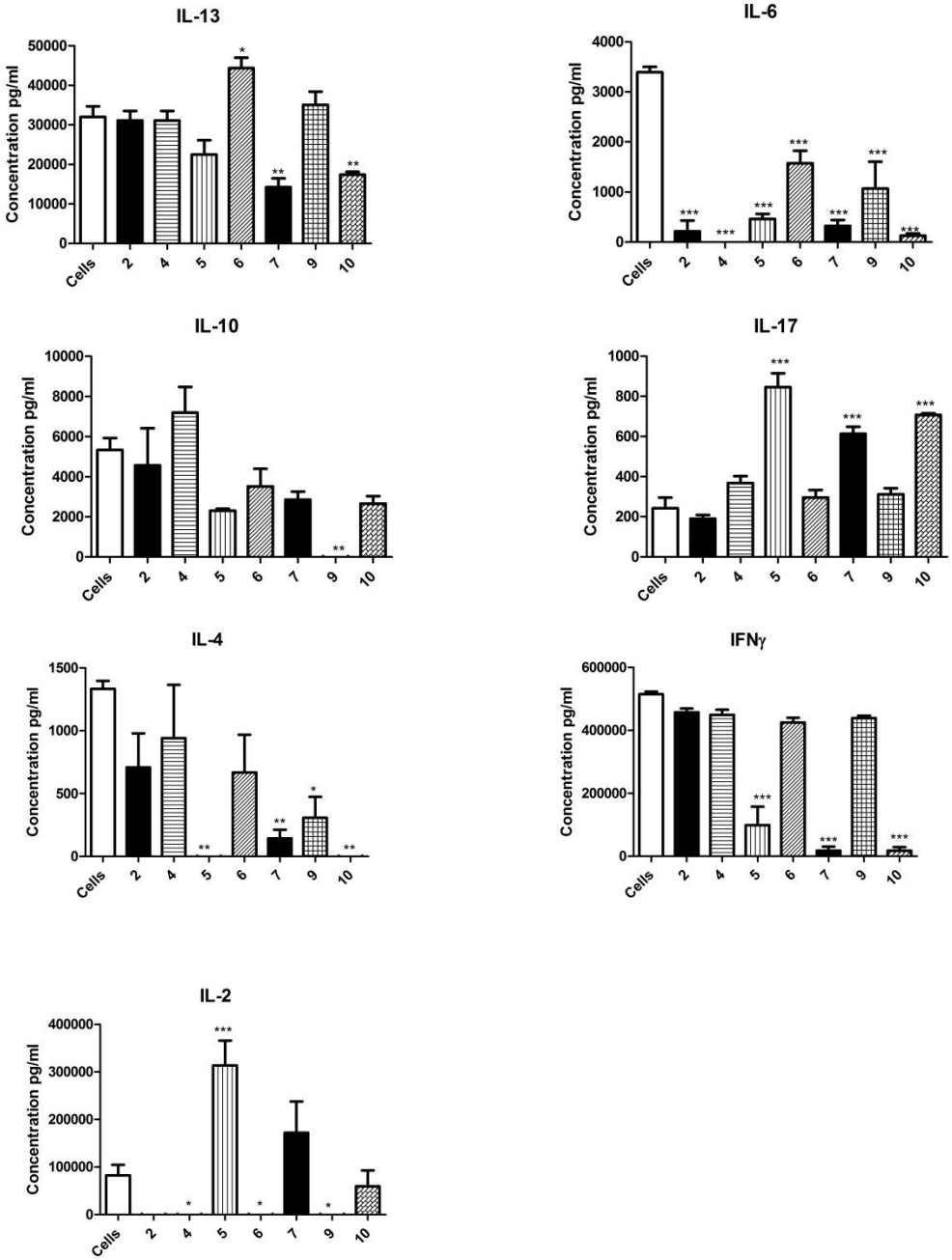

Figure 3.15 Secretion of IL-13, IL-6, IL-10, IL-17, IL-4 and IFNγ in undifferentiated CD4$^+$ T-cells treated with *Palmaria palmata* hydrolysates 2-10. CD4$^+$ T-cells were isolated from the spleens of Balb/c mice using EasySep CD4$^+$ Isolation (Stemcell). Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates (1 mg/mL) and incubated for 72 hours. Cytokine secretion was measured using ELISA (R&D Duoset). One way ANOVA with a Post test Newman Keuls was used to assess differences between groups. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to cells alone.

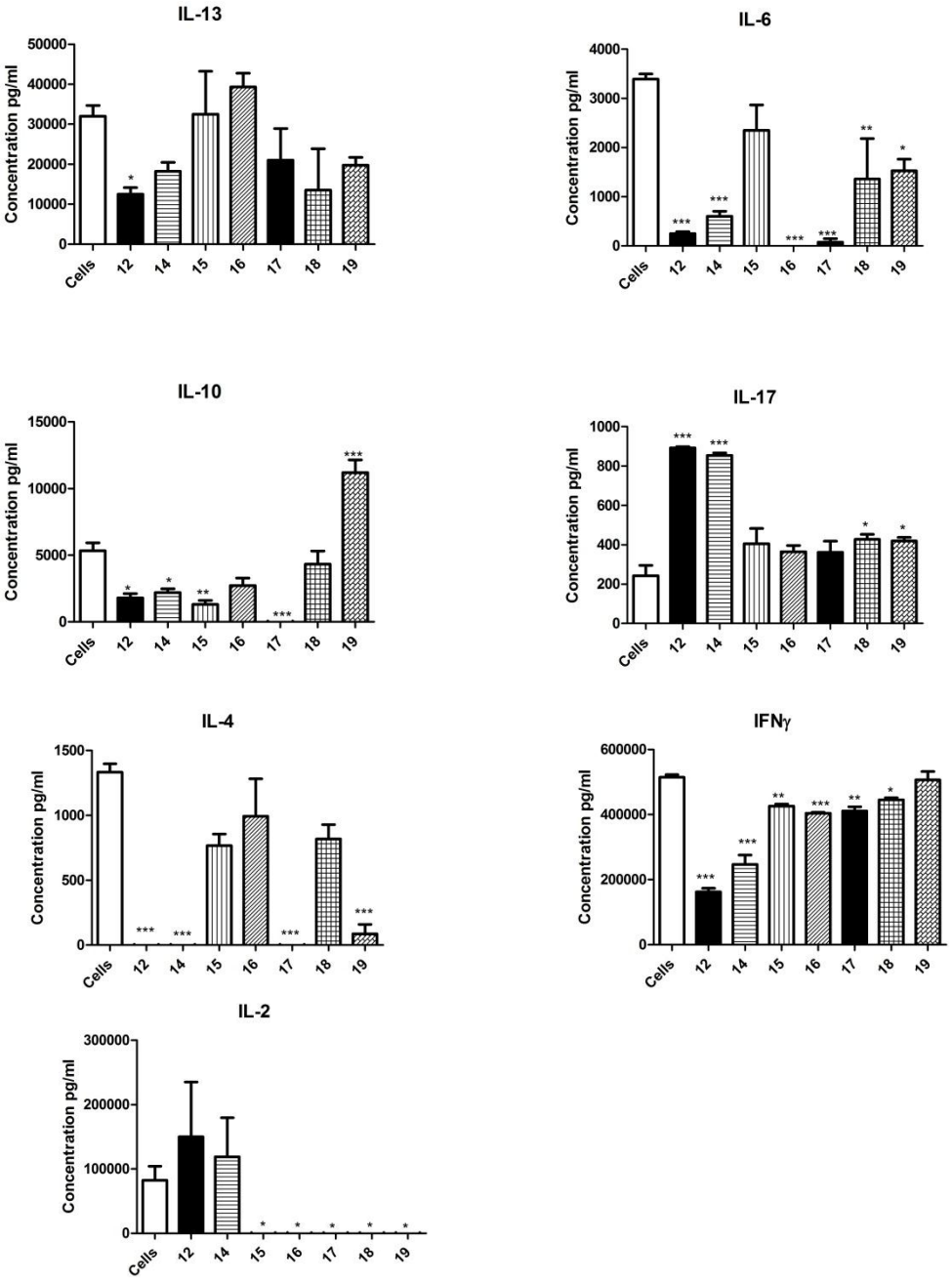

Figure 3.16 Secretion of IL-13, IL-6, IL-10, IL-17, IL-4, IFNγ and IL-2 in undifferentiated CD4$^+$ T-cells treated with *Palmaria palmata* hydrolysates 12 – 19. CD4$^+$ T-cells were isolated from the spleens of Balb/c mice using EasySep CD4$^+$ Isolation (Stemcell). Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates (1 mg/mL) and incubated for 72 hours. Cytokine secretion was measured using ELISA (R&D Duoset). One way ANOVA with a Post test Newman Keuls was used to assess differences between groups. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to cells alone.

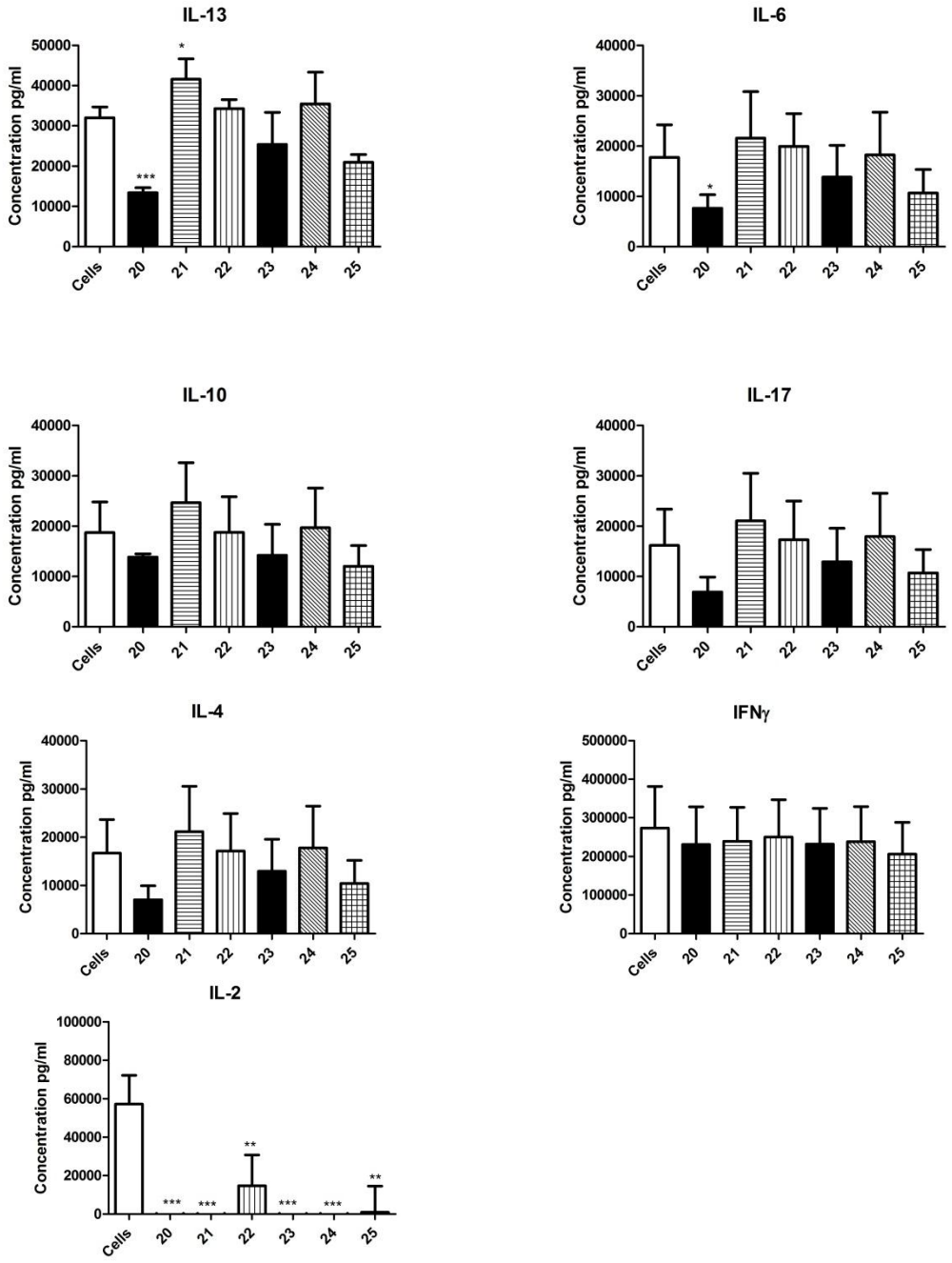

Figure 3.17 Secretion of IL-13, IL-6, IL-10, IL-17, IL-4, IFNγ and IL-2 in undifferentiated CD4$^+$ T-cells treated with *Palmaria palmata* hydrolysates 20 - 25. CD4$^+$ T-cells were isolated from the spleens of Balb/c mice using EasySep CD4$^+$ Isolation (Stemcell). Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates (1 mg/mL) and incubated for 72 hours. Cytokine secretion was measured using ELISA (R&D Duoset). One way ANOVA with a Post test Newman Keuls was used to assess differences between groups. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to cells alone.

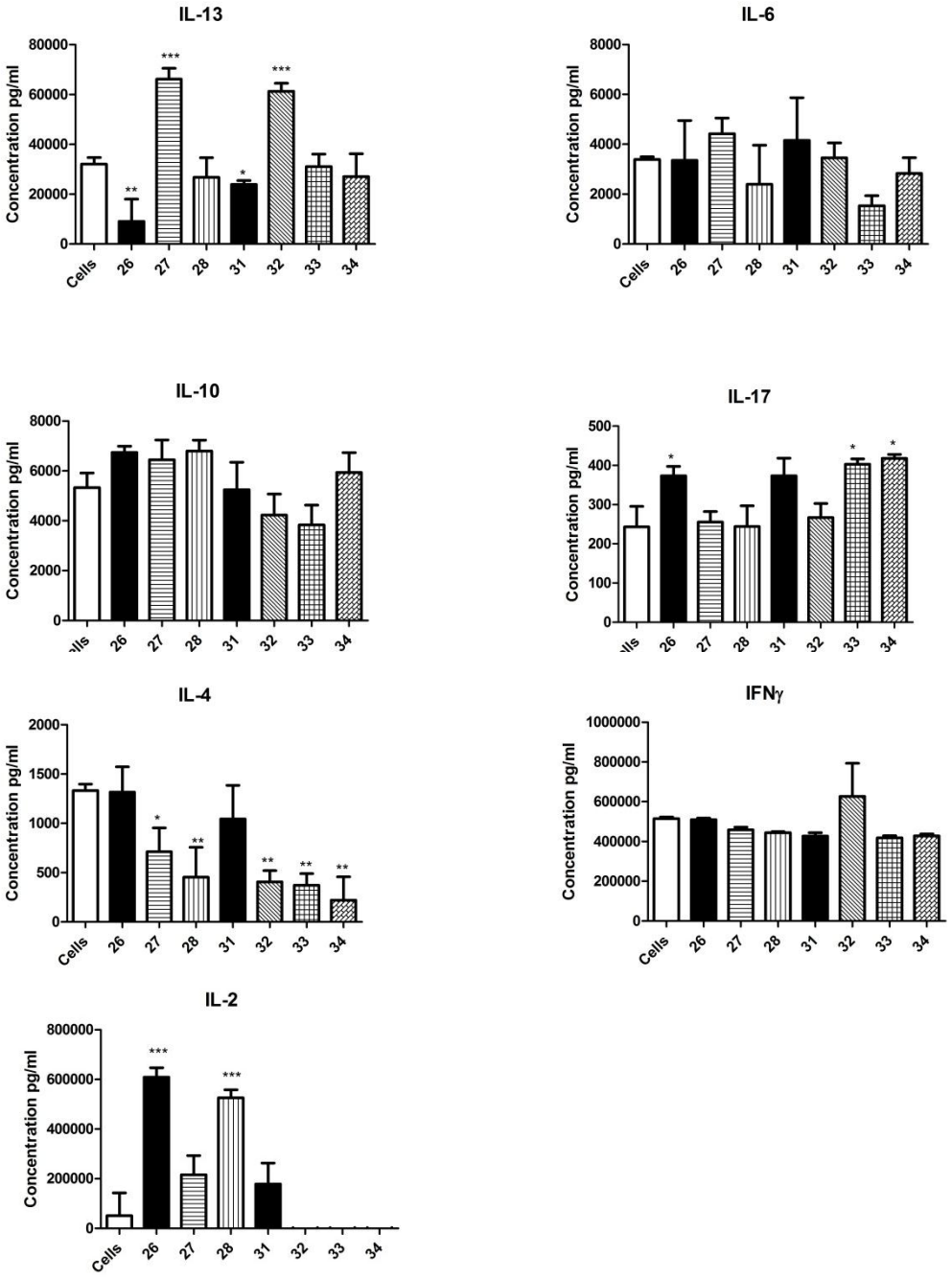

Figure 3.18 Secretion of IL-13, IL-6, IL-10, IL-17, IL-4, IFNγ and IL-2 in undifferentiated CD4$^+$ T-cells treated with *Palmaria palmata* hydrolysates 26 - 34. CD4$^+$ T-cells were isolated from the spleens of Balb/c mice using EasySep CD4$^+$ Isolation (Stemcell). Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates (1 mg/mL) and incubated for 72 hours. Cytokine secretion was measured using ELISA (R&D Duoset). One way ANOVA with a Post test Newman Keuls was used to assess differences between groups. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to cells alone.

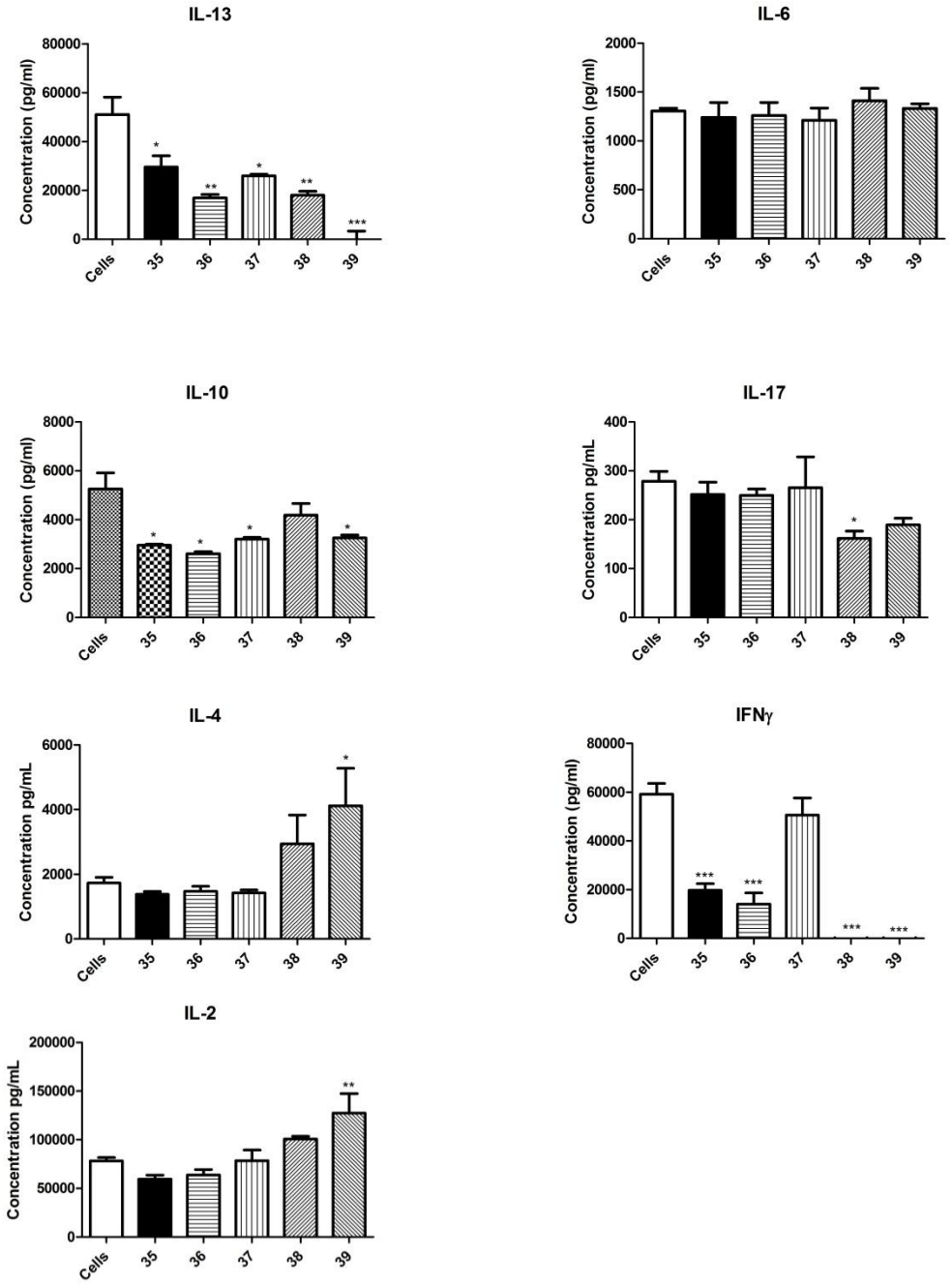

Figure 3.19 Secretion of IL-13, IL-6, IL-10, IL-17, IL-4, IFNγ and IL-2 in undifferentiated CD4$^+$ T-cells treated with *Palmaria palmata* hydrolysates 35 - 39. CD4$^+$ T-cells were isolated from the spleens of Balb/c mice using EasySep CD4$^+$ Isolation (Stemcell). Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates (1 mg/mL) and incubated for 72 hours. Cytokine secretion was measured using ELISA (R&D Duoset). One way ANOVA with a Post test Newman Keuls was used to assess differences between groups. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to cells alone.

3.2.7 T cell subsets when treated with selected *Palmaria palmata* protein hydrolysates

Hydrolysates which had been identified as potential candidates in **Section 3.2.5** and **Section 3.2.6** were then brought forward for analysis on T cell subsets. Th1, Th2 and Th17 cells were chosen for this screening as they each play a key role in the inflammatory response. Table 3.3 outlines which hydrolysates were screened in which T cell subset.

Table 3.3 Hydrolysates screened in T cell subsets

Hydrolysate	Th1	Th2	Th17
H10	✓	✓	-
H19	✓	✓	✓
H21	✓	✓	✓
H22	-	-	✓
H25	✓	✓	✓
H36	✓	✓	✓
H38	✓	✓	-
H39	✓	✓	-

3.2.7.1 Cytokine secretion of T-helper cell 1 (Th1) subset treated with selected *Palmaria palmata* protein hydrolysates.

Hydrolysates which had been identified in **Section 3.2.5** and **Section 3.2.6,** shown in Table 3.2, were first screened in a Th1 cell T cell subset. Th1 cells were isolated using an EasySep Mouse $CD4^+$ T cell Enrichment Kit and the media was supplemented with 20 ng/mL IL-2, 20 ng/mL IL-12 and 10 µg/mL anti-IL- 4 antibody for Th1 polarising conditions. Cells were treated with hydrolysates H10, H19, H21, H25, H36, H38 and H39 along with their whole protein controls, WPC7, WPC23, WPC17, WPC20 and WPC35 at a dose of 1 mg/mL and incubated for 72 hours before re-stimulation in fresh media for a further 24 hours. Secretion of cytokines (IL-13, IL-6, IL-10, IL-17, IL-4, IFNγ and IL-2) was then assessed using ELISA (R&D Duoset).

Figure 3.20 shows cytokine secretion by Th1 cells when treated with H10, WPC7, H25, WPC23, H19, WPC17, H21, WPC20, WPC35, H36, H38 and H39. H10 decreased levels of IL-13 ($p<0.001$), IL-4 ($p<0.01$), IFNγ ($p<0.01$), IL-2 ($p<0.05$) and increased IL-17 ($p<0.01$). Its control WPC7 reduced production of IL-4 ($p<0.01$) and IFNγ ($p<0.01$) whilst increasing production of IL-10 ($p<0.001$). H19 reduced IL-13 ($p<0.01$) and IFNγ ($p<0.001$). Its control WPC17 increased production of IL-13 ($p<0.01$) and IL-2 ($p<0.001$) and decreased and IL-4 ($p<0.01$). H21 decreased IL-13 ($p<0.001$), IL-4 ($p<0.01$) and IFNγ ($p<0.001$). Its control WPC20 increased levels of IL-4 ($p<0.01$) and reduced levels of IFNγ ($p<0.001$). H25 reduced IL-13 ($p<0.01$, IL-4 ($p<0.01$), IFNγ ($p<0.001$) and IL-2 ($p<0.01$) and increased production of IL-10 ($p<0.001$). Its control, WPC23 decreased IL-13 ($p<0.001$), IL-4 ($p<0.01$), IFNγ ($p<0.05$) and increased levels of IL-17 ($p<0.001$). WPC35 reduced IL-4 ($p<0.001$) and IFNγ ($p<0.001$). Its hydrolysates H36, H38 and H39 modulated the cytokine response as follows; H36 decreased levels of IL-13 ($p<0.001$), IL-4 ($p<0.01$), IFNγ ($p<0.001$) and IL-2 ($p<0.01$). H38 decreased levels of IL-4 ($p<0.01$), IFNγ ($p<0.001$) and IL-2 ($p<0.01$). H39 reduced IL-13 ($p<0.001$), IL-4 ($p<0.01$), IFNγ ($p<0.001$) and IL-2 ($p<0.01$).

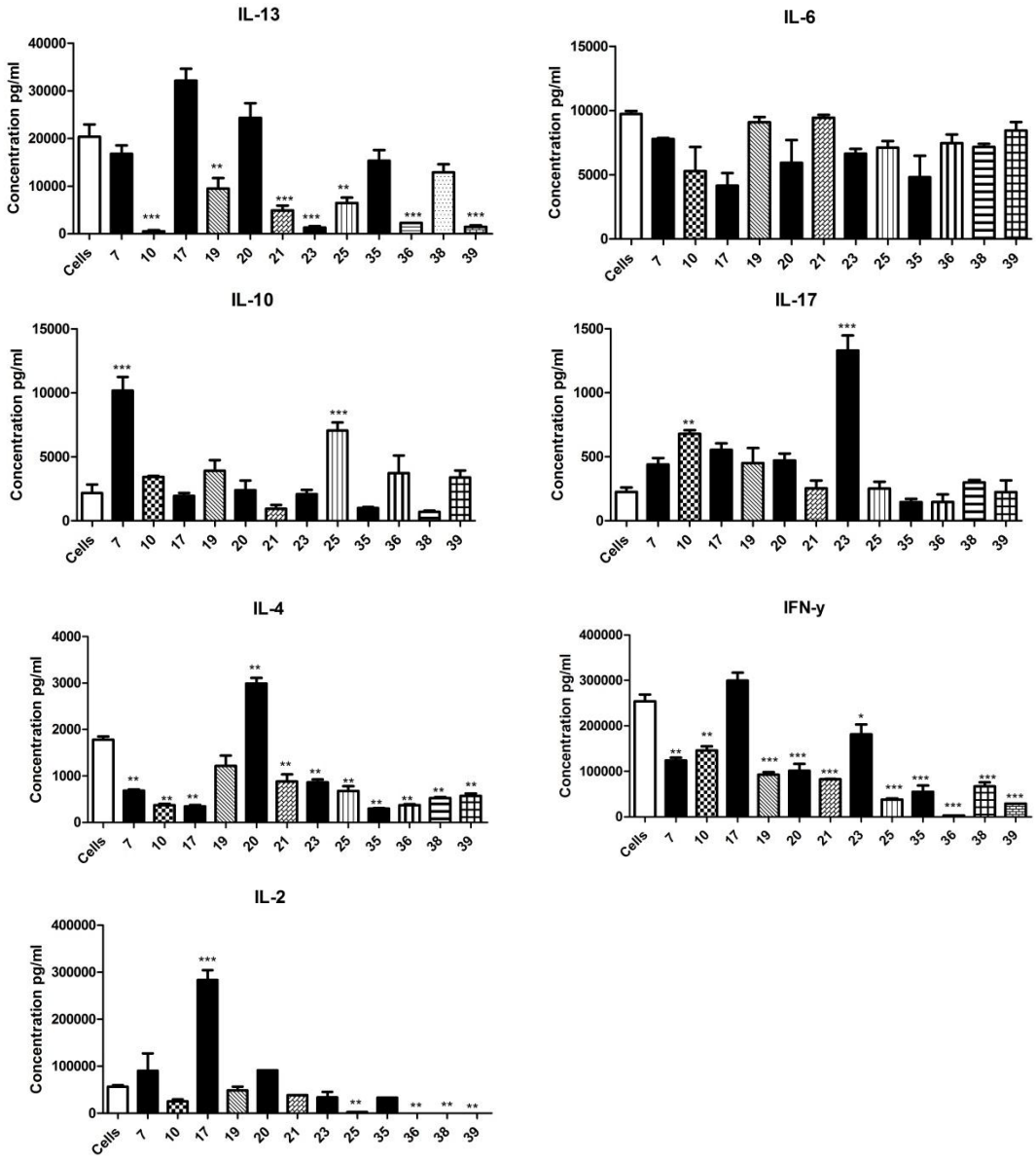

Figure 3.20 Secretion of IL-13, IL-6, IL-10, IL-17, IL-4, IFNγ and IL-2 by Th1 cells treated with selected *Palmaria palmata* hydrolysates. $CD4^+$ T-cells were isolated from the spleens of Balb/c mice using EasySep $CD4^+$ Isolation (Stemcell). Cells were plated at 1 x 10^6 cells / mL on a 96 well plate. Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates (1 mg/mL). The media was supplemented with 20 ng/mL IL-2, 20 ng/mL IL-12 and 10 μg/mL anti-IL-4 antibody for Th1 polarising conditions. Cells were incubated for 72 hours and then re-stimulated in fresh media for 24 hours. Cytokine secretion was measured using ELISA (R&D Duoset). One way ANOVA with a Post test Newman Keuls was used to assess differences between groups. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to cells alone.

3.2.7.2 Cytokine secretion by T helper cell 2 (Th2) subset treated with selected *Palmaria palmata* protein hydrolysates.

Following their assessment on Th1 cells (**Section 3.2.7.1**), hydrolysates H10, H19, H21, H25, H36, H38 and H39 were then screened on a Th2 subset. Th2 cells were isolated using an EasySep Mouse $CD4^+$ T cell Enrichment Kit and the media was supplemented with 10 µg/mL neutralising IFNγ antibody and 10 mg/mL IL-4 to induce Th2 polarising conditions. Cells were treated with hydrolysates H10, H19, H21, H25, H36, H38 and H39 along with their whole protein controls, WPC7, WPC23, WPC17, WPC20 and WPC35 at a dose of 1 mg/mL and incubated for 72 hours before re-stimulation in fresh media for a further 24 hours.

Figure 3.21 shows secretion of IL-13, IL-6, IL-10, IL-17, IL-4, IFNγ and IL-2 from Th2 cells when treated with H10, WPC7, H19, WPC17, H21, WPC20, WPC35, H36, H38 and H39. H10 decreased IL-6 ($p<0.001$), IL-10 ($p<0.001$), IL-17 ($p<0.01$), IL-4 ($p<0.001$), IFNγ ($p<0.001$). Its control WPC7 reduced IL-13 ($p<0.01$), IL-6 ($p<0.05$), IL-17 ($p<0.01$), IL-4 ($p<0.001$), IFNγ ($p<0.001$) and IL-2 ($p<0.01$) and increased levels of IL-10 ($p<0.01$). H25 decreased production of IL-13 ($p<0.001$), IL-6 ($p<0.05$), IL-4 ($p<0.01$), IFNγ ($p<0.001$) and IL-2 ($p<0.01$) and increased levels of IL-10 ($p<0.01$). WPC23, control of H25, reduced levels of IL-13 ($p<0.01$), IL-10 ($p<0.05$), IL-17 ($p<0.01$), IL-4 ($p<0.001$), and IFNγ ($p<0.001$). H19 decreased IL-13 ($p<0.001$), IL-6 ($p<0.001$), IL-10 ($p<0.01$), IL-17 ($p<0.01$), IFNγ ($p<0.001$) and IL-2 ($p<0.01$). Its control, WPC17, decreased levels of IL-13 ($p<0.01$), IL-10 ($p<0.01$), IL-4 ($p<0.001$), IFNγ ($p<0.01$) and increased levels of IL-17 ($p<0.001$). H21 reduced IL-13 ($p<0.01$), IL-10 ($p<0.01$), IL-4 ($p<0.001$) and IFNγ ($p<0.001$). Its control WPC20 decreased IL-10 ($p<0.001$), IL-4 ($p<0.001$), IFNγ ($p<0.001$) and increased levels of IL-6 ($p<0.05$) and IL-2 ($p<0.01$). WPC35, the control for H36, H38 and H39, decreased levels of IL-17 ($p<0.001$), IL-4 ($p<0.001$) and IFNγ ($p<0.001$) and increased IL-10 ($p<0.001$) and IL-2 ($p<0.01$). H36 increased levels of IL-10 ($p<0.001$) and reduced IL-13 ($p<0.001$), IL-6 ($p<0.01$), IL-17 ($p<0.01$), IL-4 ($p<0.001$), IFNγ ($p<0.001$) and IL-2 ($p<0.01$). H38 reduced IL-13 ($p<0.001$), IL-6 ($p<0.01$), IL-17 ($p<0.01$), IL-4 ($p<0.001$), IFNγ ($p<0.001$), IL-2 ($p<0.01$) and increased IL-10 ($p<0.01$). H39 reduced levels of IL-13 ($p<0.001$), IL-6 ($p<0.05$), IL-4 ($p<0.001$), IFNγ ($p<0.001$) and IL-2 ($p<0.01$).

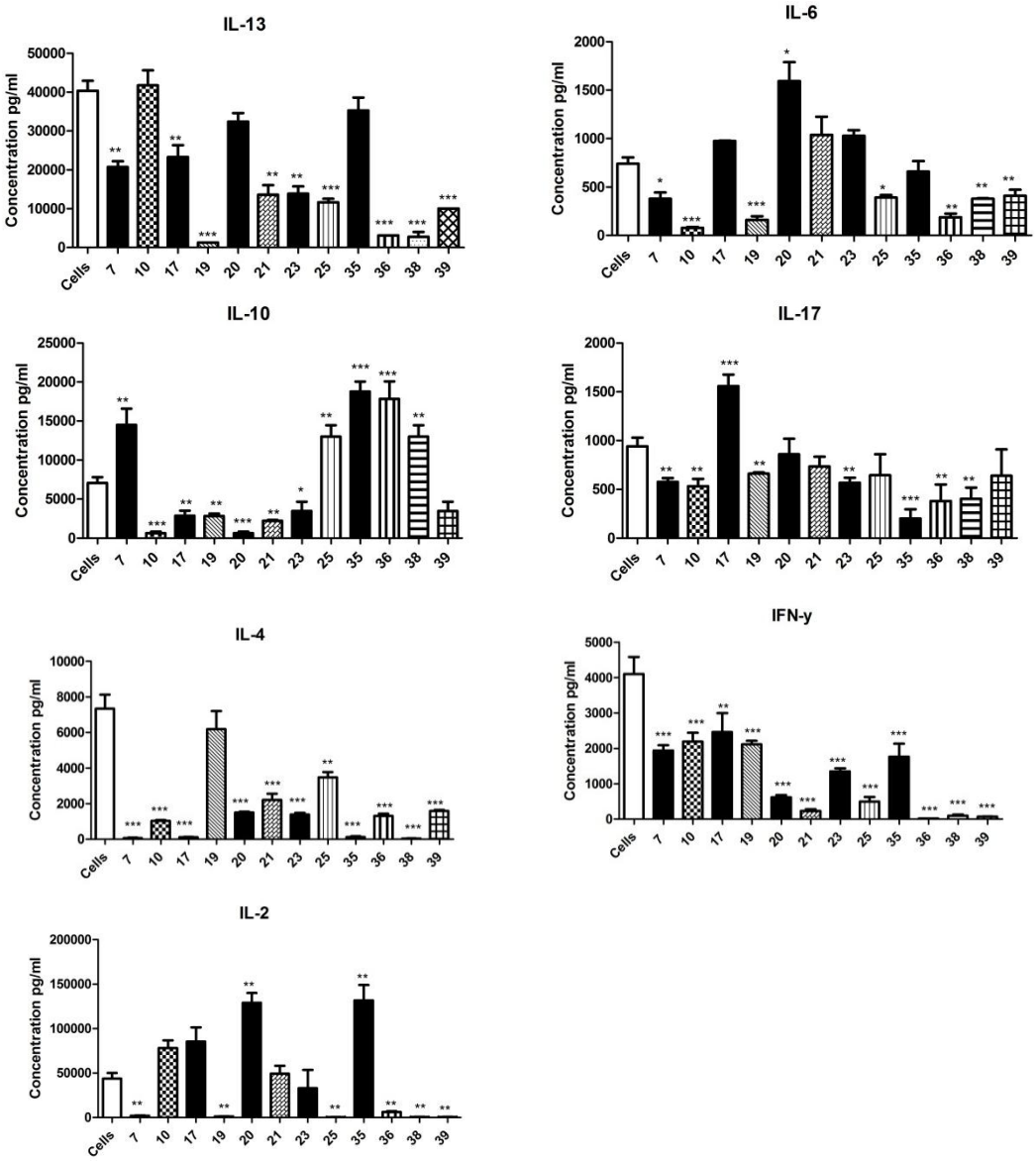

Figure 3.21 Secretion IL-13, IL-6, IL-10, IL-17, IL-4, IFNγ and IL-2 by Th2 cells treated with selected *Palmaria palmata* hydrolysates. $CD4^+$ T-cells were isolated from the spleens of Balb/c mice using EasySep $CD4^+$ Isolation (Stemcell). Cells were plated at 1 x 10^6 cells / mL on a 96 well plate. Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates (1 mg/mL). The media was supplemented with 10 μg/mL neutralizing IFNγ antibody and 10 mg/mL IL-4 for Th2 polarising conditions. Cells were incubated for 72 hours and then re-stimulated in fresh media for 24 hours. Cytokine secretion was measured using ELISA (R&D Duoset). One way ANOVA with a Post test Newman Keuls was used to assess differences between groups. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to cells alone.

3.2.7.3 Cytokine secretion by T helper cell 17 (Th17) subset treated with selected *Palmaria palmata* protein hydrolysates.

In order to complete the immunomodulatory profile of the hydrolysates, the effects of hydrolysates H10, H19, H21, H22, H25 and H36 on the cytokine secretion of Th17 cells were assessed. Th17 cells were isolated using an EasySep Mouse $CD4^+$ T cell Enrichment Kit and the media was supplemented with 10 μg/mL anti-IFNγ antibody, 10 μg/mL IL-4, 20 ng/mL IL-6, 10 ng/mL IL-23 and 2 ng/mL TGFβ to induce Th17 polarising conditions.

Cells were treated with hydrolysates H19, H21, H22, H25 and H36 along with their controls WPC23, WPC17, WPC20 and WPC35 at a dose of 1 mg/mL and incubated for 72 hours before re-stimulation in fresh media for a further 24 hours. Secretion of cytokines (IL-17, IL-6, IL-4, IFNγ and IL-2) was then assessed using ELISA (R&D Duoset).

Figure 3.22 shows secretion of IL-13, IL-6, IL-10, IL-17, IL-4, IFNγ and IL-2 from Th17 cells when treated with H19, WPC17, H21, H22, WPC20, WPC35 and H36. WPC17 displayed pro-inflammatory effects in a Th17 subset through the promotion of IL-17 ($p<0.001$). Its hydrolysate H19 did not have this effect on IL-17 levels or any significant ability to modulate a cytokine secreted from Th17 cells. WPC20 decreased levels of IL-6 ($p<0.001$) and IL-4 ($p<0.001$). H21 showed significant anti-inflammatory properties through the reduction of IL-17 ($p<0.001$) production from Th17 cells, this hydrolysate also increased levels of IL-4 ($p<0.01$) from this subset. H22, similarly to WPC 17, displayed pro-inflammatory abilities through promotion of IL-17 ($p<0.001$) production from a Th17 subset, this hydrolysate also decreased levels of IL-6 ($p<0.001$), IL-4 ($p<0.05$) and IL-2 ($p<0.05$) from the same subset. WPC23 significantly decreased IL-17 ($p<0.001$) production showing potential as an anti-inflammatory candidate whilst also reducing levels of IL-6 ($p<0.01$) from Th17 cells. H25 increased levels of IL-4 ($p<0.001$) and IL-2 ($p<0.05$). WPC35 had no significant effect on any cytokine whilst its hydrolysate 36 increased levels of IL-4 ($p<0.05$) and IL-2 ($p<0.05$).

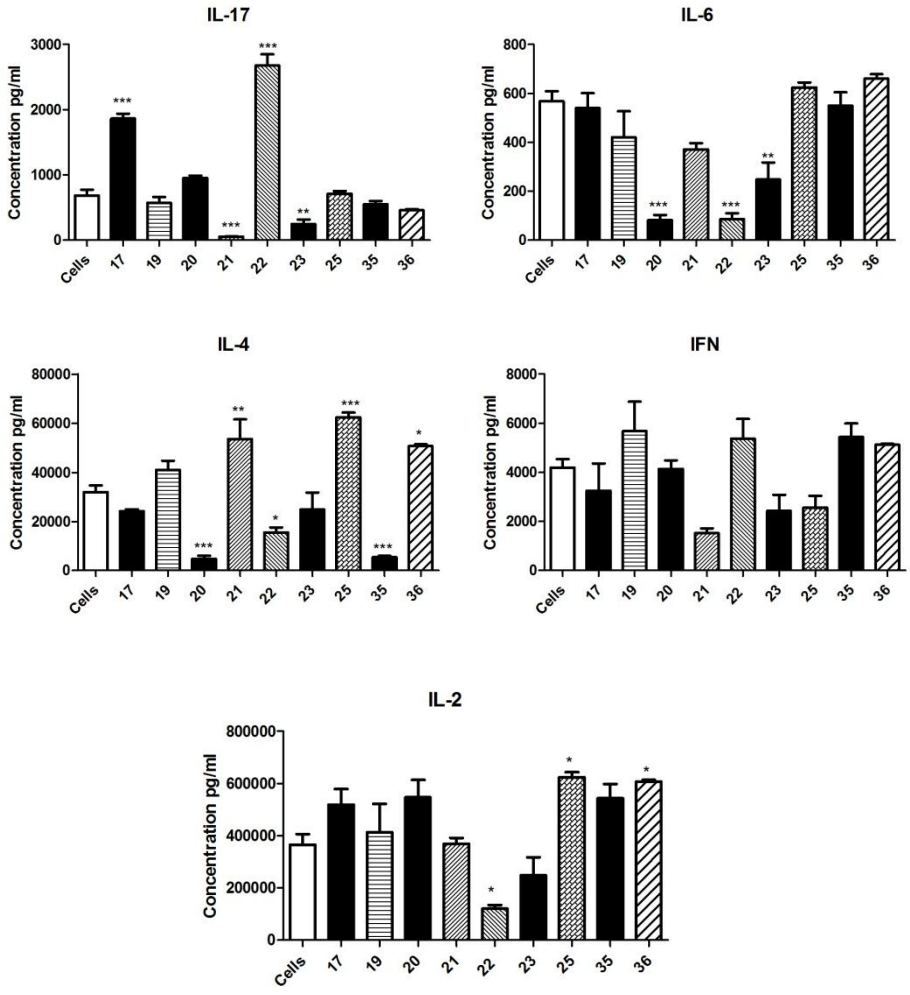

Figure 3.22 Secretion of IL-17, IL-6, IL-4, IFNγ and IL-2 by Th17 cells treated with selected *Palmaria palmata* hydrolysates. $CD4^+$ T-cells were isolated from the spleens of Balb/c mice using EasySep $CD4^+$ Isolation (Stemcell). Cells were plated at 1 x 10^6 cells / mL on a 96 well plate. Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates (1 mg/mL). The media was supplemented with 20 ng/mL IL-6, 10 ng/mL IL-23, 2 ng/mL TGFβ, 10 μg/mL anti IFNγ antibody and 10 μg/mL anti IL-4 antibody for Th17 polarising conditions. Cells were incubated for 72 hours and then re-stimulated in fresh media for 24 hours. Cytokine secretion was measured using ELISA (R&D Duoset). One way ANOVA with a Post test Newman Keuls was used to assess differences between groups. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to cells alone.

3.2.8 Time of harvest of *Palmaria palmata* influenced the effect of the hydrolysates on cytokine secretion.

Following confirmation that PP hydrolysates had the ability to modulate cytokine secretion (**Section 3.2.5** and **Section 3.2.6)**, we next assessed the potential of any variances. PP had been harvested at three distinct times during the course of a year; April, July and October. The data from **Section 3.2.5** and **Section 3.2.6** is rearranged and presented in **Figure 3.23**, **Figure 3.24** and **Figure 3.25** in order to assess any impact of harvest time.

Figure 3.23 and **Figure 3.24** show the cytokine secretion by BMDCs following treatment with PP hydrolysates: April harvest control (WPC17), April harvest hydrolysate (H18), July harvest control (WPC23), July harvest hydrolysate (H24), October harvest control (WPC2) and October harvest hydrolysate (H4). April control (H17) significantly reduced TNFα ($p<0.001$) and IL-23 ($p<0.001$) whilst promoting secretion of IL-12p70 ($p<0.05$) and IL-6 ($p<0.05$). April hydrolysate (H18) decreased levels of pro-inflammatory cytokines TNFα ($p<0.001$) and IL-23 ($p<0.001$). July control (WPC23) reduced TNFα ($p<0.01$), IL-23 ($p<0.001$) and IL-10 ($p<0.05$) secretion. July hydrolysate (H24) similarly reduced TNFα ($p<0.01$), IL-23 ($p<0.001$) and IL-10 ($p<0.01$) but also decreased levels of IL-12p70 significantly ($p<0.001$). October control (WPC2) reduced TNFα ($p<0.01$) and IL-23 ($p<0.001$) and increased IL-6 ($p<0.01$) whilst October hydrolysate (H4) similarly reduced TNFα ($p<0.01$) and IL-23 ($p<0.01$) but also promoted levels of IL-10 ($p<0.001$) and IL-6 ($p<0.01$).

Figure 3.25 show the cytokine secretion by $CD4^+$ T cells following treatment with PP hydrolysates: April harvest control (WPC17), April harvest hydrolysate (H18), July harvest control (WPC23), July harvest hydrolysate (H24), October harvest control (WPC2) and October harvest hydrolysate (H4). April control (WPC17) significantly decreased levels of IL-6 ($p<0.001$), IL-10 ($p<0.05$), IL-4 ($p<0.001$) and IFNγ ($p<0.001$). April hydrolysate (H18) reduced IL-4 ($p<0.01$) and IFNγ ($p<0.001$) whilst it increased levels of IL-17 ($p<0.05$). July control (WPC23) increased levels of IL-17 ($p<0.05$) and decreased secretion of IL-13

($p<0.01$) and IL-4 ($p<0.01$). July hydrolysate (H24) decreased levels of IL-13 ($p<0.01$) and IL-4 ($p<0.001$). October control (WPC2) decreased levels of IL-6 ($p<0.001$) and IL-4 ($p<0.05$). October hydrolysate (H4) reduced secretion of IL-6 ($p<0.001$) and IL-4 ($p<0.05$).

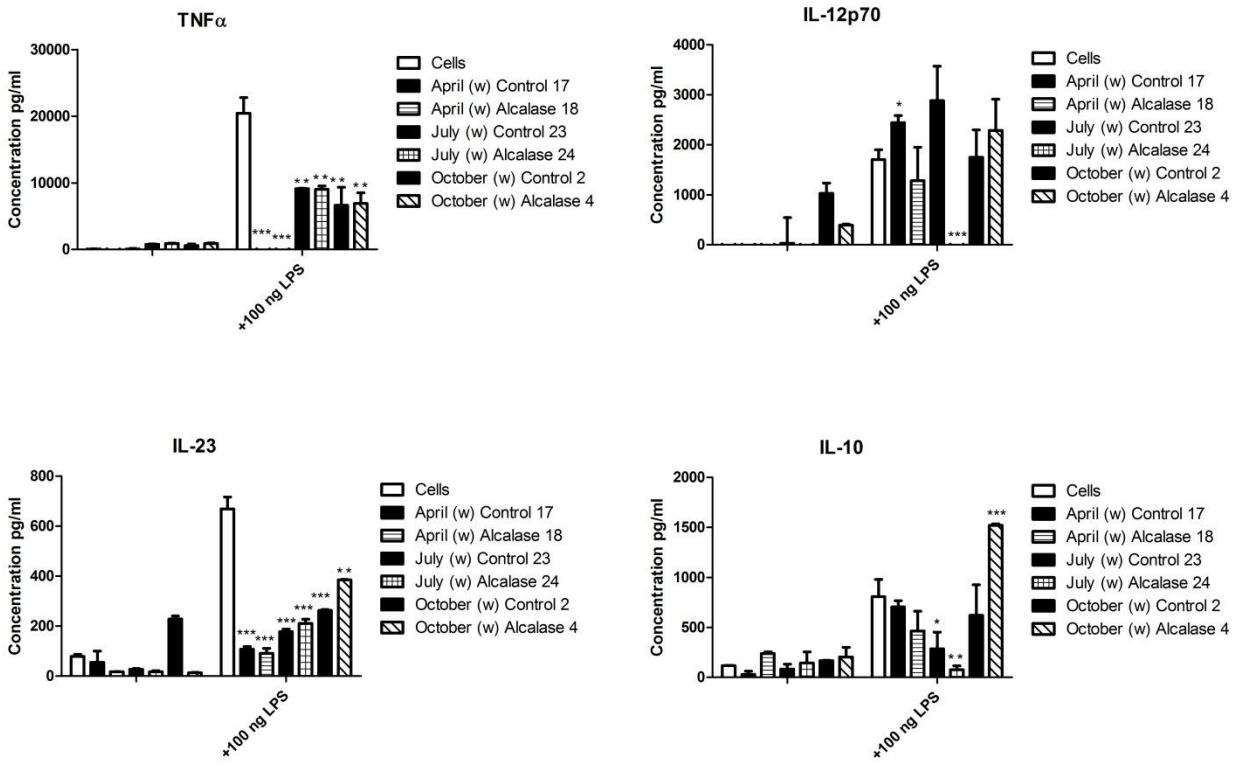

Figure 3.23 Time of harvest alters the effect of *Palmaria palmata* hydrolysates on the secretion of cytokines by BMDCs. BMDCs were isolated from Balb/c mice and differentiated in rGMCSF for seven days. On day 7 cells were treated with hydrolysates and stimulated with 100 ng/mL LPS. Secretion of TNFα, IL-12p70, IL-23 and IL-10 was measured using ELISA (R&D Duoset). Alcalase is the enzyme used in hydrolysis and (W) indicates they are wild samples. One way ANOVA with a Post test Newman Keuls was used to assess differences between groups. Cells treated with hydrolysate only were compared to cells alone, cells treated with hydrolysates + LPS were compared to cells alone + LPS. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to untreated cells (no hydrolysate).

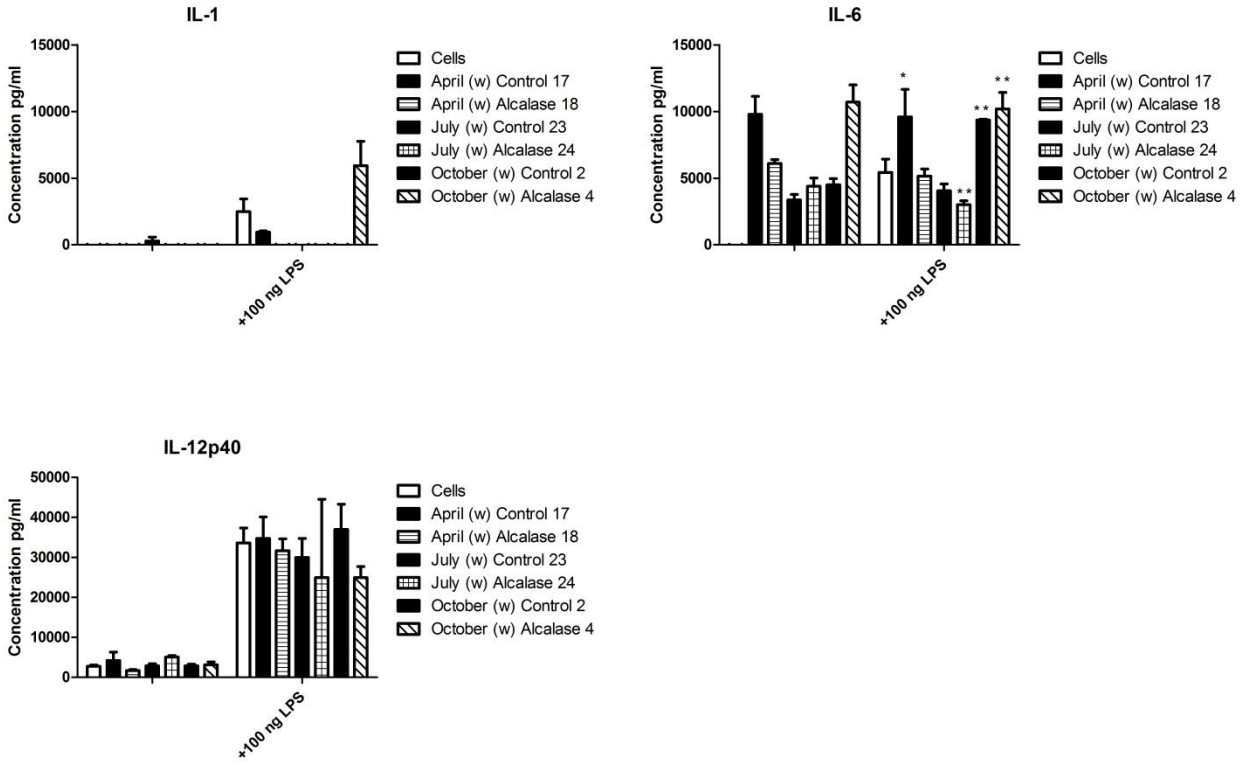

Figure 3.24 Time of harvest alters the effect of *Palmaria palmata* hydrolysates on the secretion of cytokines by BMDCs. BMDCs were isolated from Balb/c mice and differentiated in rGMCSF for seven days. On day 7 cells were treated with hydrolysates and stimulated with 100 ng/mL LPS. Secretion of IL-1β, IL-6 and IL-12p40 was measured using ELISA (R&D Duoset). Alcalase is the enzyme used in hydrolysis and (W) indicates they are wild samples. One way ANOVA with a Post test Newman Keuls was used to assess differences between groups. Cells treated with hydrolysate only were compared to cells alone, cells treated with hydrolysates + LPS were compared to cells alone + LPS. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to untreated cells (no hydrolysate).

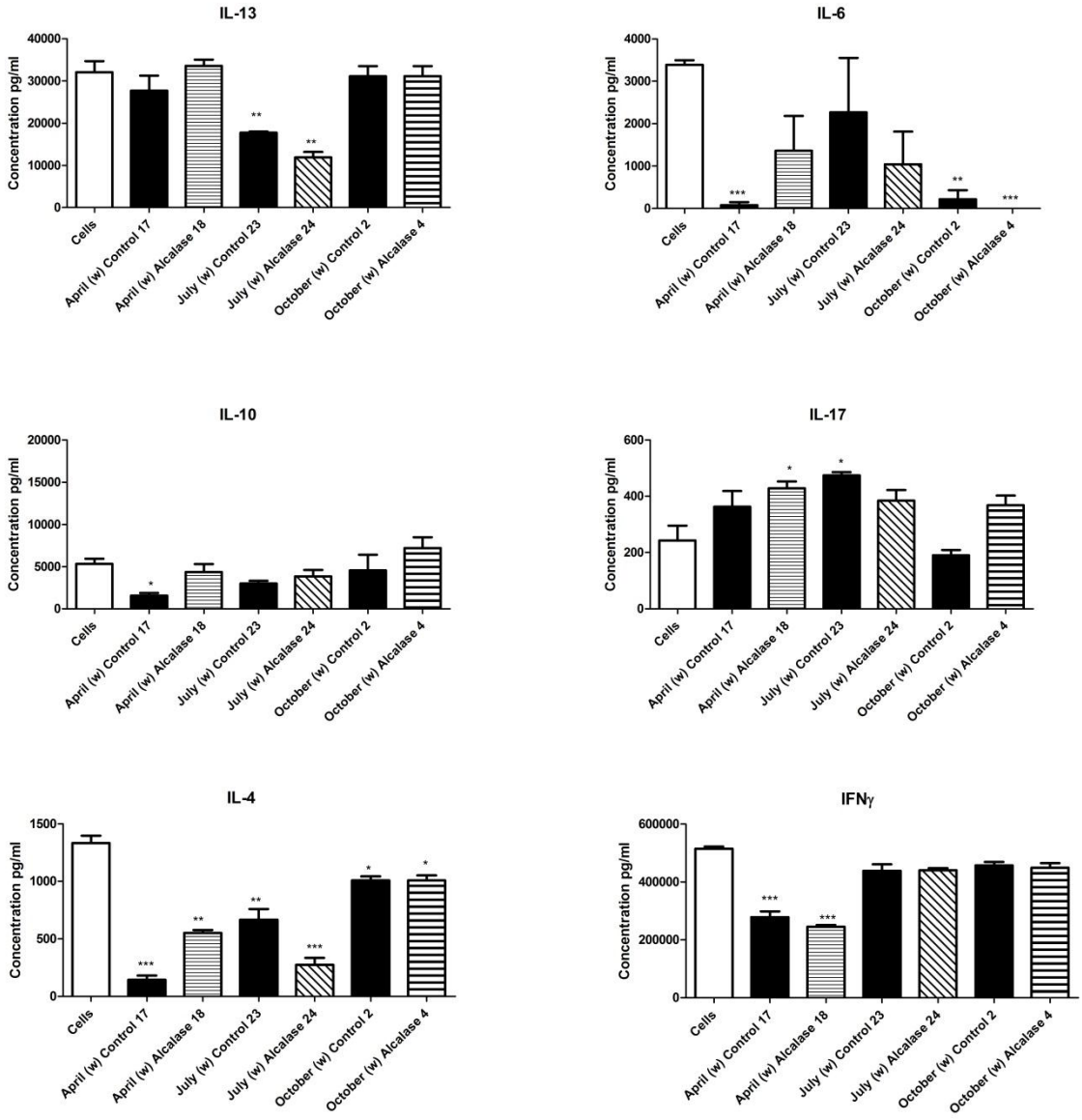

Figure 3.25 Time of harvest alters the effect of *Palmaria palmata* hydrolysates on cytokine secretion by CD4$^+$ T-cells. CD4$^+$ T-cells were isolated from the spleens of Balb/c mice using EasySep CD4$^+$ Isolation (Stemcell). Cells were plated at 1 x 10^6 cells / mL on a 96 well plate. Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates (1 mg/mL) and incubated for 72 hours. Secretion of IL-13, IL-6, IL-10, IL-17, IL-4 and IFNγ was measured using ELISA (R&D Duoset). Alcalase is the enzyme used in hydrolysis and (W) indicates they are wild samples. One way ANOVA with a Post test Newman Keuls was used to assess differences between groups. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to untreated cells (no hydrolysate).

3.2.9 The enzyme used in the enzymatic hydrolysis of *Palmaria palmata* influences the effect of the hydrolysates on cytokine secretion.

After examining the effect that the time of harvest of PP had on cytokine secretion by BMDCs and T cells, the effect of the enzyme used to enzymatically hydrolyse PP into hydrolysates was next investigated. Three different enzymes were used on the same starting material to create various hydrolysates. These include alcalase, flavourzyme and corolase. Material harvested in October (WPC2) was enzymatically hydrolysed by alcalase to create hydrolysate H4, flavourzyme to create H5 and corolase to create H6. The data from **Section 3.2.5** and **Section 3.2.6** is rearranged and presented in **Figure 3.26** and **Figure 3.27** in order to assess any impact of the enzyme used in hydrolysis

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Figure 3.26 shows the cytokine secretion by BMDCs when treated with WPC2 (black bar) and hydrolysates H4, H5 and H6. October control (WPC2) decreased TNFα ($p<0.01$), IL-23 ($p<0.001$) and increased IL-6 ($p<0.01$). Alcalase hydrolysates (H4) also decreased TNFα ($p<0.01$) and IL-23 ($p<0.01$) and increased IL-6 ($p<0.01$) but also increased IL-10 production ($p<0.001$). Flavourzyme hydrolysate (H5) decreased TNFα and IL-23 ($p<0.05$). Corolase hydrolysates (H6) reduced levels of TNFα ($p<0.01$) and IL-23 ($p<0.001$) and increased IL-12p70 ($p<0.001$) and IL-10 ($p<0.001$).

Figure 3.28 shows the cytokine secretion by $CD4^+$ T cells when treated with WPC2 (black bar) and hydrolysates H4, H5 and H6. WPC2 reduced levels of IL-6 ($p<0.001$), IL-4 ($p<0.01$) and IFNγ ($p<0.05$). H4 (alcalase hydrolysate) decreased IL-6 ($p<0.001$), IL-4 ($p<0.01$) and IFNγ ($p<0.05$). H5 (flavourzyme hydrolysate) reduced IL-13 ($p<0.05$), IL-6 ($p<0.001$), IL-10 ($p<0.05$), IL-4 ($p<0.01$) and IFNγ ($p<0.001$) and promoted secretion of IL-17 ($p<0.001$). H6 (corolase hydrolysate) increased IL-13 production ($p<0.01$) and reduced levels of IL-4 ($p<0.01$), IL-6 ($p<0.001$) and IFNγ ($p<0.05$).

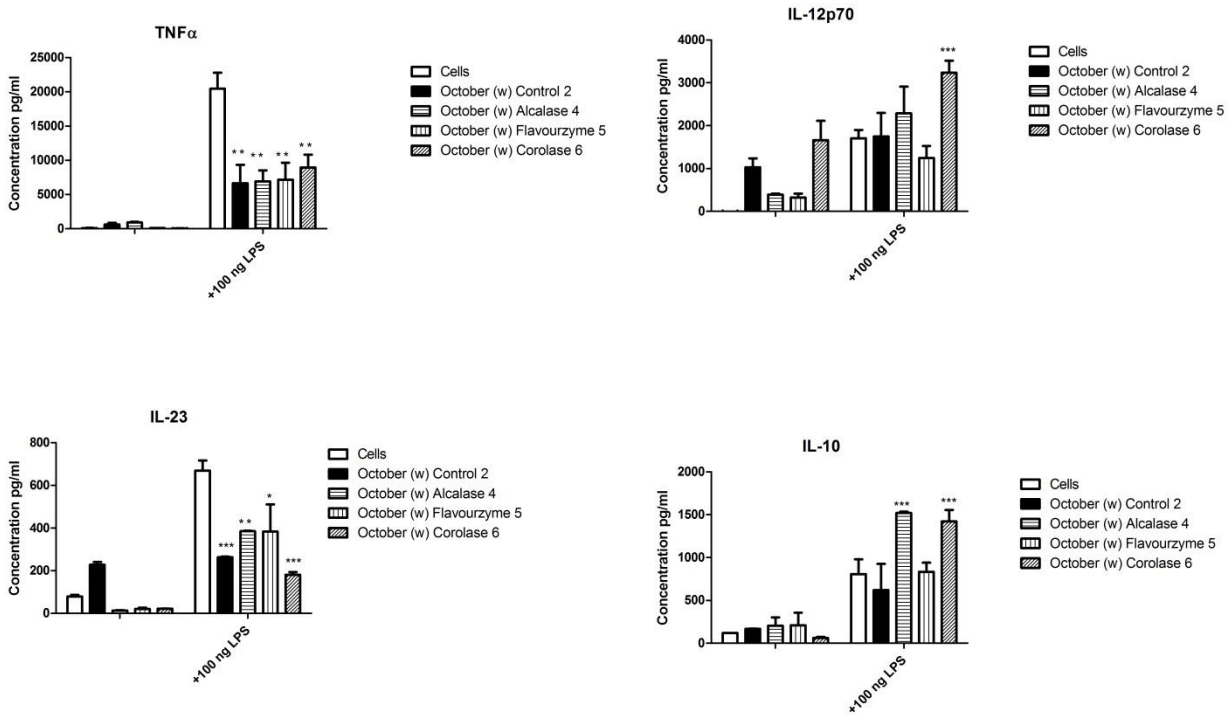


Figure 3.26 The enzyme used in the hydrolysis impacts on the ability of *Palmaria palmata* hydrolysates to modulate the cytokine secretion by BMDCs. BMDCs were isolated from Balb/c mice and differentiated in rGMCSF for seven days. On day 7 cells were treated with hydrolysates and stimulated with 100 ng/mL LPS. Secretion of TNFα, IL-12p70, IL-23 and IL-10 was measured using ELISA (R&D Duoset). (W) indicates these are wild samples. One way ANOVA with a Post test Newman Keuls was used to assess differences between groups. Cells treated with hydrolysate only were compared to cells alone, cells treated with hydrolysates + LPS were compared to cells alone + LPS. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to untreated cells (no hydrolysate).

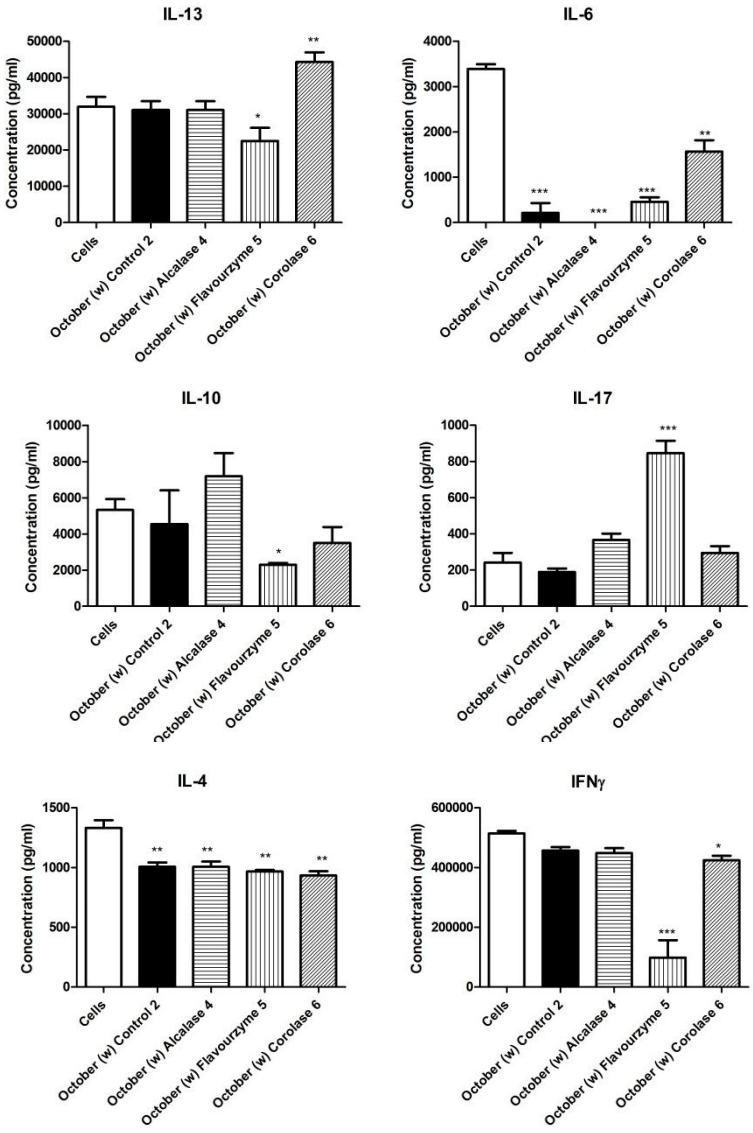


Figure 3.27 The enzyme used in the hydrolysis impacts on the ability of *Palmaria palmata* hydrolysates to modulate the cytokine secretion of undifferentiated CD4$^+$ T-cells. CD4$^+$ T-cells were isolated from the spleens of Balb/c mice using EasySep CD4$^+$ Isolation (Stemcell). Cells were plated at 1 x 10^6 cells / mL on a 96 well plate. Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates, enzymatically digested with Alcalase, Flavourzyme or Corolase, or whole protein control (both 1 mg/mL) and incubated for 72 hours. Secretion of IL-13, IL-6, IL-10, IL-17, IL-4 and IFNγ was measured using ELISA (R&D Duoset). One way ANOVA with a Post test Newman Keuls was used to assess differences between groups. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to untreated cells (no hydrolysate).

3.2.10 The source of *Palmaria palmata* (wild or farmed) alters the ability of the hydrolysates to modulate cytokine secretion.

The source of the PP and whether it affect's the ability of hydrolysate to modulate cytokine production was next examined. Wild PP was sourced from Mweenish Island Co. Galway and farmed from long line cultivation in Ard Bay Co. Galway. The data from **Section 3.2.5** and **Section 3.2.6** is rearranged and presented in **Figure 3.28** and **Figure 3.29** in order to assess any impact of source.

Figure 3.28 shows the cytokine secretion by BMDCs when treated with wild WPC17 (black bar) and its hydrolysate H18 and farmed WPC20 (black bar) and hydrolysate H21. TNFα was potently suppressed by H18 ($p<0.001$) and its WPC17 ($p<0.001$) but not as reduced by its farmed counterpart H21 ($p<0.05$) and its WPC20 ($p<0.05$). IL-12p70 was promoted by WPC17 ($p<0.001$) and H18 ($p<0.001$) and also its farmed hydrolysate counterpart H21 ($p<0.001$) but suppressed by the WPC20 ($p<0.01$). All samples suppressed IL-23 ($p<0.001$).

Figure 3.29 shows the cytokine secretion of BMDCs when treated with wild WPC17 (black bar) and hydrolysate H18 and farmed WPC20 (black bar) and hydrolysate H21. Wild control WPC17 reduced levels of IL-6 ($p<0.001$), IL-10 ($p<0.05$), IL-4 ($p<0.001$) and IFNγ ($p<0.001$). Its hydrolysate H18 suppressed IL-6 ($p<0.05$), IL-4 ($p<0.01$) and IFNγ ($p<0.001$). The farmed control WPC20 reduced levels of IL-4 ($p<0.01$) and its hydrolysate H21 increased levels of IL-10 ($p<0.001$) whilst reducing IL-4 ($p<0.01$).

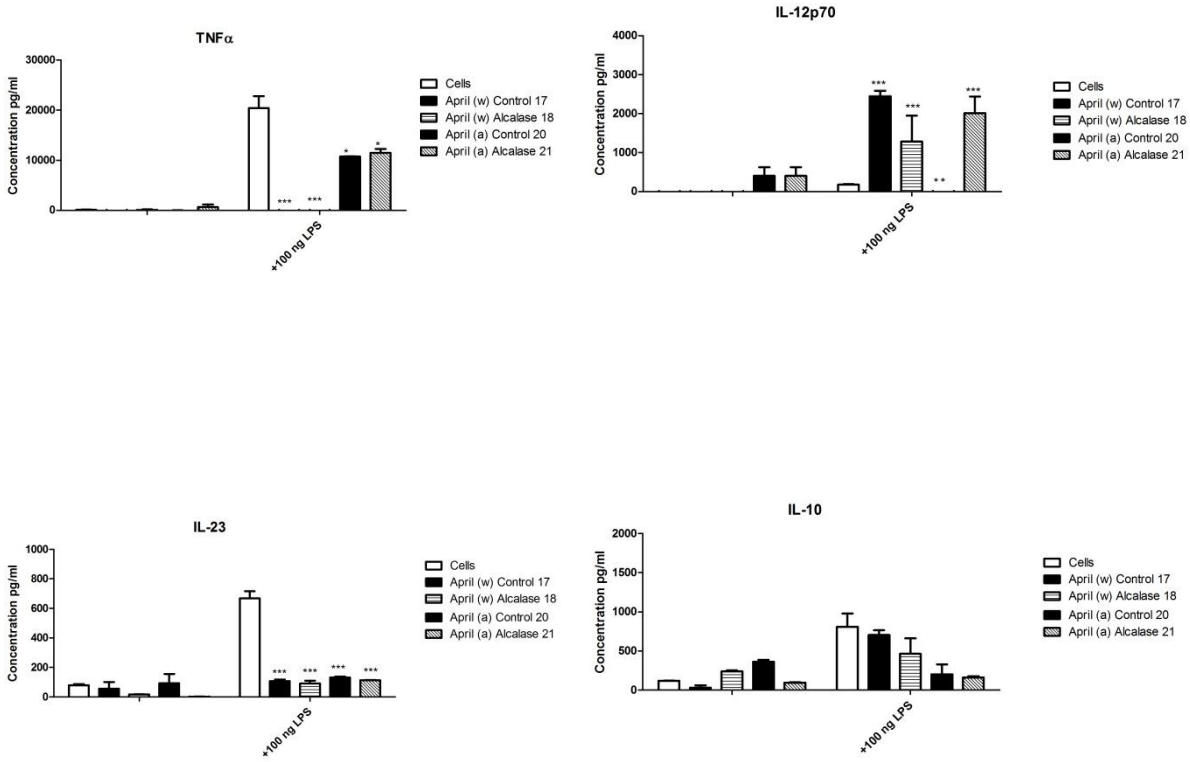


Figure 3.28 The effect of source (wild or farmed) on the ability of *Palmaria palmata* hydrolysates to modulate the cytokine secretion by BMDCs. BMDCs were isolated from Balb/c mice and differentiated in rGMCSF for seven days. On day 7 cells were treated with hydrolysates and stimulated with 100 ng/mL LPS. Secretion of TNFα, IL-12p70, IL-23 and IL-10 was measured using ELISA (R&D Duoset). (w) indicates they are wild samples, (a) indicates they were harvested in aquaculture. One way ANOVA with a Post test Newman Keuls was used to assess differences between groups. Cells treated with hydrolysate only were compared to cells alone, cells treated with hydrolysates + LPS were compared to cells alone + LPS. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to untreated cells (no hydrolysate).

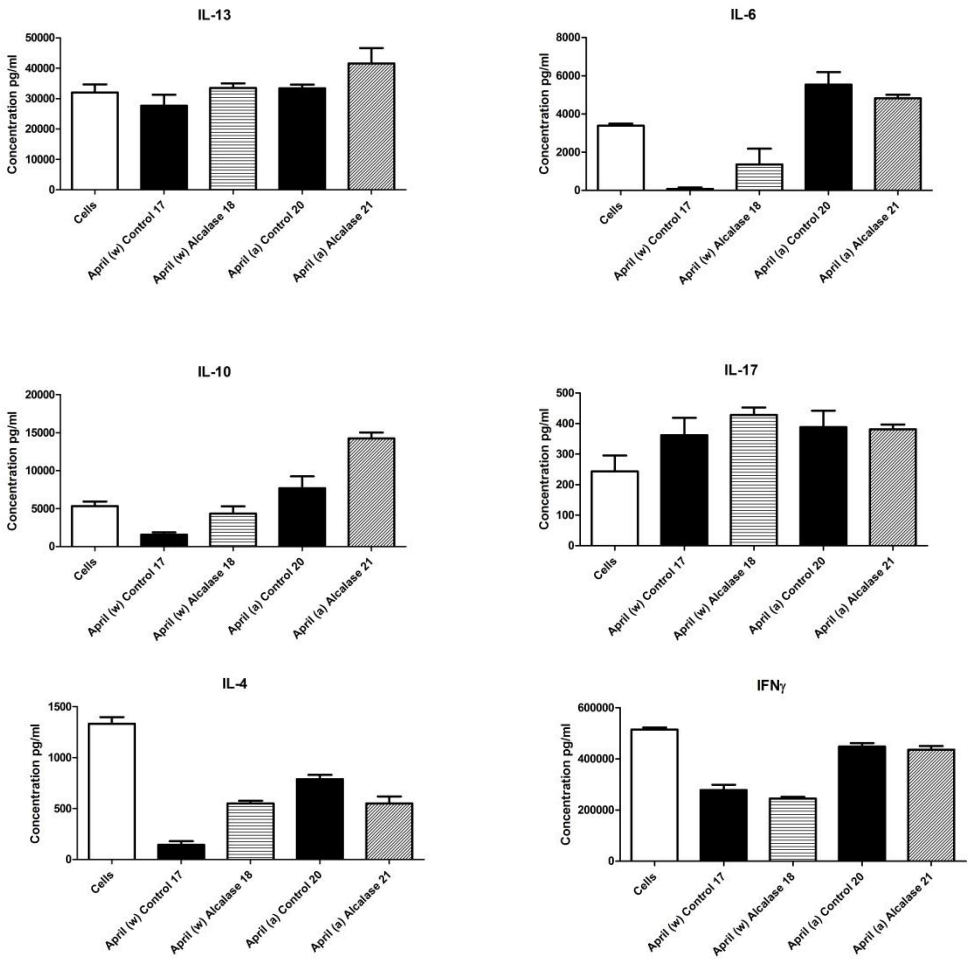


Figure 3.29 The effect of source (wild or farmed) on the ability of *Palmaria palmata* hydrolysates to modulate cytokine secretion by undifferentiated CD4$^+$ T cells. CD4$^+$ T-cells were isolated from the spleens of Balb/c mice using EasySep CD4$^+$ Isolation (Stemcell). Cells were plated at 1 x 10^6 cells / mL on a 96 well plate. Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates either from fared or wild sources and incubated for 72 hours. Secretion of IL-13, IL-6, IL-10, IL-17, IL-4 and IFNγ was measured using ELISA (R&D Duoset). (w) indicates they are wild samples, (a) indicates they were harvested in aquaculture. One way ANOVA with a Post test Newman Keuls was used to assess differences between groups. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to untreated cells (no hydrolysate).

3.3 Discussion

Inflammaging is the term used to describe the chronic low-grade inflammation experienced as humans age. This persistent inflammatory response has been shown to be at the core of many age-related diseases such as atherosclerosis, type II diabetes and many cancers (Xia *et al.*, 2016). Increases in pro-inflammatory cytokines such as IFNγ, IL-1β, TNFα and IL-6 have been implicated in this phenomenon (Akira *et al.*, 1990; Pes *et al.*, 2004). Similarly, an increase in levels of the anti-inflammatory cytokine IL-10 has been shown to reduce inflammaging and delay the possible onset of age-related disease (Adams *et al.*, 2008). This association between cytokine secretion and inflammaging offers the opportunity for a possible target.

As previously discussed, inflammaging is associated with many age-related diseases such as atherosclerosis and type II diabtes, however it has also been linked with the decline in gut health associated with aging (Biagi *et al.*, 2010). It is believed that inflammaging can occur in the gastro-intestinal tract, disturbing the delicate balance between gut health and the microbiota present in an individual causing increased progression of age-related disease and frailty amongst the older generations (Guigoz, Doré and Schiffrin, 2008). It may be possible that through modulation of the immune response towards a less inflammatory state, the balance between gut immunity and the microbiota of the gastrointestinal tract of older people.

Chronic inflammation is also the underlying cause of many diseases including IBD, RA and psoriasis. An increase in pro-inflammatory cytokines has been shown to be present in all of these diseases; IL-17, TNFα, IL-6 and IL-1β in IBD (Strober and Fuss, 2011), TNFα, IL-1β and IL-6 in RA (Feldmann, Brennan and Maini, 1996), and TNFα, IFNγ, IL-6, IL-12 and IL-17 in psoriasis (Arican *et al.*, 2005). This increase in pro-inflammatory cytokines has been identified as a target for the treatment of many inflammatory diseases. Monoclonal antibody (mAb) therapies and protein therapies which target specific pro-inflammatory cytokines are already on the market such as ustekinumab, a fully human mAb, which

targets IL-12p40 in a number of inflammatory diseases such as psoriasis (Miossec and Kolls, 2012).

Macroalgae is a term used to describe many species. They are a valuable source of chemically-diverse compounds which have been shown to display many health benefits. These benefits include anti-allergy (Vo *et al.,* 2012), anti-bacterial (Lima-Filho *et al.,* 2002), anti-fungal (Morales *et al.,* 2006), anti-hypertensive (Tierney *et al.,* 2010), anti-oxidant (Zubia *et al.,* 2007) and ACE inhibitory (Fitzgerald *et al.,* 2012). Macroalgae are a popular snack food across the world and are traditionally eaten on the west coast of Ireland. They are a rich source of fibre, protein, vitamins and minerals, including omega-3 oils (Pereira *et al.,* 2012). They have been used in many functional foods and cosmetics and are often used in agriculture as fertilisers.

Macroalgae protein hydrolysates are also rich in bioactivity. Protein hydrolysis involves the extraction of proteins from a source and the breakdown of these proteins using either boiling in a strong acid/strong base or through the addition of an enzyme. These processes break the protein down into smaller peptide or amino acid chains. This process often releases bioactive sequences which did not display activity before hydrolysation occurred (Udenigwe and Aluko, 2012). Macroalgae protein hydrolysates have been reported to have many bioactive properties. Hydrolysates from *Saccharina longicruris* displayed anti antibacterial effects (Beaulieu *et al.,* 2015.). Lee *et al.* (2013) found hydrolysates from *Costaria costata*, *Crateloupia filicina, Porphyra tenera and Enteromorpha prolifera* all to have anti-oxidant, anti-tumour, anti-tyrosinase and ACE inhibitory capabilities. Other species inlcluding *Ecklonia cava* (Kim *et al*., 2008), *Hizikia fusiformis* (Siriwardhana *et al*., 1998) and *Sargassum coreanum* (Heo *et al*., 2005) were also found to have bioactive protein hydrolysates.

PP is a macroalgae found on the northern coasts of both the Pacific and Atlantic Oceans. It is commonly referred to as Dulse, but is also known by Dillisk, sea lettuce or *creathnach* in Irish. PP has a high protein content, the second highest of all common seaweeds, as high

as 35% (Harnedy and Fitzgerald, 2013). This makes it an ideal candidate for protein hydrolysation. Whole PP has been shown to be anti-oxidant by Yuan *et al.* (2004) who found the seaweed to be capable of reducing free radicals including ROS. Yuan and Walsh (2006) furthered this study finding PP to have anti-proliferation effects on the HeLa cell line. PP's antibacterial properties were discovered by Hellio *et al.* (2001) as it displayed inhibitory effects against the growth of marine bacteria. Allsopp *et al.* (2016) found that bread enriched with whole PP had immunostimulatory effects in a randomised human trial and also saw increases in human IL-8 *in vitro.*

PP protein hydrolysates have been reported in the literature as exhibiting different bioactive behaviour to that of its whole protein or seaweed counterparts. Collaborators on this project, Harnedy *et al.* (2015), found PP hydrolysates capable of inhibiting DPP-IV, an enzyme which when blocked stimulates insulin secretion through the increase of hormones Glucagon-Like Peptide-1 (GLP-1) and Glucose-dependent Insulinotropic Polypeptide (GIP). Similarly, Harnedy *et al.* (2013) also found PP hydrolysates to have anti-oxidant activity with the ability to reduce both FRAP and ORAC activity. Anti-oxidant behaviour is a good indicator of anti-inflammatory properties due to the role oxidation plays in inducing inflammation. These hydrolysates were chosen to be analysed in this project due to their proven ability to display potent *in vitro* activity. During the course of this project Harnedy *at al.,* (2017) identified a single peptide (SDITRPGGQM) which exhibits both ORAC and FRAP anti-oxidant activity isolated from PP hydrolysates using ultra performance liquid chromatography-electron spray ionisation-tandem mass spec (UPLC-CSI-MS/MS). This finding gave hope to finding a PP hydrolysate with anti-inflammatory abilities.

For this investigation it was first necessary to ensure the dose of hydrolysate used by collaborators in University of Limerick and University of Ulster (1 mg/mL) displayed immunomodulatory activity. BMDCs were treated increasing doses of 0.25 mg/mL - 2.0 mg/mL of three PP hydrolysates 2, 7 and 12. 1 mg/mL gave the optimum response through a reduction in the levels of pro-inflammatory cytokines whilst not fully

suppressing the immune response, thus reducing inflammation but not immunocompromising the host. This concentration is also used by collaborators in *in vitro* screening thus ensuring comparability across the project.

Through the course of the dose response investigation it became apparent that no standard protocol for sample preparation was in place. Hydrolysates were provided by collaborators from the University of Limerick in a freeze-dried state and required re-suspension and sterile filtration before use in *in vitro* studies. Across the project several methods of sample preparation were being utilised, this was a feature of a previous project in the lab which also investigated hydrolysates from a different source and it was discovered that the preparation method can impact the bioactivity of the hydrolysates, hence, it was a priority of this project to ensure this was standardised throughout. Three methods were used to prepare hydrolysates as detailed in Section 3.2.2. Method A was chosen as hydrolysates prepared in this way provided ideal results through the promotion of anti-inflammatory IL-10 secretion and the reduction of pro-inflammatory cytokines IL-1β, IL-23, TNFα and IL-12p70. This method also provided ease of use with limited steps and inexpensive materials. Method A was adopted by all collaborators as the standard protocol for hydrolysate preparation for *in vitro* screening.

Following optimisation of both the dose and preparation of the hydrolysates using a small cohort of hydrolysates, all hydrolysates were then screened on BMDCs. These cells were chosen as the first cells treated with PP hydrolysates as they are key innate immune cells which are often referred to as the link between both the innate and adaptive immune systems (Fujii *et al*., 2004). They have been implicated in numerous immune disorders including chronic obstructive pulmonary disease (COPD) (Demedst *et al.,* 2007) through the over-secretion of pro-inflammatory cytokines such as TNFα and IL-1β promoting a chronic inflammatory response.

As part of this study the impact of hydrolysates on the viability of BMDCs was assessed to ensure any difference seen in cytokine secretion were not due to cytotoxicity. It was found that PP hydrolysates have no effect on the viability of BMDCs. This was the expected result as these hydrolysates have been screened *in vitro* previously by collaborators including Harnedy *et al*. (2015). Following this, the hydrolysates ability to modulate the cytokine secretion by BMDCs was assessed using ELISA. All PP hydrolysates at a dose of 1 mg/mL displayed some levels of anti-inflammatory activity namely through the suppression of TNFα with 22 out of 32 capable of reducing levels of this pro-inflammatory cytokine. Hydrolysate 10 was identified as a potential candidate for future studies due to its ability to reduce IL-12p70, TNFα, IL-12p40 and IL-6 and promote anti-inflammatory IL-10. Furthermore, this profile also differed to that of its whole protein control 7 indicating that the activity was due to the hydrolysed nature of the protein only. Hydrolysates 36, 38 and 39 were also identified as potential candidates due to their abilities to reduce pro-inflammatory cytokines including IL-12p70 and their promotion of IL-12p40 suggesting they may reduce inflammation but not the suppress the normal immune response.

To build a broader profile of the hydrolysates impact on the immune system it was necessary to study their effects on another crucial immune cell. For this study, T cells were chosen. $CD4^+$ T cells, also known as naïve undifferentiated T cells, are a central cell of the adaptive immune system. They have been heavily linked with chronic inflammation and inflammatory disorders. $CD4^+$ T cells secrete pro-inflammatory cytokines including IL-17, IFNγ and IL-6. These cells also have the ability differentiate into T cell subsets.

All hydrolysates were screened on $CD4^+$ T cells and their effects analysed and compared to that of the BMDC study before definite candidates were chosen for further study. Hydrolysate 10, identified also in BMDCs, was found to suppress IFNγ. This cytokine is associated with the Th1 subset and is a target for anti-inflammatory therapeutics. For example anti-IFN antibody therapy is used in the treatment of multiple scleorosis (MS) (Sørensen *et al.,* 2005). Hydrolysate 10 was previously identified

in BMDCs due to its ability to reduce levels of IL-12p70, IL-12p40, IL-6 and TNFα whilst increasing levels of anti-inflammatory IL-10. TNFα is a major driver of chronic inflammation and is a target for therapeutics to treat diseases such as RA, IBD and psoriasis. Anti-TNF therapeutics currently on the market include the chimeric mAb infliximab (Remicade®), the humanised pegylated monoclonal antibody (mAb) certolizumab pegol (Cimzia®) and the fusion protein etanercept (Enbrel®) (Nesbitt *et al.,* 2007). However, TNFα plays a crucial role in the normal immune system and suppression of it can cause immunocompromisation in patients. Through the reduction but not full suppression of pro-inflammatory cytokines and also the promotion of IL-10, hydrolysate 10 may reduce chronic inflammation whilst not fully suppressing immunity.

Hydrolysate 19 was found to promote IL-10 secretion in $CD4^+$ T cells. This anti-inflammatory cytokine is a potential target for therapies to treat many disorders including Crohns' disease (Herfarth and Schölmerich. 2002) and psoriasis (Asadullah *et al.,* 1998). Hydrolysate 19 was also capable of reducing pro-inflammatory cytokines TNFα and IL-23 in BMDCs. As previously discussed, TNFα is a target of commercial therapeutics for the treatment of inflammatory diseases. IL-23 has been shown to induce a pathogenic $CD4^+$ T cell population which helps drive chronic inflammation associated with autoimmune disorders such as RA and MS (Langrish *et al.,* 2005) making this cytokine a potential target in the fight against chronic inflammation. Hydrolysate 19, through its modulation of these key cytokines in both BMDCs and T cells, was chosen as a candidate for further screening.

Hydrolysates 21 and 22 were also identified as candidates due to their ability to increase IL-13 production. IL-13 is associated with the Th2 subset. Th2 cells inhibit the differentiation of the pro-inflammatory Th1 subset and are reduced in numbers during chronic inflammation (Charlton and Lafferty, 1995). The ability to promote a Th2 subset over a Th1 is a potential target for anti-inflammatory therapeutics.

Hydrolysates 36, 38 and 39 were once again identified as potential candidates, following their selection in the BMDC screen, due to their suppression of IFN. As previously discussed, IFN is the main cytokine associated with the Th1 subset and plays a major role in chronic inflammation, thus a hydrolysates ability to suppress this cytokine is very desirable. These hydrolysates also displayed anti-inflammatory properties in BMDCs with hydrolysate 36 suppressing TNFα, IL-12p70 and IL-1β, hydrolysate 38 suppressing IL-12p70, IL-23 and IL-1β and hydrolysate 39 suppressing IL-12p70 and IL-1β. Following analysis and comparison of the immunomodulatory profiles of all samples, hydrolysates 10, 19, 21, 22, 36, 38 and 39 were chosen to be brought forward into further studies. This involved examining their effects on differentiated Th subsets.

T cells have a broad range of functions and are regularly grouped based on these functions. Common subsets include cytotoxic T cells which kill and transform cells, T-regs which help dampen inflammation, Th1 cells which promote protective immunity but can over suppress IFN and play a role in chronic inflammation, Th2 cells which promote a humoral immune response and inhibit Th1 cell differentiation and Th17 cells which promote protective immunity against extracellular bacteria and fungi, but also play a role in the promotion of inflammatory disease and autoimmunity (Mosmann and Sad, 1996.). For the purpose of this project, Th1, Th2 and Th17 T cell subsets were chosen to screen the hydrolysates and build a broader profile of their immunomodulatory capabilities.

In the case of Th1 cells, secretion of IFN and IL-2 has been shown to drive a chronic inflammatory response and reduction of these pro-inflammatory cytokines has been identified as a therapeutic target (Fuss *et al.,* 1996.). Focusing on the secretion of these two cytokines, it can be seen that IFN is reduced by hydrolysates 10, 19, 21, 25, 36, 38 and 39. However, only hydrolysate 19 significantly reduces the secretion of this cytokine differently to that of its whole protein control, 17. For hydrolysates to be considered for future work, they must have differing properties to that of their whole

protein controls, as otherwise it would be not necessary to hydrolyse the starting material. Hydrolysate 19 had no impact on the secretion levels of IL-2, showing selectivity in its suppression of IFN, this is an ideal trait as hydrolysates which fully suppress the immune response could leave the individual immunocompromised. As previously discussed, this hydrolysate also promoted the secretion of anti-inflammatory IL-10 from $CD4^+$ T cells and suppressed levels of TNFα and IL-23 in BMDCs. IL-23 promotes proliferation and differentiation of Th1 cells and, hence, suppression of this cytokine could reduce Th1 levels.

In Th2 cells, secretion of IL-13, IL-4 and IL-10 is beneficial in chronic inflammatory situations due to IL-13 and IL-4's ability to promote the differentiation of a Th2 subset which has inhibitory effects on Th1 cells and IL-10's ability to prevent commitment of a Th1 phenotype. None of the hydrolysates successfully promoted secretion of IL-13 or IL-4. Hydrolysates 25, 36 and 38 all increased secretion of IL-10 with 25's activity being significantly different to that of its whole protein control 23.

Finally, the effect of the hydrolysates on a Th17 phenotype was assessed. Secretion of IL-17 is paramount in examining the effects of hydrolysates on this subset with IL-17 playing a major role in many inflammatory diseases. Hydrolysate 21 was capable of reducing levels of IL-17 secretion differently to that of its whole protein control 20. This hydrolysate also displayed anti-inflammatory capabilities in BMDCs through the decrease in secretion of TNFα, whilst its WPC fully suppressed secretion, 21 significantly reduced it but maintained levels required for an adequate immune response. 21 was also found to significantly reduce levels of IL-23 secreted from BMDCs. It was shown that IL-23 and IL-17 drive IBD (Pallone *et al.,* 2010) and hence a hydrolysate which reduces both of these cytokines could have real therapeutic potential in the treatment of IBD and chronic gut inflammation.

Unlike the other species (boarfish and blue whiting) being analysed in this project several of the PP hydrolysates were from a range of starting materials that were harvested at different times of the year. Furthermore, the enzymes used during hydrolysis were also different as was the source of the material. This was not initially considered to prevent unconscious bias.

In order to assess the impact of these variances the data was re-analysed. Three harvest times were analysed; April, July and October. The harvest times of PP had an impact on the immunomodulatory effects of the hydrolysates. In BMDCs, samples harvested in April had a far more potent effect on the suppression of TNFα than its July or October counterparts. This trend could also be seen in the reduction of IL-23 levels by the samples which lessened from April to October. In $CD4^+$ T cells, IFN is significantly reduced only by April samples. Similarly, only July samples reduce levels of IL-13. Levels of IL-10 are reduced by April samples but this reduction is not seen in October samples, this trend is also seen in BMDCs were IL-10 is increased by October samples but not samples from earlier in the year. This would indicate that samples harvested earlier in the year are more potent suppressors of pro-inflammatory cytokines but samples harvested later in the year are better at promoting the secretion of anti-inflammatory cytokines.

Differences in the bioactivity of these hydrolysates based on harvest times was also seen by our collaborators in University of Limerick. Harnedy *et al*. (2014) found that harvest time impacted on the percentage protein content isolated from the starting material, with significantly lower protein levels recovered from October samples when compared to April and July. This paper also found that bioactivity was affected by harvest time with October samples displaying the least antioxidant, tyrosinase and DPP IV inhibitory activity. July samples provided the overall best profile for their studies, showing potent anti-oxidant, tyrosinase, DPP IV inhibitory and ACE activity.

These differences in bioactivity seen both in this investigation and also by collaborators Harnedy *et al*. (2014) could be due to the reduced protein content available in October samples. These differences in protein content could be due to the environment of the plant at this time of year. Plants are harvested from the sea and the flora and fauna of the sea changes seasonally. The weather and warmth of the sea may also play a part with the sea warming as temperatures warm throughout the summer possibly impacting on the plants. A difference in hydrolysates over seasons was also seen by Wilkins *et al*. (2005), who found their hydrolysates of Valencia orange peel displayed varying bioactivity based on their time of harvest.

PP samples were mainly collected from the wild from a number of points across the west coast of Ireland. However, some samples were harvested from so-called "farmed" sources. These plants cultivated on long lines in Ard Bay, County Galway and harvested in April displayed differences in bioactivity to that of their wild counterparts also harvested in April. Wild samples displayed stronger anti-inflammatory abilities than that of the farmed plants. Wild samples fully suppressed TNFα secretion from BMDCs whilst the farmed samples reduced it but not to the same levels. In $CD4^+$ T cells, farmed plants stimulated secretion of pro-inflammatory IL-6 but also anti-inflammatory IL-10, whilst its wild counterparts either suppressed them or had no impact. Similarly, IFN levels were significantly reduced by the wild plant hydrolysates but the farmed plant hydrolysates had no impact on levels. Harnedy *et al*. (2014) also compared these hydrolysates. They isolated higher levels of protein from the wild plant to that of the farmed plant. They also found that hydrolysates from both sources had the same effects on ACE activity, however, wild samples were showed more potent inhibition of DPP IV and Tyrosinase. Farmed samples did show stronger anti-oxidant capabilities in both FRAP and ORAC assays.

Due to these differences in bioactivity due to harvest time and cultivation it was decided that PP was not a suitable species for this project as the aim is to develop a commercial product. Due to the commercial nature of the project, large amounts of sample with little

variability would be required. PP offered too much variability in terms of environment, harvest time and cultivation and ultimately it was decided to proceed with a species with less variability.

This work is novel and no other studies to-date have reported to show the impact of PP hydrolysates on the immune system. Very few *in vitro* studies showing hydrolysates, from any sources, effects on immune cells such as T cells and BMDCs exist and little is known of their impact on cytokine response. This investigation aimed to show PP hydrolysates immunomodulatory properties and their ability to modulate cytokine response for the benefit of the individual. It was shown that these hydrolysates can modulate the immune response through cytokine level manipulation in ways which have been previously reported to have therapeutic benefit. It was also shown that both harvest time and cultivation methods impact the bioactivity of this species and its hydrolysates. Ultimately, these variations forced a negative decision on the current commercial potential of the hydrolysates. There is still hope that through future studies, a peptide can be isolated from PP with immunomodulatory capabilities and identified so that it could then be synthesised in a laboratory.

Chapter 4:

Investigating the potential of Boarfish as a source of anti-inflammatory protein hydrolysates

4.1 Introduction

Boarfish (*Capros aper*) are a small, mesopelagic fish now frequently found in Irish waters. Once a rare catch in a net of mackerel, Irish fishermen have experienced a dramatic explosion in numbers of Boarfish accredited to the warming oceans (Blanchard and Vandermeirsch, 2005). These fish grow to approximately 13 cm in size, are orange in colour and swim in large shoals. Known to English fishermen as Zulu fish, they have a large tube-like mouth and feed mainly on zooplankton (Coad *et al.*, 2014). They are rough to the touch and have large spines which make up its dorsal fin. They are found at depths of between 50 and 100 metres often along the sea bed.

Due to the large increase of these fish in the last decade, the EU placed its first fishing quota on Boarfish in 2011, giving Ireland a two thirds majority of the total EU quota with a total of 27,227 tonnes for 2011. This quota then experienced a 155% increase, to 56,666 tonnes, for 2012 (Stange, 2016). In 2015, the EU began to reign in the fishing quotas for boarfish bringing in precautionary controls of the fish when the need for proper knowledge of the fish became clear. Ireland's 2018 quota for boarfish is 14,084 tonnes, a decrease of 42,582 tonnes (or 75%) (*Current Quota Uptake*, 2018).

The decrease in fishing quotas for the EU was not only due to the lack of knowledge surrounding boarfish but also due to their ultimate fate. The fish are of little commercial value due to their small weight (approximately 40 to 60 grams) and their body shape making it difficult to fillet them. The fish are primarily used as a source of animal feed and fish meal (Egerton *et al.*, 2017). In 2012, Ireland entered into a trial trade deal with China, sending 70 tonnes of boarfish to Chinese fish processing plants in order to help China meet their ever-growing demand for protein sources but also to find a solution to the problem of what to do with the boarfish which were beginning to land in Irish processing plants. This trade deal fizzled out but the boarfish continue to be caught and, therefore, finding a commercial value in boarfish became a priority (Hayes *et al.*, 2016).

A change in EU law, that is due to be brought into full effect by 2019, will increase the need to research this species' full potential even further. The landing obligation puts an

end to the practice of discarding. Discarding is the return of unwanted catches to the sea, regardless of whether the fish is dead or alive. This common practice is often due to the fish being undersized, the fisherman not having a quota for the species caught, or because of the rules surrounding catch composition. The landing obligation requires all catches of commercial species to be landed and counted against the countries quota. This introduces the problem of an increase in the numbers of boarfish being landed and if no solution is found for these fish, ending up in landfill is a distinct possibility (de Vos *et al.*, 2016).

The immune system is the host's defence against potential invasion by foreign bodies such as bacteria or viruses. It offers protection through the deployment of cells including macrophage, dendritic cells, T cells and mast cells. The immune system, whilst usually offering protection, can attack the body in a phenomenon known as auto-immunity. Auto-immunity is present in many diseases known as 'immune diseases' and the underlying characteristic of all is chronic inflammation (Atassi *et al.*, 2008). Examples of immune disease include type 1 diabetes, RA, psoriasis, IBD, Graves' disease and celiac disease.

Therapies for auto-immune disease are limited as a basic understanding of the cause of this misfiring of the immune system is lacking. In many cases, the symptoms of the disease are the target of the therapy rather than the cause. As previously mentioned, the main symptom in all immune disorders is chronic inflammation. TNFα was found to have increased secretion in RA patients and is believed to be involved in a cytokine network with many other pro-inflammatory cytokines such as IL-1β and IL-6 (Akira *et al.*, 1990) . As a result, TNFα became a target for therapeutics and an anti-TNFα therapy provided success in alleviating the disease symptoms with therapies such as infliximab and etanercept currently on the market (Mitoma *et al.*, 2008). Targeting cytokine secretion is not limited to the treatment of immune disease with two cytokines having achieved FDA approval for cancer treatment. IL-2 for metastatic melanoma and renal cell carcinoma treatment (Atkins *et al.*, 2000) and IFNα for adjuvant therapy in stage III melanoma (Kirkwood *et al.*, 2000).

Proteins and protein hydrolysates from fish sources have been found to have a broad range of bioactivity. Whole proteins, which have not undergone hydrolysis, from bass have displayed antimicrobial and antifungal properties (Shike *et al.*, 2002) with mackerel proteins showing anti-oxidant activity (Fujita and Yoshikawa, 2008). Many protein hydrolysates have also been shown to have antioxidant properties including cod (Jeon, Byun and Kim, 1999), herring (Sathivel *et al.*, 2003) and sole (Jun *et al.*, 2004).

As previously discussed in Chapter 1, several functional food products containing fish protein hydrolysates as functional ingredients have already been approved in Japan with at least one product, Valtyron®, receiving EFSA approval for use as a novel food ingredient (Harnedy and FitzGerald, 2012). Other products include an antihypertensive product PeptACE™ from Bonito peptides created by a Canadian company Natural Factors Nutritional Products Ltd. Nutripeptin™, which lowers the glycemic index, from Norwegian company Nutrimarine Life Science AS, and Seacure® which is said to improve gastrointestinal health from Proper Nutrition, a US based company (Raghavan *et al.*, 2010).Boarfish, whilst hard to process, are high in protein and have been shown to have many bioactive properties. Hayes *et al*. (2016), found antihypertensive effects of protein hydrolysates from boarfish through the inhibition of angiotensin-I-converting enzyme (ACE-I). They have been shown to have the potential to have anti-oxidant properties with Blanco *et al*. (2015) identifying theoretical anti-oxidant peptide sequences. It was also found to be a rich source of amino acids (Ojha *et al.*, 2016). To date, there are no published reports on the effects of boarfish or its protein hydrolysates on the immune system or its key immune cells such as dendritic cells or T cells.

4.1.1 Chapter Aims

The aim of this chapter is to investigate the potential of boarfish as a source of novel bioactive peptides. Boarfish was kindly donated, pre-minced, by the Killybegs Fishermen's Organisation, Killybegs, Co. Donegal, Ireland to collaborators the Fitzgerald Lab in University of Limerick. This was facilitated by Board Iascaigh Mhara (BIM, Ireland). The minced boarfish meat underwent enzymatic hydrolysis in the University of Limerick as described in **Figure 4.1**.

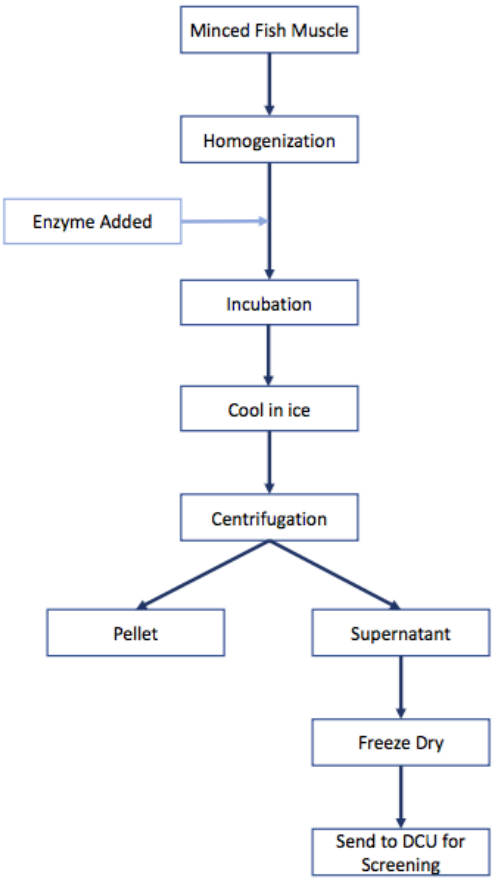


Figure 4.1 Hydrolysis process of boarfish minced meat performed in the University of Limerick by collaborators, The Fitzgerald Laboratory (UL). Boarfish protein hydrolysates screened during this project were created through a novel hydrolysis process which is the intellectual property of the Fitzgerald Laboratory.

Twenty (20) boarfish hydrolysates were produced as part of this project by varying the enzyme used during digestion and the time period which samples were digested over, as described in **Table 4.1**.

Table 4.1 Hydrolysis process of Boarfish protein samples screened in Chapter 4.

Time (Hours)	Enzyme A	Enzyme B	Enzyme C	Enzyme D	WPC
1	1	**5**	9	13	17
2	2	**6**	10	14	18
3	3	**7**	11	15	19
4	4	**8**	12	16	20

As seen in **Table 4.1**, each boarfish protein hydrolysate differed in either the enzyme used to hydrolyse protein or the length of time of the hydrolysis took place. There are four (4) whole protein controls (also known as 'no enzyme control'). These samples underwent the same process as the hydrolysates but did not have any enzyme added, therefore, their protein remains intact. For screening purposes, the hydrolysates are grouped according to the hours of hydrolysis they underwent. Hence group 1 includes hydrolysates 1, 5, 9 and 13 and their whole protein control 17.

Screening was carried out using the same protocol optimised in Chapter 3 as described in **Figure 4.2**. The cytotoxicity of hydrolysates was first investigated using an MTS assay, a colorimetric assay which measures the rate of metabolism of the cells. The viability of both BMDCs and undifferentiated $CD4^+$ T cells, when treated with the hydrolysates, was measured in this way. The cytokine secretion of BMDCs when treated with the hydrolysates was then assessed through ELISA, this was then repeated on undifferentiated $CD4^+$ T cells. All hydrolysates were assessed on both in BMDCs and undifferentiated $CD4^+$ T cells. At this point, hydrolysates were rated based on their ability to modulate inflammation. Hydrolysates were identified which had potential anti-inflammatory activity both in their suppression of pro-inflammatory cytokines and promotion of anti-

inflammatory cytokines. These candidate hydrolysates were then brought forward to be screened on T cell subsets (Th1, Th2 and Th17 cells) in order to further elucidate their effects on the immune system. Following this collaborators were consulted in order to decide if a hydrolysate was suitable for *in vivo* trials.

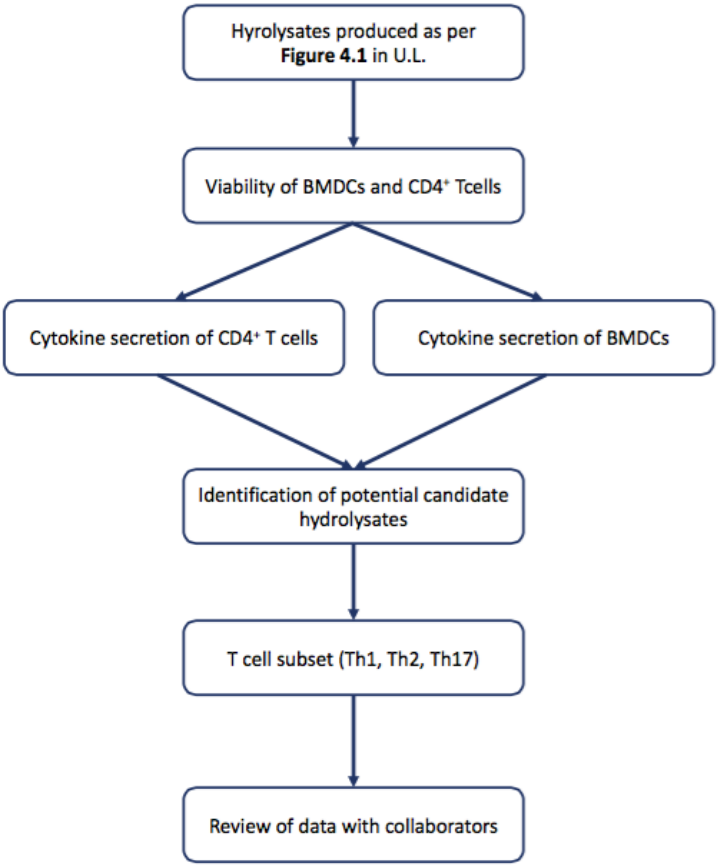


Figure 4.2 Optimised protocol for screening of marine protein hydrolysates. Protocol optimised in Chapter 3. Protocol ensures all protein hydrolysate data is comparable and is standardised across the project.

All boarfish hydrolysates underwent this screening protocol in order to identify the optimum candidates for future screening. Optimum candidates were hydrolysates found to have the ability to suppress pro-inflammatory markers such as the secretion of cytokines like TNFα, the inhibition of differentiation of Th1 or Th17 cell subsets or the ability to promote anti-inflammatory cytokines such as IL-10 or the Th2 subset which suppresses Th1 differentiation.

4.2 Results

4.2.1 The dose of boarfish hydrolysates used does not impact on dendritic cell viability.

Before examining the ability of the hydrolysates to modulate cytokine secretion, it was necessary to examine their effects on the viability of BMDCs. Cells were isolated from the bone marrow of Balb/c mice and incubated at 37°C 5% CO_2 for seven days with rGMCSF (Sigma Aldrich, Ireland). BMDCs were plated in triplicate (100 µL/well) at a concentration of $1x10^6$ cells/mL on a 96 well plate and rested. Cells were then treated with hydrolysates (1 mg/mL) or 10% DMSO (positive control) and then stimulated with LPS (100 ng/mL) and incubated for 24 hours. Viability was assessed using an MTS assay (Cell Titer 96 Aqueous One Solution – Promega) according to the manufacturer's instructions. Results were expressed as a percentage of cells alone. At 1 mg/mL hydrolysates did not have any significant effect on cell viability on BMDCs when compared to untreated cells (**Figure 4.1**).

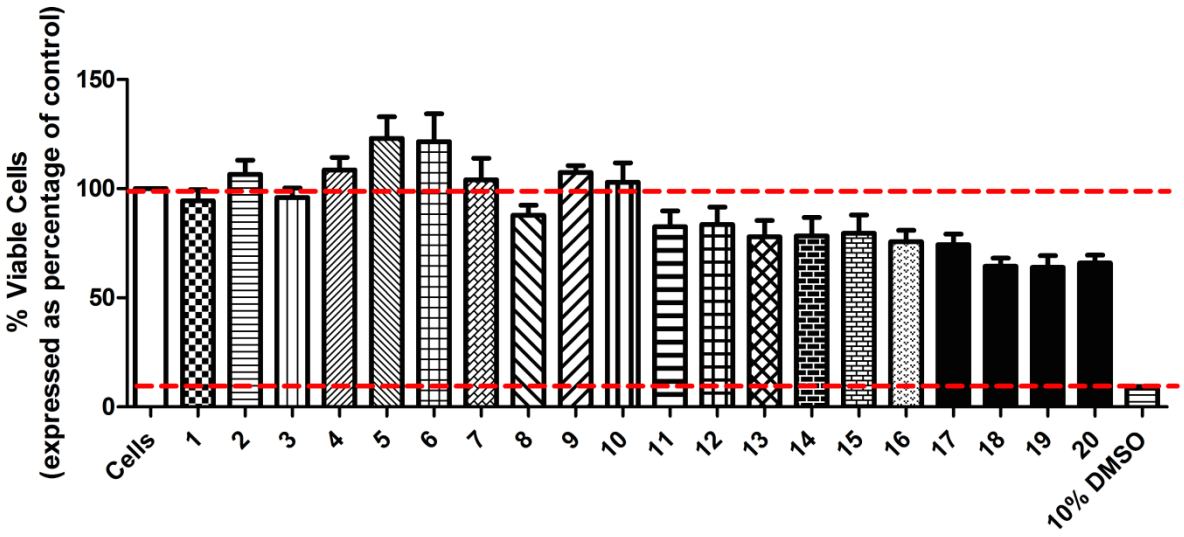


Figure 4.3 BMDC viability expressed as a percentage of cells alone following treatment with Boarfish hydrolysates and LPS stimulation. BMDCs were isolated from Balb/c mice and differentiated in rGMCSF for seven days. On day 7, cells were treated with hydrolysates and stimulated with 100 ng/mL LPS for 24 hours. DMSO (10% v/v) was included as a positive control of cytotoxicity. MTS assay (Cell Titer 96 Aqueous One Solution) was used to assess cell viability.

4.2.2 The dose of boarfish hydrolysates used does not impact on undifferentiated T cell viability.

Cell viability of naïve undifferentiated T cells treated with hydrolysate was examined prior to investigating their effects on T cell cytokine secretion. Cells were isolated from the spleens of Balb/c mice using the EasySep Mouse $CD4^+$ T cell Enrichment Kit (Stemcell Technologies). $CD4^+$ cells were plated in triplicate (100 µL/well) at a concentration of $1x10^6$ cells/mL on a 96 well plate. The cells were then stimulated with plate bound anti-CD3 (5 µg/mL) and anti-CD28 (5 µg/mL). They were then treated with hydrolysates (1 mg/mL) or 10% DMSO (positive control). Cells were incubated for 72 hours. Viability was assessed using an MTS assay (Cell Titer 96 Aqueous Solution – Promega) according to the manufacturer's instructions. Results were expressed as a percentage of cells alone. At 1 mg/mL hydrolysates did not have any significant effect on cell viability on naïve undifferentiated T cells when compared to untreated cells (**Figure 4.2**).

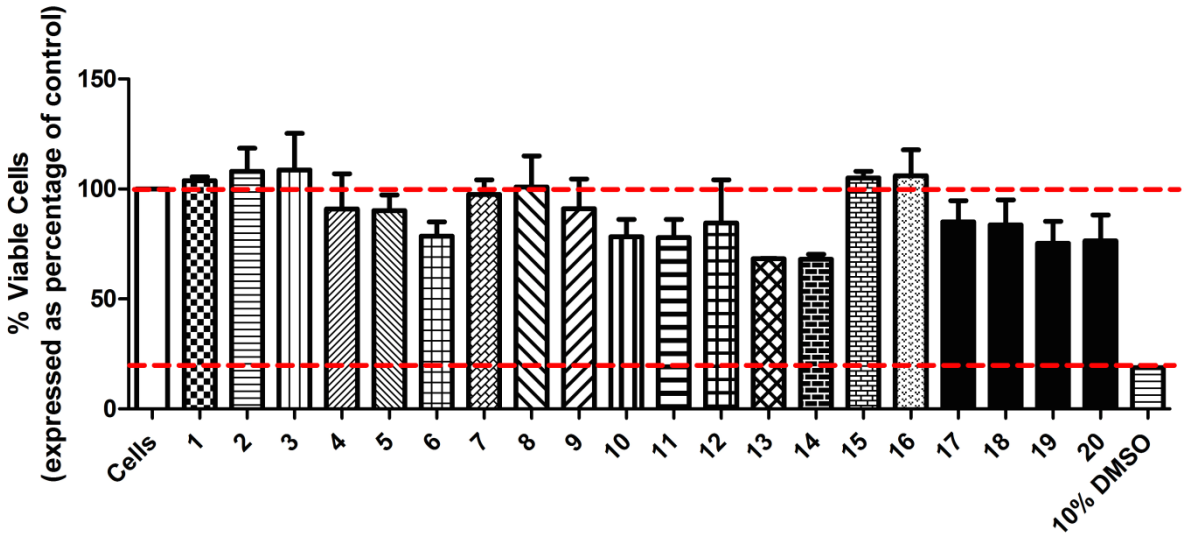


Figure 4.4 Undifferentiated CD4$^+$ T cell viability expressed as a percentage of cells alone following treatment with boarfish hydrolysate. CD4$^+$ T-cells were isolated from the spleens of Balb/c mice using EasySep CD4$^+$ Isolation (Stemcell). Cells were plated at 1 x 10^6 cells/mL on a 96 well plate. Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates (1 mg/mL) and incubated for 72 hours. DMSO (10% v/v) was included as a positive control of cytotoxicity. MTS assay (Cell Titer 96 Aqueous One Solution) was used to assess cell viability.

4.2.3 Cytokine secretion by BMDCs treated with boarfish protein hydrolysates.

Boarfish protein hydrolysates were screened on BMDCs and their effects on cytokine secretion analysed. BMDCs were isolated as in **Section 4.2.1** and treated with hydrolysates (1 mg/mL), and then stimulated with 100 ng/mL LPS. Secretion of cytokines (TNFα, IL-10, IL-6 and IL-12p40) was then assessed using ELISA (R&D Duoset).

Figure 4.5 shows cytokine secretion by BMDC treated with boarfish hydrolysates enzymatically digested for 1 hour, hydrolysates H1, H5, H9, and H13 and WPC17. H1 decreased IL-10 secretion ($p<0.001$) but had no effect on the other cytokines. H5 reduced TNFα levels ($p<0.01$) and IL-10 ($p<0.001$). H9 had no significant effect on any cytokine. H13 decreased the secretion of TNFα ($p<0.001$), IL-10 ($p<0.01$) and IL-12p40 ($p<0.01$). WPC17 significantly decreased the secretion of TNFα ($p<0.001$), IL-10 ($p<0.001$), IL-6 ($p<0.01$) and IL-12p40 ($p<0.001$). No boarfish hydrolysates enzymatically digested for 1 hour altered cytokine secretion by BMDCs differently to their whole protein control.

Figure 4.6 shows cytokine secretion by BMDC treated with boarfish hydrolysates enzymatically digested for 2 hours, hydrolysates H2, H6, H10, H14 and WPC18. H2 decreased IL-10 ($p<0.001$) but had no effect on any other cytokine. H6 decreased TNFα ($p<0.01$) and IL-10 ($p<0.001$). H10 decreased IL-10 secretion ($p<0.01$). H14 decreased TNFα ($p<0.01$), IL-10 ($p<0.01$) and IL-12p40 ($p<0.001$). WPC18 decreased TNFα ($p<0.001$), IL-10 ($p<0.001$) and IL-12p40 ($p<0.001$). H6 significantly altered cytokine secretion by BMDCs differently to WPC18 and is a potential candidate for further-screening.

Figure 4.7 shows cytokine secretion by BMDC treated with boarfish hydrolysates enzymatically digested for 3 hours, hydrolysates 3, H7, H11, H15 and WPC19. H3, H7 and H11 reduced IL-10 ($p<0.001$) only. H15 reduced IL-10 ($p<0.01$) and IL-12p40 ($p<0.001$). WPC19 decreased TNFα ($p<0.001$) and IL-10 ($p<0.001$). H15 significantly altered cytokine secretion of BMDCs differently to WPC19 and is a potential candidate for further screening.

Figure 4.8 shows cytokine secretion by BMDC treated with boarfish hydrolysates enzymatically digested for 4 hours, hydrolysates H4, H8, H12, H16 and WPC20. H4 and H8 reduced IL-10 ($p<0.01$ (H4) and $p<0.001$ (H8)) only. H12 reduced TNFα ($p<0.001$) and IL-10 ($p<0.01$). H16 decreased TNFα ($p<0.01$) and IL-12p40 ($p<0.01$). WPC20 decreased TNFα ($p<0.001$) and IL-10 ($p<0.001$). No boarfish hydrolysates enzymatically digested for 4 hours alter cytokine secretion by BMDCs differently to WPC20.

These results were then compared to the hydrolysates effects on the cytokine secretion of naïve undifferentiated T cells before potential candidates were brought forward for further screening.

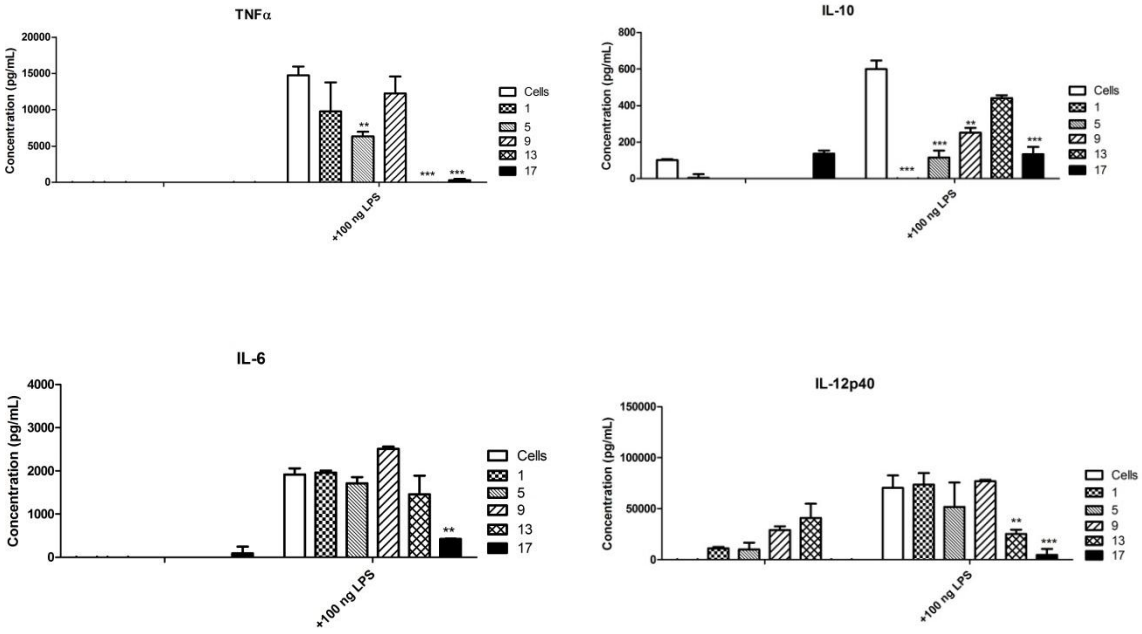


Figure 4.5 Secretion of TNFα, IL-10, IL-6 and IL-12p40 by BMDCs treated with Boarfish 1 hour hydrolysates. BMDCs were isolated from Balb/c mice and differentiated in rGMCSF for seven days. On day 7, cells were treated with hydrolysates and stimulated with 100 ng/mL LPS. Secretion of TNFα, IL-10, IL-6 and IL-12p40 was measured using ELISA (R&D Duoset). One-way ANOVA with a Post test Newman Keuls was used to assess differences between groups. Cells treated with hydrolysate only were compared to cells alone, cells treated with hydrolysates + LPS were compared to cells alone + LPS. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to untreated cells (no hydrolysate).

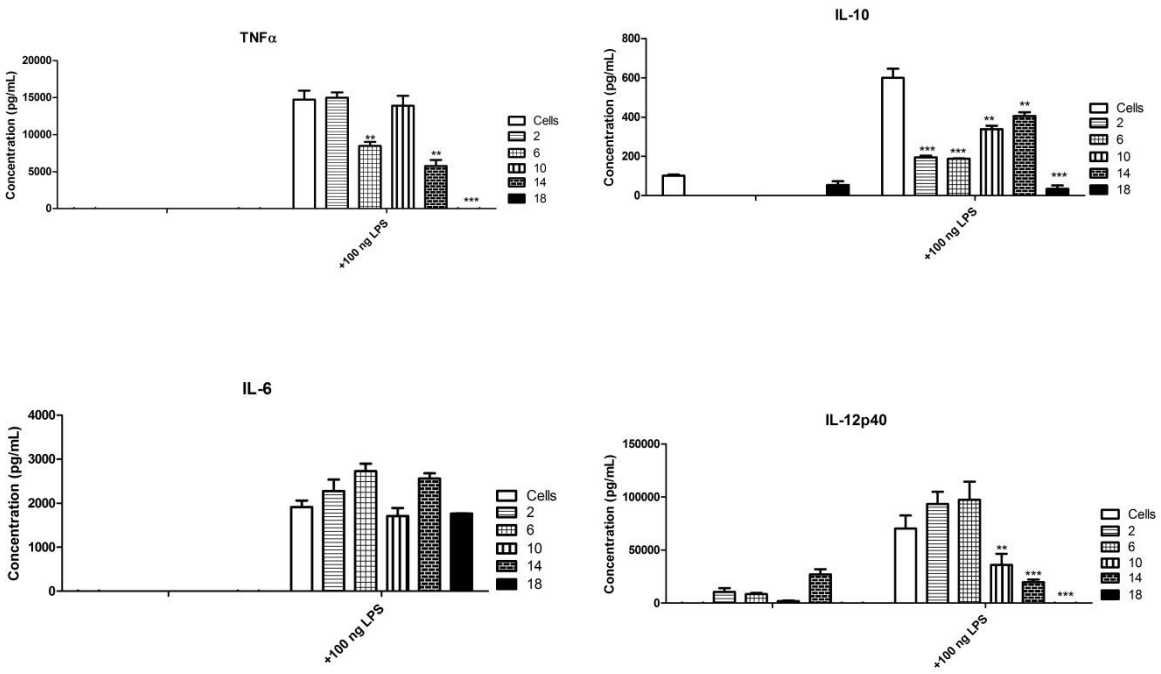


Figure 4.6 Secretion of TNFα, IL-10, IL-6 and IL-12p40 by BMDCs treated with Boarfish 2 hour hydrolysates. BMDCs were isolated from Balb/c mice and differentiated in rGMCSF for seven days. On day 7, cells were treated with hydrolysates and stimulated with 100 ng/mL LPS. Secretion of IL-12p40 and TNFα was measured using ELISA (R&D Duoset). One way Anova with a Post test Newman Keuls was used to assess differences between groups. Cells treated with hydrolysate only were compared to cells alone, cells treated with hydrolysates + LPS were compared to cells alone + LPS. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to untreated cells (no hydrolysate).

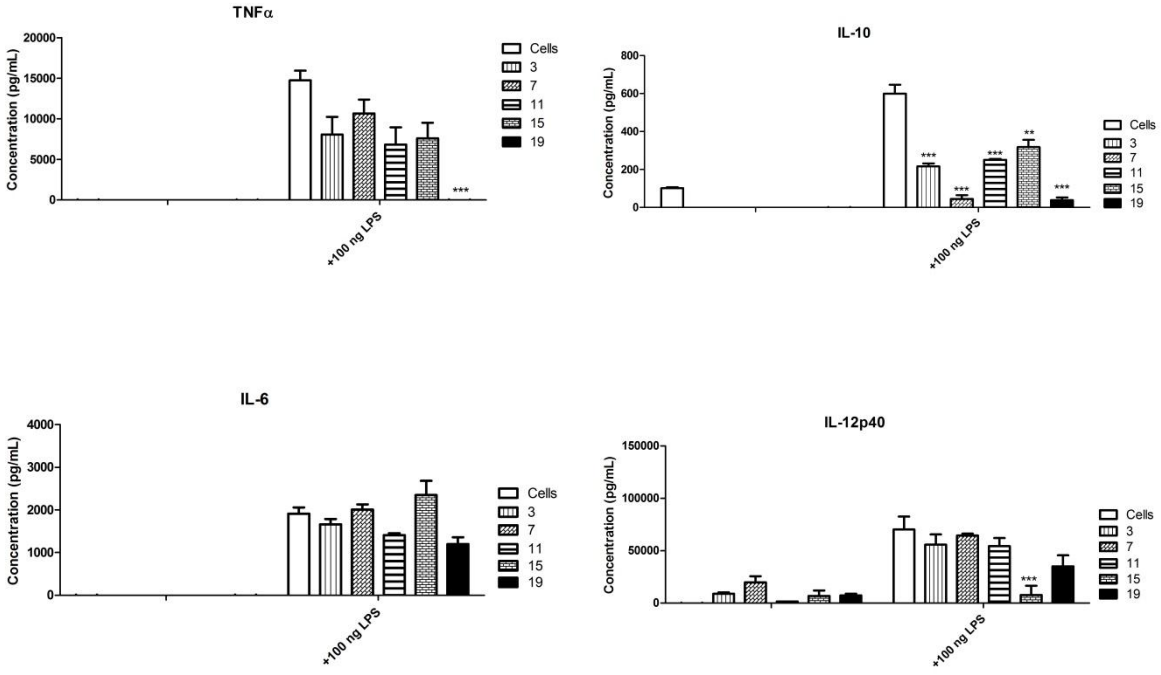


Figure 4.7 Secretion of TNFα, IL-10, IL-6 and IL-12p40 by BMDCs treated with Boarfish 3 hour hydrolysates. BMDCs were isolated from Balb/c mice and differentiated in rGMCSF for seven days. On day 7, cells were treated with hydrolysates and stimulated with 100 ng/mL LPS. Secretion of IL-12p40 and TNFα was measured using ELISA (R&D Duoset). One way Anova with a Post test Newman Keuls was used to assess differences between groups. Cells treated with hydrolysate only were compared to cells alone, cells treated with hydrolysates + LPS were compared to cells alone + LPS. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to untreated cells (no hydrolysate).

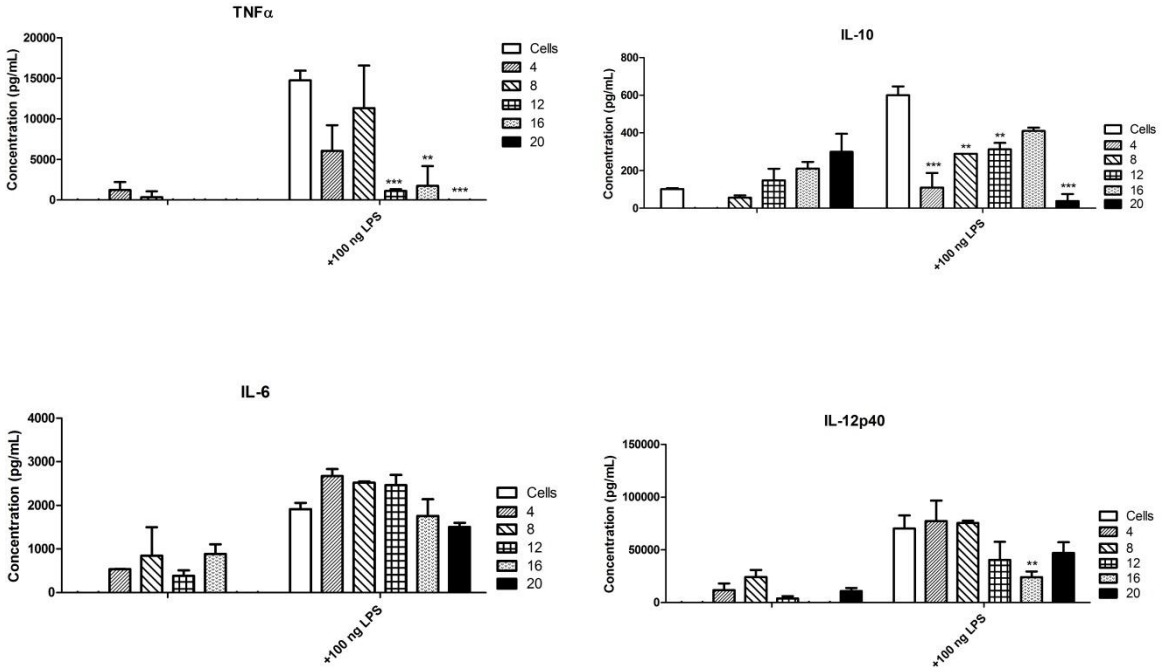


Figure 4.8 Secretion of TNFα, IL-10, IL-6 and IL-12p40 by BMDCs treated with Boarfish 4 hour hydrolysates. BMDCs were isolated from Balb/c mice and differentiated in rGMCSF for seven days. On day 7, cells were treated with hydrolysates and stimulated with 100 ng/mL LPS. Secretion of IL-12p40 and TNFα was measured using ELISA (R&D Duoset). One way Anova with a Post test Newman Keuls was used to assess differences between groups. Cells treated with hydrolysate only were compared to cells alone, cells treated with hydrolysates + LPS were compared to cells alone + LPS. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to untreated cells (no hydrolysate).

4.2.4 Cytokine secretion of undifferentiated CD4+ T cells treated with boarfish protein hydrolysates.

Boarfish protein hydrolysates were screened on undifferentiated $CD4^+$ T cells and their effects on cytokine secretion analysed. Undifferentiated $CD4^+$ T cells were isolated as before using an EasySep Mouse $CD4^+$ T cell Enrichment Kit and treated with hydrolysates at a dose of 1 mg/mL. Secretion of cytokines (IL-17, IL-2, IL-4, IL-6, IL-13, IFNγ and IL-10) was then assessed using ELISA (R&D Duoset).

Figure 4.9 and **Figure 4.10** shows secretion of IL-17, IL-2, IL-2, IL-6, IL-13, IFNγ and IL-10 by undifferentiated $CD4^+$ T cells treated with boarfish hydrolysates enzymatically digested for 1 hour, hydrolysates H1, H5, H9, H13 and WPC17. H1 did not alter any cytokine secretion. H5 decreased levels of IL-17 ($p<0.01$) and increased IL-4 ($p<0.001$) and IFNγ ($p<0.001$). H9 increased levels of IL-17 ($p<0.05$) and decreased IFNγ ($p<0.001$). H13 increased IL-4 production ($p<0.05$). WPC17 increased IL-17 ($p<0.05$) and decreased IFNγ ($p<0.001$) and IL-10 ($p<0.05$). No hydrolysate from this panel was chosen for further screening.

Figure 4.11 and **Figure 4.12** shows secretion of IL-17, IL-2, IL-2, IL-6, IL-13, IFNγ and IL-10 by undifferentiated $CD4^+$ T cells treated with boarfish hydrolysates enzymatically digested for 2 hours, hydrolysates H2, H6, H10, H14 and WPC18. H2 increased IL-17 ($p<0.001$) and decreased IL-2 ($p<0.01$). H6 reduced levels of IL-17 ($p<0.01$) and increased production of IL-4 ($p<0.01$) and IFNγ ($p<0.01$). H10 increased IL-17 ($p<0.01$) and reduced IL-2 ($p<0.01$). H14 decreased IL-17 ($p<0.01$) and increased IL-4 ($p<0.01$). WPC 18 decreased IL-2 ($p<0.001$) and IFNγ($p<0.05$). H6 significantly altered cytokine secretion of undifferentiated $CD4^+$ T cells differently to WPC18 and is a potential candidate for further screening. This hydrolysate was also identified in **Section 4.2.3** to be brought forward for further screening due to its ability to alter the cytokine secretion of BMDCs.

Figure 4.13 and **Figure 4.14** shows secretion of IL-17, IL-2, IL-2, IL-6, IL-13, IFNγ and IL-10 by undifferentiated $CD4^+$ T cells treated with boarfish hydrolysates enzymatically digested for 3 hours, hydrolysates H3, H7, H11, H15 and WPC19. H3 had no effect on cytokine secretion. H7 decreased IL-17 ($p<0.05$) and increased IL-2 ($p<0.001$). H11 reduced IL-2 ($p<0.01$). H15 increased IL-2 ($p<0.001$). WPC19 reduced levels of IL-6 ($p<0.01$) and IL-10

($p<0.05$). H7 significantly altered cytokine secretion of $CD4^+$ T cells (IL-17) differently to WPC19 and is a potential candidate for further screening in a Th17 subset.

Figure 4.15 and **Figure 4.16** shows secretion of IL-17, IL-2, IL-2, IL-6, IL-13, IFNγ and IL-10 by undifferentiated $CD4^+$ T cells treated with boarfish hydrolysates enzymatically digested for 3 hours, hydrolysates H4, H8, H12, H16 and WPC20. H4 reduced levels of IL-2 ($p<0.05$) whilst increasing levels of IL-4 ($p<0.05$). H8 increased levels of IL-17 ($p<0.05$) whilst it decreased IL-2 ($p<0.01$). H12 decreased IL-2 ($p<0.001$) and IL-6 ($p<0.01$) and increased levels of IL-4 ($p<0.001$). H16 increased IL-17 ($p<0.01$) whilst it decreased IL-2 ($p<0.001$), IL-6 ($p<0.01$) and IFNγ ($p<0.001$). WPC 20 decreased IL-2 ($p<0.05$), IL-6 ($p<0.001$) and IL-10 ($p<0.05$). No hydrolysate from this panel was chosen for further screening.

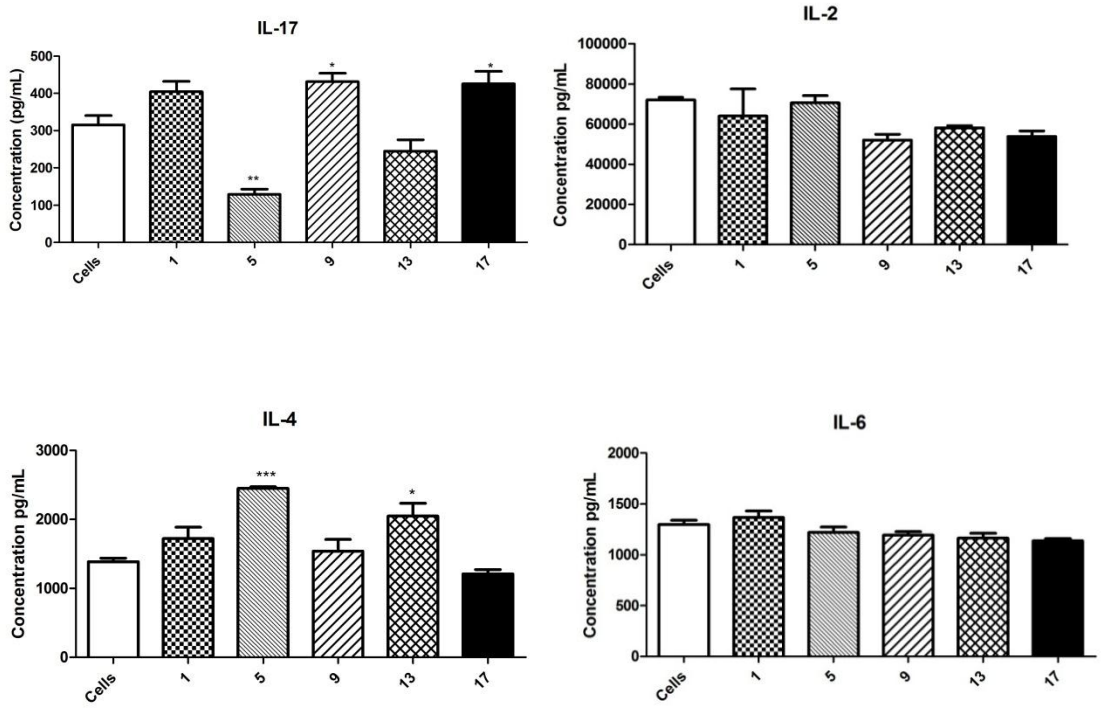


Figure 4.9 Effect of Boarfish 1 hour hydrolysates on cytokine secretion of undifferentiated CD4$^+$ T-cells. CD4$^+$ T-cells were isolated from the spleens of Balb/c mice using EasySep CD4$^+$ Isolation (Stemcell). Cells were plated at 1 x 10^6 cells / mL on a 96 well plate. Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates (1 mg/mL) and incubated for 72 hours. Secretion of IL-17, IL-2, IL-4 and IL-6 was measured using ELISA (R&D Duoset). One-way ANOVA with a Post test Newman Keuls was used to assess differences between groups. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to cells alone.

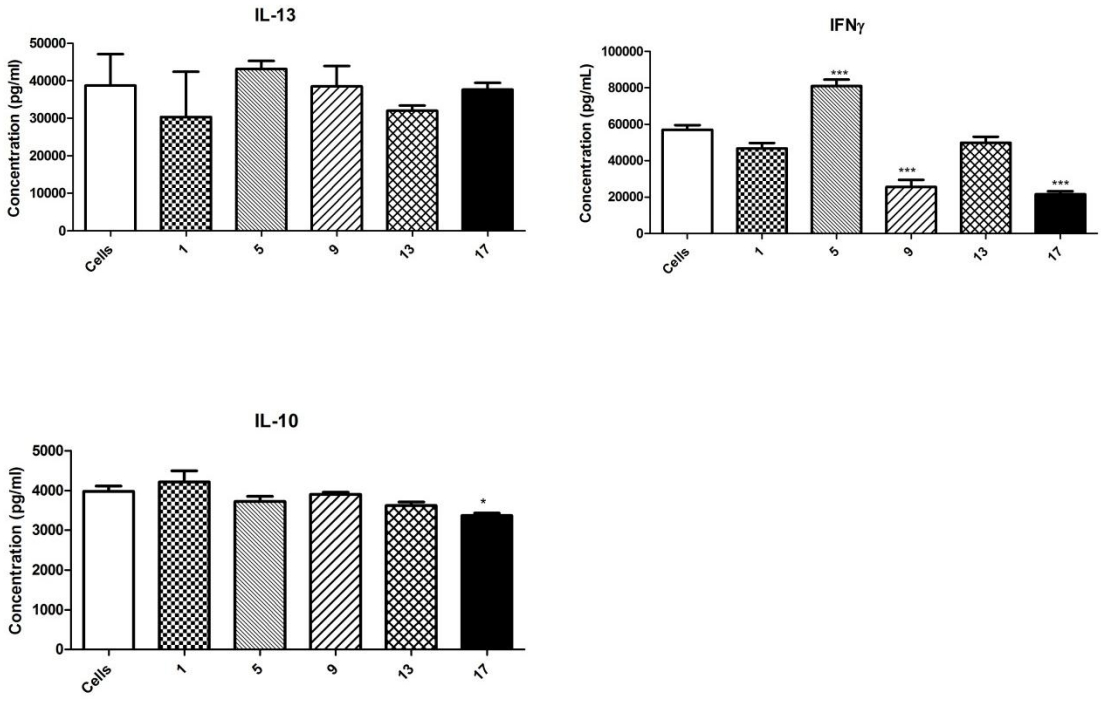


Figure 4.10 Effect of Boarfish 1 hour hydrolysates on cytokine secretion of undifferentiated CD4$^+$ T-cells. CD4$^+$ T-cells were isolated from the spleens of Balb/c mice using EasySep CD4$^+$ Isolation (Stemcell). Cells were plated at 1 x 10^6 cells / mL on a 96 well plate. Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates (1 mg/mL) and incubated for 72 hours. Secretion of IL-13, IFNγ and IL-10 was measured using ELISA (R&D Duoset). One-way ANOVA with a Post test Newman Keuls was used to assess differences between groups. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to cells alone.

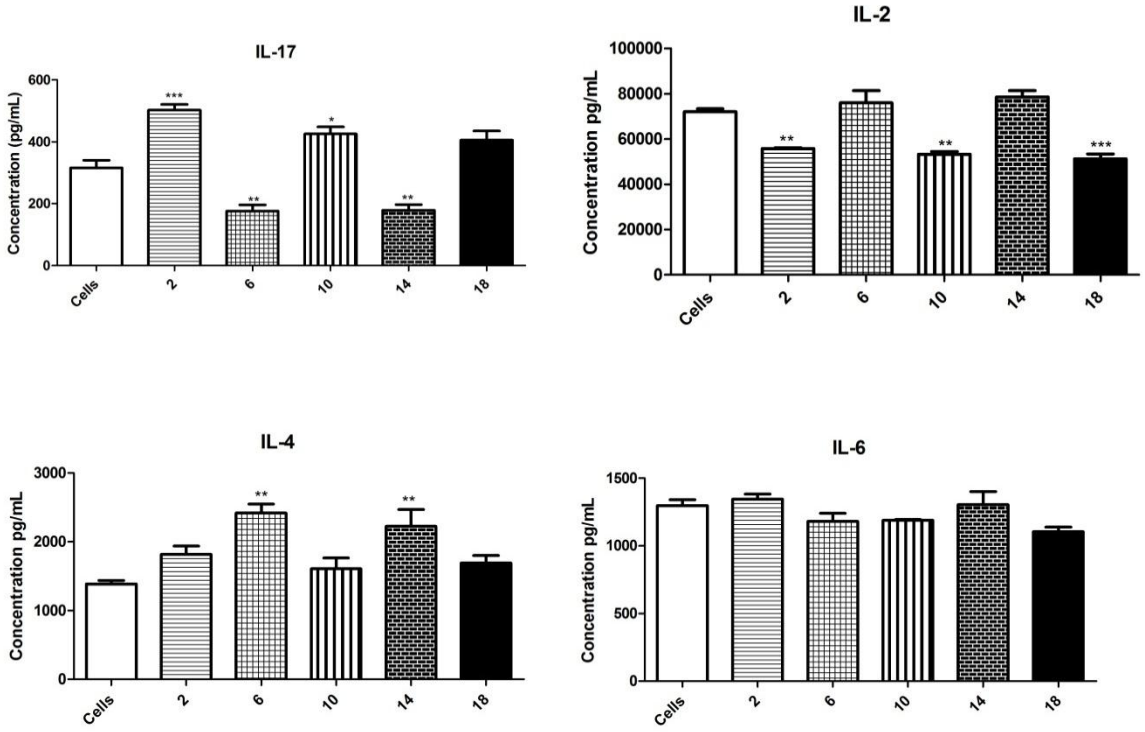


Figure 4.11 Effect of Boarfish 2 hour hydrolysates on cytokine secretion of undifferentiated CD4$^+$ T-cells. CD4$^+$ T-cells were isolated from the spleens of Balb/c mice using EasySep CD4$^+$ Isolation (Stemcell). Cells were plated at 1 x 10^6 cells / mL on a 96 well plate. Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates (1 mg/mL) and incubated for 72 hours. Secretion of IL-17, IL-2, IL-4 and IL-6 was measured using ELISA (R&D Duoset). Oneway ANOVA with a Post test Newman Keuls was used to assess differences between groups. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to cells alone.

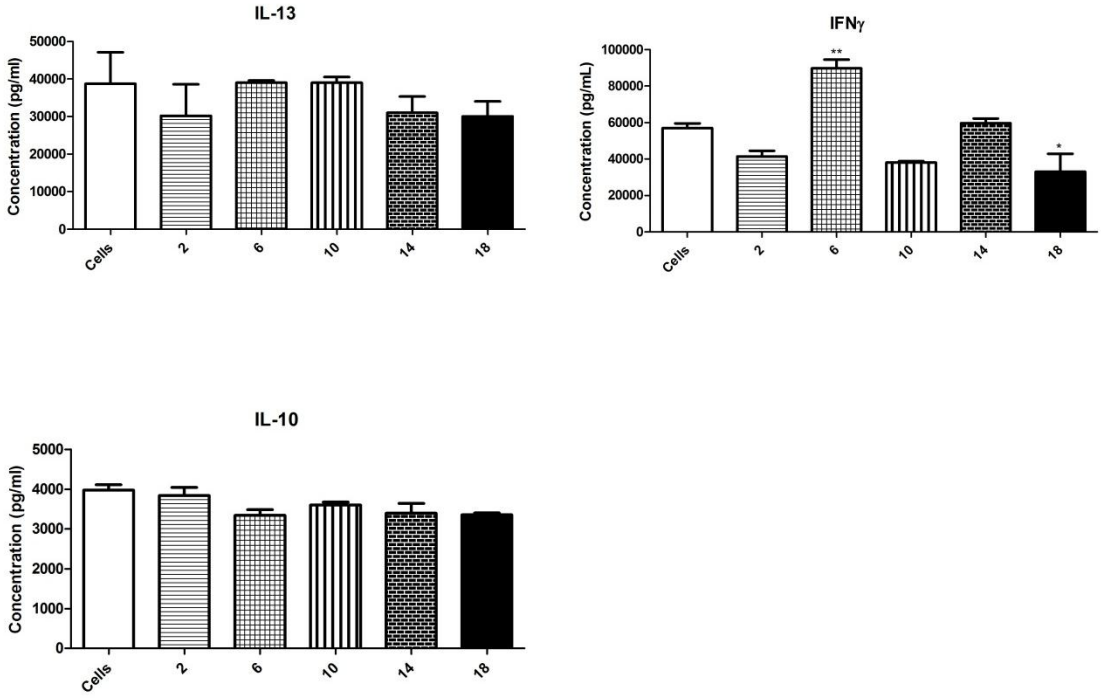


Figure 4.12 Effect of Boarfish 2 hour hydrolysates on cytokine secretion of undifferentiated CD4$^+$ T-cells. CD4$^+$ T-cells were isolated from the spleens of Balb/c mice using EasySep CD4$^+$ Isolation (Stemcell). Cells were plated at 1 x 10^6 cells / mL on a 96 well plate. Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates (1 mg/mL) and incubated for 72 hours. Secretion of IL-13, IFNγ and IL-10 was measured using ELISA (R&D Duoset). One-way ANOVA with a Post test Newman Keuls was used to assess differences between groups. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to cells alone.

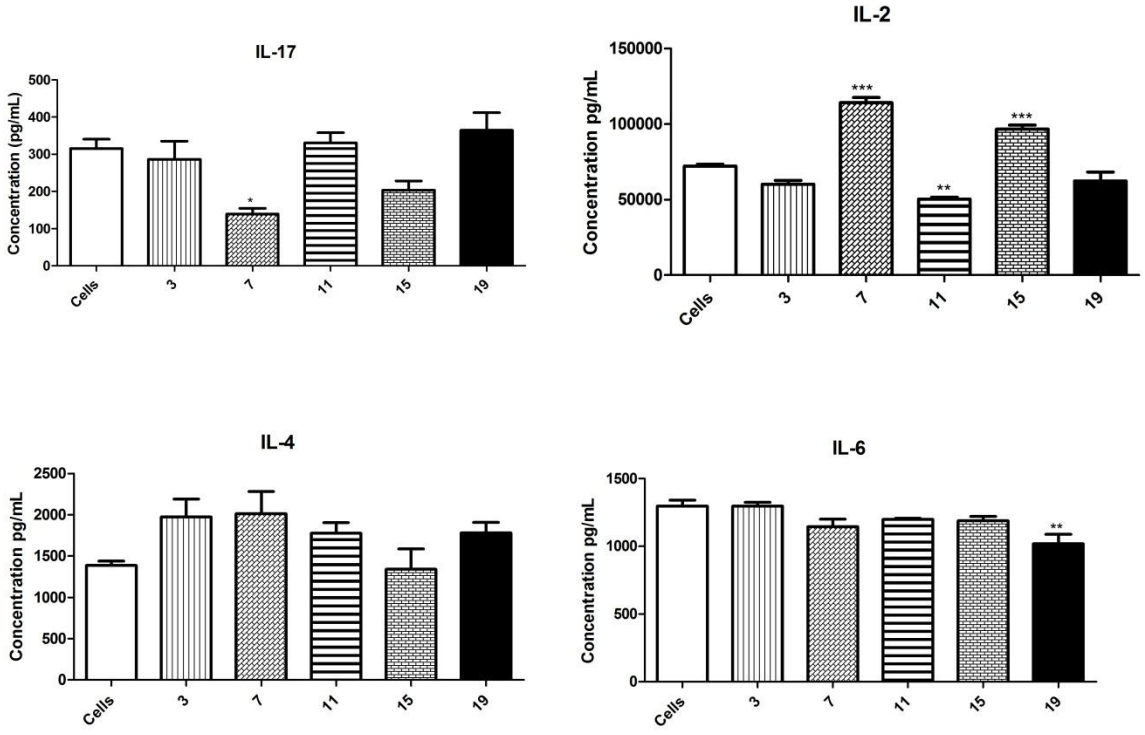


Figure 4.13 Effect of Boarfish 3 hour hydrolysates on cytokine secretion of undifferentiated CD4$^+$ T-cells. CD4$^+$ T-cells were isolated from the spleens of Balb/c mice using EasySep CD4$^+$ Isolation (Stemcell). Cells were plated at 1 x 10^6 cells / mL on a 96 well plate. Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates (1 mg/mL) and incubated for 72 hours. Secretion of IL-17, IL-2, IL-4 and IL-6 was measured using ELISA (R&D Duoset). One-way ANOVA with a Post test Newman Keuls was used to assess differences between groups. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to cells alone.

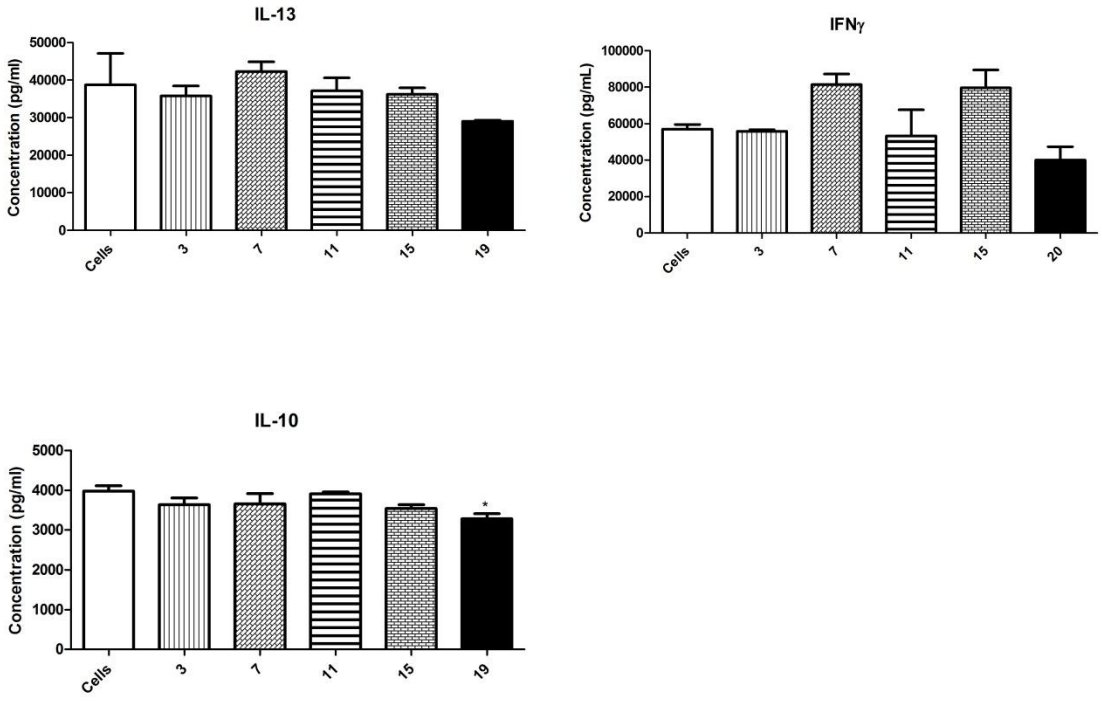


Figure 4.14 Effect of Boarfish 3 hour hydrolysates on cytokine secretion of undifferentiated CD4$^+$ T-cells. CD4$^+$ T-cells were isolated from the spleens of Balb/c mice using EasySep CD4$^+$ Isolation (Stemcell). Cells were plated at 1 x 10^6 cells / mL on a 96 well plate. Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates (1 mg/mL) and incubated for 72 hours. Secretion of IL-13, IFNγ and IL-10 was measured using ELISA (R&D Duoset). One-way ANOVA with a Post test Newman Keuls was used to assess differences between groups. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to cells alone.

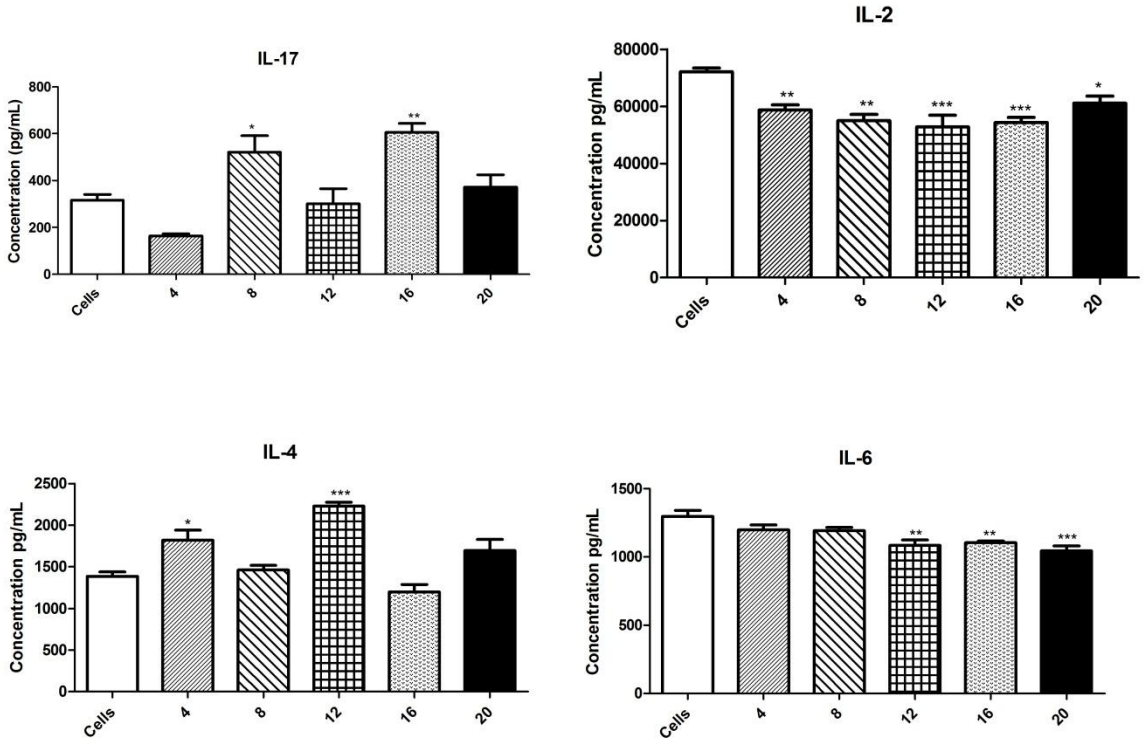


Figure 4.15 Effect of Boarfish 4 hour hydrolysates on cytokine secretion of undifferentiated CD4$^+$ T-cells. CD4$^+$ T-cells were isolated from the spleens of Balb/c mice using EasySep CD4$^+$ Isolation (Stemcell). Cells were plated at 1 x 10^6 cells / mL on a 96 well plate. Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates (1 mg/mL) and incubated for 72 hours. Secretion of IL-17, IL-2, IL-4 and IL-6 was measured using ELISA (R&D Duoset). One way ANOVA with a Post test Newman Keuls was used to assess differences between groups. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to cells alone.

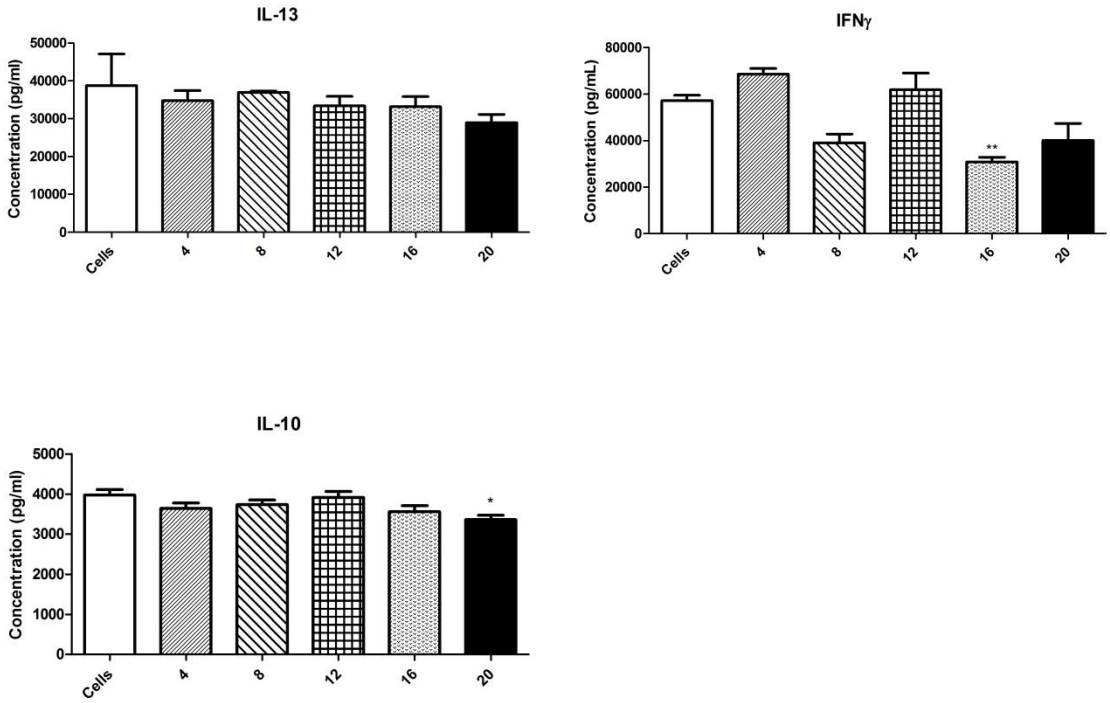


Figure 4.16 Effect of Boarfish 4 hour hydrolysates on cytokine secretion of undifferentiated CD4$^+$ T-cells. CD4$^+$ T-cells were isolated from the spleens of Balb/c mice using EasySep CD4$^+$ Isolation (Stemcell). Cells were plated at 1 x 10^6 cells / mL on a 96 well plate. Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates (1 mg/mL) and incubated for 72 hours. Secretion of IL-13, IFNγ and IL-10 was measured using ELISA (R&D Duoset). One way ANOVA with a Post test Newman Keuls was used to assess differences between groups. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to cells alone.

4.2.5 Cytokine secretion by T-helper cell 1 subset treated with selected boarfish protein hydrolysates.

Hydrolysates which had been identified as potential candidates (H6 and H7) in **Section 4.2.3 and Section 4.2.4** were then brought forward for analysis on T cell subsets. Th1 cells were isolated using an EasySep Mouse $CD4^+$ T cell Enrichment Kit and the media was supplemented with 20 ng/mL IL-2, 20 ng/mL IL-12 and 10 µg/mL anti-IL- 4 antibody for Th1 polarising conditions. Cells were treated with hydrolysates H6 and H7 along with their whole protein controls, WPC18 and WPC19 at a dose of 1 mg/mL and incubated for 72 hours before re-stimulation in fresh media for a further 24 hours. Secretion of cytokines (IL-13, IL-6, IL-10, IL-17, IL-4, IFNγ and IL-2) was then assessed using ELISA (R&D Duoset).

Figure 4.17 shows the effect of H6 and H7 and their controls WPC18 and WPC19 on cytokine secretion by Th1 cells. The key cytokines associated with Th1 cells are IFNγ and IL-2. H6 decreased levels of IFNγ ($p<0.001$). H7 reduced IL-2 secretion ($p<0.001$). WPC18 and WPC19 both decreased IL-2 production ($p<0.01$ (WPC18) and $p<0.05$ (WPC19). The hydrolysates had a number of other effects on cytokines produced by Th1 cells. H6 reduced levels of IL-6 ($p<0.01$) and increased IL-17 ($p<0.01$). H7 reduced IL-4 ($p<0.001$) and increased IL-10 ($p<0.001$) and IL-17 ($p<0.01$). H6 reduced IFNγ whilst its control WPC18 had no effect, this is indicative that this activity is as a result of hydrolysis. Before any hydrolysates are chosen for further work it is necessary to investigate their effects on other T cell subsets.

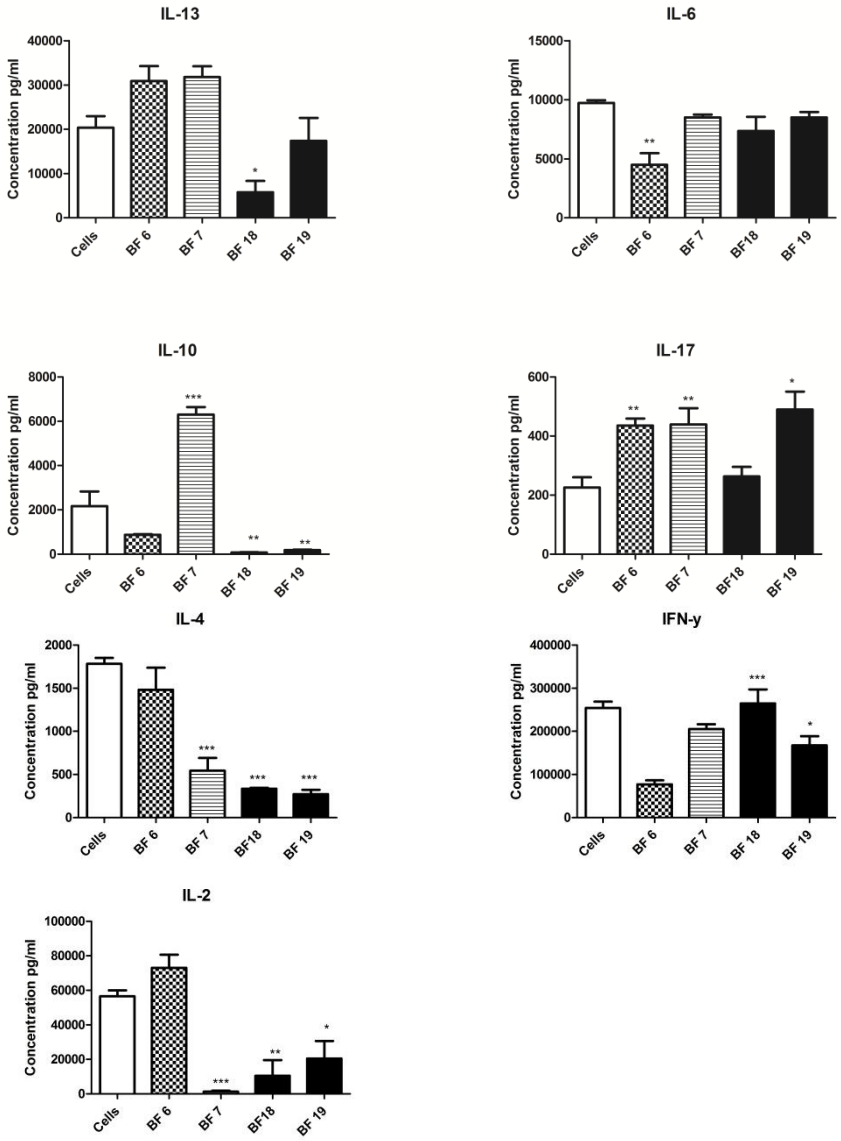


Figure 4.17 Secretion of IL-13, IL-6, IL-10, IL-17, IL-4, IFNγ and IL-2 by Th1 cells treated with selected Boarfish hydrolysates. $CD4^+$ T-cells were isolated from the spleens of Balb/c mice using EasySep $CD4^+$ Isolation (Stemcell). Cells were plated at 1 x 10^6 cells/mL on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates (1 mg/mL). The media was supplemented with 20 ng/mL IL-2, 20 ng/mL IL-12 and 10 μg/mL anti-IL-4 antibody for Th1 polarising conditions. Cells were incubated for 72 hours and then re-stimulated in fresh media for 24 hours. Cytokine secretion was measured using ELISA (R&D Duoset). One way ANOVA with a Post test Newman Keuls was used to assess differences between groups. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to cells alone.

4.2.6 Cytokine secretion by T helper cell 2 subset treated with selected boarfish protein hydrolysates.

Following their assessment on Th1 cells (**Section 4.2.5**), hydrolysates H6 and H7 were then screened on a Th2 subset in order to build a full profile of their effects on the wider immune system. Th2 cells were isolated using an EasySep Mouse $CD4^+$ T cell Enrichment Kit and the media was supplemented with 10 μg/mL neutralising IFNγ antibody and 10 mg/mL IL- 4 to induce Th2 polarising conditions. Cells were treated with hydrolysates H6 and H7 along with their whole protein controls, WPC18 and WPC19 at a dose of 1 mg/mL and incubated for 72 hours before re-stimulation in fresh media for a further 24 hours. Secretion of cytokines (IL-13, IL-6, IL-10, IL-17, IL-4, IFNγ and IL-2) was then assessed using ELISA (R&D Duoset).

Figure 4.18 shows secretion of IL-13, IL-6, IL-10, IL-17, IL-4, IFNγ and IL-2 from Th2 cells when treated with hydrolysates 6 and 7 and WPC 18 and 19. The key cytokines associated with Th2 cells are IL-13 and IL-4. H6 significantly decreased IL-13 ($p<0.05$) and IL-4 ($p<0.001$). H7 also decreased IL-13 ($p<0.001$) and IL-4 ($p<0.01$). WPC 18 and WPC19 also both reduced the secretion of these cytokines, IL-13 ($p<0.001$) and IL-4 ($p<0.001$). No hydrolysate actively promoted the Th2 subset or displayed activity different to that of their whole protein control.

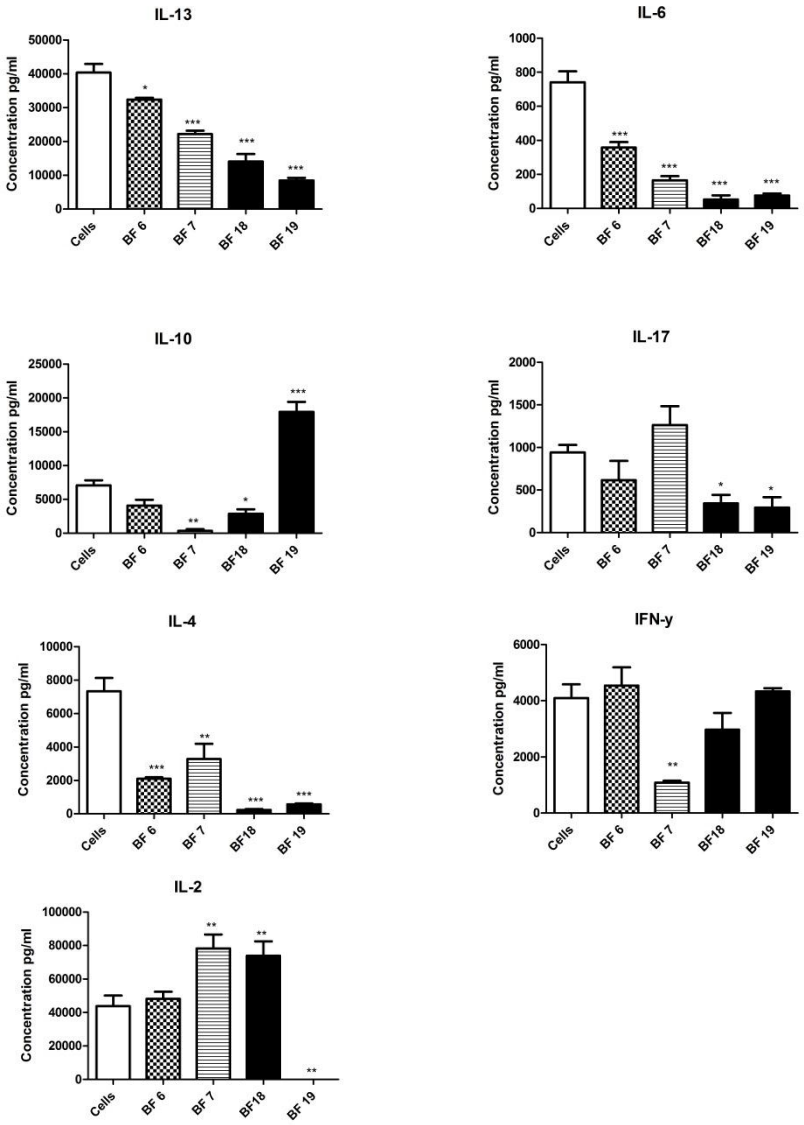


Figure 4.18 Secretion of IL-13, IL-6, IL-10, IL-17, IL-4, IFNγ and IL-2 by Th2 cells treated with selected Boarfish hydrolysates. $CD4^+$ T-cells isolated from the spleens of Balb/c mice using EasySep $CD4^+$ Isolation (Stemcell). Cells were plated at 1 x 10^6 cells/mL on a 96 well plate. Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates (1 mg/mL). The media was supplemented with 10 μg/mL neutralizing IFNγ antibody and 10 mg/mL IL-4 for Th2 polarising conditions. Cells were incubated for 72 hours and then re-stimulated in fresh media for 24 hours. Cytokine secretion was measured using ELISA (R&D Duoset). One way ANOVA with a Post test Newman Keuls was used to assess differences between groups. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to cells alone.

4.2.7 Cytokine secretion by T helper cell 17 subset treated with a selected boarfish protein hydrolysate.

In order to complete the profile of the hydrolysates effect on T cell subsets, hydrolysate H6's effects on the cytokine secretion of Th17 cells were assessed. Th17 cells were isolated using an EasySep Mouse $CD4^+$ T cell Enrichment Kit and the media was supplemented with 10 µg/mL anti-IFNγ antibody, 10 µg/mL IL-4, 20 ng/mL IL-6, 10 ng/mL IL-23 and 2 ng/mL TGFβ to induce Th17 polarising conditions. Cells were treated with hydrolysate H6 along with its whole protein control WPC 18 at a dose of 1 mg/mL and incubated for 72 hours before re-stimulation in fresh media for a further 24 hours. Secretion of cytokines (IL-17, IL-6, IL-4, IFNγ and IL-2) was then assessed using ELISA (R&D Duoset).

Figure 4.19 shows secretion of IL-17, IL-6, IL-4, IFNγ and IL-2 from Th17 cells when treated with hydrolysate H6 and WPC18. IL-17 is the key cytokine associated with Th17 cells. WPC18 increased levels of IL-17 ($p<0.001$) whilst H6 had no effect. H6 increased levels of IL-4 ($p<0.01$) and decreased IL-2 ($p<0.01$). WPC 18 decreased IL-6 ($p<0.05$) also.

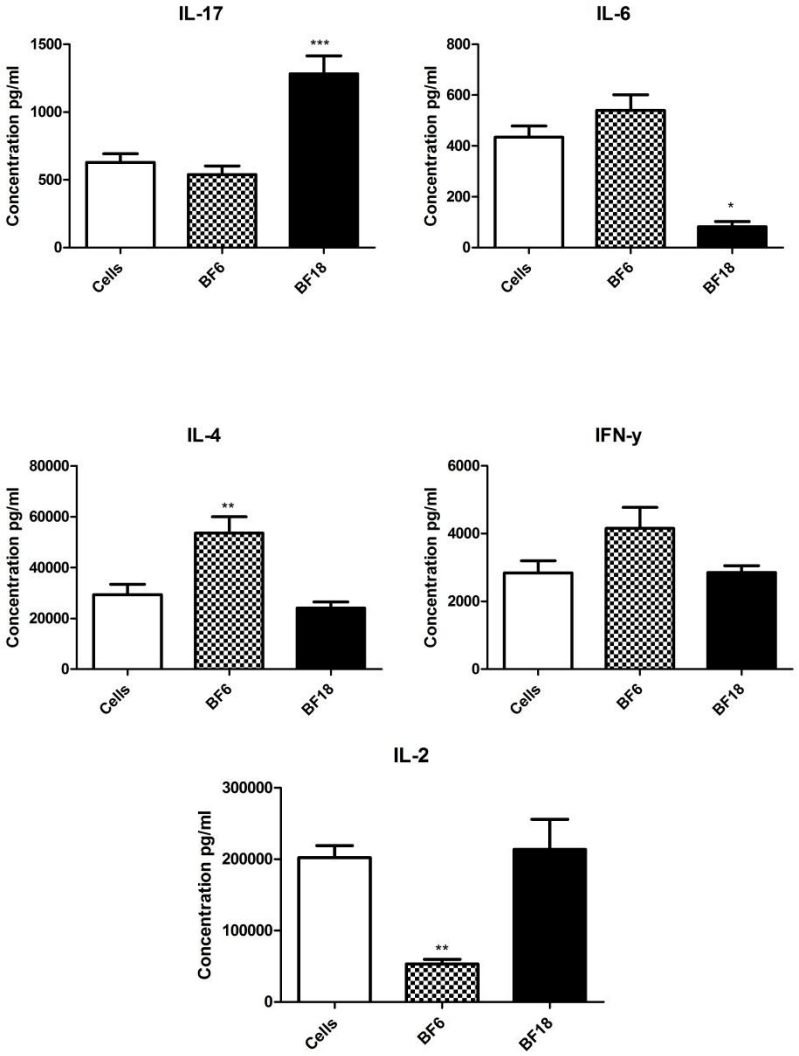


Figure 4.19 Secretion of IL-17, IL-6, IL-4, IFNγ and IL-2 by Th17 cells treated with selected Boarfish hydrolysates. $CD4^+$ T-cells isolated from the spleens of Balb/c mice using EasySep $CD4^+$ Isolation (Stemcell). Cells were plated at 1 x 10^6 cells / mL on a 96 well plate. Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates (1 mg/mL). The media was supplemented with 20 ng/mL IL-6, 10 ng/mL IL-23, 2 ng/mL TGFβ, 10 μg/mL anti IFNγ antibody and 10 μg/mL anti IL-4 antibody for Th17 polarising conditions. Cells were incubated for 72 hours and then re-stimulated in fresh media for 24 hours. Cytokine secretion was measured using ELISA (R&D Duoset). One way ANOVA with a Post test Newman Keuls was used to assess differences between groups. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to cells alone.

4.3 Discussion

Chronic inflammation is a prolonged phenomenon which can cause the destruction of tissue. It is the underlying cause of many diseases including rheumatoid arthritis (Tak *et al.,* 2000), diabetes (Donath and Shoelson, 2011) and Alzheimer's (Tuppo and Arias, 2005). Chronic inflammation can also be asymptomatic and is present in a majority of elderly people in the form of 'inflammaging'. This chronic low-grade inflammation has been said to be the leading cause of many age-related diseases (Franceschi and Campisi, 2014). Immune cells such as dendritic cells and T cells have been implicated in all forms of chronic inflammation. Dendritic cells migrate from blood to tissue and the lymph nodes. In a normal state they are present in very low numbers and the rate of migration is minimal. In inflammatory conditions, notably chronic inflammation, this rate of migration increases (Sallusto and Lanzavecchia, 1999). This sudden influx of dendritic cells, which then become activated, results in the production of pro-inflammatory cytokines such as TNFα, IL-6 and the IL-12 family. The production of these cytokines begins a cascade which attracts T cells to the site of inflammation (Langrish *et al.,* 2005).

T-cells are a type of lymphocyte at the core of the adaptive immune system. T-cells come in various subtypes such as effector, memory, cytotoxic and helper. T helper cells, such as naïve $CD4^+$ T cells, assist in many processes including maturation of B cells and activation of macrophage. They become activated when presented with MHCII molecules on the surface of APCs (Mosmann and Sad, 1996). They play a key role in inflammatory disease and chronic inflammation through their role in these processes and the secretion of pro-inflammatory cytokines such as IFNγ and IL-17 but also through their ability to differentiate into T helper subsets such as Th1 and Th17. These subsets drive inflammation and have been implicated in many inflammatory diseases (Dardalhon *et al.*, 2008). Altering the cytokine secretion of DCs and T cells can reduce chronic inflammation.

Boarfish, a small pelagic fish not usually found in Irish waters, has dramatically increased its numbers due to the warming of the Bay of Biscay and the Celtic Sea. Once a very small presence in other catches, Boarfish are now being caught in the thousands of tonnes

range. The Irish EU fishing quota increasing from 120 tonnes to 137,500 tonnes, an increase of over 137,250 tonnes (Bord Iascaigh Mhara, 2018). Unfortunately, due to their size and high lipid, bone and cartilage content they are hard to process into high quality food and are generally unappetising, hence, a use for them is required. They are, however, packed with protein with some processes being able to obtain up to 60% mean protein content from a whole milled fish. Thus, making them ideal candidates for possessing potential bioactive peptides (Ojha *et al.,* 2016).

Proteins were extracted from boarfish by collaborators in the University of Limerick and enzymatically hydrolysed using various enzymes and also various degrees of hydrolysis controlled through time. Twenty (20) overall hydrolysate samples were produced, as described in **Table 3.1**. The overall aim of this chapter is to investigate the ability of boarfish protein hydrolysates to modulate the immune system and their potential as a novel functional ingredient. In particular, the ability to switch an immune system from a state of chronic inflammation to a healthy state is of most interest. This chapter used the screening protocol optimised in Chapter 3, in order to identify candidate hydrolysates that can modulate the immune response away from a pro-inflammatory state to a normal steady state.

The effect of these samples on the viability of these key immune cells, dendritic cells and T cells, was first examined. This is an essential first step in the investigation as it is necessary to ensure the hydrolysates are not cytotoxic to cells at the concentration used (1 mg/mL). This concentration was used as it had been found to be the optimum dose for *Palmaria palmata* in Chapter 3 and is also the concentration used by collaborators in other applications of the hydrolysates.

Boarfish hydrolysates had no significant impact on the viability BMDCs when treated at a concentration of 1 mg/mL. This was as expected as likewise PP had no effect on the viability of these cells at this concentration. Hydrolysates 1 – 10 have no negative impact on the viability of BMDCs. Hydrolysates 11 – 15 slightly decreased viability but not

significantly. Hydrolysate 16 and WPCs 17 – 20 decreased viability of the BMDCs but did not decrease it beyond 70%. This displays how hydrolysing the whole proteins causes the boarfish to become less cytotoxic to the cells. The effect of these hydrolysates, or boarfish in general, on cell viability has never been reported hence it was necessary to examine this before continuing with the investigation. Similarly, the impact of the hydrolysates on the viability of $CD4^+$ T cells was examined. The overall majority of hydrolysates displayed no significant cytotoxic effect. Hydrolysates 6, 10 and 11 reduced viability to approximately 85% and 13 and 14 to roughly 70%. This reduction, whilst notable, does not indicate the hydrolysates are particularly harmful as the positive control (10% DMSO) reduces levels to 20%.

The ability of the hydrolysates to modulate the cytokine secretion of BMDCs was then examined. Levels of the pro-inflammatory cytokines TNFα, IL-6 and IL-12p40 and anti-inflammatory IL-10 were analysed by ELISA. From this study, it was clear that boarfish hydrolysates can modulate the cytokine secretion of BMDCs. Hydrolysates were grouped in order of length of digestion, as described in Table 3.1. Treatment of BMDCs by hydrolysates 5/6/12/13/14/16 caused a reduction in TNFα production and hydrolysates 10/13/14/15/16 reduced IL-12p40 significantly, the key cytokines which drive inflammation in BMDCs. However, many of these hydrolysates also decreased levels of anti-inflammatory IL-10.

A number of hydrolysates displayed the ability to reduce the secretion of TNFα by BMDCs. H6 also reduced TNFα but did not completely suppress it like its whole protein control 18. Increased levels of TNFα are found in both acute and chronic inflammatory conditions. In acute inflammatory situations TNFα protects against bacterial endotoxin and invasion from other microorganisms. In chronic inflammation, increased levels of this cytokine has been shown to cause tissue damage (Berrrrpohl *et al.*, 2007), insulin resistance development (Saghizadeh *et al.*, 1996) and atherosclerotic changes (Bruunsgaard *et al.*, 2000), amongst many other changes. Suppression of this pro-inflammatory cytokine in chronic inflammation has been shown to improve symptoms of many chronic

inflammatory diseases including RA and other arthritis', IBD (both ulcerative colitis and Crohns' disease) and psoriasis (Rutgeerts, Assche and Vermeire, 2004). These current treatments include monoclonal antibodies such as the chimeric infliximab (Remicade®), the humanised adalimumab (Humira®) and the humanised certolizumab pegol (Cimzia®) as well as the fusion protein etanercept (Enbrel®). A major side effect of these current therapies is increased susceptibility to infection due to the complete suppression of TNFα, which as described above, provides protection against invasion of microorganisms in acute inflammatory responses. This has led to patients being at increased risk of opportunistic infections including tuberculosis (Dixon *et al.*, 2010). This may be due to current therapies completely suppressing TNFα in the patient. Hydrolysate 6 reduces secretion of TNFα but, unlike its whole protein control (18), does not suppress its secretion fully. The ability to reduce, but not get rid, of TNFα could provide relief from chronic inflammation and its symptoms caused by TNFα and reduce its effects such as tissue damage whilst maintaining the immune system of the patient. This ability to reduce a pro-inflammatory cytokine such as TNFα but not completely suppress it could also be beneficial in the treatment of inflammaging. Inflammaging, as discussed previously, is chronic low-grade inflammation. During inflammation, pro-inflammatory cytokines are increased, but not to the same levels as inflammatory disease (Sergio, 2008). Hence, the ability of H6 to reduce cytokine secretion of TNFα, but not completely suppress like its WPC, could be a therapeutic target for inflammaging therapies.

Hydrolysate 6 also reduced levels of IL-12p40. IL-12p40 is a member of the IL-12 cytokine family, namely IL-12p70 and IL-23. This subunit acts as a feedback loop in the IL-12 family and has been shown to be increased in chronic inflammatory states (Cooper and Khader, 2007). The IL-12 family play a major role in driving inflammation and have been implicated in numerous inflammatory diseases including IBD (Oppmann *et al.,* 2000). IL-23, of which IL-12p40 is a component, has been considered vital in the onset and progression of IBD, particularly Crohn's disease (Neurath, 2007). IL-12p40 has been identified as a potential target for therapeutics that combats inflammation (Kikly *et al.,* 2006). Hence, a

hydrolysate which reduces this cytokine could be beneficial in the fight against inflammation.

The hydrolysates effect on the cytokine secretion of $CD4^+$ naïve undifferentiated T cells was then investigated as per the screening protocol. $CD4^+$ T cells produce cytokines such as IL-17, IL-2, IL-4, IL-6, IL-13, IFNγ and IL-10. Treatment of $CD4^+$ T cells by hydrolysates 5/6/7/14 decreased levels of IL-17, a cytokine vital for differentiation of the pro-inflammatory Th17 subset, whilst hydrolysates 9 and 16 both decreased levels of Th1 promoter IFNγ. Hydrolysates 4/5/7/8/9/12/16 all promoted levels of IL-4 and IL-2 necessary for driving a Th2 subset which inhibits a Th1 differentiation and promotes a balanced immune system when in a pro-inflammatory state. Hydrolysate 6 and 7 both reduced the levels of IL-17 from undifferentiated $CD4^+$ T cells. IL-17 is a pro-inflammatory cytokine which stimulates the production of TNFα (Sergio, 2008). IL-17 is produced by T cells and is the key cytokine produced by Th17 cells. This cytokine has been implicated in pathogenesis of several inflammatory diseases including psoriasis and RA (Miossec and Kolls, 2012). There are a large number of therapies currently in the pipeline which target IL-17 and the IL-17 pathway. This includes 13 monoclonal antibodies including Novartis' Secukinumab, a fully human mAb which has reached phase III clinical trials for psoriatic arthritis, RA and ankylosing spondylitis, phase II for asthma and MS and is in the pre-registration phase for psoriasis. Due to their ability to reduce $CD4^+$ T cells secretion of IL-17, a recognised target in the fight against chronic inflammation, and hydrolysates 6 ability to reduce TNFα, both hydrolysate 6 and 7 were chosen to be brought forward for further analysis.

The next step was to assess the effects of hydrolysates 6 and 7 on T cell subsets. For the purpose of this project, as per protocol (**Figure 4.2**); Th1, Th2 and Th17 cells were screened. Hydrolysate H6 reduced production of IFNγ from Th1 cells, whilst its whole protein control promoted the secretion of this pro-inflammatory cytokine. Hydrolysate 6 whilst decreasing IL-13 in Th2 cells, had no major significant impact on cytokines which

drive Th2 or Th17 subsets, suggesting a level of selectivity in the mechanism through which it works.

Hydrolysate 7 similarly reduced IFNγ secreted from Th1 cells, although not as potently as hydrolysate 6 was still a significant reduction. 7 also reduced levels of IL-13, IL-10 and IL-4 from Th2 cells. This reduction in levels of Th2 differentiation is not ideal and ultimately could result in the driving of inflammation as the Th1/Th2 balance is offset into a pro-inflammatory Th1 state. This is a significant finding as IFNγ is thought to play a major role in both the onset and severity of systemic autoimmunity, being implicated in many inflammatory based diseases such as lupus erythematosus (Berrrrpohl *et al.*, 2007). Clinical trials of anti-IFNγ antibodies for treatment of lupus are ongoing and showing positive results (Furie *et al.*, 2017) Secretion of IFNγ has also been shown to be increased in Crohn's disease (Fuss *et al.*, 1996) and it is hoped that anti-IFNγ therapies could succeed in reducing disease onset. Hence, the potent reduction of secretion of IFNγ from Th1 cells by hydrolysate 6 could be of therapeutic benefit to many patients. IFNγ plays an important role in the immune system mediating both anti-viral and anti-bacterial immunity whilst activating macrophage and inducing MHC expression and activating the innate immune system (Tau and Rothman, 1999). Due to this vital role, complete suppression of IFNγ could lead to detrimental side effects including immunosuppression. Hydrolysate 7 reduces levels of IFNγ whilst maintaining the hosts defence and could be a useful potential candidate for treatment in IFNγ driven disease.

Hydrolysate 7 also reduced secretion of IL-10, IL-13 and IL-4 from Th2 cells. Th2 cells have been shown to inhibit the differentiation of the pro inflammatory Th1 cell and, thus, reduce inflammation. The Th1/Th2 balance hypothesis states that immune homeostasis or "immunostasis" is achieved through the balance of Th1 and Th2 T helper cells (Kidd P, 2003). In cases of chronic inflammation this immunostasis is not present due to an increase of Th1 cells. Through increasing differentiation of Th2 cells it is hypothesised that Th1 differentiation would be inhibited through the secretion of anti-inflammatory cytokine IL-10 by Th2 cells (Nicholson and Kuchroo, 1996). Therefore the suppression of

IL-10 from Th2 cells by hydrolysate 7 would not be ideal and as such this candidate was not chosen to be brought forward for *in vivo* work.

The overall aim of this project was to identify novel marine hydrolysates which would be suitable for commercialisation as functional food ingredients that reduce chronic inflammation, both high and low grade. This was a collaborative project with the University of Limerick who isolated and hydrolysed the boarfish samples (as per **Figure 4.1**). As such, it was necessary to ensure the candidate samples were suitable for both further analysis by the University of Limerick and large-scale commercial production. Upon analysis of hydrolysate 6 it was decided that this was not a suitable candidate for this project due to the nature of their hydrolysis and the problems one would face in its large-scale commercial production.

The results from this investigation demonstrate for the first time that boarfish protein hydrolysates have no cytotoxic effects against key immune cells, T cells and BMDCs. It also showed that whilst no candidate hydrolysate was chosen to be brought forward, Boarfish hydrolysates do possess the ability to modulate the immune system. Hydrolysates were capable of suppressing an inflammatory response in both T cells and BMDCs, which have been implicated in chronic inflammation and inflammatory diseases.

Chapter 5:

Investigating the potential of Blue Whiting as a source of anti-inflammatory protein hydrolysates

5.1 Introduction

Irish waters have experienced an increase in many fish populations in the past two decades due to warming temperatures as a result of climate change and a rise in the fauna from which fish feed (Perry *et al*., 2005). Blue whiting is one such fish. Blue whiting is a member of the cod family found in the North Atlantic ocean, more commonly in the North East Atlantic from Morocco to Iceland (Hatun *et al.,* 2009). Its feeding grounds are in the Norwegian Sea and once mature it migrates to the waters off the west coast of Ireland and Scotland to spawn, then returning to its feeding grounds. Spawning occurs between February and May each year and only when the fish reach maturity, which is between 2 and 4 years (Coombs *et al.,* 1981). It is a pelagic species meaning it lives near the coast in mid-level waters, it is also a shoaling species and can often be found in large, dense shoals (Slotte *et al.,* 2004).

Blue whiting is a relatively unknown fish in Ireland, however the quota for Irish fishermen for 2018 stood at 47,451 tonnes. This quota was exceeded by 2% with 48,595 tonnes caught in Ireland in 2018 (Bord Iascaigh Mhara, 2018). Large scale processing of blue whiting occurs in Killybegs County Donegal, with the vast majority being either exported to Africa and China, where a shortage of quality protein sources exists or made into fish meal for aquaculture. 74% of blue whiting exports in 2011 were to Nigeria (Miller *et al.,* 2012). As a result of this, blue whiting is considered a low value species in Ireland.

Ireland became a member of the European Union (EU) in 1973 and as a result is subject to the European Common Fisheries Policy (ECFP). Reforms in this policy known as the Landing Obligation are due to take full effect in January 2019 and will require all EU fishermen to land all fish caught (Alzorriz *et al.,* 2016). This new obligation will effectively put an end to the practice of discarding unwanted, low value fish such as blue whiting. As a result, a significant rise in the numbers of blue whiting caught and landed in Ireland is expected. This has created a demand on Irish fish processors to find ways to utilise these extra fish, as otherwise they may end up in landfill (Iñarra *et al*., 2016). Hence, research

into these fish and their processing streams being a source of bioactive peptides has increased.

There have been few studies on the bioactive potential of blue whiting, but those that have occurred have presented exciting results. Proteins isolated from this fish have been shown to display bioactive properties. Water soluble proteins have been found to lower liver and serum cholesterol concentrations in obese rats (Drotningsvik *et al*., 2018). Protein hydrolysates generated from blue whiting have displayed anti-oxidant activity (Egerton *et al.,* 2018). Blue whiting hydrolysates have also been shown to be cardioprotective, inhibiting ACE production (Geirsdottir *et al.,* 2011), anti-proliferative on cancer cell lines (Picot *et al.,* 2006) and antidiabetic (Harnedy *et al.,* 2018). Due to these results blue whiting has been identified as a potential source of functional ingredients which improve the health of individuals (Egerton *et al.,* 2018).

Functional foods and ingredients provide added benefits beyond just nutrition and have experienced a surge in popularity in recent years due to a growing health conscious population. The marine world has long been a source of functional foods, with omega-3 fish oils used as a functional ingredient for many years (Wildman, 2002). Fish protein and protein hydrolysates provide an interesting source of functional ingredients with a broad range of bioactivity reported from ACE-inhbitory (Nasri *et al.,* 2013) to antioxidant (Ngo *et al.,* 2011) to antitumour (Wang *et al.,* 2010). Marine hydrolysates have been researched for their potential in adding function to bread and pasta products (Kadam and Prabhasankar, 2010). These hydrolysates have also been investigated for their potential in treating chronic diseases such as inflammatory conditions like R.A. (Goldberg and Katz, 2007), diabetes (Lunn and Theobald, 2006) and IBD (Lordan *et al.,* 2011).

IBD is a term used to describe inflammatory diseases of the gastrointestinal tract where no underlying cause is evident other than auto-immunity such as ulcerative colitis

and Crohns' disease (Podolsky, 1991). They involve systemic chronic inflammation of just the colon (as seen in UC) or in any place along the tract (as seen in Crohns') and are accompanied by a range of symptoms including diarrohea, fatigue, fecal occult blood, loss of appetite, weight loss and severe abdominal pain (Simrén *et al.,* 2002). A milder form of IBD or chronic gut inflammation is often seen in elderly people suffering from inflammaging. Inflammaging, the chronic inflammation associated with ageing, has been shown to be a cause of numerous inflammatory conditions including an increase in gut inflammation displaying symptoms similar to that of IBD (Francheschi and Capisisi, 2014).

The current treatments for IBD are both invasive and in many cases immunocompromising. Fecal transplants, involving the transplantation of fecal matter from a healthy donor into the gut of IBD patients has been successful in some cases through the introduction of a diverse microbiome to the individual (Borody and Khoruts, 2012). Other treatments include use of the mAb infliximab which inhibits TNFα and is known to cause immunocompromisation (Mackey *et al.,* 2007). Similarly, treatment with immunosuppressive azathioprine and 6-mercaptopurine, display similar results (Kandiel *et al.,* 2005). Current treatment for mild cases of IBD involves the use of corticosteroids and are not suitable for long term treatment (Hanauer and Present, 2003). Hence a need for a long term therapeutic suitable for treating both IBD and inflammaging associated gut inflammation is needed.

5.1.1 Chapter Aims

The aim of this chapter is to investigate the potential of blue whiting as a source of novel bioactive peptides. Blue whiting was kindly donated by the Killybegs Fishermen's Organisation, Killybegs, Co. Donegal, Ireland to collaborators, the Fitzgerald Lab, in the University of Limerick as facilitated by Board Iascaigh Mhara (BIM, Ireland). The University of Limerick collaborators then isolated protein from the minced sample and performed enzymatic hydrolysis in order to generate protein hydrolysates. Following screening of these hydrolysates, two candidate

hydrolysates were chosen and further fractionated using high performance liquid chromatography (HPLC) fractionation, as described in **Figure 5.1**.

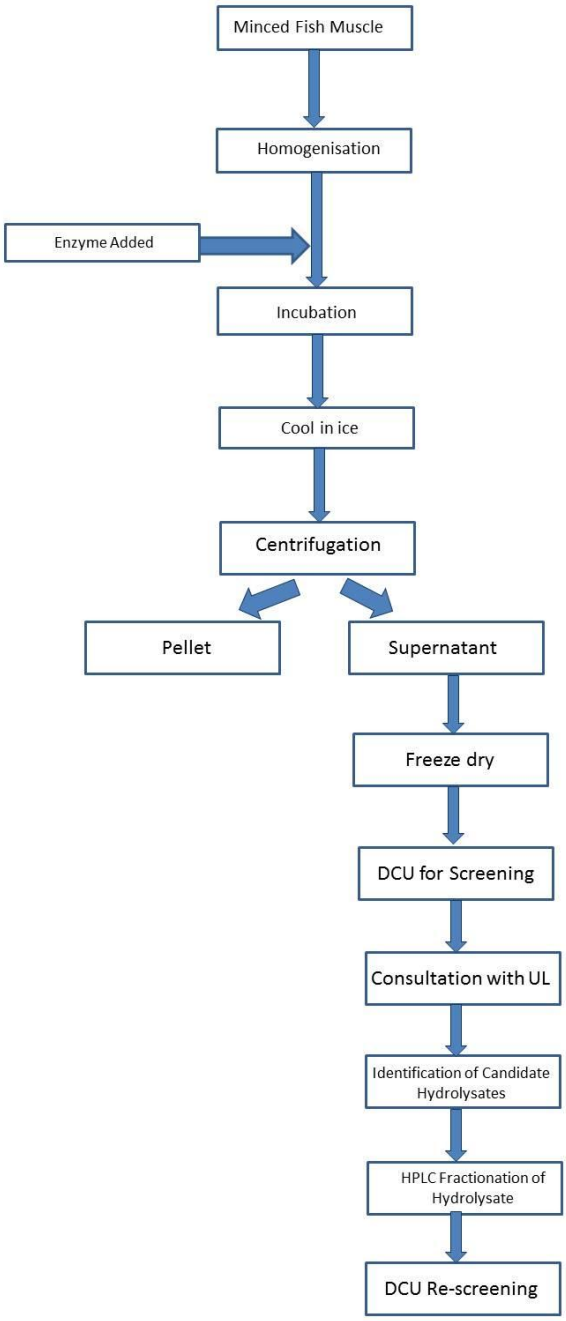


Figure 5.1 Hydrolysis process of blue whiting minced meat performed in the University of Limerick by collaborators The Fitzgerald Laboratory. Blue whiting protein hydrolysates screened during this project were created through a novel hydrolysis process which is the intellectual property of the Fitzgerald Laboratory.

Twenty (20) blue whiting hydrolysates were produced as part of this project by varying the enzyme used during digestion and the time period that samples were digested over as described in **Table 5.1.**

Table 5.1 Hydrolysis process of blue whiting protein samples screened in Chapter 5

Time (Hours)	Enzyme A	Enzyme B	Enzyme C	Enzyme D	WPC
1	21	25	29	33	37
2	22	26	30	34	38
3	23	27	31	35	39
4	24	28	32	36	40

As seen in **Table 5.1,** each blue whiting protein hydrolysate differed in either the enzyme used to perform the hydrolysis or in the length of time hydrolysis was performed. There are four (4) whole protein controls. These controls underwent the same process as the hydrolysates without the addition of any enzyme, hence they remain a whole protein. For screening purposes, the hydrolysates are grouped according to the length of time of hydrolysis. Hence group 1 includes hydrolysates 21, 25, 29 and 33 and their whole protein control 37.

All hydrolysates and whole protein controls were screened as per the protocol optimised in Chapter 3 and shown in **Figure 4.1**. The effect of the hydrolysates on cell viability of both BMDCs and undifferentiated $CD4^+$ T cells was first studied using an MTS assay. The ability of hydrolysates to modulate cytokine secretion by BMDCs and undifferentiated $CD4^+$ T cells was then assessed using ELISA. Hydrolysates were then rated on their anti-inflammatory activity and hydrolysates which displayed interesting anti-inflammatory properties were identified as candidate hydrolysates. These chosen hydrolysates were then screened on T helper cell subsets (Th1, Th2 and Th17 cells)

in order to build a broader profile of their immunomodulatory effects. Following this, collaborators were consulted in order to decide if a hydrolysate was suitable for *in vivo* trials.

Hydrolysates which were deemed suitable for *in vivo* trials were then fractionated in the University of Limerick using HPLC fractionation. Four fractions of each hydrolysate were obtained, as seen in **Table 5.2**. Fractions and hydrolysates then underwent the same screening process again in order to identify the fraction of hydrolysate displaying optimum bioactive activity. Optimum fractions were fractions found to suppress pro-inflammatory cytokines secreted by BMDCs and undifferentiated $CD4^+$ T cells and inhibit Th cell subset (Th1 or Th17) differentiation or the ability to increase secretion of IL-10 by BMDCs and undifferentiated $CD4^+$ T cells or promote the Th2 cell subset.

Table 5.2 Fractionations of hydrolysates 26 and 27 screened in Chapter 5

Hydrolysate	Fractions			
26	F1	F2	F3	F4
27	F1	F2	F3	F4

5.2 Results

5.2.1 The dose of blue whiting used does not impact on BMDC viability

As with the boarfish hydrolysates in **Section 4.2.1**, the effects of blue whiting hydrolysates on cell viability of BMDCs was first investigated prior to further analysis. BMDCs were isolated as before and plated in triplicate at a concentration of $1x10^6$ cells/mL on a 96 well plate, 100 µL/well and rested. Cells were then treated with hydrolysates (1 mg/mL) or 10% DMSO (negative control) and then stimulated with LPS (100 ng/mL) and incubated for 24 hours. Viability was assessed using an MTS assay (Cell Titer 96 Aqueous One Solution – Promega) according to the manufacturer's instructions. Results were expressed as a percentage of cells alone. Cells alone are shown as a solid white bar, controls (whole protein control) are shown as a solid black bar and hydrolysates are patterned bars. At 1 mg/mL hydrolysates did not have any significant effect on cell viability on BMDCs when compared to untreated cells (**Figure 5.2**).

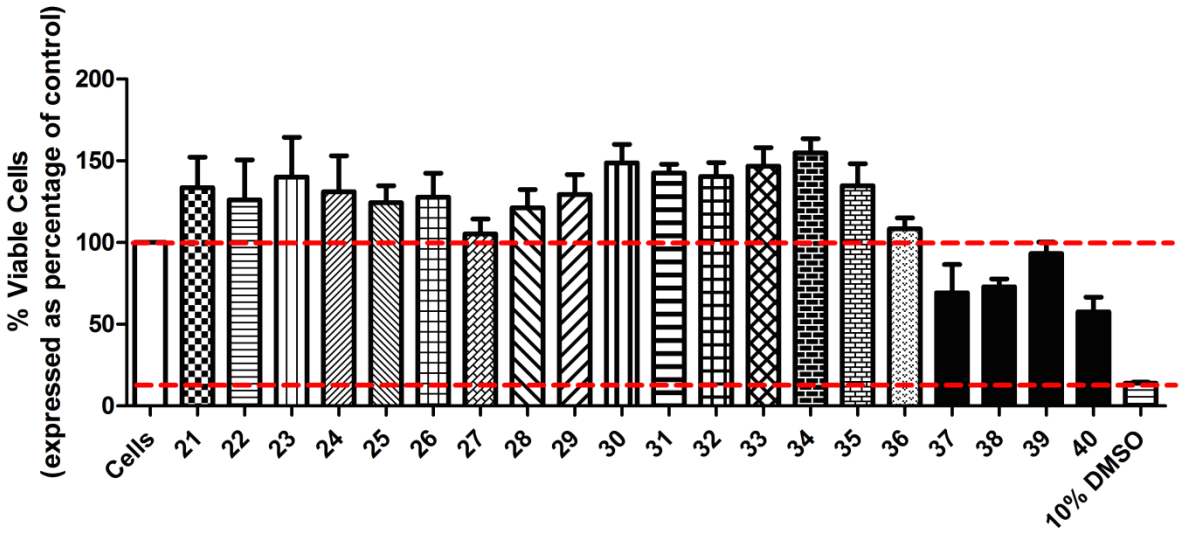


Figure 5.2 BMDC viability expressed as a percentage of cells alone following treatment with Blue Whiting hydrolysates and LPS stimulation. BMDCs were isolated from Balb/c mice and differentiated in rGMCSF for seven days. On day 7 cells were treated with hydrolysates and stimulated with 100 ng/mL LPS for 24 hours. DMSO (10% v/v) was included as a positive control of cytotoxicity. MTS assay (Cell Titer 96 Aqueous One Solution) was used to assess cell viability.

5.2.2 The dose of blue whiting hydrolysate used does not impact the viability of CD4+ T cells.

Blue whiting hydrolysates were screened on $CD4^+$ T cells and their effects on viabilty. $CD4^+$ T cells were isolated as before using an EasySep Mouse $CD4^+$ T cell Enrichment Kit. They were then treated with hydrolysates (1 mg/mL) or 10% DMSO (negative control) and incubated for 72 hours. Viability was assessed using an MTS assay (Cell Titer 96 Aqueous Solution – Promega) according to the manufacturer's instructions. Results were expressed as a percentage of cells alone. Cells alone are shown as a solid white bar, controls (whole protein control) are shown as a solid black bar and hydrolysates are patterned bars. At 1 mg/mL hydrolysates did not have any significant effect on cell viability on naïve undifferentiated T cells when compared to untreated cells (**Figure 5.3**).

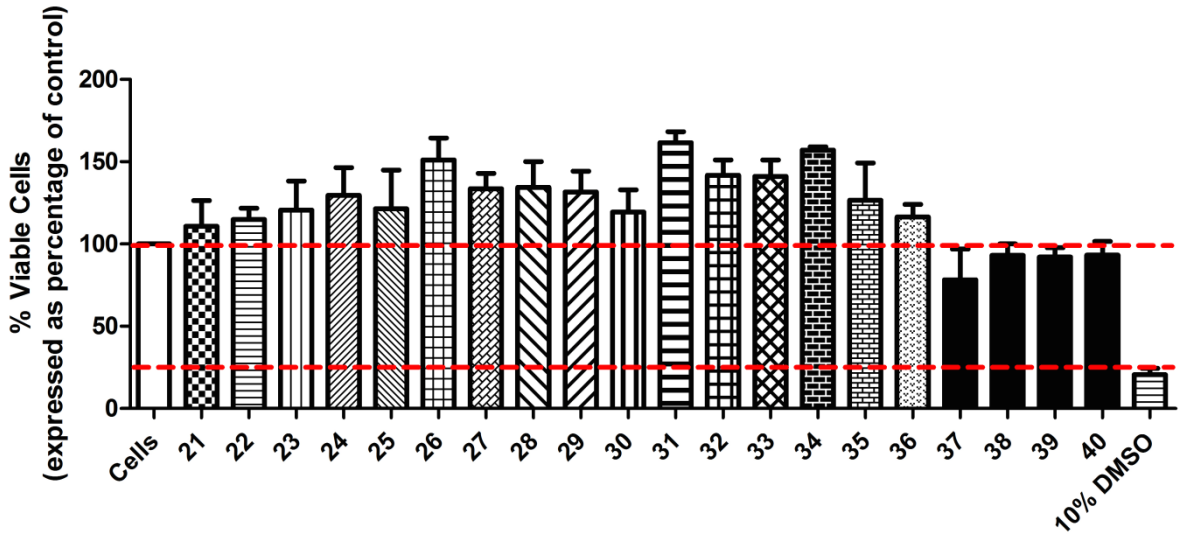


Figure 5.3 Undifferentiated CD4$^+$ T cell viability expressed as a percentage of cells alone following treatment with Blue Whiting hydrolysate. CD4$^+$ T-cells isolated from the spleens of Balb/c mice using EasySep CD4$^+$ Isolation (Stemcell). Cells were plated at 1 x 10^6 cells / mL on a 96 well plate. Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates (1 mg/mL) and incubated for 72 hours. DMSO (10% v/v) was included as a positive control of cytotoxicity. MTS assay (Cell Titer 96 Aqueous One Solution) was used to assess cell viability. Results are expressed as a percentage of cells alone.

5.2.3 Cytokine secretion by BMDCs treated with blue whiting hydrolysates.

Blue whiting hydrolysates were screened on BMDCs and their effects on cytokine secretion analysed. BMDCs were isolated as before and treated with hydrolysates at a dose of 1 mg/mL, some of these (marked +100 ng LPS) were then stimulated with 100 ng/mL LPS. Secretion of cytokines (TNFα, IL-10, IL-6 and IL-12p40) was then assessed using ELISA (R&D Duoset). Cells alone are shown as a solid white bar, controls (whole protein control) are shown as a solid black bar and hydrolysates are patterned bars.

Figure 5.4 shows BMDC cytokine secretion following treatment with blue whiting hydrolysates enzymatically hydrolysed for 1 hour. Hydrolysates H21, H25, H29 and H33 and their WPC37 are shown. H21 selectively increased levels of IL-10 ($p<0.01$) whilst having no effect on other cytokines. H25 decreased TNFα ($p<0.001$) and IL-12p40 ($p<0.001$) and increased levels of IL-10 ($p<001$). H29, behaved in a similar fashion to H21, selectively increasing IL-10 ($p<0.01$). H33 increased IL-10 ($p<0.01$) and decreased levels of TNFα ($p<0.01$) and WPC37 similarly increased IL-10 ($p<0.01$) whilst decreasing TNFα ($p<0.001$). Hydrolysate H25, due to its anti-inflammatory profile of increasing anti-inflammatory cytokine IL-10 and decreasing pro-inflammatory IL-12p40 and TNFα, was identified as a possible candidate for further screening.

Figure 5.5 displays BMDC cytokine secretion following treatment with blue whiting hydrolysates enzymatically hydrolysed for 2 hours. Hydrolysates H22, H26, H30, H34 and their WPC38 are shown. H22 increased levels of IL-10 ($p<0.001$) but did not impact any other cytokine. H26 increases IL-10 ($p<0.001$) but also decreased levels of TNFα ($p<0.001$), IL-6 ($p<0.01$) and IL-12p40 ($p<0.001$). H30 decreased IL-6 ($p<0.01$) whilst increasing IL-10 ($p<0.01$). H34 reduced TNFα ($p<0.01$), IL-6 ($p<0.001$) and IL-12p40 ($p<0.001$). WPC38 decreased levels of TNFα ($p<0.001$). H22 displayed the ability to selectively promote anti-inflammatory cytokine IL-10 hence was chosen as a candidate for possible further screening. H26 displayed significant anti-inflammatory properties in decreasing TNFα, IL-6 and IL-12p40 whilst simultaneously increasing levels of anti-inflammatory cytokine IL-10 and was identified as a potential candidate for further screening.

Figure 5.6 shows BMDC cytokine secretion following treatment with blue whiting hydrolysates enzymatically hydrolysed for 3 hours. Hydrolysates H23, H27, H31, H35 and WPC39 are shown. H23 increased levels of IL-10 ($p<0.001$). H27 decreased levels of TNFα ($p<0.001$), IL-6 ($p<0.001$) and IL-12p40 ($p<0.001$) and also increased levels of anti-inflammatory cytokine IL-10 ($p<0.01$). H31, similar to H23, increased levels of IL-10 ($p<0.01$) only. H35 reduced levels of IL-6 ($p<0.001$) and IL-12p40 ($p<0.001$). WPC39 increased IL-10 ($p<0.001$) and decreased IL-6 ($p<0.001$) and IL-12p40 ($p<0.001$). H27, due to its potent anti-inflammatory properties, was chosen as a candidate for possible further screening along with 23, which behaved in a similar fashion to H22 in **Figure 5.5,** selectively promoting IL-10 levels.

Figure 5.7 displays the BMDC cytokine secretion following treatment with blue whiting hydrolysates enzymatically hydrolysed for 3 hours. Hydrolysates H24, H28, H32, H36 and WPC40 are shown. H24 decreased levels of TNFα ($p<0.001$) and IL-12p40 ($p<0.001$) whilst increasing levels of IL-10 ($p<0.001$). H28 decreased IL-6 ($p<0.01$). H32 decreased TNFα ($p<0.01$). H36 reduced levels of IL-6 ($p<0.01$) and IL-12p40 ($p<0.001$). WPC40 decreased TNFα ($p<0.01$) and increased IL-10 ($p<0.001$). H28 was chosen as a potential candidate for further screening due to anti-inflammatory properties displayed.

These results were then compared to the hydrolysates effect on the cytokine secretion of naïve undifferentiated T cells before potential candidates were brought forward for screening on T cell subsets.

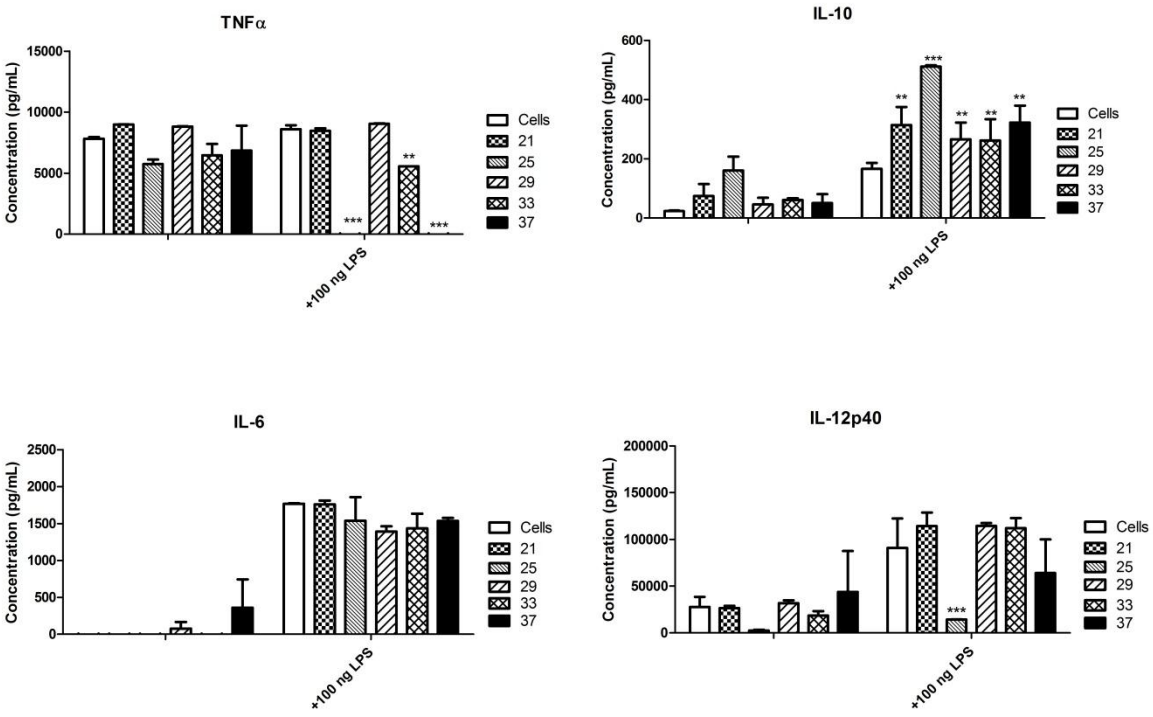


Figure 5.4 Secretion of TNFα, IL-10, IL-6 and IL-12p40 by BMDCs treated with Blue Whiting 1 hour hydrolysates. BMDCs were isolated from Balb/c mice and differentiated in rGMCSF for seven days. On day 7 cells were treated with hydrolysates and stimulated with 100 ng/mL LPS. Secretion of IL-12p40, IL-6, IL-10 and TNFα was measured using ELISA (R&D Duoset). One way Anova was used to assess differences between groups. Cells treated with hydrolysate only were compared to cells alone, cells treated with hydrolysates + LPS were compared to cells alone + LPS. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to untreated cells (no hydrolysate).

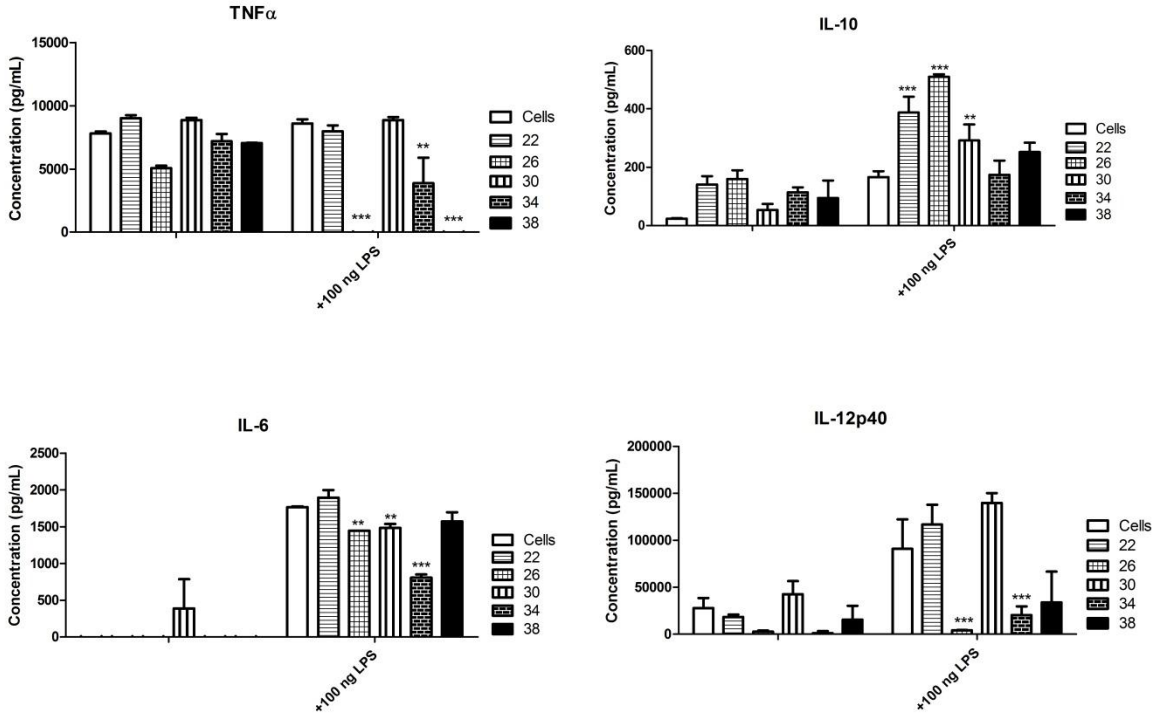


Figure 5.5 Secretion of TNFα, IL-10, IL-6 and IL-12p40 by BMDCs treated with Blue Whiting 2 hour hydrolysates. BMDCs were isolated from Balb/c mice and differentiated in rGMCSF for seven days. On day 7 cells were treated with hydrolysates and stimulated with 100 ng/mL LPS. Secretion of TNFα, IL-10, IL-6 and IL-12p40 was measured using ELISA (R&D Duoset). One way Anova was used to assess differences between groups. Cells treated with hydrolysate only were compared to cells alone, cells treated with hydrolysates + LPS were compared to cells alone + LPS. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to untreated cells (no hydrolysate).

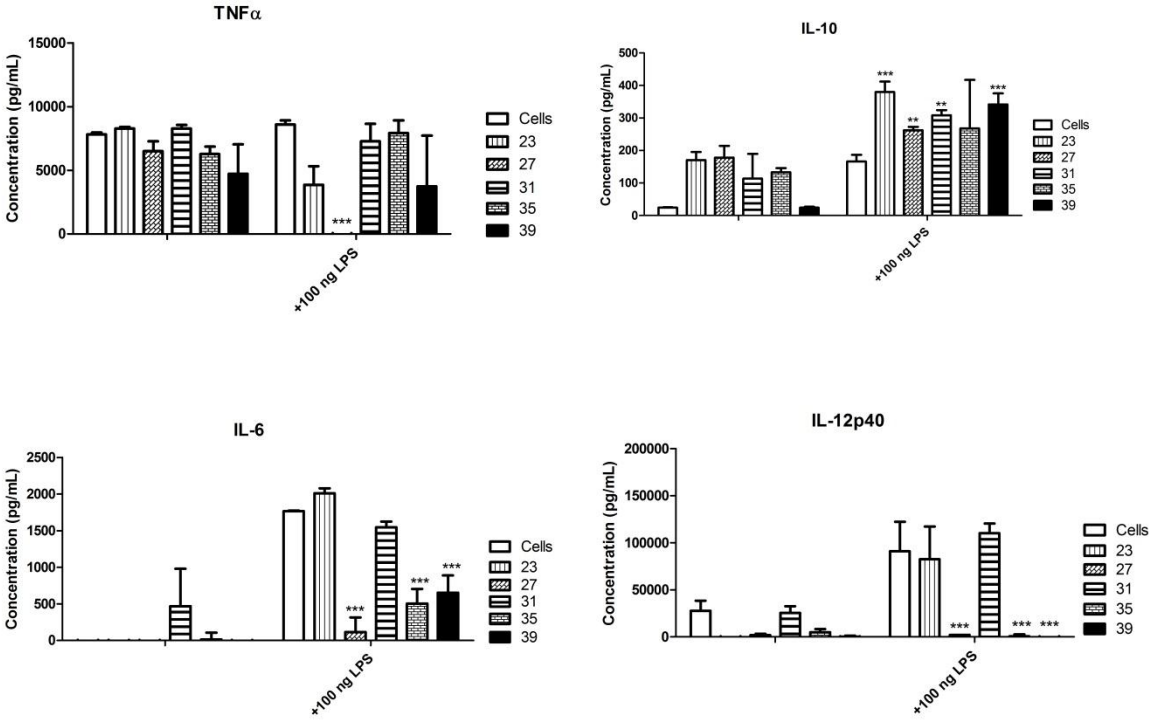


Figure 5.6 Secretion of TNFα, IL-10, IL-6 and IL-12p40 by BMDCs treated with Blue Whiting 3 hour hydrolysates. BMDCs were isolated from Balb/c mice and differentiated in rGMCSF for seven days. On day 7 cells were treated with hydrolysates and stimulated with 100 ng/mL LPS. Secretion of TNFα, IL-10, IL-6 and IL-12p40 was measured using ELISA (R&D Duoset). One way Anova was used to assess differences between groups. Cells treated with hydrolysate only were compared to cells alone, cells treated with hydrolysates + LPS were compared to cells alone + LPS. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to untreated cells (no hydrolysate).

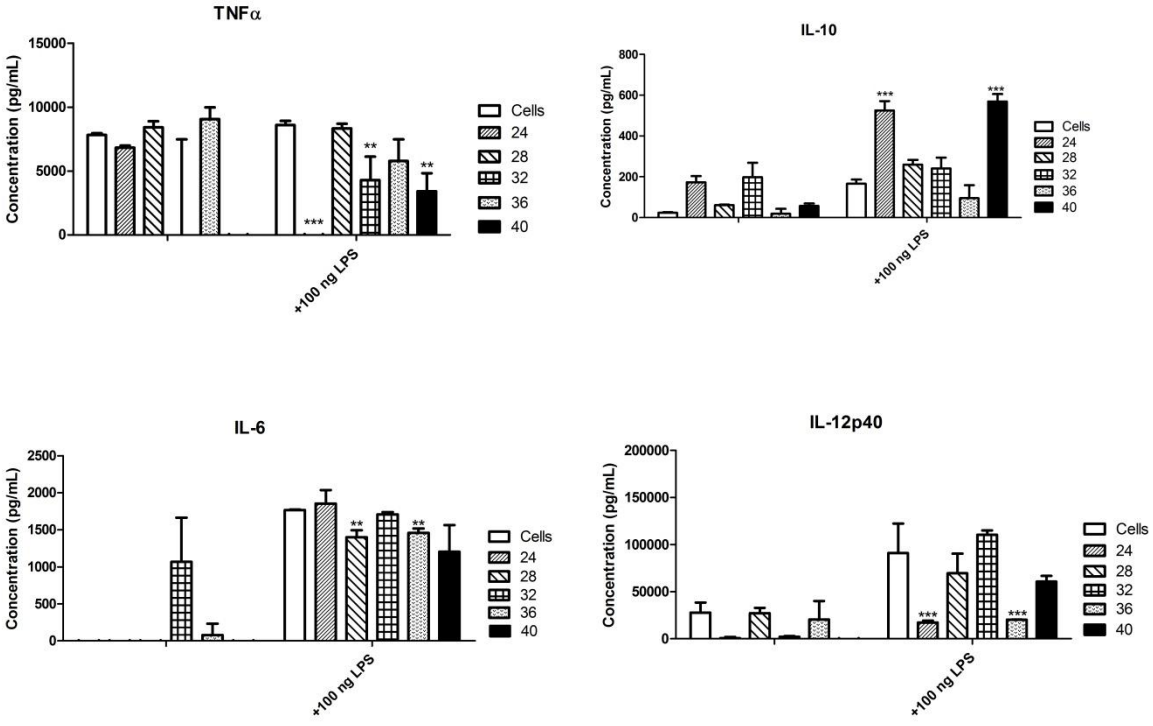


Figure 5.7 Secretion of TNFα, IL-10, IL-6 and IL-12p40 by BMDCs treated with Blue Whiting 4 hour hydrolysates. BMDCs were isolated from Balb/c mice and differentiated in rGMCSF for seven days. On day 7 cells were treated with hydrolysates and stimulated with 100 ng/mL LPS. Secretion of TNFα, IL-10, IL-6 and IL-12p40 was measured using ELISA (R&D Duoset). One way Anova was used to assess differences between groups. Cells treated with hydrolysate only were compared to cells alone, cells treated with hydrolysates + LPS were compared to cells alone + LPS. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to untreated cells (no hydrolysate).

5.2.4 Cytokine secretion by undifferentiated CD4+ T cells treated with blue whiting hydrolysates.

Blue whiting hydrolysates were screened on undifferentiated $CD4^+$ T cells and their impact on cytokine secretion analysed. $CD4^+$ T cells were isolated as before using an EasySep Mouse $CD4^+$ T cell Enrichment Kit and treated with hydrolysates at a dose of 1 mg/mL. Secretion of cytokines (IL-17, IL-2, IL-4, IL-6, IL-10, IL-13 and IFNγ) was then assessed using ELISA (R&D Duoset).

Figure 5.8 shows undifferentiated $CD4^+$ T cell cytokine secretion treated with blue whiting hydrolysates enzymatically digested for 1 hour. Hydrolysates H21, H25, H29, H33 and WPC37 are shown. H21 reduced levels of IL-6 ($p<0.05$), IL-13 ($p<0.001$) and IFNγ ($p<0.001$). H25 decreased IL-13 ($p<0.01$) and increases levels of IL-2 ($p<0.001$) and IL-4 ($p<0.001$). H29 reduced secretion of IL-17 ($p<0.05$), IL-6 ($p<0.05$) and IL-13 ($p<0.001$). H33 promoted production of IL-2 ($p<0.01$) whilst reducing levels of IL-17 ($p<0.05$), IL-6 ($p<0.05$), IL-13 ($p<0.001$) and IFNγ ($p<0.001$). WPC37 increased IL-2 ($p<0.05$) whilst decreasing IL-6 ($p<0.01$), IL-13 ($p<0.001$) and IFNγ ($p<0.01$). No hydrolysates were chosen from this cohort when compared to results in **Section 5.2.3** and **Figure 5.4.**

Figure 5.9 shows undifferentiated $CD4^+$ T cell cytokine secretion treated with blue whiting hydrolysates enzymatically digested for 2 hours. Hydrolysates H22, H26, H30, H34 and WPC38 are shown. H22 decreased secretion of IL-6 ($p<0.001$), IL-13 ($p<0.05$) and IFNγ ($p<0.05$). H26 increased IL-4 ($p<0.01$) production and decreased IL-6 ($p<0.001$), IL-13 ($p<0.05$) and IFNγ ($p<0.001$). H30 decreased IL-6 ($p<0.001$), IL-13 ($p<0.01$) and IFNγ ($p<0.05$). H34 reduced IL-6 ($p<0.001$), IL-13 ($p<0.01$) and IFNγ ($p<0.05$) and increased IL-4 ($p<0.05$). WPC38 decreased IFNγ ($p<0.001$), IL-6 ($p<0.001$) and IL-13 ($p<0.001$). Due to their ability to reduce levels of pro-inflammatory cytokine IFNγ and also when compared to results in **Section 5.2.3** and **Figure 5.5**, hydrolysates H22 and H26 were chosen as candidates for further screening.

Figure 5.10 shows undifferentiated $CD4^+$ T cell cytokine secretion treated with blue whiting hydrolysates enzymatically digested for 3 hours. Hydrolysates H23, H27, H31, H35 and WPC39 are shown. H23 significantly reduces secretion of IL-13 ($p<0.001$)

and IFNγ ($p<0.001$). H27 increases levels of IL-2 ($p<0.01$) and IL-4 ($p<0.01$) whilst decreasing IL-13 ($p<0.01$) and IFNγ ($p<0.01$). H31 reduces IL-13 ($p<0.001$) production. H35 increases IL-2 ($p<0.01$) whilst decreasing IL-13 ($p<0.01$). WPC39 reduced levels of IL-6 ($p<0.05$), IL-13 ($p<0.001$) and IFNγ ($p<0.001$). Due to their ability to reduce levels of pro-inflammatory cytokine IFNγ and also when compared to results in **Section 5.2.3** and **Figure 5.6**, hydrolysates H23 and H27 were chosen as candidates for further screening.

Figure 5.11 shows undifferentiated $CD4^+$ T cell cytokine secretion treated with blue whiting hydrolysates enzymatically digested for 4 hours. Hydrolysates H24, H28, H32, H36 and WPC40 are shown. H24 increased secretion of IL-17 ($p<0.01$) and IL-2 ($p<0.001$) and decreased levels of IL-13 ($p<0.001$) and IFNγ ($p<0.05$). H28 reduced levels of IL-17 ($p<0.01$), IL-6 ($p<0.01$), IL-13 ($p<0.001$) and IFNγ ($p<0.001$). H32 increased IL-2 ($p<0.01$) and decreased IL-6 ($p<0.01$), IL-13 ($p<0.001$) and IFNγ ($p<0.05$). H36 reduced levels of IL-17 ($p<0.05$), IL-6 ($p<0.001$), IL-10 ($p<0.05$), IL-13 ($p<0.001$) and IFNγ ($p<0.001$). WPC40 decreased IL-17 ($p<0.05$), IL-6 ($p<0.001$), IL-13 ($p<0.001$) and IFNγ ($p<0.001$). Results displayed in **Figure 5.10** were then compared to that in **Section 5.2.3** and **Figure 5.7,** due to their lack of selectivity, no hydrolysates from this cohort were chosen for further screening.

Hydrolysates H22, H23, H25, H26 and H27 were all chosen as candidates for further screening and brought forward to investigate their impact on specific T cell subsets.

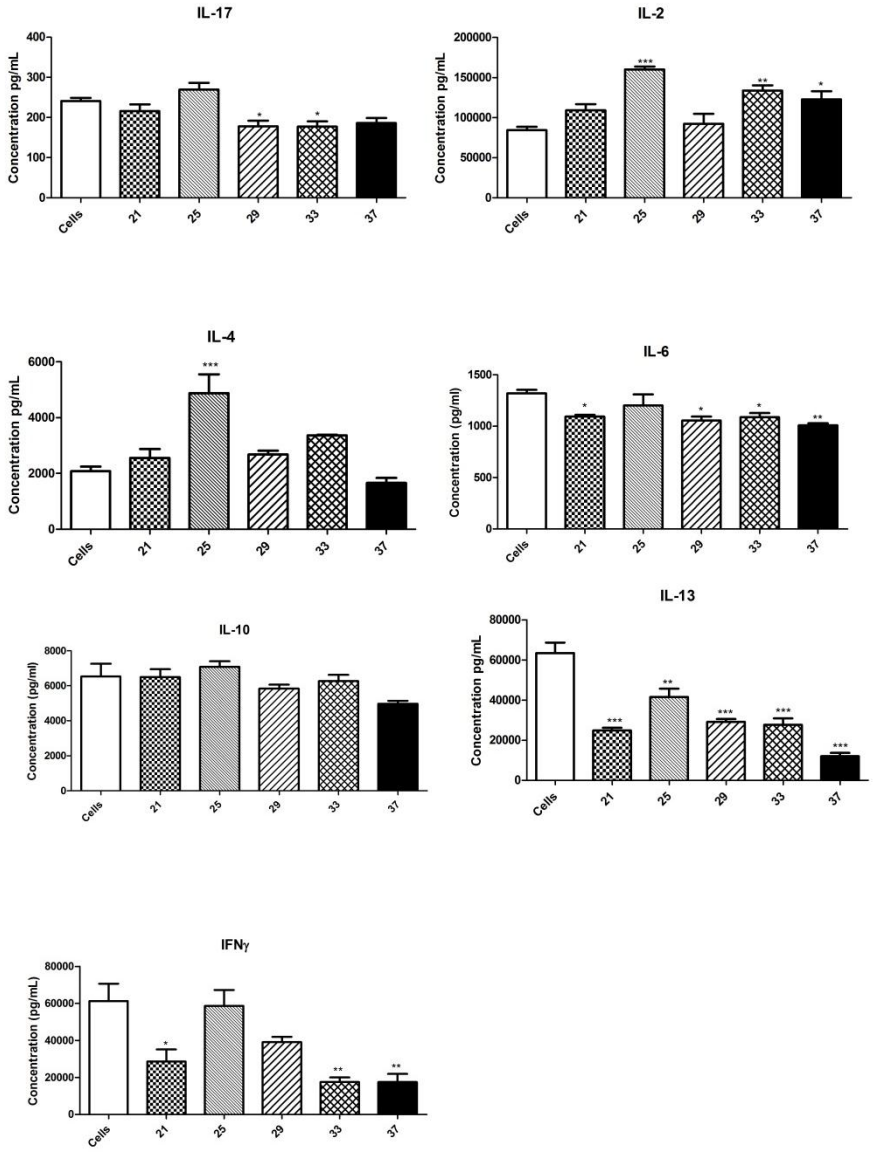


Figure 5.8 Secretion of IL-17, IL-2, IL-4, IL-6, IL-10, IL-13 and IFNγ by undifferentiated CD4$^+$ T cells treated with Blue Whiting 1 hour hydrolysates. CD4$^+$ T-cells were isolated from the spleens of Balb/c mice using EasySep CD4$^+$ Isolation (Stemcell). Cells were plated at 1 x 10^6 cells / mL on a 96 well plate. Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates (1 mg/mL) and incubated for 72 hours. Secretion of IL-17, IL-2, IL-4, IL-6, IL-10, IL-13 and IFNγ was measured using ELISA (R&D Duoset). One way Anova was used to assess differences between groups. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to cells alone.

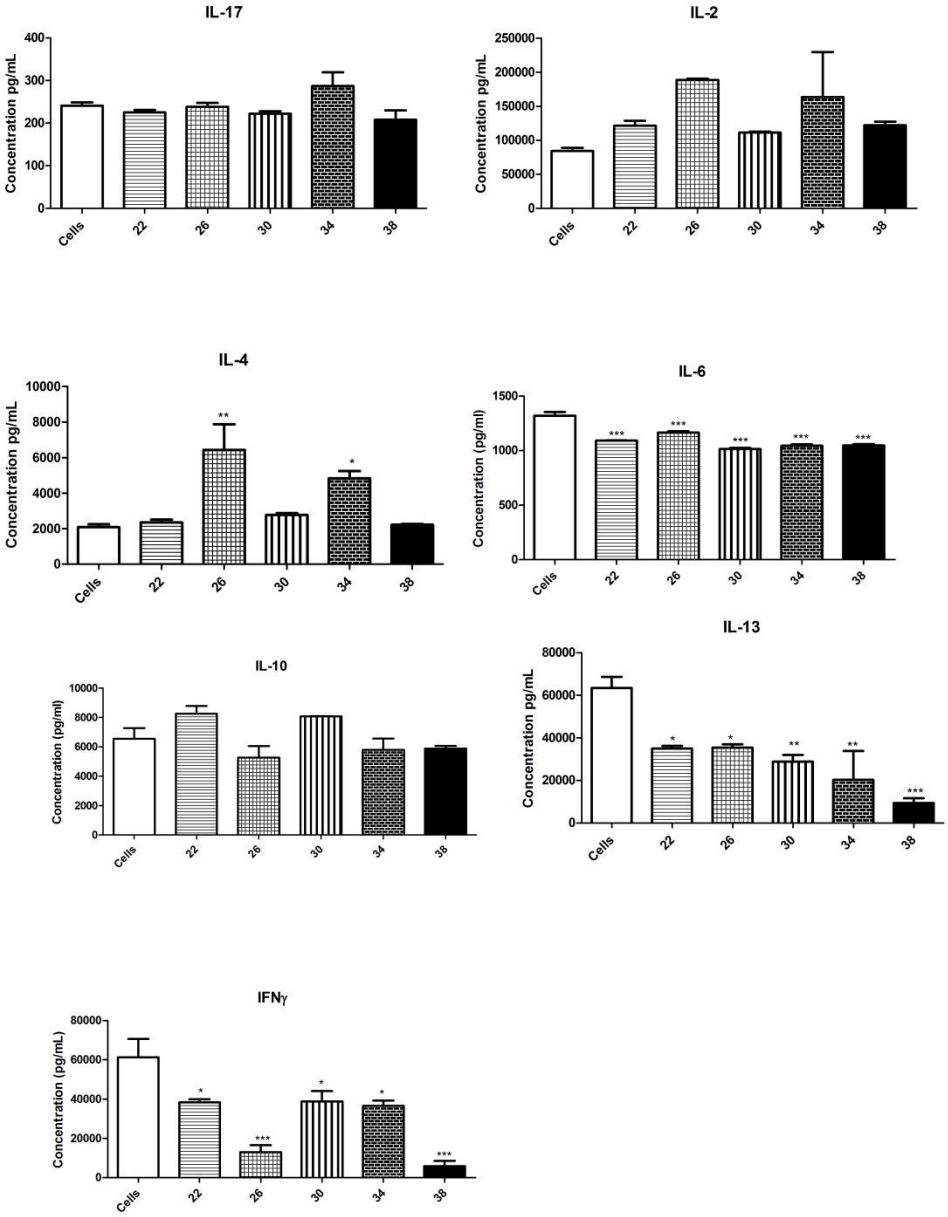


Figure 5.9 Secretion of IL-17, IL-2, IL-4, IL-6, IL-10, IL-13 and IFNγ by undifferentiated CD4$^+$ T cells treated with Blue Whiting 2 hour hydrolysates. CD4$^+$ T-cells were isolated from the spleens of Balb/c mice using EasySep CD4$^+$ Isolation (Stemcell). Cells were plated at 1 x 10^6 cells / mL on a 96 well plate. Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates (1 mg/mL) and incubated for 72 hours. Secretion of IL-17, IL-2, IL-4, IL-6, IL-10, IL-13 and IFNγ was measured using ELISA (R&D Duoset). One way Anova was used to assess differences between groups. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to cells alone.

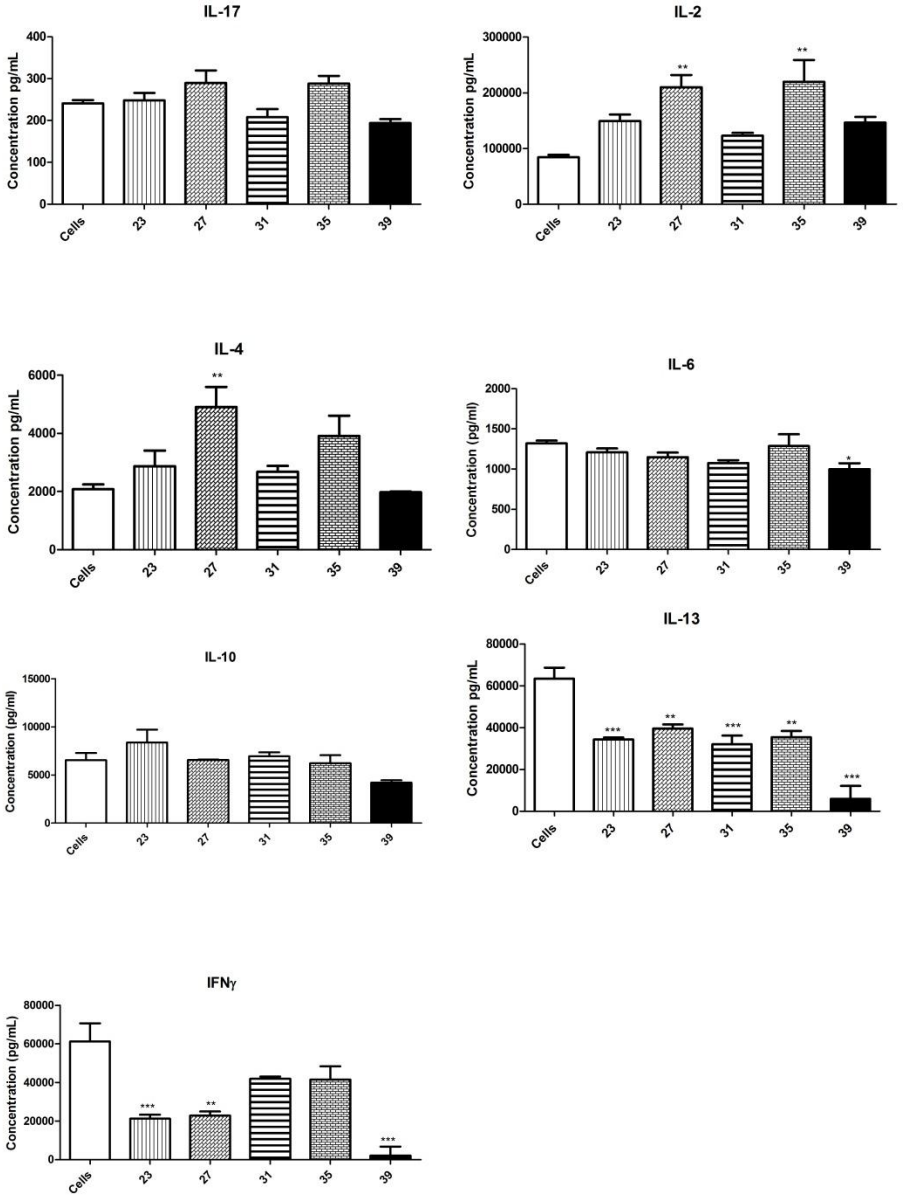


Figure 5.10 Secretion of IL-17, IL-2, IL-4, IL-6, IL-10, IL-13 and IFNγ by undifferentiated CD4$^+$ T cells treated with Blue Whiting 3 hour hydrolysates. CD4$^+$ T-cells were isolated from the spleens of Balb/c mice using EasySep CD4$^+$ Isolation (Stemcell). Cells were plated at 1 x 10^6 cells / mL on a 96 well plate. Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates (1 mg/mL) and incubated for 72 hours. Secretion of IL-17, IL-2, IL-4, IL-6, IL-10, IL-13 and IFNγ was measured using ELISA (R&D Duoset). One way Anova was used to assess differences between groups. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to cells alone.

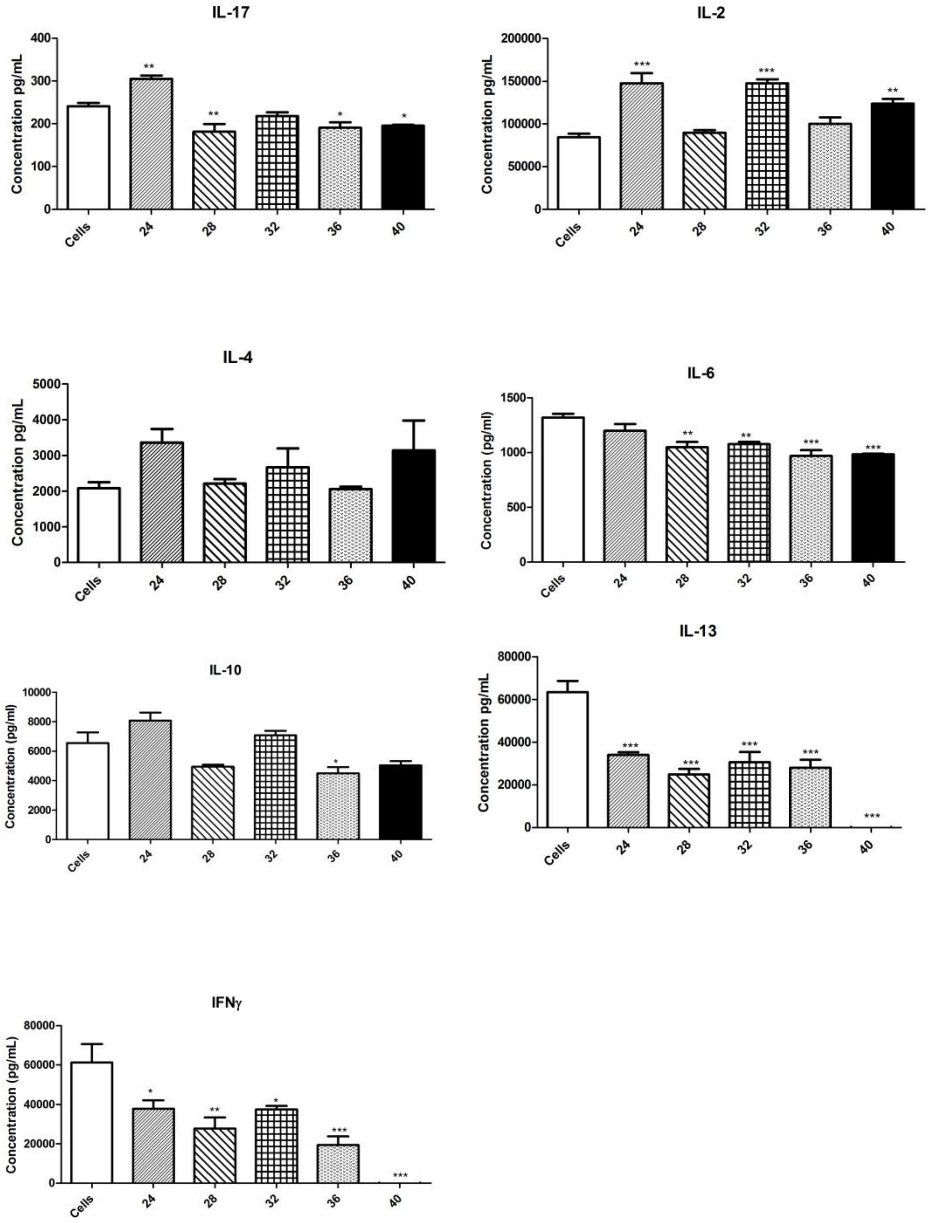


Figure 5.11 Secretion of IL-17, IL-2, IL-4, IL-6, IL-10, IL-13 and IFNγ by undifferentiated CD4$^+$ T cells treated with Blue Whiting 4 hour hydrolysates. CD4$^+$ T-cells were isolated from the spleens of Balb/c mice using EasySep CD4$^+$ Isolation (Stemcell). Cells were plated at 1 x 10^6 cells / mL on a 96 well plate. Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates (1 mg/mL) and incubated for 72 hours. Secretion of IL-17, IL-2, IL-4, IL-6, IL-10, IL-13 and IFNγ was measured using ELISA (R&D Duoset). One way Anova was used to assess differences between groups. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to cells alone.

5.2.5 Cytokine secretion by T-helper cell 1 subset treated with selected blue whiting hydrolysates.

Hydrolysates which had been identified as potential candidates in **Section 5.2.3 and Section 5.2.4** were then brought forward for analysis on specific T cell subsets. Th1 cells were isolated using an EasySep Mouse $CD4^+$ T cell Enrichment Kit and the media was supplemented with 20 ng/mL IL-2, 20 ng/mL IL-12 and 10 µg/mL anti-IL- 4 antibody for Th1 polarising conditions. Cells were treated with hydrolysates H22, H23, H25, H26 and H27 along with their whole protein controls, at a dose of 1 mg/mL and incubated for 72 hours before re-stimulation in fresh media for a further 24 hours. Secretion of cytokines (IL-13, IL-6, IL-10, IL-10, IL-17, IL-4, IFNγ and IL-2) was then assessed using ELISA (R&D Duoset).

Figure 5.12 shows Th1 cell cytokine secretion when treated with hydrolysates H22, H23, H25, H26, H27 and WPC37, WPC38 and WPC39. IFNγ and IL-2 are the key cytokines associated with a Th1 cell subset. H25 increased IL-2 ($p<0.001$) levels. H26,H27,WPC37 and WPC38 decreased IFNγ ($p<0.001$).

Other cytokines were also measured. H22 significantly increased levels of IL-10 ($p<0.001$) and decreased IL-4 ($p<0.05$) secreted by Th1 cells. H23 decreased IL-4 ($p<0.01$) and increased IL-13 ($p<0.01$) and IL-10 ($p<0.001$). H25 increased IL-17 ($p<0.01$) levels. H26 increased IL-10 ($p<0.001$), IL-17 ($p<0.001$) and IL-4 ($p<0.001$). H27 increased IL-13 ($p<0.01$). WPC38 decreased IL-6 ($p<0.001$) and IL-4 ($p<0.05$). WPC39 did not alter any cytokine secretion significantly.

These hydrolysates were then screened in Th2 and Th17 cells in order to build a profile of their effects on the wider immune system.

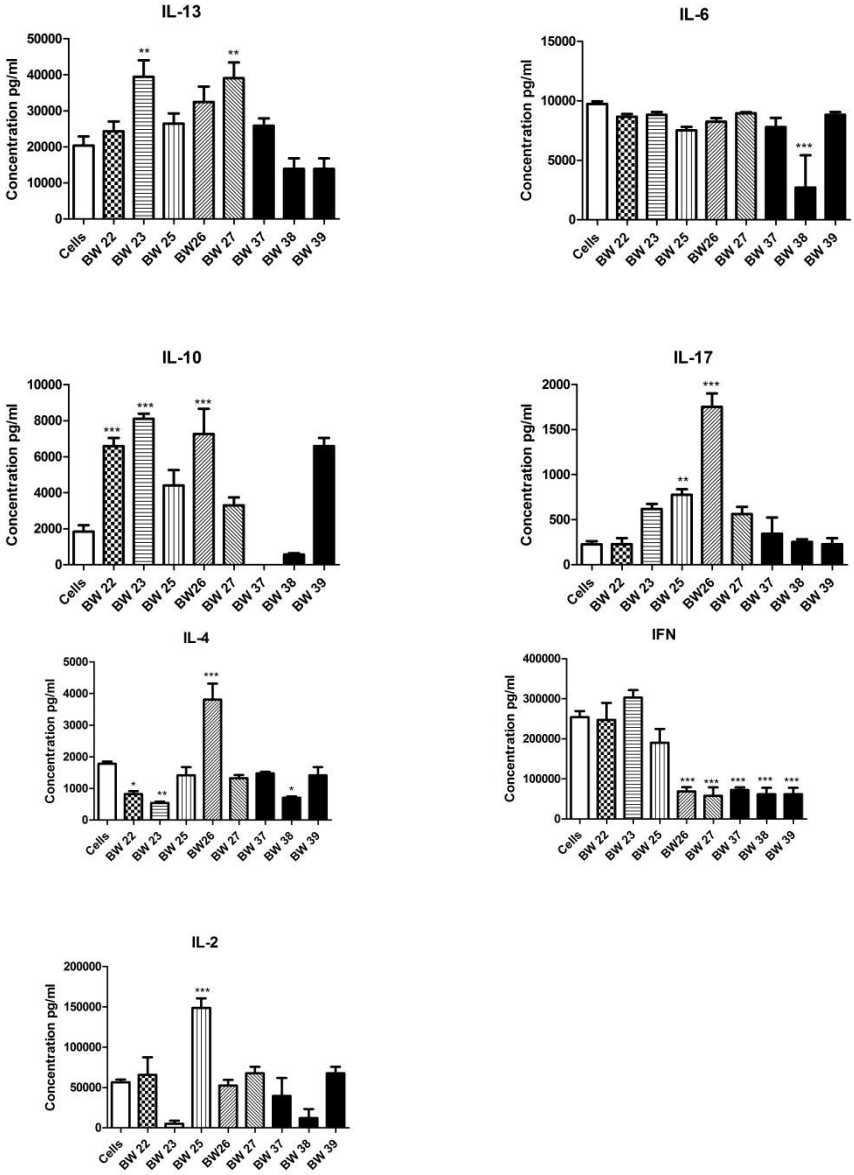


Figure 5.12 Secretion of IL-13, IL-6, IL-10, IL-17, IL-4, IFNγ and IL-2 by Th1 cells treated with selected Blue Whiting hydrolysates. $CD4^+$ T-cells isolated from the spleens of Balb/c mice using EasySep $CD4^+$ Isolation (Stemcell). Cells were plated at 1 x 10^6 cells / mL on a 96 well plate. Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates (1 mg/mL). The media was supplemented with 20 ng/mL IL-2, 20 ng/mL IL-12 and 10 μg/mL anti-IL-4 antibody for Th1 polarising conditions. Cells were incubated for 72 hours and then re-stimulated in fresh media for 24 hours. Cytokine secretion was measured using ELISA (R&D Duoset). One way Anova was used to assess differences between groups. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to cells alone.

5.2.6 Cytokine secretion of T helper cell 2 subset treated with selected blue whiting hydrolysates.

Following their assessment on Th1 cells (**Section 5.2.5**), hydrolysates H22, H23, H25, H26 and H27 were then screened on a Th2 subset in order to build a full profile of their effects on the wider immune system. Th2 cells were isolated using an EasySep Mouse $CD4^+$ T cell Enrichment Kit and the media was supplemented with 10 µg/mL neutralising IFNγ antibody and 10 mg/mL IL- 4 to induce Th2 polarising conditions. Cells were treated with hydrolysates H22, H23, H25, H26 and H27 along with their whole protein controls, at a dose of 1 mg/mL and incubated for 72 hours before re-stimulation in fresh media for a further 24 hours. Secretion of cytokines (IL-13, IL-6, IL-10, IL-17, IL-4, IFNγ and IL-2) was then assessed using ELISA (R&D Duoset).

Figure 5.13 displayed Th2 cell cytokine secretion when treated with hydrolysates H22, H23, H25, H26, H27 and WPC37, WPC38 and WPC39. The key cytokines associated with a Th2 cell subset are IL-4 and IL-13. H22 increased IL-13 ($p<0.001$) and decreased IL-4 ($p<0.001$). H27 decreased IL-4 ($p<0.001$). WPC37, WPC38 and WPC39 decreased IL-13 ($p<0.01$).

Other cytokines secreted by Th2 cells were also modulated by the hydrolysates. H22 increased IL-10 ($p<0.001$) and IL-2 ($p<0.001$) whilst decreasing levels of IL-6 ($p<0.001$), IL-17 ($p<0.001$) and IFNγ ($p<0.001$). H23 decreased IL-6 ($p<0.01$), IL-17 ($p<0.01$) and IFNγ ($p<0.01$) and increased levels of IL-10 ($p<0.05$). H25 decreased IL-6 ($p<0.001$), IL-10 ($p<0.05$), IFNγ ($p<0.01$) and IL-2 ($p<0.001$). H26 decreased IL-6 ($p<0.001$), IL-10 ($p<0.05$), IL-17 ($p<0.001$), and increases IL-2 ($p<0.05$). H27 decreased IL-6 ($p<0.001$) and IFNγ ($p<0.01$). WPC37 decreased IL-6 ($p<0.001$), IL-10 ($p<0.05$), IFNγ ($p<0.01$) and IL-2 ($p<0.01$). WPC38 decreased IL-6 ($p<0.001$), IL-10 ($p<0.05$), IL-17 ($p<0.05$) and IFNγ ($p<0.01$). WPC39 decreased IL-6 ($p<0.001$), IL-10 ($p<0.01$) and IL-17 ($p<0.05$).

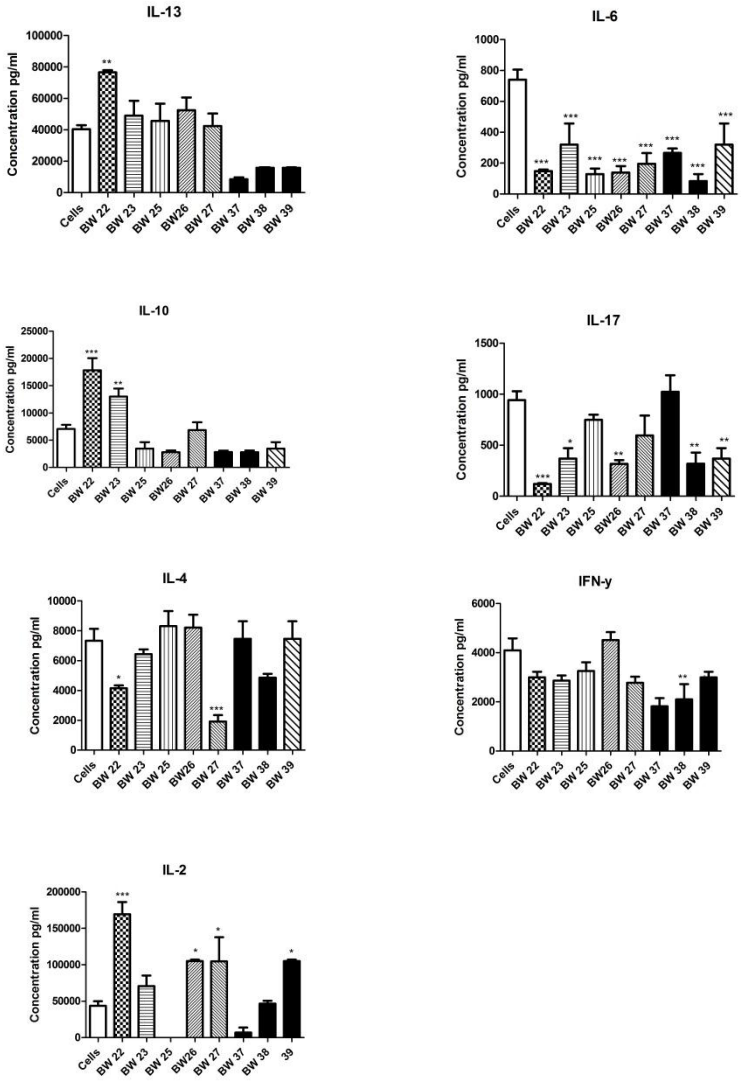


Figure 5.13 Secretion of IL-13, IL-6, IL-10, IL-17, IL-4, IFNγ and IL-2 by Th2 cells treated with selected Blue Whiting hydrolysates. $CD4^+$ T-cells isolated from the spleens of Balb/c mice using EasySep $CD4^+$ Isolation (Stemcell). Cells were plated at 1 x 10^6 cells / mL on a 96 well plate. Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates (1 mg/mL). The media was supplemented with 10 μg/mL neutralizing IFNγ antibody and 10 mg/mL IL-4 for Th2 polarising conditions. Cells were incubated for 72 hours and then re-stimulated in fresh media for 24 hours. Cytokine secretion was measured using ELISA (R&D Duoset). One way Anova was used to assess differences between groups. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to cells alone.

5.2.7 Cytokine secretion by T helper cell 17 subset treated with selected blue whiting hydrolysates.

In order to complete the profile of the hydrolysates effect on T cell subsets, the effect of hydrolysates H22, H23, H25, H26 and H27 on the cytokine secretion by Th17 cells were assessed. Th17 cells were isolated using an EasySep Mouse $CD4^+$ T cell Enrichment Kit and the media was supplemented with 10 µg/mL anti-IFNγ antibody, 10 µg/mL IL-4, 20 ng/mL IL-6, 10 ng/mL IL-23 and 2 ng/mL TGFβ to induce Th17 polarising conditions. Cells were treated with hydrolysates H22, H23, H25, H26 and H27 along with their whole protein controls, at a dose of 1 mg/mL and incubated for 72 hours before re-stimulation in fresh media for a further 24 hours. Secretion of cytokines (IL-17, IL-6, IL-4, IFNγ and IL-2) was then assessed using ELISA (R&D Duoset).

Figure 5.14 shows Th17 cell cytokine secretion when treated with hydrolysates H22, H23, H25, H26, H27 and WPC37, WPC38 and WPC39. IL-17 is the key cytokine associated with Th17 cells. H22 increased IL-17 ($p<0.001$). H23, H26 and H27 reduced IL-17 ($p<0.05$).

Other cytokines secreted by Th17 cells were also modulated by the hydrolysates. H22 increased IL-2 ($p<0.05$). H23 reduced IL-6 ($p<0.05$) and increased IL-2 ($p<0.05$). H25 decreased IL-6 ($p<0.001$), IL-4 ($p<0.05$) and IFNγ ($p<0.05$). H26 decreased IL-6 ($p<0.01$), IL-4 ($p<0.05$) and IL-2 ($p<0.05$). H27 decreases IL-4 ($p<0.05$). WPC37 reduces levels of IFNγ ($p<0.01$) and IL-2 ($p<0.05$). WPC38 increases IL-17 ($p<0.01$) and decreases IL-4 ($p<0.001$). WPC39 decreased IL-4 ($p<0.01$).

These results were then compared to **Section 5.2.5** and **Section 5.2.6**, hydrolysates 26 and 27 were chosen to undergo HPLC fractionation to investigate the section of peptide which contained optimal bioactivity.

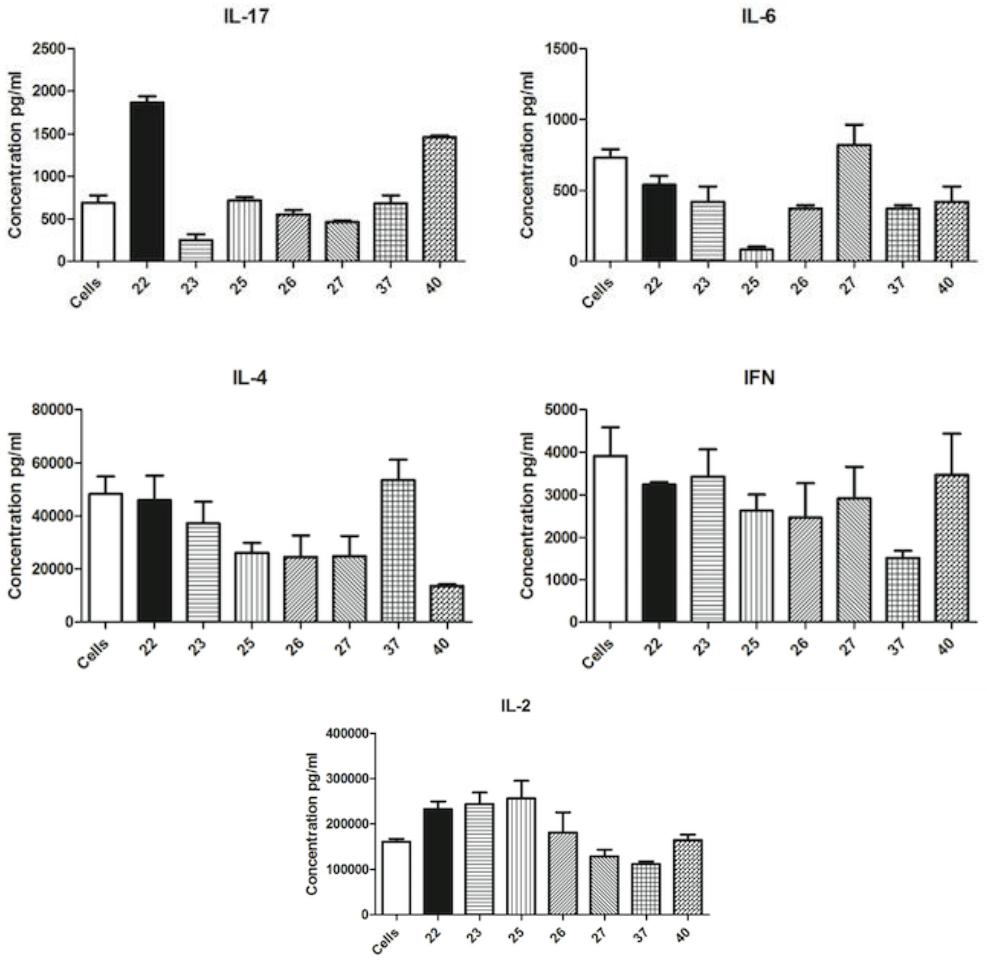


Figure 5.14 Secretion of IL-17, IL-6, IL-4, IFNγ and IL-2 by Th17 cells treated with selected Blue Whiting hydrolysates. $CD4^+$ T-cells isolated from the spleens of Balb/c mice using EasySep $CD4^+$ Isolation (Stemcell). Cells were plated at 1 x 10^6 cells / mL on a 96 well plate. Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates (1 mg/mL). The media was supplemented with 20 ng/mL IL-6, 10 ng/mL IL-23, 2 ng/mL TGFβ, 10 μg/mL anti IFNγ antibody and 10 μg/mL anti IL-4 antibody for Th17 polarising conditions. Cells were incubated for 72 hours and then re-stimulated in fresh media for 24 hours. Cytokine secretion was measured using ELISA (R&D Duoset). One way Anova was used to assess differences between groups. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to cells alone.

5.2.8 Cytokine secretion by BMDCs treated with HPLC fractions of blue whiting hydrolysate 26

Following screening in the above sections of hydrolysates on BMDCs, $CD4^+$ T cells and T cell subsets and in previous chapters, blue whiting hydrolysates H26 and H27 were chosen to be brought forward for HPLC fractionation in order to delve deeper into the optimal bioactive peptide sequence. Hydrolysate H26 was HPLC fractionated into four fractions, F1, F2, F3 and F4. These fractions along with their whole hydrolysate control were screened using the same process previous hydrolysates had undergone.

BMDCs were harvested and grown as before, from the bone marrow of Balb/c mice and incubated at 37° C 5% CO_2 for 8 days in the presence of rGMCSF (Sigma), with media replaced on day 4. Cells were then plated at 1 x 10^6 cells/mL, treated with hydrolysate/fractionate and some (marked +100 ng/mL LPS) stimulated with 100 ng LPS. Supernatants were then analysed for levels of cytokines (TNFα, IL-12p70, IL-23, IL-10, IL-1β, IL-6 and IL-12p40) using ELISA (R&D Duoset).

Figure 5.15 and Figure 5.16 shows the cytokine secretion (TNFα, IL-12p70, IL-23, IL-10 IL-1β, IL-6 and IL-12p40) by BMDCs treated with hydrolysate H26 and its HPLC fractions F1, F2, F3 and F4. Hydrolysate H26 decreased TNFα ($p<0.001$), IL-23 ($p<0.05$), IL-1β ($p<0.01$) and IL-12p40 ($p<0.01$). Fraction F1 decreased TNFα ($p<0.001$), IL-12p70 ($p<0.05$), IL-1β ($p<0.01$) and IL-12p40 ($p<0.001$). Fraction F2 decreased TNFα ($p<0.001$) and IL-12p40 ($p<0.05$). Fraction F3 promoted production of IL-10 ($p<0.05$) and IL-6 ($p<0.05$) and decreased IL-1β ($p<0.01$) and IL-12p40 ($p<0.01$). Fraction F4 increased IL-10 ($p<0.01$) whilst decreasing TNFα ($p<0.001$), IL-23 ($p<0.01$), IL-1β ($p<0.05$) and IL-12p40 ($p<0.001$).

Results from **Figure 5.15** and **Figure 5.16** were then assessed and potential candidate fractions chosen. Fraction 1 was identified as a possible candidate due to its anti-inflammatory activity in decreasing TNFα, IL-12p70, IL-1β and IL-12p40. Fraction 4 was also identified as a potential candidate due to its anti-inflammatory abilities in decreasing TNFα, IL-23, IL-1β and IL-12p40 but also for its ability to promote anti-inflammatory cytokine IL-10.

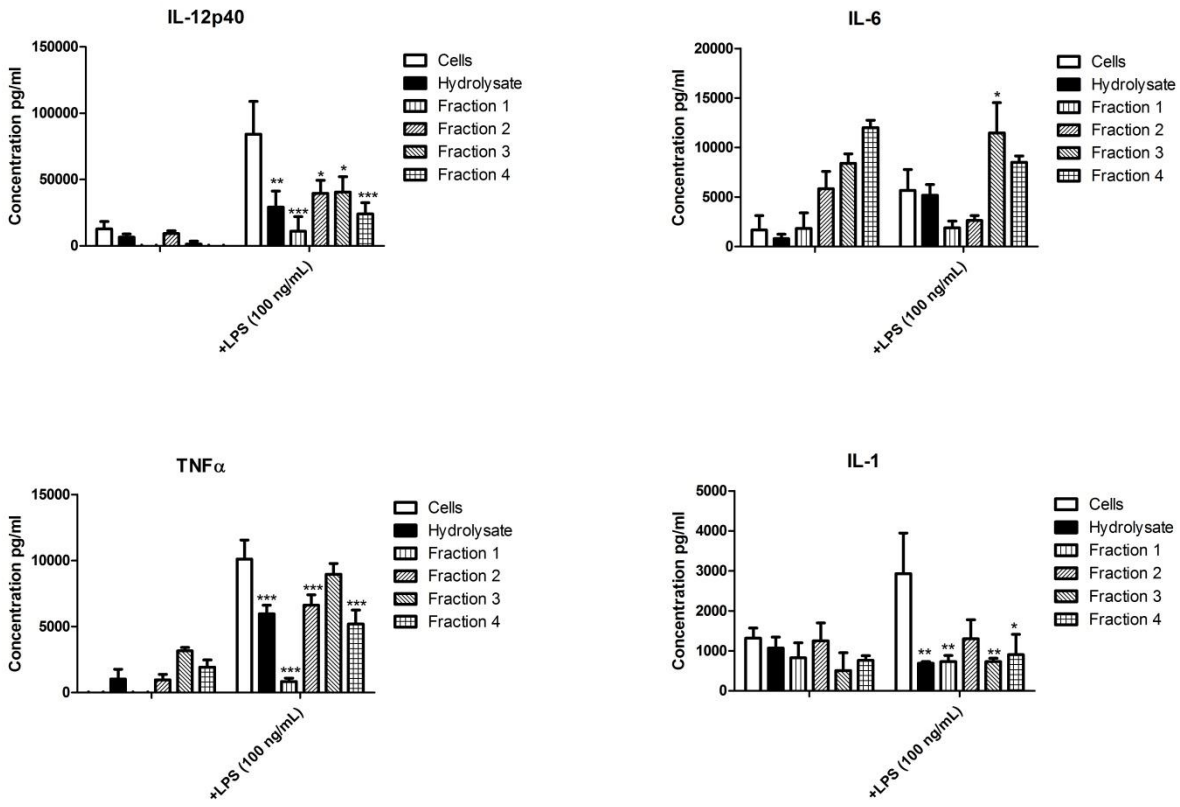


Figure 5.15 Secretion of IL-12p40, IL-6, TNFα and IL-1 by BMDCs treated with Blue Whiting Sample 26 and HPLC fractions of hydrolysate. BMDCs were isolated from Balb/c mice and differentiated in rGMCSF for seven days. On day 7 cells were treated with hydrolysates and stimulated with 100 ng/mL LPS. Secretion of TNFα, IL-12p70, IL-23 and IL-10 was measured using ELISA (R&D Duoset). One way Anova was used to assess differences between groups. Cells treated with hydrolysate only were compared to cells alone, cells treated with hydrolysates + LPS were compared to cells alone + LPS. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to untreated cells (no hydrolysate)

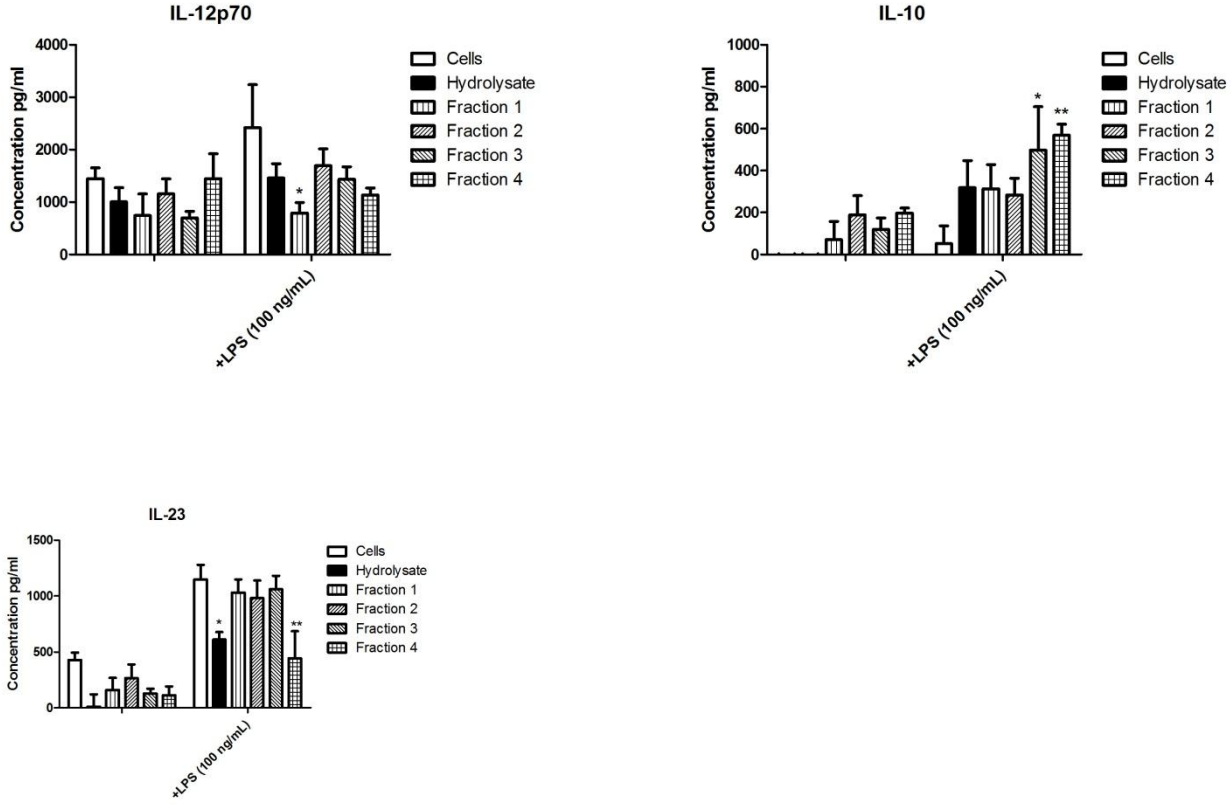


Figure 5.16 Secretion of IL-12p70, IL-10 and IL-23 by BMDCs treated with Blue Whiting Sample 26 and HPLC fractions of hydrolysate. BMDCs were isolated from Balb/c mice and differentiated in rGMCSF for seven days. On day 7 cells were treated with hydrolysates and stimulated with 100 ng/mL LPS. Secretion of IL-6, IL-1β and IL-10 was measured using ELISA (R&D Duoset). One way Anova was used to assess differences between groups. Cells treated with hydrolysate only were compared to cells alone, cells treated with hydrolysates + LPS were compared to cells alone + LPS. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to untreated cells (no hydrolysate).

5.2.9 Cytokine secretion by undifferentiated CD4+ T cells treated with HPLC fractions of blue whiting hydrolysate 26

HPLC fractions of hydrolysate H26 were next screened on undifferentiated $CD4^+$ T cells and their impact on the cytokine secretion of these cells were analysed. Undifferentiated $CD4^+$ T cells were isolated as before using an EasySep Mouse $CD4^+$ T cell Enrichment Kit and treated with hydrolysates at a dose of 1 mg/mL. Secretion of cytokines (IL-13, IL-10, IL-17, IL-2, IL-4, IFNγ and IL-2) was then assessed using ELISA (R&D Duoset).

Figure 5.17 shows $CD4^+$ T cell cytokine secretion treated with HPLC fractions of blue whiting hydrolysate H26. Fractions F1, F2, F3 and F4 along with hydrolysate H26 are shown. Hydrolysate H26 decreased IL-6 ($p<0.01$), IL-10 ($p<0.01$) and IL-4 ($p<0.001$). Fraction F1 reduced levels of IL-6 ($p<0.01$), IL-10 ($p<0.01$), IL-4 ($p<0.01$) and IFNγ ($p<0.05$). Fraction F2 similarly reduced levels of IL-6 ($p<0.05$) and IL-4 ($p<0.01$) but also decreased levels of IL-17 ($p<0.05$). Fraction F3 decreased IL-6 ($p<0.05$) and IL-4 ($p<0.001$) but also increased IL-2 ($p<0.001$) levels. Finally fraction F4 decreased IL-10 ($p<0.001$), IL-4 ($p<0.001$), IFNγ ($p<0.001$) and IL-2 ($p<0.01$).

This data was then compared to that of **Figure 5.15** and **Figure 5.16,** and once again fractions 1 and 4 were identified as possible candidates, both significantly reducing levels of IFNγ in $CD4^+$ T cells. Fraction 2 was also identified as a potential candidate for its ability to modulate IL-17 secretion. However, in order to build a full profile of the fractions ability to modulate the immune system, all fractions were brought forward for screening on T cell subsets.

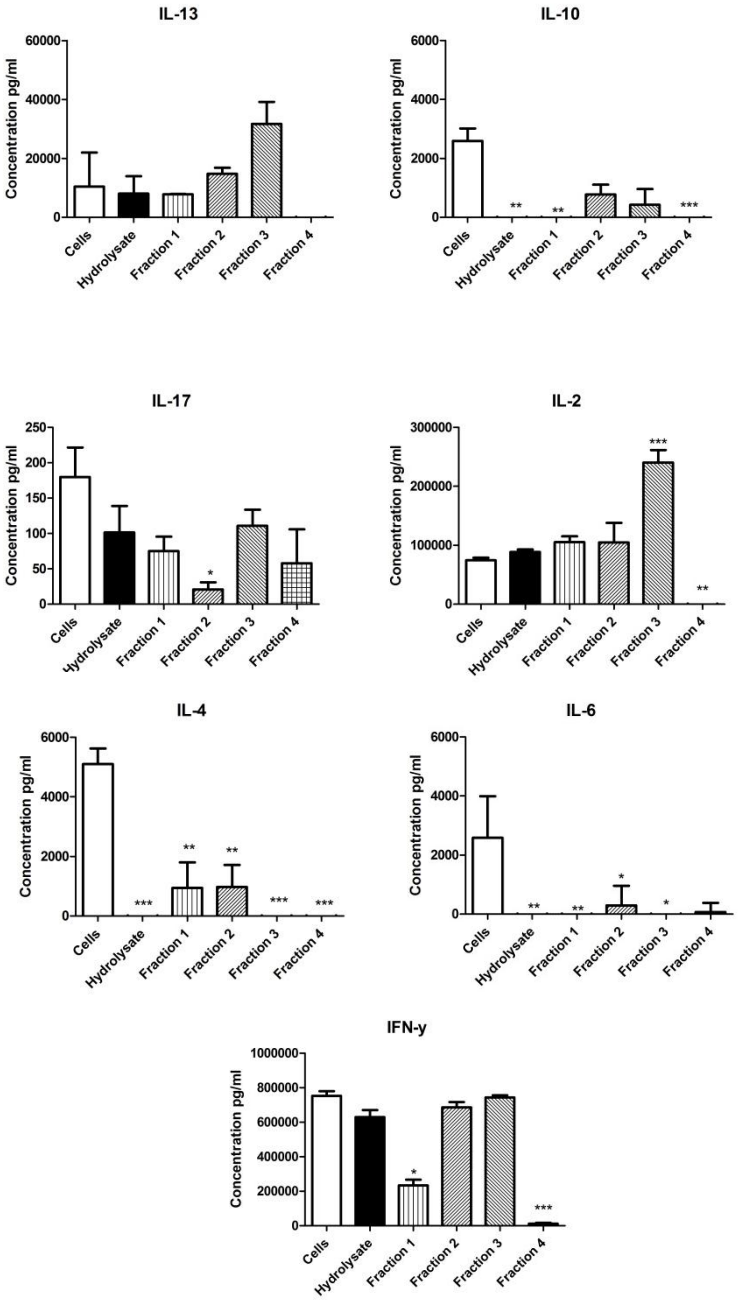


Figure 5.17 Secretion of IL-13, IL-10, IL-17, IL-2, IL-4, IL-6 and IFNγ by undifferentiated CD4$^+$ T cells treated with Blue Whiting Sample 26 and HPLC fractions of hydrolysate. CD4$^+$ T-cells were isolated from the spleens of Balb/c mice using EasySep CD4$^+$ Isolation (Stemcell). Cells were plated at 1 x 10^6 cells / mL on a 96 well plate. Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates (1 mg/mL) and incubated for 72 hours. Secretion of IL-13 and IL-10 was measured using ELISA (R&D Duoset). One way Anova was used to assess differences between groups. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to cells alone.

5.2.10 Cytokine secretion by T-helper cell 1 subset treated with HPLC fractions of blue whiting hydrolysate 26.

Hydrolysate H26 and its HPLC fractions F1, F2, F3 and F4 were then brought forward for analysis on T cell subsets. Th1 cells were isolated using an EasySep Mouse $CD4^+$ T cell Enrichment Kit and the media was supplemented with 20 ng/mL IL-2, 20 ng/mL IL-12 and 10 µg/mL anti-IL- 4 antibody for Th1 polarising conditions. Cells were treated with fractions (1, 2, 3 and 4) or hydrolysate 26, at a dose of 1 mg/mL and incubated for 72 hours before re-stimulation in fresh media for a further 24 hours. Secretion of cytokines (IL-13, IFNγ, IL-17, IL-10, IL-4) was then assessed using ELISA (R&D Duoset).

Figure 5.18 displayed Th1 cell cytokine secretion when treated with hydrolysate H26 or HPLC fractions F1, F2, F3 and F4. IFNγ and IL-2 are the key cytokines associated with Th1 cells. Fraction F1 selectively decreased IFNγ (<0.01). Fraction 4 decreased IFNγ ($p<0.001$). Hydrolysate 26 had no significant impact on the cytokine secretion of Th1 cells.

Other cytokines secreted by Th1 cells were also modulated. Fraction 2 increased IL-4 ($p<0.05$). Fraction 3 decreased IL-17 ($p<0.05$). Fraction 4 decreased IL-13 ($p<0.05$) and increased levels of IL-10 ($p<0.001$). All T cell subsets and the fractions ability to modulate their cytokine secretion were assessed and compared before candidate fractions were chosen.

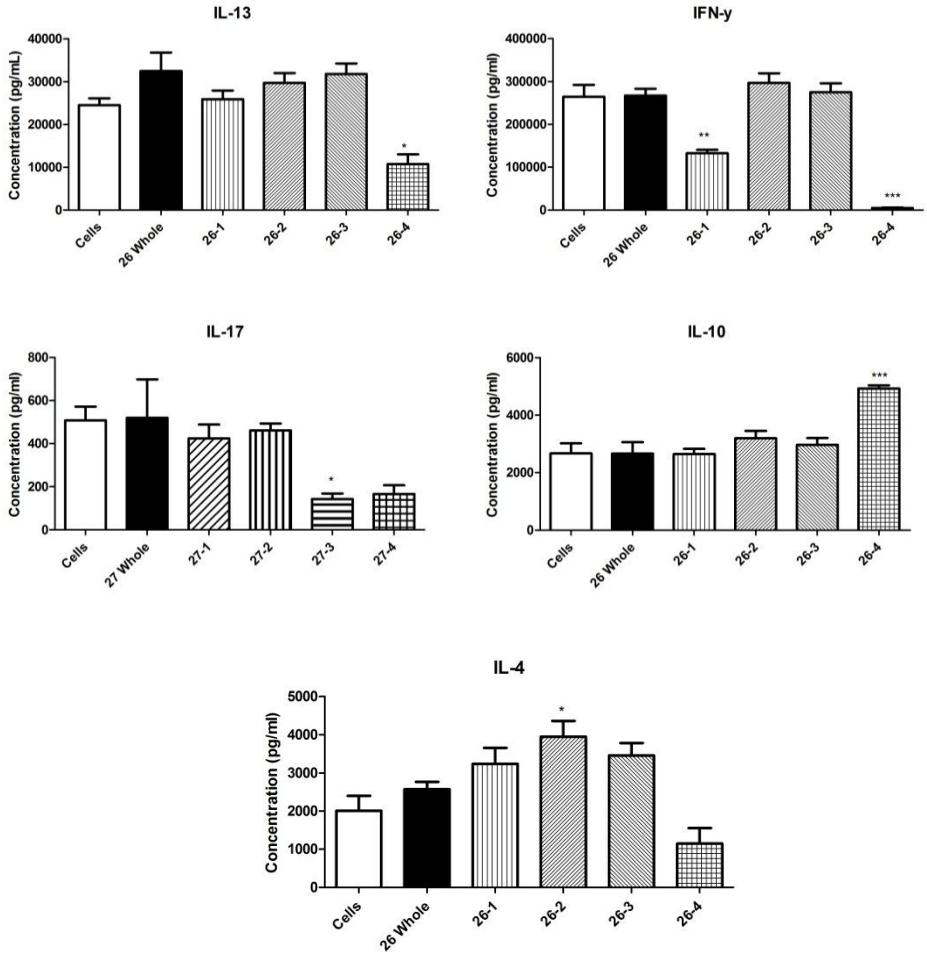


Figure 5.18 Secretion of IL-13, IFNγ, IL-17, IL-10 and IL-4 by Th1 cells treated Blue Whiting HPLC fractions of hydrolysate 26. $CD4^+$ T-cells were isolated from the spleens of Balb/c mice using EasySep $CD4^+$ Isolation (Stemcell). Cells were plated at 1 x 10^6 cells / mL on a 96 well plate. Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates (1 mg/mL). The media was supplemented with 20 ng/mL IL-2, 20 ng/mL IL-12 and 10 μg/mL anti-IL-4 antibody for Th1 polarising conditions. Cells were incubated for 72 hours and then re-stimulated in fresh media for 24 hours. Cytokine secretion was measured using ELISA (R&D Duoset). One way Anova was used to assess differences between groups. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to cells alone.

5.2.11 Cytokine secretion of T helper cell 2 subset treated treated with HPLC fractions of blue whiting hydrolysate 26.

Following their assessment on Th1 cells (**Section 5.2.9**), HPLC fractions F1, F2, F3 and F4 along with hydrolysate H26 were then screened on a Th2 subset. Th2 cells were isolated using an EasySep Mouse $CD4^+$ T cell Enrichment Kit and the media was supplemented with 10 µg/mL neutralising IFNγ antibody and 10 mg/mL IL-4 to induce Th2 polarising conditions. Cells were treated with hydrolysate 26 or its fractions 1, 2, 3 and 4, at a dose of 1 mg/mL and incubated for 72 hours before re-stimulation in fresh media for a further 24 hours. Secretion of cytokines (IL-13, IFNγ, IL-17, IL-10, IL-4) was then assessed using ELISA (R&D Duoset).

Figure 5.19 shows Th2 cell cytokine secretion when treated with hydrolysate H26 and its HPLC fractions F1, F2, F3 and F4. IL-13 and IL-4 are they key cytokines associated with Th2 cells. Fraction F1 decreased IL-13 ($p<0.001$) and IL-4 ($p<0.01$). Fraction F2, F3 and F4 reduced IL-13 ($p<0.01$).

Other cytokines secreted by Th2 cells were also modulated. Hydrolysate H26 reduced levels of IL-17 ($p<0.05$). Fraction F1 increased levels of IL-17 ($p<0.01$) and IL-10 ($p<0.001$). Fraction F2 reduced IL-10 ($p<0.001$) levels. Fraction 3 decreased IL-17 ($p<0.01$) and IL-10 ($p<0.01$). Fraction 4 decreased IFNγ ($p<0.001$).

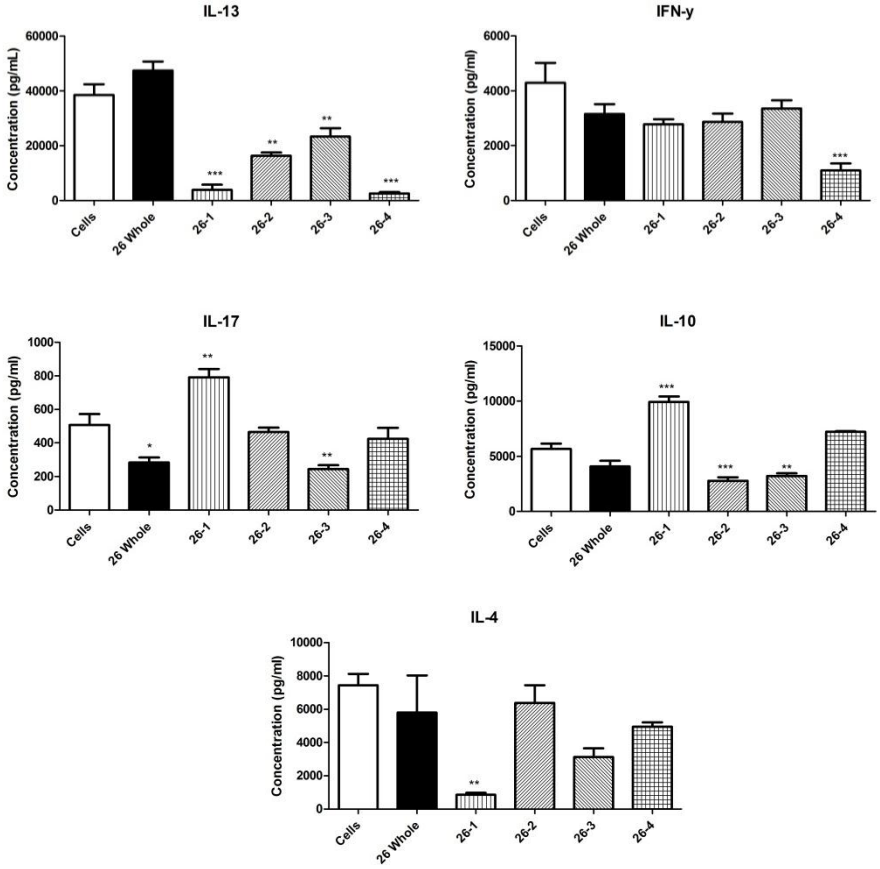


Figure 5.19 Secretion of IL-13, IFNγ, IL-17, IL-10 and IL-4by Th2 cells treated Blue Whiting HPLC fractions of hydrolysates. $CD4^+$ T-cells were isolated from the spleens of Balb/c mice using EasySep $CD4^+$ Isolation (Stemcell). Cells were plated at 1 x 10^6 cells / mL on a 96 well plate. Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates (1 mg/mL). The media was supplemented with 10 μg/mL neutralizing IFNγ antibody and 10 mg/mL IL-4 for Th2 polarising conditions. Cells were incubated for 72 hours and then re-stimulated in fresh media for 24 hours. Cytokine secretion was measured using ELISA (R&D Duoset). One way Anova was used to assess differences between groups. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to cells alone.

5.2.12 Cytokine secretion by T helper cell 17 subset treated treated with HPLC fractions of blue whiting hydrolysate 26.

Th17 cells and their cytokine secretions were then assessed in the investigation of HPLC fractions of hydrolysate H26. Th17 cells were isolated using an EasySep Mouse $CD4^+$ T cell Enrichment Kit and the media was supplemented with 10 µg/mL anti-IFNγ antibody, 10 µg/mL IL-4, 20 ng/mL IL-6, 10 ng/mL IL-23 and 2 ng/mL TGFβ to induce Th17 polarising conditions. Cells were treated with HPLC fractions 1, 2, 3, and 4 or hydrolysate 26 at a dose of 1 mg/mL and incubated for 72 hours before re-stimulation in fresh media for a further 24 hours. Secretion of cytokines (IL-13, IFNγ, IL-17, IL-10, IL-4) was then assessed using ELISA (R&D Duoset).

Figure 5.20 shows Th17 cell cytokine secretion when treated with HPLC fractions F1, F2, F3 and F4 and also hydrolysate H26. IL-17 is the key cytokine secreted by Th17 cells. Fractions F2 and F4reduced IL-17 ($p<0.05$; $p<0.001$).

Hydrolysate H26 significantly reduced IL-13 ($p<0.001$) and IFNγ ($p<0.05$) secreted from Th17 cells. Fraction 1 and fraction 3 had no significant impact on cytokine secretion from Th17 cells. Fraction 4 reduced IL-13 ($p<0.05$), IFNγ ($p<0.01$), and IL-4 ($p<0.001$) and also increased IL-10 ($p<0.05$) levels.

Data from **Section 5.2.8, Section 5.2.9, Section 5.2.10, Section 5.2.11** and **Section 5.2.12** was then assessed together in order to choose optimum HPLC fractions to be brought forward into *in vivo* studies. F4 was identified as the prime candidate due to its potent anti-inflammatory effects on BMDCs with the ability to reduce levels of pro-inflammatory TNFα, IL-23, IL-1β and IL-12p40 whilst increasing anti-inflammatory IL-10, its ability to decrease IFNγ in both $CD4^+$ T cells and Th1 cells, and reduce IL-17 in Th17 cells. Fraction F1 was identified as a candidate due its selective nature in reducing pro-inflammatory TNFα, IL-12p70, IL-1β and IL-12p40 from BMDCs and IFNγ in both $CD4^+$ T cells and Th1 cells whilst having no effect on Th17. Furthermore, its ability to also reduce IL-13 in Th2 cells may also identify it as a potential anti-allergy compound. Finally, fraction F2 was identified as a third possible candidate due to its ability to modulate IL-17 levels in Th17 cells.

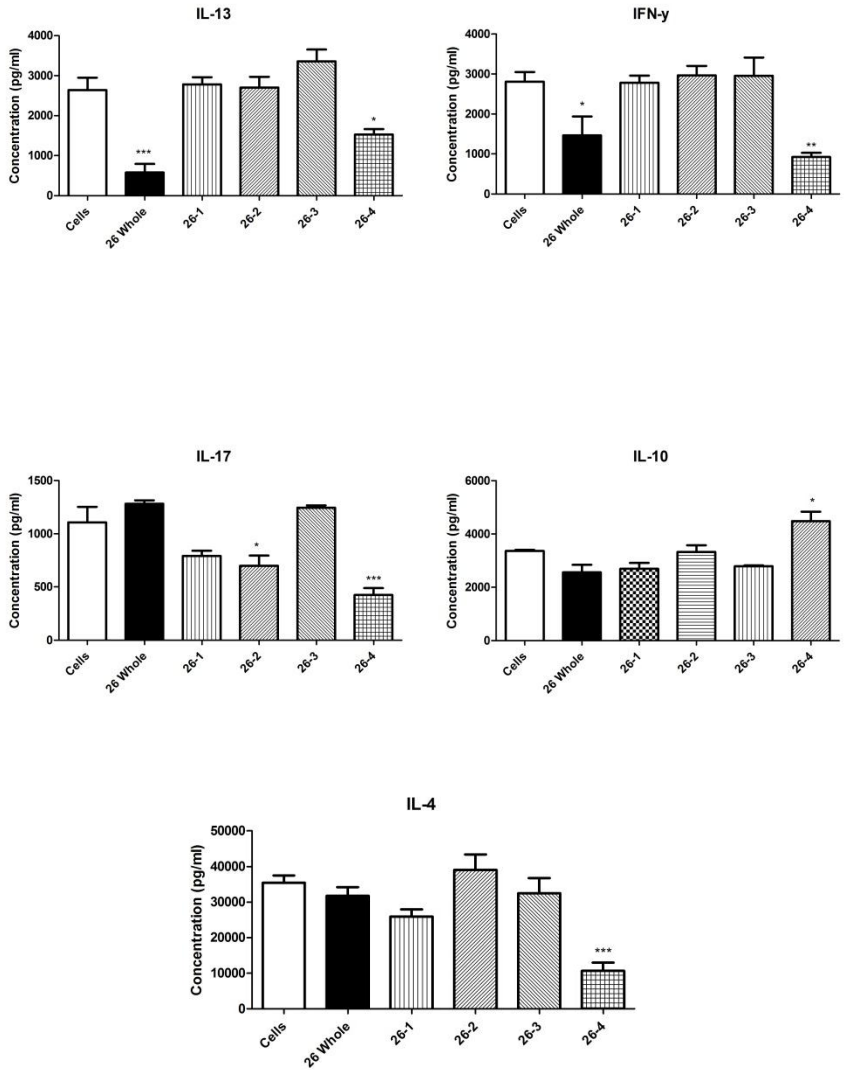


Figure 5.20 Secretion of IL-13, IFNγ, IL-17, IL-10 and IL-4 by Th17 cells treated Blue Whiting HPLC fractions of hydrolysates. $CD4^+$ T-cells isolated from the spleens of Balb/c mice using EasySep $CD4^+$ Isolation (Stemcell). Cells were plated at 1 x 10^6 cells / mL on a 96 well plate. Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates (1 mg/mL). The media was supplemented with 20 ng/mL IL-6, 10 ng/mL IL-23, 2 ng/mL TGFβ, 10 μg/mL anti IFNγ antibody and 10 μg/mL anti IL-4 antibody for Th17 polarising conditions. Cells were incubated for 72 hours and then re-stimulated in fresh media for 24 hours. Cytokine secretion was measured using ELISA (R&D Duoset). One way Anova was used to assess differences between groups. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to cells alone.

5.2.13 Cytokine secretion by BMDCs treated with HPLC fractions of blue whiting hydrolysate 27

Hydrolysate H27 was also chosen to be brought forward for HPLC fractionation in order to mine the peptide sequence for a bioactive peptide chain with anti-inflammatory abilities. Hydrolysate H27, similarly to H26, was HPLC fractionated into four fractions, F1, F2, F3 and F4. These fractions along with their whole hydrolysate control were screened using the same process previous hydrolysates and fractions had undergone.

BMDCs were harvested and grown as before in the presence of rGMCSF (Sigma) for 8 days and fed on day 4. Cells were plated at 1 x 10^6 cells/mL, treated with hydrolysate/fraction and some (marked +100 ng/mL LPS) stimulated with 100 ng LPS. Supernatants were then analysed for levels of cytokines (TNFα, IL-12p70, IL-23, IL-10, IL-1β, IL-6 and IL-12p40) using ELISA (R&D Duoset).

Figure 5.21 and **Figure 5.22** show the cytokine profile (TNFα, IL-12p70, IL-23, IL-10, IL-1β, IL-6 and IL-12p40) of BMDCs treated with hydrolysate H27 and its HPLC fractions F1, F2, F3 and F4. Hydrolysate H27 significantly reduced TNFα ($p<0.05$), IL-10 ($p<0.001$), IL-6 ($p<0.01$) and IL-12p40 ($p<0.001$) whilst increasing levels of IL-23 ($p<0.001$). Fraction F1 decreased TNFα ($p<0.05$), IL-12p70 ($p<0.05$), IL-10 ($p<0.001$), IL-6 ($p<0.01$), IL-12p40 ($p<0.001$) and IL-1β ($p<0.01$). Fraction F2 and F3 decreased IL-12p70 ($p<0.05$) and IL-10 ($p<0.001$). Fraction F4 decreased IL-12p70 ($p<0.05$), IL-10 ($p<0.001$, IL-6 ($p<0.01$) and IL-12p40 ($p<0.001$) and also IL-1β ($p<0.01$).

Figure 5.21 and **Figure 5.22** were then compared and all fractions were identified as potential candidates with further analysis of their ability to modulate the immune system needed.

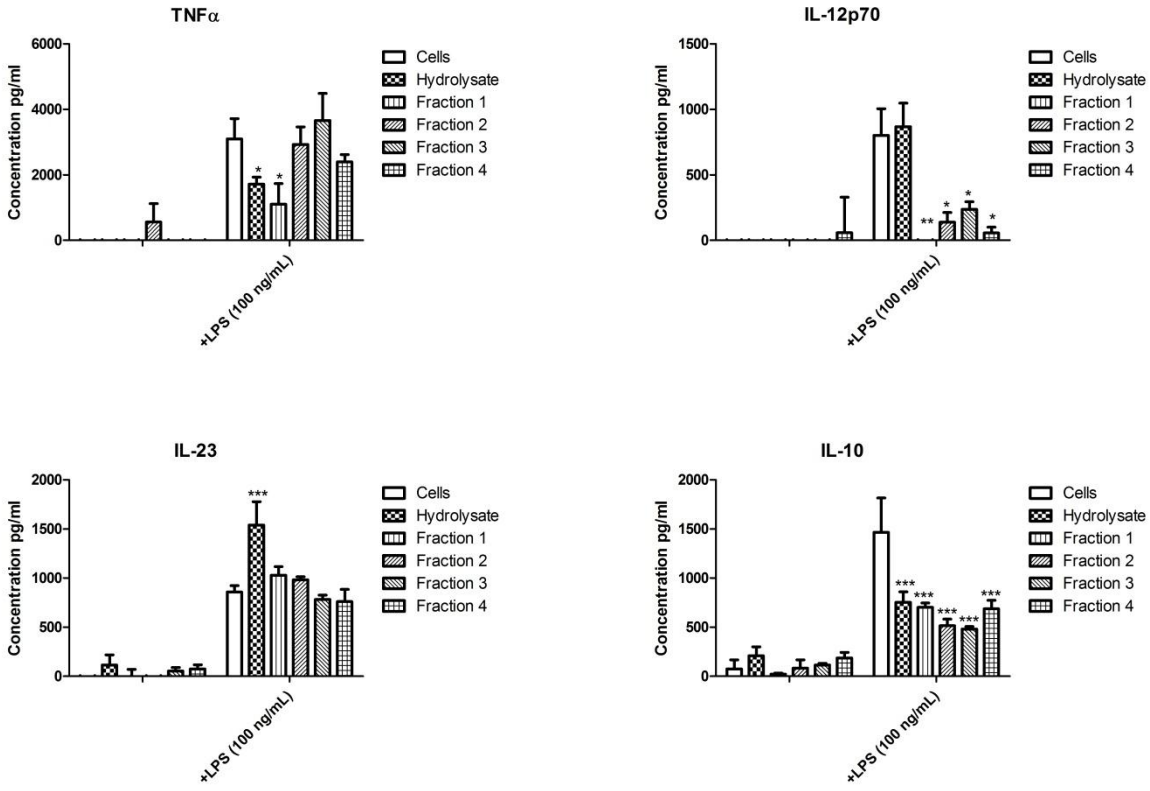


Figure 5.21 Secretion of TNFα, IL-12p70, IL-23 and IL-10 by BMDCs treated with Blue Whiting Sample 27 and HPLC fractions of hydrolysate. BMDCs were isolated from Balb/c mice and differentiated in rGMCSF for seven days. On day 7 cells were treated with hydrolysates and stimulated with 100 ng/mL LPS. Secretion of TNFα, IL-12p70, IL-23 and IL-10 was measured using ELISA (R&D Duoset). One way Anova was used to assess differences between groups. Cells treated with hydrolysate only were compared to cells alone, cells treated with hydrolysates + LPS were compared to cells alone + LPS. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to untreated cells (no hydrolysate).

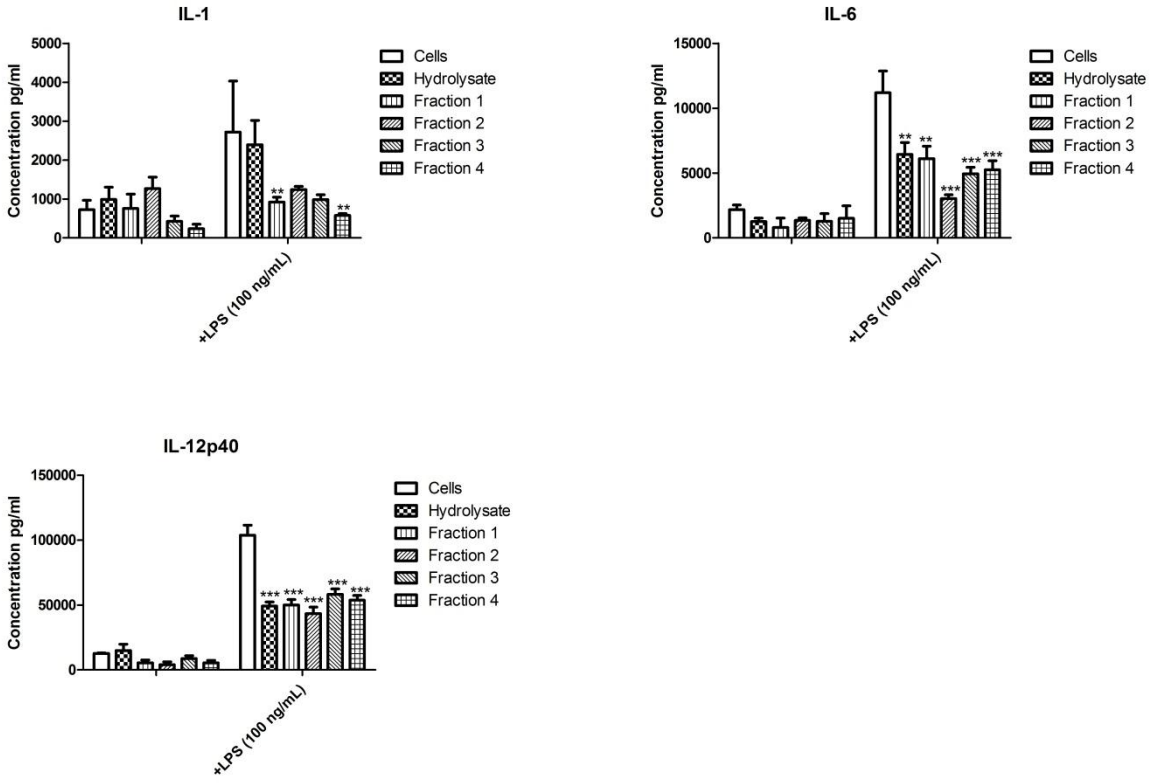


Figure 5.22 Secretion of IL-1β, IL-6 and IL-12p40 by BMDCs treated with Blue Whiting Sample 27 and HPLC fractions of hydrolysate. BMDCs were isolated from Balb/c mice and differentiated in rGMCSF for seven days. On day 7 cells were treated with hydrolysates and stimulated with 100 ng/mL LPS. Secretion of IL-6, IL-1β and IL-10 was measured using ELISA (R&D Duoset). One way Anova was used to assess differences between groups. Cells treated with hydrolysate only were compared to cells alone, cells treated with hydrolysates + LPS were compared to cells alone + LPS. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to untreated cells (no hydrolysate).

5.2.14 Cytokine secretion by undifferentiated CD4+ T cells treated with HPLC fractions of blue whiting hydrolysate 27

As with hydrolysate H26 and its fractions, hydrolysate H27 and its HPLC fractions were next screened on undifferentiated $CD4^+$ T cells and their impact on the cytokine secretion of these cells were analysed. $CD4^+$ T cells were isolated as before using an EasySep Mouse $CD4^+$ T cell Enrichment Kit and treated with hydrolysates at a dose of 1 mg/mL. Secretion of cytokines (IL-13, IL-6, IL-10, IL-17, IL-4, IFNγ and IL-2) was then assessed using ELISA (R&D Duoset).

Figure 5.23 shows $CD4^+$ T cell cytokine secretion following treatment with HPLC fractions of blue whiting hydrolysate 27. Fractions 1, 2, 3 and 4 and hydrolysate 27 are shown. Hydrolysate 27 reduced levels of IL-13 ($p<0.01$), IL-6 ($p<0.001$), IL-10 ($p<0.01$), IL-17 ($p<0.001$) and IL-4 ($p<0.001$). Fraction 1 decreased IL-6 ($p<0.001$), IL-10 ($p<0.05$), IL-17 ($p<0.001$) and IL-4 ($p<0.01$). Fraction 2 reduced IL-6 ($p<0.05$) and IL-17 ($p<0.001$). Fraction 3 and 4 both selectively reduced IL-17 ($p<0.001$) only. In order to follow the same screening process as hydrolysate 26 and its fractions, hydrolysate 27 and all of its fractions (1, 2, 3 and 4) were brought forward for screening on T cell subsets.

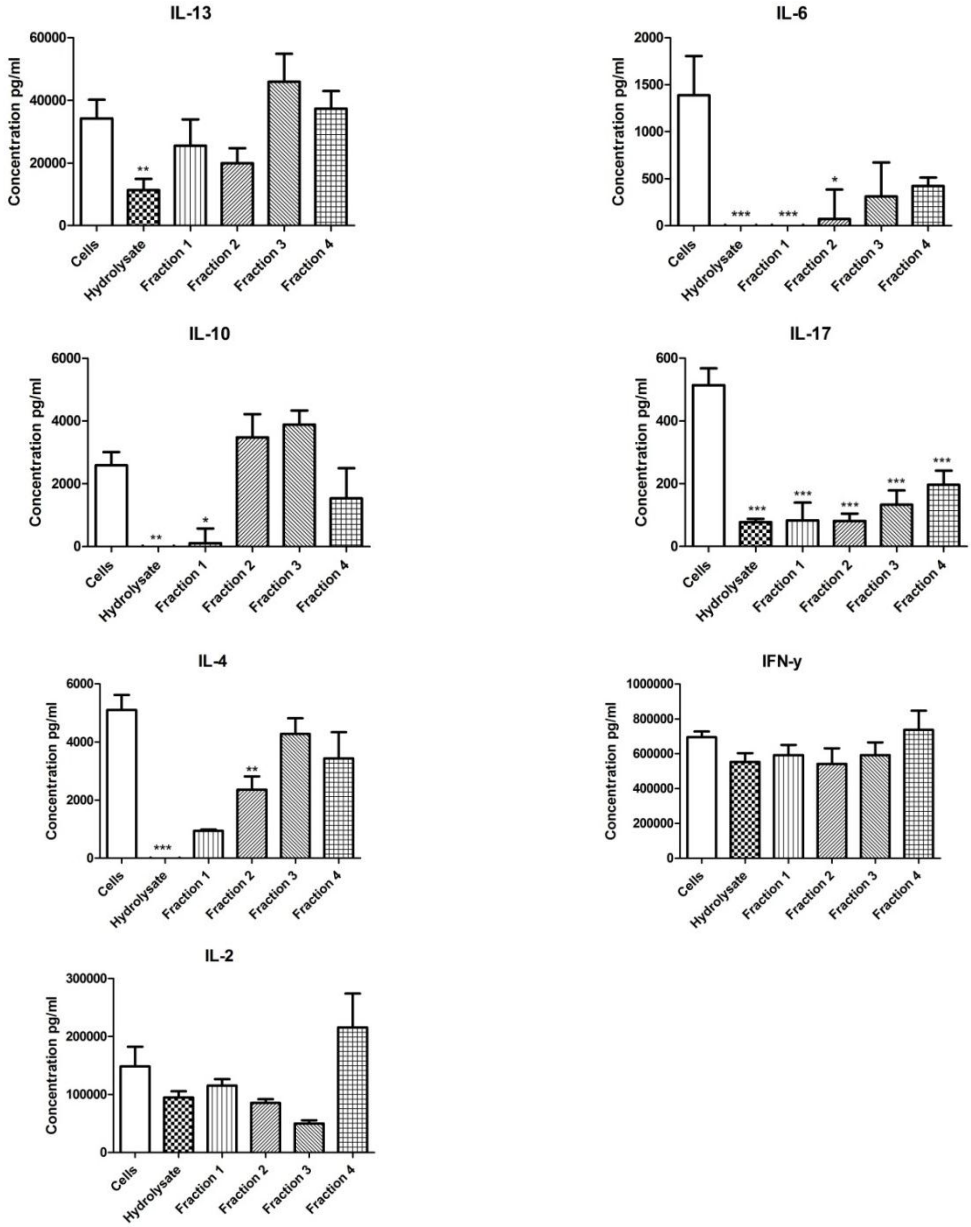


Figure 5.23 Secretion of IL-13, IFNγ, IL-17, IL-10 and IL-4 by undifferentiaed CD4$^+$ T-cells treated with Blue Whiting Sample 27 and HPLC fractions of hydrolysate. CD4$^+$ T-cells were isolated from the spleens of Balb/c mice using EasySep CD4$^+$ Isolation (Stemcell). Cells were plated at 1 x 10^6 cells / mL on a 96 well plate. Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates (1 mg/mL) and incubated for 72 hours. Cytokine secretion was measured using ELISA (R&D Duoset). One way Anova was used to assess differences between groups. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to cells alone.

5.2.15 Cytokine secretion by T-helper cell 1 subset treated with HPLC fractions of blue whiting hydrolysate 27.

Hydrolysate H27 and its HPLC fractions F1, F2, F3 and F4 were assessed for their effects on T cell subsets. Th1 cells were isolated using an EasySep Mouse $CD4^+$ T cell Enrichment Kit and the media was supplemented with 20 ng/mL IL-2, 20 ng/mL IL-12 and 10 µg/mL anti-IL- 4 antibody for Th1 polarising conditions. Cells were treated with fractions (1, 2, 3 and 4) or hydrolysate 27, at a dose of 1 mg/mL and incubated for 72 hours before re-stimulation in fresh media for a further 24 hours. Secretion of cytokines (IL-13, IFNγ, IL-17, IL-10 and IL-4) was then assessed using ELISA (R&D Duoset).

Figure 5.24 shows Th1 cell cytokine secretion when treated with hydrolysate H27 or HPLC fractions F1, F2, F3 and F4. IFNγ and IL-2 are the key cytokines secreted by Th1 cells. Hydrolysate H27 and fraction F1 reduced levels of IFNγ ($p<0.01$). Fraction F2 had no significant impact on cytokine secretion from Th1 cells. Fraction F3 reduced IFNγ ($p<0.01$).

Other cytokines secreted by Th1 cells were also modulated. Hydrolysate H27 reduced levels of IL-13 ($p<0.05$) and IL-10 ($p<0.05$). Fraction F1 decreased IL-13 ($p<0.01$), IL-10 ($p<0.01$) and IL-4 ($p<0.05$). Fraction F3 reduced IL-13 ($p<0.05$), IL-17 ($p<0.05$), IL-10 ($p<0.05$) and IL-4 ($p<0.001$). Fraction F4 reduced IL-13 ($p<0.01$), IL-10 ($p<0.01$) and IL-4 ($p<0.001$). As with hydrolysate H26 and its fractions, the fractions ability to modulate the cytokine secretion of a range of T cell subsets was assessed and compared before candidate fractions were chosen.

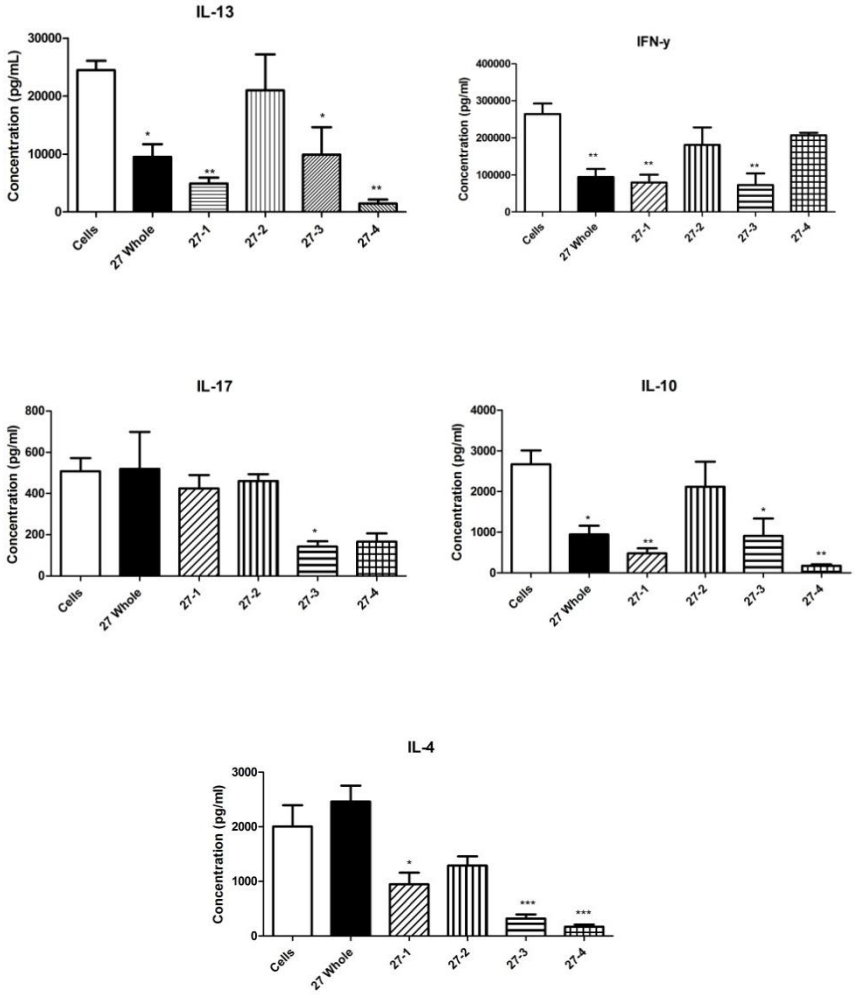


Figure 5.24 Secretion of IL-13, IFNγ, IL-17, IL-10 and IL-4 by Th1 cells treated Blue Whiting HPLC fractions of hydrolysates. $CD4^+$ T-cells were isolated from the spleens of Balb/c mice using EasySep $CD4^+$ Isolation (Stemcell). Cells were plated at 1 x 10^6 cells / mL on a 96 well plate. Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates (1 mg/mL). The media was supplemented with 20 ng/mL IL-2, 20 ng/mL IL-12 and 10 μg/mL anti-IL-4 antibody for Th1 polarising conditions. Cells were incubated for 72 hours and then re-stimulated in fresh media for 24 hours. Cytokine secretion was measured using ELISA (R&D Duoset). One way Anova was used to assess differences between groups. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to cells alone.

5.2.16 Cytokine secretion by T helper cell 2 subset treated with HPLC fractions of blue whiting hydrolysate 27.

Hydrolysate H27 and its HPLC fractions F1, F2, F3 and F4 were next screened on a Th2 subset. Th2 cells were isolated using an EasySep Mouse $CD4^+$ T cell Enrichment Kit and the media was supplemented with 10 μg/mL neutralising IFNγ antibody and 10 mg/mL IL- 4 to induce Th2 polarising conditions. Cells were treated with HPLC fractions 1, 2, 3 and 4 and hydrolysate 27 at a dose of 1 mg/mL and incubated for 72 hours before re-stimulation in fresh media for a further 24 hours. Secretion of cytokines (IL-13, IFNγ, IL-17, IL-10, IL-4) was then assessed using ELISA (R&D Duoset).

Figure 5.25 shows Th2 cell cytokine secretion when treated with HPLC fractions F1, F2, F3 and F4 and hydrolysate H27. IL-13 and IL-4 are the key cytokines associated with a Th2 cell subset. Hydrolysate H27 had no impact on the cytokine secretion of Th2 cells. Fraction F1 decreased IL-13 ($p<0.01$) and IL-4 ($p<0.01$). Fraction F2 decreased IL-13 ($p<0.05$). Fraction F3 reduced levels of IL-13 ($p<0.001$). Fraction F4 decreased IL-13 ($p<0.001$) and IL-4 ($p<0.05$).

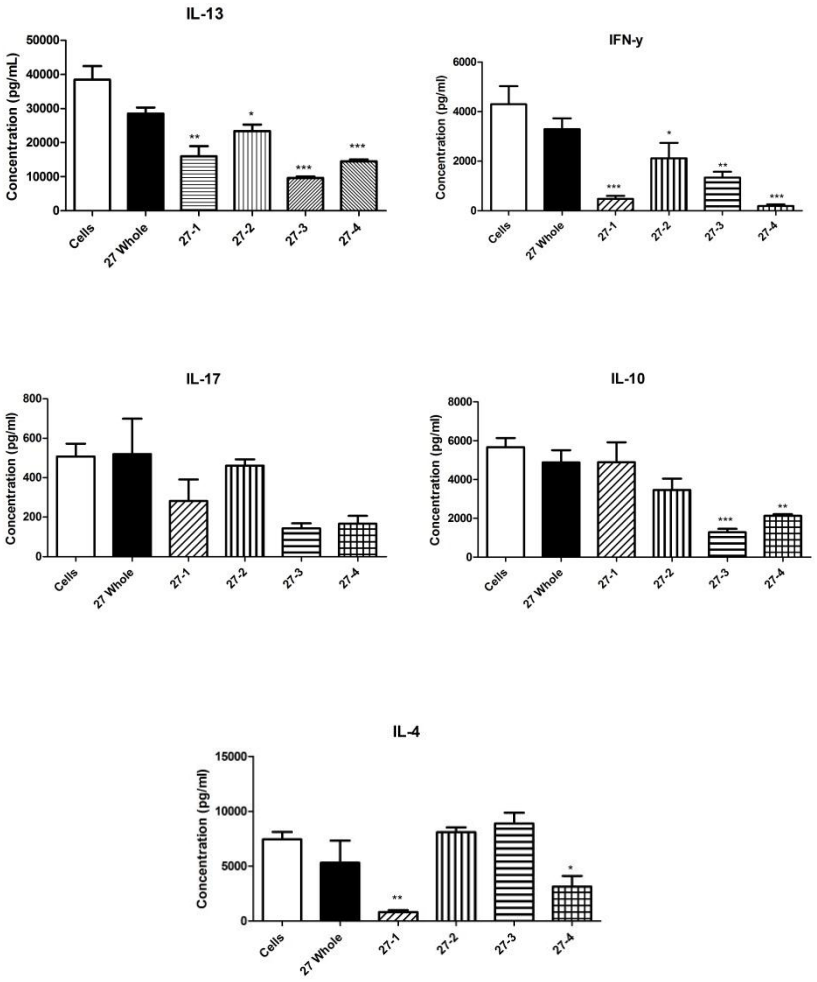


Figure 5.25 Secretion of IL-13, IL-6, IL-10, IL-17, IL-4, IFNγ and IL-2 by Th2 cells treated Blue Whiting HPLC fractions of hydrolysates. $CD4^+$ T-cells were isolated from the spleens of Balb/c mice using EasySep $CD4^+$ Isolation (Stemcell). Cells were plated at 1 x 10^6 cells / mL on a 96 well plate. Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates (1 mg/mL). The media was supplemented with 10 μg/mL neutralizing IFNγ antibody and 10 mg/mL IL-4 for Th2 polarising conditions. Cells were incubated for 72 hours and then re-stimulated in fresh media for 24 hours. Cytokine secretion was measured using ELISA (R&D Duoset). One way Anova was used to assess differences between groups. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to cells alone.

5.2.17 Cytokine secretion of T helper cell 17 subset treated with HPLC fractions of blue whiting hydrolysate 27.

Finally, the cytokine secretion of Th17 cells when treated with HPLC fractions of hydrolysate H27 were then screened. Th17 cells were isolated using an EasySep Mouse $CD4^+$ T cell Enrichment Kit and the media was supplemented with 10 µg/mL anti-IFNγ antibody, 10 µg/mL IL- 4, 20 ng/mL IL-6, 10 ng/mL IL-23 and 2 ng/mL TGFβ to induce Th17 polarising conditions. Cells were treated with HPLC fractions F1, F2, F3, and F4 or hydrolysate H27 at a dose of 1 mg/mL and incubated for 72 hours before re-stimulation in fresh media for a further 24 hours. Secretion of cytokines (IL-13, IFNγ, IL-17, IL-10, IL-4) was then assessed using ELISA (R&D Duoset).

Figure 5.26 shows Th17 cell cytokine secretion when treated with HPLC fractions F1, F2, F3 and F4 and also hydrolysate H27. IL-17 is the key cytokine associated with Th17 cells. Fraction 1 decreased IL-17 ($p<0.01$).

Other cytokines secreted by Th17 cells were also modulated. Hydrolysate H27 reduced levels of IL-13 ($p<0.05$), IFNγ ($p<0.001$) and IL-4 ($p<0.01$) whilst increasing IL-10 ($p<0.001$). Fraction F1 decreased IL-13 ($p<0.01$), IFNγ ($p<0.001$), IL-10 ($o<0.001$) and IL-4 ($p<0.01$). Fraction F2 decreased IL-10 ($p<0.001$). Fraction F3 decreased IFNγ ($p<0.001$), IL-10 ($p<0.001$) and IL-4 ($p<0.01$). Fraction F4 decreased IL-13 ($p<0.05$), IFNγ ($p<0.001$), IL-10 ($p<0.001$) and IL-4 ($p<0.05$).

Data from **Section 5.2.13, Section 5.2.14, Section 5.2.15, Section 5.2.16** and **Section 5.2.17** was then assessed together in order to choose optimum HPLC fractions to be brought forward into *in vivo* studies. F1 was identified as an ideal candidate to be brought forward for further screening including *in vivo* work. Fraction F1 displayed significant anti-inflammatory abilities across all cells investigated including reducing pro-inflammatory cytokines TNFα, IL-12p70, IL-6 and IL-12p40 in BMDCs, IL-6 and IL-17 in $CD4^+$ T cells, IFNγ in Th1 cells and IL-17 in Th17 cells whilst also displaying anti-allergy potential in the reduction of IL-13 and IL-4 in Th2 cells.

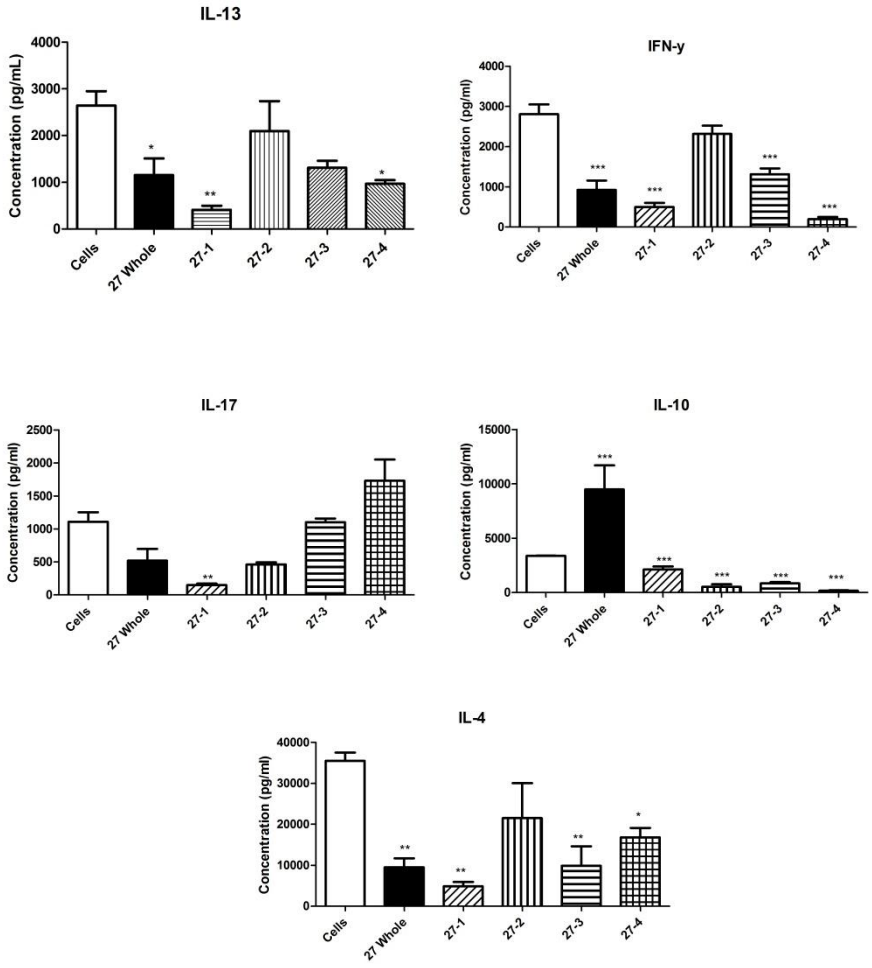


Figure 5.26 Secretion of IL-13,IFNγ, IL-17, IL-10 and IL-4in Th17 cells treated Blue Whiting HPLC fractions of hydrolysates. $CD4^+$ T-cells isolated from the spleens of Balb/c mice using EasySep $CD4^+$ Isolation (Stemcell). Cells were plated at 1 x 10^6 cells / mL on a 96 well plate. Cells were stimulated with plate bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL) then treated with hydrolysates (1 mg/mL). The media was supplemented with 20 ng/mL IL-6, 10 ng/mL IL-23, 2 ng/mL TGFβ, 10 μg/mL anti IFNγ antibody and 10 μg/mL anti IL-4 antibody for Th17 polarising conditions. Cells were incubated for 72 hours and then re-stimulated in fresh media for 24 hours. Cytokine secretion was measured using ELISA (R&D Duoset). One way Anova was used to assess differences between groups. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to cells alone.

5.3 Discussion

Micromesistius poutassou, also known as blue whiting, is a member of the cod family found in the North East Atlantic. Commonly found between North Africa and Iceland, its numbers in Irish waters have increased in the past two decades, mainly due to a rise in plankton, on which they feed, off the coast of Ireland (Hátún *et al.,* 2009). Blue whiting are approximately 30 cm in length, silver in colour and are a long, narrow fish. They are considered a low value, under-utilised fish in Ireland, not often a feature on the Irish dinner table. An increase in the worldwide demand for protein has seen an increased interest in low value fish and the side streams of fish processing currently used in producing animal feed. This is due to the realisation that many low value fish have relatively high protein concentrations and as a result are a potential rich source of bioactive protein hydrolysates and peptides. This provides an opportunity for fish processors to add value to already established processes (García-Morena *et al*., 2017).

These bioactive compounds could then be used as functional food ingredients which have experienced a huge increase in value due to an ever-growing health conscious population. This has come at an ideal time for the marine industry, as already discussed in Chapter 4, reforms to the European Common Fisheries Policy, also known as the landing obligation, will see large increases in the quantities of low value fish in Irish marine processing plants (Veiga *et al.,* 2016). These reforms, which will be fully implemented by 2019, provide an opportunity to utilise these extra numbers as potential protein sources for bioactive hydrolysates and peptides.

Blue whiting is one such low value species which will experience an increase in numbers landed in Irish fish processing plants as of 2019. Blue whiting has a 19% (w/w) protein content (Yoshie-Stark *et al.,* 2009). Protein hydrolysates generated from this fish have already been reported to have exciting bioactive activity. These include appetite reducing activity which has been seen both *in vitro* and *in vivo* (both in human and animal) through stimulation of the appetite suppressing glucagon-like peptide 1 (GLP-

1) (Cudennec *et al.,* 2012) (Nobile *et al.,* 2016). Protein hydrolysates from blue whiting have also been shown to have anti-diabetic properties by our project collaborators Harnedy *et al.* (2018) who reported DPP-IV inhibition and enhancement of insulin stimulated glucose uptake both *in vitro* and *in vivo* in a murine model using hydrolysates generated in the same way as those screened in this project. However, little is known about their effects on the immune system, hence blue whiting was chosen as the final species investigated in this project.

Blue whiting hydrolysates were generated by collaborators in the University of Limerick using the same method as Harnedy *et al.* (2018). A total of 16 protein hydrolysates were generated along with 4 whole protein controls (**Table 5.1**). Hydrolysates were screened using the same *in vitro* screening protocol as optimised in **Chapter 3,** to ensure comparability of results. The effect on cell viability of both $CD4^+$ T cells and BMDCs when treated with blue whiting protein hydrolysate was first investigated. No hydrolysates were found to be cytotoxic to either cell type at the dose of 1 mg/mL. This was as expected, as these hydrolysates had previously been investigated both *in vitro* and *in vivo* by Harnedy *et al.* (2018).

Hydrolysates were next screened in BMDCs to study their ability to modulate the cytokine secretion by these cells. Hydrolysates were grouped, as described in **Table 5.1,** for this investigation. During this study several hydrolysates displayed anti-inflammatory properties either through the suppression of pro-inflammatory cytokines different to that of their whole protein control indicating that the activity was due to the hydrolysed nature of the protein only.

A number of hydrolysates decreased the secretion of key anti-inflammatory cytokines TNFα, IL-6 and IL-12p40 by BMDCs whilst others increased levels of the anti-inflammatory cytokine IL-10. Hydrolysates H24/ H25/ H26/ H27/ H32/ H33/H34 all reduced levels of TNFα, WPC37/ WPC38/ WPC40 also reduced this cytokine. The above seven hydrolysates

all significantly decreased the pro-inflammatory cytokine TNFα, however only H27 did so differently to that of its whole protein control (WPC39). This indicates that this anti-inflammatory activity is due to the hydrolysed nature of the protein, as it is not present in the whole protein. All other hydrolysates behave similar to that of their whole protein controls.

TNFα is a key pro-inflammatory cytokine which has been implicated in a large number of inflammatory disorders including inflammaging (Lencel and Magne, 2011). An upregulation of TNFα is associated with age and has even been shown by Mooradian *et al.* (1991) to be a possible indicator of mortality. TNFα in normal immunity plays a role in the regulation of immune cells, in chronic inflammation, however, it is often over secreted resulting in this systemic inflammation. It has been identified as a target for anti-inflammatory therapeutics to combat a number of inflammatory diseases such as RA, IBD and psoriasis. All current therapies, including such monoclonal antibodies such infliximab, are administered intravenously (IV) in hospitals (Hanaeur *et al.,* 2002). A hydrolysate which can be incorporated into a functional ingredient which reduces levels of TNFα could help combat inflammation whilst removing the need for such invasive therapies or could act as an adjunct therapy.

Hydrolysates H26/H27/H28/H30/H34/H35/H36 decreased levels of IL-6 secretion, only WPC39 decreased this cytokine. This suggests that H26/H28/H30/H34/H36 all reduced IL-6 whilst their whole protein controls had no effect on this pro-inflammatory cytokine once again showing that this ability to modulate IL-6 secretion is due to the hydrolysis of the protein and not the whole protein itself. IL-6 is a pro-inflammatory cytokine secreted by many immune cells, including BMDCs and undifferentiated $CD4^+$ T cells. This cytokine has been linked to chronic gut inflammation (Mudter and Neurath, 2007) particularly IBD and has been shown to be required for the development of Th1 mediated colitis in murine models (Yamamoto *et al.,* 2000). Inflammaging, the chronic inflammation associated with aging, has been shown to particularly impact the gut, with many elderly people

experiencing gastric problems (Rehman, 2012). A hydrolysate, such as H26/H28/H30/H34/H36 which all reduced levels of IL-6, could potentially reduce the severity of chronic gut inflammation experienced in inflammaging.

IL-12p40 was reduced by H24/H25/H26/H27/H34/H35/H36 and again the only WPC which decreased this cytokine was WPC39. H24/H25/H26/H34/H36 all suppressed IL-12p40 secretion from BMDCs differently to that of their whole protein controls. IL-12p40 is a member of the IL-12 family which has been associated with IBD. In *in vivo* studies of IL-10 knockout mice that spontaneously develop IBD, treatment with anti-IL-12p40 treated the disease (Xavier and Podolsky, 2007). Furthermore, IL-23, a cytokine which the IL-12p40 subunit is part of, has been deemed a master regulator of Crohns disease, activating a Th17 T cell subset response and driving chronic gut inflammation (Neurath, 2007). Hence a hydrolysate which targets this subunit which plays a major role in gut inflammation is highly advantageous.

The anti-inflammatory cytokine IL-10 was increased by a large number of hydrolysates; H21/H22/H23/H24/H25/H26/H27/H29/H30/H31/H33 and the whole proteins WPC37/WPC39/WPC40. This means H22/H26/H30 are the only hydrolysates that increased IL-10 whilst their whole protein control does not affect its secretion. Once again, this indicates that the hydrolysed nature of the samples caused this activity and not the protein itself. IL-10 is a regulatory cytokine which has been identified as a potential therapy for the treatment of IBD. Many studies have been published linking IL-10 deficiency with onset and severity of IBD (Tagore *et al.,* 1999) and numerous studies both *in vivo* murine models and human clinical trials have shown IL-10 treatment to be an exciting prospect in IBD treatment (Steidler *et al.,* 2000). Current therapies however have all resulted in not enough IL-10 being able to be delivered to the gut in a safe and efficient manner (Li and He, 2004). Functional ingredients fortified with marine hydrolysates which actively enhance IL-10 production may be the solution to this problem due to their natural path through the gastro-intestinal tract. Two hydrolysates (H26 and H30) both decreased pro-inflammatory cytokines (IL-6

and IL-12p40) whilst increasing anti-inflammatory IL-10. This dual approach of both decreasing pro-inflammatory cytokines secreted and increasing anti-inflammatory could have a greater effect in tackling chronic inflammation such as that seen in IBD.

All blue whiting hydrolysates were next screened on another key immune cell, T cells, in order to build a broader profile of their effect on the overall immune response. For this study, naïve undifferentiated $CD4^+$ T cells were chosen, due to the central role they play in the adaptive immune system. These cells have been implicated in chronic inflammation and secrete pro-inflammatory cytokines such as IFNγ, IL-17 and IL-6. These results were then compared to the hydrolysates effect on cytokine secretion by BMDCs before candidates for further screening in T cell subsets were chosen.

IL-17 is a pro-inflammatory cytokine secreted primarily by the T cell subset Th17 cells. Hydrolysates H24/H28/H29/H33/H36 and WPC40 all reduced IL-17 secretion from naïve undifferentiated $CD4^+$ T cells. Only H29 and H33 did so differently to that of their whole protein controls. As previously discussed, when a hydrolysate displays differing activity to that of its whole protein control this indicates that this activity is as a result of hydrolysis and not the whole proteins normal activity. This is a result of the whole protein being broken down into various peptides which can unlock bioactive sequences normally hidden within the protein. IL-17 enhances T cell priming and stimulates a number of immune cells including macrophages to produce pro-inflammatory markers such as TNFα, IL-6, NOS-2 and chemokines (Sarra *et al*., 2010). An increase in IL-17 has been found in patients suffering from numerous inflammatory diseases including asthma, RA and IBD. It has been identified as a possible target for anti-inflammatory therapeutics to combat these diseases (Gaffen *et al*., 2014) therefore, a hydrolysate which reduces levels of IL-17 could be useful in the treatment of these diseases.

A number of hydrolysates increased levels of IL-2, which is commonly secreted by the T cell subset Th1 cells. H24/H25/H27/H32/H33/H35 all increased levels of this cytokine.

Whilst secreted by Th1 cells and indicative of their presence in the undifferentiated $CD4^+$ T cells analysed as part of this study, IL-2 does play a regulatory role in the immune system promoting differentiation of T regulatory cells in the thymus and the differentiation of T cells into effector and memory T cells (Austin *et al.,* 1999). Hence, hydrolysates which display potent anti-inflammatory activity elsewhere but also increase levels of IL-2 may be effective in reducing chronic inflammation whilst maintaining the patient's immune response.

IL-4 is a cytokine secreted by the T cell subset Th2 cells. Th2 cells can regulate Th1 cells and are often diminished in numbers in chronic inflammation where an influx of Th1 cells occurs. Hence a hydrolysate which increases levels of these cells may be beneficial in combatting Th1 driven chronic inflammation. H25/H26/H27/H34 increased levels of IL-4 and all did so differently to that of their whole protein controls. These hydrolysates may be useful in promoting a Th2 cell which inhibits Th1 differentiation and could reduce inflammation as a result.

Hydrolysates H21/H22/H26/H28/H29/H30/H32/H33/H34/H36 all decreased levels of IL-6 secreted by naïve undifferentiated T cells whilst all whole protein controls also decreased this cytokine, meaning no hydrolysates displayed anti-IL-6 activity differently to that of their whole protein control and as a result this activity is likely due to the protein and not of the hydrolysis. IL-6 is a cytokine which is secreted by both undifferentiated $CD4^+$ T cells and BMDCs and as previously discussed, has been linked to IBD. Whilst no hydrolysates modulated IL-6 secretion by undifferentiated $CD4^+$ T cells differently to that of the control, a number of these hydrolysates (H26/H28/H30/H34/H36) did reduce levels of IL-6 secreted by BMDCs differently to that of their whole protein controls.

IL-10 is an anti-inflammatory cytokine which promotes levels of T regulatory cells. T regulatory cells play an important role in the suppression of chronic inflammation and

auto-immunity. IL-10 is secreted by BMDCs and $CD4^+$ T cells. No hydrolysates increased levels of IL-10 secreted by undifferentiated $CD4^+$ T cells.

IL-13, like IL-4, is secreted by the Th2 cell subset which inhibits the differentiation of Th1 cells. Hence promotion of this cytokine would be ideal, however all hydrolysates and their whole protein controls reduced levels of IL-13 secreted by undifferentiated $CD4^+$ T cells.

Hydrolysates H21/H22/H23/H24/H26/H27/H28/H30/H32/H33/H34/H36 all reduced levels of IFNγ secreted by undifferentiated $CD4^+$ T cells along with all whole protein controls (WPC37/WPC38/WPC39/WPC40). IFNγ is a proinflammatory cytokine which has been shown to be essential for disease progression of IBD (Zenewicz *et al.,* 2008). Increased levels of IFNγ have been shown to be present in patients suffering from Crohn's disease, as opposed to those with ulcerative colitis (Fuss *et al.,* 1996). Crohn's disease is a type of IBD which causes chronic inflammation throughout the gastro intestinal tract whilst ulcerative colitis occurs in only the colon (Greenstein *et al.,* 1976). A hydrolysate which reduces levels of IFNγ could be a potential therapeutic for patients suffering from Crohn's disease, particularly a hydrolysate as a functional food, as Crohn's occurs along the gastro-intestinal tract which the food would pass through, thus allowing access of the hydrolysate to the sites of inflammation.

Following the analysis of all hydrolysates in both BMDCs and undifferentiated $CD4^+$ T cells, all results were assessed to identify candidates for further screening in T cell subsets. Candidates were chosen for their ability to modulate cytokines associated with inflammation and their ability to do so differently to that of their whole proteins. H22 was chosen a candidate hydrolysate as it increased levels of anti-inflammatory IL-10 secreted by BMDCs and decreased pro-inflammatory IL-6 secreted by undifferentiated $CD4^+$ T cells. H23 was identified for its ability to promote IL-10 from BMDCs and suppress IFNγ secreted by $CD4^+$ T cells. H25 decreased levels of TNFα and IL-12p40 whilst increasing IL-10 all secreted by BMDCs and increased levels of Th2 promoting IL-4 in $CD4^+$ T cells. H26

increased IL-10 and decreased TNFα, IL-6 and IL-12p40 secreted from BMDCs and decreased pro-inflammatory IFNγ and IL-6 whilst it increased IL-4 from $CD4^+$ T cells. H27 in BMDCs suppressed TNFα and IL-6, IL-12p40 and promoted levels of IL-10, in $CD4^+$ T cells this hydrolysate increased IL-4 whilst it reduced IFNγ. All of these hydrolysates were chosen for their ability to modulate cytokines which play a role in inflammation and do so differently to that of their whole proteins. These hydrolysates were then brought forward for screening in the T cell subsets, Th1, Th2 and Th17 cells in order to gain a deeper understanding of their effects on the immune system and the mechanisms through which they act.

Chosen candidate hydrolysates were first screened in a Th1 cell. Th1 cells are a T cell subset which drive inflammation and have been heavily linked to inflammatory disease. Th1 cells have been implicated in IBD with a rise in IFNγ being reported in many studies (Fuss, 2008). IFNγ and IL-2 are the key cytokines secreted by a Th1 subset. Hydrolysates H26 and H27 suppressed secretion of IFNγ by Th1 cells thus displaying anti-inflammatory activity which could be beneficial cases of chronic inflammation such as inflammaging. H26 and H27 were flagged as potential candidates for future work, but no decision was made until their effects on Th2 and Th17 cells were analysed. H25 increased production of IL-2 from Th1 cells, this is indicative of driving a Th1 response and inflammation, as a result it was decided that H25 was not a suitable candidate for future studies in *in vivo* models.

Candidate hydrolysates were next analysed for their effects on a Th2 cell T cell subset. Th2 cells are involved in a delicate balance with Th1 cells, often referred to as the Th1/Th2 balance or the Th1/Th2 paradigm (Romagnani, 1997). In certain chronic inflammatory conditions this balance is shifted towards Th1 cells, with an increase of Th1 cells present driving autoimmune inflammation. Th2 cells whilst playing a role in allergy and allergic inflammation, also inhibit the differentiation of Th1 cells (Kidd, 2003). In chronic inflammation, where an increase in Th1 occurs, a hydrolysate which increases Th2 differentiation could inhibit the Th1 influx and reduce inflammation. The key

cytokines secreted by Th2 cells are IL-13 and IL-4. IL-13 acts a central regulator of chronic inflammation whilst IL-4 induces Th2 cell differentiation whilst also being secreted by Th2 cells in a positive feedback loop (Grünig *et al.*, 1998). Hydrolysate H22 increased levels of IL-13 whilst also decreasing levels of IL-4, H27 decreased IL-4. No other hydrolysates significantly modulated cytokine secretion or a Th2 cell.

Finally, candidate hydrolysates were screened on a Th17 cell subset. Th17 cells drive inflammation and have been associated with IBD. Studies have suggested IBD is a Th17 driven inflammatory disease (Iwakura and Ishigame, 2006). IL-17 is the key cytokine secreted by Th17 cells and has been identified as a therapeutic target in inflammatory diseases (Hu *et al.,* 2011). Hydrolysate H22 increased levels of IL-17 secreted by Th17 cells, displaying pro-inflammatory activity, as a result it was decided H22 was no longer a potential candidate for further studies. Hydrolysate H23/H26/H27 all decreased levels of IL-17, displaying anti-inflammatory activity in suppressing the Th17 cell subset.

Following T cell subset studies, H25 and H22 were eliminated as possible candidates for further investigations due to driving Th1 and Th17 subsets respectively. This left H23/H26/H27 as potential candidates. H23 increased IL-10 secretion by BMDCs, decreased IFNγ in undifferentiated $CD4^+$ T cells and decreased IL-17 secretion by Th17 cells, but its whole protein control WPC 39 also displayed similar activity in all cell types. H26 reduced secretion of TNFα, IL-6 and IL-12p40 whilst increasing IL-10 by BMDCs all differently to that of its whole protein control (WPC38). It also decreased IL-6 and IFNγ and promoted IL-4 production in undifferentiated $CD4^+$ T cells. In a Th1 cell subset it decreased IFNγ levels secreted and in Th17 also reduced IL-17 levels secreted, for the majority displaying activity different to that of its whole protein control. H27 also decreased TNFα, IL-6 and IL-12p40 whilst promoting IL-10 secretion by BMDCs. In undifferentiated $CD4^+$ T cells, H27 reduced levels of IFNγ and also showed this anti-IFNγ activity in the Th1 cell subset. Finally, in Th17 cells H27 decreased levels of IL-17 produced. Due to H26 and H27 displaying potent anti-inflammatory activity across a number of cell types and doing so differently to that of their whole protein controls they were chosen to

be brought forward to the next round of screening. H23 was not chosen due to its limited anti-inflammatory activity in both BMDCs and T cells and its similarity to its whole protein control.

The next round of investigations for these hydrolysates, H26 and H27, involved collaborators in the University of Limerick using HPLC to fractionate each sample into four (4) fractions. Fractionation involves the hydrolysate being further broken down into separate sections of the protein. Fractionation is required in order to delve deeper into the peptides and try to uncover which peptide sequence provides the bioactivity displayed during the screening. Both hydrolysates and their fractions were screened again using the same protocol as before.

Hydrolysate H26 and its four fractions (F1/F2/F3/F4) were first screened on BMDCs and their effect on the cytokine secretion of these cells analysed using ELISA. All fractions and their hydrolysate H26 successfully modulated cytokine secretion of BMDCs. IL-12p40 was decreased by H26 and F1/F2/F3/F4. As previously discussed this subunit which is present in members of the IL-12 cytokine family plays a major role in chronic inflammation and has been identified as a therapeutic target for IBD (Guan *et al.,* 2018). Suppression of IL-12p40 by a fraction is indicative of potent anti-inflammatory activity. IL-23, a cytokine which possesses the IL-12p40 subunit, was next analysed. H26 and F4 both reduced secretion of IL-23 by BMDCs. IL-23 has been shown to be essential in driving the inflammatory response seen in IBD (Yen *et al.,* 2006) therefore a fraction which reduces secretion of this cytokine could help modulate inflammation in IBD. TNFα was also decreased by H26 and F1/F2/F4. IL-12p70 is another member of the IL-12 cytokine family and is also a pro-inflammatory cytokine. IL-12p70 secretion is increased in Crohn's disease and a hydrolysate which decreases this could be advantageous in treating this disease. The only fraction which suppressed IL-12p70 was F1, H26 itself had no effect. TNFα is another pro-inflammatory cytokine which has been heavily implicated in chronic inflammatory diseases and is a current target for commercial therapeutics such as infliximab (Bongartz *et al.,* 2006). Another pro-inflammatory cytokine which has been

linked to chronic inflammation and inflammatory disease is IL-1β. This cytokine up regulates HIF-1, COX-2 and NF-κB pathways (Jung *et al.,* 2003) and has been shown to be critical in driving systemic inflammation (Dinarello, 2005). H26 and F1/F3/F4 all suppressed secretion of IL-1β by BMDCs.

The fractions were next screened in undifferentiated $CD4^+$ T cells. IL-6 was reduced by H26 and F1/F2/F3. IL-6 is a pro-inflammatory cytokine which has been shown to mediate inflammation in IBD alongside IL-17, hence reducing this cytokine may reduce the inflammation associated with this disease. IL-17, as discussed is a pro inflammatory cytokine and was reduced by F2. Both F1 and F4 reduced levels of IFNγ which plays a major role in promoting chronic inflammation through the differentiation of a Th1 cell subset. F4 also reduced levels of IL-2 which is also secreted by Th1 cells, indicating F4 is a potent anti-inflammatory fraction.

All fractions were then brought forward to be screened in the T cell subsets Th1, Th2 and Th17 cells with their hydrolysate H26. Th1 cells, which drive Th1 mediated inflammation, were screened and the results showed F1 and F4 to significantly reduce levels of the key Th1 cytokine IFNγ. Th2 cells, which inhibit Th1 cell differentiation thus ameliorating inflammation, were next screened. It was found that no fraction promoted a Th2 cell type through the increase of key cytokines IL-13 and IL-4. Finally, a Th17 cell subset was analysed and levels of the IL-17 cytokine were reduced by F1 and F4. Before fractions were compared and potential candidates for *in vivo* screening identified, hydrolysate 27 and its fractions were studied using the same protocol.

Hydrolysate 27 and its fractions F1/F2/F3/F4 were screened on BMDCs and their effect on cytokine secretion analysed. TNFα, a major pro-inflammatory cytokine, was reduced by H27 and F1. The cytokine subunit IL-12p40 was reduced by H27 and F1/F2/F3/F4. Two pro-inflammatory cytokines, IL-6 was suppressed by H27 and F1/F2/F3/F4 and IL-12p70 by F1/F2/F3/F4. Overall, fractions of H27 showed significant anti-inflammatory effects

against BMDC cytokine secretion. The HPLC fractions of H27 were then screened on undifferentiated $CD4^+$ T cells and their ability to modulate cytokine secretion by these cells analysed. IL-17, the cytokine secreted by Th17 cells and implicated in IBD, was suppressed by H27 and F1/F2/F3/F4. IL-6, a cytokine which drives inflammation, was reduced by H27 and F1/F2. The fractions had no significant effects on other cytokines, however H27 did decrease levels of IL-13, IL-10 and IL-4.

All fractions were then brought forward into the T cell subsets, Th1, Th2 and Th17 cells. Th1 cells were first analysed and it was found that the key Th1 cytokine IFNγ was decreased by H27, F1 and F3. Th2 cells and their key cytokines IL-13 and IL-4 were not increased by any hydrolysate or fraction. Th17 cells were finally analysed and it was found that F1 decreased levels of IL-17.

All fractions and hydrolysate (H26 and H27) data was then compared in order to identify possible candidates for an *in vivo* murine model of IBD. It was decided not to continue with H27 and its fractions as they failed to display potent specific anti-inflammatory activity different to that of their controls. For hydrolysate H26, F1 and F4 were chosen as candidate fraction for further analysis. F4 was the first choice candidate for an *in vivo* model due to its potent suppression of IFNγ in Th1 cells and also its ability to reduce IL-17 in Th17 cells. F1 was chosen as a secondary candidate as it displayed similar activity to F4, but was not as potent in decreasing levels of IFNγ from the Th1 cell subset. However, following a discussion with collaborators it was found that F4 was not a commercially viable option due to the extremely small amounts of fraction obtained during HPLC and the large amount needed for an *in vivo* murine model. It was decided to continue with F1 as this displayed the same anti-inflammatory activity, even if not as potently. Hydrolysate 26 Fraction 1 was then scaled up and generated by the University of Limerick for further studies.

Chapter 6:

Investigating the potential of F1 as an anti-inflammatory hydrolysate fraction

6.1 Introduction

Inflammatory bowel disease (IBD) is used to describe a collection of idiopathic inflammatory disorders which affect the gastrointestinal tract. It is classified by chronic relapsing inflammation and includes such diseases as ulcerative colitis (CD) and Crohn's disease (CD) (Geboes *et al*., 2018). IBD has no known cause, however genetic predisposition to the disease has been shown (Satsangi *et al*., 1996). IBD manifests most commonly early in life and incidences of childhood IBD are increasing worldwide (Chan *et al.*, 2018). IBD is diagnosed using clinical examination, endoscopy, enteroscopy, radiology, biopsy inspection and tissue examination (Ohtsuka, 2018; Lennard-Jones, 1989). Disease symptoms include diarrhoea, abdominal pain, lesions, bloody stool, bloating, fatigue and weight loss, and can range from moderate to severe (Carter *et al.*, 2004).

Ulcerative colitis is a subcategory under the umbrella term IBD. UC is classified by chronic inflammation of the inner lining of the colon and distinguishable from CD which mainly affects the ileum but can occur anywhere along the intestinal tract (Head and Jurenka, 2004). However, UC symptoms are similar to those of CD and are often indistinguishable in early diagnoses, in these cases indeterminate colitis (IC) or inflammatory bowel disease unclassified (IBDU) diagnoses are used (Wells *et al*., 1991).

Pro-inflammatory cytokines are believed to be involved in both the onset and progression of UC and cytokines are a target of current therapies to treat the disease. Anti-TNFα antibodies infliximab and adalimumab are commonly used therapies, with an increase in TNFα being a characteristic of the disease (Sands *et al*., 2001). Many other cytokines including IL-6, IL-17, IL-1β and IFNγ have also been associated with disease onset and progression and have been identified as potential therapeutic targets (Murata *et al.*, 1995).

Other common therapies of UC and IBD include corticosteroids (Lichtenstein *et al.,* 2006), aminosalicylates (Hanauer, 2008), antibiotics and probiotics (Gionchetti *et al*., 2006) and immunosuppressants (Lewis *et al.*, 2008). Recent research exploring the potential of functional foods as a treatment for IBD have been promising (Larussa *et al*., 2017). Curcumin polymer has been shown to alleviate symptoms and reduce pro-

inflammatory cytokine levels in a dextran sodium sulphate (DSS) induced model of colitis (Strimpakos and Sharma, 2008). Similarly, punicalagin isolated from pomegranate juice has displayed the ability *in vivo* to reduce severity of the disease and suppress secretion of cytokines such as TNFα and IL-1β (Shah *et al*., 2016). Dietary interventions using functional foods may be beneficial in IBD treatment particularly in remission stages where relapse is common, but the use of long term of current therapies are not advised (Ghattamaneni *et al.*, 2018).

6.2.1 Chapter Aims

This chapter aims to explore the potential of the Blue Whiting hydrolysate HPLC fraction, F1, as a functional ingredient for use in UC and IBD treatment. Blue Whiting was kindly donated pre-minced by the Killybegs Fishermen's Organisation, Killybegs, Co. Donegal, Ireland to collaborators the Fitzgerald Lab in the University of Limerick as facilitated by Bord Iascaigh Mhara (BIM, Ireland). The Fitzgerald Lab isolated protein from the minced meat and performed enzymatic hydrolysis in order to generate protein hydrolysates, following the screening described in **Chapter 5** of this thesis, 2 hydrolysates were chosen to be fractionated using HPLC fractionation. These fractions then underwent another round of screening as outlined also in **Chapter 5**, and one fraction was chosen (F1 from H26) as a potential for *in vivo* models.

This chapter aims to further examine F1's suitability for use in an *in vivo* murine model of colitis. This was achieved through studying its impact on BMDCs when they are activated using immune stimuli that target other Toll like receptors. In the screening to date LPS was used which activates the cells through TLR4. In this study F1's ability to suppress the cytokine response when cells are stimulated with agonists to TLR1, TLR2, TLR3 TLR5, TLR6 and TLR7 was examined (Table 6.1). Its ability to modulate the viability and cytokine response of macrophage cells, another key immune cell, was also studied.

Once F1 was deemed suitable for *in vivo* use, a DSS-induced colitis model was performed. In order to investigate whether F1's *in vitro* anti-inflammatory activity translated to *in vivo* models.

6.2 Results

6.2.1 Dose response of F1 on BMDCs

Following the selection of F1 as a candidate for *in vivo* screening, it was necessary to investigate the optimal dose at which F1 of H26 had the greatest immunomodulatory effects on BMDCs. This was achieved by analysing the cytokine secretion by BMDCs treated with a dose response of F1 and its hydrolysate H26, using a range from 0.25 mg/mL to 2.0 mg/mL.

Figure 6.2 and **Figure 6.3** show the cytokine secretion by BMDCs treated with a dose range of F1 and H26. H26 (shown in black bars) at a dose of 0.25 mg/mL reduced TNFα ($p<0.01$) and IL-12p40 ($p<0.01$). H26 (0.50 mg/mL) decreased TNFα ($p<0.001$) and IL-6 ($p<0.001$). H26 (1.00 mg/mL) decreased TNFα ($p<0.001$), IL-6 ($p<0.001$) and IL-23 ($p<0.001$). H26 (1.50 mg/mL) reduced TNFα ($p<0.001$), IL-6 ($p<0.001$), IL-23 ($p<0.001$), IL-12p40 ($p<0.01$) and IL-12p70 ($p<0.001$). H26 (2.00 mg/mL) decreased TNFα ($p<0.001$), IL-6 ($p<0.001$) and IL-12p70 ($p<0.001$). F1 (shown in striped bars) at a dose of 0.25 mg/mL has no significant effect on cytokine secretion by BMDCs. F1 (0.50 mg/mL) decreased TNFα ($p<0.001$). F1 (1.00 mg/mL) reduced TNFα ($p<0.001$), IL-6 ($p<0.001$), IL-23 ($p<0.001$), IL-12p40 ($p<0.01$) and IL-12p70 ($p<0.001$). F1 (1.50 mg/mL) decreased TNFα ($p<0.001$), IL-12p40 ($p<0.001$) and IL-12p70 ($p<0.001$). F1 (2.00 mg/mL) reduced TNFα ($p<0.001$), IL-23 ($p<0.001$) and IL-12p70 ($p<0.001$).

F1 (1.00 mg/mL) displayed significant anti-inflammatory effects, reducing the secretion of pro-inflammatory cytokines TNFα, IL-6, IL-23, IL-12p40 and IL-12p70 more than any other dose.

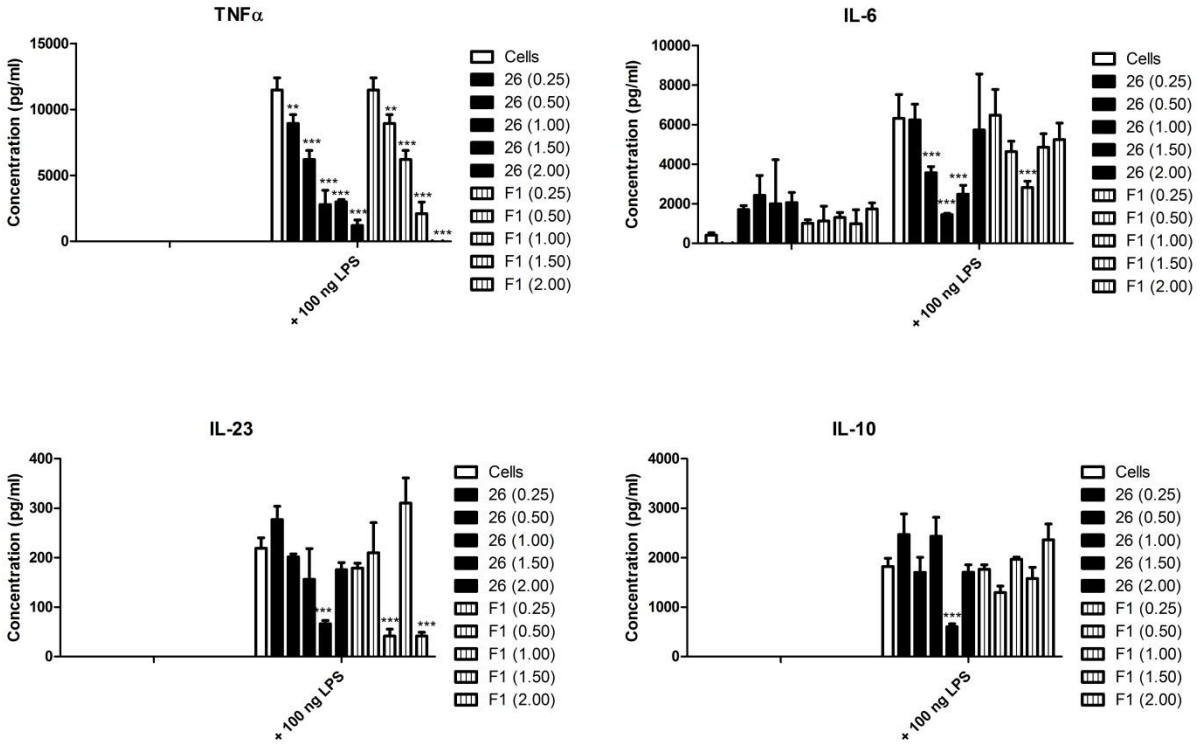


Figure 6.2 Secretion of TNFα, IL-6, IL-23 and IL-10 by BMDCs when treated with a range of doses of H26 and F1 (0.25 – 2.00 mg/mL) BMDCs were isolated from Balb/c mice and differentiated in rGMCSF for seven days. On day 7 cells were treated with H26 orF1 in a dose range 0.25 - 2.00 mg/mL. Cells marked +LPS were then stimulated with 100 ng/mL LPS. Secretion of TNFα, IL-6, IL-23 and IL-10 was measured using ELISA (R&D Duoset). One way Anova was used to assess differences between groups. Cells treated with hydrolysate only were compared to cells alone, cells treated with hydrolysates + LPS were compared to cells alone + LPS. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to untreated cells (no hydrolysate).

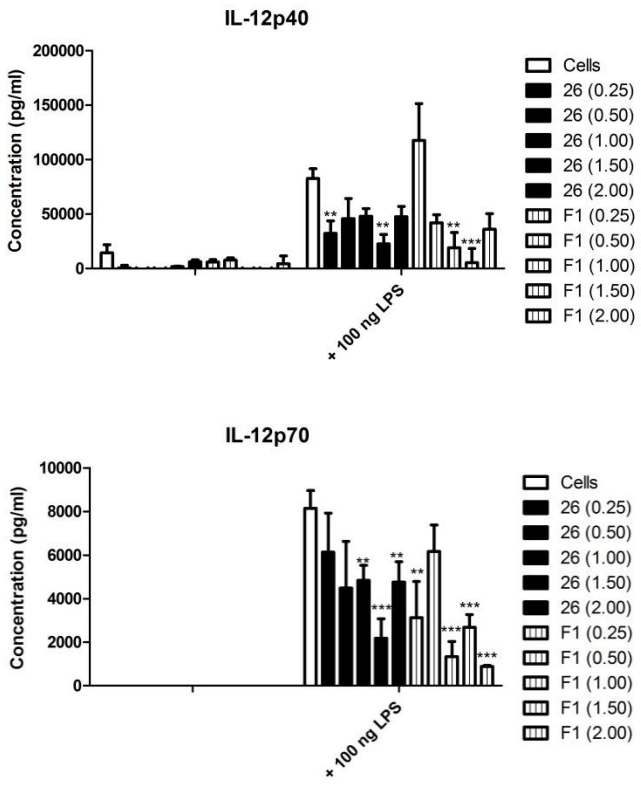


Figure 6.3 Secretion of IL-12p40 and IL-12p70 by BMDCs when treated with a range of doses of H26 and F1 (0.25 – 2.00 mg/mL) BMDCs were isolated from Balb/c mice and differentiated in rGMCSF for seven days. On day 7 cells were treated with hydrolysate or F1 in a dose range 0.25 - 2.00 mg/mL. Cells marked +LPS were then stimulated with 100 ng/mL LPS. Secretion of IL-12p40 and IL-12p70 was measured using ELISA (R&D Duoset). One way Anova was used to assess differences between groups. Cells treated with hydrolysate only were compared to cells alone, cells treated with hydrolysates + LPS were compared to cells alone + LPS. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to untreated cells (no hydrolysate).

6.2.2 F1 stimulated with various toll ligands does not impact viability of BMDC cells.

After a dose of 1 mg/mL was found to be the optimal dose of F1, the effect of this fraction on the wider immune response was investigated through the use of various toll ligand agonists. It was first necessary to ensure F1 when stimulated with the various toll ligands did not affect cell viability. Cells were isolated from the bone marrow of Balb/c mice and incubated at 37°C 5% CO_2 for seven days with rGMCSF (Sigma). BMDCs were plated in triplicate at a concentration of $1x10^6$ cells/mL on a 96 well plate, 100 µL/well and rested. Cells were then treated with H26 (1 mg/mL), F1 (1 mg/mL or 10% DMSO (negative control) and then stimulated with the relevant toll ligands (Table 6.1) and incubated for 24 hours. Viability was assessed using an MTS assay (Cell Titer 96 Aqueous One Solution – Promega) according to the manufacturer's instructions. Results were expressed as a percentage of cells alone. At 1 mg/mL H26 and F1 stimulated with toll ligands did not have a significant negative effect on cell viability on BMDCs when compared to untreated cells (**Figure 6.4**).

Table 6.1 Toll like receptors and corresponding toll ligands

Toll Like Receptor (TLR)	Toll Ligand
TLR 1	**PAM**
TLR 2	**PGN**
TLR 3	**Poly I:C**
TLR 4	**LPS**
TLR 5	**Flagellin**
TLR 6	**Zymozan**
TLR 7	**Loxoribine**

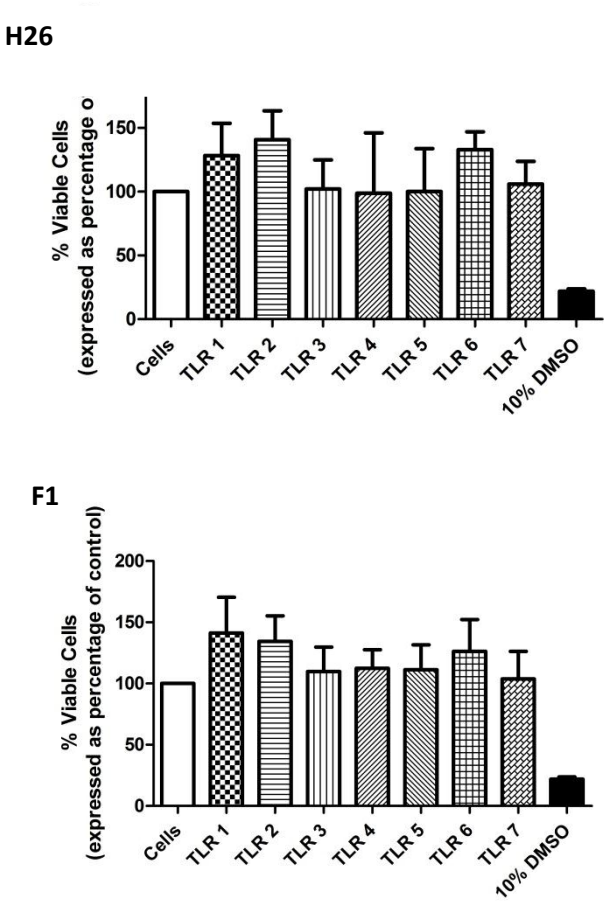


Figure 6.4 BMDC viability following treatment with H26 or F1 and stimulated with a range of toll ligands. BMDCs were isolated from Balb/c mice and differentiated in rGMCSF for seven days. On day 7 cells were treated with H26 or F1 and stimulated with a range of toll ligands for 24 hours. DMSO (10% v/v) was included as a positive control of cytotoxicity. MTS assay (Cell Titer 96 Aqueous One Solution) was used to assess cell viability. Results are expressed as a percentage of cells alone (untreated cells).

6.2.3 Cytokine and chemokine secretion by BMDCs treated with F1 and H26, and stimulated with various toll ligands.

In order to identify whether F1 had other effects on the overall immune system, its ability to modulate cytokine and chemokine secretion by BMDCs when stimulated with various toll ligands (Table 6.1) was assessed. BMDCs were isolated as before and treated with F1 (1 mg/mL) or H26 (1 mg/mL) and then stimulated with various toll ligands. Secretion of cytokines (TNFα, IL-6, IL-23, IL-10 and IL-12p40) and chemokines (MIP-1, MIP-2 and MCP-1) was then assessed using ELISA (R&D Duoset). H26 is shown in black. F1 in striped and cells alone as a white bar.

Figure 6.5 and **Figure 6.6** show cytokine and chemokine secretion by BMDCs treated with F1 and H26 and then stimulated with a range of toll ligands.

TLR1 on BMDCs was stimulated with the toll ligand 1 (PAM) following treatment with F1 and H26 and cytokine/chemokine secretion measured. TNFα secretion was not altered. IL-6 was reduced by both F1 and H26 ($p<0.001$). IL-23, IL-10, IL-12p40 and MIP-1 were all decreased by F1 ($p<0.001$). MCP-1 was also reduced by F1 ($p<0.01$).

TLR2 on BMDCs was treated with F1 and H26 and then stimulated with the toll ligand 2 (PGN) before cytokine and chemokine secretion was measured. TNFα was reduced by F1 ($p<0.01$). IL-6, IL-23 and IL-12p40 were decreased by both F1 and H26 ($p<0.001$). IL-10 and MCP-1 was reduced by F1 ($p<0.01$). MIP-1 was also reduced by F1 ($p<0.001$). MIP-2 was increased by F1 ($p<0.001$) and H26 ($p<0.01$).

TLR3 on BMDCs was stimulated with the toll ligand 3 (Poly I:C) following treatment with F1 and H26. IL-12p40 was reduced by F1 ($p<0.001$). The chemokines MIP-2 was increased by F1 ($p<0.001$) and H26 ($p<0.01$) and MCP-1 was reduced by F1 ($p<0.01$).
TLR4, whilst already widely researched throughout this investigation, was also included in this study and stimulated with LPS. TNFα was decreased by F1 ($p<0.001$) and H26 ($p<0.01$). IL-6 was also decreased by F1 ($p<0.001$) and H26 ($p<0.001$). IL-23 was reduced by F1 ($p<0.001$) as was IL-10 ($p<0.01$). IL-12p40 was decreased by both F1 ($p<0.001$) and H26

($p<0.001$). The chemokines MIP2 ($p<0.001$) and MCP-1 ($p<0.01$) were also decreased by F1.

TLR5 on BMDCs was stimulated with the toll ligand 5 (Flagellin) after treatment with F1 and H26. The only cytokine or chemokine altered was IL-12p40 which was reduced by F1 ($p<0.01$).

TLR6 on BMDCs was stimulated with the toll ligand 6 (zymozan) following treatment with F1 and H26. TNFα was decreased by both F1 ($p<0.001$) and H26 ($p<0.01$). IL-6 was also reduced by F1 ($p<0.001$) and H26 ($p<0.001$). IL-23 secretion was reduced by F1 ($p<0.001$). IL-12p40 was decreased by both F1 ($p<0.001$) and H26 ($p<0.001$). MIP-1 and MCP-1 secretion were both decreased by F1 ($p<0.001$) and MIP-2 was increased by H26 ($p<0.001$).

TLR7 on BMDCs was stimulated with the toll ligand 7 (loxoribine) following treatment with F1 and H26. TNFα was reduced by both F1 ($p<0.01$) and H26 ($p<0.01$). IL-6 secretion was decreased by F1 ($p<0.001$) and H26 ($p<0.001$). IL-12p40 was decreased by F1 ($p<0.001$) and H26 ($p<0.01$). MIP-1 was reduced by F1 ($p<0.001$) and MCP-1 reduced by F1 ($p<0.01$) and H26 ($p<0.01$).

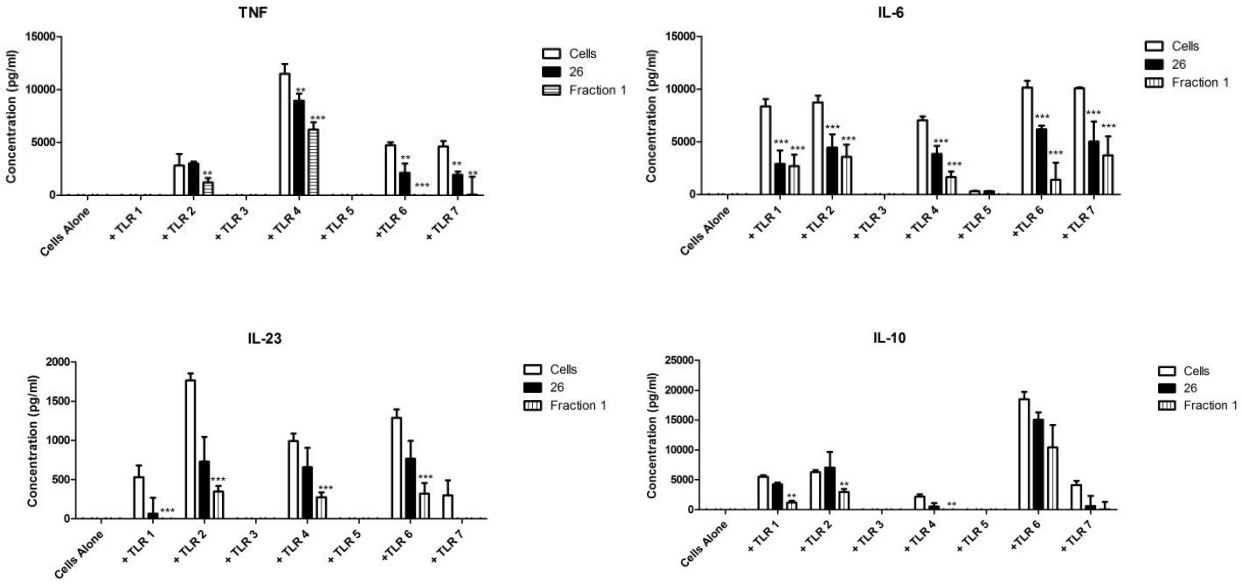


Figure 6.5 Secretion of TNFα, IL-6, IL-23 and IL-10 by BMDCs treated with H26 or F1 and stimulated with a range of toll ligands. BMDCs were isolated from Balb/c mice and differentiated in rGMCSF for seven days. On day 7 cells were treated with hydrolysates and stimulated with a range of toll ligands. Secretion of TNFα, IL-6, IL-23 and IL-10 was measured using ELISA (R&D Duoset). One way Anova was used to assess differences between groups. Cells treated with hydrolysate only were compared to cells alone, cells treated with hydrolysates + toll ligand were compared to cells alone + toll ligand. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to untreated cells (no hydrolysate).

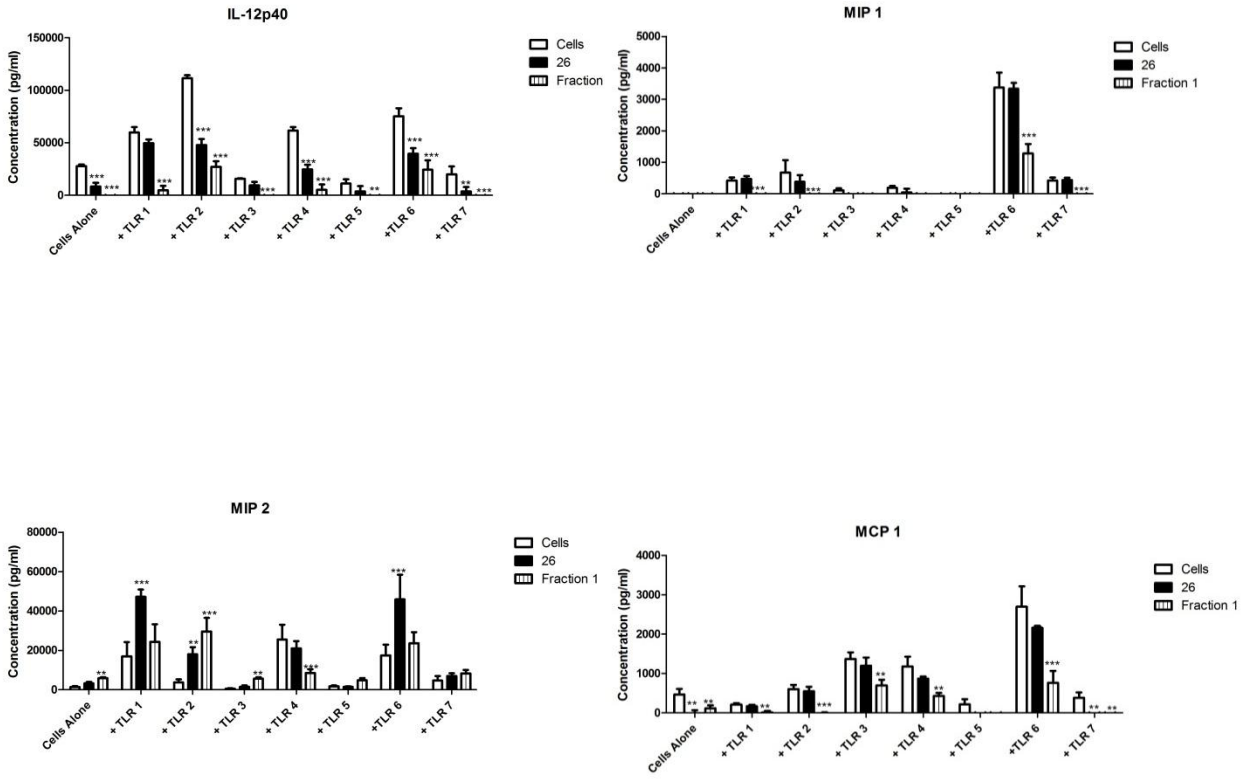


Figure 6.6 Secretion of IL-12p40, MIP-1, MIP-2 and MCP-1 by BMDCs treated with H26 or F1 and stimulated with a range of toll ligands. BMDCs were isolated from Balb/c mice and differentiated in rGMCSF for seven days. On day 7 cells were treated with hydrolysates and stimulated with a range of toll ligands. Secretion of IL-12p40, MIP-1, MIP-2 and MCP-1 was measured using ELISA (R&D Duoset). One way Anova was used to assess differences between groups. Cells treated with hydrolysate only were compared to cells alone, cells treated with hydrolysates + toll ligand were compared to cells alone + toll ligand. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to untreated cells (no hydrolysate).

6.2.4 The cell viability of J774A.1 macrophage cell line when treated with F1 or H26.

Following the investigation of F1's effect on BMDCs and T cells, it was necessary to study its effect on the wider immune response before *in vivo* screening began. For this study, J774A.1 macrophage cells were chosen. The impact of F1 and H26 on the viability of these cells was first studied. Cells were plated at a concentration of 1 x 10^6 cells/mL on a 96 well plate, 100 µL/well and rested. Cells were then treated with F1, H26 (1 mg/mL) or 10% DMSO (negative control) and then stimulated with LPS (100 ng/mL) and incubated for 24 hours. Viability was assessed using an MTS assay (Cell Titer 96 Aqueous One Solution – Promega) according to the manufacturer's instructions. Results were expressed as a percentage of cells alone. At 1 mg/mL, F1 or its hydrolysate H26 did not have a negative impact on cell viability on J774A.1 macrophage when compared to untreated cells (**Figure 6.7**).

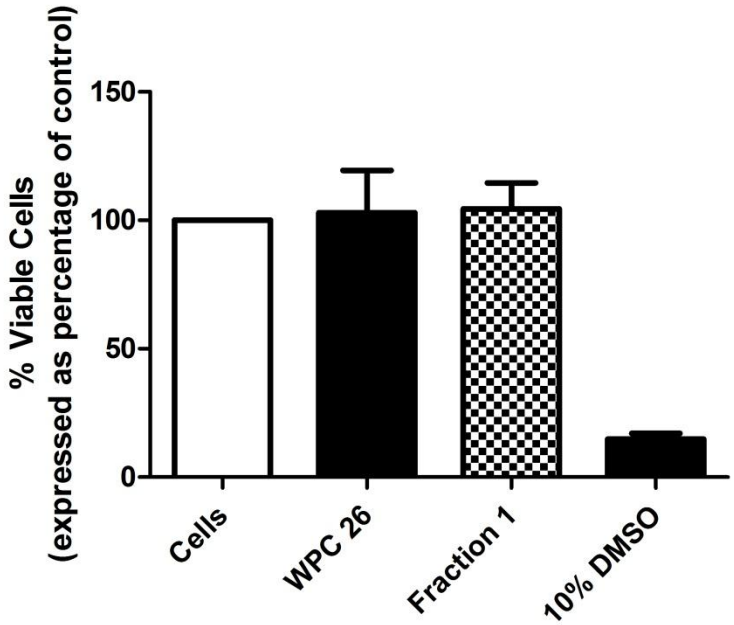


Figure 6.7 J774A.1 macrophage viability following treatment with H26 or F1 and stimulated with LPS. J774A.1 cells were treated for 24 hours with H26 or F1 at a concentration of 1 mg/mL, with LPS stimulation (100 ng/mL). DMSO (10% v/v) was included as a positive control of cytotoxicity. MTS assay (Cell Titer 96 Aqueous One Solution) was used to assess cell viability. Results are expressed as a percentage of cells alone (untreated cells).

6.2.5 The cytokine and chemokine secretion of J774A.1 macrophage when treated with F1 or H26.

After ensuring F1 did not negatively impact the viability of macrophage cells, their effect on the secretion of cytokines and chemokines by macrophage cells was investigated. Cells were plated at a concentration of 1 x 10^6 cells/mL on a 96 well plate and rested. Cells were then treated with F1 or H26 (1 mg/mL) and then some (marked +100 ng LPS) were stimulated with LPS (100 ng/mL). Cells were then incubated for 24 hours before supernatants were collected. Secretion of IL-6, TNFα, IL-12p70, IL-1β, IL-12p40, IL23, MIP-1, MIP-2, MCP-1 and IL-10 in supernatants were then analysed using ELISA (R&D Duoset).

Figure 6.8 and **Figure 6.9** show the cytokine and chemokine secretion by J774A.1 macrophage when treated with F1 or H26. IL-6 was decreased by H26 ($p<0.05$). MIP-2 secretion was increased by H26 ($p<0.05$) and F1 ($p<0.01$). No other cytokine was modulated by F1 or H26.

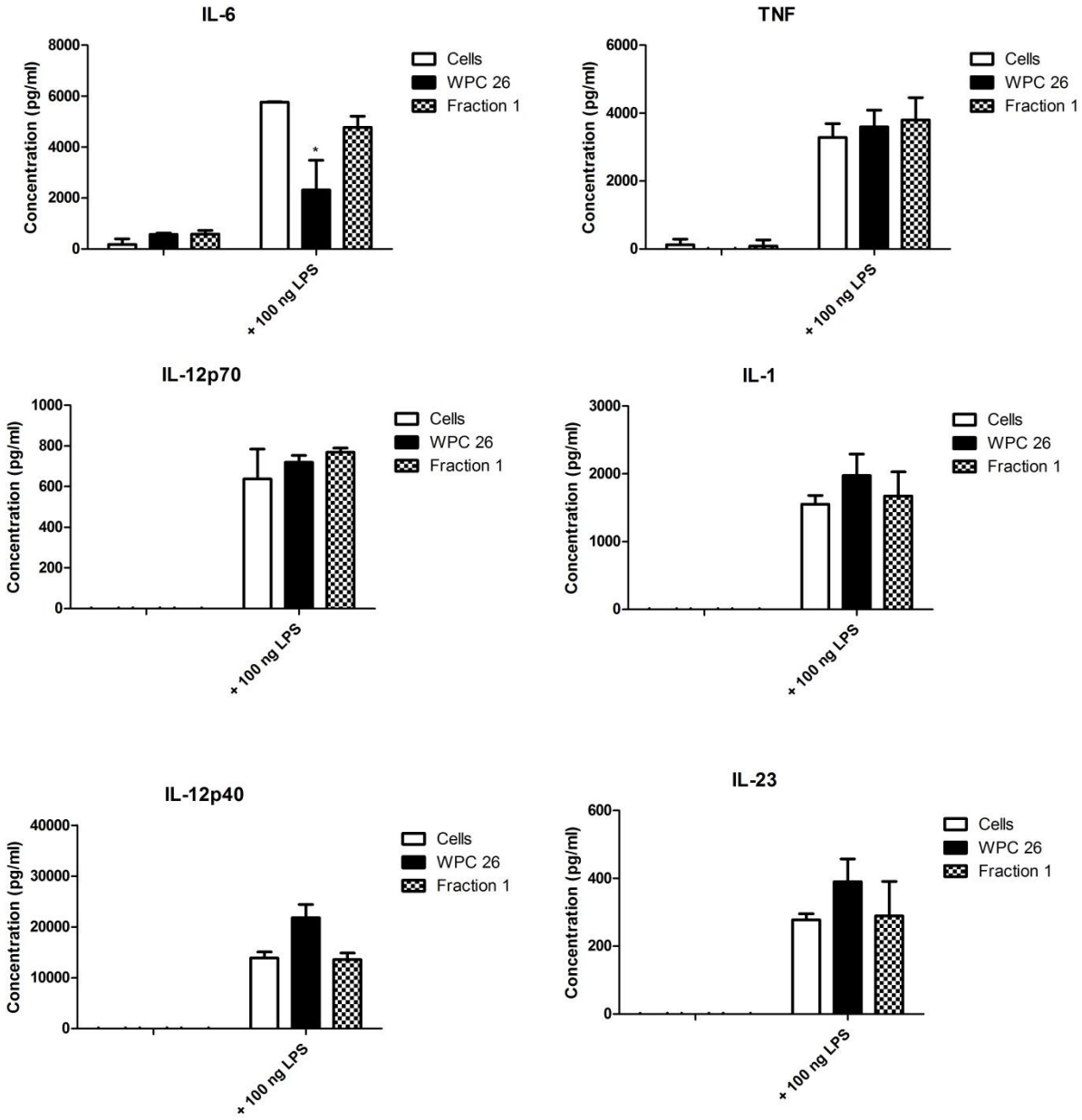


Figure 6.8 Secretion of IL-6, TNFα, IL-12p70, IL-1β, IL-12p40 and IL-23 by J774A.1 cell line treated with H26 or F1. J774A.1 cells were treated for 24 hours with H26 or F1 at a concentration of 1 mg/mL, with or without LPS stimulation (100 ng/mL). Secretion of IL-6, TNFα, IL-12p70, IL-1β, IL-12p40 and IL-23 was measured using ELISA (R&D Duoset). One way Anova was used to assess differences between groups. Cells treated with hydrolysate only were compared to cells alone, cells treated with hydrolysates + LPS were compared to cells alone + LPS. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to untreated cells (no hydrolysate).

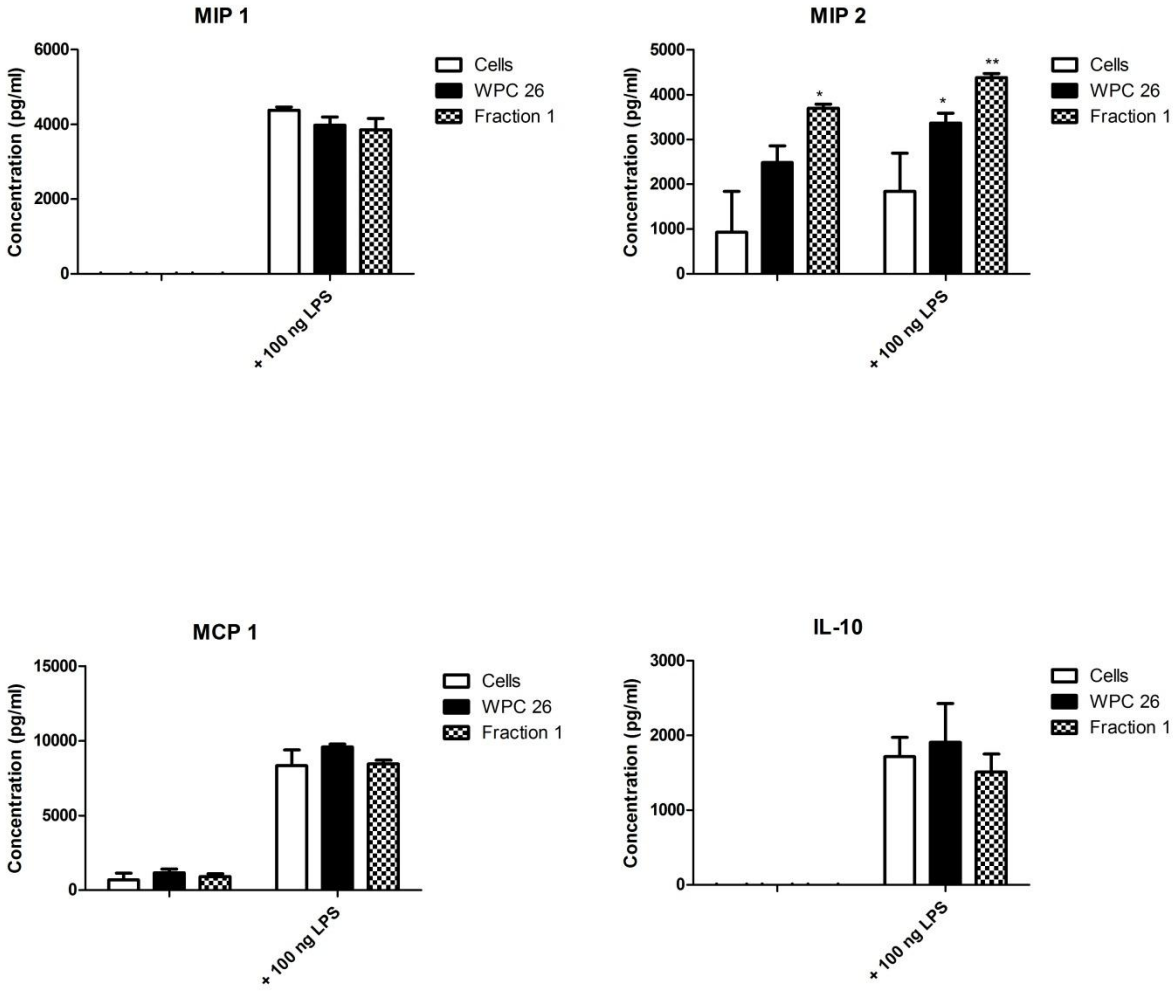


Figure 6.9 Secretion of MIP-1, MIP-2, MCP-1 and IL-10 by J774A.1 cell line treated with H26 or F1. J774A.1 cells were treated for 24 hours with H26 or F1 at a concentration of 1 mg/mL, with or without LPS stimulation (100 ng/mL). Secretion of MIP-1, MIP-2, MCP-1 and IL-10 was measured using ELISA (R&D Duoset). One way Anova was used to assess differences between groups. Cells treated with hydrolysate only were compared to cells alone, cells treated with hydrolysates + LPS were compared to cells alone + LPS. Significance $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** all relative to untreated cells (no hydrolysate).

6.2.6 DSS-induced pilot model of colitis

In order to determine the ability of F1 to modulate chronic inflammation *in vivo*, a murine model of DSS colitis was carried out. A pilot study with two groups (control and DSS) was first performed to ensure disease progression with 4 mice in each group. DSS mice were administered DSS (5% w/w) in their drinking water for 7 days. All mice were culled on the final day. DSS is a well-studied model of gut inflammation and a number of studies have associated an increase in Th1, Th17 cells and cytokine secretion by BMDCs to onset and progression of the disease.

6.2.6.1 Clinical assessment of DSS induced colitis (pilot)

The DSS murine model was carried out in the Biological Resource Unit (BRU) at Dublin City University, as described in **Section 2.2.11**. Mice were weighed and scored every day for daily disease activity index (DDAI) based on percentage body weight loss, stool consistency, presence of occult blood, stool composition, fur texture and posture. **Table 6.2** outlines the scoring system used for the comparative analysis.

Table 6.2 Scoring system to determine DDAI in the DSS model of colitis

Score	Stool Consistency	Bleeding	% Body Weight loss
0	**Normal**	Negative hemoccult	<2%
1	Soft but still formed	Positive hemoccult	2-5%
2	Very soft	Blood traces visible in stool	5-10%
3	Diarrhoea	Rectal bleeding	10-15%
4	-	-	>15%

Figure 6.10 (A) shows a change in body weight percentage over the course of the pilot study (Day 0 – Day 6). A steady decrease in weight can be seen from Day 1 onwards which reached its maximum on Day 5 and 6. A steady weight was maintained in control mice. **Figure 6.10 (B)** shows the maximum weight loss on Day 6 of the pilot study. A

significant ($p<0.001$) difference in weight loss occurred between control (healthy) mice and the DSS disease group.

The DDAI also showed disease progression alongside body weight percentage change. **Figure 6.11 (A)** shows the DDAI over the course of the pilot study (Day 0 – Day 6). No disease activity was observed in the control group whilst an increase in disease activity scores was seen as soon as Day 1 for the DSS disease group. **Figure 6.11 (B)** shows the maximum disease activity on Day 7. As can be seen from **Figure 6.11 (A)**, disease activity peaked on Day 7. This increase was a significant different to that of the control group ($p<0.001$).

On the final day, all mice were culled and colons and spleens harvested for analysis. Colons were removed, then measured and weighed as an indicator of inflammation. **Figure 6.12 (A)** shows the colon length of each group. Colons from the DSS disease group were shorter than that of the healthy group. **Figure 6.12 (B)** shows the colon weight of each group, the disease group was heavier than that of the control healthy mice average. This is due to the infiltration of immune cells and inflammation. However, neither of these results (**Figure 6.12 (A) or (B)**) were significant.

Sections of the distal colon (0.5 cm) were then removed and rolled for histological examination and stained with hematoxylin and eosin (H&E). **Figure 6.13 (A)** shows an image of the colon of a mouse belonging to the control (healthy) group, **Figure 6.13 (B)** shows the colon of a mouse belonging to the DSS disease group. These images show that DSS exposure led to a decrease in the number of goblet cells present, destruction of the crypt structure of the colon, a disturbed epithelial layer and a mass infiltration of inflammatory cells. The control (healthy) colon shows the normal morphology of the colon in a healthy state with the correct crypt structure, the presence of goblet cells and far less inflammatory cells.

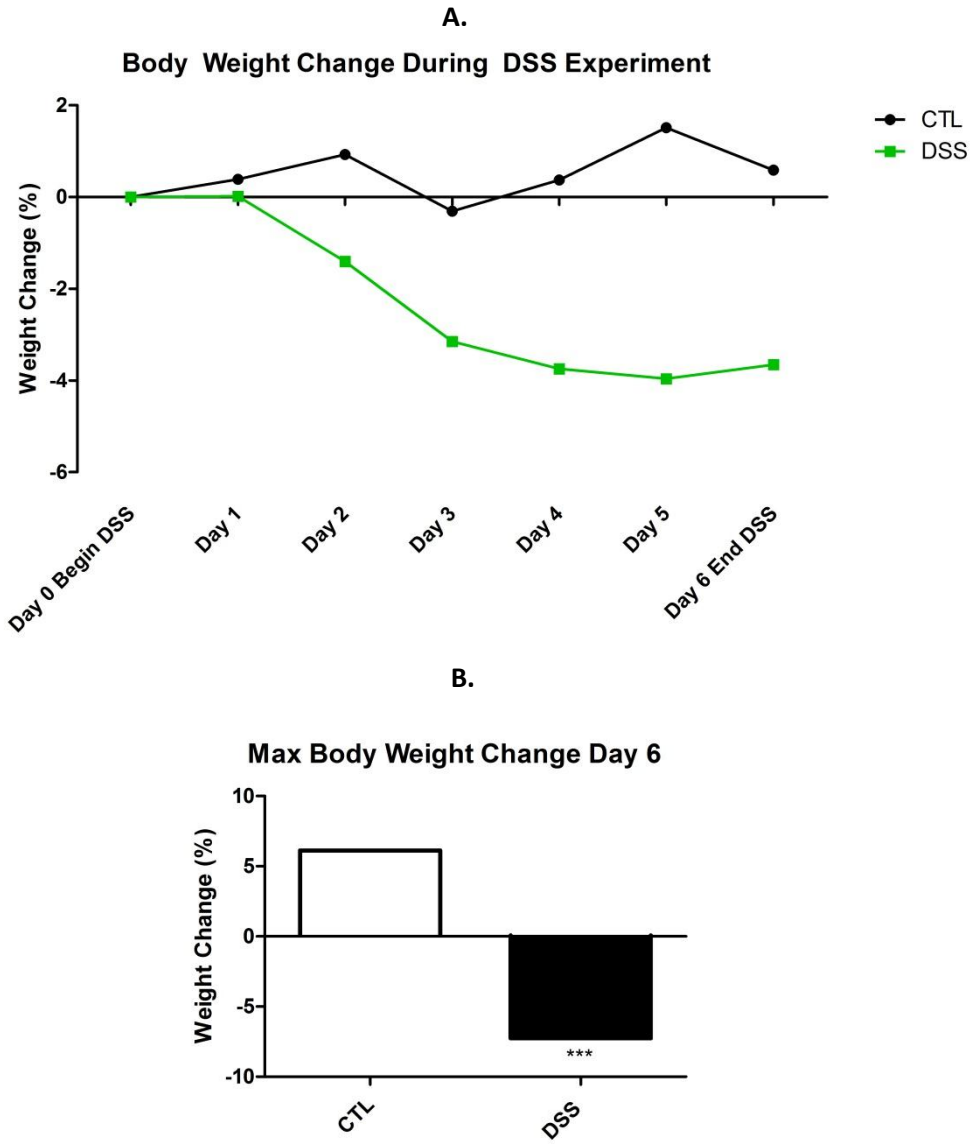


Figure 6.10 Disease associated symptoms in the DSS colitis model. 8 – 10 week old female Balb/c mice were placed into a control group of 4 mice and an experimental group of 4 mice and early acute colitis was induced in the experimental group. DSS was administered to mice in the drink water for 7 days at a concentration of 5%. **(A)** Mice were weighed every day and the average % weight change of the two groups is plotted to the end point for each group. The starting weight on Day 0 is plotted as 100% of the weight with the rest of the weight relative to this value. All mice were culled on day 7. **(B)** Maximum weight loss was recorded on Day 6. Data presented indicate the mean ±SEM (n=4). An unpaired T-test was used to determine if differences between groups were significant (*p<0.05, **p<0.01 and ***p<0.001).

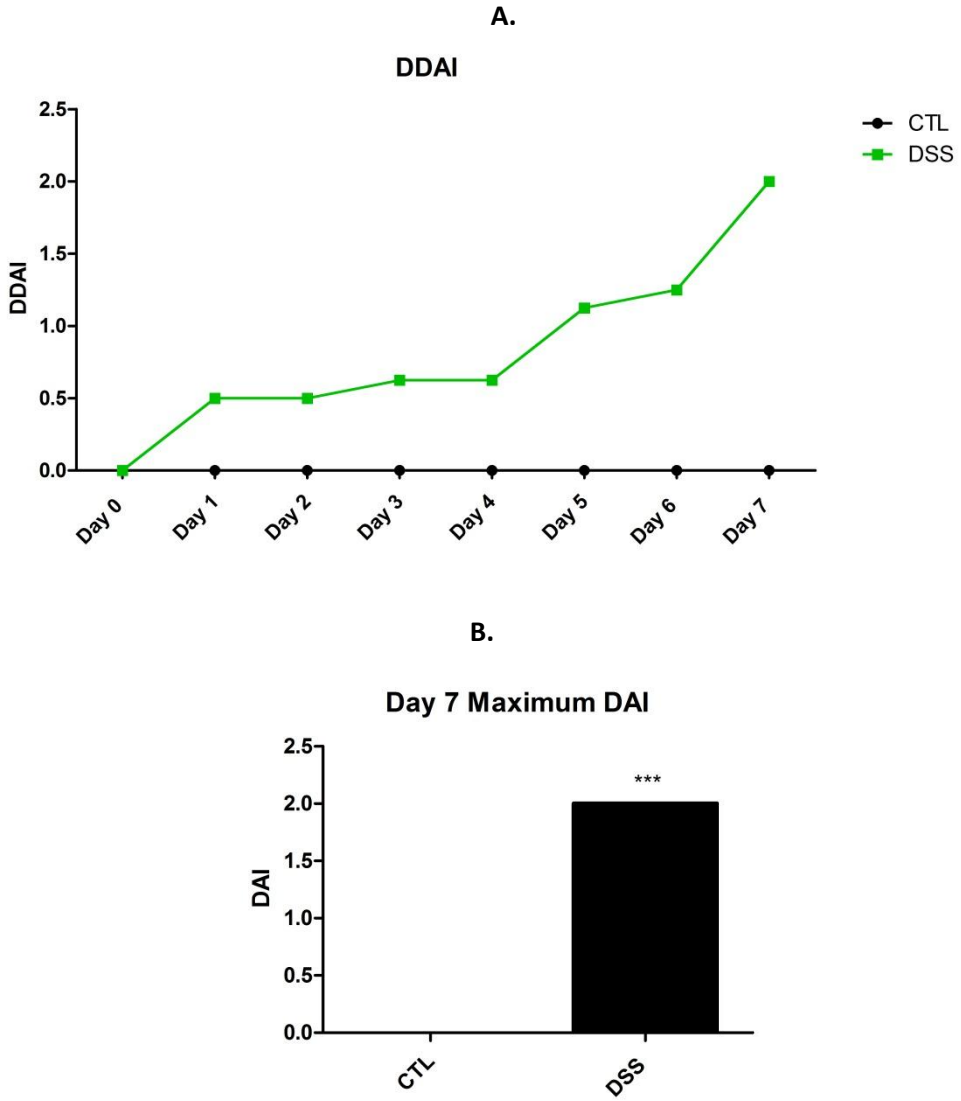


Figure 6.11 Disease associated symptoms in the DSS colitis model. 8 – 10 week old female Balb/c mice were placed into a control group of 4 mice and an experimental group of 4 mice and early acute colitis was induced in the experimental group. DSS was administered to mice in the drink water for 7 days at a concentration of 5%. **(A)** Mice were weighed every day and disease scored based on a composite measure of weight loss, stool consistency and blood in stool for daily disease index (DDAI). **(B)** Maximum DAI was recorded on Day 7. Data presented indicate the mean ±SEM (n=4). An unpaired T-test was used to determine if differences between groups were significant (*p<0.05, **p<0.01 and ***p<0.001).

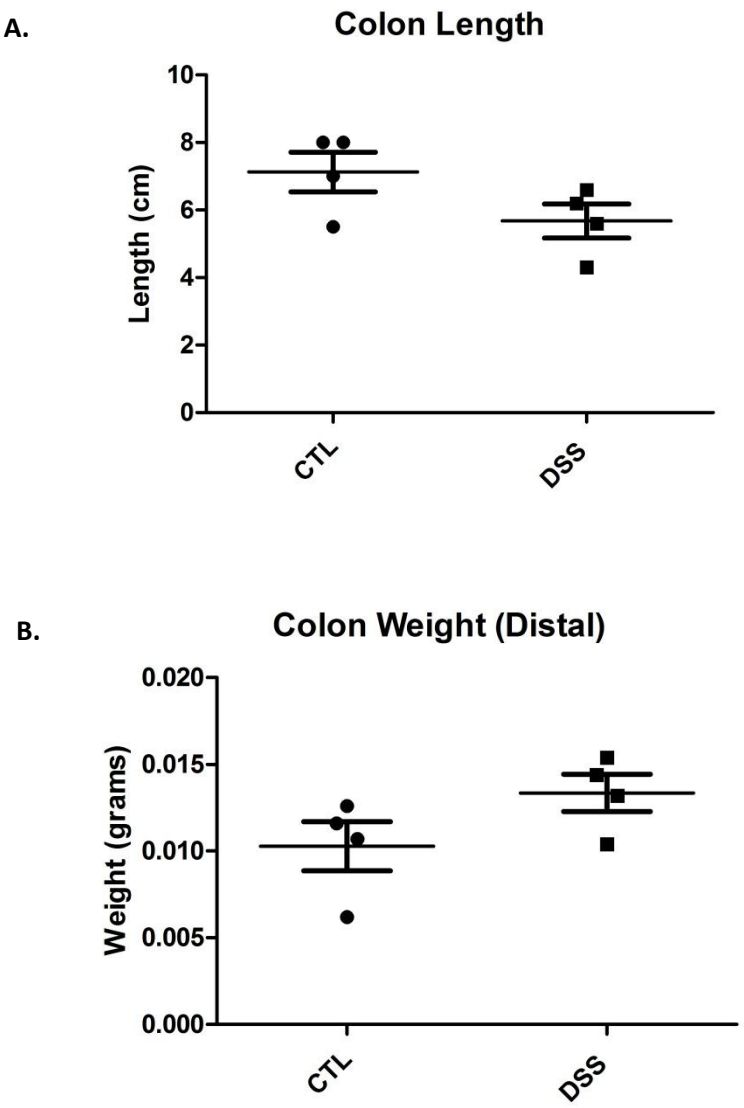
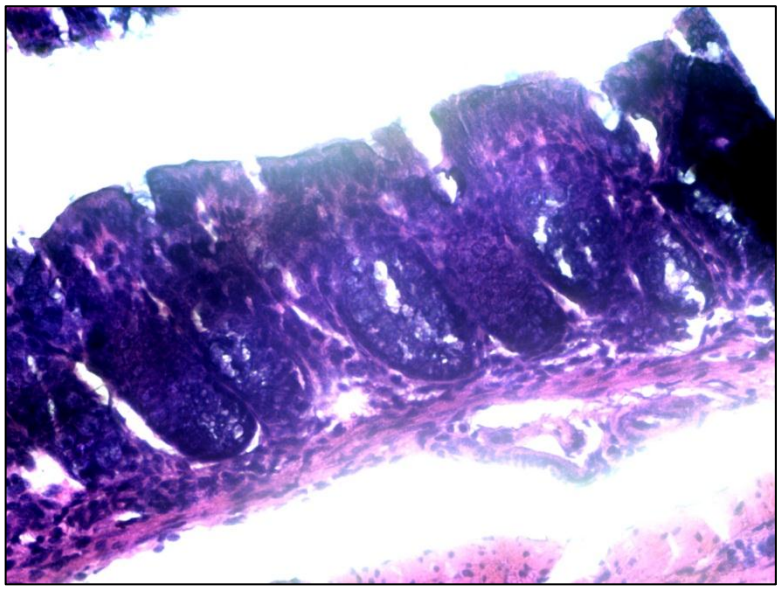


Figure 6.12 Colon length and weight in the DSS colitis model. 8 – 10 week old female Balb/c mice were placed into a control group of 4 mice and an experimental group of also 4 mice and early acute colitis was induced in the experimental group. DSS was administered to mice in the drinking water for 7 days at a concentration of 5% and mice were culled on day 7. At the end point of each group the colon was removed and washed and (A) the length and (B) the weight was measured as an indication of inflammation.

CTL



DSS

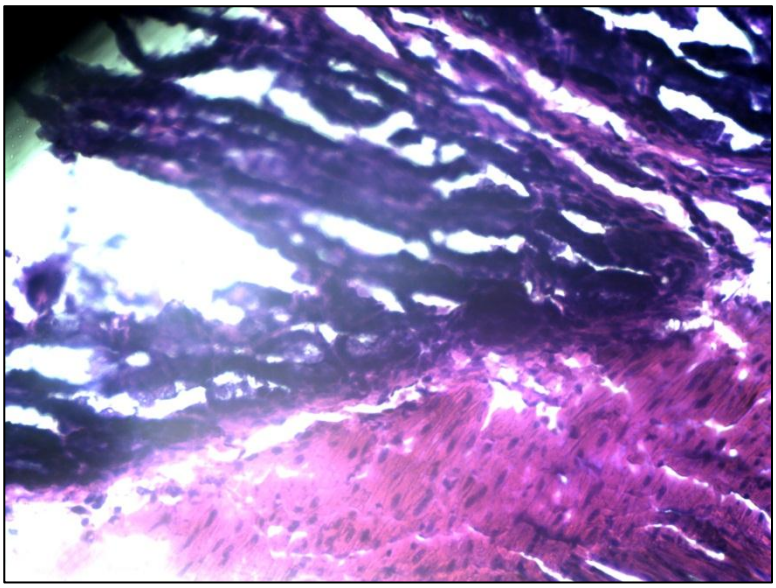


Figure 6.13 Histology of the colon in the DSS model of colitis 8 – 10 week old female Balb/c mice were placed into a control group of 4 mice and an experimental group of 4 mice and early acute colitis was induced in the experimental group. DSS was administered to mice in the drinking water for 7 days at a concentration of 5% and mice were culled on day 7. Sections of the distal colon were removed for histology and H&E staining in order to confirm inflammation. The control shows a healthy colon while infiltration and loss of crypt structure is evident in the DSS slides.

6.2.6.2 Cytokine secretion by unstimulated splenocytes of DSS induced mice

Spleens were removed from the mice following culling and splenocytes cultured as an increase in immune responses in the spleen have been reported in DSS colitis model. Spleens were collected, red blood cells lysed and splenocytes plated at a concentration of 5 x 10^6 cells/mL on a 24 well plate. Cells were left unstimulated for 72 hours before supernatants were collected. Supernatants were measured using ELISA (R&D Duoset) for the presence of cytokines IL-10, IL-6, IL-1β, TNFα, IL-2, IFNγ and IL-17.

Figure 6.14 shows the cytokine secretion by unstimulated splenocytes of DSS-induced colitis mice versus the control healthy mice. DSS mice displayed increased levels of pro-inflammatory cytokines IL-6 ($p<0.001$), IL-1β ($p<0.001$), TNFα ($p<0.001$) and IL-17 ($p<0.001$) and reduced secretion of IL-2 ($p<0.001$).

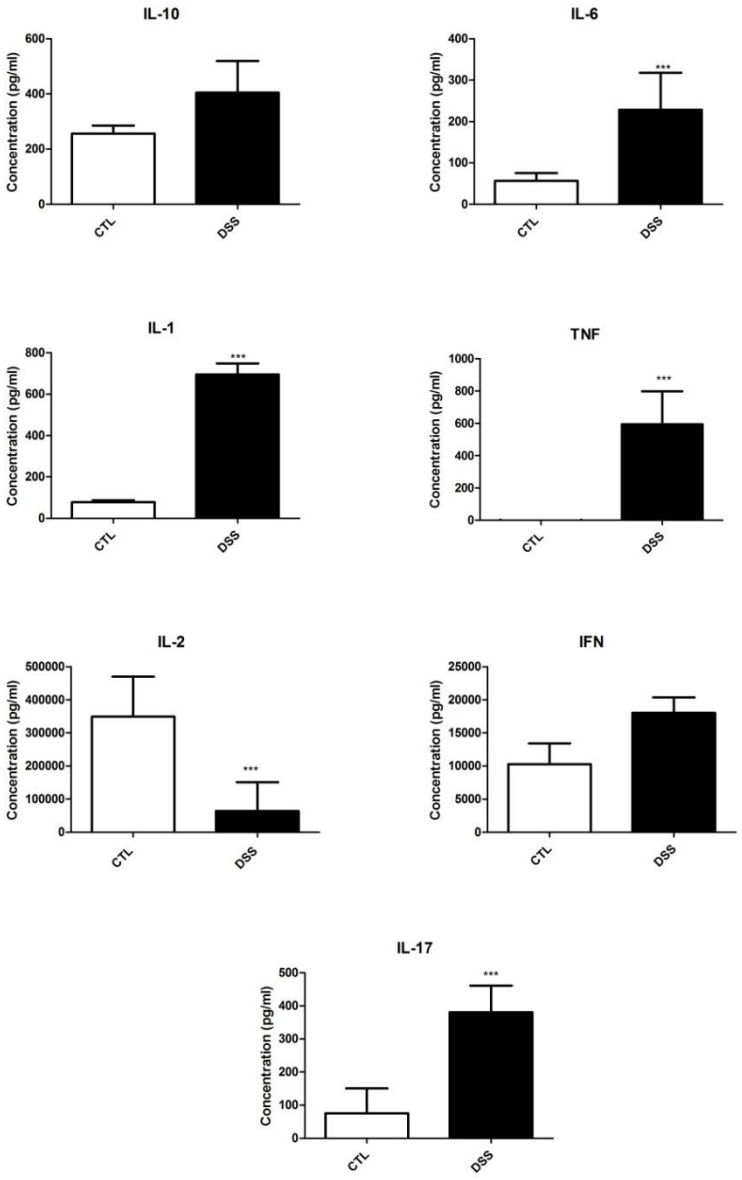


Figure 6.14 Cytokine secretion by unstimulated splenocytes in a mouse model of colitis. 8 – 10 week old female Balb/c mice were placed into a control group of 4 mice and an experimental group of 4 mice and early acute colitis was induced in the experimental group. DSS was administered to mice in the drinking water for 7 days at a concentration of 5% and mice were culled on day 7. Spleens were collected and splenocytes were plated at 5 x 10^6 cells/mL on a 24 well plate. Cells were incubated for 72 hours and supernatants collected and measured for IL-10. IL-6, IL-1β, TNFα, IL-2, IFNγ and IL-17 secretion using ELISA (R&D Duoset). Data presented indicate the mean ±SEM (n=4). An unpaired T-test was used to determine if differences between DSS and Control group were significantly different (*p<0.05, **p<0.01 and ***p<0.001).

6.2.6.3 Cytokine secretion by stimulated splenocytes of DSS induced mice

Spleens were collected, red blood cells lysed and splenocytes plated a concentration of 5 x 10^6 cells/mL on a 24 well plate. Cells were stimulated with plate bound anti-CD3 (5 µg/mL) and anti-CD28 (5 µg/mL) for 72 hours before supernatants were collected. Supernatants were then analysed using ELISA (R&D Duoset) for cytokines IL-10, IL-6, IL-1β, TNFα, IL-2, IFNγ and IL-17.

Figure 6.15 shows the cytokine secretion by stimulated splenocytes from DSS-induced colitis mice versus healthy control mice. DSS mice displayed increased levels of IL-1β ($p<0.001$) and TNFα ($p<0.001$) when compared to healthy control mice.

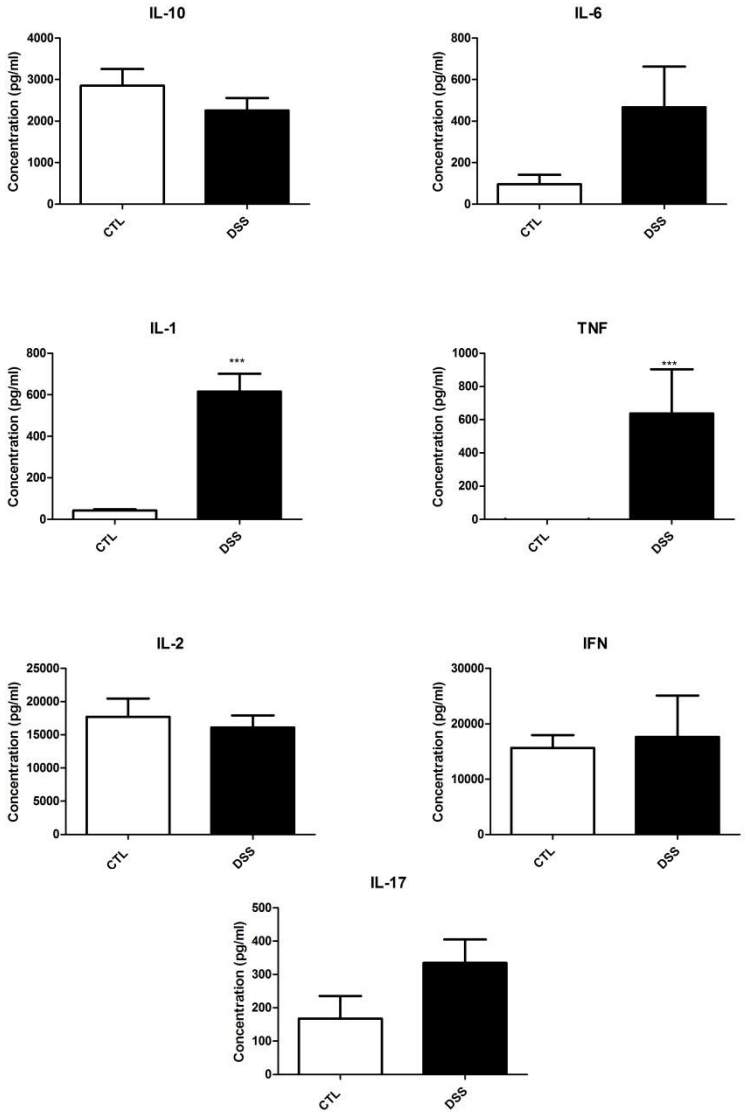


Figure 6.15 Cytokine secretion by stimulated splenocytes in a mouse model of colitis. 8 – 10 week old female Balb/c mice were placed into a control group of 4 mice and an experimental group of 4 mice and early acute colitis was induced in the experimental group. DSS was administered to mice in the drinking water for 7 days at a concentration of 5% and mice were culled on day 7. Spleens were collected and splenocytes were plated at 5 x 10^6 cells/mL on a 24 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/mL) plus anti-CD28 (5 µg/mL). Cells were incubated for 72 hours and supernatants collected and measured for IL-10. IL-6, IL-1β, TNFα, IL-2, IFNγ and IL-17 secretion using ELISA (R&D Duoset). Data presented indicate the mean ±SEM (n=4). An unpaired T-test was used to determine if differences between DSS and Control group were significantly different (*p<0.05, **p<0.01 and ***p<0.001).

6.2.7 DSS induced colitis model treated with F1

The DSS pilot model results (**Section 6.2.6**) indicated that the DSS induced model of colitis in Balb/c mice was successful and the disease was induced. It was then necessary to assess the ability of F1 to treat the disease and reduce inflammation in a mouse model of intestinal inflammation. Mice were split into 4 groups (Control (healthy mice), DSS (No DSS only), H26 (DSS and H26) and F1 (DSS and Fraction1) with 6 mice per group. Mice were administered DSS (5% w/w) for 7 days and then treatment groups received oral gavage of H26 or F1 for the final 7 days, control groups received oral gavage of PBS.

6.2.7.1 Clinical assessment of DSS induced colitis treated with F1

Mice were weighed and scored every day for daily disease activity index (DDAI) based on percentage body weight loss, stool consistency, presence of occult blood, stool composition, fur texture and posture. **Table 6.2** outlines the scoring system used for the comparative analysis.

Figure 6.16 (A) shows the body weight change percentage over the course of the 14 days of the experiment. A decrease in body weight was seen in all DSS induced groups (DSS alone, DSS + H26 and DSS + F1) from day 3 onwards. Treatment began on day 7 and body weight began to increase in the group treated with F1 (DSS + F1) from day 7. The group treated with H26 (DSS + H26) experienced an increase in body weight from day 9 onwards. DSS control group (DSS alone) experienced no real increase in body weight, maintaining the loss experienced during DSS treatment in the 7 days post treatment. Control healthy mice (CTL) maintained their body weight throughout the experiment.

Figure 6.16 (B) shows the maximum body weight change observed on day 6 of the model following DSS administration before treatment began. The maximum in body weight change experienced by DSS induced groups (DSS alone, DSS + H26 and DSS + F1) is significantly different to that of control healthy mice ($p<0.001$). All DSS induced groups experienced a decrease in body weight. Control healthy mice experienced an increase.

Figure 6.16 (C) shows the maximum body weight change observed on day 14 (final day) of the model following DSS administration and treatment. The maximum in body weight change is significantly different in that of the DSS alone mice ($p<0.001$) and H26 (DSS + H26) ($p<0.001$) treated mice to that of the control healthy mice (CTL) and the F1 (DSS + F1) treated mice. F1 treated mice experienced an increase in body weight change percentage, whilst H26 mice remained in a negative value.

The DDAI was then scored based on **Table 6.2** and followed a similar pattern to the body weight percentage change seen in **Figure 6.16**.

Figure 6.17 (A) shows the average DDAI score over the course of the 14 days of the model. An increase in DAI can be seen in all DSS induced groups (DSS alone, DSS + H26 and DSS + F1) from day 1 onwards, control healthy mice (CTL) remains 0 throughout. DAI reached its maximum in all DSS groups on day 6, the final day of DSS administration. A decrease in DAI is the observed in F1 (DSS + F1) treated mice from day 7 onwards following the start of treatment with F1, this is reduced to control healthy (CTL) levels by day 12 and maintained. H26 (DSS + H26) treated mice experience a decrease in DAI from day 9 onwards, but unlike F1, they do not return to control healthy levels by the end of the experiment (day 14). DSS alone mice experience a slight decrease in DAI after DSS administration stops but remain the highest scored group at the end of the experiment.

Figure 6.17 (B) shows the maximum DAI observed on day 7 (end of DSS inducing) in each group. All DSS induced groups experienced a DAI of over 1.5 on day 7, significantly different ($p<0.001$) to that of the control healthy group.

Figure 6.17 (C) shows the DAI observed on day 14 (final day) of the model. As is evident in **Figure 6.17 (A)** DSS alone mice experienced the highest DAI on day 14, a significant increase ($p<0.001$) to that of control healthy mice. H26 treated mice also experienced a significant increase to that of the healthy control mice by day 14 ($p<0.001$). F1 treated mice returned to the healthy control mice level by day 14.

On the final day, all mice were culled and colons and spleens harvested for analysis. Colons were removed, then measured and weighed as an indicator of inflammation. **Figure 6.18 (A)** shows the colon length of each group. The colons of DSS-induced mice (DSS Alone) were shorter than that of the control healthy mice. Mice treated with both F1 (DSS + F1) and H26 (DSS + H26) displayed no change in colon length. **Figure 6.18 (B)** shows the colon weight of each group. No change in colon weight was experienced in any group.

Sections of the distal colon (0.5 cm) were removed and rolled for histological examination following H&E staining. **Figure 6.19 (A)** shows the H&E staining of a healthy colon from a mouse from the control group (CTL) with goblet cells present and the normal crypt structure visible. **Figure 6.19 (B)** shows the H&E staining of a disease colon from a mouse from the DSS induced group (DSS alone). In this image there has been a reduction of goblet cells, loss of the crypt structure and a mass infiltration of inflammatory cells. **Figure 6.19 (C)** shows the colon of a DSS induced mouse treated with H26 (DSS + H26), which shows a similar pattern to that of the DSS alone group. The crypt structure and goblet cells are still absent and an infiltration of inflammatory cells present. **Figure 6.19 (D)** shows the colon of a DSS induced mouse treated with F1 (DSS + F1) which shows a restoration of the crypt structure and goblet cells present towards a healthy colon state and also displayed a decrease in inflammatory cells.

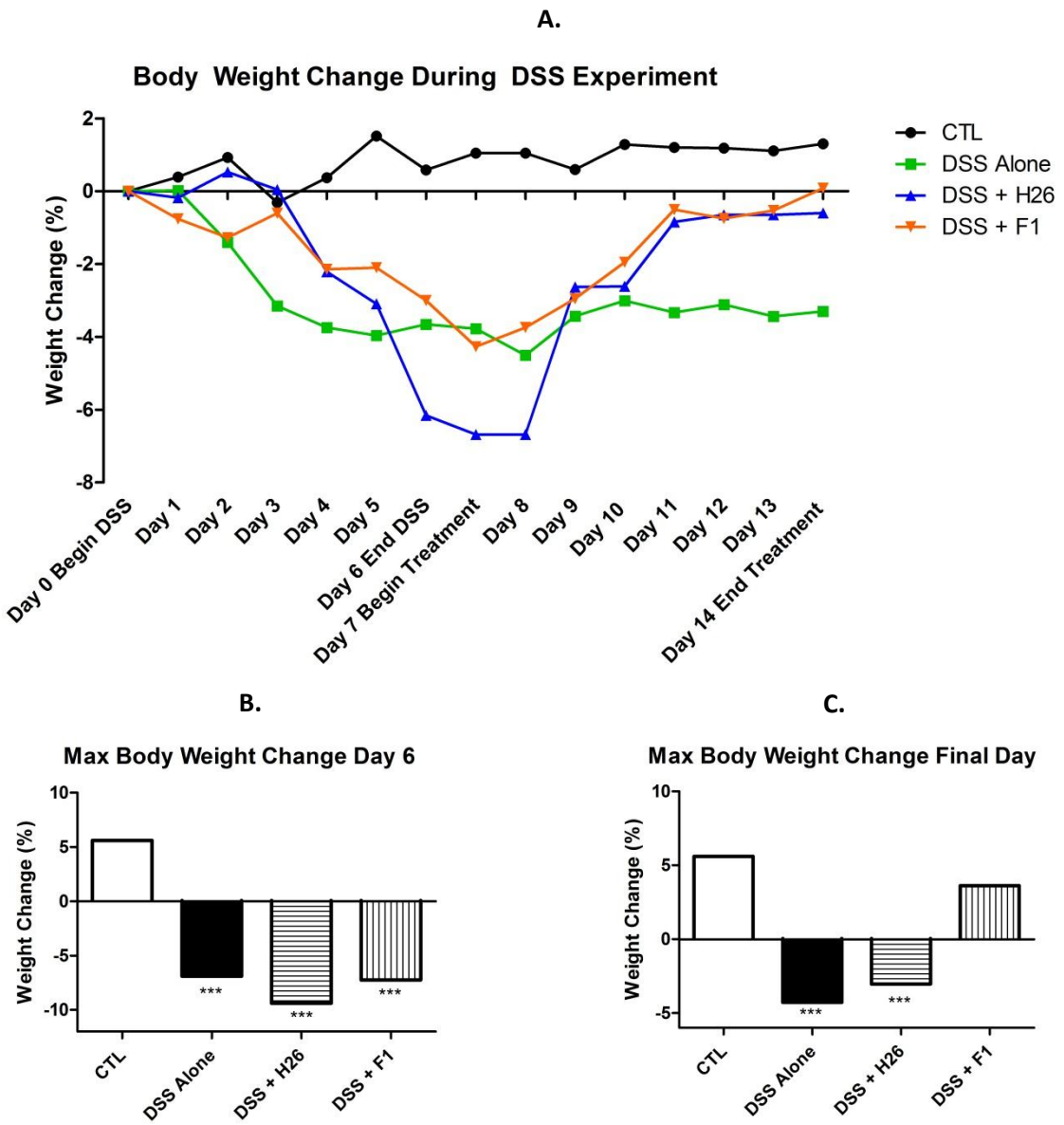


Figure 6.16 Body weight change in the DSS colitis model treated with F1 and H26. DSS was administered to test groups in the drinking water for 7 days at a concentration of 5%. Mice were treated orally with PBS (control and DSS groups), H26 or F1 in PBS every day for 7 days after DSS administration. **(A)** Mice were weighed and the average % weight change of the 5 groups is plotted to the end point for each group. The starting weight on Day 0 is plotted as 100% of the weight with the rest of the weight relative to this value. **(B)** Maximum weight loss was recorded on Day 7. **(C)** Weight loss values on final day of study were also recorded. Data presented indicate the mean ±SEM (n=6). A One way Anova was used to determine if differences between groups were significant (*p<0.05, **p<0.01 and ***p<0.001).

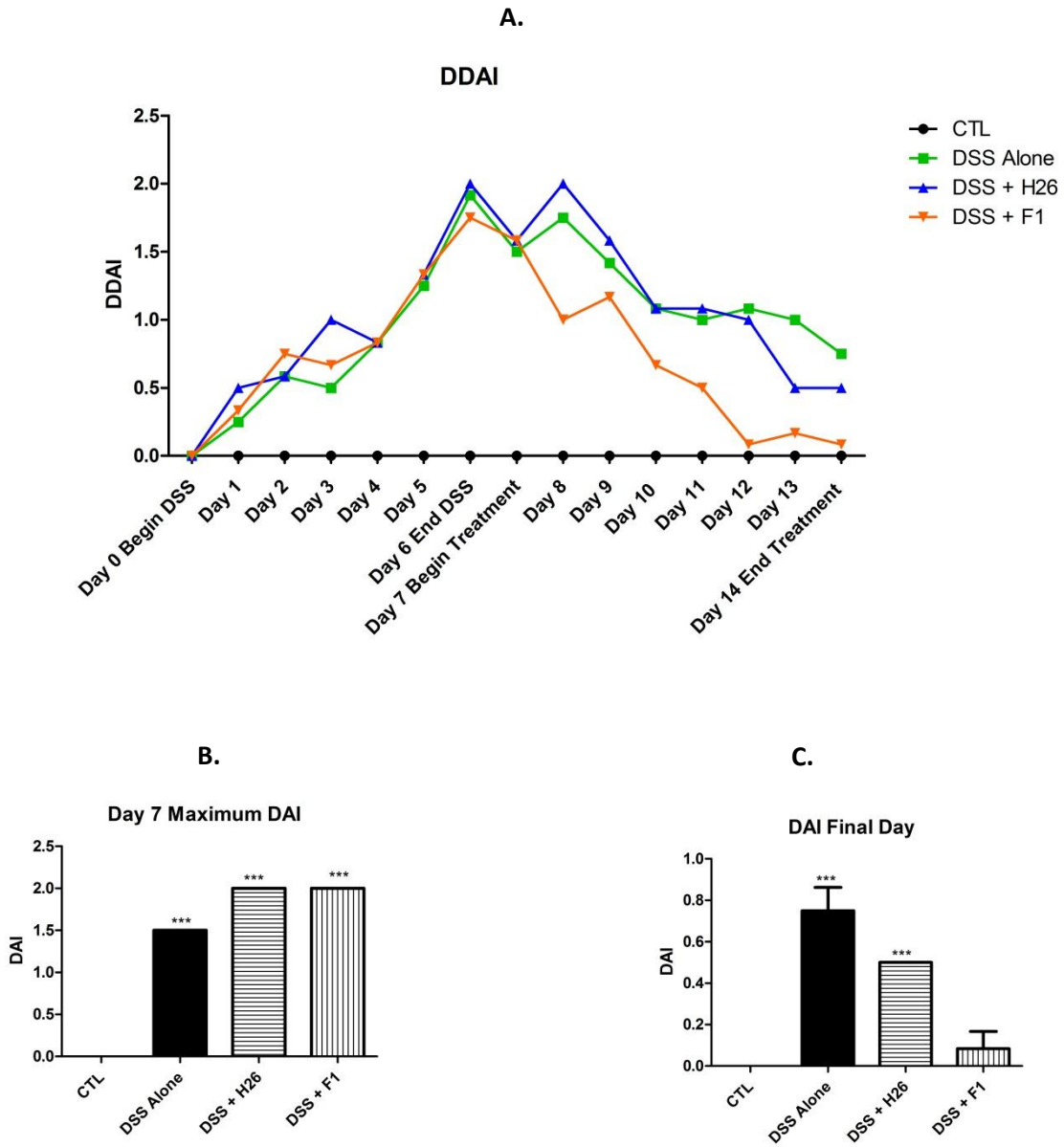


Figure 6.17 Daily disease activity index in the DSS colitis model treated with F1 and H26. DSS was administered to test groups in the drinking water for 7 days at a concentration of 5%. Mice were treated orally with PBS (control and DSS groups), H26 or F1 in PBS every day for 7 days after DSS administration. **(A)** Mice were weighed and disease scored based on a composite measure of weight loss, stool consistency and blood in stool for daily disease activity index (DDAI). **(B)** Maximum DAI was recorded on Day 7. **(C)** Final day DAI plotted with significant values. Data presented indicate the mean ±SEM (n=6). A One way Anova was used to determine if differences between groups were significant (*p<0.05, **p<0.01 and ***p<0.001).

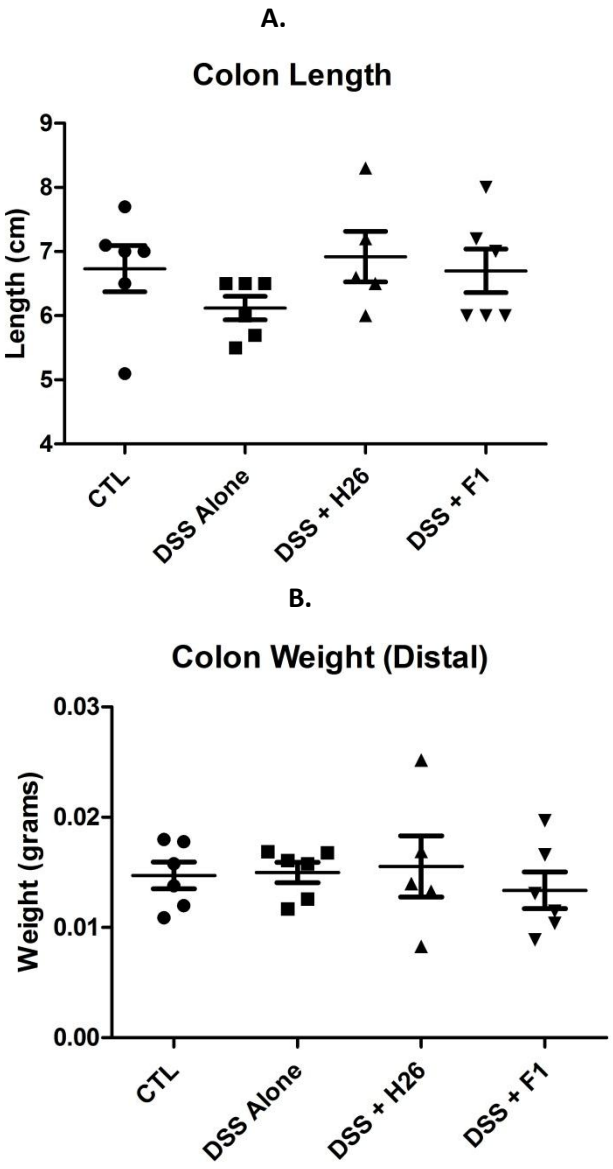


Figure 6.18 Colon length and weight in the DSS colitis model treated with F1 and H26. DSS was administered to test groups in the drinking water for 7 days at a concentration of 5%. Mice were treated orally with PBS (control and DSS groups), H26 or F1 in PBS every day for 7 days after DSS administration. At the end point of each group the colon was removed and washed and **(A)** the length and **(B)** the weight was measured as an indication of inflammation Data presented indicate the mean ±SEM (n=6). A One way Anova was used to determine if differences between groups were significant (*p<0.05, **p<0.01 and ***p<0.001).

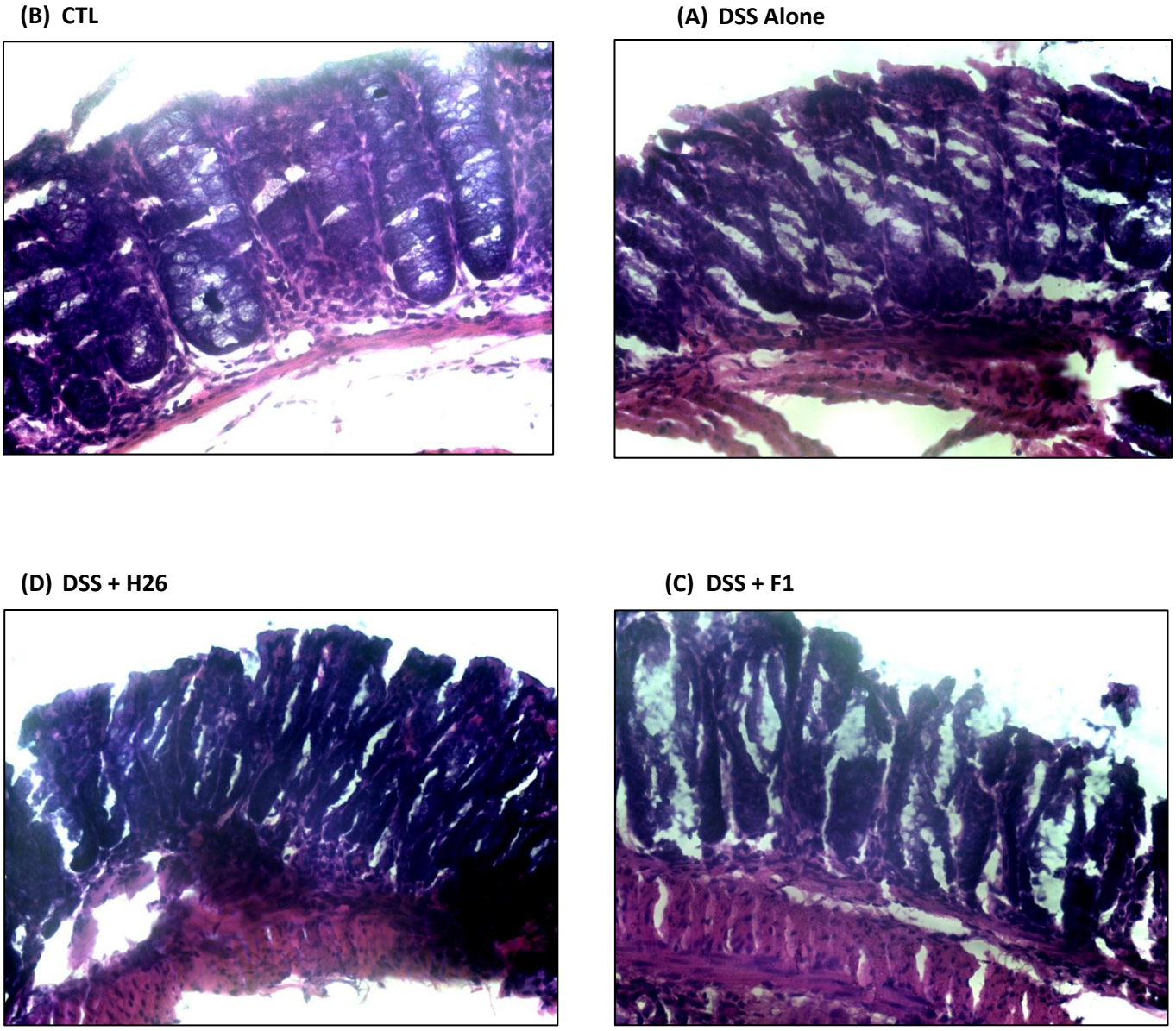


Figure 6.19 Effect of F1 and H26 on the histopathology of colon tissue in the DSS colitis model. DSS was administered to test groups in the drinking water for 7 days at a concentration of 5%. Mice were treated orally with PBS (control and DSS groups), H26 or F1 in PBS every day for 7 days after DSS administration. At the end point of each group sections of the distal colon were removed for histology and H&E staining. Representative images of the H&E staining of colonic tissue from each group were taken at 40x magnification.

6.2.7.2 Cytokine expression in colon of DSS induced mice treated with F1 and H26

RNA was isolated from colonic tissue from mice following culling on day 14. cDNA was generated from RNA using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, USA). The levels of IL-1β, IL-17, IL-6, TNFα, IL-10, and IFNγ mRNA were quantified using qPCR. The expression levels were normalised to Gus-b levels. The levels of gene expression in control healthy mice (CTL) were normalised to one and the other groups analysed were calculated as a fold change in expression relative to the control group.

Figure 6.20 shows the mRNA cytokine expression from colonic tissue harvested from the DSS model. DSS treated mice were found to have increased levels of IL-6 ($p<0.01$), IFNγ ($p<0.001$) and IL-1β ($p<0.01$) compared to the control group (CTL). DSS + H26 (mice with DSS induced colitis who had been treated with H26) had increased levels of IL-17 ($p<0.01$) and IL-10 ($p<0.01$) when compared to both CTL and DSS groups. DSS + H26 also displayed decreased levels of IL-6 ($p<0.01$), IFNγ ($p<0.001$) and IL-1β ($p<0.01$) compared to the DSS alone group. DSS + F1 (mice with DSS induced colitis who had been treated with F1) had increased levels of IL-10 ($p<0.01$) compared to both CTL and DSS alone groups, and decreased IL-6 ($p<0.001$), IFNγ ($p<0.001$) and IL-1β ($p<0.01$) compared to DSS alone groups.

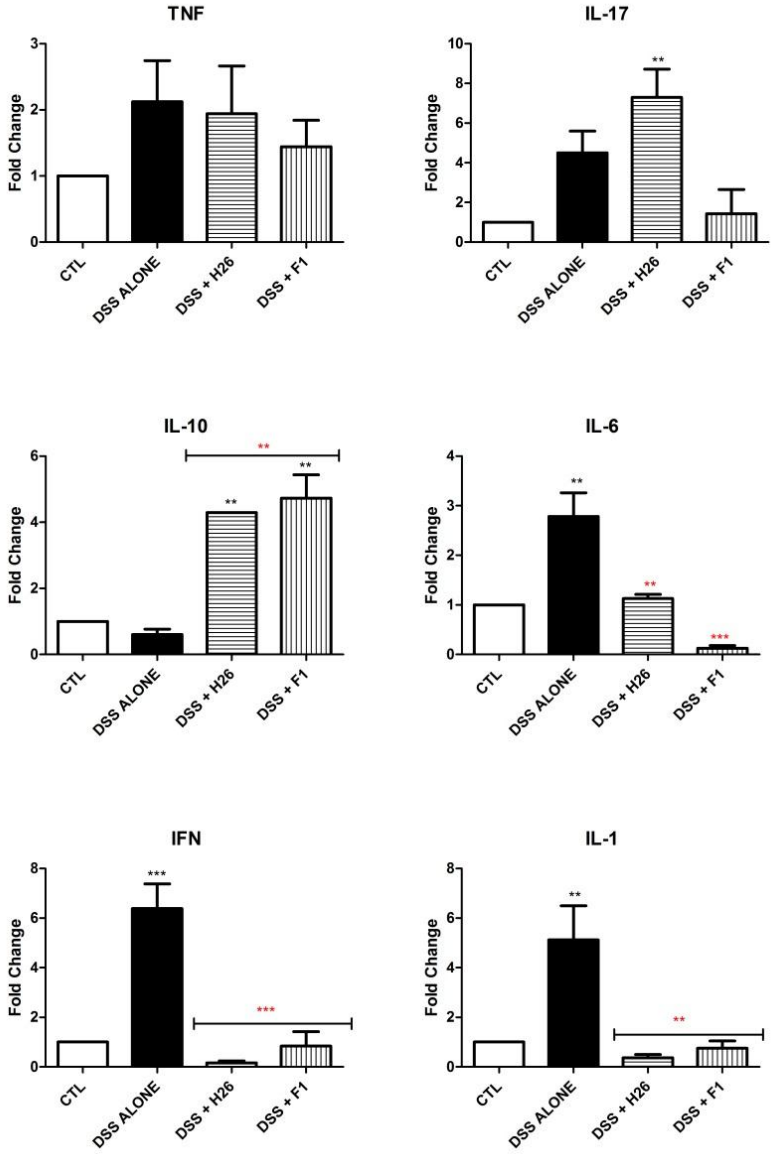


Figure 6.20 mRNA expression in a mouse model of DSS colitis treated with F1 and H26. Colonic tissue from each sample was homogenized and RNA extracted. Equalised amounts of RNA (2 µg) were converted to cDNA. The cDNA was mixed with primers (IDT) *for IL-1β, IFNγ, TNFα, IL-6* and *IL-17* and Taqman Gene Expression Mastermix (Applied Biosystems) before analyzing samples on the Lightcycler 96 system (Roche) in triplicate. Groups were compared using relative quantitation; after normalising gene expression of samples to *GusB*, the control group was normalized to 1.0 and the expression in other groups is shown relative to this value. The mean relative gene expression was calculated using the 2-ΔΔCt method. Data presented indicate the mean ±SEM (n=4). A One way Anova was used to determine if differences between groups were significant (*p<0.05, **p<0.01 and ***p<0.001). Groups compared to control (black) and to DSS alone (red).

6.2.7.3 Cytokine secretion by unstimulated splenocytes from DSS induced mice treated with F1 and H26

Spleens were also taken from the mice following culling and splenocytes were cultured as an increase in the immune response in the spleen has been reported in the DSS colitis model. Spleens were collected on day 14, red blood cells lysed and splenocytes plated at a concentration of 5 x 10^6 cells/mL on a 24 well plate. Cells were left unstimulated for 72 hours before supernatants were collected. Supernatants were measured using ELISA (R&D Duoset) for the presence of cytokines IL-10, IL-6, IL-1β, TNFα, IL-2, IFNγ and IL-17.

Figure 6.21 shows the cytokine secretion by unstimulated splenocytes from control healthy mice (CTL), DSS induced mice (DSS alone), DSS induced mice treated with H26 (DSS + H26) and DSS induced mice treated with F1 (DSS + F1). DSS alone experienced increased IL-10 ($p<0.001$), IL-6 ($p<0.01$), IL-1β ($p<0.05$), TNFα ($p<0.05$), IFNγ ($p<0.01$) and IL-17 ($p<0.05$) levels compared to CTL. DSS + H26 displayed increased IL-10 ($p<0.001$), TNFα ($p<0.05$) and IFNγ ($p<0.001$) compared to CTL and decreased IL-6 ($p<0.01$) and IL-17 ($p<0.05$) compared to DSS alone. DSS + F1 displayed reduced IL-10 ($p<0.05$), IL-6 ($p<0.01$), TNFα ($p<0.05$), IFNγ ($p<0.01$) and IL-17 ($p<0.05$) and increased IL-2 ($p<0.05$) compared to DSS alone.

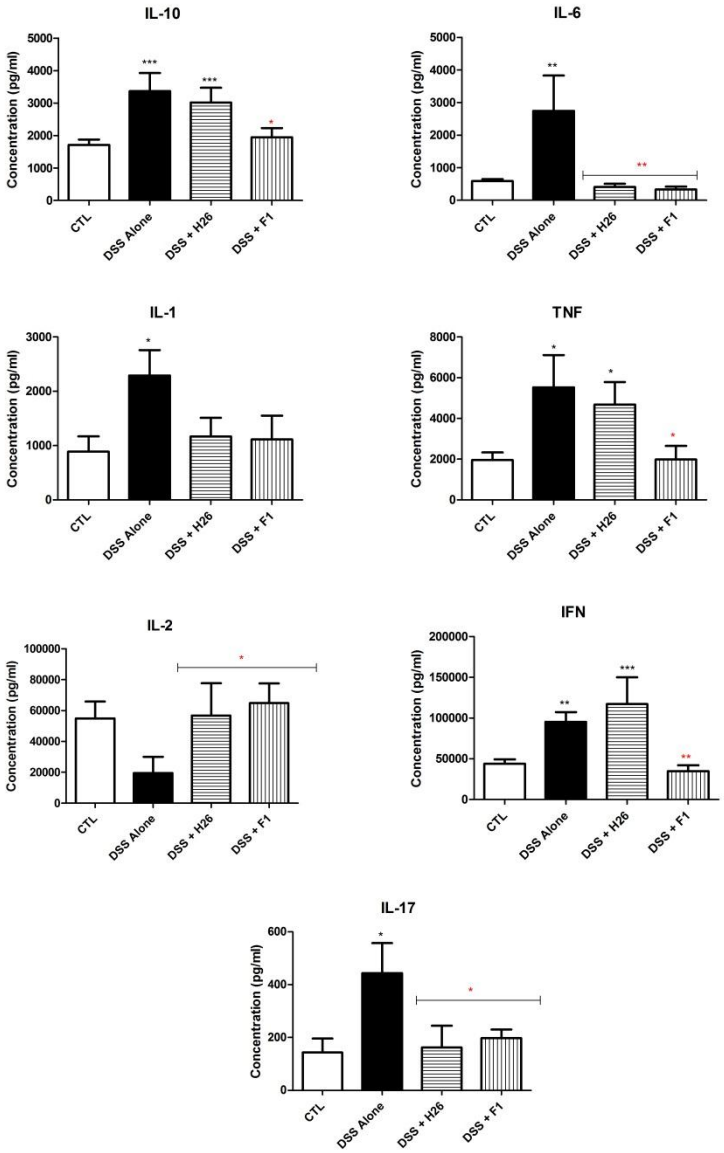


Figure 6.21 Cytokine secretion by unstimulated splenocytes in a DSS colitis model treated with F1 and H26. Balb/c mice were placed into a control group, a DSS only group, a H26 treated group and a F1 treated group (n=6). DSS was administered to mice in the drink water for 7 days at a concentration of 5% and and mice were treated orally with PBS (control and DSS groups), H26 or F1 in PBS every day for 7 days after DSS administration. Cells were stimulated with plate-bound anti-CD3 (5 μg/mL) plus anti-CD28 (5 μg/mL). Cells were incubated for 72 hours and supernatants collected and measured for IL-10. IL-6, IL-1β, TNFα, IL-2, IFNγ and IL-17 secretion using ELISA (R&D Duoset). Data presented indicate the mean ±SEM (n=4). A One way Anova was used to determine if differences between groups were significant (*p<0.05, **p<0.01 and ***p<0.001). Groups compared to control (black) and to DSS alone (red).

6.2.7.4 Cytokine secretion by stimulated splenocytes from DSS induced mice treated with F1 and H26

Spleens were collected, red blood cells lysed and splenocytes plated at a concentration of 5 x 10^6 cells/mL on a 24 well plate. Cells were stimulated with plate bound anti-CD3 (5 μg/mL) and anti-CD28 (5 μg/mL) for 72 hours before supernatants were collected. Supernatants were then analysed using ELISA (R&D Duoset) for cytokines IL-10, IL-6, IL-1β, TNFα, IL-2, IFNγ and IL-17.

Figure 6.22 shows the cytokine secretion by unstimulated splenocytes from control healthy mice (CTL), DSS induced mice (DSS alone), DSS induced mice treated with H26 (DSS + H26) and DSS induced mice treated with F1 (DSS + F1). DSS alone mice experienced increased levels of IL-1β ($p<0.05$), TNFα ($p<0.05$) and IL-17 ($p<0.05$) and decreased IL-10 ($p<0.05$) compared to CTL. DSS + H26 displayed reduced levels of IL-6 ($p<0.05$) and IL-1β ($p<0.05$) and increased IL-2 ($p<0.01$) compared to DSS alone. DSS + H26 also showed decreased IL-10 ($p<0.05$) and increased TNFα ($p<0.05$), IL-17 ($p<0.05$) and IL-2 ($p<0.01$) compared to CTL levels. DSS + F1 showed a reduction in IL-10 ($p<0.05$) compared to CTL and decreased IL-6 ($p<0.05$), IL-1β ($p<0.05$), TNFα ($p<0.01$) and IL-17 ($p<0.05$).

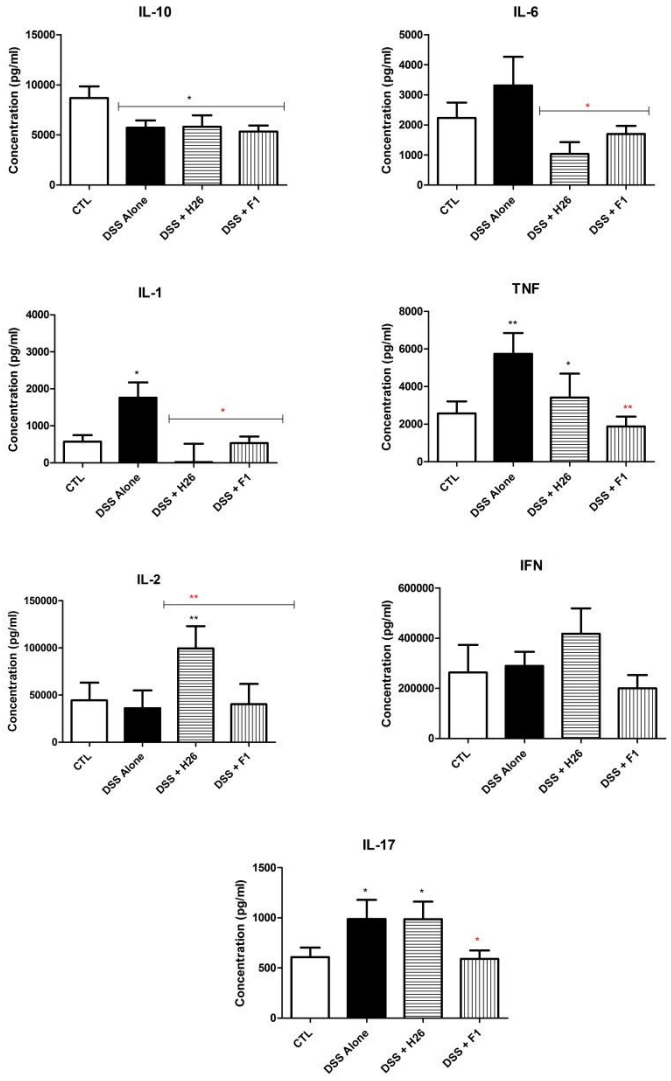


Figure 6.22 Cytokine secretion by stimulated splenocytes in a DSS colitis model treated with F1 and H26. Balb/c mice were placed into a control group, a DSS only group, a H26 treated group and a F1 treated group (n=6). DSS was administered to mice in the drink water for 7 days at a concentration of 5% and and mice were treated orally with PBS (control and DSS groups), H26 or F1 in PBS every day for 7 days after DSS administration. Spleens were collected and splenocytes were plated at 5 x 10^6 cells/mL on a 24 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/mL) plus anti-CD28 (5 µg/mL). Cells were incubated for 72 hours and supernatants collected and measured for IL-10. IL-6, IL-1β, TNFα, IL-2, IFNγ and IL-17 secretion using ELISA (R&D Duoset). Data presented indicate the mean ±SEM (n=4). A One way Anova was used to determine if differences between groups were significant (*p<0.05, **p<0.01 and ***p<0.001). Groups compared to control (black) and to DSS alone (red).

6.3 Discussion

Inflammatory bowel disease (IBD) affects approximately 40,000 people in Ireland (Irish Society for Colitis and Crohn's disease, 2018) and is characterised by chronic inflammation of the gastro-intestinal tract. There are two common forms, Ulcerative Colitis (UC) and Crohn's Disease (CD). Whilst no definitive underlying cause has been found, factors which initiate the disease or contribute to the pathogenesis have been identified. These include genetic factors such as increased secretion of cytokines and chemokines, autophagy and the presence of anti-microbial peptides in the gut. Environmental factors have also been identified such as an unhealthy diet, excessive stress, overuse of anti-biotics, smoking, having your appendix removed and long-term use of NSAIDs (Neurath, 2014). As no cause has been discovered, IBD is identified by its typical features such as the presence of ulcers in the colon when examined with an endoscope. Other clinical features include fistulas, diarrohea, the passage of mucus and an abdominal mass which can be present alongside pathological symptoms such as the destruction of the crypt architecture (Baumgart and Sandborn, 2007).

Cytokines have been implicated in the initiation and pathogenesis of IBD and many other chronic inflammatory disorders. In IBD, they have been shown to drive inflammation of the gastro-intestinal tract, increase symptoms such as diarrohea and also play a crucial role in the pathogenesis of destructive forms of the disease which often cause infection such as intestinal stenosis, rectal bleeding, abscess and fistula formation (Jürgens *et al.,* 2010). Therefore, cytokines have been identified as a therapeutic target for treatment of IBD.

The standard therapy at present for treatment of IBD is anti-TNFα antibodies. Anti-TNFα antibodies have been shown to have a marked clinical benefit for patients as TNFα plays an important role in the pathogenesis of the disease and inhibition of this cytokine may induce mucosal healing (Papadakis and Targan, 2000). This therapy involves the transfusion of the antibodies in-clinic and it is an invasive therapy and can leave the patient immunosuppressed (Rutgeerts *et al.,* 2004). IL-1β is another pro-inflammatory cytokine also shown to play a prominent role in IBD and is thought to be crucial for initiation of the disease (Cassini-Raggi *et al.,* 1995). Deficiency of the enzyme caspase-1,

which converts IL-1β to an active cytokine, was found to protect mice from DSS-induced colitis in a murine model (Siegmund *et al.,* 2001) thus making IL-1β a target for preventing the onset of IBD. Similarly, inhibition of IL-6 in IBD has been shown to suppress inflammation, induce T cell apoptosis and reduce the production of pro-inflammatory cytokines such as TNFα (Neurath and Finotto, 2011).

Studies of the anti-inflammatory cytokine IL-10 have shown this cytokine to be beneficial in the treatment of CD but not UC (Papadakis and Targan, 2000). A loss of function in the genes encoding IL-10 and the IL-10 receptor has been associated with early onset IBD in children (Glocker *et al.,* 2009). Furthermore, IL-10 gene knockout mice develop spontaneous colitis. This indicates that IL-10 plays a regulatory role in the disease and may prevent initiation of the disease (Rennick and Fort, 2000) and is a significant potential therapeutic target. However, not all cytokine therapies have been shown to be beneficial in the treatment of IBD and several cytokines identified as potential targets have failed to reduce symptoms of the disease in patients. Inhibition of IFNγ has been shown to have no clinical effect on the symptoms of disease (Kojouharoff *et al.,* 1997). However, the most surprising has been the failure of the antibody secukinumab, the anti-IL-17 antibody, in a human trial, which was found to aggravate rather than treat the disease (Hueber *et al.,* 2012). This suggests that a specific intricate cytokine network is involved in IBD, therefore therapies that target multiple cytokines are a new approach being explored for treating the disease. Tofacitinib, a JAK inhibitor which blockades IL-17 and IFNγ is one such therapy (Danese *et al.,* 2015).

A total inhibition of cytokines using single cytokine inhibitors has been shown to cause immunosuppression (Kroesen *et al.,* 2003), however, through the reduction, but not complete inhibition, of multiple cytokines, it may be possible to avoid this effect. This has been shown in Tofacitinib (Garber, 2011). Hence, F1 of H26 of blue whiting, which has been shown to reduce multiple cytokines secreted by both T cells and BMDCs, but not completely inhibit them, may be a possible therapeutic and work in a similar manner to Tofacitinib.

In order to investigate F1's potential as a therapeutic it was first necessary to study its effects on the overall immune response. The optimum dose of F1 was first determined on BMDCs using a dose range of 0 – 2.0 mg/mL and found to be 1 mg/mL. At this dose, F1 reduced but did not fully suppress pro-inflammatory cytokines TNFα IL-6, IL-23, IL-12p40 and IL-12p70. It did so either equal to or better than all other doses, including the highest of 2 mg/mL.

Following dose optimisation, the fraction's impact on the broader immune response of BMDCs was then investigated. In previous investigations, TLR4 was only examined, hence it was necessary to analyse F1's impact on the cells response to other toll like receptors. TLR1 was stimulated using the TLR1/2 agonist PAM(3)CSK(4) (PAM) a triacylated lipoprotein. PAM is a TLR1/2 ligand as TLR1 and TLR2 often form a heterodimer and hence are present together (De Luca *et al*., 2009). Stimulation of TLR1 causes the activation of the MyD88/IRAK pathways and the secretion of pro-inflammatory cytokines TNFα, IL-1, IL-6 and IFNγ as well as anti-inflammatory IL-10 (Liu *et al.,* 2007). TLR1/2 becomes activated by ligands on the surface of gram positive bacteria and initiate the immune response to this type of invasion (Medzhitov, 2001). F1 reduced a number of cytokines and chemokines secreted by BMDCs following activation with toll ligand PAM, including IL-6, IL-23, IL-10, MCP-1, IL-12p40 and MIP-1. Hence F1 may be useful in reducing TLR1 cytokine secretion. Toll like receptor 2 (TLR2) was stimulated using PGN-BS, a peptidoglycan isolated from *Bacillus subtilis*. TLR2 forms a heterodimer with TLR1, and also with TLR6, and is stimulated by gram positive bacteria (Takeuchi *et al.*, 1999). F1 reduced secretion of TNFα, IL-6, IL-23, IL-12p40, IL-10, MCP-1, MIP-1 and MIP-2 following TLR2 activation. The other TLR which forms a heterodimer with TLR2 is toll like receptor 6 (TLR6). TLR6 was stimulated with zymosan. Zymosan is a glucan found on the surface of fungi and TLR6 provides protection from fungal infection. F1 reduced levels of TNFα, IL-6, IL-23, IL-12p40, MIP-1 and MCP-1 in BMDCs with TLR6 stimulated. TLR2 has been found to be upregulated in the ileum of UC patients (Frolova *et al.,* 2008). TLR1, TLR2 and TLR6 have also been identified as influencing disease extension in IBD and reducing their response to activation and the subsequent inflammatory response, may be beneficial in limiting the disease (Pierik *et al.,* 2006).

TLR3 was stimulated by Poly I:C, to simulate a viral infection. TLR3 is stimulated by viral ligands and provides protection to the host against viral infection (Zhang *et al.,* 2007). F1 reduced secretion of IL-12p40, MIP-2 and MCP-1 following TLR3 activation. TLR3 was found to be strongly down-regulated in patients suffering from IBD (Cario and Podolsky, 2000). F1 minimally affected the immune response of this TLR.

TLR4 was stimulated by LPS. TLR4 provides protection against infection from Gram negative bacteria (Lorenz *et al.,* 2002) and has been extensively studied in this project. F1 showed similar results to previous studies in reducing secretion of pro-inflammatory cytokines in response following TLR4 activation. TLR4 has been associated with IBD and has been found to be upregulated during the disease (Cario and Podolsky, 2000). Similarly, Szebeni *et al.* (2008) also found TLR4 expression to be increased in the mucosa of children suffering from IBD. TLR4 upregulation has been linked to the pathogenesis of both UC and CD in IBD (Fukata and Abreu, 2007) hence reducing the secretion of pro-inflammatory cytokines released through TLR4 pathways could be beneficial in the treatment of IBD. F1 displayed this activity in reducing pro-inflammatory cytokines, including TNFα, IL-1β, IL-6, IL-23 and IL-12p40.

TLR5 was stimulated with flagellin. Flagellin is a hollow, cylindrical protein which forms the filament in bacterial flagellum. TLR5 provides host protection against invading motile bacteria (Gewirtz *et al*., 2001). F1 reduced secretion of IL-12p40 following stimulation of TLR5, hence displaying anti-inflammatory activity. TLR5 has been associated with colitis, with TLR5 knockout mice developing spontaneous colitis (Vijay-Kumar *et al*., 2007). Reduction of pro-inflammatory cytokines secreted through the activation of this toll like receptor may help in the reduction of the pathogenesis of this disease.

TLR7 was stimulated with LOX. LOX is a guanosine analog and a powerful stimulator of the immune response. TLR7 protects against single stranded RNA viruses such as HIV (Schlaepfer *et al*., 2006). TLR7 agonists have been identified as potential therapeutics in IBD, as activation of TLR7 has been shown to reduce symptoms of the disease (Karlsson *et al*., 2007). F1 reduced secretion of a number of cytokines following activation of TLR7

including TNFα, IL-6, IL-12p40 and the chemokines MIP-1 and MCP-1. F1 would not be useful in promoting TLR7 expression, as it suppressed TLR7 instead of signalling.

Following examination of the effect of F1 on toll like receptors in BMDCs, the effects on a different immune cell to determine whether F1 could also suppress inflammatory cytokines produced by other cells was investigated. For this study the J774A.1 macrophage cell line was chosen as they are a robust and well-studied cell line. The impact of F1 on the viability of macrophage was first analysed using the MTS assay and it was found to have no negative impact Next the ability of F1 to modulate the cytokine and chemokine secretion of J774A.1 macrophage was examined. F1 and its hydrolysate H26 were shown to have very little effect on the secretion. F1 decreased MIP-1 secretion and H26 modulated MIP-1 and IL-6.

F1 of H26 displayed potent anti-inflammatory effects *in vitro*. It reduced secretion of TNFα, IL-12p40, IL-1β and IL-12p70 from BMDCs. It decreased IL-6, IL-10 and IFNγ secretion by undifferentiated $CD4^+$ T cells and IFNγ levels secreted by Th1 cells. It also suppressed the immune response of various toll like receptors including TLR1, TLR2, TLR4, TLR5 and TLR6, which all have been associated with IBD initiation and pathogenesis. It also displayed selective anti-inflammatory effects, having no impact on the immune response of macrophage cells, resulting in the potential to maintain the immune response of an individual whilst modulating chronic inflammation. Its ability to suppress inflammation *in vivo* was then examined using an *in vivo* murine DSS-induced model of colitis. This model has previously been used in the research group and involves the induction of colitis in mice using DSS. Furthermore, DSS-induced colitis is a widely used murine model of colitis due to its simplicity and its many similarities to human UC (Chassaing *et al*., 2014). It displays similarities to human UC in pathology, pathogenesis and therapeutic response (Solomon *et al.*, 2010). DSS is toxic to the epithelial layer of the colon, compromising the integrity of the intestinal barrier. This results in erosions and alters the expression of tight junction proteins, increasing the epithelial permeability. This increased permeability results in an infiltration of pro-inflammatory cytokines and intestinal bleeding typically follows (Cooper *et al*., 1993). Pathology of the disease typically presents in the distal colon of the large intestine. For this reason, analysis was focused on tissue from the distal colon

of mice. Clinical symptoms such as weight loss, diarrhoea, hemo-occult blood and histological features induced by DSS in murine models accurately represent human ulcerative colitis (Laroui *et al.*, 2012). DSS was also the chosen model as it does not require the harmful physical stressors such as hypoxia or hypothermia required by other models. DSS-induced colitis can be acute or chronic depending on the length of DSS exposure. The length of exposure is also characterised by the T helper response. Short term exposure in mice drives acute inflammation and a Th1 or Th17 response which results in a cytokine cascade of TNFα, IL-1β, IL-6, IL-10, IL-12, IL-17 and IFNγ. Long term exposure is characterised by a Th2 response with a large increase in IL-4 and IL-5 secretion (Yan *et al.*, 2009). An acute model of DSS-induced colitis was chosen for this project in order to examine the ability of F1 to suppress the pro-inflammatory cytokines secreted *in vivo*.

A successful 7-day pilot model of DSS-induced colitis was first carried out in order to ensure onset and pathogenesis of the disease. Following this a full 14-day model was undertaken, and mice were administered 5% (w/w) DSS for 7 days in order to elicit the typical clinical symptoms of the disease, including weight loss, diarrhoea, hemo-occult blood, loss of crypt architecture, destruction of the epithelial layer, reduction in colon length and increase in colon weight, an infiltration of inflammatory cells and an increase secretion of pro-inflammatory cytokines. These clinical symptoms were successfully reversed by treatment with F1. The percentage body weight change was returned to day 0 levels by F1 and H26 by day 11, however the maximum change on the final day of the model was still significantly different in mice treated with H26 compared to control, whereas F1 successfully returned to control levels. DAI was reduced to control levels by F1 by day 11 also and was maintained until the end point of the model. H26 did not reduce DAI back to control levels. On the final day the maximum DAI of mice treated with F1 was the same as that of control mice, the max DAI of mice treated with H26 was still significantly different to that of the control mice on the final day. Sections of the distal colon were removed, H&E stained and imaged in order to assess the histopathology of the colon. From these images there is a clear difference between control mice and DSS alone treated mice, with a loss of crypt architecture and goblet cells, an influx of inflammatory cells and the destruction of the epithelial layer in the DSS treated mice. In F1 treated mice there is visible restoration of the crypt structures and epithelial layer, a return of goblet

cells and a decrease in inflammatory cells. This restoration was not seen in the H26 treated mice.

F1 demonstrated consistent anti-inflammatory activity in suppressing the pro-inflammatory cytokine response associated with the DSS model. F1's effect on mRNA cytokine expression levels in the colon was first examined. Treatment with F1 reversed the Th17 -associatedincrease in IL-1β and IL-6 mRNA gene expression. An increase of IL-1β and an imbalance between it and its receptor in the mucosa, have been associated with the onset of IBD. (Casini-Raggi *et al.*, 1995). IL-6 plays a key role in the disease severity of IBD (Atreya and Neurath, 2005) and in the polarisation of Th17 cells. F1 also increased expression of IL-10 compared to both DSS alone and control mice. IL-10 has been identified as a potential therapeutic target for treatment of colitis (Li and He, 2004) and upregulation of the expression of this gene could be beneficial in the treatment of the disease. Both F1 and H26 also decreased IFNγ expression. IFNγ has also been associated with disease progression and inhibition of this cytokine has been shown to treat the disease in murine models (Singh *et al*., 2003).

F1 treatment of DSS-induced mice did not suppress the ability of the splenocytes of these mice to mount an immune response to an external stimulus. Cytokines examined (IL-6, IL-1β, TNFα, IL-2, IL-17 and IFNγ) in splenocytes stimulated with plate bound CD3 and CD28 from mice treated with F1 displayed the same levels of secretion to that of the control mice. This indicates that F1 is not immunosuppressive. Immunosuppression is a major side effect of cytokine therapies and is seen in many commonly used treatments for colitis and IBD (Bebb *et al.*, 2002). The use of immunosuppressors in IBD treatment has also been linked to an increased risk of cancer, including lymphoma (Beaugerie *et al.*, 2014). Treatment with F1 of DSS-induced mice showed further anti-inflammatory effects in the unstimulated splenocytes isolated from the mice. It reduced levels of IL-6, IL-17 and TNFα secreted. IL-17 has been shown to play a major role in the pro-inflammatory activity of Th17 cells (Hundorfean *et al*., 2011). It has also been associated with severity of the disease and hence decreasing this cytokine could reduce symptoms in patients with severe disease (Yen *et al*., 2006). TNFα has also been strongly associated with IBD and anti-TNFα antibodies are a common treatment for the disease. Inhibition of this pro-

inflammatory cytokine has been shown in both DSS-induced colitis models and humans to reduce chronic gut inflammation (Kojouharoff *et al*., 1997). F1 displayed potent anti-inflammatory effects different to that of H26. H26 did not successfully reduce levels of TNFα or IFNγ, indicating that this activity is a result of fractionation.

Pro-inflammatory cytokine such as TNFα, IFNγ, IL-17, IL-6 and IL-1β play a pivotal role in the pathogenesis of IBD and the development of colitis associated cancers (Francescone *et al*., 2015), hence the potential of the blue whiting hydrolysate fraction to suppress these pro-inflammatory cytokines has particular relevance to IBD. Whilst therapeutics are currently available for IBD, relapse and further inflammation is common amongst recovered patients. The identification of natural immunomodulatory compounds, such as F1, may offer an alternative which can prevent recurring inflammation as they can be taken as part of a balanced diet (Pan *et al.,* 2009).

This chapter demonstrates the consistent anti-inflammatory activity displayed both *in vitro* and *in vivo* by a blue whiting hydrolysate fraction, F1. This fraction displayed potent anti-inflammatory abilities with the suppression of pro-inflammatory cytokines from a range of immune cells. This activity was then shown to translate into *in vivo* models, where F1 successfully reduced clinical symptoms of colitis and restored cytokine secretion to healthy control levels. This fraction has the potential to be a novel therapy for the treatment of IBD and gut inflammation and its potential as an anti-inflammatory food ingredient should be explored.

Chapter 7:
General Discussion

7.1 General Discussion

Inflammation plays a crucial role in pathogen clearance and provides the individual with protection against infection but it can become dysregulated and result in chronic inflammation which can be harmful (Szabo *et al.*, 2007). Chronic inflammation is characterised by an excessive, prolonged inflammatory response (Ferrero-Miliani *et al.*, 2007). It is an underlying cause of many diseases including RA, IBD and diabetes, and has been associated with the onset of many cancers (Baecklund *et al.*, 2006; Hussain *et al*., 2000; Sanderson, 1986). Incidences of chronic inflammation have been shown to increase as people age, with the term inflammaging being used to describe this phenomenon (Franceschi *et al*., 2000). Whilst chronic inflammation and inflammaging affect a large number of people worldwide, long term therapies to combat inflammation remain elusive. Current therapies include anti-bodies which inhibit pro-inflammatory cytokines such as TNFα, however these often leave the individual immunocompromised and also require invasive in-clinic transfusions in order to be administered.

The aim of the research detailed in this thesis was to investigate the potential of protein hydrolysates from marine sources as anti-inflammatory therapeutics and/or functional ingredients. This involved screening numerous marine species in order to identify a hydrolysate fraction that can be used as a functional ingredient or therapeutic for the treatment of the chronic inflammation associated with inflammatory disease and inflammaging. Three marine species, *Palmaria palmata* (PP), blue whiting and boarfish, were harvested from the Atlantic Ocean by Killybegs Fishermen's Organisation, Killybegs, County Donegal facilitated by Bord Iascaigh Mhara, Ireland and proteins isolated and hydrolysed by project collaborators in the University of Limerick (UL). Whilst protein hydrolysates from all three sources displayed anti-inflammatory activity in a range of key immune cells, including BMDCs, undifferentiated $CD4^+$ T cells and T helper cell subsets, some species hydrolysates were not viable options for commercial scale up.

PP was chosen for this study as it was already shown to have antihypertensive (Fitzgerald *et al.*, 2012) properties and by project collaborators to have antioxidant (Harnedy *et al*., 2017), antidiabetic (Harnedy and Fitzgerald, 2013) and cardioprotective

(Harnedy *et al*., 2015) activity. This species was used to optimise the chosen screening protocol for all hydrolysates. A multi-cellular screening protocol was adopted in order to gain as much insight into the impact of hydrolysates on the immune response as possible. As part of this protocol the effect of hydrolysates on the viability and cytokine responses of BMDCs and undifferentiated $CD4^+$ T cells was examined, hydrolysates which displayed potent anti-inflammatory effects were then chosen to be brought forward to investigate their impact on T cell subsets; Th1, Th2 and Th17 cells.

BMDCs were chosen for this project as they are widely described as the link between the innate and adaptive immune systems (Steinman, 2007), they play a crucial role in both systems, through capturing and processing antigens, signaling T cell responses and secreting cytokines to initiate an immune response (Banchereau and Steinman, 1998). They were also chosen as they have been associated with chronic inflammation (Lambrecht *et al.,* 1998) and linked to a range of inflammatory diseases including IBD (Hart *et al.,* 2005). Hydrolysates were also screened on undifferentiated $CD4^+$ T cells, this cell type was selected as part of this project as they are the naïve precursor to T helper cell subsets and provide crucial information on the hydrolysates potential to modulate T cells (Barrat *et al.*, 2002). PP hydrolysates displayed potent anti-inflammatory effects on both BMDCs and undifferentiated CD4+ T cells, successfully suppressing the cytokine secretion of a number of pro-inflammatory cytokines such TNFα, IFNγ, IL-12p70, IL-12p40 and IL-6 and promoting the secretion of anti-inflammatory IL-10 and IL-13. Hydrolysates chosen from this cohort were then studied on T cell subsets and it was found that PP hydrolysates were capable of reducing IFNγ secretion by Th1 cells and IL-17 secretion by Th17 cells.

PP hydrolysates appeared promising as potential candidates for *in vivo* model, however it was discovered that due to a number of variances in their harvest procedures which impacted their ability to modulate the immune response they were no longer a commercially viable option. PP for consumption is harvested both from the wild and in cultivation in aquaculture farms on the west coast of Ireland. Hydrolysates were found to be from starting material obtained from both wild and farmed sources. When the results of the *in vitro* screening of these hydrolysates were analaysed it was found that starting

material of PP obtained in the wild displayed more potent anti-inflammatory activity than its farmed counterpart. It was also discovered that starting material had been harvested at different times of the year. Through a comparison study on the *in vitro* data generated using these hydrolysates it was also found that the time of year affects the ability of hydrolysates to modulate cytokine secretion. Hydrolysates harvested earlier in the year (April – June) successfully suppressed pro-inflammatory cytokines with little impact on anti-inflammatory IL-10, whereas hydrolysates harvested later in the year (October) displayed the ability to promote IL-10, but not suppress pro-inflammatory cytokines. Due to these variations it was decided to no longer continue investigating PP as a potential source of commercially viable functional ingredients.

Following the discovery of the differences in bioactivity seen in PP harvested at different times of the year and in different places, it was clear that the starting material before hydrolysis was crucial to the bioactivity displayed by the resultant hydrolysates. In seaweed these differences in starting material may be due to sunlight exposure, indeed differences in antioxidant bioactive compounds in seaweed due to seasonal affected sunlight exposure have previously been reported (Sampath-Wiley *et al.*, 2008) alongside variations in minerals presents (Riget *et al*., 1995). As a result, it was decided that fish such as blue whiting and boarfish, may be better options for commercially viable hydrolysates. Fish have less seasonal variations compared to seaweed because of the strict fishing European Common Fisheries Policy regulations Irish fishermen must adhere to. Due to Ireland being an EU member state, its fishing industry is tightly regulated, with specific fishing seasons and strict fishing quotas in place (Daw and Gray, 2005). This means boarfish and blue whiting are only caught at specific times of year, from usually the same fishing grounds, unlike PP which is harvested for 7 months of the year, in many locations across the west and north coasts of Ireland. Fish are also less affected by sunlight exposure, unlike seaweed which photosynthesize and therefore are less impacted by the seasonal changes in sunlight.

Capros aper, or boarfish, was chosen as a more controlled source of marine protein hydrolysates with potential for anti-inflammatory activity. This species of fish was chosen as proteins isolated from it have been reported to have antihypertensive (Hayes *et al.*, 2016), antidiabetic (Parthsarathy *et al*., 2016) and antioxidant (Ojha *et al.*, 2016) properties. It was also selected as in Ireland it is considered a low value or under utilised fish, mainly ending up in animal feed, and is often discarded by fishermen. Due to changes in the European Common Fisheries Policy (ECFP) the tradition of discarding catches of low value fish back into the ocean will be banned from 2019 and all fish caught must be landed in a new reform known as the Landing Obligation (Dorothee *et al*., 2016). This change in EU law means a large increase in the number of boarfish caught in Ireland is expected and rather than fish end up in landfill, their potential as sources of bioactive peptides is being investigated (Hayes *et al*., 2016).

Protein was isolated from minced boarfish and hydrolysates generated using enzymatic hydrolysis by collaborators in the University of Limerick. Boarfish hydrolysates then underwent the same screening protocol as PP hydrolysates, with their effects on the viability and cytokine secretion of BMDCs and undifferentiated $CD4^+$ T cells investigated. Boarfish hydrolysates decreased secretion of TNFα and IL-12p40 by BMDCs and IL-17 and IFNγ by undifferentiated $CD4^+$ T cells, displaying significant anti-inflammatory effects in doing so. Selected hydrolysates were then brought forward and screened on T helper cell subsets; Th1, Th2 and Th17 cells where a hydrolysate was found to significantly decreased secretion of IFNγ by the pro-inflammatory Th1 cell subset. However, upon discussion with collaborators it was decided to focus on blue whiting hydrolysates, as this species hydrolysates displayed more commercially interesting activity than that of the boarfish hydrolysates. The two species of fish underwent the same methods of hydrolysis and screening for bioactivity, with blue whiting having a much larger number of hydrolysates identified as potential candidates than boarfish.

Whilst no PP or boarfish hydrolysates were chosen to be brought forward into *in vivo* models, the work detailed in Chapter 3 and Chapter 4 of this thesis demonstrates for the first time the ability of hydrolysates from these species to modulate the cytokine secretion by key immune cells, BMDCs and T cells and their impact on the viability of these

cells. The effect of harvest time and whether wild or cultivated on PP hydrolysates ability to modulate cytokine secretion of immune cells was also reported for the first time, this work compliments Harnedy *et al.* (2014) who reported differences in the antioxidant and antidiabetic properties of hydrolysates harvest at different times and in different places.

Following the screening of both PP and boarfish hydrolysates it was clear that Irish marine species are a rich source of anti-inflammatory protein hydrolysates, whether commercially viable or not. This led the project to investigate another fish commonly caught off the west coast of Ireland and landed in Killybegs, blue whiting, in the search for commercially viable anti-inflammatory hydrolysates. Blue whiting is similar to boarfish as it is considered a low value or under utilised fish (Geirsdottir *et al.,* 2011), it is not commonly eaten in Ireland and will also be subject to large increases in numbers being landed due to the ECFP reforms (Prellezo *et al*., 2016). Protein hydrolysates generated from blue whiting have previously been shown to be anti-proliferative (Picot *et al.*, 2006), appetite reducing (Cuddenec *et al*., 2012), antihypertensive (Nakajima *et al*., 2009) and also shown by project collaborators to be antidiabetic (Harnedy *et al*., 2018). The effects of blue whiting hydrolysates on the viability and inflammatory response of immune cells has not yet been reported on. The research in Chapter 5 of this thesis aimed to investigate the effect of hydrolysates from this species on the viability and cytokine secretion of BMDCs and T cells.

Blue whiting protein hydrolysates were shown to have no negative impact on the cell viability of BMDCs or undifferentiated $CD4^+$ T cells. Hydrolysates successfully modulated the secretion of pro-inflammatory cytokines by both BMDCs and naïve undifferentiated $CD4^+$ T cells, decreasing levels of IL-6, TNFα, IL-12p40, IL-17, IL-2 and IFNγ whilst increasing anti-inflammatory IL-10 and IL-4. Candidate hydrolysates which were then screened in T helper cell subsets also successfully modulated the cytokine response of T helper cells which play key roles in inflammatory disease. In Th1 cells, hydrolysates 26 (H26) and 27 (H27) decreased IFNγ secretion whilst also reducing secretion of IL-17 from Th17 cells. These hydrolysates displayed potent anti-inflammatory activity in all cell types, showing dual activity in reducing pro-inflammatory TNFα, IL-6, IL-12p40 secretion whilst increasing

anti-inflammatory IL-10 levels by BMDCs. This dual activity was also seen in undifferentiated $CD4^+$ T cells where H26 and H27 decreased IFNγ secretion whilst promoting Th2 associated IL-4 levels. As a result of the potent anti-inflammatory properties shown by these hydrolysates and upon consultation with collaborators, it was decided to bring these hydrolysates forward to be further studied.

H26 and H27 were then fractionated into smaller peptide sequences using HPLC fractionation into four fractions each by collaborators in the University of Limerick, before being screened using the same protocol optimised in Chapter 3 on PP hydrolysates and used throughout this work. Two fractions, both from H26, were identified to be potentially brought forward for *in vivo* work due to their anti-inflammatory activity displayed in BMDCs, undifferentiated $CD4^+$ T cells, Th1 cells and Th17 cells. However, following consultation with collaborators in the University of Limerick, it was discovered that F4 of H26 was not a viable option for *in vivo* work as it could not be commercially scaled up. F1 was chosen as it was a viable option for scale up and it displayed potent anti-inflammatory activity better than that of its hydrolysate H26.

Chapter 6 describes the effect of F1 treatment on DSS-induced colitis in an *in vivo* murine model. F1 successfully reduced the clinical and pathological symptoms of DSS-induced colitis in mice within 7 days of treatment back to that of the healthy control mice. H26 did not reduce all clinical and pathological symptoms of the disease in DSS-induced colitis within the 7 days of treatment, and signs of the disease were still present at day 14 compared to healthy control mice. The results in Chapter 5 describe F1's ability *in vitro* to reduce pro-inflammatory cytokines secreted by BMDC's such as TNFα and IL-1β, by undifferentiated $CD4^+$ T cells such as IFNγ and IL-6 and by Th1 cells such as IFNγ. These results were also seen *in vivo* where mRNA expression of IL-6, IFNγ and IL-1β was reduced to healthy control levels by F1 and cytokine secretion by splenocytes with reductions in IL-6, IL-1β, TNFα and IFNγ all seen in unstimulated and stimulated splenocytes. This correlation of anti-inflammatory effects from *in vitro* work to *in vivo* models shows the efficacy of F1 and its ability to modulate inflammation. Furthermore it also demonstrated the validity of our screening platform and showed that the effect of hydrolysates in vitro translated into their effects in vivo. F1 also displayed the ability, both *in vitro* and *in*

vivo to suppress multiple pro-inflammatory cytokines simultaneously. Current research in therapeutics for IBD has focused on compounds which target multiple cytokines (Vuitton *et al.*, 2013), as oppose to common therapies, such as the anti-TNFα antibody infliximab, which target, and completely inhibit, single cytokines (Lim and Hanauer, 2004). This multi-cytokine approach has been found to be advantageous over single cytokine inhibition in IBD, due to the complexity of the disease. Despite the number of biological agents currently on the market for the treatment of IBD, a significant proportion of patients do not respond to treatment or lose response over time (Yanai and Hanauer, 2011). The percentage of nonresponders varies depending on biological agent and also for the type of IBD being treated. Clinical trials of infliximab had nonresponse in 28% of patients with Crohn's disease (Ricart *et al*., 2001). Therapeutic trials measure 'absolute benefit' which refers to the percentage difference between the effectiveness of treatment in patients receiving active compound versus the placebo. Anti-TNF compounds, including infliximab and adalimumab, in IBD treatment have an absolute benefit rating of approximately 30% (Targan, 2006). Infliximab has an efficacy of induction of remission at only 25-30% with this number dropping below 20% in long term use, with the best long term biologics only able to maintain 25% of patients over 1 year (Sann *et al*., 2013). This low efficacy rating highlights the need for research into novel therapeutics for IBD.

IBD is a collection of idiopathic diseases of the gastrointestinal tract which can present similar symptoms in individuals (Prenzel and Uhlig, 2009). As a result of this, individuals are commonly misdiagnosed with either Crohn's Disease (CD) or Ulcerative Colitis (UC) or diagnosed as having indeterminate colitis (IC) as specific disease symptoms are indistinguishable in the patient. Individuals with IBD can also respond differently to treatments, even when diagnosed with the correct disorder or can experience a loss of response to a therapy which previously worked (Yanai and Hanauer, 2011). Multiple pro-inflammatory cytokines have been implicated in the onset and pathogenesis of IBD (Strober and Fuss, 2011), hence, a therapeutic which can target multiple cytokines such as F1 would be advantageous in the treatment of the disease.

Before F1 was examined *in vivo*, its effect on the wider immune response was analysed. F1's effect on another key immune cell, macrophage cells, was

investigated and it was found that F1 had little effect on the cytokine secretion or viability of macrophage cells. Macrophage cells are often described as the first line of defence, being the first cell type many pathogens will meet during infection (Dunn *et al.*, 1987). Their ability to provide this protection to individuals is key to maintaining an adequate immune response, and therapeutics which inhibit this response may leave the individual immunosuppressed (Stobbo, 1977). Hence F1's ability to modulate various immune cell responses such as BMDCs and T cells, but also maintain a complete macrophage cytokine response is indicative of the overall effect it may have on the immune system and a lack of immunosuppressive side effects. This extra in-depth screening of F1 on the broader immune response further demonstrated F1's potential as an anti-inflammatory therapeutic which preserved the immune response of the individual.

The current commonly used multi-cytokine targeted therapeutic Tofacitinib (Xeljanz ™) in the treatment of IBD has been shown to have systemic immunosuppressive activity which leaves the individual susceptible to a range of infection (Löwenberg and D'Haens, 2015). This has also been reported in single cytokine inhibitors such as anti-TNF therapy which has seen a significant number of patients develop infectious diseases including tuberculosis (TB) (Dixon *et al*., 2009). A significant number of anti-TNF treated patients also developed severe opportunistic non-TB related infections due to the treatment (Salmon-Céron *et al*., 2011). This severe immunosuppression has been shown to be especially apparent in the first 6 months of treatment but is a risk throughout the course of the therapy (Galloway *et al*., 2010). The ability of F1 to maintain host protection against infection in the individual through preservation of macrophage cytokine secretion is advantageous over current therapies.

Another advantage F1, and the hydrolysates produced in this project in general, have on current therapeutics is their cost. Biological therapeutics have traditionally been expensive both to develop and manufacture. The estimated cost of antibody development from idea to phase III clinical trial is estimated at $1 billion, with many failing to reach the consumer market (Nelson *et al*, 2010). Protein hydrolysates and fractions generated from marine sources, such as F1, have the advantage of being naturally occurring and in abundance. Another advantage of F1 is its source; blue whiting. Blue whiting is considered

a low value and under utilised fish and as a result is often discarded or considered waste. This project provides potential commercial value to this waste stream. This waste stream is also set to see a large rise in its amount of waste due to the ECFP reform the Landing Obligation (García *et al.*, 2017), meaning adding value to this stream would be of great benefit to the fishing industry.

As F1 is isolated from a food source it has the potential to be used as an anti-inflammatory functional food ingredient. Anti-inflammatory diets and the use of functional foods with anti-inflammatory activity have been reported as useful alternative or complimentary therapies for IBD (Al Mijan and Lim, 2018). Probiotics, such as *Lactobaccilus sp*., have traditionally been used in the promotion of gut health (Lee *et al*., 2009) and have been successful in restoring the microbiota balance of moderate IBD patients (Currò *et al.*, 2017). Plant extracts have also been shown to display strong therapeutic effects in the treatment of IBD. Extracts from the Chinese mushroom, *Coriolus versicolor*, have displayed the ability to decrease pro-inflammatory cytokines TNFα, IFNγ, IL-1β and IL-6 in a murine model of colitis (Lim, 2011). Bioactive peptides from food sources have also displayed potent anti-inflammatory effects in models of IBD, with peptides isolated from Salmon shown to reduce clinical and pathological symptoms of disease in a DSS-induced colitis model (Grimstad *et al*., 2013). The current trend in research into food as sources of anti-inflammatory therapeutics and functional ingredients specifically for treatment of IBD has been triggered by a lack of efficacy in current IBD therapies (Kaplan and Ng, 2017). Natural sources, such as food, of bioactive substances offers safe and effective anti-inflammatories with fewer known side effects compared to traditional therapies (Grzanna *et al.*, 2005). Dietary intervention and the use of functional ingredients have emerged as promising treatments for IBD in recent years and compounds such as F1 which display potent anti-inflammatory effects both *in vitro* and *in vivo*, offer exciting potential for future IBD therapies.Current immunomodulating functional foods include Wellmune®, a β-glucan from oats and barley, which boosts the immune response and decreases susceptibility to upper respiratory tract infection (Talbot *et al*., 2010). However, there is a lack of food currently on the market which display anti-inflammatory activity.

The aim of this project was to investigate the potential of Irish marine organisms as sources of anti-inflammatory protein hydrolysates. This research also aimed to uncover a marine protein hydrolysate with the ability to suppress inflammation *in vitro* and *in vivo*. As part of this project Irish marine sources have been shown to be rich sources of anti-inflammatory protein hydrolysates and a protein hydrolysate which can potently suppress inflammation was discovered. This hydrolysate was then fractionated, in order to try unlock the bioactive peptide sequence behind this activity. Through these studies, F1 was shown to actively suppress inflammation *in vitro*. This activity was then translated into *in vivo* models, where F1 displayed significant therapeutic properties in the treatment of IBD. This work has discovered a protein hydrolysate fraction with real potential as a therapeutic and functional ingredient for the treatment of inflammatory disease.

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