Immunomodulatory Protein Hydrolysates for the Management of Intestinal Immune Disorders in Infants

A thesis submitted for the degree of Ph. D.

by

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DECLARATION

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy is entirely my own work, that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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TABLE OF CONTENTS

Contents

DECLARATION i
ACKOWLEDGEMENTS ii
TABLE OF CONTENTS iv
LIST OF FIGURES xii
LIST OF TABLES xvi
ABBREVIATIONS xvii
PUBLICATIONS xx
ABSTRACT xxii
GENERAL INTRODUCTION 1
1 INTRODUCTION 2
1.2 Gut Homeostasis and Mucosal Immunity in Infancy 3
1.2.1 Interaction of The Host Immune System and Gut Microbiota 4
1.2.2 Gut Microbiota Dysbiosis and Life Course Disease Risk 6
1.3 Innate and Adaptive Immunity in Healthy Hosts 8
1.3.1 Role of Innate Immunity in Immune Homeostasis 8
1.3.2 Adaptive Immunity 10
1.3.2.1 T-Helper 1 Cells 11
1.3.2.2 T-Helper 2 Cells 12
1.3.2.3 T-Helper 17 Cells 12
1.3.2.4 Regulatory T-Cells 13
1.3.2.5 T-cell Imbalance – Autoimmunity and Allergy 13
1.4 Food Allergy 15
1.4.1 Allergy Begins During Infancy 15
1.4.1.1 Poor ability to digest proteins 16
1.4.1.2 High permeability of the epithelial barrier 16
1.4.1.3 Larger proteins that cross the epithelial barrier promote allergic Th2 response 16
1.4.1.4 Atopic infants can fail to generate immune homeostasis 17
1.4.1.5 Failure to develop Th1/Th2 balance 18
1.4.1.5 Failure to develop Treg Cells 19
1.4.1.6 NEC Recovery in Preterm Infants 19
1.4.2 Early Intervention Necessary for the Generation of Immune Homeostasis and Prevention of Sensitisation 20

1.5 Necrotizing Enterocolitis in Preterm Infants 20
1.5.1 NEC and 'The Cross-Switching Hypothesis' 21
1.5.2 Pro-Inflammatory Cytokines Associated with NEC 23
1.5.2.1 IL-1β 23
1.5.2.2 TNF-α 24
1.5.2.3 IL-6 24
1.5.2.4 IL-17 25
1.5.2.5 IFN-γ 25
1.5.2.6 IL-10 25
1.5.2.7 TGF-β 26
1.5.3 $CD4^+$ T-Cells and NEC Progression 26
1.5.4 Additional Factors Affecting NEC Development 27
1.6 Cow's Milk Protein Hydrolysates for The Management of Intestinal Immune Disorders in Infants 27
1.6.2 Immunomodulatory Protein Hydrolysates and Immune Homeostasis 29
1.7 FHI Approach and thesis outline 30
1.8 Aims and Objectives 34
CHAPTER 2 36
MATERIALS AND METHODS 36
2.1 MATERIALS 37
2.2 PREPARATION OF THE MILK PROTEIN HYDROLYSATES 41
2.2.1 MILK PROTEIN HYDROLYSATE RESUSPENSION FOR CELL CULTURE 41
2.3 CELL CULTURE 42
2.3.1 CELL COUNTING 42
2.4 PRIMARY CELL ISOLATION 43
2.4.1 MICE 43
2.4.2 ISOLATION OF $CD4^+$ T-CELLS 43
2.4.2.1 SPLENOCYTE ISOLATION 43
2.4.2.2 $CD4^+$ T-CELL MAGNETIC PARTICLE ISOLATION 43
2.4.2.3 REGULATORY T-CELL ISOLATION 44
2.4.2.4 POLARISATION OF TH1, TH2 AND TH17 $CD4^+$ T-CELLS 45
2.5 MTS VIABILITY ASSAY 45
2.6 ENZYME-LINKED IMMUNO-SORBENT ASSAY (ELISA) 46

2.6.1 BASIC PRINCIPLES OF ELISA 46
2.6.2 CYTOKINE ELISA 47
2.6.3 IL-1β, IFN-γ and IL-2 ELISA 48
2.6.4 TGF-β ELISA 48
2.6.5 IgG1 ELISA 48
2.7 FLOW CYTOMETRY 48
2.7.1 BASIC PRINCIPLES OF FLOW CYTOMETRY 48
2.7.2 CELL SURFACE STAINING 50
2.7.3 STAINING ANTIBODY TITRATION 51
2.7.4 INTRACELLULAR TRANSCRIPTION FACTOR AND CYTOKINE STAINING 51
2.8 RNA ANALYSIS 52
2.8.1 RNA ISOLATION FROM COLONIC TISSUE 52
2.8.2 CDNA SYNTHESIS 52
2.8.3 BASIC PRINCIPLES OF QUANTITATIVE REAL TIME PCR (qPCR) 53
2.8.3.1 TAQMAN® ASSAY 53
2.8.4 QPCR PROTOCOL 54
2.8.5 PCR DATA ANALYSIS 54
2.8.6 DNA PRODUCT ANALYSIS BY GEL ELECTROPHORESIS 55
2.9 IN VIVO MURINE MODELS 55
2.9.1 DEXTRAN SULFATE SODIUM (DSS) INDUCED MODEL OF COLITIS 55
2.9.2 OVA INDUCED MODEL OF ALLERGY 57
2.9.3 COLONIC TISSUE SECTIONING 58
2.9.4 HAEMOTOXYLIN AND EOSIN STAINING 59
2.9.5 SERUM PREPARATION 59
2.10 STATISTICAL ANALYSIS 59
CHAPTER 3 60
ISOLATION AND POLARISATION OF CD4$^+$ T-CELLS IN VITRO 60
3.1 INTRODUCTION 61
3.2 RESULTS 65
3.2.1 ISOLATION AND CONFIRMATION OF CD4$^+$ T-HELPER CELL POPULATION FROM BALB/C MICE. 65
3.2.1.1 EXAMINATION OF CD3 EXPRESSION ON ISOLATED CD4$^+$ T-HELPER CELL IN THE SPLEEN TO DETERMINE POPULATION PURITY. 65

3.2.1.2 ANTIBODIES DIRECTED TO CD3 AND CD28 ARE ESSENTIAL FOR IN VITRO ACTIVATION OF CD4+ CELLS. *66*

3.2.2 ACTIVATION/POLARISATION AND CONFIRMATION OF CD4$^+$ T-HELPER CELL SUBTYPES FROM BALB/C MICE. **67**

3.2.2.1 TIME COURSE ANALYSIS FOR THE OPTIMISATION OF TH1, TH2, TH17 AND REGULATORY CD4+ T-CELL POLARISATION IN VITRO – VIABILTY AND CYTOKINE SECRETION *67*

3.2.2.2 INTRACELLULAR MARKER STAINING FOR THE OPTIMISATION OF TH1 AND TH2 T-CELL POLARISATION IN VITRO *69*

3.2.3 FURTHER OPTIMISATION OF REGULATORY T-CELL POLARISATION IN VITRO. **69**

3.2.3.1 TIME COURSE ANALYSIS OF A TGF-β DOSE RESPONSE FOR THE OPTIMISATION OF REGULATORY CD4+ T-CELL POLARISATION IN VITRO – CYTOKINE SECRETION AND INTRACELLULAR STAINING 70

3.2.3.2 REGULATORY T-CELL ISOLATION KIT COMPARISON *71*

3.2.3.3 CD25 EXPRESSION ANALYSIS IN REGULATORY T-CELLS *72*

3.2.3.4 INTRACELLULAR MARKER STAINING FOR THE OPTIMISATION OF REGULATORY T-CELL POLARISATION IN VITRO *73*

3.2.3.5 COMPARISON OF IN VITRO CYTOKINE PROFILES BETWEEN EFFECTOR T-CELL SUBSETS AND OPTIMISED REGULATORY T-CELL ISOLATION KIT *74*

3.3 DISCUSSION **93**

CHAPTER 4 ..... **100**

IMMUNOMODULATORY PROPERTIES OF PROTEIN HYDROLYSATES FOR APPLICATION IN INFANT ALLERGIES **100**

4.1 INTRODUCTION **101**

4.2 RESULTS **106**

4.2.1 ASSESSMENT OF ALLERGY SUPPRESSING ABILITY OF FIRST-ROUND HYDROLYSATES IN VITRO. **106**

4.2.1.1 THE DOSES OF FIRST-ROUND WHEY AND CASEIN PROTEIN HYDROLYSATES USED HAVE NO SIGNIFICANT EFFECT ON CELL VIABILITY. *106*

4.2.1.2 FIRST-ROUND HYDROLYSATES SUPPRESS THE CYTOKINES REQUIRED TO DRIVE AN ALLERGIC TH2 RESPONSE FROM UNDIFFERENTIATED T-CELLS IN VITRO. *106*

4.2.1.3 FIRST-ROUND HYDROLYSATES CAN PREVENT DIFFERENTIATION OF T-HELPER 2 CELL SUBSET IN VITRO. *107*

4.2.1.4 ASSESSMENT OF FIRST-ROUND HYDROLYSATES IN A T-HELPER CELL 1 SUBSET IN VITRO. *108*

4.2.2 ASSESSMENT OF ALLERGY SUPPRESSING ABILITY OF SECOND-ROUND REGENERATED HYDROLYSATES IN VITRO. ... **109**

4.2.2.1 THE DOSES OF SECOND-ROUND REGENERATED HYDROLYSATE USED HAVE NO SIGNIFICANT AFFECT ON CELL VIABILITY. *109*

4.2.2.2 SECOND-ROUND REGENERATED HYDROLYSATES OF UL-2-40/41/42/47 DISPLAY SIMILAR ACTIVITY AS PARENT SAMPLES IN UNDIFFERENTIATED CD4$^+$ T-CELLS IN VITRO. *110*

4.2.2.3 SECOND-ROUND REGENERATED HYDROLYSATES OF UL-2-40/41/42/47 DISPLAY ALLERGY SUPPRESSING ACTIVITY, SIMILAR TO PARENT SAMPLES IN TH2 CELLS IN VITRO. *111*

4.2.3 ASSESSMENT OF ALLERGY SUPPRESSING ABILITY OF THIRD-ROUND OPTIMISED HYDROLYSATES IN VITRO. **111**

4.2.3.1 THE DOSES OF THIRD-ROUND OPTIMISED UL-2-42 and UL-2-47 PROTEIN HYDROLYSATES USED ARE NOT TOXIC TO CELL VIABILITY. *113*

4.2.3.2 THIRD-ROUND UL-2-42 AND REGENERATED AND OPTIMISED HYDROLYSATES DISPLAY ALLERGY SUPPRESSING PROPERTIES IN UNDIFFERENTIATED T CELLS AND TH2 CELLS IN VITRO. *113*

4.2.3.3 THIRD-ROUND UL-2-47 AND REGENERATED AND OPTIMISED HYDROLYSATES DO NOT DISPLAY ALLERGY SUPPRESSING PROPERTIES IN UNDIFFERENTIATED T CELLS AND TH2 CELLS IN VITRO. *114*

4.2.3.4 ASSESSMENT OF THIRD-ROUND HYDROLYSATES IN A REGULATORY T-CELL SUBSET IN VITRO. *115*

4.2.3.5 OPTIMISED HYDROLYSATE, UL-2-147, DOSE DEPENDENTLY INHIBITS THE ALLERGIC TH2 RESPONSE IN VITRO. *116*

4.2.4 UPSCALED UL-2-147 SUPPRESSES THE CHARACTERISTIC TH2 CYTOKINES IN VITRO, CONFIRMING BIOACTIVITY FOR IN VIVO OVA MURINE MODEL. **117**

4.2.5 OVA-INDUCED MODEL OF FOOD ALLERGY **118**

4.2.5.1 CLINICAL ASSESSMENT OF OVA-INDUCED ALLERGY *119*

4.2.5.2 CYTOKINE SECRETION FROM EX VIVO SPLENOCYTE CULTURE IN OVA-INDUCED ALLERGY *120*

4.2.5.3 CYTOKINE EXPRESSION IN COLON IN OVA-INDUCED ALLERGY *121*

4.2.5.4 IMMUNOGLOBULIN ANTIBODY LEVELS IN SERUM IN OVA-INDUCED ALLERGY IN VIVO. *121*

4.2.6 EFFECT OF UL-2-147 WHEY HYDROLYSATE TREATMENT IN A MOUSE MODEL OF ALLERGY. **122**

4.2.6.1 CLINICAL ASSESSMENT OF OVA-INDUCED ALLERGIC MICE TREATED WITH UL-2-147 ..... *122*

4.2.6.2 CYTOKINE SECRETION FROM EX VIVO SPLENOCYTE CULTURE IN OVA-INDUCED ALLERGIC MICE TREATED WITH UL-2-147 ..... *124*

4.2.6.3 CYTOKINE EXPRESSION IN COLON IN OVA-INDUCED ALLERGIC MICE TREATED WITH UL-2-147 ..... *126*

4.2.6.4 IMMUNOGLOBULIN ANTIBODY LEVELS IN SERUM IN OVA-INDUCED ALLERGIC MICE TREATED WITH UL-2-147 126

4.2.7 ASSESSMENT OF TOLERISING ABILITY OF FIRST-ROUND HYDROLYSATES IN VITRO. 126

4.2.7.1 FIRST-ROUND HYDROLYSATES INDUCE THE CYTOKINE REQUIRED TO DRIVE A TOLEROGENIC TREG RESPONSE FROM UNDIFFERENTIATED T-CELLS IN VITRO. 127

4.2.7.2 FIRST-ROUND HYDROLYSATES CAN MODULATE DIFFERENTIATION OF T HELPER 2 T CELL SUBSET IN VITRO. 127

4.2.7.3 FIRST-ROUND HYDROLYSATES CAN DRIVE DIFFERENTIATION OF REGULATORY T CELL SUBSET IN VITRO. 128

4.2.7.4 FIRST-ROUND HYDROLYSATES DO NOT AFFECT POLARISATION OF T HELPER 1 T CELL SUBSET IN VITRO. 128

4.2.8 ASSESSMENT OF TOLERISING ABILITY OF SECOND & THIRD-ROUND REGENERATED AND OPTIMISED HYDROLYSATES IN VITRO. 129

4.2.8.1 THE DOSES OF SECOND & THIRD-ROUND UL-2-20 REGENERATED AND OPTIMISED HYDROLYSATES USED HAVE NO SIGNIFICANT AFFECT ON CELL VIABILITY. 129

4.2.8.2 SECOND & THIRD-ROUND UL-2-20 REGENERATED AND OPTIMISED HYDROLYSATES DISPLAY SIMILAR ACTIVITY AS PARENT SAMPLE IN UNDIFFERENTIATED $CD4^+$ T-CELLS IN VITRO. 130

4.2.8.3 SECOND & THIRD-ROUND UL-2-20 REGENERATED AND OPTIMISED HYDROLYSATES DRIVE DIFFERENTIATION OF REGULATORY T CELL SUBSET AND INHIBIT DIFFERENTIATION OF T HELPER 2 T CELL SUBSET IN VITRO. 131

4.3 DISCUSSION 169

CHAPTER 5 185

IMMUNOMODULATORY PROPERTIES OF PROTEIN HYDROLYSATES FOR APPLICATION IN INFANT NECROTISING ENTEROCOLITIS 185

5.1 INTRODUCTION 186

5.2 RESULTS 190

5.2.1 ASSESSMENT OF ANTI-INFLAMMATORY ACTIVITY OF FIRST-ROUND HYDROLYSATES IN VITRO. 190

5.2.1.1 THE DOSES OF FIRST-ROUND WHEY AND CASEIN PROTEIN HYDROLYSATES USED HAVE NO SIGNIFICANT AFFECT ON CELL VIABILITY. 190

5.2.1.2 FIRST-ROUND HYDROLYSATES SUPPRESS THE CYTOKINES REQUIRED TO DRIVE AN INFLAMMATORY TH1 RESPONSE FROM UNDIFFERENTIATED T-CELLS IN VITRO. 191

5.2.1.3 FIRST-ROUND HYDROLYSATES CAN PREVENT DIFFERENTIATION OF T-HELPER 1 CELL SUBSET BUT DO NOT SUPPRESS DIFFERENTIATION OF A T-HELPER 17 SUBTYPE IN VITRO. 191

5.2.2 ASSESSMENT OF ANTI-INFLAMMATORY ABILITY OF SECOND-ROUND REGENERATED HYDROLYSATES IN VITRO. ... 192

5.2.2.1 THE DOSES OF SECOND-ROUND WHEY AND CASEIN PROTEIN REGENERATED HYDROLYSATES USED HAVE NO SIGNIFICANT AFFECT ON CELL VIABILITY. 193

5.2.2.2 SECOND-ROUND REGENERATED HYDROLYSATES DISPLAY SIMILAR ACTIVITY AS PARENT SAMPLES IN UNDIFFERENTIATED $CD4^+$ T-CELLS AND TH1 CELLS IN VITRO. 193

5.2.3 ASSESSMENT OF ANTI-INFLAMMATORY ACTIVITY OF THIRD-ROUND OPTIMISED HYDROLYSATES IN VITRO. 194

5.2.3.1 THE DOSES OF THIRD-ROUND OPTIMISED UL-2-34 AND UL-2-35 HYDROLYSATES USED HAVE NO SIGNIFICANT AFFECT ON CELL VIABILITY. 195

5.2.3.2 THIRD-ROUND UL-2-34 & UL-2-35, REGENERATED AND OPTIMISED HYDROLYSATES DISPLAY CONSISTENT ANTI-INFLAMMATORY PROPERTIES IN UNDIFFERENTIATED T CELLS IN VITRO. 195

5.2.3.3 THIRD-ROUND UL-2-34 & UL-2-35, REGENERATED AND OPTIMISED HYDROLYSATES DISPLAY CONSISTENT ANTI-INFLAMMATORY PROPERTIES IN TH1 CELLS IN VITRO. 196

5.2.3.4 UL-2-34 AND UL-2-132 DOSE DEPENDENTLY REDUCE IFN-γ SECRETION FROM TH1 CELLS. 197

4.2.3.5 UPSCALED UL-2-34 & UL-2-132 SUPPRESS THE CHARACTERISTIC TH1 CYTOKINE IN VITRO, CONFIRMING BIOACTIVITY PRIOR TO IN VIVO DSS MURINE MODEL. 198

5.2.4 DSS-INDUCED MODEL OF COLITIS 199

5.2.4.1 CLINICAL ASSESSMENT OF DSS-INDUCED COLITIS 199

5.2.4.2 CYTOKINE EXPRESSION IN COLON IN DSS-INDUCED COLITIS 201

5.2.5 EFFECT OF UL-2-34 & UL-2-132 CASEIN HYDROLYSATE TREATMENTS IN A MOUSE MODEL OF COLITIS. 201

5.2.5.1 CLINICAL ASSESSMENT OF DSS-INDUCED MICE TREATED WITH UL-2-34 & UL-2-132 201

5.2.5.2 CYTOKINE EXPRESSION IN COLON IN DSS-INDUCED MICE TREATED WITH UL-2-34 & UL-2-132 203

5.2.5.3 CYTOKINE SECRETION FROM UNSTIMULATED SPLENOCYTES OF DSS-INDUCED MICE TREATED WITH UL-2-34 & UL-2-132 204

5.2.5.4 CYTOKINE SECRETION FROM STIMULATED SPLENOCYTES OF DSS-INDUCED MICE TREATED WITH UL-2-34 & UL-2-132 205

5.3 DISCUSSION 229
CHAPTER 6 242
GENERAL DISCUSSION 242
6.1 GENERAL DISCUSSION 243
CHAPTER 7 259
BIBLIOGRAPHY 259
8.1 APPENDICES A.1
Appendix A - Media and Buffers A.1
Appendix B - Chapter 4 Additional Data B.1
Appendix C - Primer Sequences and PCR Gels C.1

LIST OF FIGURES

Figure	Name	Page
1.1	Classical $CD4^+$ T-cell differentiation	11
1.2	Overview of the cellular mechanisms during sensitisation and allergic reaction	17
1.3	Diagrammatic representation of the outline of this project	33
2.1	Schematic representation of sandwich ELISA	47
2.2	Basic outline of a flow cytometer	49
3.1.1	Overview of the three signals required for the activation and polarisation of undifferentiated $CD4^+$ T-cells	62
3.1	CD3 and CD4 surface staining on $CD4^+$ T-cells	75
3.2	Comparison of viability of stimulated and unstimulated T-cells at 24 and 72 hours	76
3.3	Comparison of cytokine secretion from stimulated and unstimulated T-cells at 24 and 72 hours	77
3.4	Comparison of CD25 expression on stimulated and unstimulated T-cells at 24 hours	78
3.5	Comparison of CD25 expression on stimulated and unstimulated T-cells at 72 hours	79
3.6	Comparison of the viability of undifferentiated, Th1, Th2 and Treg cells at 24 hours, 72 hours and 96 hours	80
3.7	Time course analysis of cytokine secretion from undifferentiated $CD4^+$ T-cells, Th1, Th2 and Treg cells	81
3.8	Time course analysis of cytokine secretion from Th17 cells	82
3.9	Intracellular staining for Th1 and Th2 cells	83
3.10	Time course analysis of cytokine secretion from Treg cells cultured with a range of TGF-β concentrations	84
3.11	FoxP3 transcription factor staining for Regulatory T-cells over a time course	85
3.12	CD4, CD25 and FoxP3 antibodies were titrated to optimise concentrations used for FoxP3 staining protocol	86
3.13	Comparison of cytokine secretion from Treg cells isolated from two Treg isolation kits	87
3.14	CD25 expression of splenocytes pre-isolation	88
3.15	CD25 expression of isolated CD4 Tcells	89
3.16	CD25 expression of isolated Regulatory T-cells	90
3.17	Intracellular transcription factor staining for Treg cells	91
3.18	Cytokine secretion from undifferentiated CD4 T-cells, Th1, Th2, TH17 and Treg cells	92
4.1	The concentrations of protein hydrolysates used do not affect the viability of undifferentiated T-cells	132
4.2	First-round hydrolysates suppress the cytokines required to drive an allergic Th2 response from undifferentiated T-cell *in vitro*	133

4.3	First round hydrolysates can prevent differentiation of Th2 cells *in vitro* by suppressing the characteristic Th2 cytokines	134
4.4	Assessment of first-round hydrolysates in a Th1 subset *in vitro*	135
4.5	The concentration of protein hydrolysate regenerates used do not affect the viability of undifferentiated T-cells	136
4.6	Second-round hydrolysates suppress the cytokines required to drive an allergic Th2 response from undifferentiated T-cells *in vitro*	137
4.7	Second-round hydrolysates and regenerates can prevent differentiation of Th2 cells *in vitro* by suppressing the characteristic Th2 cytokines	138
4.8	The concentrations of optimised protein hydrolysates from UL-2-42 and UL-2-47 used do not affect the viability of undifferentiated T-cells	139
4.9	Third-round UL-2-42 hydrolysate, regenerate and optimised hydrolysates suppress the cytokines required to drive an allergic Th2 response from undifferentiated T-cells *in vitro*	140
4.10	Third-round UL-2-42 hydrolysate, regenerate and optimised hydrolysates can prevent differentiation of Th2 cells *in vitro* by suppressing the characteristic Th2 cytokines	141
4.11	Third-round UL-2-47 hydrolysate, regenerate and optimised hydrolysates suppress the cytokines required to drive an allergic Th2 response from undifferentiated T-cells *in vitro*	142
4.12	Third-round UL-2-47 hydrolysate, regenerated and optimised hydrolysates can prevent differentiation of Th2 cells *in vitro* by suppressing the characteristic Th2 cytokines	143
4.13	UL-2-42 and UL-2-147 do not drive or inhibit Treg cells *in vitro*	144
4.14	UL-2-147 inhibits the allergic Th2 response in dose dependent manner *in vitro*	145
4.15	Bioactivity of upscaled UL-2-147 was confirmed for *in vivo* OVA murine model, suppresses the characteristic Th2 cytokines	146
4.16	Disease associated symptoms in the OVA allergy model	147
4.17	Disease associated symptoms in the OVA allergy model	148
4.18	Disease associated symptoms in the OVA allergy model	149
4.19	Cytokine secretion from *ex vivo* splenocyte culture in OVA-induced allergy *in vivo*	150
4.20	mRNA expression of the Th2 dominant cytokine genes in colon of OVA-sensitised mice	151
4.21	Immunoglobulin antibody levels in serum in OVA-induced allergy *in vivo*	152
4.22	Disease associated symptoms (body weight change) in the OVA allergy model	153
4.23	Disease associated symptoms (DDAI) in the OVA allergy model	154
4.24	Effect of UL-2-147 hydrolysate on disease associated symptoms (histopathological changes of the colon tissue) in the OVA allergy model	155
4.25	UL-2-147 attenuates the Th2 dominant cytokine spike in OVA stimulated splenocytes from OVA-sensitised mice	156

4.26	UL-2-147 attenuates the Th2 dominant cytokine spike in CD3/CD28 stimulated splenocytes from OVA-sensitised mice	157
4.27	UL-2-147 attenuates the Th2 dominant cytokine spike in unstimulated splenocytes from OVA-sensitised mice	158
4.28	UL-2-147 and WPC80 inhibit the mRNA expression of the Th2 dominant cytokine genes in colons of OVA-sensitised mice	159
4.29	IgG1 levels from serum of OVA-sensitised mice treated with UL-2-147	160
4.30	First-round hydrolysates modulate secretion of cytokines associated with Th2 and Treg cells from undifferentiated T-cells *in vitro*	161
4.31	First-round hydrolysates modulate the allergic response Th2 response *in vitro*	162
4.32	First round UL-2-20 drives a Treg response *in vitro*	163
4.33	First round UL-2-20 has no effect on Th1 polarisation *in vitro*	164
4.34	The concentration of optimised protein hydrolysates from UL-2-20 used do not affect the viability of undifferentiated T-cells	165
4.35	Third-round UL-2-20 hydrolysate, regenerate and optimised hydrolysates modulate cytokine secretion from undifferentiated T-cells *in vitro*	166
4.36	Third-round UL-2-195 induces Treg cells *in vitro*	167
4.37	The effect of third-round UL-2-20 hydrolysate, regenerated and optimised hydrolysates on Th2 cells *in vitro*	168
5.1	The concentrations of protein hydrolysates used do not affect the viability of undifferentiated T-cells	206
5.2	First-round hydrolysates suppress the cytokine required to drive an inflammatory Th1 response from undifferentiated Tcells *in vitro*	207
5.3	First-round hydrolysates suppress the cytokines required to drive an inflammatory Th17 response from undifferentiated T-cells *in vitro*	208
5.4	First-round hydrolysates can prevent differentiation of Th1 cells *in vitro* by suppressing the characteristic Th1 cytokines	209
5.5	First-round hydrolysates promote differentiation of Th17 cells *in vitro* by inducing the characteristic Th17 cytokine	210
5.6	The concentration of protein hydrolysates used do not affect the viability of undifferentiated T-cells	211
5.7	Second-round regenerated hydrolysates suppress the Th1 cytokines from undifferentiated and Th1 cells *in vitro*	212
5.8	The concentrations of optimised protein hydrolysates from UL-2-34 and Ul-2-35 do not affect the viability of undifferentiated T-cells	213
5.9	Third-round UL-2-34/35, regenerated and optimised hydrolysates suppress the cytokine required to drive an inflammatory Th1 response from undifferentiated T-cells *in vitro*	214
5.10	Third-round UL-2-34/35, regenerated and optimised hydrolysates prevent differentiation of a Th1 subset *in vitro*	215
5.11	UL-2-34 and UL-2-132 inhibit the inflammatory Th1 response in dose dependent manner *in vitro*	216
5.12	Upscaled UL-2-34 and UL-2-132 continue to inhibit the Th1 response *in vitro*	217

5.13	Disease associated symptoms in the DSS colitis model	218
5.14	Disease associated symptoms in the DSS colitis model	219
5.15	Disease associated symptoms in the DSS colitis model continued	220
5.16	Cytokine mRNA expression in a mouse model of colitis	221
5.17	Disease associated symptoms (body weight change) in the DSS colitis model treated with UL-2-34 and U-2-132 hydrolysates	222
5.18	Disease associated symptoms (DDAI) in the DSS colitis model treated with UL-2-34 and UL-2-132 hydrolysates	223
5.19	Disease associated symptoms (colon length and weight) in the DSS colitis model treated with UL-2-34 and UL-2-132 hydrolysates	224
5.20	Effect of UL-2-34 and UL-2-132 hydrolysates on disease associated symptoms (histopathological changes of the colon tissue) in the DSS colitis model	225
5.21	Cytokine mRNA expression in a mouse model of colitis treated with UL-2-34 and UL-2-132 hydrolysates	226
5.22	Cytokine secretion from unstimulated splenocytes in a mouse model of colitis treated with UL-2-34 and Ul-2-132 hydrolysates	227
5.23	Cytokine secretion from stimulated splenocytes in a mouse model of colitis treated with UL-2-34 and UL-2-132 hydrolysates	228
8.1	Assessment of IL-17 secretion in undifferentiated T-cells treated with UL-2-147	B.1
8.2	RNA product analysis by gel electrophoresis	C.3
8.3	RNA product analysis by gel electrophoresis	C.3
8.4	RNA product analysis by gel electrophoresis	C.4
8.5	RNA product analysis by gel electrophoresis	C.4
8.6	RNA product analysis by gel electrophoresis	C.5
8.7	RNA product analysis by gel electrophoresis	C.5

LIST OF TABLES

Table	Name	Page
1.1	Overview of diseases associated with a dysregulated immune response in the gut	5
1.2	Summary of cytokines and transcription factors involved in the differentiation and commitment of T-helper subsets and associated disease associated with dysregulation in the gut	14
1.3	Overview of protein hydrolysates screened in the first round of this project	32
2.1	Cell culture materials	37
2.2	Differentiating cytokines and antibodies	37
2.3	ELISA Reagents	38
2.4	Flow cytometry reagents	38
2.5	RNA Isolation and cDNA synthesis materials	39
2.6	Quantitative PCR reagents	39
2.7	Gel electrophoresis reagents	40
2.8	Immunohistochemistry reagents	40
2.9	OVA and DSS reagents	40
2.10	List of polarising cocktails and concentrations	45
2.11	Thermal cycling conditions	53
2.12	Thermal cycling conditions	54
2.13	Scoring system used to determine intestinal bleeding in the DSS model	56
2.14	OVA model template welfare sheet	58
3.1	Summary of cytokines and transcription factors involved in the differentiation and commitment of T-helper subsets	63
4.1	Overview of parent and regenerated hydrolysates brought forward into round 2 screening	109
4.2	Overview of parent, regenerated and optimised hydrolysates brought forward into round 3 screening for allergy suppression	112
4.3	Summary of *in vitro* results from allergy and immunity infant nutrition FH1 work-package	118
4.4	OVA model template welfare sheet	120
4.5	Overview of parent, regenerated and optimised hydrolysates brought forward into round 3 screening for tolerance induction	129
5.1	Overview of parent and regenerated hydrolysates in round 2 screening	192
5.2	Overview of parent, regenerated and optimised hydrolysates in round 3 screening	194
5.3	Overview and summary of hydrolysates being brought forward for upscaling into animal trial	196
5.4	Summary of *in vitro* results from allergy and immunity infant nutrition FHI work-package	198
5.5	Scoring system used to determine intestinal bleeding in the DSS model	199

ABBREVIATIONS

ANOVA	Analysis of Variance
APC	Antigen presenting cell
BSA	Bovine Serum Albumin
cDNA	Complimentary Deoxyribonucleic Acid
CM	Cow's milk
CMPA	Cow's milk protein allergy
DDAI	Daily Disease Activity Index
DC	Dendritic Cell
dNTP	Deoxyribonucleotide Triphosphate
DSS	Dextran sodium sulphate
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immuno-Sorbent Assay
EPEC	Enteropathogenic *E. coli*
FACS	Fluorescence Activated Cells Sorting
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
Foxp3	Forkhead box P3
GALT	Gut Associated Lymphoid Tissue
GF	Germ free
GUSB	Glucuronidase-β
HRP	Horseradish Peroxidase

H&E	Haematoxylin and Eosin Staining
IBD	Inflammatory Bowel Disease
IEC	Intestinal epithelial cell
IMF	Infant milk formula
IFN-γ	Interferon Interferon-gamma
IL	Interleukin
IP	Intraperitoneal Injection
LP	lamina propia
LPS	Lipopolysaccharide
MHC	Major Histocompatibility Complex
mRNA	Messenger Ribonucleic Acid
NK	Natural killer
MLN	Mesenteric Lymph Node
MMP	Matrix metalloproteinase
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NaCN	Sodium caseinate (casein)
NK	Natural killer
OIT	Oral immunotherapy
PBS	Phosphate-Buffered Saline
PRR	Pattern recognition receptor
RORγ	Retinoid orphan receptor gamma

RT-qPCR	Reverse Transcriptase quantitative Polymerase Chain Reaction
Runx1	Runt-related transcription factor 1
STAT	Signal transducer and activator of transcription
TBST	Tris-buffered Saline with Tween
TCR	T-cell receptor complex
TGF	Transforming growth factor
Th	T-helper
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Treg	Regulatory T-cell
UC	Ulcerative Colitis
WHO	World Health Organisation
WPC80	Whey protein concentrate

PUBLICATIONS

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Niamh Hunt

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Clarification on Common Misconceptions about Dairy Consumption: Dairy, Asthma and Mucus Production
Niamh Hunt

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A Formula for Tolerance**:** Could cow's milk be a novel solution to milk allergy?
Niamh Hunt

PRESENTATIONS

School of Biotechnology Annual Research Day, Dublin, January 2014
A Formula for Tolerance to Cow's Milk Protein Allergy – Oral
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A Formula for Tolerance to Cow's Milk Protein Allergy - Oral
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A Formula for Tolerance to Cow's Milk Protein Allergy - Oral
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Hypoallergenic and Immunomodulatory Hydrolysates for Infants with Cow's Milk Protein Allergy - Poster
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Immunomodulatory Protein Hydrolysates for the Management of Intestinal Immune Disorders in Infants - Oral
***Niamh Hunt**, Christine Loscher, Alice Nongonierma and Richard Fitzgerald*

ABSTRACT

Niamh Hunt

Immunomodulatory Protein Hydrolysates for the Management of Intestinal Immune Disorders in Infants

Newborn infants have specific dietary needs. Breastfeeding, being the optimal feeding source, provides the infant with a natural form of nutrition whilst also aiding the development of the infant's mucosal immune system and providing protective factors against infectious disease. However, failure to develop mucosal immunity and establish immune balance in the infant gut, in circumstances where breastfeeding is not possible, is the cause of many immune disorders, such as food allergy and inflammatory bowel disease. These conditions are associated with an imbalance of immune cells in the infant gut, characterised by excessive increases in allergic or pro-inflammatory mediators. Development of effective treatments for these conditions in infants has declined. Our understanding of protein hydrolysate diets is growing and currently much research is focussed on their potential health benefits. By tailoring the hydrolysis conditions it is possible to yield novel protein hydrolysates with specific bioactivity. The overall aim of this project was thus, to identify novel cow's milk-derived protein hydrolysates with beneficial immunomodulatory properties, that can be of benefit in early postnatal life, primarily to prime the immune system from birth and induce immune homeostasis in the gut, which would be beneficial for infants at risk of developing cow's milk protein allergy as well as infants who suffer from inflammatory bowel conditions, particularly necrotising enterocolitis.

Through *in vitro* studies, we identified novel candidate cow's milk protein hydrolysates that specifically modulated the immune response away from an allergic type 2 helper T-cell (Th2). We also identified hydrolysates that specifically acted on and inhibited the pro-inflammatory type 1 helper T-cell (Th1). We then examined the effects of these candidate hydrolysates in *in vivo* mouse models of OVA allergy and DSS-induced inflammatory bowel disease in order to assess their therapeutic application in a disease setting. We observed a complete reduction of the clinical symptoms associated with both diseases. One hydrolysate modulated the immune response away from an allergic Th2 response and more towards a regulatory T-cell phenotype, which allows the immune system to develop immune homeostasis. Furthermore, we demonstrated the consistent anti-inflammatory bioactivity of a second hydrolysate in an inflammatory disease setting.

In this research we contribute novel and useful scientific evidence to the current knowledge in the field regarding the bioactivity of cow's milk hydrolysates Throughout this project we focussed on the processes of food allergy and inflammation, in particular in the context of the immune response in the gut and describe how a number of novel cow's milk protein hydrolysates act against the allergic and inflammatory processes according to both *in vitro* and *in vivo* studies. Finally, we indicated how these cow's milk protein hydrolysates could be useful as novel therapeutic strategies for the prevention and/or therapy of intestinal food allergy and intestinal inflammation in infants and children. While our focus was on developing hydrolysates for infant nutrition, the hydrolysates identified in this project also possess potential to be beneficial for other life threatening food allergies and inflammatory conditions.

CHAPTER 1

GENERAL INTRODUCTION

1 INTRODUCTION

It is well known that our health and the development of certain diseases is impacted by our genetics and the environmental factors which we are exposed to, diet being a major factor. Protein is an essential nutrient required within the diet. While protein has become increasingly popular and known for its positive effects in sports nutrition, as a means to build muscle and improve post exercise recovery, dietary protein is also a key player in the development of the immune system, aiding the defence against infection by inhibiting microbial growth (antimicrobial) or by stimulating or dampening the immune system (immunomodulatory) (Van Loon, 2007; and Raikos and Dassios, 2014). Dairy is a key source of nourishment from which we receive an abundance of essential proteins which implement this antimicrobial and immunomodulatory behaviour. Milk, being a primary source of dairy protein in the diet, provides us with the highest-quality protein, and it is for this reason that it demonstrates many advantages and is an essential form of nourishment in the infant diet.

It is estimated that between 95 and 120 g of protein is consumed daily. During the digestion process, protein becomes hydrolysed and broken down into peptides. Although physiological actions are known to be regulated by hormones or cytokines, dietary protein peptides are also thought to play a role as gastrointestinal and immune modulators with various mechanisms of action (Hernández-Ledesma et al. 2014). Proteins can also be pre-digested or hydrolysed into peptides *in vitro* and are known as protein hydrolysates. Proteins that undergo hydrolysis can result in the release of biologically active peptides that are inactive in the precursor protein sequences. These peptides can be released in three ways; enzymatic hydrolysis with digestive enzymes like pepsin, trypsin and chymotrypsin; fermentation of milk with proteolytic starter cultures; or proteolysis by enzymes (proteolytic enzymes) derived from proteolytic microorganisms (Mohanty et al. 2016). The bioactive peptides found in hydrolysates possess additional immunological properties to the whole protein and have been demonstrated to modulate the immune system for benefit. They have been shown to display anti-inflammatory effects and stimulate the intestinal epithelial barrier, which may be useful in the setting of food allergy and intestinal inflammatory conditions (Kiewiet et al. 2015). Recent findings indicate that hydrolysed dietary peptides, such as milk, pea, rice and soy peptides are known to affect major systems such as the

cardiovascular, nervous, digestive, and immune system and can have additional physiological effects such as antioxidant activity or may even possess functional significance, which is still not fully understood to date (Raikos and Dassios, 2014). Therefore, the identification and use of these bioactive peptides as a functional food incorporated into the diet could prove extremely beneficial in the future for the food industry as well as contribute to the overall health of society. A particular area of interest for the development and use of dietary protein hydrolysates currently, is in infant milk formula (IMF), which is the key aim of this project. Hydrolysed peptide formulas are a suitable method for babies to easily digest and absorb protein. Therefore, when produced in the right way, through enzymatic hydrolysis (using selected enzymes for hydrolysis); they may be beneficial for infants who fail to establish immune balance, which can result in allergy or inflammatory conditions, as a consequence.

1.2 Gut Homeostasis and Mucosal Immunity in Infancy

Newborns have specific dietary needs. Breastfeeding, being the optimal feeding source, provides the infant with a natural form of nutrition whilst also aiding the development of the infant's mucosal immune system and providing protective factors against infectious disease. Breast milk is an abundant source of protein, which is an essential part of an infant's diet, serving to achieve optimal growth, development and health in the first few months of life (Jackson and Nazar, 2006). Breast milk promotes the colonisation of healthy bacteria in the intestinal tract, which promotes the development of immune balance and tolerance and thus, a correctly functioning immune system. Many studies suggest that infants who are breastfed are less susceptible than formula-fed infants to developing intestinal diseases including respiratory diseases and diarrhoea (Hsieh et al. 2015; and Newburg, 2009). This indicates that a lack of human milk during this vulnerable period in infancy may result in a failure to develop an adequate immune response in the gut. Breast milk is, therefore, advised as the best source of nourishment for the first six months of life. Failure to develop mucosal immunity and establish immune balance in the infant gut is the cause of many immune disorders, such as food allergy and intestinal inflammatory disease (IBD). For this reason, most research focuses on targeting the

gastrointestinal tract of infants when developing therapeutics linked to immune dysregulation during infancy (Kiewiet et al. 2015). The overall aim of this project is to identify novel cow's milk (CM) protein hydrolysates that can be beneficial for infants with cow's milk protein allergy (CMPA) as well as infants who suffer from inflammatory bowel conditions, particularly necrotising enterocolitis (NEC). These conditions are both linked to a dysregulated immune response in the gastrointestinal tract and will be discussed in further detail.

1.2.1 Interaction of The Host Immune System and Gut Microbiota

A vast array of beneficial microorganisms, also known as microbes, live inside us and influence our health. These microorganisms, referred to as "microbiota" (the organisms) or the "microbiome" (the organisms and their overall genetic makeup), play an important role from birth in promoting the development and activation of the mucosal immune system as well as neural pathways in the infant. Microorganisms can be found on the skin, in the oral cavity, and the urogenital tract, however, it is the gut that boasts the most diverse and abundant population of microbes, the distal gut being populated with an average of 10^{13} bacteria (Peterson et al. 2015). The human gut is also home to the largest compartment of the immune system. A healthy gut microbiome is important for developing and regulating the immune system as it works closely with the host's mucosal immune system of the gastrointestinal (GI) tract to promote oral tolerance to food antigens and overall immune homeostasis, as well as having protective effects acting as a barrier against the proliferation of pathogenic organisms. Under normal physiological conditions, a properly functioning immune system in a healthy individual maintains a balanced state of immune homeostasis, meaning it has established an appropriate balance between self-tolerance to the commensal (harmless) gut microbiota and oral tolerance to harmless food antigens, yet has the ability to induce a response to a potential pathogen (Crimeen-Irwin et al. 2005). Immune homeostasis can take up to three years to be fully established in an infant, leaving them vulnerable during this time of development (Koenig et al., 2011; Weng & Walker, 2013; and Yang et al. 2016). In infants who develop poor microbial colonisation or an imbalance of gut microbiota between pathogenic and commensal bacteria, known as dysbiosis, immune balance is not achieved and immune

responses can either under or over react to foreign antigens, resulting in increased disease risk such as IBD or allergy against harmless food proteins. Many factors influence the colonisation of commensal gut microbiota and thus, the development of immune homeostasis or the potential to impact life course disease risk, which we will discuss in greater detail (Yang et al. 2016). A general overview of diseases associated with a dysregulated immune response in the gut is summarized in **Table 1.1**. This project will focus on NEC and CMPA. Understanding these influences would be highly beneficial and crucial for the development of novel therapeutic strategies for disease involving the human gut. The interaction between the gut microbiota and host involves participation of both innate and adaptive responses which will also be discussed.

Disease	Description	Demographic	Current Treatments
IBD (Crohn's Disease)	Chronic inflammation of the gastrointestinal tract	Commonly starts between ages 15-35 in both men and women equally	Antibiotics, corticosteroids, immunomodulators, surgery to remove affected section of intestine
IBD (Ulcerative Colitis)	Chronic inflammation of the large intestine (colon and rectum)	Commonly starts between ages 15-30 in both men and women equally	Antibiotics, corticosteroids, immunomodulators, surgery: proctocolectomy
NEC	Lethal disease of the gastrointestinal tract	Preterm infants: increased expression of TLR4 in the premature gut	Discontinuation of enteral feeding. Medical management, such as nasograstric decompression, intravenous fluids and broad-spectrum antibiotics
CMPA	Most common food allergy in infancy	2% of infants under the age of four	Strict avoidance of cow's milk using extensively hydrolysed cow's milk based forrmula
Celiac Disease	Autoimmune disorder of the small bowel induced by the ingestion of gluten	Affects 0.5%-1% of global population. Diabetics & Individuals with HLA-DQ2 and/or HLA-DQ8 genes are most at risk	Strict life-long gluten-free diet, Gluten modification

Table 1.1: Overview of diseases associated with a dysregulated immune response in the gut.

1.2.2 Gut Microbiota Dysbiosis and Life Course Disease Risk

The gastrointestinal immune system plays a major role in the development of oral tolerance and immune homeostasis and it is understood that a healthy intestinal microbiome displays beneficial immunomodulatory activity that leads to this development and maintenance of immune tolerance (Pabst and Mowat, 2012). As mentioned, some diseases have been associated with the failure to develop the intestinal mucosal defence system in infants and children, such as allergy, asthma, eczema and allergic rhinitis as well as inflammatory conditions, such as NEC. Many factors affect the infant's ability to develop optimum immunity and, moreover, the risk of developing the associated diseases.

The health benefits of breast milk are well known, in particular its ability to promote immune homeostasis and natural tolerance. The risk of developing the diseases caused by intestinal dysbiosis and a dysregulated immune response is greatly reduced in exclusively breastfed infants. Many Immunoglobulins can be found in breast milk, IgA being the most abundant, making up more than 90% of the total antibody content. Immunoglobulins are antibodies, formed or acquired over time following exposure to infectious agents. IgA can be found throughout the gut and respiratory system of adults and functions to neutralise infectious agents and prevents tissue damage by inflammation that can occur with other antibody types (Jackson and Nazar, 2006; and Newman, 1995). IgA, in particular, plays a key role in the development and regulation of healthy gut flora. Infants cannot produce their own IgA antibodies until several months of age. One-year old infants possess only 20% of adult serum IgA levels (Weemaes et al. 2003; and Stiehm and Fudenberg, 1966). The mother's acquired IgA antibodies can, however, be passed to the infant through breast milk when required. IgA antibodies prevent the spread of infection in the infant by blocking attachment of external pathogens to the cells in the gastrointestinal tract. They do this by binding to the external antigens themselves (Jackson and Nazar, 2006). IgA antibodies from human milk have been identified that target bacterial pathogens such as *Escherichia coli*, *Vibrio cholerae*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Clostridium difficile*, and *Salmonella*; viruses such as rotavirus, cytomegalovirus, HIV, influenza virus, and respiratory syncytial virus; and yeasts such as *Candida albicans*, some of which are present in high levels in formula-fed infants (Lönnerdal, 2003; and Goldman, 1993). Breast milk also contains a wealth of

healthy bacteria, such as *Bifidobacteria,* and a large amount of galactooligosaccharides, which influence strong microbial gut colonisation within the infant. *Bifidobacterium* make up the bulk of the gut microbiota (60-70%) in breastfed infants, however, a much lower proportion is observed in formula-fed infants along with increased numbers of *streptococci* and *staphylococci*, which are usually targeted by the IgA antibodies in breast-fed infants. Formula-fed infants have a heightened risk of developing conditions such as CMPA and NEC when compared to breast-fed infants as a result of this altered microbiome. Furthermore, it has been observed that poor microbial colonisation of the infant gut, as a result of formula-feeding, can potentially lead to a predominant allergic (atopic) Th2 cell type environment, resulting in allergy, which will be discussed in more detail (Hendaus et al 2016). Many studies have also shown, that children with food allergies have a much smaller population of *Bifidobacteria* and *Lactobacilli* and increased counts of harmful bacteria such as *E. coli* and *Clostridium difficile (*Mazmanian et al. 2005; and Round et al. 2010*),* which are targeted by IgA antibodies in breast milk. Moreover, Yel, (2010) also claims that people who are susceptible to food allergies are also associated with IgA deficiency, which once again highlights the importance of breastfeeding. Aghamohammadi et al., (2009) report that 84% of individuals (infants and adults) with IgA deficiency also had food allergy.

Niño et al. (2016) describes another risk factor for intestinal disease progression, suggesting the existence of a link between intestinal dysbiosis in premature infants and the development of NEC. Preterm infants are susceptible to NEC development. It has been shown that infants born preterm have a much lower proportion of *Bifidobacteria* and *Bacteroidetes* and higher levels of harmful *Clostridiaceae* and *Enterobacteriaceae* compared to infants born at term. Studies have also demonstrated that preterm infants with NEC display much lower diversity in gut microbiota when compared to healthy controls, which may be responsible for their increased risk of NEC. However, it remains to be elucidated whether this irregular gut flora is linked to NEC onset or whether it is a consequence of the disease itself. As preterm infants have heightened levels of *Clostridiaceae* and *Enterobacteriaceae* they, therefore, have an increased requirement for IgA antibodies in human milk in order to reduce these populations and thus, NEC risk. As IgA levels in formula-fed infants are poor, Torrazza et al. (2013) and Tanner et al. (2015) suggest that this may also contribute to

NEC development. Collectively, these studies highlight the importance of breastfeeding and indicate that IgA antibodies and healthy bacteria, such as *Bifidobacteria*, from human milk play key roles in the development and regulation of healthy gut flora and thus, immune homeostasis and oral and self-tolerance, preventing both sensitisation to harmless food proteins and an excessive inflammatory response resulting in NEC.

1.3 Innate and Adaptive Immunity in Healthy Hosts

The gut microbiome shapes and develops both the innate and adaptive immune responses. A healthy microbiome supports the development of immune homeostasis (Wu and Wu, 2012). Understanding of the underlying mechanisms that control immune homeostasis and the development of disease upon failure to develop this balance represents an important step in our ability to reliably modulate the gastrointestinal immune system in order to develop novel therapeutics for infants who suffer with immunological intestinal disorders, such as allergy and NEC.

1.3.1 Role of Innate Immunity in Immune Homeostasis

Colonising bacteria and ingested proteins first encounter a layer of epithelial cells. These cells are connected by tight junctions resulting in low permeability (Shen and Turner, 2006). The binding of food antigens or harmless bacteria to pattern recognition receptors (PRRs) on the epithelial layer can result in higher or lower permeability. When permeability is increased, food antigens and colonising bacteria can enter the lamina propria and are taken up by antigen presenting cells (APCs), mainly dendritic cells (DCs) located in the Peyer's patches in the Gut Associated Lymphoid Tissue (GALT). When immune homeostasis is established, innate intestinal APCs protect the body against infection, while remaining inactive and maintaining immune tolerance to the normal gut microbiota (Schmidt-Weber and Blaser, 2002). In healthy individuals, epithelial cells then secrete IL-10, TGF-β and TSLP which results in differentiation of DCs into tolerogenic DCs, which then play a key role in the development of oral tolerance to harmless food allergens, such as CM proteins, or self-tolerance to commensal gut microbiota (Zeuthen et al. 2008). They process the food or bacterial

antigen and present it on a major histocompatibility complex (MHC) class II receptor to T-cells in the mesenteric lymph nodes (MLNs), resulting in immunological tolerance by the induction of regulatory T-cells (Treg) and/or by clonal anergy or deletion of T-cells in the adaptive immune response (Maldonado and von Andrian, 2010; and Raker et al. 2015). Secretion of IL-10 and TGF-β from DCs promotes Treg induction. Treg cells, in turn, secrete TGF-β, which inhibits differentiation of the effector adaptive T-helper cells - T helper 1 (Th1), Th2 and Th17 cells, which will be discussed in more detail. Another method of tolerance induction, as mentioned, is clonal anergy. When the immune system mounts a response to a harmful antigen, the activated APC, usually a DC, interacts with a naïve $CD4^+$ T-cell. The peptide-MHC-II-complex from the DC binds the T-cell receptor (TCR) and co-stimulatory molecules interact between both cells types, resulting in the activation of effector T-cells and an immune response. When commensal gut bacteria are encountered or a harmless food antigen is ingested and tolerance is achieved, this co-stimulatory molecule interaction does not occur alongside TCR engagement, which leads to an unresponsive T-cell or T-cell clonal anergy (Goubier et al. 2008; and Tuettenberg et al. 2009).

Macrophages of the gut also display a non-inflammatory profile when they encounter microbial stimuli in a homeostatic environment also via the production of IL-10. For example, intestinal macrophages do not produce pro-inflammatory cytokines in response to microbial stimuli such as Toll-like receptor (TLR) ligands, a set of microbe-associated molecular patterns (Smith et al. 2011). Natural killer (NK) cells and neutrophils play a key role in intestinal barrier protection against potential pathogenic bacteria via secretion of IL-22 (Denning and Parkos, 2013).

Many studies have demonstrated the importance of gut microbiota in influencing and shaping innate APC responses. A study carried out on germ-free (GF) animals observed lower levels of intestinal DCs but normal levels of systemic DCs. Both gut and systemic macrophages were decreased in GF pigs. Furthermore, GF mice displayed compromised phagocytosis and microbicidal activities, associated with normal macrophage function, from peritoneal macrophages. Another study reported a loss of MHC II on macrophages in GF mice. Decreased phagocytic function was also observed in the peripheral blood neutrophils of GF rats. Collectively, these studies indicate that microbiota play a key role in regulating the development of innate immunity.

1.3.2 Adaptive Immunity

$CD4^+$ T-cells are key cells of the adaptive immune response which recognise peptides presented on MHC class II molecules by APCs. Intestinal $CD4^+$ T-cells are mostly located in the lamina propia (LP) of the intestine. Crosstalk between the gut microbiota and $CD4^+$ T-cells in the LP is essential for regulating and shaping adaptive immune responses and for the generation of immune homeostasis (Wu and Wu, 2012). Macpherson et al. (2002) and Mazmanian et al. (2005) demonstrated that GF animals display a decrease in $CD4^+$ cell populations in the LP, as well as systemically, in the mesenteric lymph nodes (MLNs) and spleens. GF mice were also observed to have a Th1/Th2 imbalance: their immune response is biased toward the Th2 response; which will be discussed in more detail in the next section. $CD4^+$ T-cells have many important roles, functioning to enhance microbicidal activity by macrophages, recruit neutrophils, eosinophils, and basophils to sites of infection and inflammation and induce antibody production from B cells. They exert these functions mainly by the secretion of cytokines and chemokines (Zhu and Paul, 2008). Depending on the type of stimulation, naive $CD4^+$ T-cells can differentiate into one of four T-cell subtypes: Th1, Th2, Th17, or Treg. Each $CD4^+$ T-cell subset has a unique role, characterised by distinctive transcription factors and cytokine profiles, summarised in **Figure 1.1**.

Cytokines are small signalling proteins secreted primarily by immune cells upon activation. Cytokine signalling heavily influences the development and function of the infant immune system. The cytokine profile to be activated is dependent on the type of pathogen and thus, determines the type of immune response to be activated. $CD4^+$ T cell differentiation has been shown to be regulated by the cytokine milieu present during activation (Shiner et al. 2014). Cytokines and their receptors participate in a diverse array of functions beyond innate and adaptive immunity including inflammation, immune cell differentiation, angiogenesis, tumorigenesis, development, neurobiology, and viral pathogenesis (Cohen and Cohen 1996). Dysregulation of cytokine expression is a cause of immunological and inflammatory diseases as well as other disease states (Tracey and Cerami 1993; Finkel et al. 1992; Rogler and Andus 1998). Cytokines and their patterns were the main focus throughout this project.

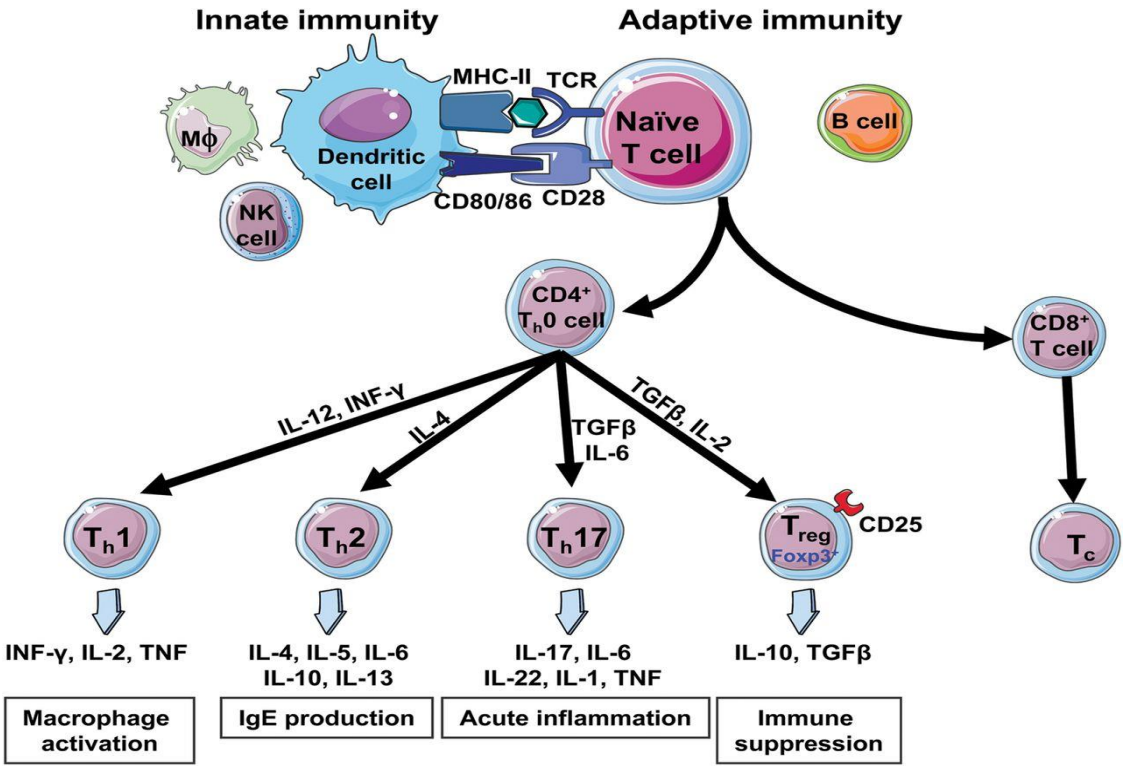


Figure 1.1: Classical CD4$^+$ T-cell Differentiation. The type of cytokine miliu secreted from APCs and undifferentiated CD4$^+$ T-cells determines the type of T-cell subset to be activated. Each T-cell subset has a unique role and secretes a defined cytokine profile specific to the type of pathogen present, leading to an effective immune response.

1.3.2.1 T-Helper 1 Cells

Th1 cells provide protection against intracellular pathogens. They activate cell-mediated immunity and phagocyte-dependent protective responses. They have an additional role in humans, targeting mycobacterial infections. Interferon-gamma (IFN-γ), interleukin (IL)-2, and tumour necrosis factor (TNF)-β are the characteristic cytokines secreted from Th1 cells (Mosmann and Coffman, 1989). Th1 secreted IFN-γ activates macrophages and enhances their microbicidal activity (Suzuki et al. 1988). IFN-γ from Th1 cells is a stimulatory factor for Th1 differentiation and acts as the positive feedback amplifier, whilst also directly inhibiting the polarisation of Th2 cells (Lighvani et al. 2001). IL-2 plays a role in generating CD4 T-cell memory. T-bet is the master regulator in Th1 cells. STAT1 is a transcription factor essential for the upregulation of T-bet through increasing IFN-γ production (Lighvani et al. 2001). STAT4 is also important for Th1 differentiation. Not only does STAT4 induce IL-12

secretion but it also promotes IFN-γ from CD4 T-cells, creating a positive feedback loop for Th1 differentiation (Zhu and Paul, 2008; and Bacon et al. 1995).

1.3.2.2 T-Helper 2 Cells

Th2 cells are responsible for host defence against helminths and extracellular parasite infections. Unlike Th1 cells, they activate phagocyte-independent protective responses. They do so by inducing eosinophil activation and antibody production and inhibiting macrophage function (Romagnani, 1999). They secrete IL-4, IL-5, IL-10 and IL-13. IL-4 is also required for Th2 differentiation, creating a positive feedback loop for this phenotype (Le Gros et al. 1990; and Swain et al. 1990). IL-4 secreted from Th2 cells is responsible for activating IgE class switching in B cells (Kopf et al. 2007). IgE then binds to and cross-links with the high-affinity IgE receptor (FcεRI) on mast cells and basophils, which results in the secretion of cytokines, such as IL-4, IL-13, and TNF-α, as well as histamine and serotonin. IL-5 plays a critical role in recruiting eosinophils (Coffman et al. 1989). IL-10, produced by Th2 cells, prevents differentiation of the Th1 subset, possibly through suppression of dendritic cell function (Zhu and Paul, 2008; and Moore et al. 2001). IL-13 is the main cytokine responsible for the removal of helminths and cause of airway hypersensitivity (Wynn, 2003). Similar to IL-4, IL-13 also regulates IgE production but, unlike IL-4, it has no role in T cell differentiation to Th2 cells and T lymphocytes do not respond to IL-13 (Deo et al. 2010). IL-4 also activates the transcription factor, STAT6, essential for IL-4–mediated Th2 differentiation (Kaplan et al. 1996). STAT6 is also crucial for the upregulation of GATA-3, the Th2 master regulator (Kurata et al. 1999). GATA-3 expression is sufficient for Th2 commitment (Zheng and Flavell, 1997). While STAT6 is not absolutely essential for Th2 differentiation, the absence of GATA-3 results in failure for $CD4^+$ cells to differentiate into a Th2 subset both *in vitro* and *in vivo* (Pai et al. 2004). Moreover, IL-5 and IL-13 secretion is abolished in Th2 cells following GATA-3 deletion (Zhu et al. 2004). STAT5 signalling is also deemed important for Th2 differentiation and is activated by IL-2.

1.3.2.3 T-Helper 17 Cells

Th17 cells are critical for the host defence against extracellular bacteria, fungi and parasites. They produce IL-17, IL-21, and IL-22 (Littman and Rudensky, 2010). Expression of the Th17 master regulator and transcription factor, retinoid-related

orphan receptor (RORγt), is enhanced during Th17 differentiation and induces secretion of IL-17 (Ivanov et al. 2006). IL-17a and IL-17f are genetically linked and both signal through the IL-17RA chain (Hymowitz et al. 2001). IL-17a is important for activating inflammatory responses via enhancement of inflammatory cytokine secretion, such as IL-6, and chemokine secretion, such as IL-8. In an attempt to expel extracellular bacteria and fungi, both IL-17a and IL-17f recruit and activate neutrophils to aid in this response (Zhu and Paul, 2008). The Th17 signal transducer, STAT3, regulates the production of IL-6, IL-21 and IL-23. IL-6 and IL-23 in turn activate IL-22, which stimulates dermal inflammation. Th17 secreted IL-21 is a stimulatory factor for Th17 polarisation and functions as the positive feedback amplifier (Korn et al. 2007; and Zhou et al. 2007). STAT3 is also critical for IL-17 production (Harris et al. 2007).

1.3.2.4 Regulatory T-Cells

Treg cells are essential for the regulation and maintenance of immune homeostasis and self-tolerance (Sakaguchi, 2004). They do this by secreting cytokines, such as TGF-β, IL-10, and IL-35, which suppress the functions of the effector T-cells. Treg produced TGF-β can further induce iTreg cells from naive $CD4^+$ T-cells when required (Zhu and Paul, 2008). IL-10 secreted from Treg cells is responsible for its therapeutic effects in the prevention or treatment of inflammatory bowel disease (Asseman et al. 1999). IL-35 contributes to the overall suppressive activity of Treg cells (Collison et al. 2007). Foxp3 is the key transcription factor associated with Treg cell activity and its expression is upregulated by TGF-β (Hori et al. 2003; and Fontenot et al. 2003). Foxp3 is essential for orchestrating the suppressive activity of Treg cells and for maintaining their phenotype (Williams and Rudensky, 2007). It is suggested that STAT5, which is important for Th2 differentiation, may also play a role in upregulating Foxp3 expression (Davidson et al. 2007; and Yao et al. 2007).

1.3.2.5 T-cell Imbalance – Autoimmunity and Allergy

Establishment of healthy gut flora and achievement of immune homeostasis in the intestine results in a dynamic balance of the T-cell subsets with Treg cells (Smith and Garrett, 2011). An imbalance in this regulation, in compromised individuals, results in underactive or excessive and uncontrolled T-cell activation, which can lead to deleterious effects and often results in disease (Zhu et al. 2010). As Treg cells are

essential for proper regulation and the maintenance of immune homeostasis; their dysfunction can be pathological, resulting in failure to induce tolerance followed by the development of autoimmune disorders or allergy. Overactive Th1 and Th17 responses have been associated with the onset of organ-specific autoimmune diseases while the Th2 response has been linked to the development of allergic disease (Mosmann et al. 1986). Development of novel treatments for these conditions is the overall key focus of this PhD project. A summary of the cytokines and transcription factors involved in the differentiation and commitment of T-helper subsets as well as the diseases associated with their regulation is provided in **Table 1.2**.

Type	Priming cytokines	Transcription factors	Effector cytokines	Functions	Disease associated with dysregulation	Disease in the Gut
Th1	IL-12 IFN-γ	T-bet STAT1 STAT4	IFN-γ	Intracellular pathogens	Autoimmune Inflammation cell-mediated response	IBD NEC
Th2	IL-4 IL-6 IL-10	GATA-3 STAT5 STAT6	IL-4 IL-5 IL-10 IL-13	Extracellular pathogens	Asthma allergy atopy	Food allergy
Th17	TGF-β IL-6	ROR-γ STAT3	IL-17 IL-22	Extracellular bacteria	Autoimmune inflammation	IBD NEC
Treg	TGF-β IL-2	Foxp3 STAT5	TGF-β IL-10 IL-35	Immune homeostasis	Autoimmunity allergy metabolic dysfunction	IBD NEC Food allergy

Table 1.2: Summary of cytokines and transcription factors involved in the differentiation and commitment of T-helper subsets and associated disease associated with dysregulation in the gut.

1.4 Food Allergy

The prevalence of allergic disease has risen in developing countries, and even more substantially in developed countries. Significant global increases in all allergic diseases, such as atopic dermatitis, asthma, and food allergy, have been observed (Prescott et al. 2013). Although family history and genetic predisposition play a key role in the risk and onset of allergy, these global increases are more likely associated with changes in environmental and antigenic exposure. Food allergy is a major burden on society, impairing the quality of life for patients and their families. The economic impact of food allergy is considerable. Financial costs include direct (treatments, emergency department visits, hospitalisations) and indirect (absence of a parent from work or child from school, reduced productivity and performance) associated healthcare costs (Hendaus et al, 2016). It is estimated that the annual economic cost of food allergy in the United States is 24.8 billion dollars (Gupta et al, 2013).

Food antigens are the most common antigens that can cause the onset of atopic disease in infancy and for this reason it is believed that food allergy during infancy may mark the beginning of lifelong atopy, resulting in the development of multiple allergic manifestations in later life, such as asthma, atopic dermatitis and rhinitis (Vanto et al. 2004; Vandenplas et al. 2007). This process is known as the "atopic march" (Zheng et al. 2011). CMPA is the most common food allergy associated with infants (Savilahti and Savilahti, 2013). There is an urgent requirement for the development of novel treatments and even more so for primary prevention strategies to counter the rise in food allergy incidence in infancy, in the hope of reducing and preventing the burdens it places on patients in later life.

1.4.1 Allergy Begins During Infancy

Food allergies or impaired natural tolerance is most common in infants. Oral tolerance allows the infant's immune system to remain in a non-activated state when it encounters harmless food proteins, yet it can retain its ability to mount an immune response to a potential pathogen. As mentioned, the gastrointestinal tract plays a critical role in the development of oral tolerance (Chehade and Mayer, 2005). The intestinal tract and immune system of infants are still not fully developed, resulting in

a higher risk for sensitisation during first exposure to food proteins, such as CM proteins (Savilahti, 2013). It is important to note that the first induction of an allergic immune response following exposure to an allergen is referred to as '*allergic sensitisation' (*Valenta et al. 2015*).* There are five main risk factors that result in failure to induce oral tolerance and contribute to the development of food allergy in infants. These include the stability of the allergen against digestion, the permeability of the epithelial barrier, the size or the amount of ingested allergen, atopy risk and the inability to induce immune homeostasis (Th1/Th2-paradigm and Treg induction) (Valenta et al. 2015). These risk factors are discussed below:

1.4.1.1 Poor ability to digest proteins

Food allergy is most common during infancy due to their poor ability to digest proteins, resulting in higher risk of being exposed to more allergenic food antigens than adults (Perrier and_Corthésy, 2011). Infants do not produce enough of the enzymes required to digest proteins and have a high stomach pH (Henderson et al. 2001; Washington et al. 1999). Pepsin is an important enzyme required for the complete digestion of food proteins and is active at a low pH. The pH of an adult's stomach is optimal for pepsin, ranging from 1.5 to 2.5, whereas a newborn's stomach pH is much higher ranging from 4.5 to 5, which results in proteins not being fully digested or hydrolysed (Dotevall, 1961; Becker et al. 1976; and Dallas et al. 2012). This results in larger proteins being absorbed.

1.4.1.2 High permeability of the epithelial barrier

Permeability of infants' epithelial cells is also known to be higher than that of adults (Vickery et al. 2011). Collectively, these factors enable an increased number of larger proteins to pass the gastrointestinal barrier and influence the development of allergy in the infant.

1.4.1.3 Larger proteins that cross the epithelial barrier promote allergic Th2 response

The allergic potential of peptides is dependent on the level of exposure and their size, larger peptides being more allergenic and increasing risk of sensitisation to food proteins. Once crossed over the gastrointestinal barrier, these larger peptides can activate and over-express a type 2 helper T-cell (Th2) phenotype. Th2 cells are known to be the key drivers of allergy. Upon secretion of IL-4, IL-10, IL-13 and IL-5;

cytokines that aid in the persistence of allergic reactions, Th2 cells interact with and activate B-cells. Once activated, B-cells secrete immunoglobulin E (IgE). Secreted IgE antibody binds to receptors on mast cells and results in the release of histamine and the onset of the clinical symptoms of allergy, as can be seen in **Figure 1.2** on the next page (Kiewiet et al. 2015; Takastu 1997).

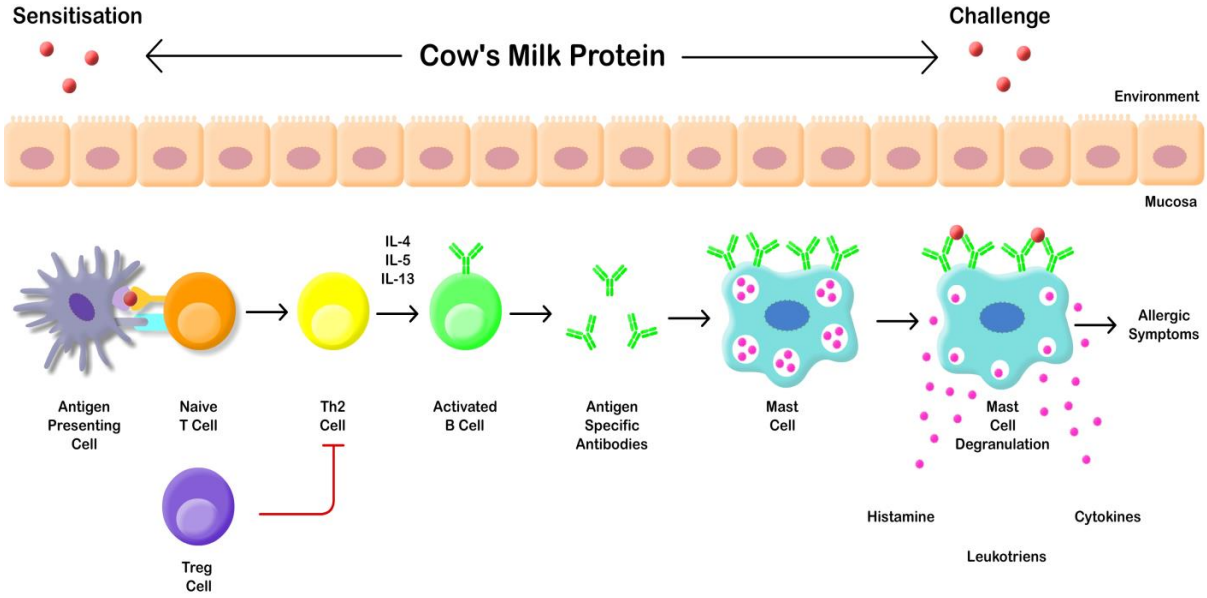


Figure 1.2: Overview of the cellular mechanisms during sensitisation and allergic reaction. Once crossed over the gastrointestinal barrier, proteins can activate an allergic Th2 subset, rsulting in IgE secretion from B-cells and the release of histamine from mast cells, leading to the onset of the clinical symptoms of allergy.

1.4.1.4 Atopic infants can fail to generate immune homeostasis

The ability to generate immune balance is flawed in atopic infants, who are predisposed toward developing IgE-associated allergies. Following first time exposure to a food protein, healthy infants induce the production of Treg cells and/or driving clonal anergy or deletion of T-cells upon APC presentation of the harmless allergen to naïve CD4 T-cells. In atopic infants, however, allergen-specific Th2 cells become activated and release IL-4, IL-5, IL-10 and IL-13 cytokines. These cytokines subsequently induce class switching and production of allergen-specific IgE and the clinical symptoms of allergy (Romagnani, 1997; and Vercelli and Geha, 1992). This

then leads to the development T-cell memory and upon secondary exposure to the same allergen; a more immediate immune response is activated (Niederberger et al. 2007; Naclerio et al. 1997; and Mojtabavi et al. 2002). Upon exposure to a primary food allergen, healthy infants produce allergen-specific IgG and IgA, which do not drive an allergic reaction, in addition to preventing the activation of T-cells (Valenta et al. 2015).

1.4.1.5 Failure to develop Th1/Th2 balance

Fetal Th1 cells are dampened during pregnancy as they are toxic to the placenta and also to inhibit maternal antigens from triggering an immune response (Szekeres-Bartho et al. 1996; Krishnan et al. 1996). As a result, the immune system of infants is Th2 dominant at birth (Prescott et al. 1998; Vighi et al. 2008). IFN-γ secretion from undifferentiated $CD4^+$ T-cells in newborns is much lower than IFN-γ secretion from the same cells in adults *in vitro*. Human adult lymphocytes produce 10-fold more IFN-γ than neonatal cells when stimulated with Con A and PMA (White et al. 2002). Th1 cells mediate a cellular immune response in order to expel bacteria and viruses, while suppressing Th2 functions, whereas Th2 cells drive a humoral response, resulting in IgE secretion (Zeiger 1999). If the immune system favours either of these patterns, as it does in infants at birth, it can result in disease, and either pathway can down-regulate or inhibit the other (Kidd 2003). Genetic and/or environmental factors may result in failure of some infants to develop a Th1/Th2 balance, which may result in allergy (Prescott, Sly & Holt 1998, Prescott et al. 1998, Prescott et al. 1999, Neaville et al. 2003). The shift in the Th1/Th2-paradigm in allergic individuals can be seen in a study where peripheral blood mononuclear cells (PBMCs) from patients with IgE-mediated CMPA and healthy controls were assessed. Following CM stimulation, an increase in Th2 cytokines and less IFN-γ from Th1 cells was observed in allergic individuals compared to healthy control subjects. Patients who had induced tolerance to CM following CMPA showed an increase in Th1 cytokines, establishing immune-balance (Vocca et al. 2011). As mentioned, the gut microbiota plays an important role in bringing about immune homeostasis, establishing a Th1/Th2 balance preventing the onset of disease. Furthermore, a Th1/Th2 imbalance was observed in GF mice, characterised by a Th2 biased response (Mazmanian et al. 2005; and Wu and Wu, 2012). Durkin et al. 1989 also observed a Th2 dominant phenotype in GF mice following. They observed a local increase of IgE in the intestine, as well as a systemic

increase in the serum of GF rats. These observations indicate the importance of microbial colonisation in balancing the Th2 favoured phenotype at birth.

1.4.1.5 Failure to develop Treg Cells

Treg cells are a critical part in the tolerance induction process. They are involved in maintaining homeostasis in the immune system. It is the secretion of large amounts of TGF-β and IL-10, mostly from Treg cells, that ultimately results in the induction of immune balance and oral tolerance to food antigens and colonizing commensal bacteria (Hori et al. 2003). An increase in Treg cells and the characteristic cytokines of Treg cells has been associated with the generation of immune balance and tolerance in both *in vitro* and *in vivo* studies. A decrease in Treg numbers or dysregulation in Treg activity is often associated with the development of allergy and inflammatory disorders, such as NEC. A healthy diverse gut microbiome is critical for the production of Treg cells, which is of particular importance for generating immune balance in newborns, balancing the Th1/Th2-paradigm. Growth of Treg populations has been shown to be induced, mostly in the colon, by short-chain fatty acids, such as propionate and butyrate, produced by bacteria upon fermentation when they break down dietary fiber (Smith et al. 2013). Additionally, Furusawa et al. (2013) found that in the presence of gut microbiota, butyrate induces naive T-cells to differentiate into Treg cells. Both of these studies also observed an increase in IL-10 levels in parallel with the increase in Treg populations.

1.4.1.6 NEC Recovery in Preterm Infants

It is also understood that a link between NEC and CMPA progression exists (Lapillonne et al. 2016). Feeding is stopped during NEC recovery. Breastfeeding is recommended during the refeeding process as infants develop an increased risk of sensitisation during first time exposure to intact CM proteins after eliminating them from the diet during an important time of development. Case studies have shown that NEC recovered infants have increased susceptibility of sensitisation to intact proteins upon refeeding (Walther and Kootstra, 1983). Furthermore, an *in vitro* study, carried out by Abdelhamid et al. (2011 and 2013), also showed that PBMCs from preterm infants with NEC had increased sensitisation to CM protein during exposure.

1.4.2 Early Intervention Necessary for the Generation of Immune Homeostasis and Prevention of Sensitisation

The first 6 months of life represent a crucial window of opportunity for shaping the development of the gastrointestinal tract and immune system and is the most critical time for establishing immune balance, as once sensitisation has occurred it is difficult to correct. This is due to the differentiation of Th2 cells into memory T-cells, which continue to favour an allergic Th2 phenotype and are boosted upon repeated allergen contact to elicit unnecessary immune responses (Galli et al. 2008; and Vickery et al. 2011). This contributes to the development of long-term immune memory, associated with patients with chronic allergy. Studies have shown that children with food allergy are two to four times more likely to develop other atopic diseases throughout life, such as asthma, allergic rhinitis, and atopic dermatitis (Henson and Burks, 2012; and Branum and Lukacs 2008). Environmental exposures and gut microbiome development during infancy, affect the ability to develop immune homeostasis and thus, the likelihood of developing the childhood and adult diseases, such as food allergy, inflammatory bowel disease and obesity (Houghteling and Walker, 2015). For this reason it is critical to prime the newborn immune system as early as possible in order to balance the Th1/Th2-paradigm and prevent the sensitisation to harmless food proteins and the problems associated with allergy in later life. As mentioned, *Bifidobacterium* predominate the gut microbiota of breast-fed infants, unlike formula-fed infants. Recent findings indicate that exposure to maternal gut microbiota in breast milk, can skew this Th2 favoured environment toward a Th1 phenotype through production of Treg cells, bringing about natural Th1/Th2 balance and in most cases immune tolerance to food antigens, such as CM (Mazmanian et al. 2005; Round and Mazmanian 2010; and Walker and Iyengar, 2014). Unfortunately, in cases where infants cannot breastfeed, this exposure is not the same in IMF. In circumstances where breastfeeding is not possible, we aim to identify novel CM hydrolysates that can mimic the effect of breast milk to promote the induction of immune homeostasis.

1.5 Necrotizing Enterocolitis in Preterm Infants

Necrotizing Enterocolitis (NEC) is a form of severe intestinal inflammation, which generally presents in premature low birth weight infants (Gephart et al. 2012). While

understanding of its pathogenesis is poor, it is known that NEC typically affects the terminal ileum, the point at which the small intestine intersects with the large intestine. The disease is characterised by coagulation necrosis, acute and chronic inflammation, bacterial overgrowth, formation of lesions and tissue repair (Claud, 2009; and Ballance et al. 1990). Inflammation tends to occur in the mucosa and submucosa of the intestine, however, in more severe cases it can also present in all layers of the gut. Targeting mainly the intestinal barrier, NEC disrupts the tight junctions between intestinal epithelial cells (IECs). The epithelial layer is a permeable barrier which selectively allows fluids and solutes to cross over while maintaining a protective barrier against other contents of the intestinal lumen. The tight junctions are controlled by many proteins which aid in this protective process. Occludin and claudin-3 are amongst some of these proteins that have been shown *in vivo* to be targets of NEC (Clark et al. 2006). Cell death due to oxidative stress, apoptosis, or necrosis also plays a role in the intestinal barrier disruption process (Jilling et al 2004).

Most research carried out to date, to develop our understanding as to why premature infants are highly susceptible to developing NEC, has been focused on the infant intestinal tract. Many differences between the premature and the full-term gut have been observed which contribute to our understanding of the pathogenesis of the disease. Alterations in the maturity of the gastrointestinal immune system and bacterial colonisation seem to be the key differences and, therefore, major players in the onset of the disease (Tanner et al. 2015).

1.5.1 NEC and 'The Cross-Switching Hypothesis'

The intestinal epithelium of healthy term infants is pre-programmed to prevent activation of an excessive inflammatory response upon colonisation of healthy bacteria and their microbial associated molecular pattern molecules (MAMPs), which is known as immune-maturation. This pre-programmed response in healthy infants involves increased expression of negative regulators (both intracellular and on the surface) that prevent upregulation of Toll-like receptors (TLRs), particularly TLR2 and TLR4, which subsequently prevents the activation of nuclear factor kappaB (NFκB) as well as the onset of an inflammatory cytokine cascade beginning with IL-6, TNF-α and IL-1β (Lotz et al. 2006; Xiao et al. 2007; Melmed et al. 2003; and Claud

et al. 2004). It is believed, however, that preterm infants fail to develop this protective mechanism to colonising bacteria and respond inappropriately upon exposure (Nanthakumar et al. 2011). *In vitro* studies have demonstrated the different responses between term and preterm infants when exposed to healthy bacteria. Claud et al. (2004) reported the occurrence of an inflammatory response when primary fetal enterocyte cultures were exposed to healthy bacteria, however, no response was observed in studies when mature enterocytes, such as Caco-2 cells, were exposed to the same bacteria (Nanthakumar et al. 2000; and Fusunyan et al. 2001).

'The cross-switching hypothesis' provides a noble explanation as to why preterm infants fail to develop this protective mechanism and why NEC onset occurs upon bacterial colonisation. TLR4, an innate immune receptor that recognises lipopolysaccharide (LPS) on gram-negative bacteria, is required for gut differentiation and regulation of normal gut development *in utero* and is, therefore, expressed at high levels in the developing gut *in utero*. Premature infants are born before the gut has had time to fully develop and TLR4 levels remain elevated after birth. Colonisation of the intestinal tract begins immediately after birth, resulting in direct exposure of the distal ileum and colon to a substantial number of MAMPs. The immature mucosal immune system of preterm infants who failed to develop immune maturation responds inappropriately to this sudden exposure of MAMPs. The excessive TLR4 expression, following MAMP exposure, in the postnatal period of the premature gut promotes the release of inflammatory mediators and the onset of an inflammatory cascade and potentially the development of NEC. When *in utero* TLR4 signalling leads to destruction in the postnatal period it is referred to as 'the cross-switching hypothesis'. This hypothesis has been demonstrated in many rodent models of NEC, which suggest that overactive NFκB signalling and TLR expression, in particular TLR4, are the main culprits for NEC development in preterm infants (Jilling et al. 2006). Nanthakumar et al. (2011) observed a downregulation of the negative regulators that control NFκB signalling in NEC enterocytes (a type of IEC). Sodhi et al. (2010) observed that TLR4 deficient mice were protected against NEC, which suggests that upregulation of enterocyte TLR4 expression is required for intestinal inflammation. TLR4 upregulation in the premature intestine, upon subsequent colonisation of and exposure to gram-negative bacteria, results in reduced protection and increased exposure to pathogenic bacteria and to the contents of the lumen which may be toxic. This occurs

as a result of a decreased population of mucus-producing goblet cells in the premature intestinal tract (Sodhi et al. 2010; and Deplancke and Gaskins, 2011). Preterm infants also have immature tight junctions on the epithelial layer, increasing its permeability and allowing the entry of luminal contents, increasing potential for inflammatory signalling (Anand et al. 2007; and Liu et al. 2005). Moreover, immature enterocytes in preterm infants inhibit the ability of the intestine to clear the luminal contents, causing tissue damage. This can result in reduced blood flow and promote the development of intestinal ischaemia and necrosis. Severe NEC can induce overwhelming sepsis and even death (Neu et al. 2008; and Patole, 2007).

1.5.2 Pro-Inflammatory Cytokines Associated with NEC

As discussed, activation of TLR4 on the lining of the premature gut leads to enhanced pro-inflammatory cytokine release (Lu et al. 2014). Many cytokines and chemokines have been linked to an involvement in NEC progression, triggering a state of necrosis. The IL-8 chemokine directly activates neutrophils and controls the expression of neutrophil adhesion molecules, playing a key role in driving inflammation in NEC. Edelson et al. (1999) demonstrated the occurrence of an upregulation of serum IL-8 levels in the first 24 hours in cases of severe NEC. Following specimen collection, Nadler et al. 2001 also demonstrated an increase in IL-8 mRNA expression in the intestinal epithelium of infants with NEC compared to healthy infants or those with other inflammatory conditions. The pro-inflammatory cytokines, IL-1β, TNF-α, IL-6, IL-17 and IFN have also been shown to play a key role in disease onset and progression, as witnessed by their increase in serum concentrations and intestinal tissue in infants with NEC (Claud, 2009; Morecroft et al. 1994; Maheshwari et al. 2014; Edelson et al. 1999; and Ng et al. 2003). TNF-α and IL-1β contribute to the inflammatory state by activating the transcription factor, NF-κB, thereby, leading to increased expression of inflammatory genes (Claud, 2009).

1.5.2.1 IL-1β

The pro-inflammatory IL-1 cytokine has been linked to the systemic inflammatory response syndrome (SIRS), which, if not treated correctly, can result in sepsis, shock

and subsequently organ failure (Dinarello, Gelfand and Wolff, 1993). Shock is often associated with NEC and therefore, many studies on NEC in infancy have closely examined IL-1 for its role in the development and progression of the disease. IL-1β, particularly, has been found to be strongly associated with inflammatory disease and has been found in the intestinal tissue in patients with NEC and IBD (Viscardi et al. 1997; and McCabe et al. 1993). Human studies have demonstrated a 10-fold increase in ileal *IL-1β* mRNA in infants with NEC compared to healthy controls (Weitkamp et al. 2013). Many studies have shown that *IL-1β* mRNA is most abundant in stage III NEC infants compared with surgical controls (Edelson, Bagwell and Rozycki, 1999).

1.5.2.2 TNF-α

TNF-α is a pro-inflammatory cytokine known to have a knock-on effect and increase the circulation of other cytokines in the inflammatory cascade (Cho et al. 2016). It has also been indicated to have a key role in NEC progression. Rat models of NEC carried out demonstrated an immediate increase in both ileal and systemic TNF-α mRNA, following feeding (Seitz et al. 2005; and Cetinkaya et al. 2013). Many experimental models have demonstrated a link between matrix metalloproteinases (MMPs) and TNF-α. MMPs are significantly increased following TNF-α release in NEC (Pender et al. 2003 and 1998; and Markel et al, 2006). MMPs are proteolytic enzymes that degrade the extracellular matrix, resulting in tissue injury following necrosis of the intestine.

1.5.2.3 IL-6

IL-6 is also involved in the pathogenesis and progression of NEC. Moreover, IL-6 has been demonstrated to be a useful biomarker in NEC (Cho et al. 2016). A single nucleotide polymorphism (SNP), known as IL-6 rs1800795, that results in raised IL-6 plasma levels, has been identified in Caucasian neonates of 32 weeks gestation or less (Kilpinen et al. 2001). Neonates who had this SNP were six times more likely to develop NEC after birth and seven times more likely to progress to stage III of the disease when compared to those without the SNP (Franklin et al. 2015). Following examination of umbilical cord IL-6 concentration, Goepfert et al. (2004) demonstrated and proved the existence of a strong link between elevated IL-6 levels and the development of neonatal inflammatory disease such as NEC and SIRS. Analysis of cord blood IL-6 levels has also been useful as a predictive biomarker for determining a preterm infant's risk of NEC development. Furthermore, intestinal tissue of infants

with stage III NEC showed significantly enhanced IL-6 mRNA expression and protein levels when compared to healthy controls (Chan et al. 2012 and 2014). Therefore, IL-6 also reflects the clinical severity of NEC (Sharma et al. 2007; and Maheshwari et al. 2014). Morecroft et al. (1994) state that plasma concentration of IL-6, rather than TNF-α, is associated with and may dictate the severity of the disease and its symptoms.

1.5.2.4 IL-17

IL-17, the predominant Th17 pro-inflammatory cytokine, also contributes to the pathogenesis of NEC. Raised levels of intestinal IL-17A and IL-17 receptor A (IL-17RA) were observed in mice as well as human infants with NEC (Egan et al. 2016). Furthermore, destruction of tight junctions and crypt cells was observed in newborn mice who received IP injection of recombinant IL-17A. Namachivayam and colleagues (2013) also report that ileal *IL17A* mRNA expression was 5-fold higher in formula-fed preterm baboons with NEC when compared to healthy preterm controls.

1.5.2.5 IFN-γ

IFN-γ is another known cytokine which plays a role in NEC. This has been proven in an IFN-γ knock-out model, as IFN-γ knock-out mice do not develop symptoms, such as epithelial cell destruction, when exposed to the NEC model (Leaphart et al. 2007). Leaphart and colleagues (2008) further discovered that IFN-γ inhibits enterocyte migration, which may allow for the entry of invasive enteric pathogens to the epithelium and impairs healing of the damaged mucosa.

1.5.2.6 IL-10

As mentioned, IL-10 is essential for the regulation of the immune response and is essential for maintaining immune balance in the intestine, particularly in infants who have an immature gut, rendering them susceptible to inflammatory bowel disorders. It has been shown in infants and mice, that the onset of inflammatory bowel disease results from IL-10 deficiency. Emami et al. (2012) observed, in an experimental model of IL-10-deficient mice, that administration with IL-10 before the induction of NEC, prevented intestinal injury. Bioactive IL-10 is found in abundance in human breast milk. Moreover, increased NEC development in infants has been associated with reduced IL-10 levels in breast milk (Fituch et al. 2004). Although IL-10 deficiency has led to the pathogenesis of NEC, conflicting studies exist when it comes to its role in NEC progression. Despite the many observations of the beneficial role of IL-10 to

counterbalance the inflammatory response, increased levels of IL-10 have also been associated with severe NEC and increased morbidity (Edelson, Bagwell and Rozycki, 1999). It is, therefore, questionable whether administration of exogenous IL-10, or natural bioactive compounds that enhance IL-10 levels in NEC, would be harmful or beneficial for an infant with or recovering from NEC. It is a possibility that the immune system upregulates IL-10 during severe NEC in an attempt to switch off the excessive inflammatory cascade.

1.5.2.7 TGF-β

TGF-β has been demonstrated, in a range of animal models, to play a beneficial role in preventing the onset of inflammatory disorders and, therefore, also as an effective treatment for NEC infants. It is well known that NEC patients display low blood TGF-β levels (Maheshwari et al. 2011). Oz et al. (2004) reported improved clinical symptoms, reduction in inflammatory markers and resolved tissue pathology in the intestine in a mouse model of colitis, following oral administration with TGF-β alone. TGF-β suppresses the Th1 and Th2 populations in the gut by promoting apoptosis in activated T-cells, blocking T-cell trafficking, and enhancing Treg development (Wahl, 2007). TGF-β deficiency has also been shown to promote NEC onset (Maheshwari et al. 2011; and Namachivayam et al. 2013). Studies have shown that TGF-β-deficient mice develop mucosal and systemic inflammation within a few weeks after birth (Yoshinaga et al.2008). Blood TGF-β_1 is, therefore, now the primary predictive biomarker used to determine an infant's risk of developing NEC several weeks before progression. This allows for much earlier intervention and a more targeted therapeutic approach.

1.5.3 $CD4^+$ T-Cells and NEC Progression

Studies have identified a role for T lymphocytes in NEC development. It is understood that a Th17/Treg imbalance in the intestinal mucosa is involved in NEC progression (Niño et al. 2016). It is believed that TLR4 activation results in subsequent activation of Th17 cells and a parallel inhibition of Treg cells, leading to an imbalance between the two subsets. Inhibition of STAT3, essential for Th17 polarisation, in a murine model of NEC reduced Th17 cell numbers and increased Treg populations, supporting

the role of the Th17/Treg imbalance in NEC progression (Egan et al. 2016). Furthermore Cho et al. (2016) suggests that the Th17/Treg imbalance may in fact be essential for the onset of NEC as the Th17/Treg ratio was restored in TLR4 deficient mice in the study by Egan et al. (2016). This was accompanied by reduced infiltration of $CD4^+$ T cells that was associated with NEC mice. Despite these studies, there is currently insufficient evidence to support the role and relevance of Th polarisation in NEC.

1.5.4 Additional Factors Affecting NEC Development

As mentioned, poor bacterial colonisation and thus, an underdeveloped gut microbiome may have a role to play in NEC progression in premature infants (Collado et al. 2015; Anand et al. 2007; Vongbhavit et al. 2016; and Carlisle et al. 2013). It is understood that the more premature the infant, the slower the progression of bacterial colonisation (La Rosa et al. 2014). Many studies have demonstrated this decreased diversity in the gut microbiota of infants diagnosed with NEC when compared with healthy infants born at term (Carlisle et al. 2013). Vongbhavit and Underwood (2016) claim that higher numbers of harmful *Clostridiaceae* and *Enterobacteriaceae* and smaller populations of *Bifidobacteria* and *Bacteroidetes* (found in breast milk) are associated with preterm infants when compared with healthy infants born at term. However, it remains to be validated whether this gut dysbiosis in preterm infants is associated with NEC onset or whether it is a consequence of the disease itself.

1.6 Cow's Milk Protein Hydrolysates for The Management of Intestinal Immune Disorders in Infants

In newborns, immune homeostasis is prompted by breast milk constituents, such as IgA, and beneficial microbiota (*Bifidobacteria* and *Lactobacillus*). Although it is considered the gold standard nutrition source for infants in the first six months of life, breastfeeding is not always an option. When breast milk is limited or impossible, infant formula is the only alternative that fulfils the nutritional requirements of newborns during the first months of life. CM is the most popular choice and preferred source of

infant formula, as it is made up of many of the components found in breast milk that are required to support the developmental process of the infant and are abundant in all the essential amino acids (Bouma and Strober, 2003).

CM protein hydrolysates can also be of support and often used during neonatal development. Extensively hydrolysed milk formulas (EHF), which contain extensively hydrolysed casein or whey derived CM protein peptides, have been used for more than 50 years for infants who are unable to breastfeed and/or who suffer with intestinal immune disorders, such as CMPA and NEC (Baker et al. 2000). These hydrolysed formulas are produced by enzymatic or chemical hydrolysis of proteins, which leads to the pre-digestion or hydrolysis of protein into peptides, known as protein hydrolysates. Hydrolysis retains the high nutritional quality of the proteins, whilst also reducing their allergenic properties, which allows the infant to easily digest and absorb the protein. Hydrolysed formulas are beneficial and currently used for infants who are allergic to one or more of the intact proteins in CM, as they prevent allergic reactions in 90% of infants with CMPA (Brill, 2008). The allergic potential of a peptide is dependent on its size, as mentioned previously. The smaller the protein that is ingested, the less allergenic it will be to the infant (Kiewiet et al. 2015). Another use for hydrolysed formulas is in the refeeding process for infants recovering from NEC. While it is well known that maternal breast milk is the optimal feeding source for the refeeding process following NEC recovery, when it is unavailable EHF are used as the replacement. As infants recovering from NEC are at increased risk of developing CMPA, EHF, therefore, allow the infant to safely ingest protein without developing sensitisation to the protein and eliciting an allergic response (Thurreen, 1993).

Although hydrolysed formulas have been proven to be beneficial as an avoidance strategy in cases such as these, when produced in the right way, using selected enzymes for hydrolysis, they may in fact also be beneficial as a therapeutic prevention strategy in infants at high risk of developing allergy or relapse to NEC via modulation of the immune system. Some studies suggest that certain hydrolysates possess potential immunomodulatory ability to induce tolerance. As newborns are at high risk of developing allergy and preterm infants are susceptible to NEC and NEC relapse, they are a key focus group for the area of protein hydrolysate development for the induction of immune homeostasis via modulation of the immune response.

1.6.2 Immunomodulatory Protein Hydrolysates and Immune Homeostasis

Protein hydrolysates can possess various immunomodulatory activities. They have been shown to display protective effects against infection as well as aid in the development of immune tolerance, via modulation of the infant's immune system (Hernández-Ledesma et al. 2014). Previous studies have identified hydrolysates that have similar beneficial effects to breast milk on gut colonisation and display potential ability to promote oral tolerance. Among these hydrolysates, both *in vitro* and *in vivo* studies have shown that CM derived whey, casein and lactoferrin hydrolysates have positive modulatory effects on the immune system (Cross and Gill, 2000). Gauthier et al. (2009) observed an increase in IgA levels in non-infected as well as *E. coli* infected mice following administration of whey protein and its peptide hydrolysates, potentially increasing the infant's ability to acquire immune homeostasis and oral and self-tolerance. Some hydrolysates have been proven to improve mucosal immunity, which again, is the key to driving immune balance and oral tolerance. Lactoferrin is a major protein in breast milk, which provides support against infections through various biological activities, such as immunomodulation, and can also be found in CM (Lönnerdal, 2003). Hydrolysis of bovine lactoferrin with pepsin gives rise to peptides which demonstrate antibacterial activity (Liepke et al. 2002). Studies have shown that immunocompromised mice, treated orally with a pepsin lactoferrin hydrolysate, acquired increased levels of intestinal IgA, thereby supporting the prevention of the entry and growth of potential pathogens and promoting immune homeostasis (Debbabi et al. 1998; and Actor et al. 2009). Additionally, research has established that lactoferricin, a peptide derived from the hydrolysis of lactoferrin, can prevent the growth of bacteria and additionally, possesses the ability to kill bacteria (Tomita et al. 1991). This peptide is also known to inhibit gram-negative bacterial infection by damaging their outer membrane. It has been shown that lactoferricin prevents the attachment of enteropathogenic *E. coli* (EPEC), which results in diarrhoea, to intestinal cells by degrading the protein structures on its surface **(**Yamauchi et al. 1993; Edde et al. 2001; and Lönnerdal, 2003**).** Lactoferrampin, another bovine lactoferrin hydrolysate, has shown activity against *Bacillus subtilis, Escherichia coli* and *Pseudomonas aeruginosa* (Van der Kraan et al. 2004). Furthermore, a bovine lactoferricin-lactoferrampin hydrolysate mixture raised serum levels of IgA, IgG and IgM in piglets following administration, contributing further evidence to their support

in developing gut health and inducing immune homeostasis and oral tolerance (Tang et al. 2008).

1.7 FHI Approach and thesis outline

Food for Health Ireland (FHI) is an intelligent milk mining programme, which involves the collaboration of the Irish dairy industry and a number of Irish universities across the country. The overall aim of FHI is to produce and identify novel milk ingredients with proven health benefits. An extensive bioassay screening platform has led to the identification of a number of bioactive CM protein hydrolysates, which have potential to be beneficial in an array of health areas. The contribution of the fields of academia and industry yield maximum potential for this programme, due to a wealth of combined knowledge and expertise. In order to identify bioactive peptides that would be useful in the setting of food allergy or NEC, a range of CM hydrolysates were screened throughout this project. These hydrolysates were obtained by the technology team in the Department of Life Sciences, University of Limerick (UL), following enzymatic hydrolysis of sodium caseinate (NaCN) (89.2% (w/w) protein, Kerry, Listowel, Ireland) or whey protein concentrate (WPC80) (81% (w/w) protein, Carbery Food Ingredients (Ballineen, Ireland), as described by Nongonierma & FitzGerald, (2012). A list of first round hydrolysates and details of their hydrolysis conditions can be seen in **Table 1.3.** Once bioactivity was demonstrated at lab scale, FHI scaled the sample to pre-commercial quantities and bioactivity was confirmed again before the addition of sensory elements, food structure design and sometimes encapsulation to the research approach.

In this project, *in vitro* bioactivity of hydrolysates was assessed in three separate screening rounds. Round one involved an initial broad screen of all hydrolysates produced (known as parent hydrolysates) in undifferentiated $CD4^+$ T-cells. Based on their ability to modulate the relevant key T-cell subset priming cytokines involved in inflammation and allergy, candidate hydrolysates were chosen and further tested in *in vitro* assays of differentiated T-helper cell subsets; Th1, Th2 and Th17. In Round two, promising candidates were regenerated and re-screened in the relevant T-cell subset assays in order to confirm the reproducibility of their bioactivity. Following this confirmation, the technology team in UL liaised closely with the industry partners to

discuss potential optimisation strategies of the hydrolysates being brought forward for Round three screening. During the optimisation process a number of parameters were varied such as regulation of the pH during hydrolysis, utilization of different bases to regulate the pH (NaOH vs. KOH), variation in duration of the hydrolysis reaction and variation of the enzyme to substrate ratio. Optimisation plans were tailored for each hydrolysate and optimised hydrolysates were produced and tested in undifferentiated T-cells the relevant T-cell subsets as well as Treg cells once again. Finally, candidate hydrolysates were upscaled in larger quantities at industry level and their bioactivity was examined in *in vivo* murine models, to confirm their efficacy and assess their capacity to modulate the immune response and bring about immune homeostasis in models of allergy and intestinal inflammation. A diagrammatic representation of the outline of this project can be seen in **Figure 1.3**. This project was carried out as part of the Allergy and Immunity stream of the Infant Nutrition work package within FHI. This research stream was also made up of another research group in Dublin City University and a research group in the National University of Maynooth. Collectively the *in vitro* results from each of the research groups informed our decision of which hydrolysates to bring forward for *in vivo* testing.

Sample Short Code	*Degree of Hydrolysis (%)*	*Starting Substrate*	*Sample Description*	*Enzyme/Bacterial Source*
WPC80	*0.0*	*-*	*Negative Control*	*-*
NaCN	*0.0*	*-*	*Negative Control*	*-*
UL-2-01	*20.9*	*WPC80*	*Hydrolysate*	*Microbial*
UL-2-02	*22.0*	*WPC80*	*Hydrolysate*	*Aspergillus*
UL-2-03	*14.5*	*WPC80*	*Hydrolysate*	*Bacillus*
UL-2-05	*41.7*	*WPC80*	*Hydrolysate*	*Aspergillus*
UL-2-06	*18.2*	*WPC80*	*Hydrolysate*	*Bacillus*
UL-2-07	*0.0*	*WPC80*	*Hydrolysate*	*Rhizomucor*
UL-2-09	*8.2*	*WPC80*	*Hydrolysate*	*Bacillus*
UL-2-10	*15.4*	*WPC80*	*Hydrolysate*	*Aspergillus*
UL-2-11	*13.4*	*WPC80*	*Hydrolysate*	*Plant*
UL-2-12	*20.4*	*WPC80*	*Hydrolysate*	*Plant*
UL-2-13	*22*	*WPC80*	*Hydrolysate*	*Bacillus*
UL-2-14	*25*	*WPC80*	*Hydrolysate*	*Bacillus*
UL-2-15	*13.5*	*WPC80*	*Hydrolysate*	*Bacillus*
UL-2-16	*44.3*	*WPC80*	*Hydrolysate*	*Aspergillus*
UL-2-17	*33.2*	*WPC80*	*Hydrolysate*	*Aspergillus*
UL-2-18	*20.7*	*NaCN*	*Hydrolysate*	*Microbial*
UL-2-19	*7.9*	*NaCN*	*Hydrolysate*	*Plant*
UL-2-20	*39.4*	*NaCN*	*Hydrolysate*	*Aspergillus*
UL-2-21	*12.9*	*NaCN*	*Hydrolysate*	*Plant*
UL-2-22	*24*	*NaCN*	*Hydrolysate*	*Bacillus*
UL-2-23	*15.9*	*NaCN*	*Hydrolysate*	*Bacillus*
UL-2-24	*19.8*	*NaCN*	*Hydrolysate*	*Bacillus*
UL-2-25	*16.7*	*NaCN*	*Hydrolysate*	*Bacillus*
UL-2-26	*26.8*	*NaCN*	*Hydrolysate*	*Aspergillus*
UL-2-27	*17.8*	*NaCN*	*Hydrolysate*	*Bacillus*
UL-2-28	*0.0*	*NaCN*	*Hydrolysate*	*Rhizomucor*
UL-2-29	*33.1*	*NaCN*	*Hydrolysate*	*Aspergillus*
UL-2-30	*14.4*	*NaCN*	*Hydrolysate*	*Bacillus*
UL-2-31	*15.9*	*NaCN*	*Hydrolysate*	*Aspergillus*
UL-2-32	*27.3*	*NaCN*	*Hydrolysate*	*Aspergillus*
UL-2-33	*29.3*	*NaCN*	*Hydrolysate*	*Bacillus*
UL-2-34	*48.2*	*NaCN*	*Hydrolysate*	*Aspergillus*
UL-2-35	*18.8*	*NaCN*	*Hydrolysate*	*Aspergillus*
UL-2-36	*23.2*	*WPC80*	*Hydrolysate*	*Bacillus*
UL-2-37	*33.5*	*WPC80*	*Hydrolysate*	*Aspergillus*
UL-2-38	*14.5*	*WPC80*	*Hydrolysate*	*Aspergillus*
UL-2-39	*20.4*	*WPC80*	*Hydrolysate*	*Bacillus*
UL-2-40	*2.3*	*WPC80*	*Hydrolysate*	*Aspergillus*
UL-2-41	*2*	*WPC80*	*Hydrolysate*	*Aspergillus*
UL-2-42	*15.3*	*WPC80*	*Hydrolysate*	*Bacillus*
UL-2-43	*1.9*	*WPC80*	*Hydrolysate*	*Aspergillus*
UL-2-44	*19.8*	*NaCN*	*Hydrolysate*	*Bacillus*
UL-2-45	*0.0*	*NaCN*	*Hydrolysate*	*Aspergillus*
UL-2-46	*0.0*	*NaCN*	*Hydrolysate*	*Aspergillus*
UL-2-47	*12.8*	*NaCN*	*Hydrolysate*	*Bacillus*
UL-2-48	*0.6*	*NaCN*	*Hydrolysate*	*Aspergillus*

Table 1.3: Overview of protein hydrolysates screened in the first round of this project.

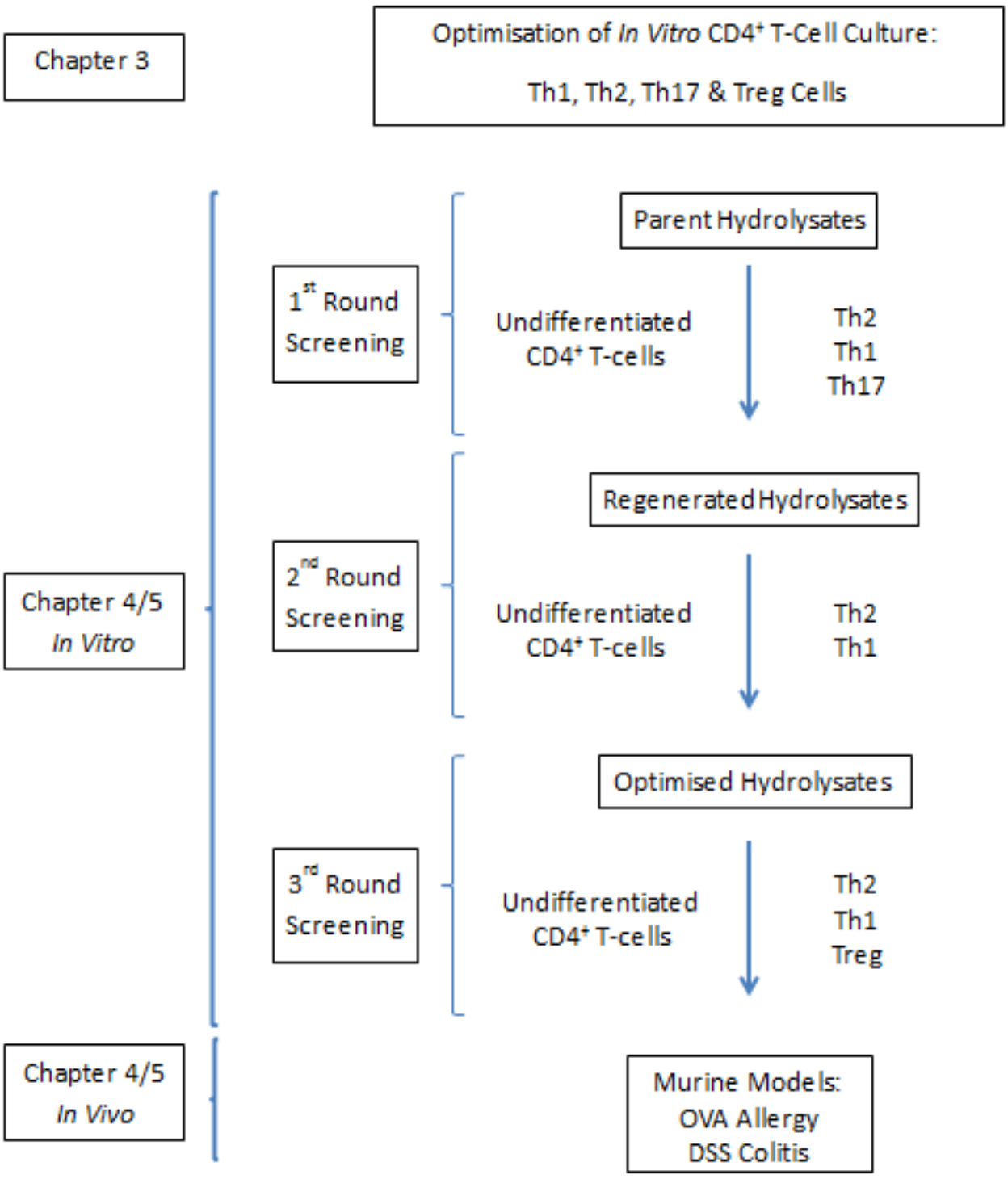


Figure 1.3: Diagrammatic representation of the outline of this project.

1.8 Aims and Objectives

Hydrolysed peptide formulas are a suitable method for infants to easily digest and absorb protein. Therefore, when produced in the right way, using selected enzymes for hydrolysis, they may be beneficial for infants who fail to establish immune balance and are at risk of developing allergy or inflammatory conditions, such as NEC. The removal of allergy causing and/or presence of certain immunomodulatory peptides in the hydrolysate can modulate the infant immune system for benefit so that they can safely ingest the protein and generate immune balance. Whilst the importance of breast milk is understood to be "the ideal source of nutrition for infants", according the World Health Organisation (WHO), they also believe in the development of breastfeeding substitutes when it is not possible. The WHO regard these human milk alternatives as safe and nutritious (World Health Organization, 1981).

The overall aim of this project is to identify novel candidate CM protein hydrolysates with positive immunomodulatory properties, which can prime the infant immune system from birth and thus, bring about immune homeostasis, preventing onset of subsequent disease associated with failed tolerance. Understanding of the underlying mechanisms that control homeostasis and dysbiosis of the gut microbiota represents an important step in our ability to reliably modulate the immune response and prevent the onset of the associated diseases with positive clinical outcomes. As it is well known that T-cells play critical effector roles in the development of immune disease, our objective is to observe T-cells in an over-active state, in order to assess the ability of CM hydrolysates to modulate this response. Candidate hydrolysates will be examined in *in vivo* mouse models, which will involve gut-associated pathology and mimic allergy and IBD. Their capacity as a treatment and ability to bring about immune balance and suppress the clinical symptoms associated with these conditions will be assessed. The outcome of this project is hoped to improve the nutrition, health and quality of life of infants, by improving the composition of infant milk formulas (IMF) with the addition of novel functional hydrolysates.

The main aims of this thesis were to:

- Optimise *in vitro* assays for each of the $CD4^+$ T-cell subsets to be used in the identification of CM hydrolysates which possess potential ability to modulate cytokine secretion and inhibit the polarisation of cells involved in allergy (Th2 cells) and NEC progression (Th1/Th17).

- Examine the ability of CM hydrolysates to suppress the priming and characteristic cytokines of the allergic Th2 subset *in vitro,* followed by confirmation of chosen candidate hydrolysates in an *in vivo* OVA food allergy model.

- Investigate the ability of CM hydrolysates to suppress the polarising and characteristic cytokines of the inflammatory Th1 and Th17 subsets *in vitro,* followed by confirmation of the bioactivity of chosen candidate hydrolysates in an *in vivo* DSS model of IBD.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

Cell Culture Reagents

Material	Source
Foetal Calf Serum	Gibco
RPMI-1640	Gibco
Penicillin Streptomycin/Glutamine	Gibco
Trypan blue (0.4% w/v)	Sigma-Aldrich
PBS	Gibco
Beta-mercaptoethanol	Sigma-Aldrich
Sterile Water	Sigma-Aldrich
Red Blood Cell Lysis Buffer	Sigma-Aldrich
Sterile Petri dishes	Nunc
6, 24 & 96 well tissue culture plates	Nunc
96 Well round bottomed plate	Starstedt
0.2 µm Cellulose Acetate Filters	Sigma-Aldrich
EDTA	Sigma-Aldrich
CellTiter 96™ Aqueos One Solution	Promega

Table 2.1 Cell Culture Materials. All cell culture materials/reagents used and corresponding sources.

T-Cell Differentiation Reagents

Material	Source
rmIL-2	R&D Systems
rmIL-4	R&D Systems
rmIL-6	R&D Systems
rmIL-12	R&D Systems
rmIL-23	R&D Systems
rhTGF-β	R&D Systems
Anti-mouse IL-4 neutralising antibody	R&D Systems
Anti-mouse IFN-γ neutralising antibody	R&D Systems
Anti-mouse CD3 monoclonal antibody	BD
Anti-mouse CD28 monoclonal antibody	BD

Table 2.2 Differentiating Cytokines and Antibodies: All differentiating reagents used and corresponding sources.

ELISA Reagents

Material	Source
96-well microtiter plate	Nunc
3,3',5,5'-tetramethyl-benzidineTMB	BD
Tween 20	Sigma-Aldrich
Bovine Serum Albumin	Sigma-Aldrich
Sodium Azide (NaN3)	Sigma-Aldrich
ELISA Duoset kits	R&D Systems
TGF beta-1 Human/Mouse Uncoated ELISA Kit	eBioscience
Mouse IgG1 Uncoated ELISA kit	Invitrogen
DPBS	Gibco

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

Flow Cytometry Reagents

Material	Source
FACS Flow	BD
FACS RINSE	BD
FACS Clean	BD
37% (v/v) paraformaldehyde	Sigma-Aldrich
Sodium Azide	Sigma-Aldrich
EDTA	Sigma-Aldrich
Triton X-100	Sigma-Aldrich
Mouse Regulatory T Cell Staining Kit #2	eBioscience
Ant-CD4 antibody	eBioscience
Anti-CD25 antibody	eBioscience
Anti- Foxp3 antibody	eBioscience
Anti-IL-13 antibody	eBioscience
Anti-IFN-γ antibody	eBioscience
Viability Die	eBioscience
CompBeads	BD

Table 2.4 Flow Cytometry Reagents. All flow cytometry reagents used and corresponding sources.

RNA Isolation and cDNA Synthesis

Material	Source
Nuceleospin RNA II Columns	Macherey-Nagel
DEPC treated water	Invitrogen
High Capacity cDNA Reverse Transcription Kit	Applied Biosystems
Molecular grade ethanol	Sigma-Aldrich
RNaseZAP	Sigma-Aldrich
RNA later	Sigma-Aldrich

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

qPCR Reagents

Material	Source
Taqman® Universal Mastermix	Applied Biosystems
SYBR® Green Matermix	Roche
Microamp® Optical Adhesive film	Applied Biosystems
Microamp® Optical 96-well plate	Applied Biosystems
GUSB PrimeTime qPCR Primers	IDT
IL-1β PrimeTime qPCR Primers	IDT
TNF-α PrimeTime qPCR Primers	IDT
IL-6 PrimeTime qPCR Primers	IDT
IFN-λ PrimeTime qPCR Primers	IDT
IL-17 PrimeTime qPCR Primers	IDT
IL-4 PrimeTime qPCR Primers	IDT
IL-10 PrimeTime qPCR Primers	IDT
IL-13 PrimeTime qPCR Primers	IDT

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

Gel Electrophoresis Reagents

Material	Source
Agarose	Thermo-Fisher Scientific
SYBR® Safe DNA Gel Stain	Applied Biosystems
Gene-ruler 100 bp plus DNA ladder	Thermo-Fisher Scientific
RNA Sample Loading Buffer	Sigma-Aldrich

Table 2.7 Gel Electrophoresis Reagents. All gel electrophoresis reagents used and corresponding sources.

Immunohistochemistry Reagents (Tissue processing, paraffin embedding and H&E Staining)

Material	Source
Paraffin wax	VWR
Formalin	Sigma-Aldrich
Xylene	Sigma-Aldrich
HCL	Sigma-Aldrich
Sodium Bicarbonate	Sigma-Aldrich
Ethanol	Merck
Harris Haematoxylin	Sigma-Aldrich
Eosin	Sigma-Aldrich
Histo-clear	National Diagnostics
Histobond microscope slides	RA Lamb

Table 2.8 Immunohistochemistry Reagents. All immunohistochemistry reagents used and corresponding sources.

Reagents for *In Vivo* OVA and DSS Murine Models

Material	Source
OVA – Albumin from chicken egg white	Sigma-Aldrich
Imject Aluminium Hydroxide	Thermo-Fisher Scientific
Dextran Sulfate Sodium	Sigma-Aldrich

Table 2.9 OVA and DSS Reagents. All reagents used for *in vivo* models and corresponding sources.

2.2 PREPARATION OF THE MILK PROTEIN HYDROLYSATES

The milk protein hydrolysates were produced by Professor Richard Fitzgerald's research group: Department of Life Sciences, University of Limerick, Limerick, Ireland. Hydrolysates were obtained following enzymatic hydrolysis of sodium caseinate (89.2% (w/w) protein, Kerry, Listowel, Ireland) or whey protein concentrate (81% (w/w) protein, Carbery Food Ingredients (Ballineen, Ireland), as previously described (Nongonierma & FitzGerald, 2012). Briefly, the milk protein substrates were rehydrated in distilled water at 10% (w/w) protein at 50°C for 60 min. Their pH was adjusted to 7.0 with the addition of 0.1M NaOH (VWR, Dublin, Ireland). The commercial enzyme preparation was subsequently added to the protein solutions at an enzyme to substrate ratio of 2.0 % (w/w). Hydrolysis was conducted at 50°C for 180 min without pH regulation. The enzyme was inactivated using a water bath set at 90°C for 20 min. Samples were freeze-dried (FreeZone 18L, Labconco, Kansas City, MO, USA) and stored at -20°C prior to further analysis.

A number of physicochemical characterizations were carried out by the technology team in order to check reproducibility and maintain consistency between the hydrolysate batches produced at small scale (Nongonierma et al. 2017). The degree of hydrolysis (DH) of the hydrolysates was determined in triplicate using the 2,4,6-Trinitrobenzenesulfonic acid (TNBS) method as per Le Maux et al. (2016). The molecular mass of hydrolysates was determined by gel permeation high performance liquid chromatography (GP-HPLC) and the peptide profile was determined by reverse-phase high performance liquid chromatography (RP-HPLC). The same physicochemical checks were also carried out on the samples produced at large scale to ensure that the hydrolysis protocol could be transferred to large scale and that the hydrolysates can be remade in food companies.

2.2.1 MILK PROTEIN HYDROLYSATE RESUSPENSION FOR CELL CULTURE

Hydrolysate powders were stored at -20°C upon receipt. Before dissolving the powders, samples were allowed to equilibrate at room temperature (RT) in order to avoid water condensation on the powder. The amount of powder required (0.015g) was carefully weighed out. The powder was then dispersed in 1.5ml sterile water

(stock concentration - 10mg/ml) using gentle agitation. In order to avoid clumping of the samples in certain instances, the freeze-dried powder was added slowly to gently stirring sterile water. Samples were then put on a rotator for 15 – 30 min at RT to ensure complete dispersion. Following this, the dispersed powders were centrifuged at RT at 5000.g for 5 min and supernatant was collected. The supernatant was filtered through a 0.2 µm cellulose acetate filter. The final sample was then added at a working concentration of 1 mg/ml to cells.

2.3 CELL CULTURE

All cell culture was carried out in a class II laminar airflow unit (Holten 2010 – ThermoElectron Corporation, OH, USA). Strict aseptic techniques were adhered to at all times. The laminar flow cabinet was swabbed with 70% industrial methylated spirits (IMS) before and after use, as were all the items used in the cabinet. The cabinet was cleaned each week with industrial detergents (Virkon, Antec. International; TEGO, TH. Goldschmidt Ltd). Cell cultures were maintained in a 37°C incubator with 5% CO_2 and 95% humidified air (Model 381 -Thermo Electron Corporation OH USA). Cells were grown in complete DMEM (Sigma-Aldrich) or DMEM-glutamax (Biosciences) supplemented with 10% FBS and 2% penicillin-streptomycin/glutamine. FCS was heat-inactivated (56°C for 30 min) to inactivate complement and aliquoted for storage at -20 °C. Supplemented medium was stored at 4 °C for no longer than one month.

2.3.1 CELL COUNTING

Cell counting and viability determinations were carried out using the trypan-blue (Sigma-Aldrich) dye exclusion technique. This test is based on the ability of viable cells to actively exclude dye as a result of having an intact cell membrane. Dead cells are unable to exclude the dye and appear blue when viewed under a microscope. 100 µl of cell suspension was mixed with 150 µl PBS and 250 µl of trypan blue solution. After 3 min incubation at RT, a sample of this mixture was applied to the chamber of a haemocytometer over which a glass cover slip had been placed. Cells in the 16 squares of the four outer corner grids of the chamber were counted microscopically. An average per corner grid was calculated.

A viable cell count was determined using the following formula:

$$\text{Cell/ml} = N \times 5 \times 10^4$$

Where, N = average cell number counted, 5 = dilution factor, and 10^4 = constant.

2.4 PRIMARY CELL ISOLATION

2.4.1 MICE

BALB/c mice, 6-8 weeks old, were purchased from Charles River. Mice were kept under specific pathogen-free conditions at the Bioresource Unit, Faculty of Science and Health, Dublin City University, Ireland. All mice were maintained according to the guidelines of the Irish Department of Children and Health.

2.4.2 ISOLATION OF $CD4^+$ T-CELLS

Primary T-cells were isolated aseptically from female Balb/c mice aged 6-12 weeks. All mice were sourced from Charles River and housed in a Specific Pathogen Free (SPF) unit.

2.4.2.1 SPLENOCYTE ISOLATION

$CD4^+$ T-cells develop in the thymus and reside in the spleen and lymph nodes. In order to isolate these cells from BALB/c mice, the spleen was removed from the mouse aseptically and collected in RPMI/10% (v/v) FBS on ice. A single cell suspension was achieved by filtering each spleen through a cell strainer (40µm, BD falcon). Cells were then washed with RPMI/10% (v/v) FBS and counted. The splenocytes were then resuspended in fully supplemented RPMI and T-helper cells were isolated using the EasySep™ mouse $CD4^+$ T-cell isolation kit available from Stemcell Technologies as described in the materials and methods.

2.4.2.2 $CD4^+$ T-CELL MAGNETIC PARTICLE ISOLATION

(Stemcell Technologies - EasySep© Mouse CD4+ T-cell isolation kit #19852)
Cells were prepared in recommended medium at a concentration of $1x10^6$ cells/ml. The cell solution was then placed in a 5ml polystyrene falcon tube (BD Biosciences). The EasySep™ mouse $CD4^+$ T-cell enrichment cocktail was then added at 50µl/ml of

cells, mixed well and left at RT for 10 min. The cocktail contains a selection of antibodies targeting cell surface markers of all cells excluding T-cells. The cocktail for the CD4+ T-cell isolation consisted of antibodies directed against all non- CD4+ cells: CD8, CD11b, CD11c, CD19, CD24, CD45R, CD49b and TER119. The EasySep™ streptavidin rapidspheres™ were vortexed well, added at 75µl/ml of cells, mixed well and left at RT for 2.5 min. The suspension was then made up to 2.5mls total volume and mixed gently before placing in the EasySep™ magnet at RT for 2.5 min. The supernatant that was poured off contained the CD4+ T-cells and naïve CD4+ T-cells. These cells were centrifuged at 300g for 5min, enumerated and resuspended in cRPMI media.

2.4.2.3 REGULATORY T-CELL ISOLATION

(Stemcell Technologies - EasySep™ Mouse CD25 Regulatory T Cell Positive Selection Kit #18782)

Regulatory T-cells (Treg cells) were isolated from splenocytes using the kit above. This kit targets CD25 Treg cells for positive selection with a PE-labeled antibody directed against the CD25 surface marker. Desired cells are labeled with antibodies and magnetic particles. Cells were prepared in recommended medium at a concentration of $1x10^6$ cells/ml. The cell solution was then placed in a 5ml polystyrene falcon tube (BD Biosciences). The FcR blocker was then added at 50 µl/ml of cells, mixed well and left at RT for 5 min. The Positive Selection Cocktail was next added at 50 µl/ml of cells, mixed well and left at RT for 5 min. The PE Selection Cocktail was added at 20 µl/ml of cells, mixed well and left at RT for 5 min. This is a PE-labeled antibody which is directed against the CD25 surface marker. The Rapidspheres™ were vortexed well, added at 20 µl/ml of cells, mixed well and left at RT for 5 min. The suspension was then made up to 2.5 mls total volume and mixed gently before placing in the EasySep™ magnet at RT for 15 min. The supernatant was discarded and the suspension was made up to 2.5 mls and placed in the EasySep™ magnet at RT for 15 min. This was repeated one more time. Cells were then resuspended in the desired medium.

2.4.2.4 POLARISATION OF TH1, TH2 AND TH17 CD4⁺ T-CELLS

Cells were stimulated using agonist antibodies directed to CD3 (clone: 145-2C11 BD bioscience) and CD28 (clone: 37.51 BD). Plates were coated with 5 µg/ml anti-CD3 for 2 hours at 37ºC. 5 µg/ml anti-CD28 was added to cells. Cells were stimulated in the presence of polarising cytokines. The cocktails used to drive specific T-cell subsets and their concentrations are represented in **Table 2.10** on the next page. After 2 hours, cells were transferred to coated anti-CD3 plates and incubated for 72 hours at 37ºC. Undifferentiated T cells were harvested after 72 hours. All media was removed from cells being driven into the relevant T cell subsets and replaced with fresh media (rested). New anti-CD3 plates were coated (2.5µg/ml) for 2 hours at 37ºC. Anti-CD28 (2.5µg/ml) was added to cells which were then transferred to the coated plates (re-stimulated) and incubated for 24 hours at 37ºC. All cells were harvested.

Phenotype	Cocktail	Concentration	Source
Th1	rmIL-12	10ng/ml	R&D
	anti-IL-4	10µg/ml	R&D
Th2	rmIL-2	10ng/ml	R&D
	rmIL-4	10ng/ml	R&D
	anti-IFNγ	10ug/ml	R&D
Th17	rmIL-6	20ng/ml	R&D
	rmIL-23	10ng/ml	R&D
	rhTGF- β	2ng/ml	R&D
	anti-IFNγ	10µg/ml	R&D
	anti-IL-4	10µg/ml	R&D

Table 2.10: List of polarising cocktails and concentrations

2.5 MTS VIABILITY ASSAY

Cell viability was determined by the MTS assay (Promega). The CellTiter 96® AQueous Assay is composed of solutions of a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine methosulfate) PMS. MTS is bioreduced by cells into a formazan product that is soluble

in tissue culture medium. The absorbance of the formazan product at 490 nm can be measured directly from 96-well assay plates without additional processing. Briefly, 100 µl cell suspension (containing 1 x 106 cells/ml) was plated in a 96-well plate. At the end of each experiment the cell proliferation reagent MTS (20 µl) was added to each well, and the cells were incubated at 37°C for 1hr. A490nm was measured using a VERSA Amax microplate reader (Molecular Devices, CA, USA). Results are expressed as a percentage of control cells.

2.6 ENZYME-LINKED IMMUNO-SORBENT ASSAY (ELISA)

2.6.1 BASIC PRINCIPLES OF ELISA

Enzyme-Linked Immuno-Sorbent Assay (ELISA) is a sensitive and accurate method used to measure antigen concentration in an unknown sample. The method of ELISA used in all studies was a sandwich ELISA to measure the amount of cytokines in cell supernatants or antibodies in blood serum. To detect the antigen, the wells of 96-well microtiter plates were coated with a capture antibody specific to the target molecule (diluted in PBS). Samples containing an unknown concentration of the target antigen were then added to the wells along with a series of diluted standards of a known concentration of recombinant target protein. An antigen-specific antibody (secondary antibody) conjugated to an enzyme (usually biotin) was added to the wells. A detection reagent, streptavidin horseradish-peroxidase (HRP) was then added to bind to it. The amount of HRP is proportional to the amount of target antigen in the sample. Tetramethylbenzidine (TMB) was finally added. The HRP enzyme catalyses the oxidation of the TMB substrate, transforming it to a blue compound. The intensity of the colour is proportional to the concentration of target antigen present. Once the desired colour intensity was reached, sulphuric acid was added to stop the reaction and prevent further oxidation. This alters the pH and is distinguished by a change in colour from blue to yellow. The optical density (O.D.) of the colour was measured on a microplate reader. A standard curve was generated and the concentrations of antigen present in the unknown samples were calculated from this. This process is summarised in **Figure 2.1**.

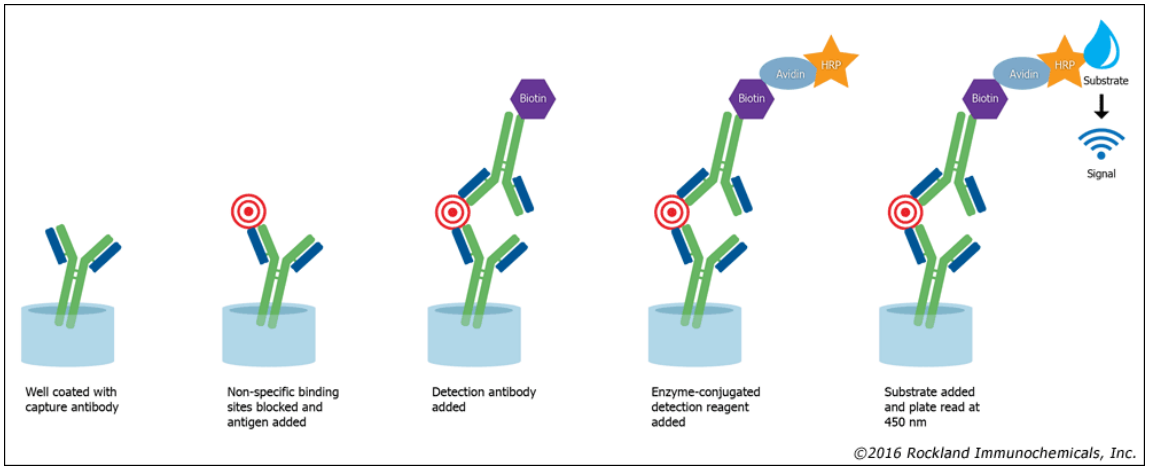


Figure 2.1 Schematic representation of sandwich ELISA. Schematic taken from ELISA-Kits on www.rockland-inc.com

2.6.2 CYTOKINE ELISA

The concentration of cytokines IFN-γ, IL-2, IL-4, IL-6, IL-10, IL-13, IL-17, IL-1β and TNF-α were examined in cell using ELISA Duoset kits from R&D Systems in accordance with the manufacturers' instructions. A kit from eBioscience was used to measure TGF-β concentration. Briefly, 100 µl/well of capture antibody (diluted to the appropriate concentration in PBS) was added to a 96-well ELISA plate (Nunc) and incubated overnight at RT. Following a washing step in Wash Buffer (PBS + 0.05% Tween-20), to remove unbound antibody, wells were then blocked in 300 µl of Blocking buffer (1% (w/v) BSA/PBS) for a minimum of 1hr at RT to prevent non-specific binding. The washing step was carried out again and 100 µl of samples and standards (diluted to appropriate concentrations) were added per well, in triplicate. Plates were then incubated at 4ºC overnight. The plates were then washed again and 100 µl of the biotinylated detection antibody was added to each well and incubated for 2hr at RT. Plates were washed and 100 µl of Streptavidin-HRP (R&D) was then added to each well. Plates were incubated for 20 min at RT. Plates were washed for a final time and 100 µl of TMB (BD Biosciences) was added to each well and plates were left in dark for 20 min or until the blue colour develops. Colour development was stopped by adding 50 µl of 1M sulphuric acid (Sigma-Aldrich) to the wells. The optical density was determined at 450nm, using a VersaMax™ microplate reader (Molecular Devices). The cytokine concentrations in the supernatants were determined from the standard curves.

2.6.3 IL-1β, IFN-γ and IL-2 ELISA

The method above was followed with two deviations: Blocking buffer used was 1% (w/v) BSA/PBS + 0.05% (w/v) NaN3. The reagent diluent was 0.1% (w/v) BSA/TBS + 0.05% (v/v) Tween-20.

2.6.4 TGF-β ELISA

The method above was followed using a TGF beta-1 Human/Mouse Uncoated ELISA Kit with the following deviations: 1X ELISA/ELISPOT Diluent was used as reagent diluent and blocking buffer. Acid Activation of Samples was carried out to activate latent TGF-ß1 to the immunoreactive form. The samples were acidified (per 100 ul of sample, 20 ul of 1N HCl was added for 10 min at RT) and then neutralized by adding 20 ul of 1N NaOH.

2.6.5 IgG1 ELISA

The concentration of IgG1 antibody in blood serum was determined using a IgG1 Mouse Uncoated ELISA Kit from Invitrogen in accordance with the manufacturers' instructions. This kit is also based on the "sandwich" ELISA method and was carried out as per protocol above.

2.7 FLOW CYTOMETRY

2.7.1 BASIC PRINCIPLES OF FLOW CYTOMETRY

Flow cytometry simultaneously measures and analyses multiple physical characteristics of single cells, as they flow in a fluid stream through a beam of light. Particle size, granularity or internal complexity, and relative fluorescence intensity are amongst the parameters that can be measured. This can be done using an optical-to-electronic coupling system that records how the cell or particle scatters incident laser light and emits fluorescence. A flow cytometer is made up of three main systems: fluidics, optics, and electronics. The fluidics system transports particles in a stream to the laser beam for interrogation. The optics system consists of lasers to illuminate the particles in the sample stream and optical filters to direct the resulting light signals to the appropriate detectors. The electronics system converts the detected light signals

into electronic signals, which is then processed by the computer. **Figure 2.2** outlines a schematic representation of these systems.

When a cell passes through the laser beam, laser light is scattered in all directions. The machine measures light that scatters in two directions: forward scatter (FSC) and side scatter (SSC). FSC is light that scatters axial to the laser beam and SSC is light that scatters perpendicular to the laser beam. FSC and SSC are related to certain physical properties of cells. FSC is proportional to the cell-surface area or size, while SSC is proportional to cell granularity or internal complexity.

Cells can be labelled with fluorescent probes to detect specific molecules internally or on their surface. These probes are typically antibodies that target specific cellular antigens with a covalently attached fluorochrome. A fluorescent compound absorbs light energy over a range of wavelengths that is characteristic for that compound. This absorption of light causes an electron in the fluorescent compound to become excited and fluoresces (emits the excess energy as a photon of light). The amount of fluorescent signal detected is proportional to the number of fluorochrome molecules on the particle.

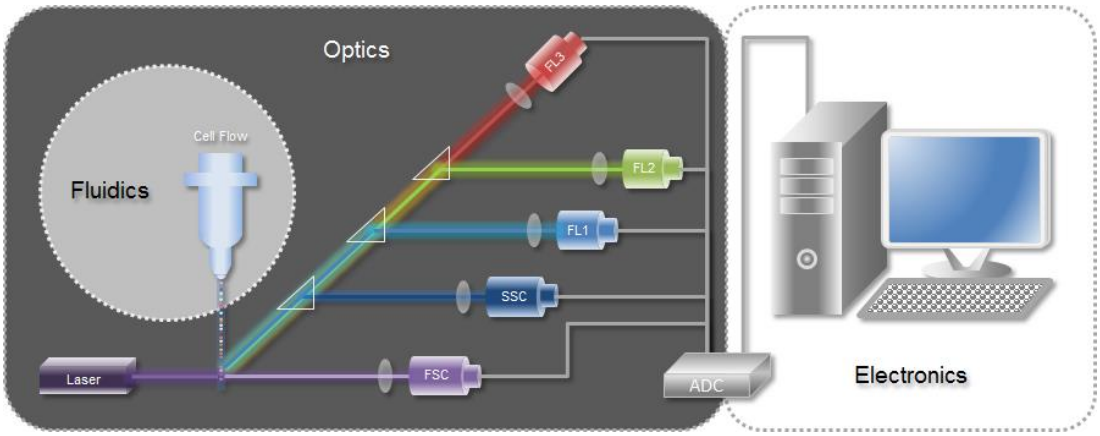


Figure 2.2 Basic outline of a flow cytometer. Schematic taken from Flow Cytometry/FACS Introduction on http://www.sinobiological.com/flow-cytometry-fcm-facs-introduction.html

2.7.2 CELL SURFACE STAINING

Cells were removed from tissue culture and placed in a 96-well round bottomed plate (~ $2.5x10^5$ cells per well) for staining. Cells were blocked with an equal amount of foetal bovine serum for 15 min at RT to prevent binding of non-specific antibodies. The plate was then centrifuged at 2000 rpm for 5 min to pellet cells. The supernatant was removed and the cell pellets were washed in PBS. The plate was centrifuged again and cells were then stained with fixable Viability Dye (eBioscience, San Diego, CA, USA) for 30 min at 4 ºC protected from light, to exclude dead cells. The cells were washed twice with Flow Cytometry Staining Buffer (supplied in eBioscience™ Mouse Regulatory T Cell Staining Kit #2). Cells were then resuspended in 100 µl of fluorochrome-conjugated antibodies for 30 min at 4 ºC protected from light. Cells were then washed twice with Flow Cytometry Staining Buffer to remove the unbound antibodies and were analysed using a FACSAria I (BD). All the flow cytometry data was analysed using FlowJo software (Treestar). Before reading cells, cytometer setup and tracking beads were ran on the cytometer. Cells were also stained with compensation beads in order to carry out compensation. These cells were also stained with Anti-Rat/Hamster FITC, PE and APC fluorophores in addition to compensation beads to prevent spectral overlap between these fluorochromes during analysis. Compensation gating was then carried out using the FlowJo software, prior to analysis. There were a number of controls used during cell staining. The first was an unstained control, in which cells were left unstained in Staining Buffer as a negative control and to measure autofluorescence. An internal negative control was also used. Here, undifferentiated T-cells, which express a much lower concentration of the marker/antigen being measured (for example, Foxp3), were stained to avoid a false positive result from non-specific antibody binding. A number of antibody controls were also used during staining. Fluorescence Minus One (FMO) controls were used for gating purposes and determining populations present. These included a FITC FMO, PE FMO and APC FMO. The final control included cells that were stained only with fixable viability to determine non-specific binding of the antibody and to gate out and exclude dead cells from analysis.

2.7.3 STAINING ANTIBODY TITRATION

All antibodies used were titrated to acquire the best possible fluorescent signal using the least amount of antibody possible. Antibody titrations result in efficient staining and a limit background detection and steric hindrance where bulky fluorophores such as Phycoerythrin (PE) can block potential binding sites when using multi-colour flow cytometry analysis. This most commonly occurs during intracellular staining. Antibodies titrated included FITC conjugated Anti-Mouse CD4, PE conjugated Anti-Mouse CD25 and APC conjugated Anti-Mouse/Rat Foxp3. Cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks. Erythrocytes were lysed using a red blood cell lysing buffer (SIGMA). Cells were stained with a range of antibody concentrations (CD4 and Foxp3: 0.125, 0.25, 0.5, 1, 2 µg/100µl; and CD25: 0.0625, 0.125, 0.25, 0.5, 1 µg/100µl). Cells were analysed on BD FACS Aria, data was analysed using Flowjo software (treestar). The Mean Fluorescence Intensity (MFI) was measured and the log MFI/the stain index was plotted for each concentration of antibody examined. The stain index is the ratio of the separation between the positive and negative population divided by two times the standard deviation of the negative population. The dilution with the best stain index results in the least amount of background and more specific staining. See **Section 3.2.3.1** for further details. Each curve shows a point of saturation and a concentration of each antibody titrated was selected after this point.

2.7.4 INTRACELLULAR TRANSCRIPTION FACTOR AND CYTOKINE STAINING

In order to stain intracellular antigens, the cell must be permeabilised to allow binding of the antibody to the intracellular antigen. The eBioscience™ Mouse Regulatory T Cell Staining Kit #2 (#88-8118-40) from Thermo-Fisher Scientific was used in accordance with the manufacturers' instructions. Cells were washed once with PBS and then removed from tissue culture plates with a cell scraper. Cells were then placed in 96-well round bottomed plates at a concentration of ~ $2.5x10^5$ cells per well. Cells were then blocked with an equal amount of foetal bovine serum for 15 min at RT to prevent binding of non-specific antibodies. The plate was then centrifuged at 2000rpm to pellet cells and cells were washed twice with Flow Cytometry Staining Buffer (from kit). Cells were surface stained, as per protocol above, prior to permeabilisation. Cells were washed twice with Flow Cytometry Staining Buffer and then permeabilised

simultaneously in Fixation/Permeabilization Diluent (from kit) for 30 min at 4°C in the dark. Following this, cells were washed twice in 1X Permeabilization Buffer (from kit). Cells were incubated with fluorescently conjugated antibodies for 30 min at 4°C. Antibodies were diluted in Permeabilisation Buffer to ensure cells remained permeable. Cells were then washed twice with Permeabilisation Buffer to remove the unbound antibodies and were resuspended in staining buffer prior to being analysed using a FACSAria I (BD). All the flow cytometry data was analysed using FlowJo software (Treestar).

2.8 RNA ANALYSIS

2.8.1 RNA ISOLATION FROM COLONIC TISSUE

Colonic tissue (20-30 mg) was homogenised in Lysis buffer RA1 (Macherey-Nagel) containing β-mercaptoethanol (β-ME) using the Qiagen TissueLyser LT for 5 min at 50 Hz. Total RNA was then isolated using a Nucleospin RNA II kit (Macherey-Nagel) according to manufacturer's instructions. Briefly, tissue was disrupted again by passing up and down through a 19.5 g needle. The viscosity of the lysate was cleared by filtration through a NucleoSpin® filter and centrifuging for 1min at 11,000 x g. 70% ethanol was added to the homogenised lysate to adjust RNA binding conditions. The NucleoSpin® RNA II column was then 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 a DNase reaction mixture at RT for 15 min. After 15 min Buffer RA2 and RA3 were added to inactivate rDNase and wash the column. The NucleoSpin® RNA II column was then placed into a nuclease free collection tube and RNA was eluted in RNase-free H_2O. The concentration of RNA was then measured using the Nanodrop® ND-1000 (Thermo-Fisher Scientific). The purity of the RNA was determined using 260nm and 280nm absorption wavelengths. All the RNA used in this study had an A280/A260 ratio between 1.8 and 2.1.

2.8.2 CDNA SYNTHESIS

Complementary DNA (cDNA) is generated from RNA using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, USA). This kit contains a master mix of dNTP, random primers and the enzyme reverse transcriptase. Reverse transcriptase

synthesises single strain cDNA using the RNA strands as a template. This is then used as the template for the subsequent qPCR experiments. 2 µg of RNA was used in each cDNA synthesis reaction. **Table 2.11** displays the settings used in the reaction on a PTC-200 PCR thermal cycler (MJ Research).

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature (°C)	25	37	85	4
Time (min)	10	120	5	∞

Table 2.11 cDNA Thermal cycling conditions

2.8.3 BASIC PRINCIPLES OF QUANTITATIVE REAL TIME PCR (qPCR)

Real-time quantitative polymerase chain reaction (qPCR) is a molecular biology method which uses PCR to amplify (copy) specific sequences within a DNA or cDNA template. Detection and quantitation of the amplified sequence are performed at the end of the reaction after the last PCR cycle in traditional PCR, and require post-PCR analysis such as gel electrophoresis and image analysis. An advantage of qPCR is that the amount of PCR product is measured at each cycle. It is, therefore, possible to accurately determine the initial amount of target due to the ability to monitor the reaction during its exponential phase. Fluorescent markers that are incorporated into the PCR product are used to measure the amount of DNA after each cycle. The increase in fluorescent signal is directly proportional to the number of PCR product generated in the exponential phase of the reaction. Fluorescent reporters used include double-stranded DNA (dsDNA)-binding dyes, or dye molecules attached to PCR primers or probes. These fluorescent reagents can be sequence specific or non-sequence specific. The Taqman® assay was used in this thesis.

2.8.3.1 TAQMAN® ASSAY

TaqMan® probes require a pair of PCR primers in addition to a probe with both a reporter and a quencher dye attached. The probe is designed to bind to the sequence amplified by the primers. During qPCR, the probe is cleaved by the 5´ nuclease activity of the Taq DNA polymerase; this releases the reporter dye and generates a fluorescent signal that increases with each cycle. Because all three components (2

primers and 1 probe) must hybridize to the target, this method allows detection of the PCR product with greater specificity and higher accuracy.

2.8.4 QPCR PROTOCOL

This technique compares the relative expression of different samples, one of which is a calibrator or control (untreated tissue). PCR was prepared in triplicate for each sample by adding cDNA, Taqman® Gene Expression Mastermix (Applied Biosystems) and RNase free water (Invitrogen). All the primers were ordered from IDT and are listed in **Table 2.6** and **Appendix C**. Samples were added to a 96-well reaction plate (Applied Biosystems) and sealed with optically clear film (Applied Biosystems). Plates were centrifuged for 1 min at 1000g and run on the Lightcycler® 96 system (Roche) under the conditions shown in **Table 2.12.** The results were analysed using the ABI Prism sequence detection software (Applied Biosystems), the Lightcycler® 96 software (Roche) and Excel Software (Microsoft).

	Step 1	**Step 2**	**Step 3a**	**Step 3b**
Temp (°C)	50	95	95	60
Time	2min	10min	15sec*	15sec*
***Repeat for 40 cycles**				

Table 2.12 PCR Thermal cycling conditions.

2.8.5 PCR DATA ANALYSIS

For gene expression analysis we used a relative quantification method in which the gene levels are expressed as a fold difference between a sample and a calibrator such as untreated tissue (from healthy control mice). An endogenous control, Glucuronidase-β (GUSB), was used to normalise samples. This is a gene that maintains consistent expression levels despite treatment. Normalised samples were then compared to the calibrator (control mice) using ΔΔCt method:

$$\Delta\Delta Ct = \Delta Ct \text{ sample}^* - \Delta Ct \text{ calibrator}^{**}$$

*ΔCt sample = Ct sample - Ct endogenous control

**ΔCt calibrator = Ct calibrator/control - Ct endogenous control.

2.8.6 DNA PRODUCT ANALYSIS BY GEL ELECTROPHORESIS

Gel electrophoresis was used to check RNA integrity following qPCR **[Appendix C]**. Samples were run on a 2% agarose gel. The gel was prepared by dissolving 2 g of agarose (Termo-Fisher Scientific) in 100 ml 1X TAE buffer and heating to boiling point. After cooling, 10 µl of SYBR® safe DNA gel stain (Applied Biosystems) was added to the gel mix before pouring into a gel mould and setting the well comb. DNA samples were mixed with loading buffer (Fermentas) and loaded straight onto the gel, together with GeneRuler 100 bp DNA ladder (Termo-Fisher Scientific). Gels were run for 1hr in 1X TAE buffer at 100 V and visualised using the G-box imaging system (Syngene, UK). Intact RNA has two clear bands, corresponding to 28S and 18S subunits. The ratio of intensity of 28S to 18S should be 2:1.

2.9 *IN VIVO* MURINE MODELS

2.9.1 DEXTRAN SULFATE SODIUM (DSS) INDUCED MODEL OF COLITIS

Dextran sodium sulphate (DSS) model of colitis is one of the most widely used chemically induced models of inflammatory bowel disease. It is directly toxic to the colonic epithelial cells in the basal crypts and induces an acute inflammatory response (Kullmann et al. 2001). 30 BALB/c female mice were housed at the Biological Resource Unit (BRU), at Dublin City University. DSS was administered to mice in drinking water. DSS was prepared fresh every day in normal tap water at a final concentration of 5% (w/v). Mice were administered DSS for 7 days followed by daily intra-gastric (IG) gavage with PBS or hydrolysed cow's milk proteins in PBS treatment for 8 days. Mice were sacrificed on day 14 and organs were harvested. Mice were split into five groups for this study:

1. Control

No DSS was administered to the control mice. On day 7 – day 14 mice were given IG gavage of PBS (once daily). Animals were sacrificed for sample and tissue processing on day 14.

2. DSS

Mice were administered DSS for 7 days (day 0 – day 6) followed by 7 days of IG gavage with PBS (once daily) and were sacrificed for sample and tissue processing on day 14.

3. DSS + NaCN

Mice were administered DSS for 7 days (day 0 – day 6) followed by 7 days of IG gavage with whole NaCN protein in PBS (50 mg in 0.2 ml) once daily and were sacrificed for sample and tissue processing on day 14.

4. DSS + NaCN hydrolysate

Mice were administered DSS for 7 days (day 0 – day 6) followed by 7 days of IG gavage with hydrolysed NaCN protein in PBS (50 mg in 0.2 ml) once daily and were sacrificed for sample and tissue processing on day 14.

To assess the development of the disease, mice were weighed and scored (every day) for daily disease activity index (DDAI) based on body weight loss, stool consistency presence of occult blood, stool composition, fur texture and posture. **Table 2.13** details the scoring system used for the comparative analysis of intestinal bleeding. At the end point of each group the length & weight of each colon was also measured and used as an indication of colitis in the mouse model. Sections of distal colon were collected for tissue homogenisation and RNA purification to determine cytokine expression and for histological analysis. Spleens were collected for analysis of cytokine secretion patterns.

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

Table 2.13: Scoring system used to determine intestinal bleeding in the DSS model.

2.9.2 OVA INDUCED MODEL OF ALLERGY

The OVA model is associated with disease in the Th2 subset and is a rapid and very reliable food allergy model with literature reports involving the food allergy topics. Sensitisation to OVA using OVA and an aluminium hydroxide (Alum) adjuvant induces a Th2 response in mice.

24 BALC/c female mice were housed at the Biological Resource Unit (BRU), at Dublin City University. These mice were grouped into a non-sensitised control group of 6 mice and 3 test groups (also 6 mice each) with OVA-induced allergy. Two OVA-sensitised groups were treated with whole WPC80 or a hydrolysed WPC80 protein. Test groups received IP injection of OVA and aluminium hydroxide (50 µg and 1.5 mg, respectively) in warm PBS on days 1 and 14. The control group received PBS only. On days 16-20 mice received IG gavage once daily of WPC80 or WPC80 hydrolysate (50 mg in 0.2 ml PBS). Control and OVA-only groups received PBS IG gavage only. On days 21, 23, 25 and 27 mice were given an oral challenge, which involved IG gavage of OVA (50 mg) on these days. Control mice received PBS only. Mice were sacrificed an hour following the final oral challenge on day 27 and organs and tissues were recovered for analysis.

To assess the development of the disease, mice were weighed and scored every day during oral challenges for DDAI. **Table 2.14** (on the next page) details the scoring system used for the determining the DDAI during oral challenges. At the end point of each group sections of distal colon were collected for tissue homogenisation and RNA purification to determine cytokine expression and for histological analysis. Spleens were collected for analysis of cytokine secretion patterns and blood serum was collected to determine immunoglobulin antibody levels.

Signs	0	1	2	3
Activity	Normal	Isolated, abnormal posture, scratching and rubbing around nose and head	Huddled/inactive OR overactive	Moribund OR fitting
Alertness/Sleeping	Normal	Dull or depressed	Little response to handling	Unconscious
Body Weight	Normal weight & growth rate	Reduced growth rate	Weight loss >15% OR failure to grow	Weight loss of >20% OR failure to grow & weight loss
Breathing	Normal	Rapid, shallow	Rapid, abdominal breathing	Laboured, irregular, skin blue
Coat	Normal	Coat rough	Unkempt, wounds, hair thinning	Bleeding or infected wounds or severe hair loss or self-mutilation
Eyes	Normal	Wetness or dullness	Discharge	Eyelids matted
Faeces	Normal	Faeces moist	Loose, soiled perineum OR abnormally dry +/- mucus	Running out on handling OR no faeces for 48hrs OR frank blood on faeces
Nose	Normal	Wetness	Discharge	Coagulated

Table 2.14: OVA Model Template Welfare Sheet. Clinical signs and severity scores assigned to symptoms triggered following the oral challenges.

2.9.3 COLONIC TISSUE SECTIONING

Tissue sections of 0.5 cm were removed from the distal part of the washed colon and arranged into swiss roles before being left in formalin overnight at RT to fix. The swiss role allows colonic pathology in extended regions to be observed compared to standard longitudinal sections (Chassaing et al. 2014). Following overnight fixation the tissue was then put in a TP1020 processor (Leica Biosystems) for paraffin tissue processing. Briefly the steps included 1hr in 70% EtOH, 1hr in 96% EtOH, 1hr in 100% EtOH followed by 2hr in 100% EtOH. This ensures adequate dehydration of the tissue. The program continued with 1hr in xylene, 2hr in xylene to remove alcohol before paraffin embedding, and finally 3 x 1hr in paraffin. The tissue is then fixed in small paraffin blocks and left to set. 0.5 μm sections were cut from the blocks and put onto slides. The slides were left to dry and then baked at 50°C for at least 40 mins.

2.9.4 HAEMOTOXYLIN AND EOSIN STAINING

Prior to staining, the tissue was immersed in Histoclear (National Diagnostics) for 2 x 10 min to de-parrafinise it. Tissue was then gradually re-hydrated by immersion in 100% EtOH (2 x 5 min), 95% EtOH (2 min) and 75% EtOH (2 min). Slides were stained with Harris haematoxylin (Sigma-Aldrich) for 10 min and washed under running tap water for 5 min. The tissue was differentiated in 1% acid/alcohol for 30 s 3 times and then washed under a tap for 1 min. After washing slides were placed in 0.1% sodium bicarbonate (Sigma-Aldrich) for 1 min followed by washing under running tap water for 5 min. The slides were then rinsed in 95% ethanol (Merck) for 10 dips before counterstaining with Eosin (Sigma-Aldrich) for 1 min (dipping up and down). Finally the slides were dehydrated again by dipping in 75% ethanol for 3 min, 95% ethanol for 3 min (x2), followed by 100% ethanol for 3 min and 3 min in Histoclear. Slides were then mounted with mounting medium (DPX) and the cover slips pushed firmly to remove bubbles.

2.9.5 SERUM PREPARATION

Following cardiac puncture whole blood was collected in a covered test tube on ice. The blood was then left to clot by leaving it undisturbed at RT for 15–30 min. The blood was then centrifuged at 1000–2000 g for 10 min at 4 ˚C and serum was recovered (supernatant). The serum was transferred into a clean polypropylene tube using a Pasteur pipette and stored at -80 ˚C until analysed for levels of IgG1.

2.10 STATISTICAL ANALYSIS

Results are presented as mean ± standard error of the mean (SEM) and groups were compared using an unpaired Student's t-test or for multiple groups, a one-way ANOVA followed by a Newman-Keuls post-hoc test. All data were analysed using Prism Software (GraphPad Software, Inc.). Values of less than $p<0.05$ were considered statistically significant.

CHAPTER 3

ISOLATION AND POLARISATION OF CD4^{+} T-CELLS *IN VITRO*

3.1 INTRODUCTION

The aim of this chapter was to optimise the isolation and culture of a pure and viable population of $CD4^+$ helper T-cells from mice *in vitro*. To achieve this, cytokine secretion and surface marker expression was assessed in order to identify the optimum culture conditions. Cell viability was also assessed under a number of conditions.

Three signals are required to activate and differentiate $CD4^+$ T-helper cells, see **Figure 3.1.1**. Antigen-presenting cells (APCs) recognise invading pathogens and present them, via major histocompatibility complex II (MHC II), to undifferentiated $CD4^+$ T-cells in the lymph nodes. MHC II interacts with the T-cell receptor (TCR) on the T-helper cell which results in, depending on the nature of the pathogen encountered, the release of a broad spectrum of cytokines and co-stimulatory factors (Goldsby et al. 2003). This is the first signal required for T-cell activation. The second signal necessary for activation involves co-stimulatory molecule interaction between the two cells. Here B7 molecules, B7-1 (CD80) and B7-2 (CD86), found on APCs interact with CD28 on the T-cell surface (Chen 2004; Hwang et al. 2000). This signal prevents cells from mounting an immune response to self antigens which results in autoimmunity (Lenschow et al. 1996). T-cell anergy will result if this signal is absent during antigen presentation (Bour-Jordan et al. 2011). At this point undifferentiated T-cells have become activated and the differentiation into each of the $CD4^+$ T-helper cell subsets is dependent on the third signal. This signal involves the signalling and activation through a cytokine milieu released both from APCs and u undifferentiated $CD4^+$ T-cells, which are released at the time of antigen presentation. The specific cytokines released during antigen presentation and T-cell activation determine the cell fate or phenotype of T-cell to be differentiated. They do this by activating signal transducers and activators of transcription (STATs) and transcription factors (Vahedi et al. 2013). The type of cytokines released during activation will, therefore, determine the cell fate and the type of T-cell subset to be activated.

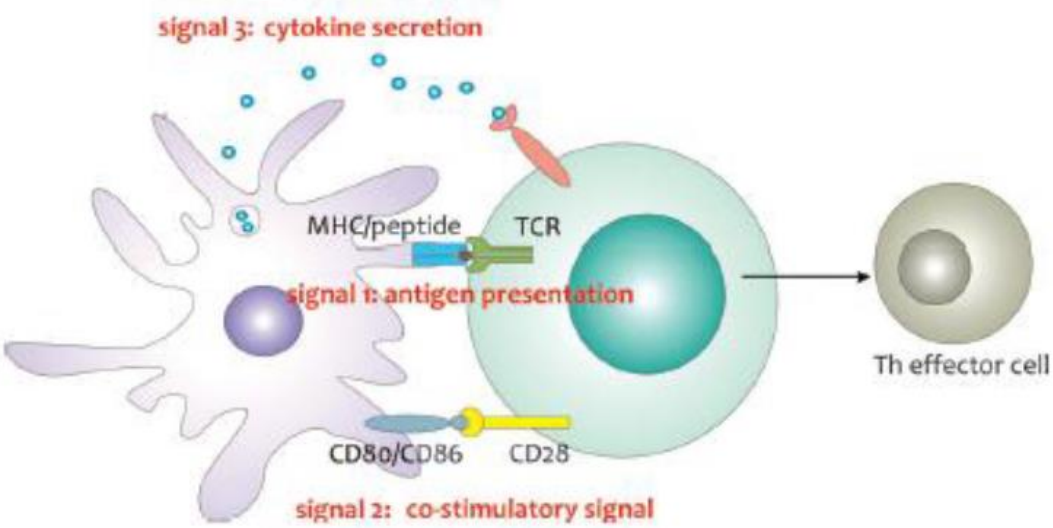


Figure 3.1.1: Overview of the Three Signals Required for the Activation and Polarisation of Undifferentiated CD4$^+$ T-Cells. Signal 1 involves the antigen presentation by MHC II and subsequent stimulation of the TCR. Signal 2 involves interaction between the co-stimulatory B7 molecules on the APC and CD28 on the T-cell surface. Signal 3 involves the release of a specific group of cytokines which will determine the type of T-cell to be activated (De Koker et al. 2011).

When all three signalling events occur in unison, CD4$^+$ T-cells become activated, which leads to the differentiation of one of four fates and clonal expansion. This differentiation is dependent on the cytokines present and each subset secretes a defined cytokine profile specific to the type of pathogen present, leading to an effective immune response, see **Figure 1.1**. The secretion of IL-12 and IFN-γ from APCs, such as dendritic cells (DCs) and macrophage, directly induces the differentiation of type 1 helper T-cells (Th1). IFN-γ, the characteristic cytokine associated with Th1 cells, inhibits the polarisation of Th2 cells. Th1 cells continue to secrete IFN-γ in order to expel the invading pathogen whilst continuing to inhibit the other effector T-cells (Szabo et al. 2000). These cells protect against infections caused by viruses and bacteria. Type 2 helper T-cells (Th2) target parasites and activate humoral immunity. IL-4 is required for the activation of this subset and is also the signature cytokine secreted from Th2 cells. IL-5, IL-13 and IL-10 are also commonly secreted from Th2 cells and up-regulate antibody production from B-cells and target parasitic organisms. Th17 cells have not been studied in as much detail as the other T-cell subsets, however,

they are known to target extracellular pathogens and fungal infections. Th17 cells are activated in the presence of IL-6, IL-1β and IL-23 and secrete large volumes of IL-17 (Kaiko et al. 2008). Regulatory T-cells (Treg cells) are associated with the induction of tolerance to harmless or self-antigens. IL-10 and TGF-β secreted from tolerogenic DCs, upon exposure to these harmless antigens, activate Treg cells. Treg cells, in turn, secrete TGF-β, which inhibits differentiation of the effector adaptive T-helper cells (Th1, Th2 and Th17) and indices tolerance (Zeuthen et al. 2008; Maldonado and von Andrian, 2010; and Raker et al. 2015).

Within the immune response, dysregulation has been shown to occur. This is observed in instances such as when infection has been cleared and the generated effector T-cells are not dampened or switched off, or in the case of autoimmunity whereby there is an exacerbated immune response resulting in disease. When Th1 and Th17 cell activity is over-activated inflammatory disease results. Th2 cells are the primary agonist in initiating allergies and asthma (Maggi, 1998)**.** Th17 cells have also been shown to have an influential role in the progression of asthma (Keisuke et al. 2008). **Table 3.1** details the origin and role of a number of T-cell subsets in allergy-based diseases.

Type	Priming cytokines	Transcription factors	Effector cytokines	Functions	Disease associated with dysregulation
Th1	IL-12 IFN-γ	T-bet STAT1 STAT4	IFN-γ	Intracellular pathogens	Autoimmunity, cell-mediated response
Th2	IL-4 IL-6 IL-10	GATA-3 STAT5 STAT6	IL-4 IL-5 IL-10 IL-13	Extracellular pathogens	Asthma, allergy, atopy
Th17	TGF-β IL-6	ROR-γ STAT3	IL-17 IL-22	Extracellular bacteria	Autoimmune inflammation
Treg	TGF-β IL-2	Foxp3 STAT5	TGF-β IL-10 IL-35	Immune homeostasis	Autoimmunity, allergy, metabolic dysfunction

Table 3.1: Summary of cytokines and transcription factors involved in the differentiation and commitment of T-helper subsets.

The signals required for T-cell activation can be replicated *in-vitro*. In this chapter, an anti-CD3 antibody was used in order to crosslink with the TCR on freshly isolated $CD4^+$ T-cells, mimicking the signal from MHC II on APCs. An anti-CD28 antibody was used to crosslink CD28 on the T-cell surface, providing the co-stimulatory signal required for activation. These two signals allow us to activate a population of undifferentiated $CD4^+$ T-cells. In order to determine the effect of cow's milk hydrolysates on undifferentiated $CD4^+$ T-cells and T-helper subsets, it was necessary to optimise their culture *in vitro*. An environment which favours the polarisation of each of the $CD4^+$ T-helper cell subsets can be mimicked *in vitro*, using recombinant cytokines and neutralising antibodies in culture with isolated $CD4^+$ T-cells. As mentioned, each of the T-cell subsets require a defined cytokine profile in order for their differentiation to take place and, once activated, they secrete various cytokines at different levels. In order to determine the optimum conditions necessary for the isolation and culture of these cells, cell viability, surface marker expression and cytokine secretion over a period of time was analysed. By examining the levels of cytokine secretion from each subset treated with protein hydrolysates *in vitro*, we can determine the effect they may have on modulating the immune system *in vivo.*

3.2 RESULTS

3.2.1 ISOLATION AND CONFIRMATION OF $CD4^+$ T-HELPER CELL POPULATION FROM BALB/C MICE.

3.2.1.1 EXAMINATION OF CD3 EXPRESSION ON ISOLATED $CD4^+$ T-HELPER CELL IN THE SPLEEN TO DETERMINE POPULATION PURITY.

As some macrophages and dendritic cells (DCs) also express CD4, it was necessary to determine the T-cell abundance in the spleen. CD3 antigen staining was therefore carried out. CD3 is a marker on all T-cells, on both $CD4^+$ and $CD8^+$ populations, and makes up part of the TCR complex. $CD4^+$ T-cells develop in the thymus and reside in the spleen and lymph nodes. The spleen was aseptically removed from a BALB/c mouse and collected in RPMI/10% (v/v) FBS. A single cell suspension was achieved by filtering the spleen through a 50µm filter. The cells were re-suspended in fully supplemented RPMI. $CD4^+$ cells were isolated using an EasySep™ kit from Stemcell Technologies. Biotinylated antibody cocktails target and remove any non-$CD4^+$ cells. Tetrameric antibody complexes that recognise biotin and dextran-coated magnetic particles then bind to these antibodies. The cell suspension is placed into a magnetic field which retains the unwanted labelled cells and the $CD4^+$ T-cells are poured off. Isolated $CD4^+$ T-cells were blocked and stained with primary PE-Cyanine 5.5 conjugated anti-CD4 and primary FITC conjugated anti-CD3 antibodies for 30 mins and washed before analysis on a BD FACS Aria. Data was analysed using Flowjo software. **Figure 3.1 (A)** shows removal of dead cells and debris based on FSC and SSC. Dead cells and debris have a small FSC and SSC and were gated out for further analysis. **Figure 3.1 (B)** shows cells stained with primary PE-Cyanine 5.5 conjugated anti-CD4 antibody. On a dot plot, each dot represents a single cell and a shift to the right indicates increased fluorescence intensity. Cells are 97.6% $CD4^+$ cells. **Figure 3.1 (C)** shows the double stained cells are 97% positive for both CD3 and CD4 markers, as seen by a shift in stained cells to the top right quadrant.

3.2.1.2 ANTIBODIES DIRECTED TO CD3 AND CD28 ARE ESSENTIAL FOR IN VITRO ACTIVATION OF CD4+ CELLS.

Three signals are essential for the activation of T-cells; TCR engagement, activation of the CD28 co-stimulation pathways, and cytokine signalling. As mentioned above, CD3 makes up the TCR. Anti-CD3 antibody (BD Clone: 145-2C11) mimics the signal provided by MHC II on APCs and interacts with CD3 on the TCR. In order to activate co-stimulatory interaction, an anti-CD28 antibody (BD) was used to crosslink and activate the CD28 pathway. This antibody mimics the B7 ligands (CD80 & CD86) on APCs. In order to confirm that the isolated $CD4^+$ T-cells were viable and capable of being activated using antibodies for CD3 and CD28, cells were either stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) or left in culture unstimulated. Cells and supernatants were collected at 24 and 72 hrs and a series of experiments were set up.

Viability was assessed using an MTS assay (Cell Titer 96 Aqueous One Solution – Promega, WI, USA). Results are expressed as a percentage of stimulated cells. Cells were only viable once activated with anti-CD3 and anti-CD28 antibodies **[Figure 3.2]**, as seen by the significant decrease ($p<0.001$) in viability in unstimulated cells compared to stimulated cells at 72 hrs.

Cytokine secretion from $CD4^+$ T-cells is increased upon TCR activation (Zhu J. et al., 2010). Supernatants were, therefore, collected and measured for IFN-γ, IL-2, IL-4, IL-13, IL-10 and IL-17 secretion using ELISA according to manufacturer's instructions (R&D duoset). Enhanced cytokine secretion was only present in cells stimulated with anti-CD3 and anti-CD28 antibodies cultured for 72 hrs **[Figure 3.3]**, which indicates that stimulation with these antibodies is sufficient for T-cell activation. Cells cultured for 72 hrs resulted in optimum cytokine secretion, evident by a significant increase in secretion for all of the cytokines examined (IFN-γ: $p<0.01$; and IL-2, IL-4, IL-13, IL10 and IL-17: $p<0.001$).

When activated, the CD25 surface marker on T-cells becomes upregulated. It also promotes proliferation of T-helper cells, being the IL-2 receptor (IL-2R). IL-2 is a T-cell growth factor which is essential for T-cell clonal expansion following activation (Corthay, 2009). For this reason, cells were stained using a primary PE conjugated antibody for CD25 (eBioscience) (/100 µl) and analysed on the BD FACS Aria. Data

was analysed using Flowjo software. CD25 expression was upregulated following stimulation with anti-CD3 and anti-CD28. Expression of CD25 at 72 hrs **[Figure 3.4]** was higher than expression at 24 hrs **[Figure 3.5]**. These results collectively confirm that $CD4^+$ T-cells can be activated *in vitro* using CD3 and CD28 antibodies at an optimum culture time of 72 hrs.

3.2.2 ACTIVATION/POLARISATION AND CONFIRMATION OF $CD4^+$ T-HELPER CELL SUBTYPES FROM BALB/C MICE.

Following the optimisation of $CD4^+$ T-cells *in vitro*, the next step was to optimise the polarisation of the $CD4^+$ T-cell subsets *in vitro*.

3.2.2.1 TIME COURSE ANALYSIS FOR THE OPTIMISATION OF TH1, TH2, TH17 AND REGULATORY CD4+ T-CELL POLARISATION IN VITRO – VIABILTY AND CYTOKINE SECRETION

A time course analysis experiment was carried out for each T-cell subset to optimise the *in vitro* culture time for each of the T-cell assays. $CD4^+$ T cells were isolated and stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml). The media was supplemented with recombinant cytokines and polarising antibodies required to drive each of the subsets. IL-12 and IFN-γ are the key cytokines associated with driving Th1 cells, IL-4 is the characteristic cytokine required for the development of Th2 cells, IL-6 and IL-23 drive Th17 cells and TGF-β and IL-10 are necessary for the activation of Treg cells (Zhu et al. 2010; Massimo et al. 2007). The media was, therefore, supplemented with 20 ng/ml IL-2, 20 ng/ml IL-12 and 10 µg/ml anti-IL-4 antibody for Th1 polarising conditions; 10 µg/ml neutralising anti-IFN-γ antibody and 10 ng/ml IL-4 for Th2 polarising conditions; 10 µg/ml neutralising anti-IFN-γ antibody and 20 ng/ml IL-6 and 20 ng/ml IL-23 for Th17 polarising conditions; or 20 ng/ml IL-2 and 5 ng/ml TGF-β for Treg cell polarising conditions. Cells were re-stimulated in fresh media on day 3 with plate-bound anti-CD3 (1 µg/ml) plus anti-CD28 (0.2 µg/ml) for Treg cells and anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) for undifferentiated, Th1, Th2 and Th17 cells for 24 hrs. This was done in order to ensure the measurement of cytokine secretion in the supernatants was coming from the cells

themselves and not from the addition of recombinant cytokines to the culture medium in order to polarise a certain subtype. As our primary aim was to examine the effect of hydrolysate treatment on $CD4^+$ T-cells and the respective subsets, this study was necessary to ensure these conditions did not have any significant effect within the experimental setup. It is understood that strong co-stimulation, in particular CD28 signaling, can result in the inhibition of Treg induction. Strong CD28 signalling activates the mTor signaling pathway, which regulates proliferation and survival of the effector T-cells and, thus prevents Treg cell activation (Geiger & Tauro 2012; and Schmidt et al. 2016). Gottschalk et al. (2010) state that TCR signal strength/anti-CD3 concentration may also affect the stability of the key Treg transcription factor, Foxp3, expression. They found that only by using low doses of strong TCR agonists to stimulate Foxp3+ cells resulted in stable Foxp3 expression. For this reason, we used lower doses of anti-CD3 and anti-CD28 for re-stimulating Treg cells *in vitro*.

We first determined the viability of each of the T-cell subsets. It was necessary to ensure that the polarising antibodies and recombinant cytokines did not have any cytotoxic effects on the cells. Viability was assessed at 24, 72 and 96 hrs using an MTS assay (Cell Titer 96 Aqueous One Solution – Promega, WI, USA). Results are expressed as a percentage of undifferentiated $CD4^+$ T-cells. Cells were only viable after being cultured for 72 hrs or 96 hrs **[Figure 3.6].**

We collected the supernatants and examined the characteristic cytokine secretory profiles for each of the T-cell subsets. IFN-γ and IL-2 are the characteristic cytokines associated with Th1 cells, IL-4, IL-10 and IL-13 are the key cytokines secreted from a Th2 subset, IL-17 is the characteristic Th17 cytokine and TGF-β and IL-10 are secreted in abundance from Treg cells (Zhu et al. 2010; Massimo et al. 2007). Supernatants were collected at 24, 72 and 96 hrs and analysed using ELISA according to manufacturer's instructions (R&D duoset). Th1, Th2 and Th17 cells yielded optimum cytokine secretions after 96 hrs and re-stimulation, as indicated by the high secretion of the characteristic cytokines associated with each subset **[Figure 3.7; Figure 3.8].** Treg cells, however, secreted a similar level of IL-10 compared to undifferentiated T-cells and high secretion of IL-17 was also observed. As Tregs are known to secrete high levels of IL-10, further optimisation was, therefore, required to characterise the Treg *in vitro* profile in more depth.

3.2.2.2 INTRACELLULAR MARKER STAINING FOR THE OPTIMISATION OF TH1 AND TH2 T-CELL POLARISATION IN VITRO

After optimising the culture conditions (time) and confirming recombinant cytokine and polarising antibody concentrations for each T-cell subset, based on cell viability and cytokine profiles, we further validated these profiles and examined intracellular cytokines for Th1 and Th2 cells to confirm their polarisation. We looked at intracellular IFN-γ expression for Th1 cells and intracellular IL-13 for Th2 cells. $CD4^+$ T cells were isolated and stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/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; 10 µg/ml neutralising anti-IFN-γ antibody and 10 ng/ml IL-4 for Th2 polarising conditions. Cells were re-stimulated in fresh media on day 3 with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) for a further 24 hrs. Cells were collected and stained for CD4 using FITC primary conjugated antibody and either intracellular IL-13 using PE primary conjugated antibody or intracellular IFN-γ using APC primary conjugated antibody. Cells were also stained with Fixable Viability Dye (eBioscience). Cells were analysed on BD FACS Aria and data was analysed using Flowjo software (treestar). Cells were gated on viable $CD4^+$ expressing cells. **Figure 3.9 (A)** shows IL-13 expression on Th1 cells (red) Th2 cells (blue) and unstained cells (filled grey). IL-13 expression is upregulated in both T-cell subtypes and a higher expression is observed in Th2 cells over Th1 cells, the mean fluorescence for Th2 being 2390 and 1785 for Th1 cells. **Figure 3.9 (B)** shows IFN-γ expression on Th1 cells (red) Th2 cells (blue) and unstained cells (filled grey). IFN-γ expression is much higher in Th1 cells (mean flurescence 11,800 1.18^4) than in Th2 cells (604).

3.2.3 FURTHER OPTIMISATION OF REGULATORY T-CELL POLARISATION *IN VITRO*.

No increase in IL-10 was observed from Treg cells. Additionally, high secretion of IL-17 was observed from Treg cells, see **Figure 3.7**. It is possible that a Th17 subset had been polarised as a result of the conditions used, as the cytokine secretion profiles observed for the Th17 and Treg cells were similar. Therefore, it was necessary to

further optimise this subset. Zhou et al. (2008) state that Th17 cell differentiation is regulated in a dose dependent manner by TGF-β.

3.2.3.1 TIME COURSE ANALYSIS OF A TGF-β DOSE RESPONSE FOR THE OPTIMISATION OF REGULATORY CD4+ T-CELL POLARISATION IN VITRO – CYTOKINE SECRETION AND INTRACELLULAR STAINING

High concentrations of TGF-β block Th17 differentiation and instead, Forkhead box P3 (Foxp3) expressing Treg cells develop (Korn T et al. 2007 and Zhou et al. 2007). For this reason a range of TGF-β concentrations in the culture of Treg cells *in vitro* was assessed. Foxp3 is the master regulator necessary for the development and function of Treg cells and this was also stained for $CD4^+$ T cells were isolated from the spleens of female BALB/c mice using EasySep $CD4^+$ Isolation (Stemcell Cat# 19752). Cells were plated at $1x10^6$/ml on a 96 well plate for ELISA and on a 24 well plate for flow cytometry. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml). The media was supplemented with 20 ng/ml IL-2 and 2 ng/ml, 5 ng/ml or 10 ng/ml TGF-β for Treg polarising conditions. Samples were taken at 24, 48, 72 and 96 hrs. Cells were re-stimulated in fresh media on day 3 with plate-bound anti-CD3 (1 µg/ml) plus anti-CD28 (0.2 µg/ml) for 24 hrs.

Supernatants were collected and measured for IL-10, IFN-γ, IL-2, IL-4, IL-13, IL-6 and IL-17 secretion using ELISA according to manufacturer's instructions (R&D duoset) to assess the ability of cells treated with TGF over a range of time points, to suppress the cytokines associated with the effector T-cells (Th1, Th2 and Th17) and to increase production of IL-10, the characteristic Treg cytokine. Each of the experimental parameters assessed failed to give satisfactory results. Treatment with 5 ng/ml TGF-β at 96 hours (rested and re-stimulated) seemed to be the most effective dose and time point, however still did not perform adequately. Amongst the TGF-β treated groups in **Figure 3.10**, IL-10 secretion was highest in cells treated with 5 ng/ml TGF-β at 96 hours (rested and re-stimulated). However, IL-10 secretion from this group was decreased compared to untreated $CD4^+$ T-cells and IFN-γ and IL-17 were also significantly increased ($p<0.05$ and $p<0.001$, respectively). The 5 ng/ml TGF-β dose at this time point did show some positive suppressive results. IL-6 is significantly reduced ($p<0.01$), at this dose, compared to untreated cells and increasing the dose

further actual inhibits the suppressive activity as the 10 ng/ml TGF-β dose reduced IL-6 to a less significant effect ($p<0.05$). IL-4 and IL-13 levels were also significantly suppressed ($p<0.001$) by the 5 ng/ml TGF-β dose compared to untreated cells and increasing the dose further had no additional effect. The highest IL-10 secretion was observed from cells treated with IL-2 alone at 96 hrs (rested and re-stimulated). All of these results indicate that the TGF-β doses and time points used were not optimal for *in vitro* differentiation of a Treg subset and further optimisation was required.

Cells were also collected at each time point and stained for CD4 using FITC primary conjugated antibody, CD25 using PE primary conjugated antibody and intracellular Foxp3 using APC primary conjugated antibody. Cells were also stained with Fixable Viability Dye (eBioscience). Cells were analysed on BD FACS Aria, data was analysed using Flowjo software (treestar). Cells were gated on viable $CD4^+$ expressing cells. The number of $Foxp3^+$ expressing cells following culture with TGF-β and IL-2 should be >60% of the cell population (Fantini et al. 2007). However, the highest expression of Foxp3 was observed in only 35% of cultured cells, seen at 96 hrs after stimulation with 5 ng/ml TGF-β **[Figure 3.11].** Collectively, our cytokine and intracellular staining data indicate that further work is required to optimise the *in vitro* differentiation of a Treg assay. CD4, CD25 and Foxp3 antibodies were titrated before use **[Figure 3.12].**

3.2.3.2 REGULATORY T-CELL ISOLATION KIT COMPARISON

As we were unsuccessful in our attempts to optimise an *in vitro* Treg cell assay using a number of TGF-β doses over various time points, we decided to examine the ability of commercial Treg isolation kits to do so. We compared the effectiveness of two Treg kits from Stemcell Technologies to undifferentiated $CD4^+$ T-cells. $CD4^+$ T-cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19752). $CD4^+$ Treg cells were also isolated using EasySep™ Mouse CD25 Regulatory T-Cell positive selection Kit (Treg Kit 1 - Stemcell Cat#18782) or using EasySep™ Mouse $CD4^+CD25^+$ Regulatory T Cell Isolation Kit II (Treg Kit 2 - Stemcell Cat#18783). Kit 1 isolates highly purified CD25+ cells from single-cell suspensions of splenocytes by positive selection, whereas Kit 2 isolates $CD4^+$ T-cells first by negative selection followed by an isolation

of CD25+ cells by positive selection. Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml). Supernatants were collected at 72 hrs and analysed using ELISA, according to manufacturer's instructions (R&D duoset). Similar cytokine secretion profiles were observed for both Treg kits **[Figure3.13]**. Both kits significantly increased IL-10 ($p<0.001$) and IL-17 ($p<0.05$) secretion, significantly decreased IL-13 and IL-6 production ($p<0.001$) and had no effect on IFN-γ levels compared to undifferentiated $CD4^+$ T-cells. Kit 1 had no effect on IL-4 secretion while Kit 2 significantly decreased IL-4 levels ($p<0.05$). Kit 1 also significantly inhibited IL-2 secretion ($p<0.01$), while kit 2 had no effect on IL-2. IL-2 is required for the proliferation of the effector T-helper cell subsets. IL-10 is a key Treg cytokine. Kit 1 had a more significant increase in IL-10 secretion than Kit 2. These results indicate a stronger Treg response from Kit 1 than Kit 2. Additionally, according to Stemcell Technologies, the Treg cell content ($CD4^+CD25^+Foxp3^+$) of the isolated fraction typically ranges from 80 - 93% for Kit 1 and from 70 - 93% for Kit 2. We, therefore, decided to assess Treg purity further using Kit 1.

3.2.3.3 CD25 EXPRESSION ANALYSIS IN REGULATORY T-CELLS

The next step of our *in vitro* Treg optimisation analysis was to examine the expression of the CD25 surface marker. As mentioned, all $CD4^+$ T-cells express CD25 when activated but it is predominantly associated with Treg cells. CD25 expression following an acute antigen-driven immune response *in vivo* is only observed temporarily for a short duration, whereas a high IL-2R phenotype is characteristic of Treg cells (O'Gorman et al. 2009; and Miyara et al. 2009). Malek and Castro (2010) suggest that purification of $CD4^+CD25^+$ T-cells is ideal for inducing $Foxp3^+$ Treg cells. For this reason, we examined CD25 expression on Treg cells isolated from Kit 1. We compared CD25 expression on pre-isolated cells, isolated $CD4^+$ cells and isolated $CD4^+$ Treg cells.

Spleens were collected from female BALB/c mice aged 8-12 weeks. Three independent experiments were set up. (1) Erythrocytes were lysed using a red blood cell lysing buffer (SIGMA), (2) $CD4^+$ T-cells using EasySep $CD4^+$ Isolation (Stemcell Cat# 19752) or (3) Treg cells were isolated or using EasySep™ Mouse CD25

Regulatory T Cell positive selection Kit (Stemcell Cat#18782). Isolated cells were plated at $1x10^6$/ml on a 6 well plate and were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) for 72 hrs. Cells were stained for CD4 using FITC primary conjugated antibody and CD25 using PE primary conjugated antibody. Cells isolated from the Treg isolation kit were not stained for CD25, as the kit labels the isolated cells with a PE labelled CD25 antibody during the isolation process. Cells were also stained with APC-Cy7 conjugated Fixable Viability Dye (eBioscience). Cells were analysed using BD FACS Aria and data was analysed using Flowjo Software (treestar). Cells were gated on viable $CD4^+$ CD25+ expressing cells.

Figure 3.14 shows pre-isolated lysed splenocytes with a $CD4^+$ $CD25^+$ cell population of 9.25%, **Figure 3.15** shows isolated $CD4^+$ T-cells with a $CD4^+$ $CD25^+$ cell population of 8.39% and **Figure 3.16** shows isolated Treg cells with a $CD4^+$ $CD25^+$ cell population of 90.9%. This confirms a successful isolation and purification of $CD4^+CD25^+$ T-cells necessary for a strong enrichment for $Foxp3^+$ Treg cells.

3.2.3.4 INTRACELLULAR MARKER STAINING FOR THE OPTIMISATION OF REGULATORY T-CELL POLARISATION IN VITRO

In order to validate the cytokine profile results, it was necessary to examine Foxp3 expression in isolated Tregs. Foxp3 is the key Treg transcription factor and is responsible for dampening the effector T-cells. $CD4^+$ T-cells and $CD4^+$ Treg cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19752) and EasySep™ Mouse CD25 Regulatory T Cell positive selection Kit (Stemcell Cat#18782), respectively. Cells were plated at $1x10^6$/ml in a 6 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml). The media was supplemented with 10 µg/ml neutralising anti-IFN-γ antibody and 10 ng/ml IL-4 for Th2 polarising conditions and 20 ng/ml IL-2, 20 ng/ml IL-12 and 10 µg/ml anti-IL-4 antibody for Th1 polarising conditions. Cells were collected after 72 hrs and stained for CD4 using FITC primary conjugated antibody and intracellular Foxp3 using APC primary conjugated antibody. Cells were also stained with Fixable Viability Dye (eBioscience). Cells were analysed on BD FACS Aria, data was analysed using Flowjo software (treestar). Cells were gated on viable $CD4^+$ expressing cells. The histogram in **Figure 3.17** shows Foxp3

expression on viable $CD4^+$ Th1 (red), Th2 (blue), Treg (orange) and unstained cells (filled grey). A ten-fold increase in the level of Foxp3 expression was observed in Treg cells compared to the effector, Th1 and Th2, cell populations, confirming/finalising the development of the *in vitro* Treg cell assay.

3.2.3.5 COMPARISON OF IN VITRO CYTOKINE PROFILES BETWEEN EFFECTOR T-CELL SUBSETS AND OPTIMISED REGULATORY T-CELL ISOLATION KIT

Now that we had optimised the Treg subset to a satisfactory level *in vitro*, the final validation of characterisation step was to repeat the comparison of cytokine profiles between the optimised polarised T-cell assays. Cytokine secretion from undifferentiated $CD4^+$ T-cells, Th1, Th2, Th17 and Treg cells was examined. $CD4^+$ T cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19752) and EasySep™ Mouse CD25 Regulatory T Cell positive selection Kit (Stemcell Cat#18782) for Treg cells. Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml). The media of the $CD4^+$ isolated cells 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; 10 µg/ml neutralising anti-IFN-γ antibody and 10 ng/ml IL-4 for Th2 polarising conditions; or 10 µg/ml neutralising anti-IFN-γ antibody and 20 ng/ml IL-6 and 20 ng/ml IL-23 for Th17 polarising conditions. Supernatants were collected at 72 (undifferentiated and Treg cells) and 96 hrs (Th1, Th2 and Th17 cells). Cells were re-stimulated in fresh media on day 3 with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) for Th1, Th2 and Th17 cells for 24 hrs. Supernatants were collected and analysed using ELISA according to manufacturer's instructions (R&D duoset).

Figure 3.18 shows a satisfactory cytokine profile for each of the T-cell subsets. When compared to undifferentiated T-cells; Th1 cells significantly enhanced ($p<0.001$) levels of its characteristic cytokine, IFN-γ; Th2 cells significantly increased ($p<0.001$) secretion of their key cytokines, IL-4, IL-10 and IL-13; Th17 cells significantly induced ($p<0.001$) IL-17 levels; and Treg cells significantly enhanced ($p<0.001$) their key regulatory cytokine, IL-10. Treg cells had higher IL-17 secretion than Th17 cells ($p<0.001$).

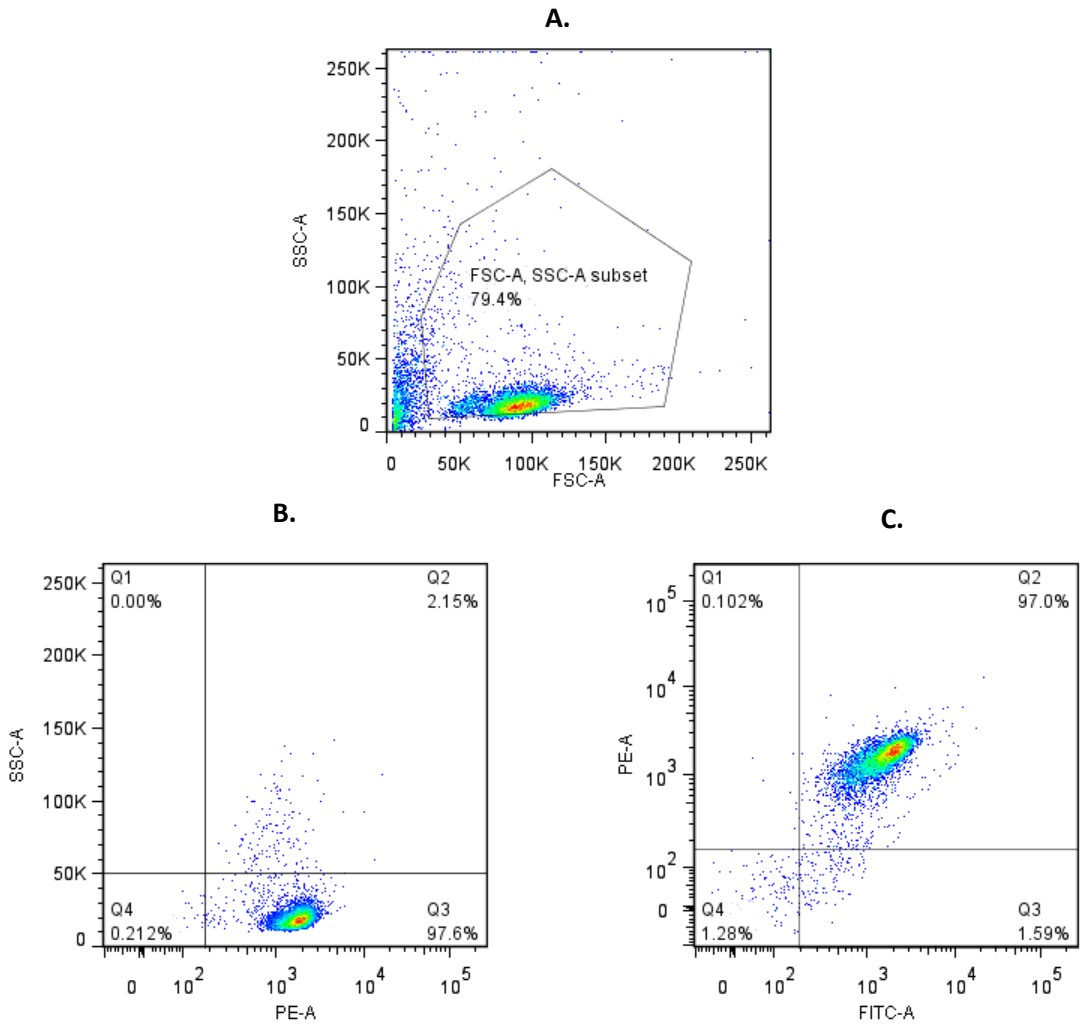


Fig 3.1: CD3 and CD4 surface staining on CD4⁺ T-Cells. CD4⁺ T-cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep CD4⁺ Isolation (Stemcell Cat# 19752). Cells were stained using anti mouse antibodies from BD. Cells were analysed using BD FACS Aria flow cytometer. **(A)** FSC and SSC gating to remove dead cells and debris from analysis. **(B)** Cells were single stained with primary PE-Cyanine 5.5 conjugated anti-CD4 antibody. Cells are 97.6% CD4⁺ cells. **(C)** Cells were double stained with primary PE-Cyanine 5.5 conjugated anti-CD4 antibody and primary FITC conjugated anti-CD3 antibody. Single stained cells were used to compensate for the fluorescent emission overlap of FITC and PE. The dot plot indicates that cells are 97% of the cells are positive for both CD3 and CD4.

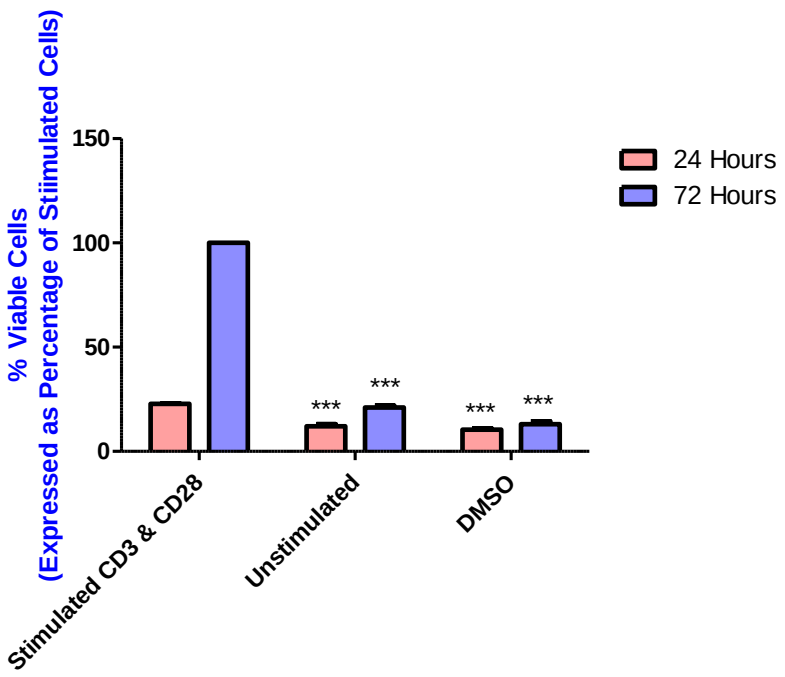


Fig 3.2: Comparison of viability of stimulated and unstimulated T-cells at 24 and 72 hrs. $CD4^+$ T-cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19752). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were either stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) or left in culture unstimulated. Cells were collected at 24 and 72 hrs. Viability was assessed using an MTS assay (Cell Titer 96 Aqueous One Solution – Promega, WI, USA). One way Anova with Newman Keuls post-hoc test was used to determine if differences between groups were significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Results are expressed as a percentage of stimulated cells at 72 hrs.

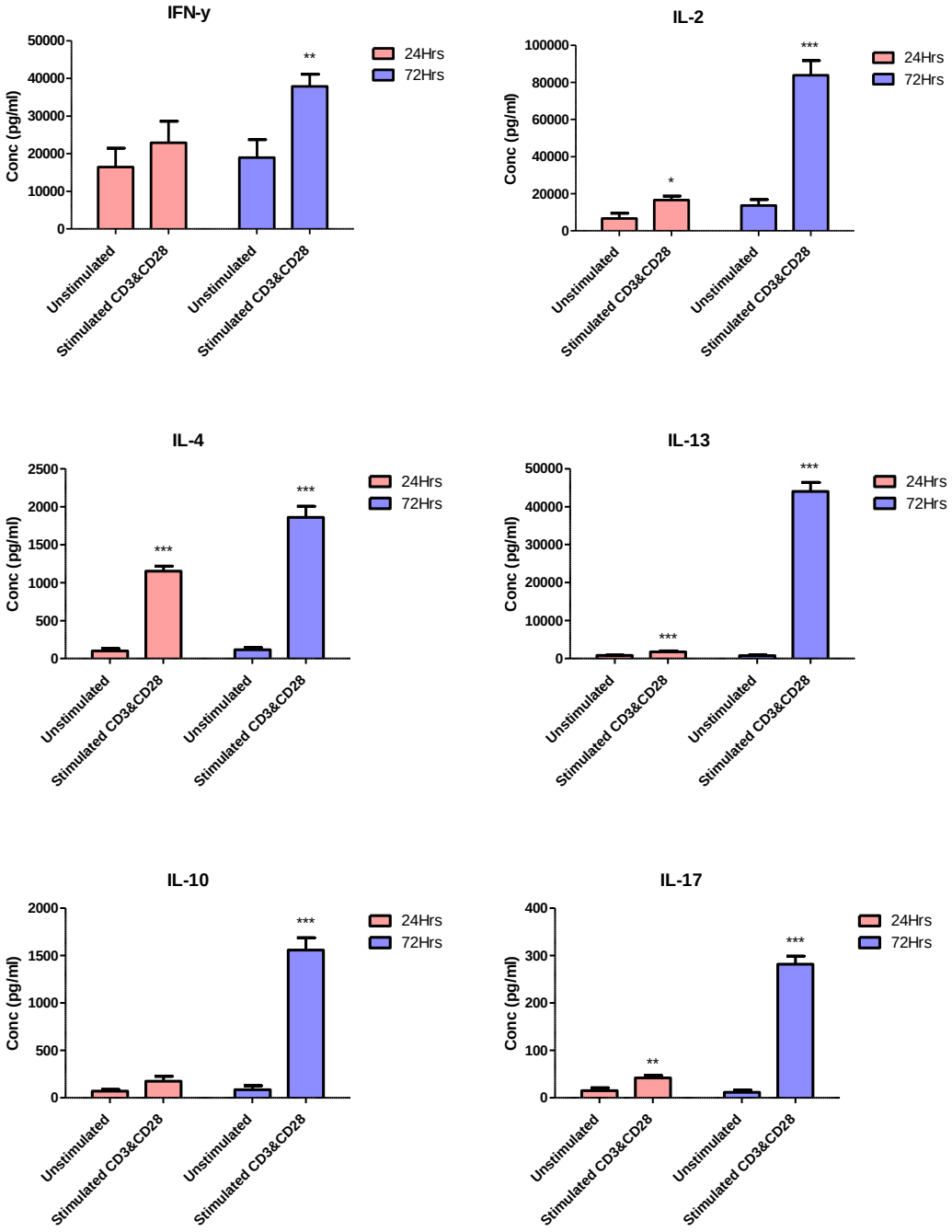


Fig 3.3: Comparison of cytokine secretion from stimulated and unstimulated T-cells at 24 and 72 hrs. $CD4^+$ T-cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19752). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were either stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) or left in culture unstimulated for 24 and 72 hrs. Cells were collected and analysed using ELISA according to manufacturer's instructions (R&D duoset). An unpaired *t*-test was used to determine if differences between groups were significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Stimulated cells are compared to unstimulated cells independently at each time point.

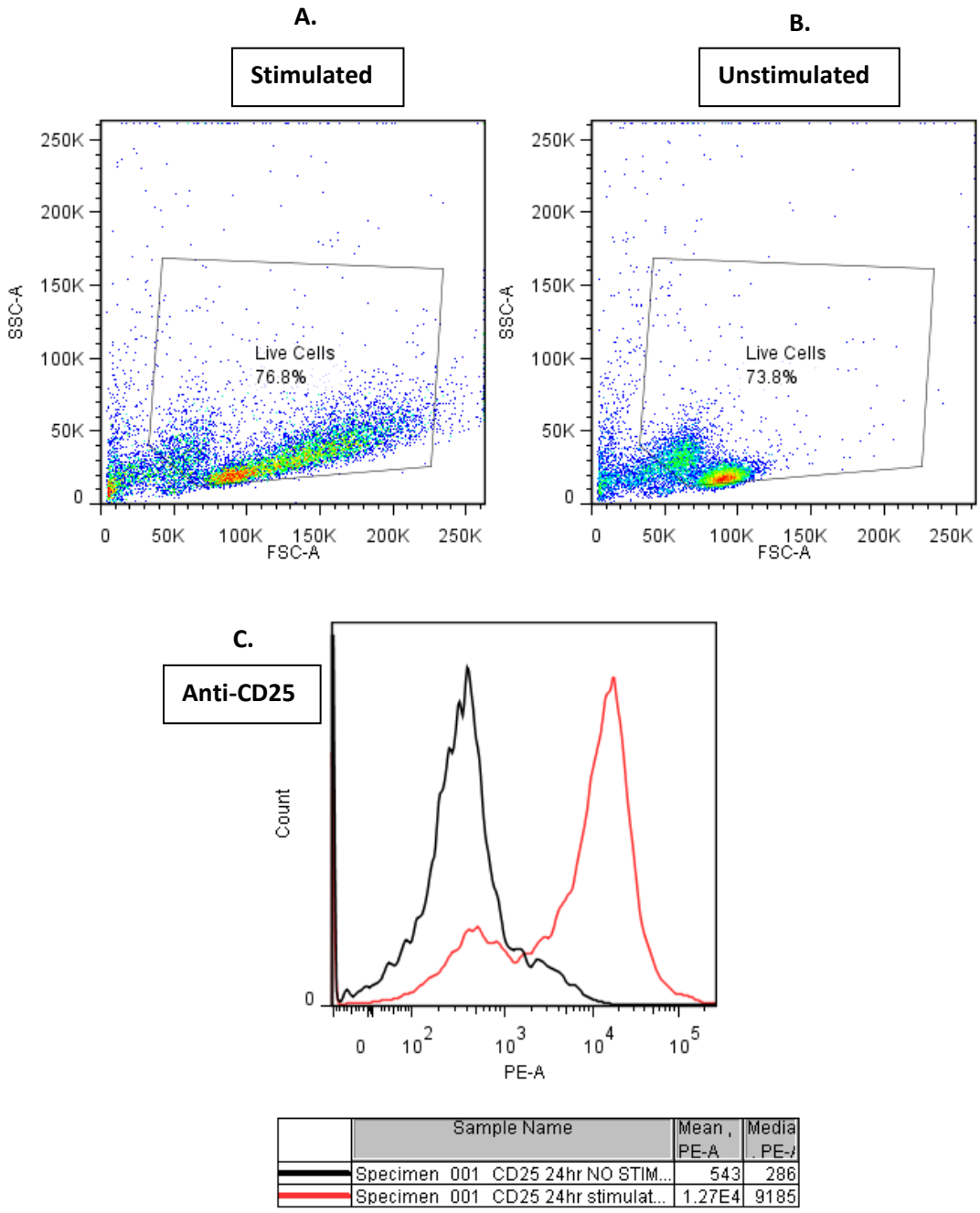


Fig 3.4: Comparison of CD25 expression on stimulated and unstimulated T-cells at 24 hrs. $CD4^+$ T-cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19752). Cells were plated at $1x10^6$/ml on a 6 well plate. Cells were either stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) **(A)** or left in culture unstimulated for 24 hrs **(B).** Cells were collected and stained for CD25 using a PE labelled primary conjugated anti-CD25 antibody (eBioscience) and CD4 using a FITC labelled primary conjugated antibody. Cells were analysed using BD FACS Aria and data was analysed using Flowjo Software (treestar). Cells were gated on $CD4^+$ expressing cells and histograms of CD25 expression are overlaid showing an increase in expression of CD25 on stimulated cells (red) compared to unstimulated cells (black) **(C).**

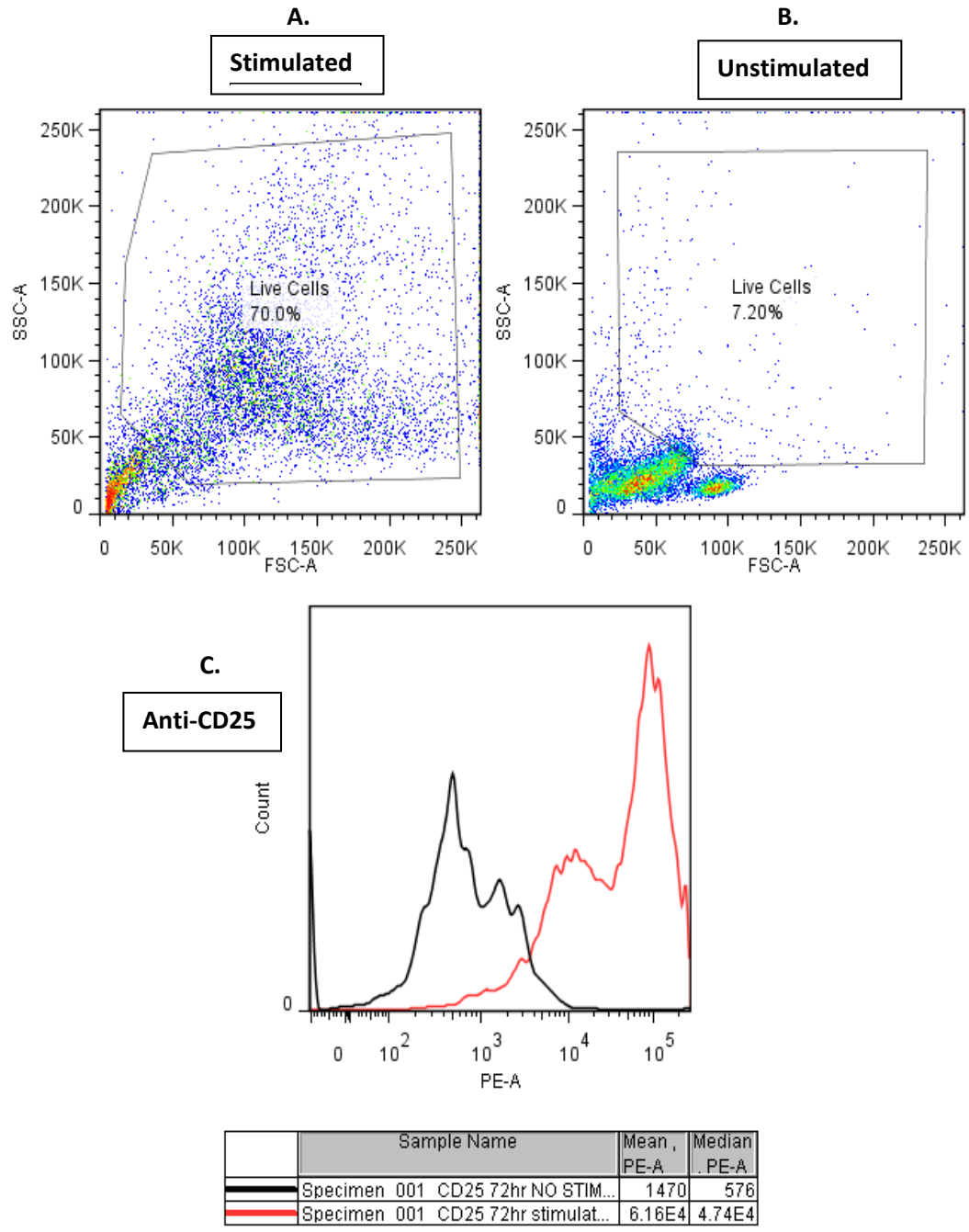


Fig 3.5: Comparison of CD25 expression on stimulated and unstimulated T-cells at 72 hrs. $CD4^+$ T-cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19752). Cells were plated at $1x10^6$/ml on a 6 well plate. Cells were either stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) **(A)** or left in culture unstimulated for 72 hrs **(B).** Cells were collected and stained for CD25 using PE labelled primary conjugated anti-CD25 antibody (eBioscience) and CD4 using a FITC labelled primary conjugated antibody. Cells were analysed using BD FACS Aria and data was analysed using Flowjo Software (treestar). Cells were gated on $CD4^+$ expressing cells and histograms of CD25 expression are overlaid showing an increase in expression of CD25 on stimulated cells (red) compared to unstimulated cells (black) **(C).**

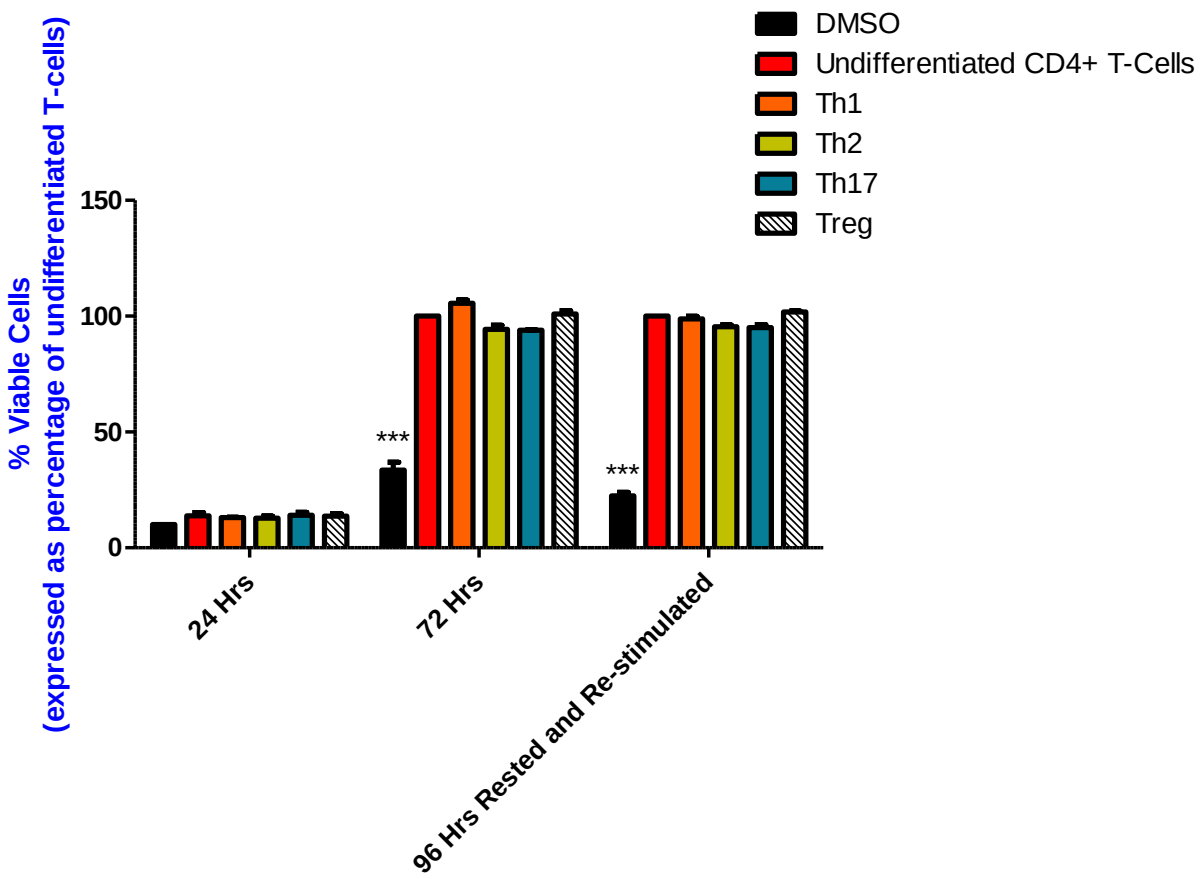


Fig 3.6: Comparison of the viability of undifferentiated, Th1, Th2 and Treg cells at 24 hrs, 72 hrs and 96 hrs. $CD4^+$ T-cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19752). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/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; 10 µg/ml neutralising anti-IFN-γ antibody and 10 ng/ml IL-4 for Th2 polarising conditions, 10 µg/ml neutralising anti-IFN-γ antibody and 20 ng/ml IL-6 and 20 ng/ml IL-23 for Th17 polarising conditions, or 20 ng/ml IL-2 and 5 ng/ml TGF-β antibody for Treg polarising conditions. Cells were collected at 24, 72 and 96 hrs. Cells were re-stimulated in fresh media on day 3 with plate-bound anti-CD3 (1 µg/ml) plus anti-CD28 (0.2 µg/ml) for Treg cells and anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) for undifferentiated, Th1 and Th2 cells for 24 hrs. Viability was assessed using an MTS assay (Cell Titer 96 Aqueous One Solution – Promega, WI, USA). Results are expressed as a percentage of undifferentiated T-cells. One way Anova with Newman Keuls post-hoc test was used to determine if differences between groups were significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Results are expressed as a percentage of undifferentiated T-cells at each time point.

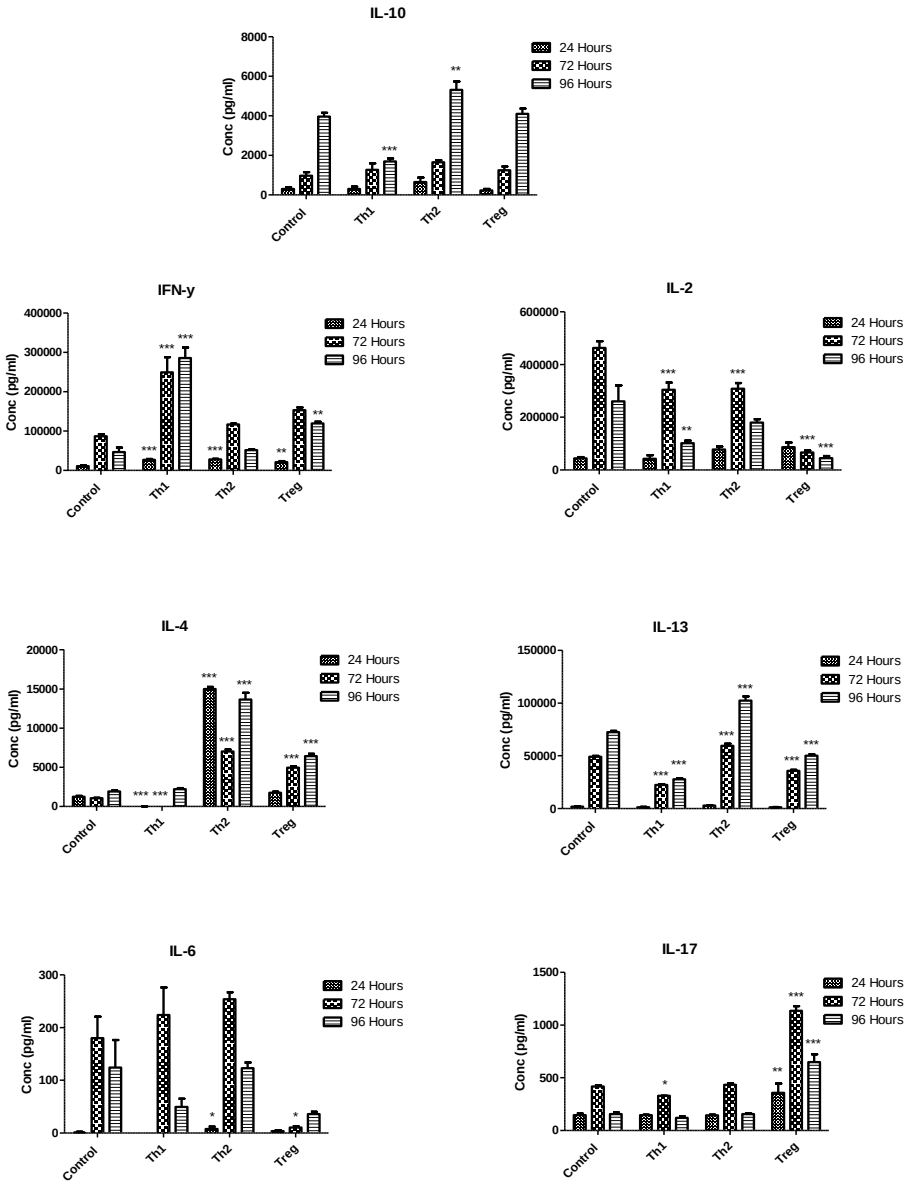


Fig 3.7: Time course analysis of cytokine secretion from undifferentiated CD4$^+$ T-cells, Th1, Th2 and Treg cells. CD4$^+$ T cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep CD4$^+$ Isolation (Stemcell Cat# 19752). Cells were plated at 1x10^6/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/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; 10 µg/ml neutralising anti-IFN-γ antibody and 10 ng/ml IL-4 for Th2 polarising conditions; or 20 ng/ml IL-2 and 5 ng/ml TGF-β antibody for Treg polarising conditions. Cells were re-stimulated in fresh media on day 3 with plate-bound anti-CD3 (1 µg/ml) plus anti-CD28 (0.2 µg/ml) for Treg cells and anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) for undifferentiated, Th1 and Th2 cells for 24 hrs. Supernatants were collected at 24, 72 and 96 hrs and analysed using ELISA according to manufacturer's instructions (R&D duoset). One way Anova with Newman Keuls post-hoc test was used to determine if differences between groups were significant (*p<0.05, **p<0.01, ***p<0.001). Groups are compared to control cells (undifferentiated CD4$^+$ T-cells) independently at each time point.

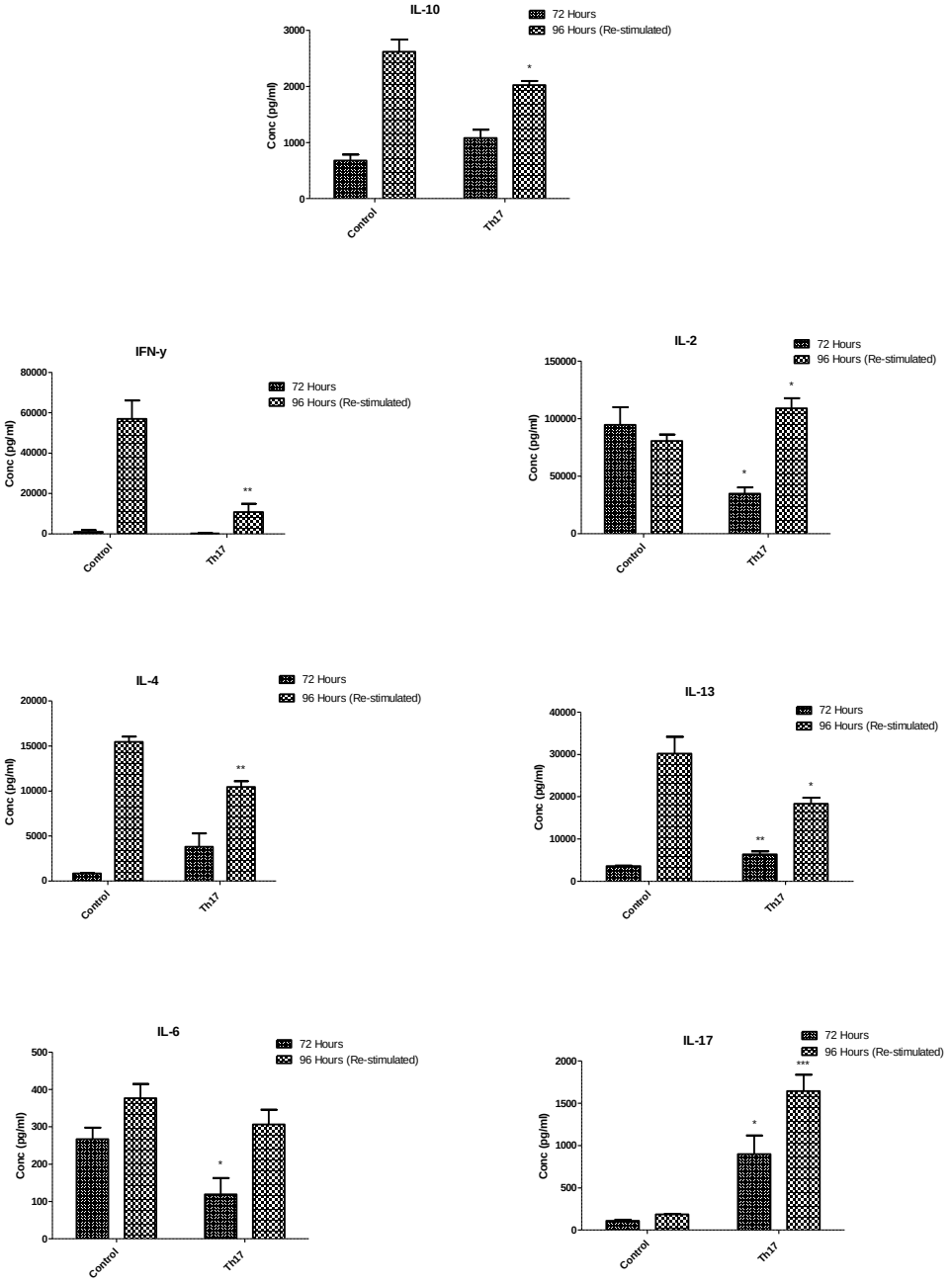


Fig 3.8: Time course analysis of cytokine secretion from Th17 Cells. $CD4^+$ T cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19752). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) in media supplemented with 10 µg/ml neutralising anti-IFN-γ antibody and 20 ng/ml IL-6 and 20 ng/ml IL-23 for Th17 polarising conditions. Supernatants were collected at 72 and 96 hrs. Cells were re-stimulated in fresh media on day 3 with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) for 24 hrs. Supernatants were collected and analysed using ELISA according to manufacturer's instructions (R&D duoset). An unpaired *t*-test was used to determine if differences between groups were significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Th17 cells are compared to control cells (undifferentiated $CD4^+$ T-cells) independently at each time point.

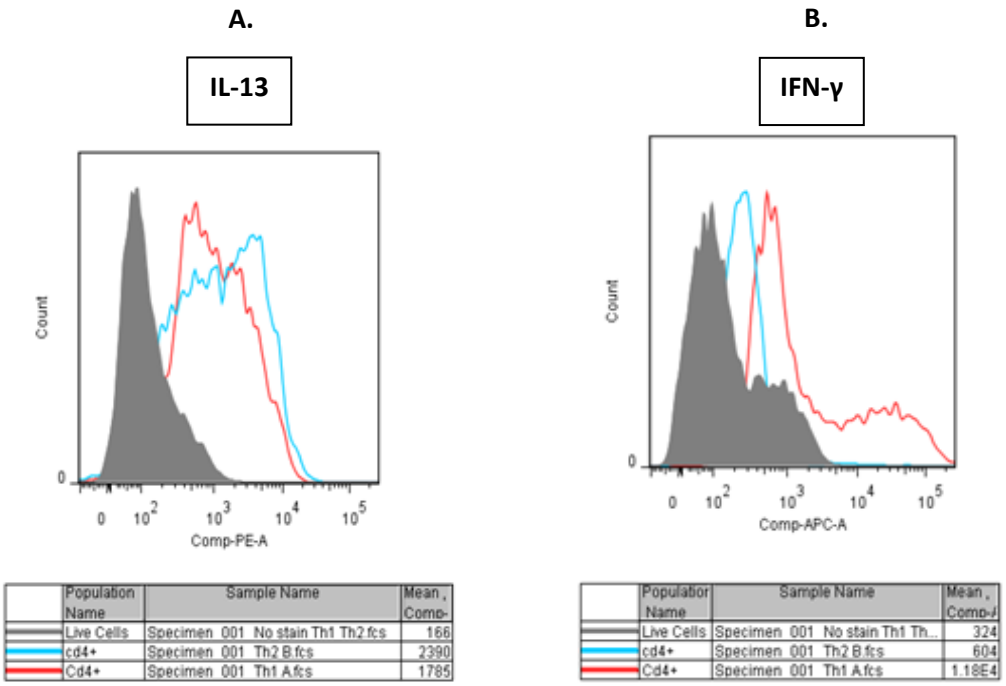


Fig 3.9: Intracellular staining for Th1 and Th2 cells. $CD4^+$ T cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19752). Cells were plated at $1x10^6$/ml on a 24 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml). The media was supplemented with 10 µg/ml neutralising anti-IFN-γ antibody and 10 ng/ml IL-4 for Th2 polarising conditions and 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 hrs and were restimulated in fresh media for a further 24 hrs. Cells were collected and stained for CD4 using FITC primary conjugated antibody and either intracellular IL-13 using PE primary conjugated antibody **(A)** or intracellular IFN-γ using APC primary conjugated antibody **(B)**. Cells were also stained with Fixable Viability Dye (eBioscience). Cells were analysed on BD FACS Aria, data was analysed using Flowjo software (treestar). Cells were gated on viable $CD4^+$ expressing cells. Histogram in **(A)** shows IL-13 expression on Th1 cells (red) Th2 cells (blue) and unstained cells (filled grey). Histogram in **(B)** shows IFN-γ expression on Th1 cells (red) Th2 cells (blue) and unstained cells (filled grey).

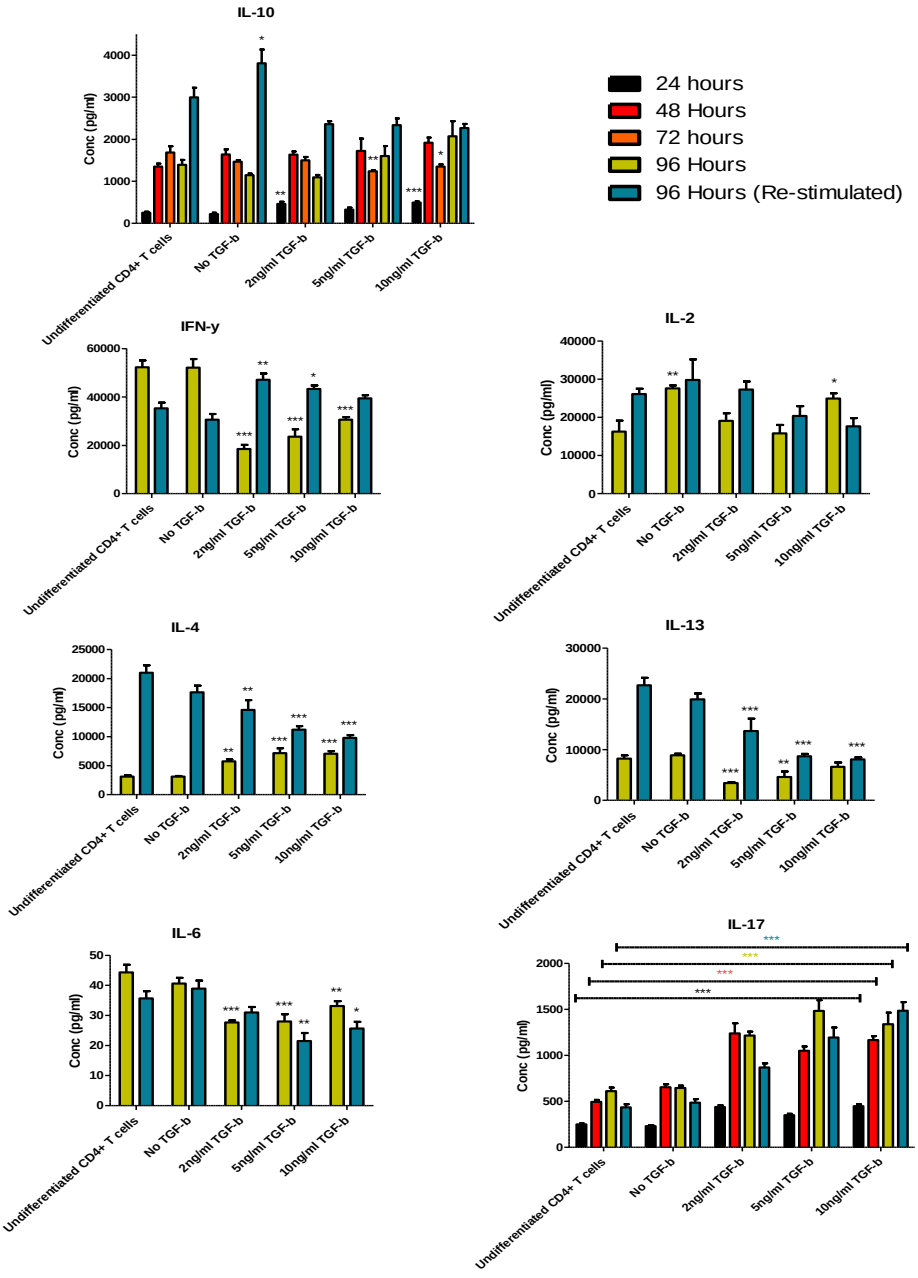
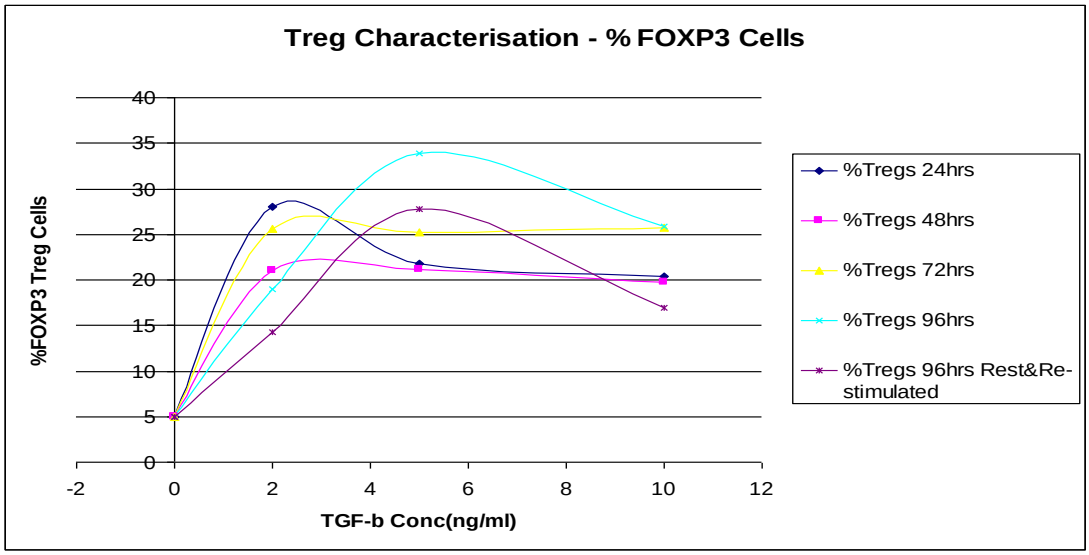


Fig 3.10: Time course analysis of cytokine secretion from Treg cells cells cultured with a range of TGF-β concentrations. $CD4^+$ T cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19752). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml). The media was supplemented with 20 ng/ml IL-2 and 2 ng/ml, 5 ng/ml, 10 ng/ml or no TGF-β antibody for Treg polarising conditions. Samples were taken at 24, 48, 72 and 96hrs. Cells were re-stimulated in fresh media on day 3 with plate-bound anti-CD3 (1 µg/ml) plus anti-CD28 (0.2 µg/ml) for 24 hrs. Supernatants were collected and analysed using ELISA according to manufacturer's instructions (R&D duoset) to confirm production of IL-10 over each time point. One way Anova with Newman Keuls post-hoc test was used to determine if differences between groups were significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Groups are compared to undifferentiated $CD4^+$ T-cells independently at each time point.

A.



B.

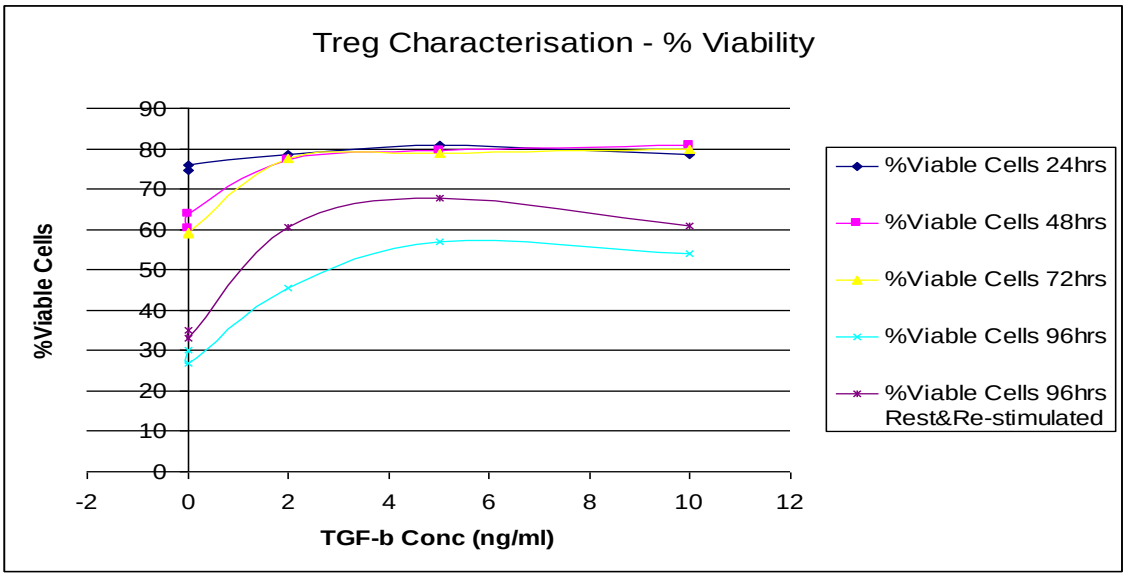
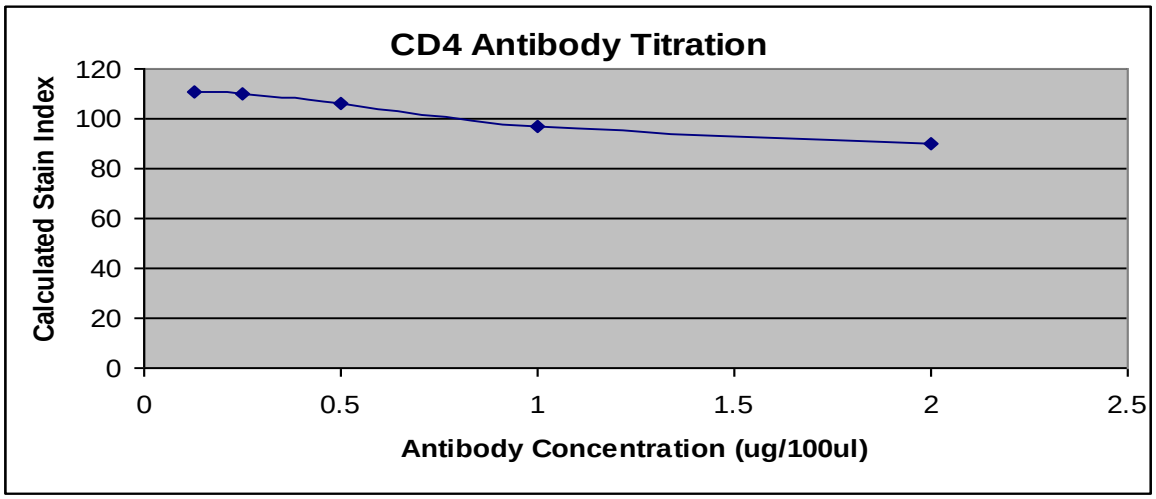
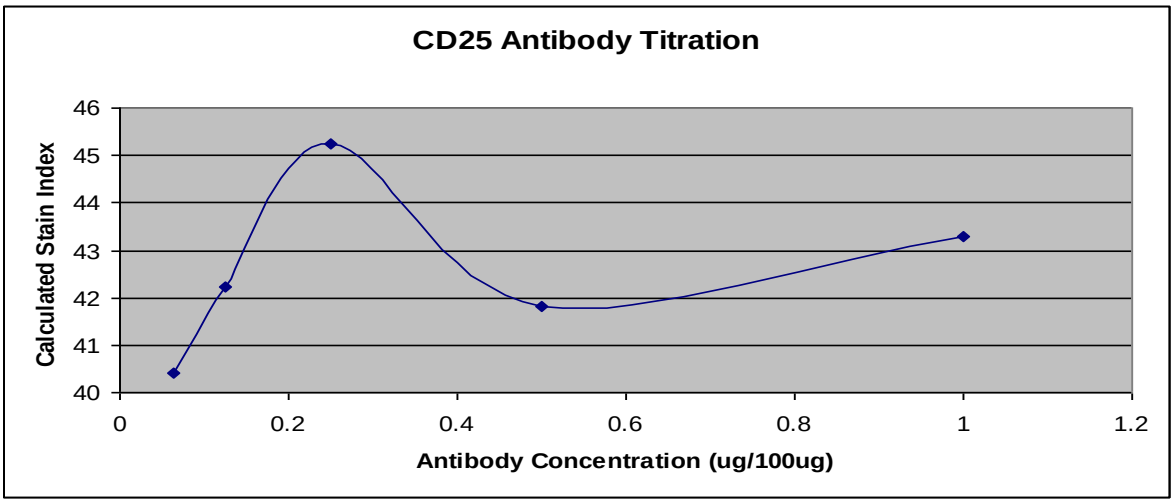


Fig 3.11: FoxP3 transcription factor staining for Regulatory T-cells over a time course. $CD4^+$ T cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19752). Cells were plated at $1x10^6$/ml on a 24 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml). The media was supplemented with 20 ng/ml IL-2 and 2 ng/ml, 5 ng/ml or 10 ng/ml TGF-β antibody for Treg cell polarising conditions. Samples were taken at 24, 48, 72 and 96 hrs. Cells were re-stimulated in fresh media on day 3 with plate-bound anti-CD3 (1 µg/ml) plus anti-CD28 (0.2 µg/ml) for 24 hrs. **(a)** Cells were collected at each time point and stained for CD4 using FITC primary conjugated antibody, CD25 using PE primary conjugated antibody and intracellular FOXP3 using APC primary conjugated antibody. **(b)** Cells were also stained with Fixable Viability Dye (eBioscience). Cells were analysed on BD FACS Aria, data was analysed using Flowjo software (treestar). Cells were gated on viable $CD4^+$ expressing cells.





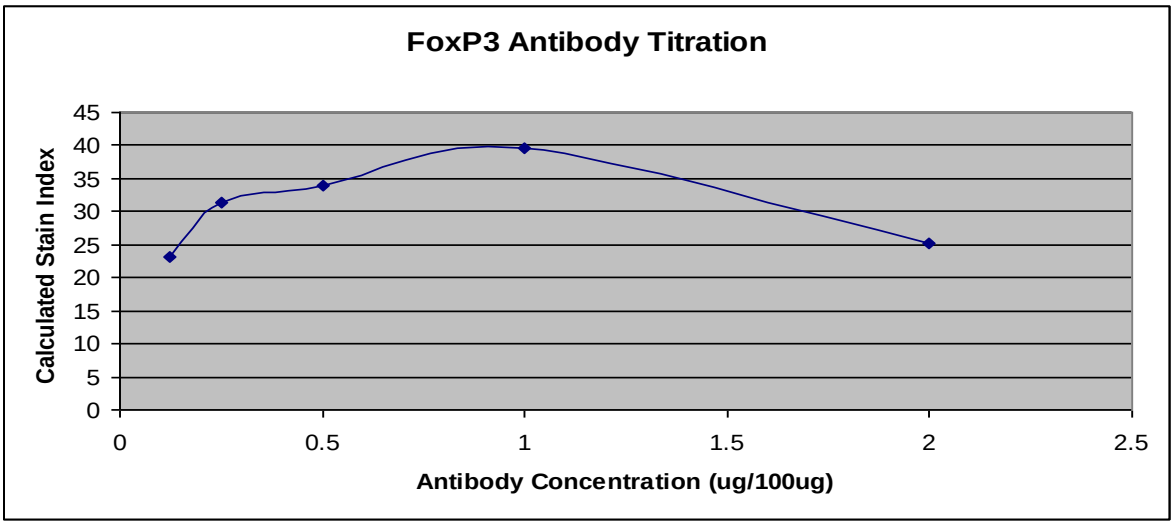


Fig 3.12: CD4, CD25 and FoxP3 antibodies were titrated to optimise concentrations used for Foxp3 staining protocol. Cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks. Erythrocytes were lysed using a red blood cell lysing buffer (SIGMA). Cells were stained with a range of concentrations of CD4 using FITC primary conjugated antibody, CD25 using PE primary conjugated antibody and intracellular $FOXP3^{+}$ using APC primary conjugated antibody. Cells were analysed on BD FACS Aria, data was analysed using Flowjo software (treestar).

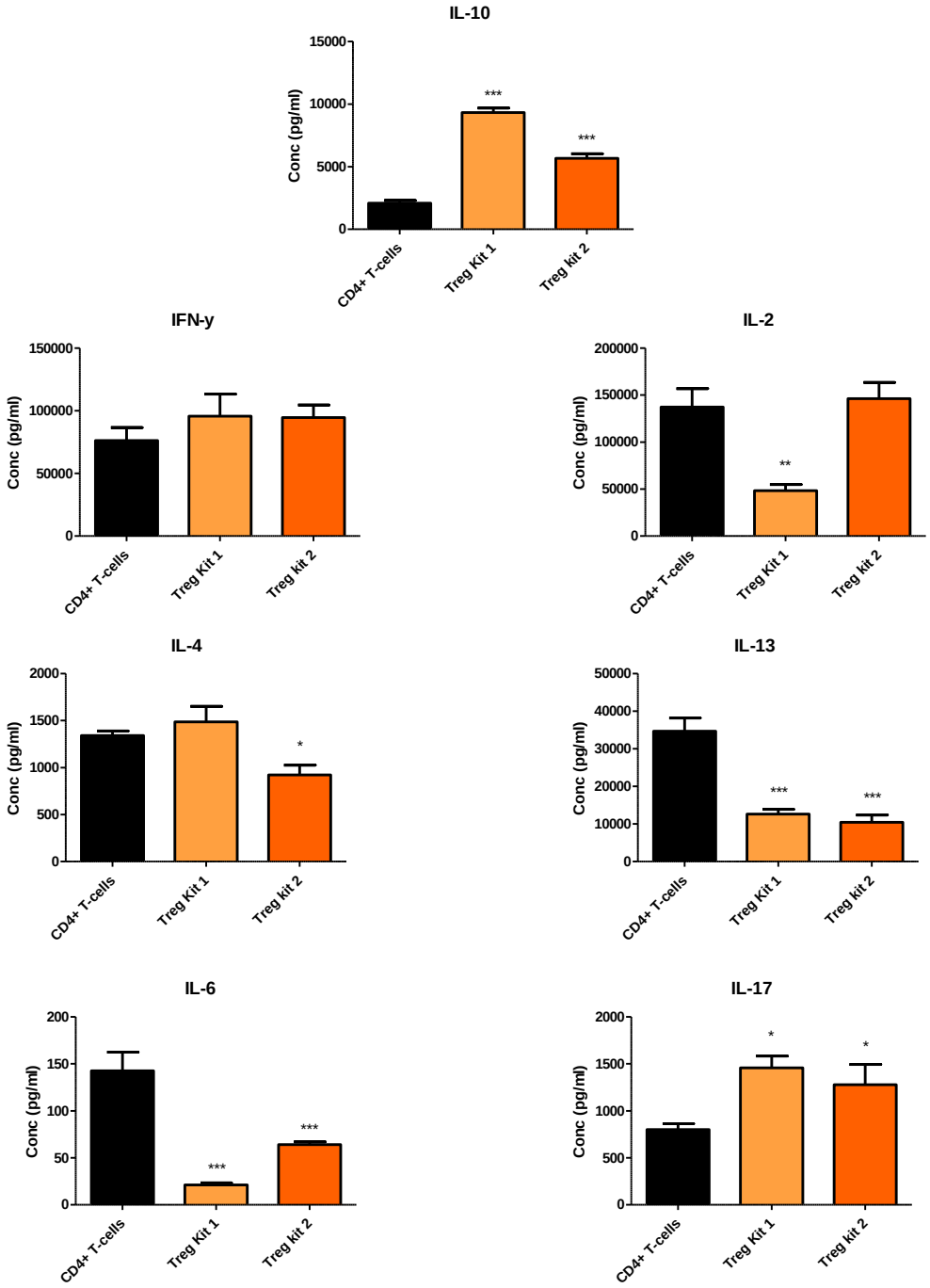


Fig 3.13: Comparison of Cytokine secretion from Treg cells isolated from two Treg isolation kits. $CD4^+$ Treg cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep™ Mouse CD25 Regulatory T Cell positive selection Kit (Treg Kit 1 - Stemcell Cat#18782) and EasySep™ Mouse $CD4^+CD25^+$ Regulatory T Cell Isolation Kit II (Treg Kit 2 - Stemcell Cat#18783). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml). Supernatants were collected at 72 and analysed using ELISA according to manufacturer's instructions (R&D duoset). One way Anova with Newman Keuls post-hoc test was used to determine if differences between groups were significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Groups were compared to undifferentiated $CD4^+$ T-cells.

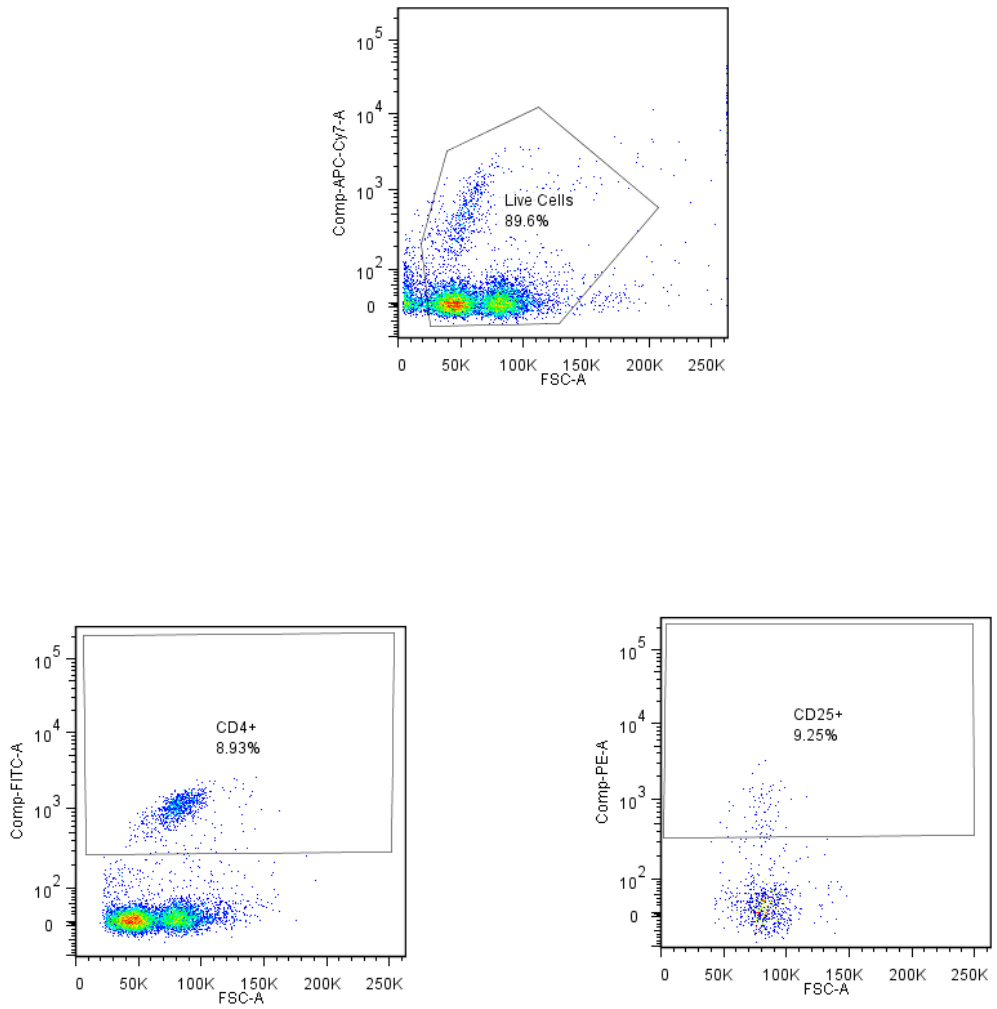


Fig 3.14: CD25 expression of splenocytes pre-isolation. Cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks. Erythrocytes were lysed using a red blood cell lysing buffer (SIGMA). Cells were stained for CD4 using FITC primary conjugated antibody and CD25 using PE primary conjugated antibody. Cells were also stained with Fixable Viability Dye (eBioscience). Cells were analysed using BD FACS Aria and data was analysed using Flowjo Software (treestar). Cells were gated on viable $CD4^+$ $CD25^+$ expressing cells (9.25%).

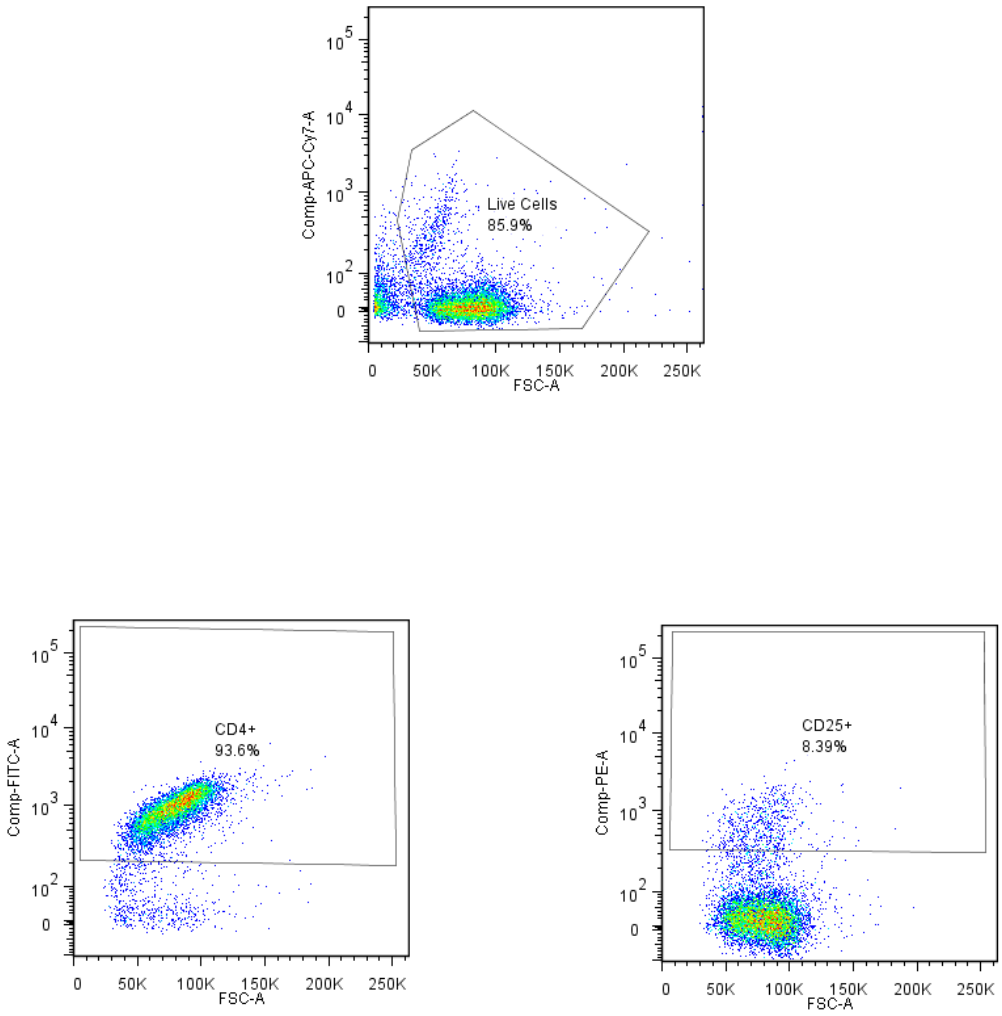


Fig 3.15: CD25 expression of isolated CD4⁺ T-cells. CD4⁺ T-cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep CD4⁺ Isolation (Stemcell Cat# 19752). Cells were plated at $1x10^6$/ml on a 6 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) for 72 hrs. Cells were collected and stained for CD4 using FITC primary conjugated antibody and CD25 using PE primary conjugated antibody. Cells were also stained with APC-Cy7 conjugated Fixable Viability Dye (eBioscience). Cells were analysed using BD FACS Aria and data was analysed using Flowjo Software (treestar). Cells were gated on viable CD4⁺ CD25+ expressing cells (8.39%).

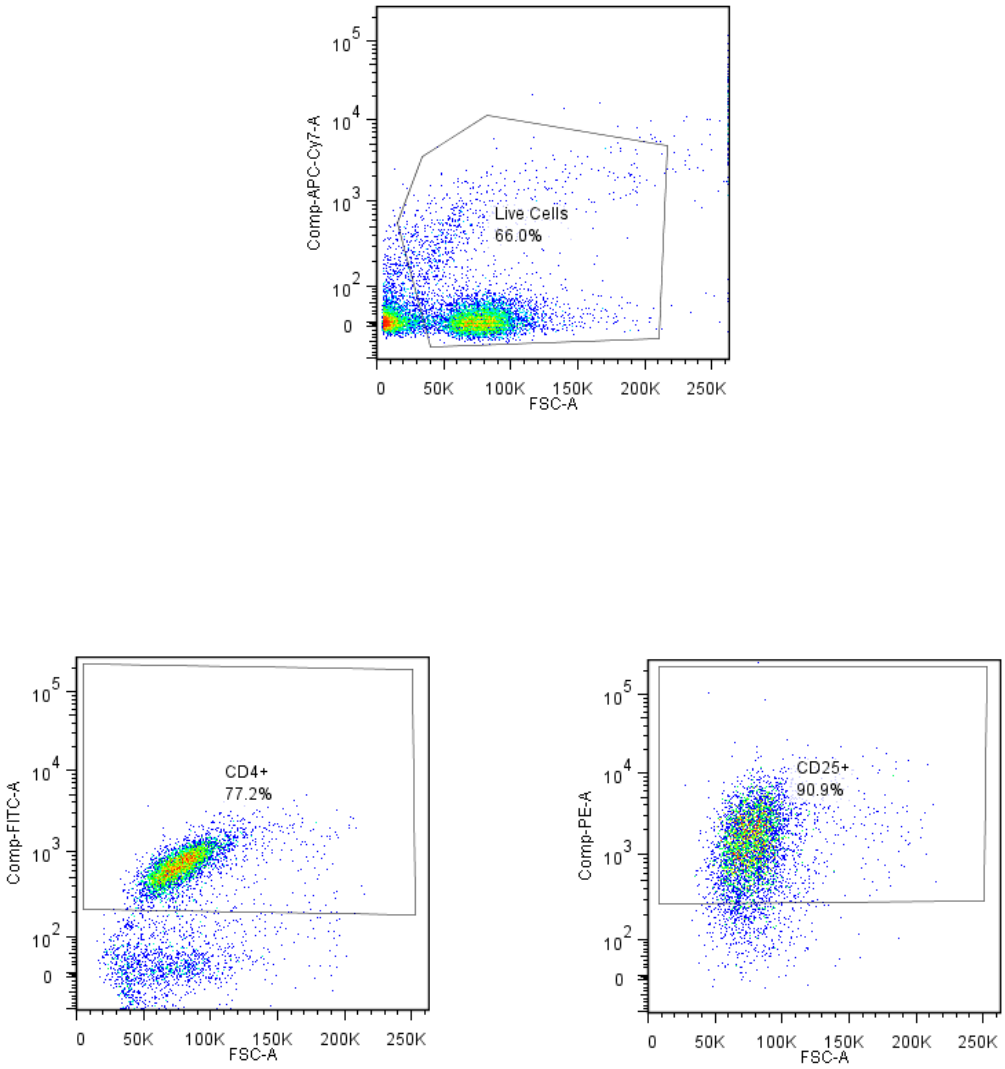


Fig 3.16: CD25 expression of isolated Regulatory T-cells. $CD4^+$ Treg cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep™ Mouse CD25 Regulatory T Cell positive selection Kit (Stemcell Cat#18782). Cells were plated at $1x10^6$/ml on a 6 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) for 72 hrs. Cells were collected and stained for CD4 using FITC primary conjugated antibody and CD25 using PE primary conjugated antibody. Cells were also stained with Fixable Viability Dye (eBioscience). Cells were analysed using BD FACS Aria and data was analysed using Flowjo Software (treestar). Cells were gated on viable $CD4^+$ $CD25^+$ expressing cells (90.9%).

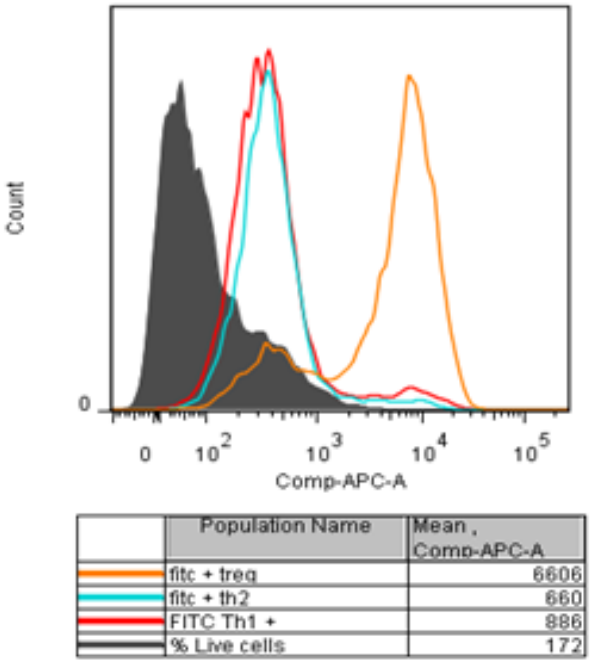


Fig 3.17: Intracellular transcription factor staining for Treg cells. $CD4^+$ T-cells and $CD4^+$ Treg cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19752) and EasySep™ Mouse CD25 Regulatory T Cell positive selection Kit (Stemcell Cat#18782), respectively. Cells were plated at $1x10^6$/ml on a 6 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml). The media was supplemented with 10 µg/ml neutralising anti-IFN-γ antibody and 10 ng/ml IL-4 for Th2 polarising conditions and 20 ng/ml IL-2, 20 ng/ml IL-12 and 10 µg/ml anti-IL-4 antibody for Th1 polarising conditions. Cells were collected after 72 hrs and stained for CD4 using FITC primary conjugated antibody and intracellular Foxp3 using APC primary conjugated antibody. Cells were also stained with Fixable Viability Dye (eBioscience). Cells were analysed on BD FACS Aria, data was analysed using Flowjo software (treestar). Cells were gated on viable $CD4^+$ expressing cells. Histogram shows Foxp3 expression on viable $CD4^+$ Th1 (red), Th2 (blue), Treg (orange) and unstained cells (filled grey).

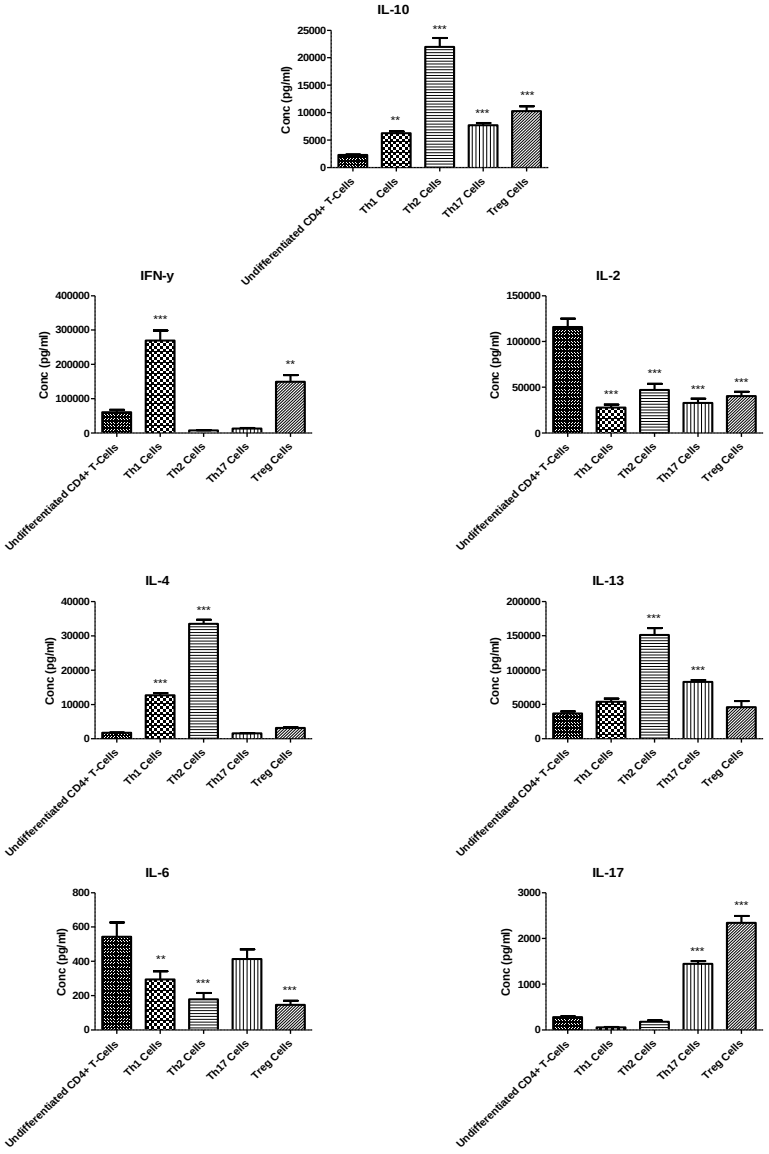


Fig 3.18: Cytokine secretion from undifferentiated CD4$^+$ T-cells, Th1, Th2, Th17 and Treg cells. CD4$^+$ T cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep CD4$^+$ Isolation (Stemcell Cat# 19752) and EasySep™ Mouse CD25 Regulatory T Cell positive selection Kit (Stemcell Cat#18782) for Treg cells. Cells were plated at 1x10^6/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/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; 10 µg/ml neutralising anti-IFN-γ antibody and 10 ng/ml IL-4 for Th2 polarising conditions; or 10 µg/ml neutralising anti-IFN-γ antibody and 20 ng/ml IL-6 and 20 ng/ml IL-23 for Th17 polarising conditions. Supernatants were collected at 72 (undifferentiated and Treg cells) and 96 hrs (Th1, Th2 and Th17 cells). Cells were re-stimulated in fresh media on day 3 with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) for Th1, Th2 and Th17 cells for 24 hrs. Supernatants were collected and analysed using ELISA according to manufacturer's instructions (R&D duoset). One way Anova with Newman Keuls post-hoc test was used to determine if differences between groups were significant (*p<0.05, **p<0.01, ***p<0.001). Groups were compared to undifferentiated CD4$^+$ T-cells.

3.3 DISCUSSION

In this chapter the culturing conditions required for the polarisation of the $CD4^+$ T-helper subsets *in vitro* were optimised. A pure and viable $CD4^+$ T-cell population was isolated. A magnetic based isolation protocol, using an EasySep™ Mouse $CD4^+$ T-cell Isolation Kit from Stemcell Technologies, was followed in order to isolate a $CD4^+$ T-cell population. This method involves the addition of antibodies that are directed against any unwanted cells in collected splenocytes. Magnetic nano-particles are then added which bind these antibodies, forming a magnetic nano-particle-antibody complex. The cells are then placed into a magnetic field which attracts and holds this complex in place, allowing the unbound $CD4^+$ cells to be poured off.

In order to confirm that the isolated cells are indeed $CD4^+$ T-cells, the cells were double stained for CD4 and CD3. As some macrophage and DCs also express the CD4 surface marker, it was necessary to also stain for CD3, which makes up a part of the TCR complex on T-cells and plays a role in antigen recognition (Krummel et al. 2000). The magnetic isolation of $CD4^+$ T-cells yielded a population of >90% positive $CD3^+/CD4^+$ T-cells.

Once a pure population of $CD4^+$ T-cells was confirmed using this isolation method, the next aim was to optimise their activation. This involved optimising the replication of the signals required for activation of $CD4^+$ T-cells *in vitro.* Anti-CD3 and anti-CD28 antibodies were used which cross-link the TCR and CD28 respectively, replicating the MHC II-TCR signal and B7-1/B7-2-CD28 co-stimulatory signals. Plate-bound CD3 for two hours prior to activation was the method of choice. Soluble anti-CD3 has proven to yield poor proliferation and promotes cell anergy and was therefore avoided. Activation of $CD4^+$ T-cells was possible using these antibodies in culture with isolated T-cells for 72 hours. Stimulation of T-cells results in the upregulation of the CD25 surface marker. This is the T-cell receptor for IL-2, which is a proliferation factor. In order to assess the ability of cells to become activated they were stained with antibodies specific for CD25. Activated T-cells expressed highest levels of CD25 when cultured with anti-CD3 and anti-CD28 antibodies *in vitro* for 72 hours. Cells were also most viable and cytokine secretion was most abundant under these conditions. This method of activation and culture was therefore chosen for future experiments involving undifferentiated $CD4^+$ T-cells.

Following optimisation of the culture of $CD4^+$ T-cells, the next step was to optimise the differentiation of these cells into the distinguished T-helper subsets. The specific cytokines and co-stimulatory factors released during antigen presentation and T-cell activation determines the cell fate or phenotype of T-cell to be differentiated. This phenotype is maintained by negative-feedback loops of cytokine secretion which prevent the development of other subsets. These cytokine-induced responses are mediated through signal transducers and activators of transcription (STAT) proteins (Zhu et al. 2010). In order to optimise the *in vitro* assays for the differentiation of $CD4^+$ T-cell subsets (Th1, Th2, Th17 and Treg cells), the cytokine environment required for the differentiation of each cell type was mimicked *in vitro.* A time course analysis was carried out involving the addition of recombinant cytokines and neutralising antibodies, required to drive each of the subsets, to the culture of isolated $CD4^+$ T-cells *in vitro*.

$CD4^+$ T-cells have a wide range of functions in the immune system. They activate B cells and promote antibody production, help maintain $CD8^+$ T-cells function in fighting pathogens, regulate macrophage activity, directly expel pathogenic microorganisms and finally suppress the immune response to maintain homeostasis and control autoimmunity (Zhu et al. 2010). Activation and clonal expansion of Th1 cells occurs in response to intracellular pathogens. APCs secrete large amounts of IL-12 when they come into contact with surrounding pathogens. This results in the production of IFN-γ from APCs which creates a feedback loop enhancing further IL-12 secretion (de Saint-Vis et al. 1998; Hsieh et al. 1993; Macatonia et al. 1995; Manetti et al. 1993). IFN-γ secretion from T-cells persists at this point, which inhibits the proliferation of Th2 cells and leads to the activation of STAT1. Following the activation of STAT1, the transcription factor, T-bet, is induced. This induces further IFN-γ secretion and the commitment of type 1 T-helper cells. Another feedback loop is generated, inducing and maintaining T-bet expression as well as promoting further differentiation of surrounding $CD4^+$ T-cells into Th1 cells (Kaiko et al. 2008). Due to the importance of IL-12 in Th1 commitment, recombinant IL-12 was added to the culture of $CD4^+$ T-cells *in vitro* to drive Th1 differentiation. IL-4 produced by Th2 cells inhibits IFN-γ secretion, and ultimately Th1 differentiation, while upregulating the Th2 transcription factor, GATA-3. GATA-3 inhibits STAT4, which is involved in

the activation of T-bet (Usui et al. 2003; Ouyang et al. 1998). For this reason, a neutralising antibody was used in the culture of Th1 cells.

Th2 cells are known to play a role in the target and removal of parasites such as worms and helminths. IL-4, IL-6, IL-10 and IL-13 are the main cytokines involved in the polarisation of the Th2 subset (Kaiko et al. 2008). STAT6 is activated in response to IL-4 secretion from natural killer T (NKT) cells, eosinophils or mast cells. This then acts by upregulating the transcription factor GATA-3 (Wang et al. 2006; Ouyang et al. 1998; Kaplan et al. 1996). GATA-3 transcription then leads to the secretion of cytokines characteristic of Th2 cells – IL-4, IL-5, IL-9, IL-10 and IL-13. GATA-3 expression is blocked by IL-12 and IFN-γ secretion from Th1 cells, subsequently inhibiting Th2 differentiation. IL-12 signalling via STAT4 in Th1 cells allows for the suppression of a Th2 Phenotype (Ouyang et al. 1998). Therefore, a neutralising anti-IFN-γ antibody was added to the culture of Th2 cells.

Th17 cells target the removal of extracellular bacteria. TGF-β and IL-6 are essential for the initiation of Th17 differentiation (Mangan et al. 2006). IL-23 supports Th17 commitment, once initiation has begun (Zhou et al. 2007). STAT3 and retinoic acid receptor-related orphan receptor (ROR)-γ, transcription factor, play key roles in the mediation of cytokine secretion. STAT3 is upregulated upon T-cell activation and allows for the release of IL-6 and differentiation of Th17 cells (Yang et al. 2007). Increased release of IL-6 results in the secretion of IL-21, which is required for the complete differentiation of Th17 cells (Nurieva et al. 2007). IL-21 and IL-23 activate RORγ, which results in IL-17 secretion with the help of STAT3. Fully activated Th17 cells secrete IL-17 and IL-22. ROR-γ expression inhibits the differentiation of Th1 and Th2 cells. IL-6, during Th17 induction, also inhibits the generation of Treg cells (Awasti et al. 2008). IFN-γ, IL-4 and IL-2, characteristic cytokines of Th1, Th2 and Treg cells respectively, inhibit Th17 differentiation (Harrington et al. 2005; Park et al. 2005; Laurence et al. 2007; Bi et al. 2007). For this reason a neutralising anti-IFN-γ antibody was added to the culture of $CD4^+$ T-cells to polarise Th17 cells.

Treg cells maintain homeostasis in the host and prevent autoimmunity by regulating the effector T-cell responses (Th1, Th2, Th17) through the secretion of TGF-β and the anti-inflammatory cytokine, IL-10. They are essential for the generation of tolerance to self- and non-self-antigens. Forkhead box P3 (Foxp3) is the transcription factor and

master regulator, which controls the development and function of Treg cells. Foxp3 transduction in undifferentiated T-cells also upregulates CD25 and cytotoxic T-cell-associated antigen-4 (CTLA-4) expression, while inhibiting the production of the effector T-cell cytokines IFN-γ, and IL-4 (Hori et al. 2003; Sakaguchi et al. 2008). CD25 is the IL-2 receptor. IL-2 is essential for the activation and maintenance of Treg cells. It allows for the differentiation of undifferentiated T-cells to Foxp3 expressing Treg cells when TGF-β is present and expands them at high doses. IL-2 also prevents the polarisation of Th17 cells by preventing IL-17 production through STAT5 (Laurence et al. 2007). For this reason TGF-β and IL-2 were used for the initial optimisation of Treg cell culture. Foxp3 is the most reliable marker of Treg cells; therefore, it was necessary to stain for both Foxp3 and CD25 to confirm this cell type *in vitro.*

A time course analysis of cytokine secretion from Th1, Th2, Th17 and Treg cells was carried out *in vitro*. The cytokine secretory profiles examined fit for Th1, Th2 and Th17 cells cultured *in vitro* for 96 hours, with a re-stimulation step at 72 hours. Th1 cells secreted a high IFN-γ profile, along with low levels of IL-4 and IL-10 secretion. As mentioned IL-4 from Th2 cells inhibits the fate of Th1 cells, therefore this is a suitable *in vitro* model for Th1 cells. Th2 cells secreted high levels of IL-4 and IL-10, while reducing IFN-γ secretion, confirming the characteristic cytokine profile associated with this subset. Th17 cells displayed an increase in secretion of IL-17 while inhibiting the other cytokines, making it an ideal *in vitro* model for Th17 cells. Undifferentiated T-cells secrete an optimum cytokine profile at 72 hours. These conditions were used for future experiments. As mentioned, it has been demonstrated that Treg cells can suppress effector T-cell cytokines *in vitro* (Hori et al. 2003). However, in this experiment, Treg cells increased secretion of IFN-γ, IL-4 and IL-17 and no increase in IL-10 was observed when compared to undifferentiated T-cells. This suggests that the culture of this subset required further optimisation.

In order to characterise a full immune profile of the effector $CD4^+$ T-cells, it was necessary to also stain for and measure the expression of characteristic intracellular cytokines in order to quantify the frequency of cytokine production inside the cell at a single cell level, in addition to cytokine secretion released from cells (ELISA). In this study, we used IFN-γ and IL-13 as representative Th1 and Th2 cytokines, respectively, to demonstrate their expression in both cell types. There was a clear difference in the

expression of these markers between both cell types. No considerable IFN-γ or IL-13 expression was observed in unstained control cells. In contrast, expression of the Th1-specific cytokine was higher in Th1 cells than in Th2 cells and similarly IL-13 expression was highest in Th2 cells when compared to Th1 cells. This data further contributes to the overall profile of these *in vitro* assays. Hydrolysates were not assessed in great detail in Th17 cells, therefore, our Th17assay was not characterised further than cytokine secretion measured by ELISA.

In a second attempt to optimise *in vitro* Treg cell culture, we examined the ability of a range of TGF-β concentrations to differentiate Treg cells. While increasing the concentration of TGF-β in culture inhibited IFN-γ, IL-4, IL-6 and IL-13 secretion compared to undifferentiated T-cells in a dose dependent manner, it did not upregulate IL-10 secretion. IL-17 secretion was also increased dose dependently with TGF-β, with 10 ng/ml TGF-β yielding the same level of IL-17 secretion as Th17 cells. This result suggests that Th17 cells, and not Treg cells, have been polarised under these conditions and have blocked the differentiation of Treg cells. Zhou et al. (2008), state that Th17 cell differentiation is regulated in a dose dependent manner by TGF-β. Low concentrations of TGF-β, along with IL-6 and IL-21, activate differentiation of Th17 cells, whereas high concentrations of TGF-β block Th17 differentiation and instead, Foxp3 expressing Treg cells develop (Korn T et al. 2007 and Zhou et al. 2007). Therefore, differentiation of either a Th17 or Treg subset is dependent upon the cytokine-regulated balance of Foxp3 and RORγt. Foxp3 staining was also carried out for the confirmation of Treg cells. CD4, CD25 and Foxp3 antibodies were titrated before use in order to optimise the most efficient concentration for their use. Maximum expression of Foxp3 was observed in cells cultured with 5 ng/ml TGF-β for 96 hours. However, only 35% of these cells expressed Foxp3. Treg cells make up 5-10% of surrounding $CD4^+$ T-cells. The number of $Foxp3^+$ expressing cells following culture with TGF-β and IL-2 should be ~60% of the cell population according to Fantini et al. (2007). This protocol suggests a culture time of 5 days in order to yield Treg cells. We attempted to repeat this exact protocol but viability was poor in cells left in culture for longer than 4 days.

Our next and final approach involved examining the use of commercial *in vitro* Treg isolation kits. We identified two Treg isolation kits from Stemcell Technologies, from which we compared cytokine profiles. Both isolation kits were capable of isolating

cells that suppressed or had no effect on the characteristic effector cell cytokines compared to undifferentiated T-cells, indicating characteristic Treg activity. Both Treg cell types also significantly enhanced IL-10 levels. It is noteworthy that IL-2 secretion was significantly suppressed in cells isolated from Treg Kit 1 but was enhanced from cells isolated from Kit 2. While high levels of IL-2 are required for the activation of Treg cells, Treg cells, themselves, do not secrete IL-2 (Malek and Castro, 2010). On the other hand, the effector $CD4^+$ and $CD8^+$ T-cells produce IL-2 upon activation. The capacity to secrete IL-2 is, therefore, a distinguishing feature between effector and Treg cells. Wu et al. (2006) and Ono et al. (2007) observed that IL-2 secretion is inhibited in Treg cells due to Foxp3 interacting with Nuclear factor of activated T-cells (NFAT) and Runt-related transcription factor 1 (Runx1), which are critical transcription factors for the differentiation of effector T-cells (Th2 and Th1/Th17 cells, respectively). Due to its ability to isolate cells which suppressed or did not drive the effector T-cell characteristic cytokines (IFN-γ, IL-4, IL-13 and IL-6), reduced IL-2 secretion and increased IL-10 levels, we made the decision to characterise the cells from Kit 1 further. IL-17 was also significantly increased by both Treg kits, which we will discuss in our final paragraph.

We assessed and compared CD25 surface marker expression on three different cell populations: splenocytes prior to carrying out the isolation protocol; isolated undifferentiated $CD4^+$ T-cells after culture; and isolated Treg cells following culture. Treg cells are characterised by their high expression of CD25 (Malek and Castro, 2010). Chen et al. (2010) report that the average number of CD25-expressing CD4 Treg cells among healthy mouse peripheral $CD4^+$ T cells is 5–10%. This was observed in both the pre-isolated lysed splenocyte and $CD4^+$ T-cell populations, as just less than 10% of these cell populations were $CD4^+CD25^+$ Treg cells. Isolated Treg cells using the Treg isolation Kit 1, however, had a much more dominant population of $CD4^+CD25^+$ T-cells, making up just over 90% of the cell population, indicating a pure population of $CD4^+CD25^+$ Treg cells.

As the effector T-cells also express CD25, although to a lesser extent than Treg cells, it was necessary to also stain for the Treg master regulator and transcription factor, Foxp3 to validate this result. Foxp3 controls the development and function of Treg cells (Hori et al. 2003; and Fontenot et al. 2003). Low expression of Foxp3 results in failure to induce the Treg subset (Wan and Flavell, 2007). Additionally, Foxp3 is

responsible for the suppression of the effector T-cells (Hori et al. 2003; Fontenot et al. 2003; and Khattri et al. 2003). It is, therefore, a key marker necessary for the complete characterisation of our *in vitro* Treg assay. We demonstrated that Foxp3 expression was ten times greater in isolated Treg cells when compared to the effector, Th1 and Th2 cell populations, which contributed further to the overall characteristic profile of the Treg cells.

The final experiment carried out to conclude this chapter involved a repeat examination of the T-cell subset cytokine profiles in order to compare cytokine secretion from Treg cells isolated from the commercial kit to the effector cell populations. The effector subsets, once again, displayed their characteristic cytokine profiles as expected. Treg cells also significantly enhanced their key regulatory cytokine, IL-10. Similar to our Treg kit comparison experiment, Treg cells also significantly increased IL-17 secretion, to an even greater extent than Th17 cells themselves. Beriou et al. (2009) state that Treg cells possess the ability to produce IL-17 and assessed their capacity to retain their suppressive function. They report that IL-17^+/$FoxP3^+$ Tregs retain a suppressive phenotype. Moreover, they display the plasticity to exert suppressive effects or secrete IL-17, depending on the nature of the stimulus provided. They conclude by stating that suppressive $FoxP3^+$ Treg cells produced *in vitro* can be encouraged to secrete IL-17 under inflammatory conditions. It is very likely that stimulation with CD3 and CD28 antibodies in our study may, therefore, have induced this Treg population with the additional ability to secrete IL-17 to combat the external stimulus they were exposed to.

In summary, this chapter describes the isolation, differentiation and characterisation of $CD4^+$ T-cells and their respective activated subtypes; Th1, Th2, Th17 and Treg cells. Using a magnetic isolation approach, highly purified viable cell populations could be isolated and their respective secretory profile of key cytokines (IFN, IL-2, IL4, IL-13, IL-17 and IL-10) and characteristic surface and intracellular markers validated. We are satisfied that we have determined their effectiveness as *in vitro* model systems. In conclusion, we have determined the effectiveness of the assays developed in this chapter as *in vitro* model systems for the screening of cow's milk hydrolysates. Chapters 4 and 5 will exploit these *in vitro* models to examine the ability of cow's milk protein hydrolysates to modulate the immune system when in a state of dysregulation.

CHAPTER 4

IMMUNOMODULATORY PROPERTIES OF PROTEIN HYDROLYSATES FOR APPLICATION IN INFANT ALLERGIES

4.1 INTRODUCTION

In recent times, there has been a significant increase in food allergy awareness, as well as its associated threats, in the Western world. Recent studies indicate that between 15-30% of this population suffer from a food allergy (van den Elsen et al. 2013). Despite this increase within the population of food allergy, the availability of treatments against such is still lacking and subsequently, food allergies are typically managed by the complete removal of the food stuff that triggers an immune response (allergen) from the diet. It is thought that a better understanding of the mechanisms involved in food allergy would promote the development of more effective and pragmatic treatments and prevention strategies. If successful, these treatments applied in the setting of infant nutrition could ablate the development of allergies in later life by reducing the time taken to induce tolerance to harmless food antigens.

A specific aim of this PhD project is to identify novel bovine milk-derived protein hydrolysates that possess positive immunomodulatory properties, which involve priming the immune system of infants to avoid allergy in later life by bringing about immune balance. Hydrolysates that can prevent sensitisation to cow's milk protein allergy (CMPA) via modulation of the immune system would beneficial in preventing the problems associated with allergy in later life. This chapter aims to identify candidate hydrolysates which can modulate the immune response away from an allergic type 2 helper T-cell (Th2) dominant phenotype *in vitro*. This will be carried out using the clearly defined T-helper cell subsets, Th1, Th2 and Treg cells, optimised in chapter three, for the screening of cow's milk (CM) hydrolysates. These selected candidate hydrolysates will then be brought forward for *in vivo* testing in a mouse model of OVA-induced allergy in order to determine their effectiveness to induce immune homeostasis in an allergy setting.

Cow's milk protein allergy (CMPA) is the most common food allergy diagnosed in young babies, affecting 2-3% of infants worldwide. CMPA can be IgE or non-IgE-mediated. IgE is an antibody secreted following an allergic response, which results in the release of histamine and clinical symptoms. Non-IgE-mediated allergy typically resolves earlier than IgE-mediated and symptoms are usually immediate and more severe in infants with IgE-mediated allergy (Savilahti and Savilahti, 2013). Breast-fed infants are less likely to develop CMPA than formula-fed infants. For this reason,

along with a plethora of health benefits associated with breast milk, breastfeeding should always be the primary option. However, when breastfeeding is not possible, IMF that can mimic the effects of breast milk would be a good substitute. Breast milk contains immune-modulators that are not present in IMF, which are essential for the development of the infant's immune system early on. In circumstances where breastfeeding is not possible, we aim to identify hydrolysates that mimic the effects of these immune-modulators found in breast milk, through the induction of immune homeostasis.

Currently there is no treatment for CMPA. Strict CM avoidance has been the dietary management for infants diagnosed with CMPA. Milk formulas containing cow's milk hydrolysates are available for infants who suffer with CMPA. Depending on the degree of hydrolysis (DH), proteins are referred to as partially hydrolysed or extensively hydrolysed proteins. Extensively hydrolysed formula (EHF) is the current formula on the market in use for infants with CMPA. These are made up of extensively hydrolysed CM proteins which are easier for the allergic infant to digest because of their small size and thus reduced allergenic properties, thereby allowing the infant to reap the full nutritional benefits of CM (Terheggen-Lagro et al. 2002; and Sampson et al. 1991). The high DH ensures destruction of more of the IgE binding epitopes resulting in lower allergenic potential of the hydrolysates. However, whilst extensive hydrolysis results in the loss of allergenicity, this also results in a loss of overall immunogenicity, which inhibits the immune system's ability to develop natural tolerance to milk proteins. It is also still possible for these formulas to elicit allergic responses. Moreover, accidental exposure to CM is common and can cause allergic reactions and even anaphylactic shock. The symptoms can be treated with anti-histamines or, for more severe reactions, such as anaphylaxis, injectable epinephrine can be used (Vandenplas et al. 2007). Most infants (90%) outgrow CMPA before their fifth year but the small percentage of children who do not develop tolerance, most likely IgE-mediated patients, are more likely to develop multiple food allergies or atopic diseases, such as atopic dermatitis, asthma and allergic rhinitis, in later life (Motala and Fiocchi, 2012; Vandenplas et al. 2007). Hendaus et al. (2016) claim that just under 700 million people worldwide suffer with respiratory allergic diseases. This indicates that there is an urgent requirement for novel treatments and prevention strategies. Further research is necessary to get a deeper understanding of food allergy

for the development of more effective treatments for the disease and to avoid subsequent allergies in later life.

In order to address to the lack of treatments available, recent studies have discovered that exposure to CM allergens may in fact be beneficial (Staden et al. 2007). Oral immunotherapy (OIT) seems to provide an alternative treatment for CMPA. OIT involves the regular oral administration of small amounts of food allergen, with the hope of generating tolerance to the intact food protein over time. OIT results in avoidance of symptoms by providing the infants with partially hydrolysed proteins. These are broken down CM protein peptides that lack the IgE binding epitopes which result in clinical symptoms (Tanabe 2008). Hydrolysed peptides possess different immunological properties, modulating the immune system in various ways (Kiewiet et al. 2015). OIT can result in oral tolerance, which enables the mucosal immune system to remain in a non-activated state when it encounters harmless proteins, such as CM, while retaining its ability to mount an immune response to a potential pathogen. Partial hydrolysis of a peptide allows it to retain enough immunogenicity to generate tolerance (Kiewiet et al. 2015). Among these hydrolysates, both *in vitro* and *in vivo* studies have identified cow's milk derived whey, casein and lactoferrin hydrolysates that have positive modulatory effects on the immune system (Hernández-Ledesma et al. 2014; Gauthier et al. 2006; and Gill et al. 2000). Studies have shown that they display similar beneficial effects to breast milk on gut colonisation, by promoting the growth of *Bifidobacterium*, which is present at lower levels in formula-fed infants and is required for generation of immune balance/homeostasis, thereby promoting oral tolerance (Brück et al. 2003; Mohanty et al. 2016; and Chirico et al. 1997). Manso and Lopez-Fandino (2004) noted that κ-Casein macropeptide (CMP), a whey hydrolysate, enhances the growth of bifidobacteria due to its carbohydrate content. Mao et al. (2007) also claim that a yak casein hydrolysate has the ability to modulate the Th1/Th2 balance following the observation that this hydrolysate modulated the differentiation of helper T cell toward a Th1 phenotype in murine splenocytes, which would be useful in the setting of allergy. Furthermore, it has been shown that partially hydrolysed whey peptides given orally prior to sensitisation can increase $Foxp3^{+}$ expressing Treg cells in mice *in vivo*. This was not observed for extensively hydrolysed whey. Foxp3 is a key transcription factor found in Treg cells and plays a role in the induction of tolerance. Prior to whey sensitisation, mice were

treated with whole, partial or extensively hydrolysed whey via oral gavage. After being challenged with whole whey, the number of $Foxp3^+$ expressing Treg cells in the mesenteric lymph nodes (MLNs) were increased in mice treated with partial whey hydrolysates, compared to untreated mice sensitised to whey. Th2 cells were also decreased in mice treated with both whole whey and partially hydrolysed whey (van Esch. et al. 2011). This suggests that partially hydrolysed peptides possess the potential to promote tolerance and could be a valuable method of OIT. We aim to develop and identify partially hydrolysed CM peptides as a prevention strategy for atopic neonates at risk of developing CMPA, which may reduce the time for allergic patients to generate tolerance and avoid subsequent allergy in later life.

As mentioned in the General Introduction (Chapter 1), fetal Th1 cells are dampened during pregnancy, which results in an allergic Th2 dominant phenotype in newborn infants (Prescott et al. 1998; Vighi et al. 2008). Failure to develop a Th1/Th2 balance early in life results in allergic disease (Prescott, Sly & Holt 1998, Prescott et al. 1998, Prescott et al. 1999, Neaville et al. 2003). For this reason, it is important to prime newborns' immune systems as early as possible in order to balance the Th1/Th2-paradigm to prevent the onset of allergy and the associated problems in later life. Exposure to the mother's gut microbiota, in healthy breast-milk fed babies, can skew this Th2 favoured environment toward a Th1 phenotype, bringing about natural Th1/Th2 balance and in most cases natural immune tolerance to food antigens, such as CM (Mazmanian et al. 2005; Round and Mazmanian 2010). In circumstances where breastfeeding is not possible, this chapter aims to identify CM hydrolysates that can mimic this balancing effect in breast milk, via modulation of cytokine secretion from $CD4^+$ T-cells *in vitro* and *in vivo*. Modulation of the immune response can switch T-cell differentiation away from a Th2 response, which is a key driver of allergy, and towards a Th1 or Treg response, which may lower the time to achieve immune balance and tolerance in allergic infants, while also preventing the development of further allergies later in life (Kiewiet et al. 2015). Treg cells are the key cells involved in maintaining homeostasis in the immune system and in tolerance induction (Hori et al. 2003).

$CD4^+$ T-cells were chosen for this study because they are the precursors to Th1, Th2, Th17 and Treg cells, the key players in the allergy and inflammation processes when dysregulated. Therefore, they may be logical targets of the CM hydrolysates in order

to suppress allergy and have health benefits. Whey protein concentrate (WPC80) and casein protein (NaCN) CM hydrolysates were prepared by our collaborators, Professor Fitzgerald's research group, in UL. The immuno-modulatory ability of these hydrolysates was assessed *in vitro* in order to identify candidates that possessed potential ability to modulate the immune system in an allergic state. The immune-modulatory and therapeutic ability of the candidate hydrolysates was then examined in an *in vivo* OVA allergy mouse model.

4.2 RESULTS

4.2.1 ASSESSMENT OF ALLERGY SUPPRESSING ABILITY OF FIRST-ROUND HYDROLYSATES *IN VITRO*.

This chapter aims to identify hydrolysates that can suppress the key allergy enhancing cell type, Th2, as well as hydrolysates that could potentially induce tolerance to allergy by inducing Treg cells. We first looked at the ability of the hydrolysates to modulate cytokine secretion from the Th2 subset *in vitro,* followed by the Treg subset.

4.2.1.1 THE DOSES OF FIRST-ROUND WHEY AND CASEIN PROTEIN HYDROLYSATES USED HAVE NO SIGNIFICANT EFFECT ON CELL VIABILITY.

Prior to examining their activity on cytokine secretion, it was necessary to first determine whether the hydrolysates were toxic to T-cells. Cells were isolated from the spleens of BALB/c mice using the EasySep Mouse $CD4^+$ T Cell Enrichment Kit (Stemcell Technologies #19752). $CD4^+$ 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) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml) or 10% DMSO (vehicle control). Cells were incubated for 72 hrs. Viability was assessed using an MTS assay (Cell Titer 96 Aqueous One Solution – Promega, WI, USA) according to the manufacturer's instructions. At 1 mg/ml, hydrolysates did not have any significant cytotoxic effect on undifferentiated T-cells when compared to untreated cells *in vitro* **[Figure 4.1].**

4.2.1.2 FIRST-ROUND HYDROLYSATES SUPPRESS THE CYTOKINES REQUIRED TO DRIVE AN ALLERGIC TH2 RESPONSE FROM UNDIFFERENTIATED T-CELLS IN VITRO.

In order to identify whether the hydrolysates possessed allergy suppressing properties, their ability to modulate the key polarising cytokines necessary to drive an allergic Th2 response was first examined in undifferentiated T-cells treated with hydrolysates *in vitro*. Cells were isolated from the spleens of BALB/c mice using the EasySep Mouse $CD4^+$ T Cell Enrichment Kit (Stemcell Technologies #19752). $CD4^+$ cells were plated in triplicate, at a concentration of $1x10^6$ cells/ml on a 96-well plate, at a final volume of 250 µl/well. The cells were then stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml). Cells were

incubated for 72 hrs. Supernatants were collected and measured for the cytokines that drive a Th2 response, IL-4, IL-6 and IL-10, using ELISA according to manufacturer's instructions (R&D duoset).

Figure 4.2 shows a significant reduction in each of the allergy driving cytokines, IL-4 (UL-2-40/41/42/43/45/46/47/48: $p<0.001$), IL-6 (UL-2-43/48: $p<0.001$; UL-2-41/42: $p<0.01$; and UL-2-46: $p<0.05$) and IL-10 (UL-2-40/41/42/43/44/45/46/47/48: $p<0.001$), from undifferentiated T-cells treated with hydrolysates. This indicates that UL-2-40/41/42/43/45/46/47/48 may potentially prevent differentiation into a Th2 subset and they were therefore brought forward for testing in Th2 cells *in vitro*. UL-2-44 was the only hydrolysate that did not inhibit IL-4 and IL-6 secretion and was, therefore, not examined further.

4.2.1.3 FIRST-ROUND HYDROLYSATES CAN PREVENT DIFFERENTIATION OF T-HELPER 2 CELL SUBSET IN VITRO.

After examining the cytokine profile secreted from undifferentiated T-cells and establishing which hydrolysates could potentially inhibit a Th2 phenotype, it was necessary to confirm this activity in a Th2 subset *in vitro*. Cells were isolated from the spleens of BALB/c mice using the EasySep Mouse $CD4^+$ T Cell Enrichment Kit (Stemcell Technologies #19752). $CD4^+$ cells were plated in triplicate at a concentration of $1x10^6$ cells/ml on a 96-well plate, 250 µl/well. The cells were then stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml). The media was supplemented with 10 µg/ml neutralising anti-IFN-γ antibody and 10 ng/ml IL-4 for Th2 polarising conditions. Cells were incubated for 72 hrs and were restimulated in fresh media for 24 hrs. Supernatants were collected and measured for IL-4, IL-10 and IL-13 secretion using ELISA according to manufacturer's instructions (R&D duoset).

Figure 4.3 shows a significant reduction in IL-13 secretion ($p<0.001$) from Th2 cells when treated with UL-2-40/41/42/43/45/46/47/48 compared to the untreated control cells (no peptide). All hydrolysates tested also reduced IL-10 secretion, although UL-2-45/46/47 were the only hydrolysates to have a significant effect ($p<0.001$). UL-2-40/41/42/43 were the only hydrolysates that significantly reduced IL-4 secretion ($p<0.01$). UL-2-47 slightly reduced IL-4 levels. UL-2-45/46/48 slightly increased IL-

4 secretion and, therefore, are not desirable candidates for suppressing Th2 cells. For this reason UL-2-40/41/42/43/47 were of interest for further testing.

4.2.1.4 ASSESSMENT OF FIRST-ROUND HYDROLYSATES IN A T-HELPER CELL 1 SUBSET IN VITRO.

Ideally candidate hydrolysates will have specific activity, acting on certain immune cells but not all, in this scenario ideally one T-cell subset. As mentioned in Chapter 3, dynamic balance between the T-cell subtypes is essential for the development of immune homeostasis. A dysregulated (excessive or underactive) response in any of the T-cell subsets, inhibits this balance and can result in disease. This chapter aims to identify hydrolysates that can specifically target and suppress the Th2 subset, having little or no effect on the other effector T-cell subsets in order to bring about immune balance. In an attempt to build a full immune profile for UL-2-40/41/42/43/45/46/47/48 and ensure that they specifically act on Th2 cells and do not result in complete immune suppression of all of the T-cell subsets, Th1 cells were also treated with these hydrolysates. Cells were isolated from the spleens of BALB/c mice using the EasySep Mouse $CD4^+$ T Cell Enrichment Kit (Stemcell Technologies #19752). $CD4^+$ cells were plated in triplicate at a concentration of $1x10^6$ cells/ml on a 96-well plate, 250 µl/well. The cells were then stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and 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 hrs and restimulated in fresh media for a further 24 hrs. Supernatants were collected and measured for secretion of the characteristic Th1 cytokines, IFN-γ and IL-2, using ELISA according to manufacturer's instructions (R&D duoset).

Figure 4.4 shows only a slight reduction in IFN-γ and IL-2 secretion from Th1 cells when treated with UL-2-40/41/42/43/47. UL-2-45/46 had an undesirable effect, driving IFN-γ secretion and reducing IL-2 secretion (UL-2-46 treated cells being significant: $P<0.001$). UL-2-48 treatment had no effect on IFN-γ secretion, however, it significantly reduced IL-2 secretion ($p<0.05$). On observation of these results, in addition to their ability to suppress the Th2 subset, UL-2-40/41/42/43/47 were of interest to be brought forward for further testing. At this point we were informed by

the production team in UL that the hydrolysis processes for UL-2-43 was considered expensive which makes this hydrolysate undesirable for future upscaling for commercialisation and industry use. For this reason, UL-2-40/41/42/47 were selected for further testing.

4.2.2 ASSESSMENT OF ALLERGY SUPPRESSING ABILITY OF SECOND-ROUND REGENERATED HYDROLYSATES *IN VITRO*.

In order to determine whether the bioactivity of the hydrolysates of interest (now referred to parent hydrolysates) is reproducible, UL-2-40/41/42/47 hydrolysates were regenerated, using identical hydrolysis conditions as the parent compounds, and named UL-2-77/78/79/151 respectively. The list of regenerated hydrolysates can be seen in **Table 4.1**.

Parent Hydrolysate	*Regenerate Hydrolysate*	*Starting Substrate*	*Activity/Reason brought forward*
UL-2-40	**UL-2-77**	**WPC80**	**Allergy Suppressing ↓ Th2**
UL-2-41	**UL-2-78**	**WPC80**	**Allergy Suppressing ↓ Th2**
UL-2-42	**UL-2-79**	**WPC80**	**Allergy Suppressing ↓ Th2**
UL-2-47	**UL-2-151**	**NaCN**	**Allergy Suppressing ↓ Th2**

Table 4.1: Overview of Parent and Regenerated Hydrolysates brought forward into Round 2 Screening.

4.2.2.1 THE DOSES OF SECOND-ROUND REGENERATED HYDROLYSATE USED HAVE NO SIGNIFICANT AFFECT ON CELL VIABILITY.

To confirm that the regenerated hydrolysates were not toxic to T-cells, cells were isolated from the spleens of BALB/c mice using the EasySep Mouse $CD4^+$ T Cell Enrichment Kit (Stemcell Technologies #19752). $CD4^+$ 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) plus anti-CD28 (5 µg/ml) and

treated with hydrolysates (1 mg/ml) or 10% DMSO (vehicle control). Cells were incubated for 72 hrs. Viability was assessed using an MTS assay (Cell Titer 96 Aqueous One Solution – Promega, WI, USA) according to the manufacturer's instructions. At 1 mg/ml, regenerated hydrolysates did not have any significant cytotoxic effect on undifferentiated T-cells when compared to untreated cells *in vitro* **[Figure 4.5].**

4.2.2.2 SECOND-ROUND REGENERATED HYDROLYSATES OF UL-2-40/41/42/47 DISPLAY SIMILAR ACTIVITY AS PARENT SAMPLES IN UNDIFFERENTIATED CD4⁺ T-CELLS IN VITRO.

In order to assess the bioactivity of regenerated hydrolysates, UL-2-77/78/79/151, they were screened, alongside the parent hydrolysates, on undifferentiated T-cells prior to Th2 cells and cytokine secretion was examined. Cells were isolated from the spleens of BALB/c mice using the EasySep Mouse $CD4^+$ T Cell Enrichment Kit (Stemcell Technologies #19752). $CD4^+$ cells were plated in triplicate at a concentration of $1x10^6$ cells/ml on a 96-well plate, 250 µl/well. The cells were then stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml). Cells were incubated for 72 hrs. Supernatants were collected and measured for IL-4, IL-6 and IL-10 secretion using ELISA according to manufacturer's instructions (R&D duoset).

UL-2-77/78/79/151, regenerates of UL-2-40/41/42/47 respectively, confirmed similar activity to their parent samples. **Figure 4.6** shows a non-significant reduction in IL-4 and IL-6 secretion from undifferentiated T-cells when treated with parent and regenerate hysrolysates. Regenerated hydrolysates, UL-2-78/79 significantly reduced ($p<0.05$) IL-10 secretion, while parent hydrolysates showed an insignificant IL-10 reduction. These results confirmed that the bioactivity of these hydrolysates is reproducible in undifferentiated T-cells.

4.2.2.3 SECOND-ROUND REGENERATED HYDROLYSATES OF UL-2-40/41/42/47 DISPLAY ALLERGY SUPPRESSING ACTIVITY, SIMILAR TO PARENT SAMPLES IN TH2 CELLS IN VITRO.

In order to confirm the bioactivity of regenerated hydrolysates in a Th2 subset, cells were isolated from the spleens of BALB/c mice using the EasySep Mouse $CD4^+$ T Cell Enrichment Kit (Stemcell Technologies #19752). $CD4^+$ cells were plated in triplicate at a concentration of $1x10^6$ cells/ml on a 96-well plate, 250 µl/well. The cells were then stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) in media supplemented with 10 µg/ml neutralising anti-IFN-γ antibody and 10 ng/ml IL-4 for Th2 polarising conditions. Cells were treated with hydrolysates (1 mg/ml). Cells were incubated for 72 hrs and restimulated in fresh media for a further 24 hrs. Supernatants were collected and measured for IL-4, IL-10 and IL-13 secretion using ELISA according to manufacturer's instructions (R&D duoset).

UL-2-77/78/79/151, regenerates of UL-2-40/41/42/47 respectively, confirmed similar activity to their parent samples in Th2 cells. **Figure 4.7** shows an insignificant reduction in IL-4, IL-10 and IL-13 secretion from Th2 cells when treated with parent hydrolysates (UL-2-40/41/42/47), with the exception of UL-2-41, which slightly increased IL-13. **Figure 4.7** also shows an insignificant reduction in IL-4 secretion from Th2 cells when treated with all regenerated hydrolysates (UL-2-77/78/79/151). There was also a non-significant reduction in IL-13 secretion when treated with UL-2-77/78 and a significant reduction in IL-13 when treated with UL-2-79/151 ($p<0.05$). Regenerated hydrolysates, significantly reduced IL-10 secretion (UL-2-77/78/79: $p<0.001$; and UL-2-151: $p<0.01$). These results confirmed that the bioactivity of these hydrolysates is reproducible in Th2 cells. UL-2-42 and UL-2-47 were chosen for further testing as their effect on IL-4 secretion was most potent.

4.2.3 ASSESSMENT OF ALLERGY SUPPRESSING ABILITY OF THIRD-ROUND OPTIMISED HYDROLYSATES *IN VITRO*.

As this project aims to produce and identify candidate cow's milk hydrolysates for commercialisation at an industry level, it was necessary to determine whether the chosen hydrolysates could be optimised further to increase their commercial value and

make them more desirable for industry, while maintaining or enhancing their bioactivity. Ultimately the most beneficial hydrolysates will be those that are cost effective, efficacious and acceptable to the broadest possible segment of the population and easy to reproduce at large scale. Optimisation was carried out with the hope to improve a number of factors. These factors include enhancing hydrolysis conditions, lowering the cost of production, improving peptide solubility and potentially enhancing overall bioactivity. Improved solubility was a particular aim for the optimisation of UL-2-42/47 as they both display poor solubility. It was hoped that their bioactivity would become more consistent and reproducible. Optimised hydrolysates, of each UL-2-42 and UL-2-47, were produced for further testing. The list of optimised hydrolysates can be seen in **Table 4.2**. The enzyme control (inactive enzyme) was produced using the same hydrolysis process as each of the parent hydrolysates but the enzyme was inactivated after hydrolysis. The purpose of this was to determine that the bioactivity observed in the cells was solely as a result of the hydrolysate treatment and not as a result of the enzyme source. Negative hydrolysate controls (UL-2-150 and UL-2-153) were produced in order to determine the importance of the enzyme source used in the hydrolysis process. They underwent the same heat variations as the hydrolysates but were not exposed to enzymes during hydrolysis.

Parent Hydrolysate	*Regenerate Hydrolysate*	*Optimised Hydrolysates*	*Substrate*	*Notes*	*Reason Brought Forward*
UL-2-42	UL-2-79	UL-2-147 UL-2-148 UL-2-150 Inactive Enzyme	WPC80	Optimisation Controls Enzyme Control	Allergy Suppressing ↓ Th2
UL-2-47	UL-2-151	UL-2-151 UL-2-152 UL-2-153 UL-2-154 Inactive Enzyme	NaCN	UL-2-153 - Control Enzyme Control	Allergy Suppressing ↓ Th2

Table 4.2: Overview of Parent, Regenerated and Optimised Hydrolysates brought forward into Round 3 Screening for Allergy Suppression.

4.2.3.1 THE DOSES OF THIRD-ROUND OPTIMISED UL-2-42 and UL-2-47 PROTEIN HYDROLYSATES USED ARE NOT TOXIC TO CELL VIABILITY.

To confirm whether the optimised hydrolysates were toxic to T-cells, cells were isolated from the spleens of BALB/c mice using the EasySep Mouse $CD4^+$ T Cell Enrichment Kit (Stemcell Technologies #19752). $CD4^+$ 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) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml) or 10% DMSO (vehicle control). Cells were incubated for 72 hrs. Viability was assessed using an MTS assay (Cell Titer 96 Aqueous One Solution – Promega, WI, USA) according to the manufacturer's instructions. At 1 mg/ml, optimised hydrolysates did not have any significant cytotoxic effect on undifferentiated T-cells when compared to untreated cells *in vitro* **[Figure 4.8].**

4.2.3.2 THIRD-ROUND UL-2-42 AND REGENERATED AND OPTIMISED HYDROLYSATES DISPLAY ALLERGY SUPPRESSING PROPERTIES IN UNDIFFERENTIATED T CELLS AND TH2 CELLS IN VITRO.

Optimised hydrolysates for UL-2-42 were examined for bioactivity in undifferentiated T-cells and the Th2 subset to determine if its activity could be enhanced or remain unchanged during the optimisation process. Cells were isolated from the spleens of BALB/c mice using the EasySep Mouse $CD4^+$ T Cell Enrichment Kit (Stemcell Technologies #19752). $CD4^+$ cells were plated in triplicate at a concentration of $1x10^6$ cells/ml on a 96-well plate, 250 µl/well. The cells were then stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml). The media of Th2 cells was supplemented with 10 µg/ml neutralising anti-IFN-γ antibody and 10 ng/ml IL-4 for Th2 polarising conditions. Cells were incubated for 72 hrs. Th2 cells were restimulated in fresh media for a further 24 hrs. Supernatants were collected and measured for IL-4, IL-6, IL-10 and IL-13 secretion using ELISA according to manufacturer's instructions (R&D duoset).

IL-4 secretion was reduced by parent (UL-2-42), regenerated (UL-2-79) and optimised hydrolysate (UL-2-147) and inactive enzyme in undifferentiated T-cells. A reduction in IL-6 was also observed when undifferentiated T-cells were treated with UL-2-

42/79/147/148 hydrolysates, inactive enzyme and UL-2-150 control, UL-2-42/79 and inactive enzyme being significant ($p<0.05$). UL-2-42/147/148 and inactive enzyme slightly reduced IL-10 levels in undifferentiated T-cells, while UL-2-79 and control, UL-2-150, had little or no effect. WPC80 increases all of these cytokines, IL-4 and IL-10 being significant ($p<0.001$) **[Figure 4.9]**. Upon examining their effect in Th2 cells, all hydrolysates and inactive enzyme control significantly reduced secretion of all of the characteristic Th2 cytokines [IL-4 and IL-13: $p<0.001$; and IL-10: $p<0.01$ (inactive enzyme) and $p<0.05$ (UL-2-42/79/147/148)]. While the UL-2-150, positive control, significantly reduced IL-4 levels ($p<0.01$), it had no effect on IL-10 and IL-13 secretion **[Figure 4.10]**.

4.2.3.3 THIRD-ROUND UL-2-47 AND REGENERATED AND OPTIMISED HYDROLYSATES DO NOT DISPLAY ALLERGY SUPPRESSING PROPERTIES IN UNDIFFERENTIATED T CELLS AND TH2 CELLS IN VITRO.

In order to determine whether the optimised hydrolysates of UL-2-47 could enhance the function of these hydrolysates, their activity was examined in undifferentiated T-cells and Th2 cells. Cells were isolated from the spleens of BALB/c mice using the EasySep Mouse $CD4^+$ T Cell Enrichment Kit (Stemcell Technologies #19752). $CD4^+$ cells were plated in triplicate at a concentration of $1x10^6$ cells/ml on a 96-well plate, 250 µl/well. The cells were then stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml). The media of Th2 cells was supplemented with 10 µg/ml neutralising anti-IFN-γ antibody and 10 ng/ml IL-4 for Th2 polarising conditions. Cells were incubated for 72 hrs. Th2 cells were restimulated in fresh media for a further 24 hrs. Supernatants were collected and measured for IL-4, IL-6, IL-10 and IL-13 secretion using ELISA according to manufacturer's instructions (R&D duoset).

IL-4 and IL-10 secretion were enhanced by parent (UL-2-47), regenerated (UL-2-151) and optimised hydrolysates (UL-2-152/154), UL-2-154 being significant ($p<0.001$ and $p<0.05$, respectively) in undifferentiated T-cells. These hydrolysates did, however, inhibit IL-6 secretion, UL-2-47/152 being significant ($p<0.05$). The inactive enzyme also significantly reduced IL-6 secretion ($p<0.05$) but also significantly increased IL-4 levels ($p<0.001$) and had no effect on IL-10 secretion. While the

positive control, UL-2-153, drove IL-4 secretion, it also significantly inhibited IL-6 and IL-10 levels ($p<0.001$ and $p<0.01$, respectively) **[Figure 4.11]**.

While UL-2-47/151/152/154 suppressed IL-13 secretion from Th2 cells (UL-2-47/151/152: $p<0.05$), they also increased IL-10 secretion. UL-2-151/152 also increased IL-4 levels while UL-2-47/154 had no effect on IL-4 secretion. Similarly, the inactive enzyme also significantly decreased IL-13 secretion ($p<0.001$) but increased IL-10 and had no effect on IL-4. The positive control, UL-2-153, on the other hand, inhibited IL-13 and IL-10, IL-13 being significant ($p<0.001$) and had no effect on IL-4 levels **[Figure 4.12]**. Bioactivity is not consistent or reproducible for UL-2-47 and optimised hydrolysates as they did not perform in the manner that we had hoped. They are therefore not desirable as candidate hydrolysates for *in vivo* studies and were not examined any further.

4.2.3.4 ASSESSMENT OF THIRD-ROUND HYDROLYSATES IN A REGULATORY T-CELL SUBSET IN VITRO.

Due to their ability to prevent differentiation of a Th2 subset *in* vitro and potential to suppress allergy in an *in vivo* mouse model, the next step was to assess whether these hydrolysates possessed the ability to potentially induce oral tolerance. To examine this, UL-2-42 and UL-2-147 were screened in Treg cells *in vitro.* $CD4^+$ Treg cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep™ Mouse CD25 Regulatory T Cell positive selection Kit (Stemcell Cat#18782). Cells were plated in triplicate, at a concentration of $1x10^6$ cells/ml on a 96 well plate, at a volume of 250 µl. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml). Cells were treated with hydrolysates (1 mg/ml). Supernatants were collected at 72 hrs and analysed using ELISA according to manufacturer's instructions (R&D duoset).

Figure 4.13 shows a significant reduction in IL-10 secretion ($p<0.001$) from Treg cells treated with UL-2-42/147. However, they have no effect on TGF-β secretion suggesting that they allow for or do not inhibit the polarisation of Treg cells *in vitro*, which may induce immune homeostasis between the T-cell subsets in an *in vivo* allergy setting. Whilst parent hydrolysate, UL-2-42, has desirable bioactivity, its

commercial value is low due to its poor solubility. As solubility for optimised UL-2-147 was improved and this hydrolysate displayed the most potent ability to suppress the Th2 phenotype it was considered the best candidate to be brought forward for *in vivo* trials. The next step was to assess whether the bioactivity of UL-2-147 was dose dependent.

4.2.3.5 OPTIMISED HYDROLYSATE, UL-2-147, DOSE DEPENDENTLY INHIBITS THE ALLERGIC TH2 RESPONSE IN VITRO.

In order to determine whether the bioactivity of UL-2-147 is dose dependent, a dose response was carried out on Th2 cells and IL-4, IL-10 and IL-13 secretion was measured. Cells were isolated from the spleens of BALB/c mice using the EasySep Mouse $CD4^+$ T Cell Enrichment Kit (Stemcell Technologies #19752). $CD4^+$ cells were plated in triplicate, at a concentration of $1x10^6$ cells/ml on a 96-well plate, at a volume of 250 µl/well. The cells were then stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and treated with UL-2-147 at various doses (0.5 mg/ml / 1 mg/ml / 2 mg/ml). The media was supplemented with 10 µg/ml neutralising anti-IFN-γ antibody and 10 ng/ml IL-4 for Th2 polarising conditions. Cells were incubated for 72 hrs and were restimulated in fresh media for a further 24 hrs. Supernatants were collected and measured for IL-4 and IL-13 secretion using ELISA according to manufacturer's instructions (R&D duoset).

Figure 4.14 shows a dose dependent inhibition of IL-4 and IL-13 secretion from Th2 cells compared to untreated cells, when treated with increasing doses of UL-2-147. UL-2-147 significantly reduced IL-4 secretion (500 µg/ml, 1 mg/ml and 2 mg/ml: $p<0.001$) and IL-13 secretion (500 µg/ml: $p<0.01$; and 1 mg/ml and 2 mg/ml: $p<0.001$).

4.2.4 UPSCALED UL-2-147 SUPPRESSES THE CHARACTERISTIC TH2 CYTOKINES *IN VITRO*, CONFIRMING BIOACTIVITY FOR *IN VIVO* OVA MURINE MODEL.

The final experiment, before examining the activity of UL-2-147 *in vivo*, was to confirm that upscaling of the hydrolysate at large scale did not affect its bioactivity. Scale up of UL-2-147 and associated protocol transfer took place at the bio-functional food engineering (BFE) (industry-large scale) plant in Teagasc, Moorepark. To date all hydrolysates had only been produced in small quantities in the lab in UCC. Scale-up is carried out firstly to produce larger quantities of hydrolysates for animal and human trials taking place within FHI, and secondly to ensure that the hydrolysis protocol could be transferred successfully and yield hydrolysates with the same bioactivity. UL-2-147 was produced in large scale at 3hr (original protocol) and 6 hr. The 6 hr duration was been requested by industry to ensure that they could transfer the protocol in their plant without losing the activity. The scaled up hydrolysate was tested in Th2 cells alongside the original sample. Cells were isolated from the spleens of BALB/c mice using the EasySep Mouse $CD4^+$ T Cell Enrichment Kit (Stemcell Technologies #19752). $CD4^+$ cells were plated in triplicate at a concentration of $1x10^6$ cells/ml on a 96-well plate, 250 µl/well. The cells were then stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and treated with original UL-2-147 and 3 hr and 6 hr upscaled UL-2-147 (1 mg/ml). The media was supplemented with 10 µg/ml neutralising anti-IFN-γ antibody and 10 ng/ml IL-4 for Th2 polarising conditions. Cells were incubated for 72 hrs and were restimulated in fresh media for a further 24 hrs. Supernatants were collected and measured for IL-4, IL-10 and IL-13 secretion using ELISA according to manufacturer's instructions (R&D duoset).

The original UL-2-147 and upscaled UL-2-147, both 3 hr and 6 hr samples, continued to significantly inhibit all three Th2 cytokines. IL-4 and IL-13 were significantly reduced by all samples ($p<0.001$) and IL-10 was significantly suppressed by the original UL-2-147 hydrolysate as well as the 6 hr sample ($p<0.01$). The 6 hr hydrolysis time resulted in the most potent effect in all three cytokines **[Figure 4.15]**. The next step in the study was to determine the ability of UL-2-147 (6 hr) to modulate the immune response *in vivo*, through suppression of a Th2 environment and assess its capacity to alleviate allergic symptoms in a mouse model of allergy for proof of

principle. A summary of *in vitro* results collected from all of research groups within the Allergy and Immunity stream of the Infant Nutrition work package which informed our decision to bring UL-2-147 forward for *in vivo* testing can be seen in **Table 4.3** below**.**

	Humanised	***Murine***
DCs/Macrophage	**-**	**↑IL-10**
		↓IL-6
		↓IL-4
Undifferentiated CD4+ T-	-	**↓IL-6**
Cells		**↓IL-10**
Th2 Cells	**↓GATA-3**	**↓IL-4**
	↓IL-4	**↓IL-10**
		↓IL-13
Mast Cells	**-**	**Slightly reduces IgE**
		Mediated Degranulation
Treg Cells	**Slight ↑ Foxp3**	**Slight ↑ TGB-β**
Th1 Cells	**Slight ↑ T-bet**	**No effect**

Table 4.3: Summary of *in vitro* results from Allergy and Immunity Infant Nutrition FHI Workpackage: Humanised and murine immune cells treated with UL-2-147 hydrolysates being brought forward for assessment in animal trials.

4.2.5 OVA-INDUCED MODEL OF FOOD ALLERGY

In order to determine the ability of UL-2-147 to modulate the immune system and suppress a Th2 environment and bring about immune balance *in vivo*, a mouse model of OVA-induced allergy was carried out. This model had not previously been carried out in our research group and, therefore, required optimisation. A pilot study was first performed to ensure disease progression prior to UL-2-147 testing. OVA is a well studied mouse model and a number of publications have identified that Th2 cells have a key role to play in the progression of the disease, making it a suitable model for us to assess the translation of our *in vitro* results into *in vivo*.

4.2.5.1 CLINICAL ASSESSMENT OF OVA-INDUCED ALLERGY

The OVA model was carried out in the Biological Resource Unit (BRU), at Dublin City University as described in **Section 2.9.2.** 12 week old female BALB/c mice were divided into a non-sensitised control group of 6 mice and a test group (also 6 mice) with OVA-induced allergy. On Day 1, the test group received IP injection of OVA and aluminium hydroxide (alum) adjuvant (50 µg and 1.5 mg, respectively) in warm PBS. This procedure was repeated on Day 14. The control group received PBS injection only. On Day 16, 17, 18, 19 and 20 both groups received IG gavage of PBS. On Day 21, 23, 25, and 27 mice were orally challenged with OVA. For the oral challenge, OVA-sensitised mice received oral gavage of OVA in PBS (50 mg/ml) once a day and control group received oral gavage of PBS only. Mice were culled on Day 28. Mice were weighed and disease scored every day during oral challenges based on activity, breathing, fur texture/posture, eyes, faeces and nose for a daily disease activity index (DDAI) **[Table 4.4]**.

No disease activity was observed in the control mice but there was a rapid increase in disease activity in OVA-sensitised mice after the first and third oral challenge **[Fig 4.16 (A)]**, with a maximum significant DAI being observed after the third oral challenge ($p<0.05$) **[Fig 4.16 (B)]**. The average % weight change of the 2 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. The weights of both groups showed a similar pattern of disease to the DDAI. There was a drop in body weight for the OVA-sensitised group following the first and third oral challenge, whereas a steady and healthy gain in weight was observed for control mice **[Fig 4.17 (A)]**. A significant weight difference ($p<0.001$) between OVA-sensitised and control mice was observed on Day 16 **[Figure 4.17 (B)].**

Food allergy plays an important role in the induction of intestinal inflammation (Li et al. 2016). For this reason, a small section of distal colon (0.5 cm) was removed for histological analysis to determine disease progression and pathology of the colon. The tissue was stained with haematoxylin and eosin (H&E), as described in **Section 2.9.4**. The H&E staining shows a healthy colon in the control group with a presence of goblet cells, good crypt formation and no infiltrating cells in the lower muscle layer of the gut **[Figure 4.18].** We can see destruction of crypt structure together with a visible reduction in goblet cells, a disturbed epithelial layer and massive infiltration of

inflammatory cells (purple spots) in the mucosa in the colons of OVA-sensitised mice when compared to the normal morphology of control colon tissue **[Figure 4.18]**.

Signs	0	1	2	3
Activity	Normal	Isolated, abnormal posture, scratching and rubbing around nose and head	Huddled/inactive OR overactive	Moribund OR fitting
Alertness/Sleeping	Normal	Dull or depressed	Little response to handling	Unconscious
Body Weight	Normal weight & growth rate	Reduced growth rate	Weight loss >15% OR failure to grow	Weight loss of >20% OR failure to grow & weight loss
Breathing	Normal	Rapid, shallow	Rapid, abdominal breathing	Laboured, irregular, skin blue
Coat	Normal	Coat rough	Unkempt, wounds, hair thinning	Bleeding or infected wounds or severe hair loss or self-mutilation
Eyes	Normal	Wetness or dullness	Discharge	Eyelids matted
Faeces	Normal	Faeces moist	Loose, soiled perineum OR abnormally dry +/- mucus	Running out on handling OR no faeces for 48hrs OR frank blood on faeces
Nose	Normal	Wetness	Discharge	Coagulated
Other				

Table 4.4: OVA Model Template Welfare Sheet. Clinical signs and severity scores assigned to symptoms triggered following the oral challenges.

4.2.5.2 CYTOKINE SECRETION FROM EX VIVO SPLENOCYTE CULTURE IN OVA-INDUCED ALLERGY

To determine the extent of Th2 activation in response to oral OVA challenge, we assessed and compared cytokine secretion from spleens of OVA-sensitised and control mice. Spleens were collected, red blood cells were lysed and splenocytes were plated at 5 x 10^6 cells/ml on a 24 well plate. Cells were left unstimulated or stimulated with either OVA (100 µg/ml) or with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) for 72 hr before harvesting. Supernatants were collected and measured for IFN-

γ, IL-4, IL-13, IL-10, IL-6, IL-2 and IL-17 secretion using ELISA according to manufacturer's instructions (R&D duoset).

There was a slight increase in all cytokines measured in OVA-sensitised mice stimulated with OVA and unstimulated OVA-sensitised mice, IL-17 being a significant increase in the latter group ($p<0.05$). There was a significant decrease in the secretion of the pro-inflammatory cytokines, IFN-γ, IL-2 and IL-17 in OVA-sensitised mice when stimulated with CD3 and CD28 antibodies ($p<0.05$, $p<0.001$ and $p<0.05$, respectively). However, a significant increase in the allergy driving cytokines, IL-6 and IL-13 ($P<0.05$) and insignificant increase in IL-10 was simultaneously observed in OVA-sensitised mice stimulated with CD3/CD28. IL-4 was also significantly increased by all OVA-sensitised groups (OVA stimulated and unstimulated: $p<0.001$; and CD3/CD28 stimulated: $p<0.05$) **[Figure 4.19].**

4.2.5.3 CYTOKINE EXPRESSION IN COLON IN OVA-INDUCED ALLERGY

RNA was isolated from distal colonic tissue from mice, as described in **Section 2.8.1**. Complementary DNA (cDNA) was then generated from RNA using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, USA). The levels of IFN-γ, IL-4, IL-13, IL-10 and IL-6 mRNA were quantified using qPCR. The expression levels were normalised to Gus-b levels. The levels of expression in control mice were normalised to one and the other groups measured were calculated as fold change in expression relative to the control group.

Increased expression in each of the cytokines measured was observed in OVA-sensitised colons, however, these results were not significant **[Figure 4.20]**. This indicates evidence of a successful allergic inflamed colon in mice sensitised with OVA.

4.2.5.4 IMMUNOGLOBULIN ANTIBODY LEVELS IN SERUM IN OVA-INDUCED ALLERGY IN VIVO.

Hansen (2007) states that using alum as an adjuvant significantly increases OVA-specific IgE and IgG1. IgG1 is a Th2 cytokine-stimulated antibody and is often

interpreted in line with IgE as an "allergic" antibody in murine allergy models (Van Halteren et al. 1997; and Meulenbroek et al. 2013). Blood serum was collected from mice and, therefore, measured for IgG1 levels using ELISA according to manufacturer's instructions (Invitrogen).

Figure 4.21 shows a significant increase in IgG1 levels in OVA-sensitised mice compared to control mice.

4.2.6 EFFECT OF UL-2-147 WHEY HYDROLYSATE TREATMENT IN A MOUSE MODEL OF ALLERGY.

Collectively these results indicated a successful OVA-induced murine model of food allergy. The next step was to examine the effect of UL-2-147 treatment in the optimised OVA allergy mouse model and assess its capacity to modulate the immune response away from a Th2 phenotype and bring about immune balance, whilst suppress/attenuate/alleviate the clinical symptoms of allergy.

4.2.6.1 CLINICAL ASSESSMENT OF OVA-INDUCED ALLERGIC MICE TREATED WITH UL-2-147

12 week old female BALB/c mice were grouped into a non-sensitised control group of 6 mice and 3 test groups (also 6 mice each) with OVA-induced allergy [one OVA-sensitised group to be treated with WPC80 and one with UL-2-147(6 hr)]. On Day 1, the 3 test groups received IP injection of OVA and alum (50 µg and 1.5 mg, respectively) in warm PBS. This procedure was repeated on Day 14. Control group received PBS injection only. On Day 16, 17, 18, 19 and 20, 1 test group received IG gavage of UL-2-147 (50 mg/ml) in PBS and another group receives IG gavage of WPC80 (50 mg/ml) once daily. Control and OVA-only group received IG gavage of PBS only. On Day 21, 23, 25, and 27 mice were orally challenged with OVA. OVA-sensitised mice received oral gavage of OVA once a day and control group received IG gavage of PBS only. Mice were culled on Day 28. To assess the development of the disease, mice were weighed and scored (every day during oral challenges) for daily

disease activity index (DDAI) based on activity, breathing, fur texture/posture, eyes, faeces and nose **[Table 4.4]**.

The average % weight change 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. A steady, healthy gain in weight was observed for control mice throughout the course of the model. There was a drop in body weight for the OVA-sensitised groups on Day 3 onwards **[Fig 4.22 (A)]**, which reached its maximum on Day 26, as seen by a significant decrease in all test groups compared to control mice ($p<0.001$) **[Fig 4.22 (B)]**. While mice treated with UL-2-147 lost weight following sensitisation, they maintained a continuous and steady weight during and following the oral challenges with OVA, following hydrolysate treatment. PBS and WPC80 treated groups, however, continued to steadily lose weight following the oral challenges **[Fig 4.22 (A)]**. Although a significant decrease in body weight was observed in all test groups on the final day compared to control mice, UL-2-147 and WPC80 treated mice showed a significant increase in body weight compared to untreated OVA-sensitised mice **[Fig 4.22 (C)]**.

No disease activity was observed in the control mice but a rapid increase in disease activity in OVA-sensitised mice (PBS and WPC80 treated groups) was observed following each of the oral challenges **[Figure 4.23 (A)]**. The final oral challenge resulted in the highest disease activity score in PBS treated OVA-sensitised mice, whereas the WPC80 treatment resulted in improved disease activity. Both PBS and WPC80 treated OVA-sensitised groups had a significant increase in DAI compared to control mice, after each oral challenge and this increase ($p<0.001$) can be observed in **Figure 4.23(B)** on the final day. UL-2-147 treated OVA-sensitised mice had a slight increase in disease activity following the first oral challenge, however, no disease activity was observed in this group for the rest of the oral challenges **[Figure 4.23(A)].** Both the UL-2-147 and WPC80 treated groups had a significantly lower DAI on the final day of the study **[Figure 4.23(B)]**.

A small section of distal colon (0.5 cm) was removed for histological analysis to determine disease progression and pathology of the colon. The tissue was stained with haematoxylin and eosin (H&E), as described in Materials and methods **Section 2.11.3**. The H&E staining shows a healthy colon in the control group with a presence of goblet

cells, good crypt formation and no infiltrating cells in the lower muscle layer of the gut **[Figure 4.24].** We can see a visible reduction in goblet cells, together with loss of crypts and infiltration of inflammatory/immune cells (purple spots) to the mucosa in the colons of OVA-sensitised mice treated with PBS and WPC80. UL-2-147 seemed to resolve superficial inflammation and restored tissue morphology in colons of OVA -sensitised mice **[Figure 4.24]**.

4.2.6.2 CYTOKINE SECRETION FROM EX VIVO SPLENOCYTE CULTURE IN OVA-INDUCED ALLERGIC MICE TREATED WITH UL-2-147

In order to assess the translation of our *in vitro* results, which were based around measuring cytokine secretion from T-cells in the spleen, into this *in vivo* scenario, splenocyte cytokine profiles were examine in OVA-sensitised mice. Spleens were collected, red blood cells were lysed and splenocytes were plated at 5 x 10^6 cells/ml on a 24 well plate. Cells were left unstimulated or stimulated with either OVA (100 µg/ml) or with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) for 72 hr before harvesting. Supernatants were collected and measured for, IL-4, IL-13, IL-6, IFN-γ, IL-10, TGF-β, IL-2 and IL-17 secretion using ELISA according to manufacturer's instructions (R&D duoset).

Following OVA stimulation, IL-4 and IL-13 secretion was significantly enhanced in all OVA-sensitised groups compared to control mice, PBS treated OVA-sensitised mice having the most potent effect (PBS and WPC80 treated mice: $p<0.001$; and UL-2-147 treated mice: $p<0.01$). Although, UL-2-147 treated mice had significantly increased IL-4 levels when compared control mice, this treatment actually significantly reduced IL-4 secretion when compared to PBS treated OVA-sensitised mice, as did WPC80 treated mice ($p<0.001$). Secretion of IL-10 and IL-6 was significantly induced in OVA-sensitised PBS treated mice compared to control mice ($p<0.001$) but this result is significantly reduced with WPC80 (IL-6: $p<0.01$; IL-10: $p<0.05$) and UL-2-147 (IL-6 and IL-10: $p<0.001$) treatments. PBS and WPC80 treated OVA-sensitised mice reduce TGF-β secretion but treatment with UL-2-147 significantly increased ($p<0.01$) TGF-β levels compared to PBS treated OVA-sensitised mice and slightly increased it compared to control mice. All OVA-sensitised

treated groups significantly reduced IFN-γ secretion ($p<0.01$), significantly enhanced IL-2 levels ($p<0.001$) and slightly increased IL-17 **[Figure 4.25].**

Upon stimulation with plate-bound anti-CD3 plus anti-CD28, a significant increase in the Th2 cytokines, IL-4, IL-13, IL-6 and IL-10, was observed in PBS treated OVA-sensitised mice (IL-4, IL-6 and IL10: $P<0.001$; and IL-13: $p<0.01$). PBS treated OVA-sensitised mice also reduced IFN-γ and TGF-β levels compared to controls. WPC80 treatment had no effect on suppressing IL-4, IL-6 and IL-10 levels in allergic mice, as they still significantly increased these cytokines compared to control mice (IL-4 and IL-6: $P<0.001$; and IL-10: $p<0.01$). WPC80 treatment did, however, slightly reduce IL-13 secretion and slightly increase TGF-β levels compared to PBS treated OVA-sensitised mice, although TGF-β was still lower than control mice. WPC80 treated OVA-sensitised mice also significantly increased IL-17 ($p<0.01$) and slightly reduced IL-2 compared to control mice. While UL-2-147 significantly increased IL-4 compared to control mice ($p<0.01$), this treatment significantly reduced IL-4, IL-13, IL-6 and IL-10 compared to PBS treated OVA-sensitised mice (IL-4: $p<0.001$; IL-10: $p<0.01$; and IL-13 and IL-6: $p<0.05$). Levels of IL-13, IL-10 and IL-6 in mice treated with UL-2-147 were almost restored to those of control mice. TGF-β secretion was increased in mice treated with UL-2-147 compared to both PBS treated OVA-sensitised mice and control mice. IFN-γ secretion was only slightly reduced in all test groups compared to control mice **[Figure 4.26]**.

Following the culture of unstimulated splenocytes, WPC80 and PBS treatments significantly increased IL-4, IL-13, IL-6, IL-10 and IL-2 levels in OVA-sensitised mice (IL-2, IL-4, IL-13 and IL-6: $p<0.001$; and IL-10: $p<0.05$). Treatment with UL-2-147 also significantly increased IL-2, IL-4 and IL-6 levels in allergic mice compared to control mice ($p<0.001$, $p<0.05$ and $p<0.01$, respectively) but significantly reduced these cytokines, as well as IL-13 and IL-10, when compared to PBS treated OVA-sensitised mice (IL-2, IL-4 and IL-13: $p<0.001$; and IL-10 and IL-6: $p<0.01$). PBS and WPC80 treatments reduced TGF-β levels compared to control mice, but UL-2-147 restored this level in OVA-sensitised mice back to levels of control mice. All OVA-sensitised test groups reduced IFN-γ and IL-17 levels compared to control mice **[Figure 4.27]**.

4.2.6.3 CYTOKINE EXPRESSION IN COLON IN OVA-INDUCED ALLERGIC MICE TREATED WITH UL-2-147

RNA was isolated from distal colonic tissue from mice, as described in **Section 2.8.1**. Complementary DNA (cDNA) was then generated from RNA using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, USA). The levels of IFN-γ, IL-4, IL-13, IL-10 and IL-6 mRNA were quantified using qPCR. The expression levels were normalised to Gus-b levels. The levels of expression in control mice were normalised to one and the other groups measured were calculated as fold change in expression relative to the control group.

Expression of the characteristic Th2 cytokines, IL-4, IL-13, IL-10 and IL-6, were increased in PBS treated OVA-sensitised mice compared to control mice, however, this result was not significant. Both WPC80 and UL-2-147 treatments reversed this increase in expression in IL-4, IL-13, IL-10 and IL-6, as levels are the same as control mice. IFN-γ expression was reduced in all OVA-sensitised groups, WPC80 having a significant effect ($p<0.05$) **[Figure 4.28]**.

4.2.6.4 IMMUNOGLOBULIN ANTIBODY LEVELS IN SERUM IN OVA-INDUCED ALLERGIC MICE TREATED WITH UL-2-147

Blood serum was collected and measured for IgG1 levels using ELISA according to manufacturer's instructions (Invitrogen). All OVA-sensitised groups showed a significant increase in IgG1 levels compared to control mice (PBS and WPC80 treated mice: $p<0.001$; and UL-2-147 treated mice: $p<0.01$). UL-2-147 treatment significantly decreased IgG1 levels when compared to PBS treated OVA-sensitised mice ($p<0.01$), as did WPC80 treated mice ($p<0.05$) **[Figure 4.29]**.

4.2.7 ASSESSMENT OF TOLERISING ABILITY OF FIRST-ROUND HYDROLYSATES *IN VITRO*.

Thus far, we have examined the ability of hydrolysates to modulate Th2 cells. The next step was to examine the hydrolysates ability to induce Treg cells, to assess their ability to potentially induce tolerance.

4.2.7.1 FIRST-ROUND HYDROLYSATES INDUCE THE CYTOKINE REQUIRED TO DRIVE A TOLEROGENIC TREG RESPONSE FROM UNDIFFERENTIATED T-CELLS IN VITRO.

In order to identify whether the hydrolysates possessed the ability to induce oral tolerance, their ability to modulate the key cytokines required to drive a tolerogenic Treg response was first examined in undifferentiated T-cells. Cells were isolated from the spleens of BALB/c mice using the EasySep Mouse $CD4^+$ T Cell Enrichment Kit (Stemcell Technologies #19752). $CD4^+$ cells were plated in triplicate at a concentration of $1x10^6$ cells/ml on a 96-well plate, 250 µl/well. The cells were then stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml). Cells were incubated for 72 hrs. Supernatants were collected and measured for IL-10, a key cytokine that drives a Treg response, and IL-4 and IL-6 using ELISA according to manufacturer's instructions (R&D duoset).

A significant increase in secretion of the anti-inflammatory cytokine, IL-10, coupled with no increase of IL-4 and IL-6 secretion was observed when treated with UL-2-20 **[Figure 4.30].** UL-2-23 increased all cytokines measured. An increase in IL-10 secretion alone indicates that these hydrolysates may potentially promote differentiation into a Treg subset which may promote tolerance to allergy. An increase in IL-10 drives Th2 cells when coupled with increased IL-4 and IL-6.

4.2.7.2 FIRST-ROUND HYDROLYSATES CAN MODULATE DIFFERENTIATION OF T HELPER 2 T CELL SUBSET IN VITRO.

In order to assess whether these hydrolysates affected Th2 polarisation their effect was examined on Th2 cells. Cells were isolated from the spleens of BALB/c mice using the EasySep Mouse $CD4^+$ T Cell Enrichment Kit (Stemcell Technologies #19752). $CD4^+$ cells were plated in triplicate at a concentration of $1x10^6$ cells/ml on a 96-well plate, 250 µl/well. The cells were then stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml). The media was supplemented with 10 µg/ml neutralising anti-IFN-γ antibody and 10 ng/ml IL-4 for Th2 polarising conditions. Cells were incubated for 72 hrs and were restimulated in fresh media for 24 hrs. Supernatants were collected and measured for IL-4, IL-10 and IL-13 secretion using ELISA according to manufacturer's instructions (R&D duoset).

Figure 4.31 shows UL-2-20 slightly reduced IL-4 and IL-13 secretion and had no effect on IL-10. UL-2-23 significantly increased IL-4 ($p<0.05$), slightly reduced IL-13 and had no effect on IL-10.

4.2.7.3 FIRST-ROUND HYDROLYSATES CAN DRIVE DIFFERENTIATION OF REGULATORY T CELL SUBSET IN VITRO.

After examining the cytokine profile secreted from undifferentiated T-cells and establishing that UL-2-20 could potentially induce a regulatory Treg phenotype while not inducing a Th2 phenotype, it was necessary to confirm this activity in a Treg subset *in vitro*. $CD4^+$ T cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep™ Mouse CD25 Regulatory T Cell positive selection Kit (Stemcell Cat#18782). Cells were plated in triplicate, at a concentration of $1x10^6$ cells/ml on a 96 well plate, at a volume of 250 µl. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml). Cells were treated with hydrolysates (1 mg/ml). Supernatants were collected at 72 hrs and supernatants were collected and measured for IL-10 and TGF-β secretion using ELISA according to manufacturer's instructions (R&D duoset).

Figure 4.32 shows an increase in TGF-β and no reduction in IL-10 in Treg cells treated with UL-2-20.

4.2.7.4 FIRST-ROUND HYDROLYSATES DO NOT AFFECT POLARISATION OF T HELPER 1 T CELL SUBSET IN VITRO.

In order to assess that UL-2-20 had specific activity, targeting Treg cells, its effect on Th1 cells was also examined. Cells were isolated from the spleens of BALB/c mice using the EasySep Mouse $CD4^+$ T Cell Enrichment Kit (Stemcell Technologies #19752). $CD4^+$ cells were plated in triplicate at a concentration of $1x10^6$ cells/ml on a 96-well plate, 250 µl/well. The cells were then stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and 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 hrs and restimulated in fresh media for a further 24 hrs. Supernatants were collected and

measured for secretion of the characteristic Th1 cytokines, IFN-γ and IL-2, using ELISA according to manufacturer's instructions (R&D duoset).

Figure 4.33 shows that UL-2-20 treatment induced a slight increase in IL-2 and IL-10 secretion in Th1 cells and had no effect on IFN-γ. This confirmed that UL-2-20 does not target Th1 cells *in vitro*.

4.2.8 ASSESSMENT OF TOLERISING ABILITY OF SECOND & THIRD-ROUND REGENERATED AND OPTIMISED HYDROLYSATES *IN VITRO.*

In order to determine whether the bioactivity of the parent hydrolysate of interest, UL-2-20, is reproducible, it was regenerated, using identical hydrolysis conditions as the parent compound. Optimised hydrolysates were also produced at the same time due to time constraints. As mentioned above, we hoped that the optimisation step would enhance hydrolysis conditions whilst maintaining or enhancing the bioactivity of the parent hydrolysate. Analysis was carried out simultaneously on regenerated and optimised samples. A list of regenerated and optimised hydrolysates can be seen in **Table 4.5**.

Parent Hydrolysate	*Regenerate Hydrolysate*	*Optimised Hydrolysates*	*Substrate*	*Reason Brought Forward*
UL-2-20	**UL-2-188**	**UL-2-189 – UL-2-197**	**NaCN**	**Pro-Tolerance ↑ Treg Allergy Suppressing ↓ Th2**

Table 4.5: Overview of Parent, Regenerated and Optimised Hydrolysates brought forward into Round 3 Screening for Tolerance Induction.

4.2.8.1 THE DOSES OF SECOND & THIRD-ROUND UL-2-20 REGENERATED AND OPTIMISED HYDROLYSATES USED HAVE NO SIGNIFICANT AFFECT ON CELL VIABILITY.

To confirm that the regenerated and optimised hydrolysates were not toxic to T-cells, cells were isolated from the spleens of BALB/c mice using the EasySep Mouse $CD4^+$

T Cell Enrichment Kit (Stemcell Technologies #19752). $CD4^+$ 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) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml) or 10% DMSO (vehicle control). Cells were incubated for 72 hrs. Viability was assessed using an MTS assay (Cell Titer 96 Aqueous One Solution – Promega, WI, USA). At 1 mg/ml, regenerated hydrolysates did not have any significant cytotoxic effect on undifferentiated T-cells *in vitro* **[Figure 4.34].**

4.2.8.2 SECOND & THIRD-ROUND UL-2-20 REGENERATED AND OPTIMISED HYDROLYSATES DISPLAY SIMILAR ACTIVITY AS PARENT SAMPLE IN UNDIFFERENTIATED $CD4^+$ T-CELLS IN VITRO.

In order to confirm the bioactivity of regenerated and optimised hydrolysates of UL-2-20, they were screened, alongside parent hydrolysate, on undifferentiated T-cells and cytokine secretion was assessed. Cells were isolated from the spleens of BALB/c mice using the EasySep Mouse $CD4^+$ T Cell Enrichment Kit (Stemcell Technologies #19752). $CD4^+$ cells were plated in triplicate at a concentration of $1x10^6$ cells/ml on a 96-well plate, 250 µl/well. The cells were then stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml). Cells were incubated for 72 hrs. Supernatants were collected and measured for IL-4, IL-6 and IL-10 secretion using ELISA according to manufacturer's instructions (R&D duoset).

Figure 4.35 shows a significant increase in IL-10 secretion when treated with optimised hydrolysates UL-2-193-196 ($p<0.001$, $p<0.01$, $p<0.001$ and $p<0.05$, respectively), which was accompanied by a significant increase in IL-4 (UL-2-193/195/196/197: $p<0.001$; and UL-2-194: $p<0.05$) and an increase in IL-6 (UL-2-195/196 significant: $p<0.001$). UL-2-20, regenerate UL-2-188 and optimised hydrolysates UL-2-189-191 had a slight increase in IL-10 secretion and no effect on IL-4 and IL-6.

4.2.8.3 SECOND & THIRD-ROUND UL-2-20 REGENERATED AND OPTIMISED HYDROLYSATES DRIVE DIFFERENTIATION OF REGULATORY T CELL SUBSET AND INHIBIT DIFFERENTIATION OF T HELPER 2 T CELL SUBSET IN VITRO.

In order to determine whether the regenerated and optimised hydrolysates of UL-2-20 display reproducible and enhanced bioactivity, their activity was next examined in Treg and Th2 cells. Treg cells were isolated from the spleens of BALB/c mice using the EasySep Mouse EasySep™ Mouse CD25 Regulatory T Cell positive selection Kit (Stemcell Technologies Cat#18782). $CD4^+$ cells were isolated from the spleens of BALB/c mice using the EasySep Mouse $CD4^+$ T Cell Enrichment Kit (Stemcell Technologies #19752). Cells were plated in triplicate at a concentration of $1x10^6$ cells/ml on a 96-well plate, 250 µl/well. The cells were then stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml). The media of CD4 T-cells was supplemented with 10 µg/ml neutralising anti-IFN-γ antibody and 10 ng/ml IL-4 for Th2 polarising conditions. Cells were incubated for 72 hrs. Treg supernatants were collected at this stage. Th2 cells were restimulated in fresh media for a further 24 hrs before collection. Supernatants from Treg cells were measured for IL-10 and TGF-β secretion and supernatants from Th2 cells were measured for IL-4, IL-10 and IL-13 secretion using ELISA according to manufacturer's instructions (R&D duoset).

Figure 4.36 shows an increase in IL-10 and significant increase in TGF-β ($p<0.01$) from Treg cells when treated withUL-2-195. All other hydrolysates examined reduced IL-10 secretion, UL-2-188/189/190/191/192/197 being significant ($p<0.01$).

Following treatment of Th2 cells with optimised hydrolysates UL-2-193/195/196/197, **Figure 4.37** shows a significant reduction in both IL-4 ($p<0.001$, $p<0.01$, $p<0.05$ and $p<0.001$, respectively) and IL-13 ($p<0.01$, $p<0.05$, $p<0.01$ and $p<0.001$, respectively). UL-2-20 and optimised hydrolysates, UL-2-188/189/190/191/192 also significantly reduced IL-13 ($p<0.01$ and $p<0.001$, respectively) but had no effect on suppressing IL-4 and little effect on IL-10.

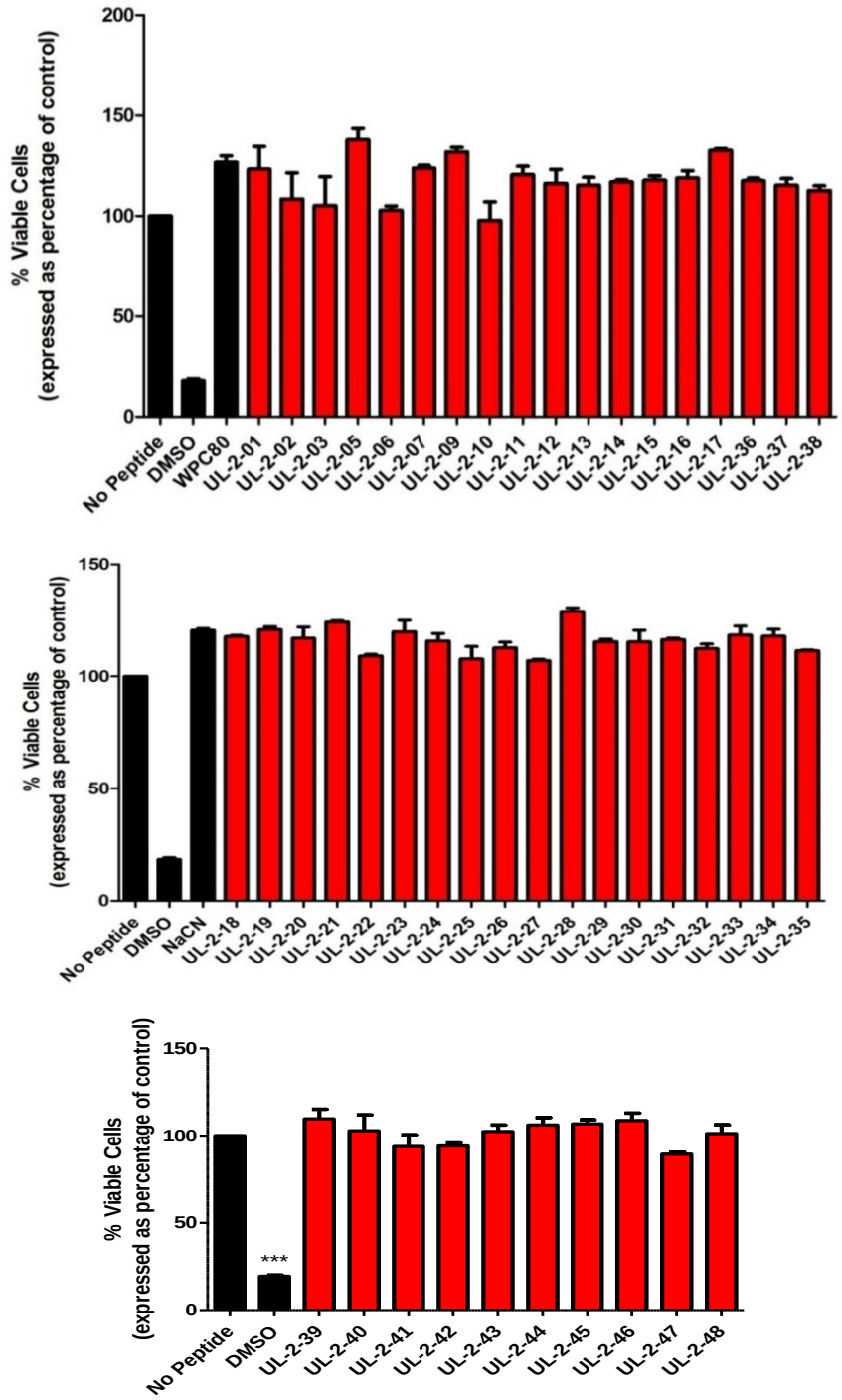


Fig 4.1: The concentrations of protein hydrolysates used do not affect the viability of undifferentiated T-cells. $CD4^+$ cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19752). Cells were plated at $1x10^6$ cells/ml, on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml) or 10% DMSO (vehicle control). Cells were incubated for 72 hrs. 20µl of the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay solution (Promega, WI, USA) was added to each well and left at 37°C 5% CO2 for 1hr. The plate was then read at a wavelength of 450 nm. Results are expressed as a percentage of the control (100%). Data are presented as mean ± SEM of three replicates and are representative of three independent experiments.

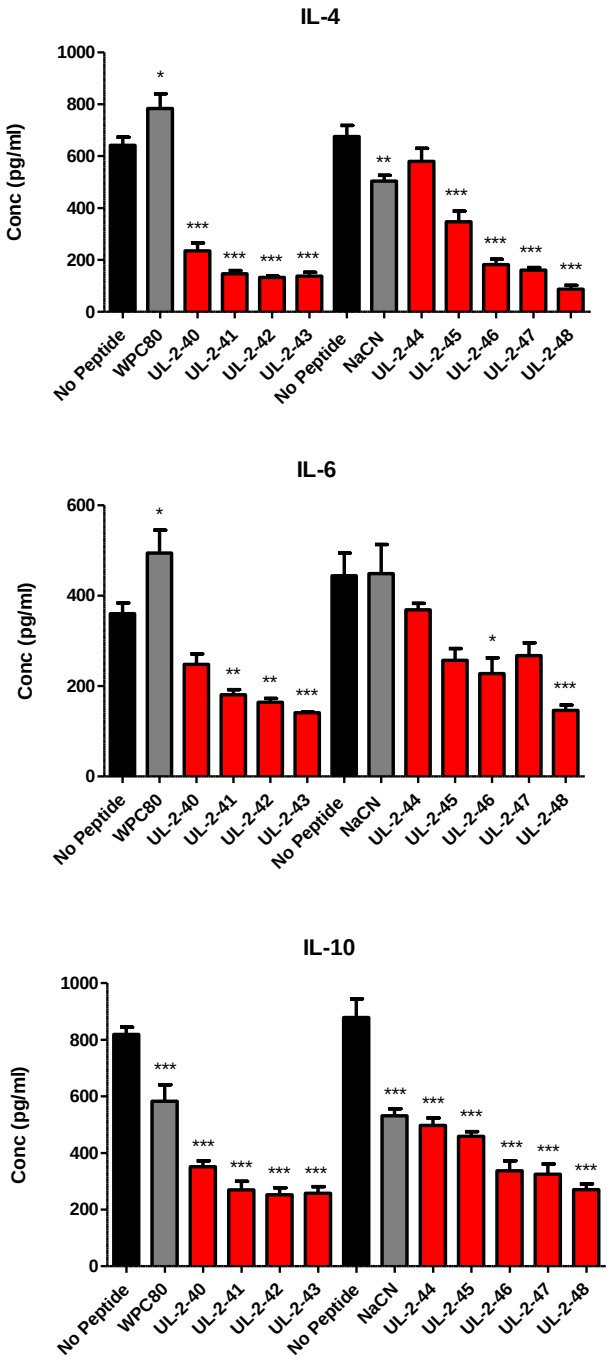


Fig 4.2: First-Round hydrolysates suppress the cytokines required to drive an allergic Th2 response from Undifferentiated T-cells *in vitro*. $CD4^+$ cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19752). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml). Cells were incubated for 72 hrs. Supernatants were collected and measured for IL-4, IL-6 and IL-10 secretion using ELISA according to manufacturer's instructions (R&D duoset). One way Anova was used to determine if differences between groups were significant (*p<0.05, **p<0.01, ***p<0.001). Hydrolysates are compared to untreated cells (No Peptide).

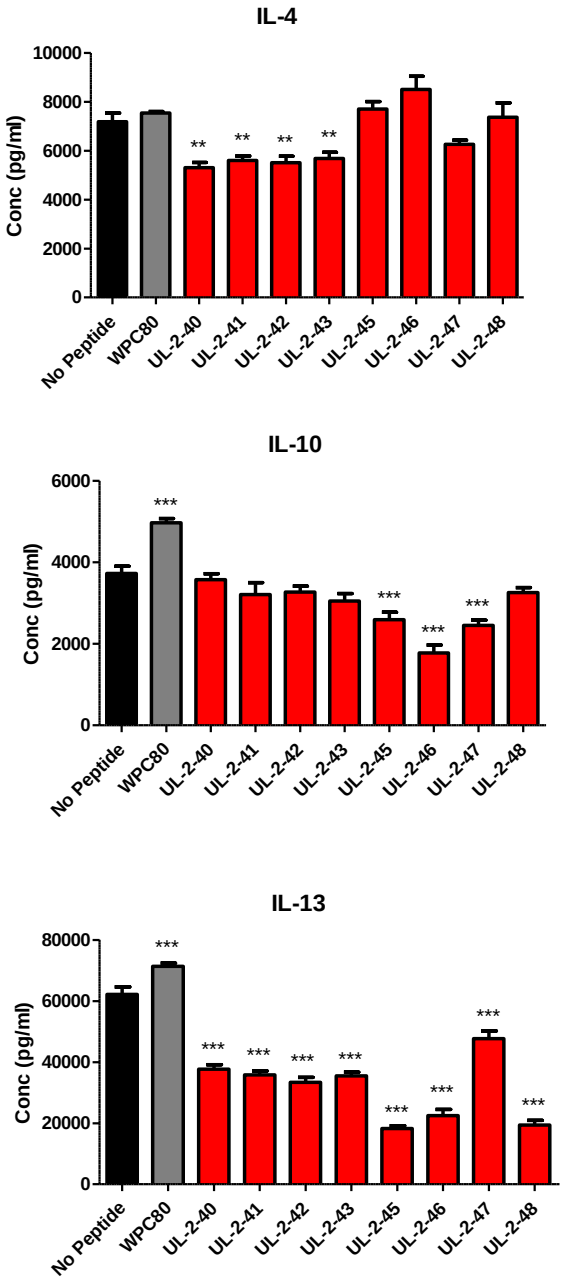


Fig 4.3: First-Round hydrolysates can prevent differentiation of Th2 cells *in vitro* by suppressing the characteristic Th2 cytokines. $CD4^+$ cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19752). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) in media supplemented with 10 µg/ml neutralising anti-IFN-γ antibody and 10 ng/ml IL-4 for Th2 polarising conditions. Cells were treated with hydrolysates (1 mg/ml). Cells were incubated for 72 hrs and were restimulated in fresh media for a further 24 hrs. Supernatants were collected and IL-4, IL-10 and IL-13 secretion was measured using ELISA according to manufacturer's instructions (R&D duoset). One way Anova was used to determine if differences between groups were significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Hydrolysates are compared to untreated cells (No Peptide).

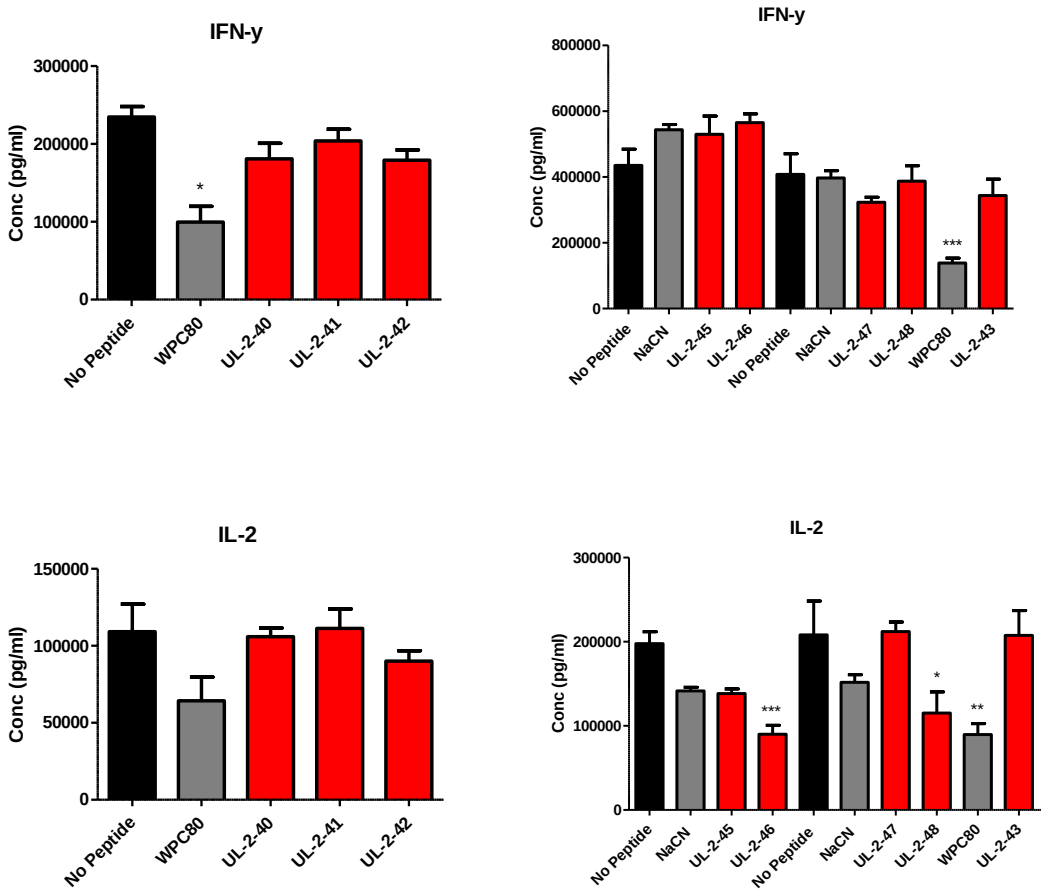


Fig 4.4: Assessment of First-Round hydrolysates in a Th1 subset *in vitro*. $CD4^+$ cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19752). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and 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 hrs and were restimulated in fresh media for a further 24 hrs. Supernatants were collected and measured for IFN-γ and IL-2 secretion using ELISA according to manufacturer's instructions (R&D duoset). One way Anova was used to determine if differences between groups were significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Hydrolysates are compared to untreated cells (No Peptide).

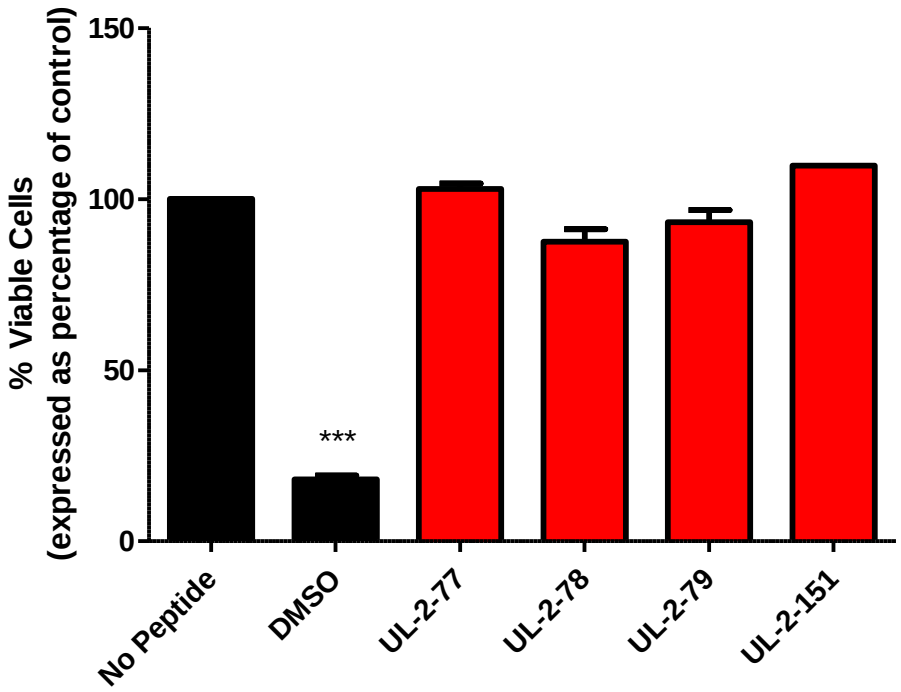


Fig 4.5: The concentrations of protein hydrolysate regenerates used to not affect the viability of undifferentiated T-cells. $CD4^+$ cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19752). Cells were plated at $1x10^5$/100 µl on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml) or 10% DMSO (vehicle control). Cells were incubated for 72 hrs. Viability was assessed using an MTS assay (Cell Titer 96 Aqueous One Solution – Promega, WI, USA). One way Anova was used to determine if differences between groups were significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Results are expressed as a percentage of untreated cells.

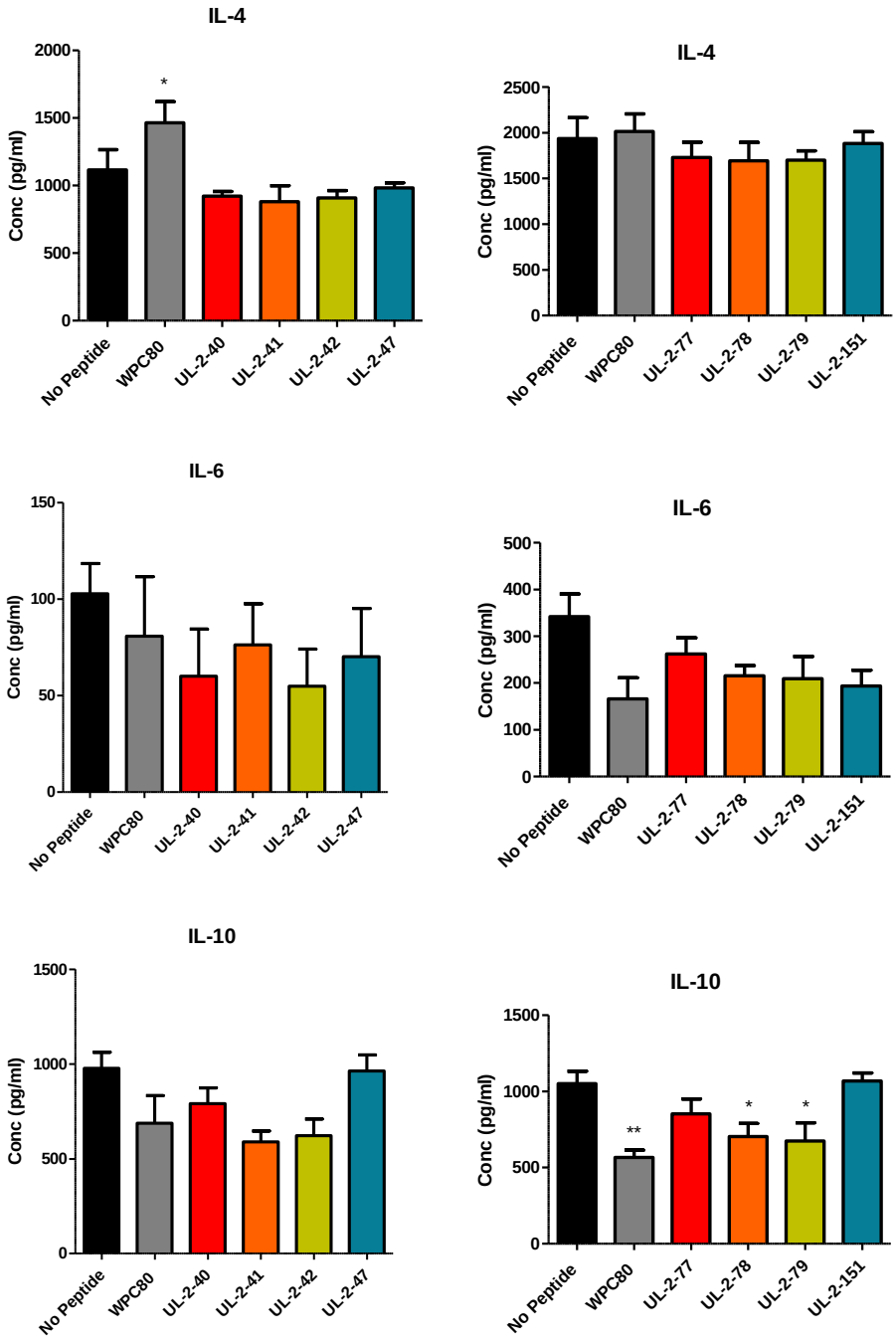


Fig 4.6: Second-Round regenerated hydrolysates suppress the cytokines required to drive an allergic Th2 response from Undifferentiated T-cells *in vitro*. $CD4^+$ cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19752). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml). Cells were incubated for 72 hrs. Supernatants were collected and IL-4, IL-6 and IL-10 secretion was measured using ELISA according to manufacturer's instructions (R&D duoset). One way Anova was used to determine if differences between groups were significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Hydrolysates are compared to untreated cells (No Peptide).

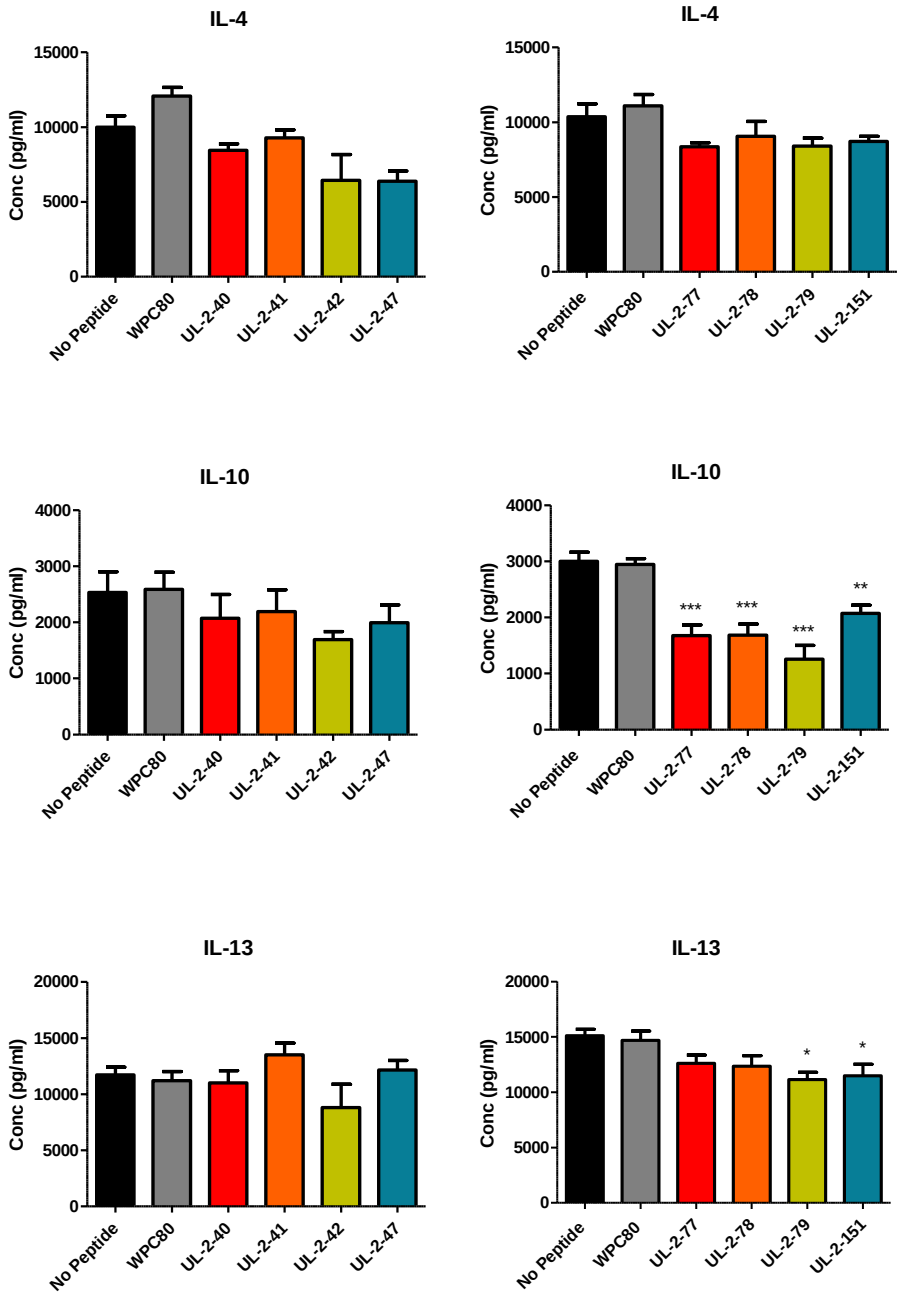


Fig 4.7: Second-Round hydrolysates and regenerates can prevent differentiation of Th2 cells *in vitro* by suppressing the characteristic Th2 cytokines. $CD4^+$ cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19752). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) in media supplemented with 10 µg/ml neutralising anti-IFN-γ antibody and 10 ng/ml IL-4 for Th2 polarising conditions. Cells were treated with hydrolysates (1 mg/ml). Cells were incubated for 72 hrs and were restimulated in fresh media for a further 24 hrs. Supernatants were collected and IL-4, IL-10 and IL-13 secretion was measured using ELISA according to manufacturer's instructions (R&D duoset). One way Anova was used to determine if differences between groups were significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Hydrolysates are compared to untreated cells (No Peptide).

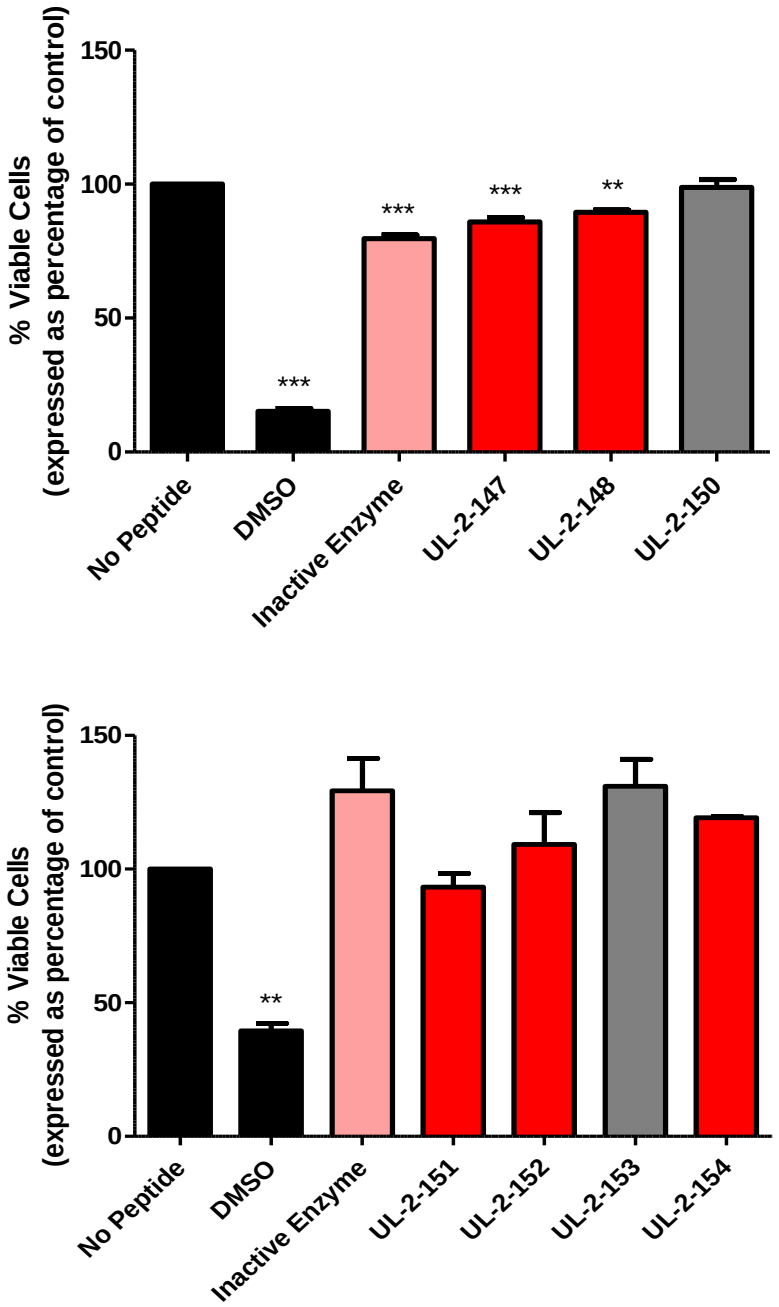


Fig 4.8: The concentrations of optimised protein hydrolysates from UL-2-42 and UL-2-47 used do not affect the viability of undifferentiated T-cells. $CD4^+$ cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19752). Cells were plated at $1x10^5$/100 µl on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml) or 10% DMSO (vehicle control). Cells were incubated for 72 hrs. Viability was assessed using an MTS assay (Cell Titer 96 Aqueous One Solution – Promega, WI, USA). One way Anova was used to determine if differences between groups were significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Results are expressed as a percentage of untreated cells.

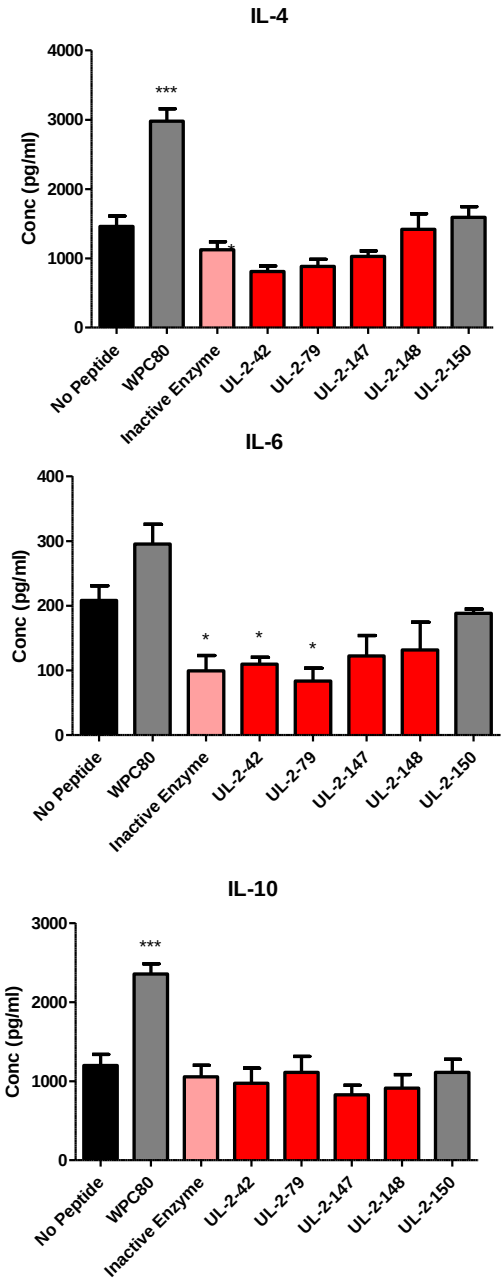


Fig 4.9: Third-Round UL-2-42 hydrolysate, regenerate and optimised hydrolysates suppress the cytokines required to drive an allergic Th2 response from Undifferentiated T-cells *in vitro*. $CD4^+$ cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19752). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml). Cells were incubated for 72 hrs. Supernatants were collected and IL-4, IL-10 and IL-13 secretion was measured using ELISA according to manufacturer's instructions (R&D duoset). One way Anova was used to determine if differences between groups were significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Hydrolysates are compared to untreated cells (No Peptide).

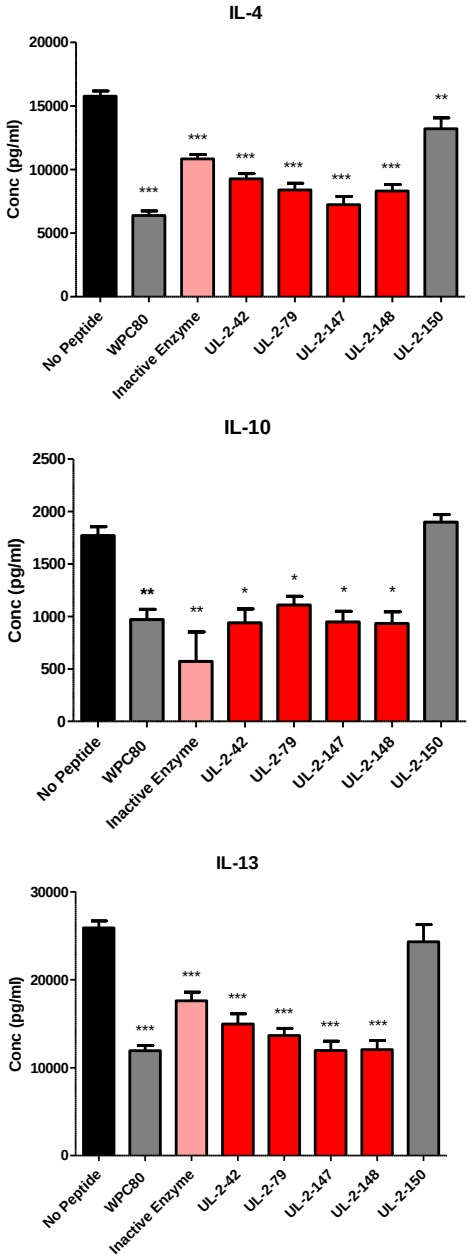


Fig 4.10: Third-Round UL-2-42 hydrolysate, regenerate and optimised hydrolysates can prevent differentiation of Th2 cells *in vitro* by suppressing the characteristic Th2 cytokines. $CD4^+$ cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19752). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) in media supplemented with 10 µg/ml neutralising anti-IFN-γ antibody and 10 ng/ml IL-4 for Th2 polarising conditions. Cells were treated with hydrolysates (1 mg/ml). Cells were incubated for 72 hrs and were restimulated in fresh media for a further 24 hrs. Supernatants were collected and IL-4, IL-10 and IL-13 secretion was measured using ELISA according to manufacturer's instructions (R&D duoset). One way Anova was used to determine if differences between groups were significant (*p<0.05, **p<0.01, ***p<0.001). Hydrolysates are compared to untreated cells (No Peptide).

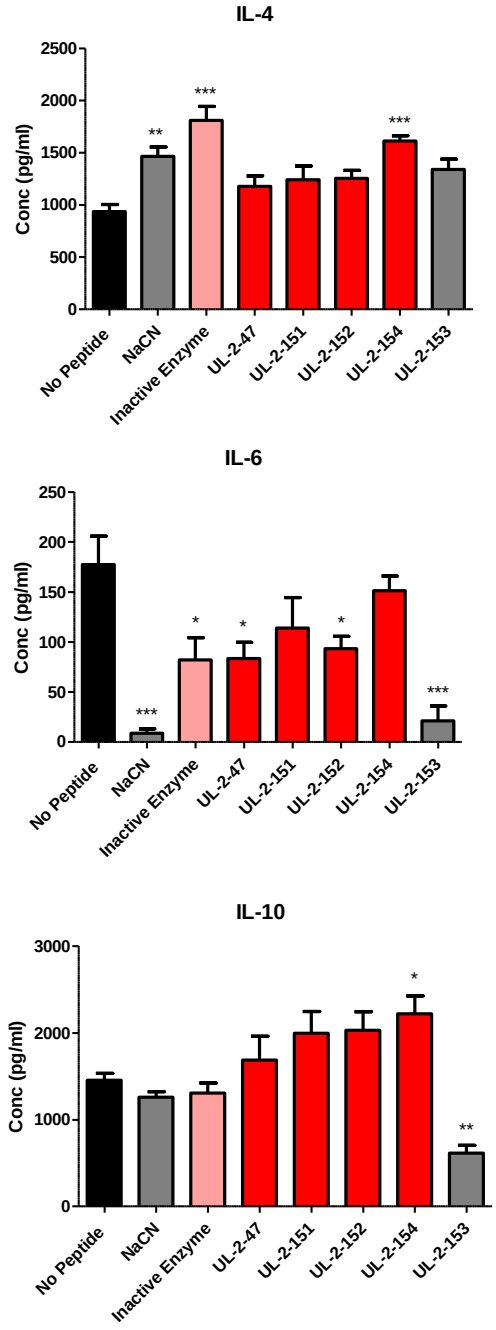


Fig 4.11: Third-Round UL-2-47 hydrolysate, regenerate and optimised hydrolysates suppress the cytokines required to drive an allergic Th2 response from Undifferentiated T-cells *in vitro*. $CD4^+$ cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19752). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml). Cells were incubated for 72 hrs. Supernatants were collected and IL-4, IL-6 and IL-10 secretion was measured using ELISA according to manufacturer's instructions (R&D duoset). One way Anova was used to determine if differences between groups were significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Hydrolysates are compared to untreated cells (No Peptide).

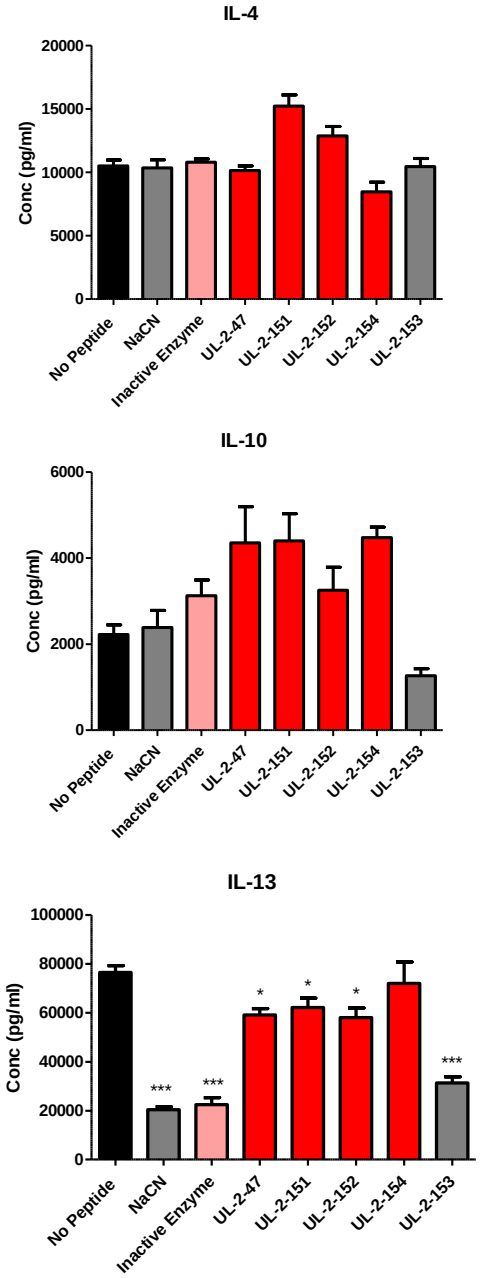


Fig 4.12: Third-Round UL-2-47 hydrolysate, regenerate and optimised hydrolysates can prevent differentiation of Th2 cells *in vitro* by suppressing the characteristic Th2 cytokines. $CD4^+$ cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19752). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) in media supplemented with 10 µg/ml neutralising anti-IFN-γ antibody and 10 ng/ml IL-4 for Th2 polarising conditions. Cells were treated with hydrolysates (1 mg/ml). Cells were incubated for 72 hrs and were restimulated in fresh media for a further 24 hrs. Supernatants were collected and IL-4, IL-10 and IL-13 secretion was measured using ELISA according to manufacturer's instructions (R&D duoset). One way Anova was used to determine if differences between groups were significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Hydrolysates are compared to untreated cells (No Peptide).

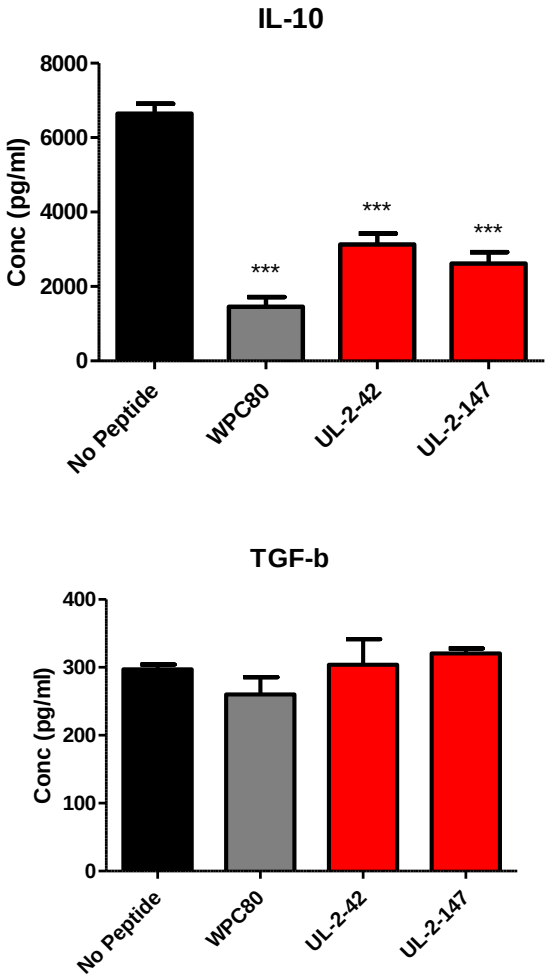


Fig 4.13: UL-2-42 & UL-2-147 do not drive or inhibit Treg cells *in vitro*. $CD4^+$ T cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep™ Mouse CD25 Regulatory T Cell positive selection Kit (Stemcell Cat#18782). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml). Cells were treated with hydrolysates (1 mg/ml). Supernatants were collected at 72 hrs and IL-10 and TGF-β were measured using ELISA according to manufacturer's instructions (R&D duoset). One way Anova was used to determine if differences between groups were significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Hydrolysates are compared to untreated cells (No Peptide).

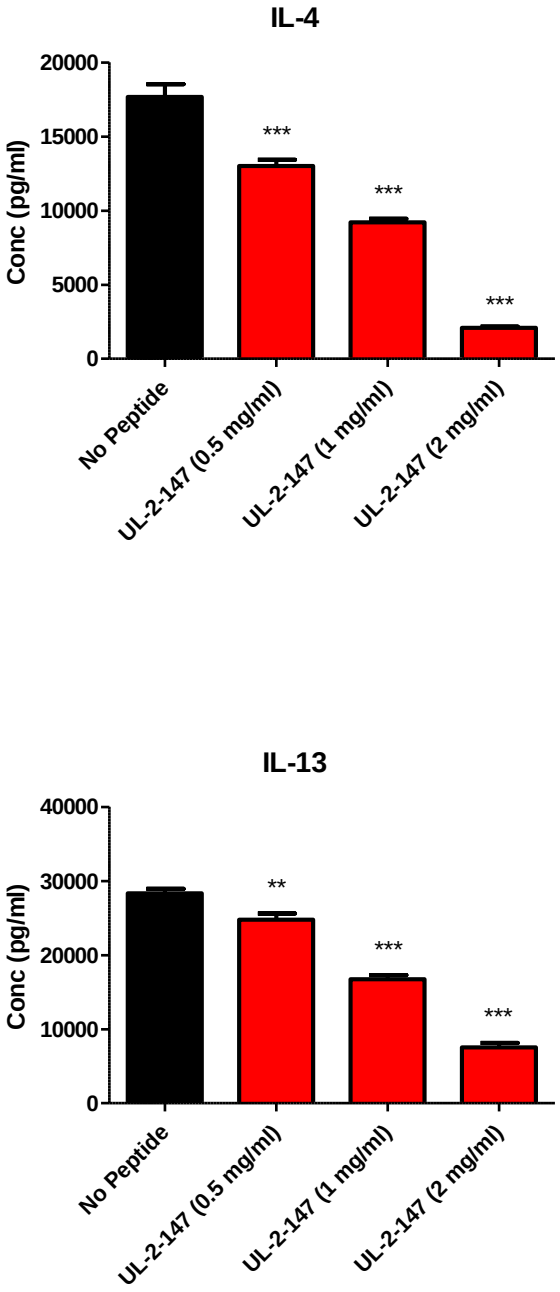


Fig 4.14: UL-2-147 inhibits the allergic Th2 response in dose dependent manner *in vitro*. $CD4^+$ cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19752). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) in media supplemented with 10 µg/ml neutralising anti-IFN-γ antibody and 10 ng/ml IL-4 for Th2 polarising conditions. Cells were treated with hydrolysates (1 mg/ml). Cells were incubated for 72 hrs and were restimulated in fresh media for a further 24 hrs. Supernatants were collected and IL-4 and IL-13 secretion was measured using ELISA according to manufacturer's instructions (R&D duoset). One way Anova was used to determine if differences between groups were significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Hydrolysates are compared to untreated cells (No Peptide).

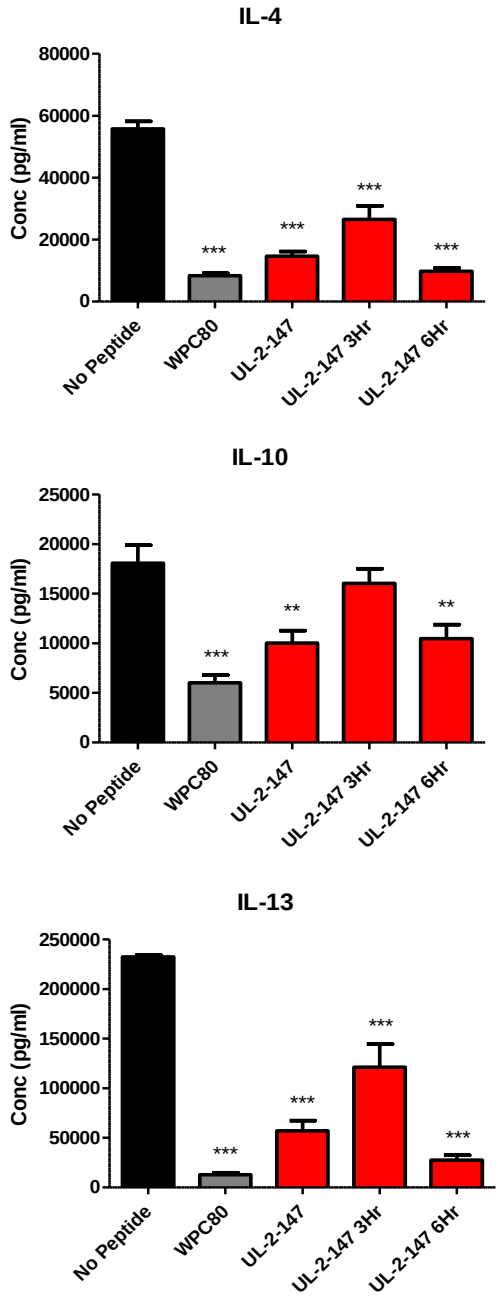
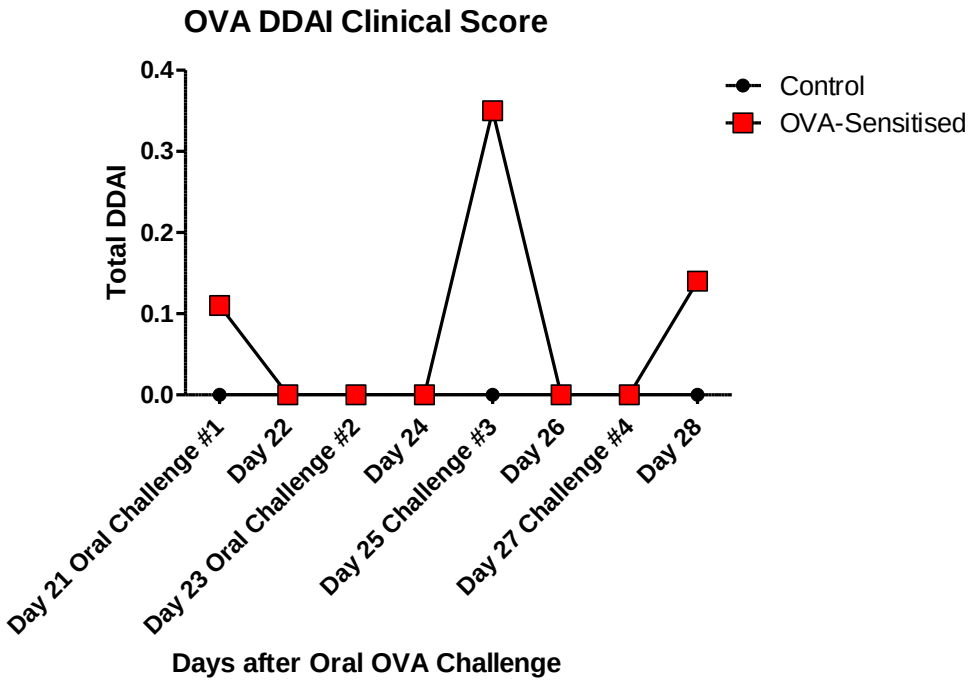


Fig 4.15: *In vitro* bioactivity of upscaled UL-2-147 was confirmed prior to *in vivo* OVA murine model - Upscaled UL-2-147 suppresses the characteristic Th2 cytokines. $CD4^+$ cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19752). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) in media supplemented with 10 µg/ml neutralising anti-IFN-γ antibody and 10 ng/ml IL-4 for Th2 polarising conditions. Cells were treated with hydrolysates (1 mg/ml). Cells were incubated for 72 hrs and were restimulated in fresh media for a further 24 hrs. Supernatants were collected and IL-4, IL-10 and IL-13 secretion was measured using ELISA according to manufacturer's instructions (R&D duoset). One way Anova was used to determine if differences between groups were significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Hydrolysates are compared to untreated cells (No Peptide).

(A)



(B)

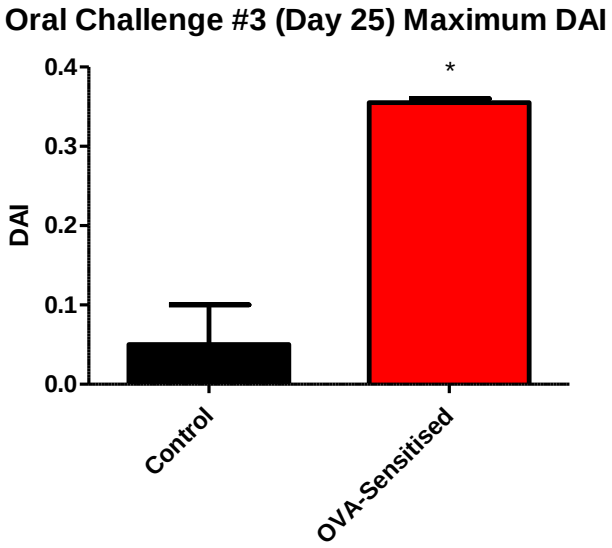
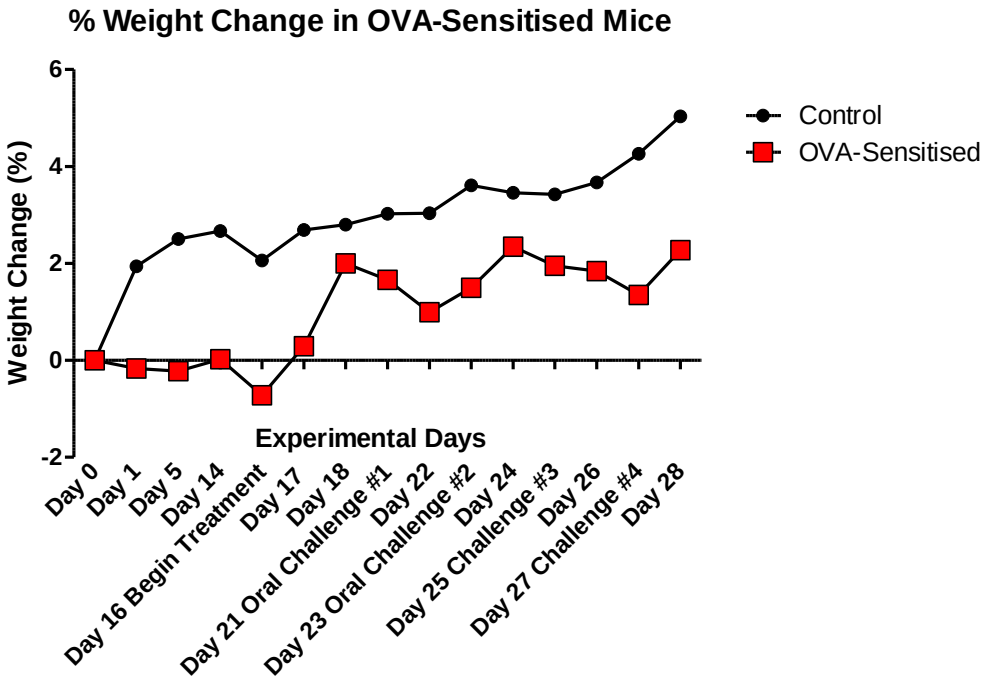


Fig 4.16: Disease associated symptoms in the OVA allergy model. 10-12 week old female BALB/c mice were grouped into a non-sensitised control group of 6 mice and a test group (also 6 mice) with OVA-induced allergy. Both groups were culled on day 28 and mice were weighed and scored (every day during oral challenges) for daily disease activity index (DDAI) based on activity, breathing, fur texture/posture, eyes, faeces and nose **(A)**. Maximum DAI was recorded on Day 25, following the third oral challenge (**B**). Data presented indicate the mean ± SEM (n = 6). An unpaired T-test was used to determine if differences between OVA and Control groups were significantly different (*p<0.05, **p<0.01, ***p<0.001).

(A)



(B)

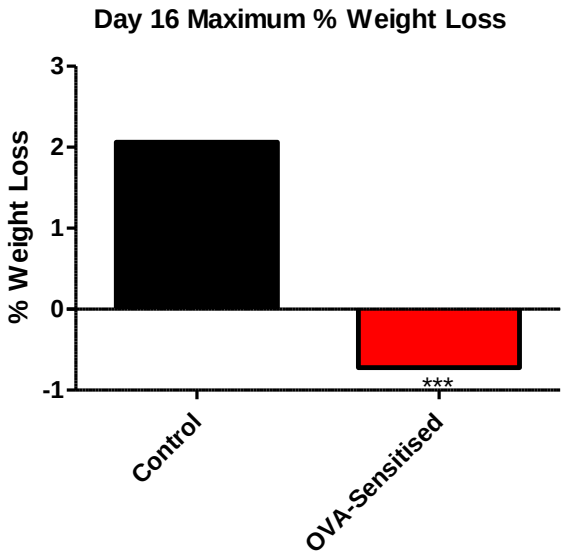


Fig 4.17: Disease associated symptoms in the OVA allergy model. 10-12 week old female BALB/c mice were grouped into a non-sensitised control group of 6 mice and a test group (also 6 mice) with OVA-induced allergy. Both groups were culled on day 28. Mice were weighed every day. The average % weight change of the 2 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 **(A)**. Maximum weight loss was recorded on Day 16 (**B**). Data presented indicate the mean ± SEM (n = 6). An unpaired T-test was used to determine if differences between OVA and Control groups were significantly different (*p<0.05, **p<0.01, ***p<0.001).

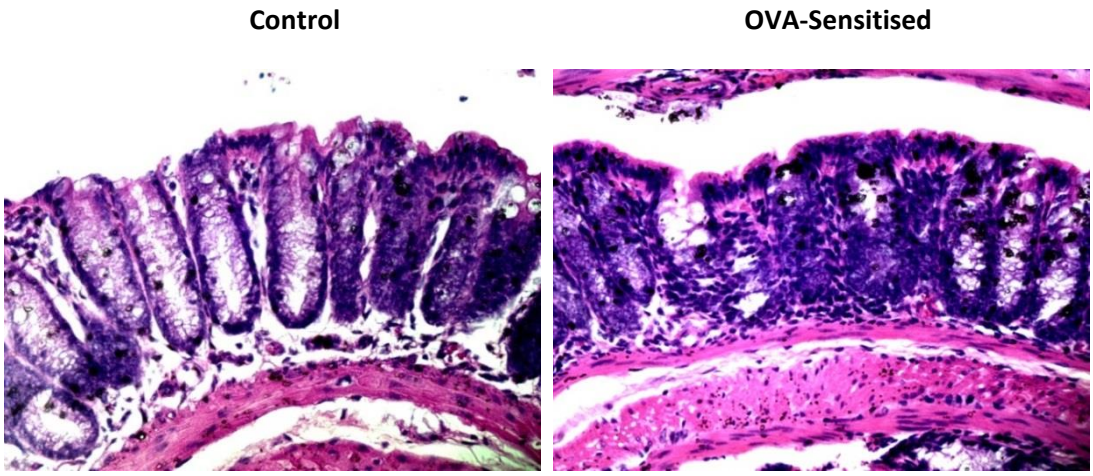


Fig 4.18: Disease associated symptoms in the OVA allergy model. 10-12 week old female BALB/c mice were grouped into a non-sensitised control group of 6 mice and a test group (also 6 mice) with OVA-induced allergy. Both groups were culled on day 28 and sections of the distal colon were removed for histology 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 OVA-sensitised slides.

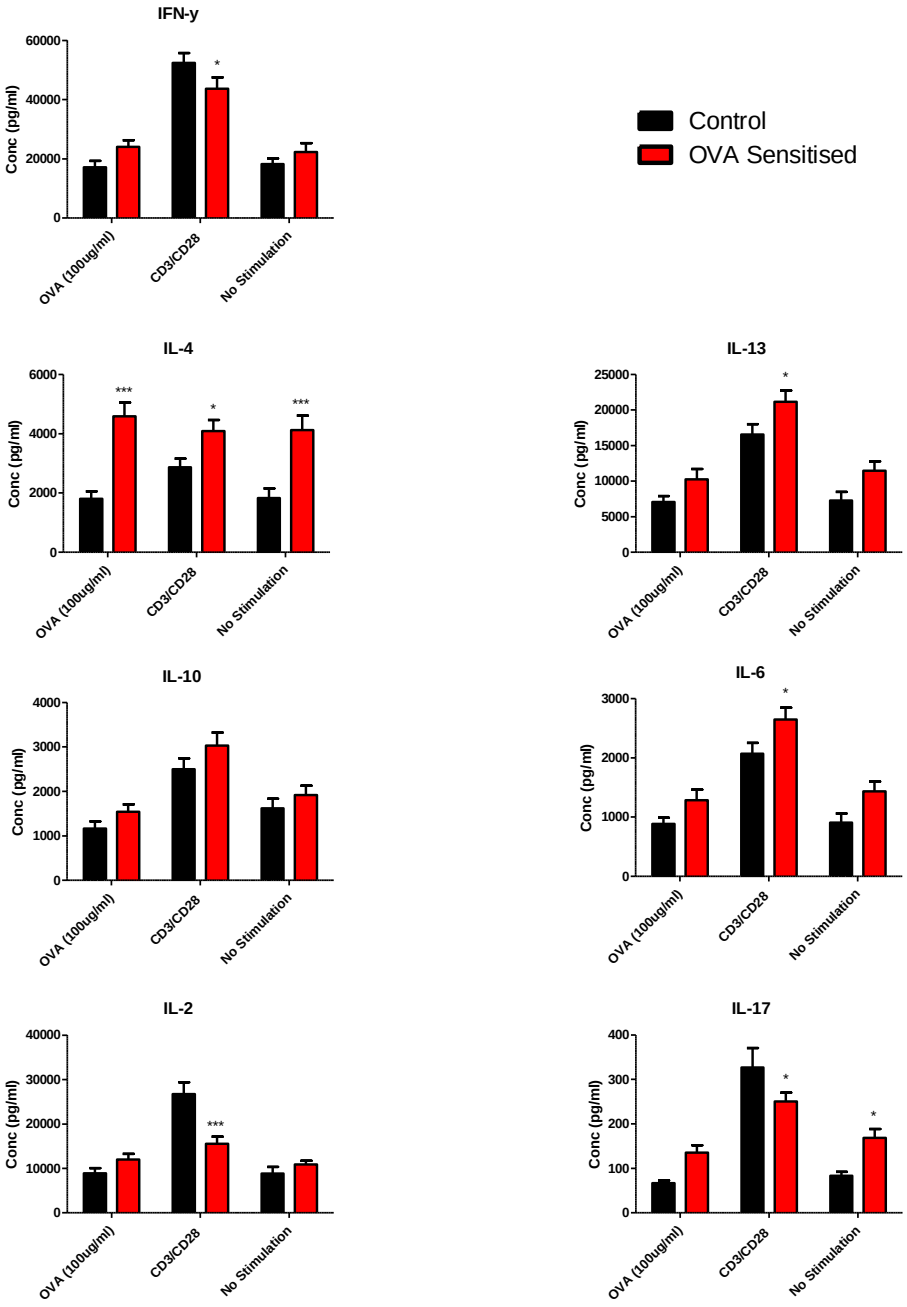


Fig 4.19: Cytokine secretion from *ex vivo* splenocyte culture in OVA-induced allergy *in vivo*. 10-12 week old female BALB/c mice were grouped into a non-sensitised control group of 6 mice and a test group (also 6 mice) with OVA-induced allergy. Both groups were culled on day 27. Spleens were harvested and splenocytes were plated at $5x10^6$ cells/ml on a 24 well plate. Cells were stimulated with OVA (100 µg/ml), plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) or left unstimulated for 72 hr before harvesting. Results are means ±SD of at least 6 mice, an unpaired T-test was used to determine if differences between groups were significant (*p<0.05, **p<0.01, ***p<0.001). All groups are compared to non-sensitised control mice. Black bars represent control group and red bars represent OVA-sensitised group.

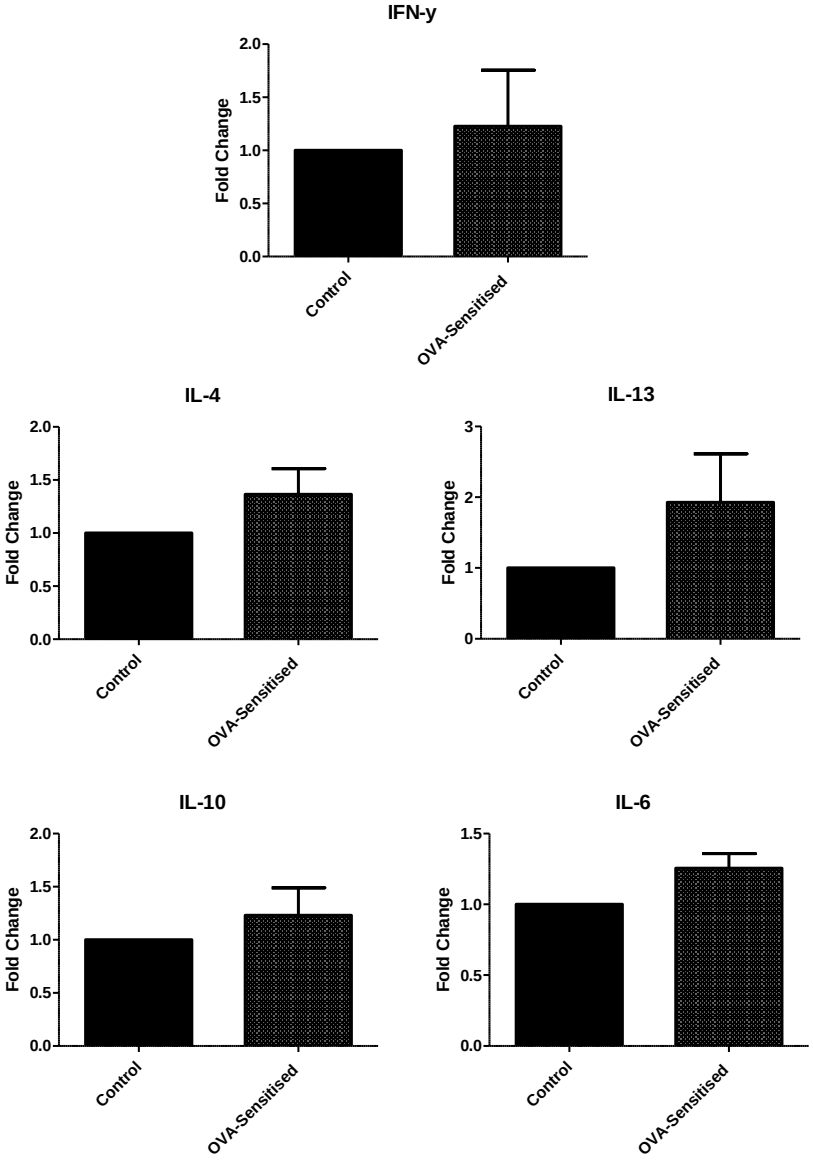


Fig 4.20: mRNA expression of the Th2 dominant cytokine genes in colons of OVA-sensitised mice. 10-12 week old female BALB/c mice were grouped into a non-sensitised control group of 6 mice and 3 test groups (also 6 mice each) with OVA-induced allergy. Two OVA-sensitised groups were treated with WPC80 or UL-2-147 (6 hr). All groups were culled on day 27. RNA was extracted and converted to cDNA. The cDNA was mixed with primers for IFNγ, IL-4, IL-13, IL-10 and IL-6 (IDT) and Taqman® Gene Expression Mastermix (Applied Biosystems) before analysing samples on the Lightcycler® 96 system (Roche). Groups were compared using relative quantitation; after normalising samples to *GusB*, the control group was normalised to 1.0 and the expression in other groups is shown relative to this value**.** Results are means ±SD of at least 6 mice measured in triplicate. An unpaired T-test was used to determine if differences between groups were significantly different (*$p<0.05$, **$p<0.01$, ***$p<0.001$). All groups are compared to non-sensitised control mice.

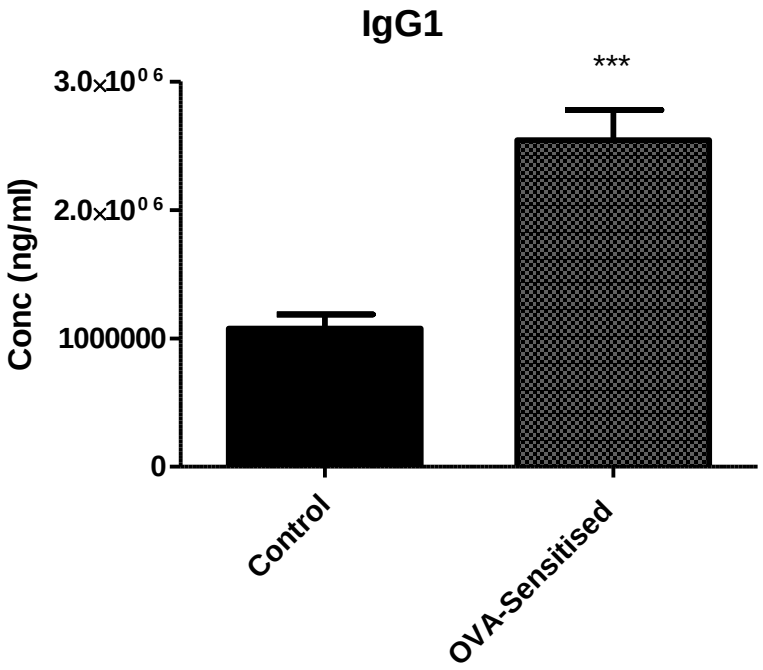


Fig 4.21: Immunoglobulin antibody levels in serum in OVA-induced allergy *in vivo*. 10-12 week old female BALB/c mice were grouped into a non-sensitised control group of 6 mice and a test group (also 6 mice) with OVA-induced allergy. Both groups were culled on day 27. Blood serum was collected and measured for IgG1 secretion using ELISA according to manufacturer's instructions (Invitrogen). Results are means ±SD of at least 6 mice, One way Anova was used to determine if differences between groups were significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

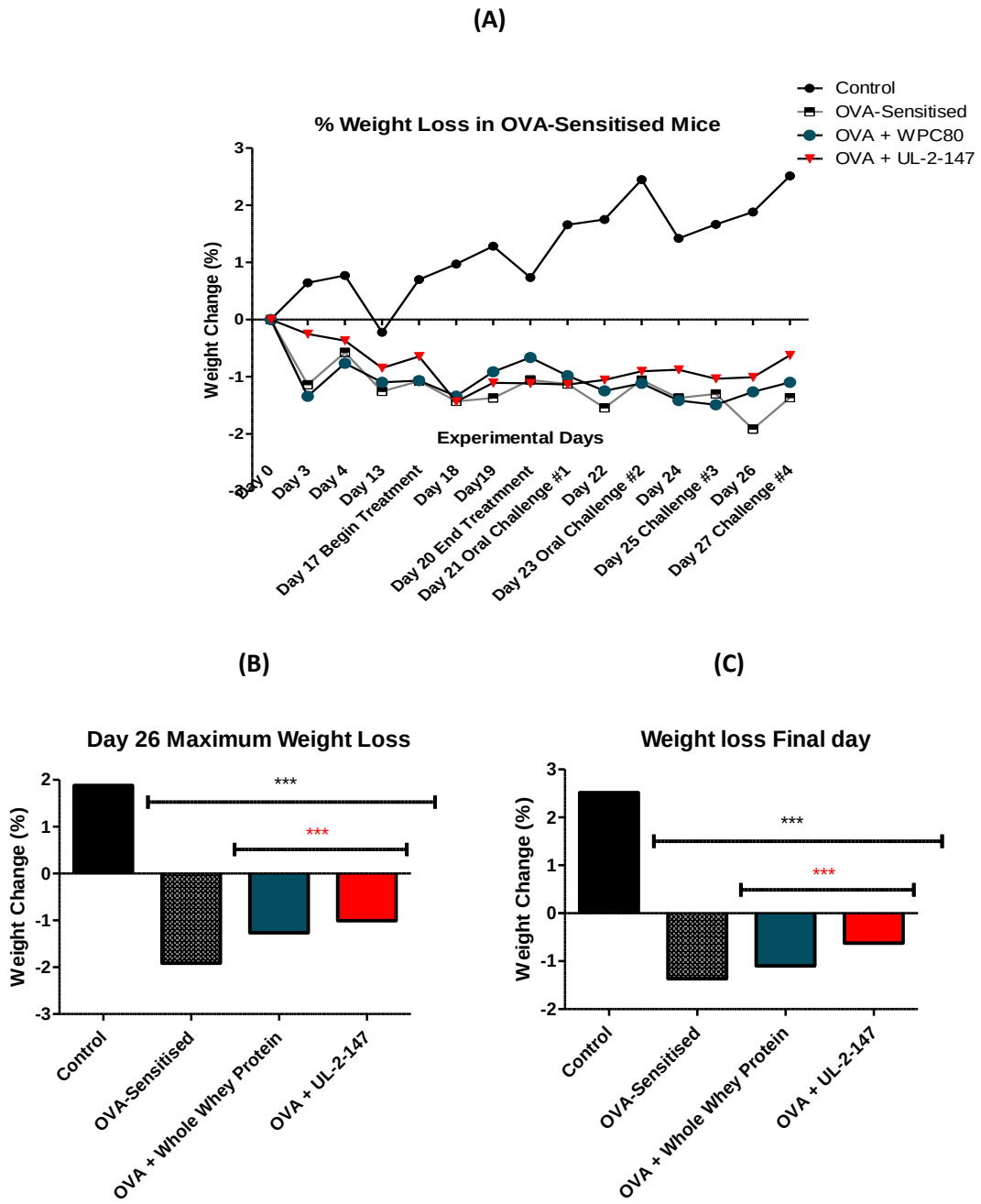
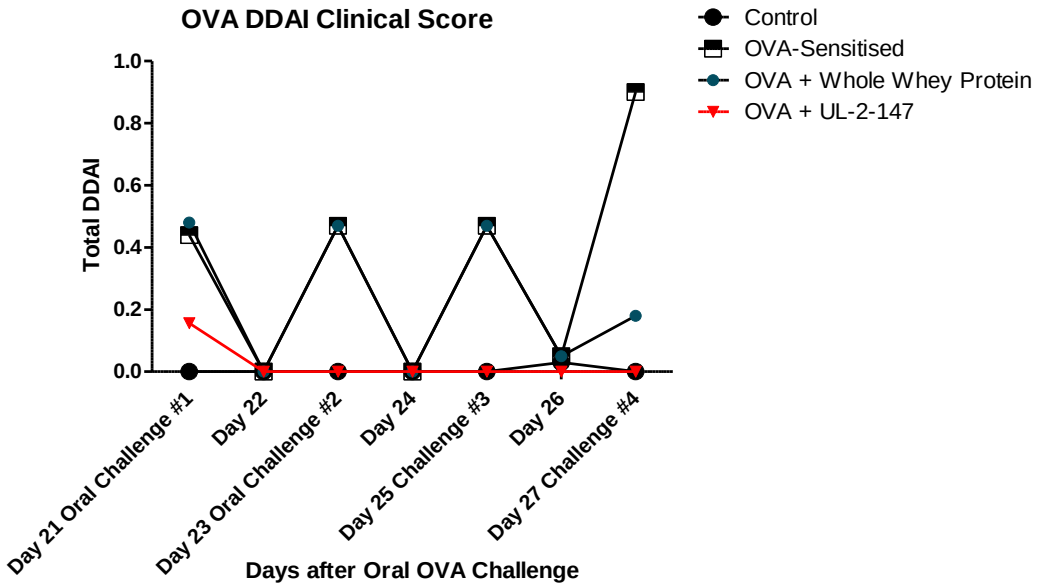


Fig 4.22: Disease associated symptoms (body weight change) in the OVA allergy model. 10-12 week old female BALB/c mice were grouped into a non-sensitised control group of 6 mice and 3 test groups (also 6 mice each) with OVA-induced allergy. Two OVA-sensitised groups were treated with WPC80 or UL-2-147 (6 hr). All groups were culled on day 27. Mice were weighed every day. The average % weight change of the 4 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 **(A)**. Maximum weight loss was recorded on day 26 (**B**). Weight loss values on final day of study (**C**). Data presented indicate the mean ± SEM (n = 6). One way Anova with Newman Keuls post-hoc test was used to determine if differences between groups [Groups compared to control (black) and groups compared to DSS alone (red)] were significant (*p<0.05, **p<0.01, ***p<0.001).

(A)



(B)

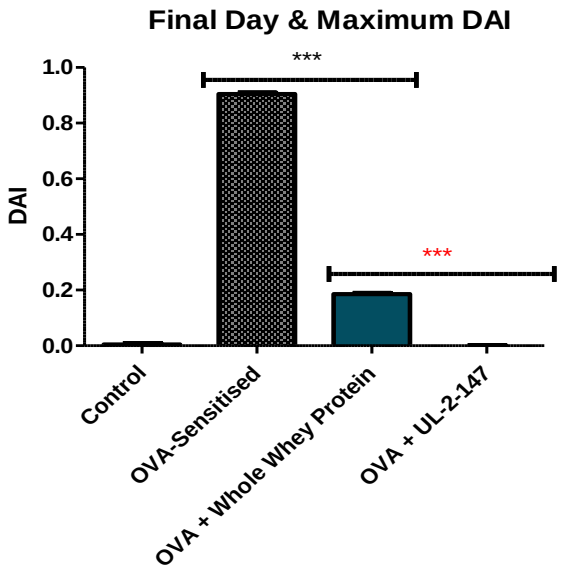


Fig 4.23: Disease associated symptoms (DDAI) in the OVA allergy model. 10-12 week old female BALB/c mice were grouped into a non-sensitised control group of 6 mice and 3 test groups (also 6 mice each) with OVA-induced allergy. Two OVA-sensitised groups were treated with WPC80 or UL-2-147 (6 hr). All groups were culled on day 27 and mice were weighed and scored (every day during oral challenges) for daily disease activity index (DDAI) based on activity, breathing, fur texture/posture, eyes, faeces and nose **(A)**. Maximum DAI was recorded on the final day, following the final oral challenge (**B).** Data presented indicate the mean ± SEM (n = 6). One way Anova with Newman Keuls post-hoc test was used to determine if differences between groups [Groups compared to control (black) and groups compared to DSS alone (red)] were significant (*p<0.05, **p<0.01, ***p<0.001).

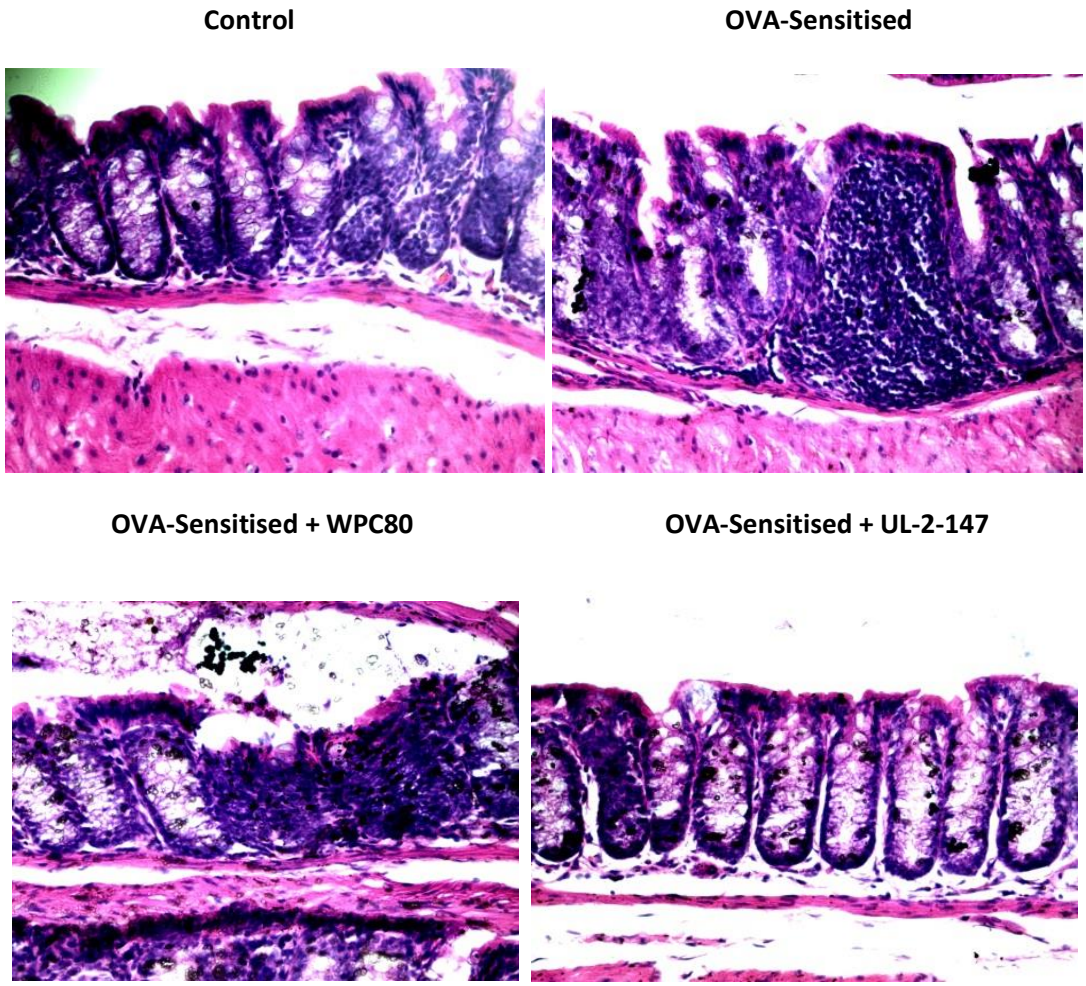


Fig 4.24: Effect of UL-2-147 hydrolysate on disease associated symptoms (histopathological changes of the colon tissue) in the OVA allergy model. 10-12 week old female BALB/c mice were grouped into a non-sensitised control group of 6 mice and 3 test groups (also 6 mice each) with OVA-induced allergy. Two OVA-sensitised groups were treated with WPC80 or UL-2-147 (6 hr). All groups were culled on day 27 and sections of the distal colon were removed for histology and H&E staining in order to confirm inflammation. Representative images of H&E staining of colonic tissue from each group are taken at 40x magnification.

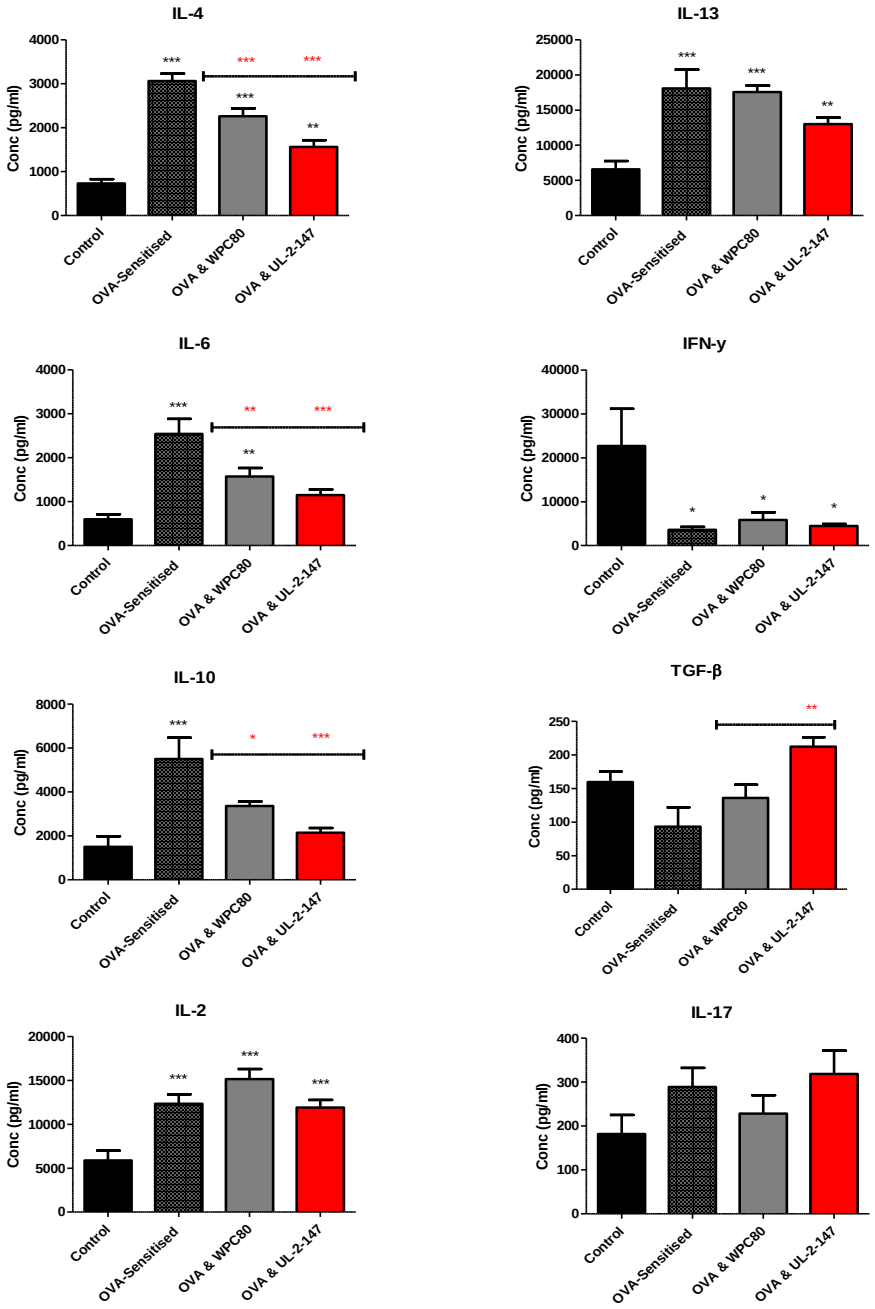


Fig 4.25: UL-2-147 attenuates the Th2 dominant cytokine spike in OVA stimulated splenocytes from OVA-sensitised mice.10-12 week old female BALB/c mice were grouped into a non-sensitised control group of 6 mice and 3 test groups (also 6 mice each) with OVA-induced allergy. Two OVA-sensitised groups were treated with WPC80 or UL-2-147 (6 hr). All groups were culled on day 27. Spleens were collected and splenocytes were plated at $5x10^6$ cells/ml on a 24 well plate. Cells were stimulated with OVA and incubated for 72 hrs and Supernatants were collected and measured for IL-4, IL-13, IL-6, IFN-γ, IL-10, TGF-β, IL-2 and IL-17 secretion using ELISA according to manufacturer's instructions (R&D duoset). Data presented indicate the mean ± SEM (n = 3) in triplicate. One way Anova with Newman Keuls post-hoc test was used to determine if differences between groups [Groups compared to control (black) and groups compared to OVA alone (red)] were significant (*p<0.05, **p<0.01, ***p<0.001).

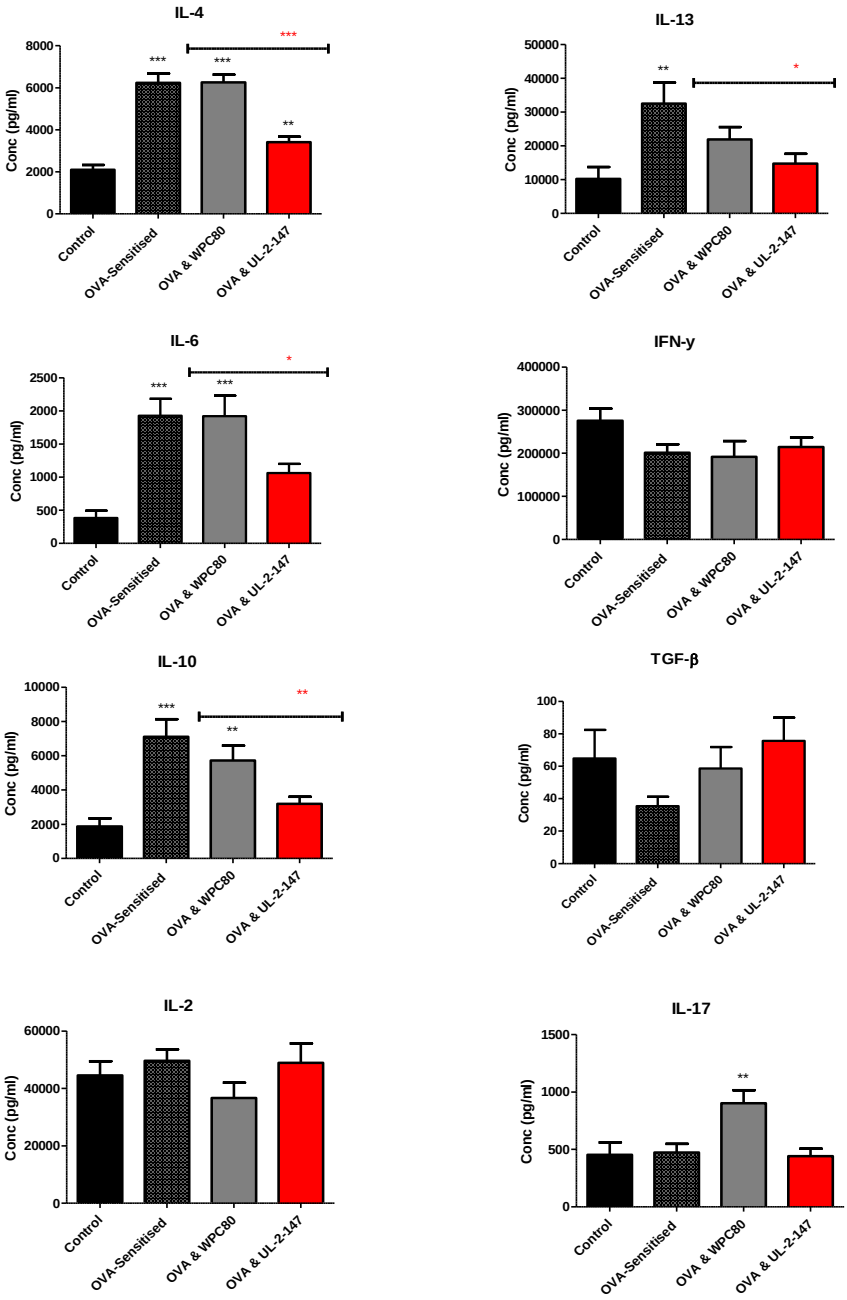


Fig 4.26: UL-2-147 attenuates the Th2 dominant cytokine spike in CD3/CD28 stimulated splenocytes from OVA-sensitised mice. 10-12 week old female BALB/c mice were grouped into a non-sensitised control group of 6 mice and 3 test groups (also 6 mice each) with OVA-induced allergy. Two OVA-sensitised groups were treated with WPC80 or UL-2-147 (6 hr). All groups were culled on day 27. Spleens were collected and splenocytes were plated at $5x10^6$ cells/ml on a 24 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) for 72 hr before harvesting. Supernatants were collected and measured for IL-4, IL-13, IL-6, IFN-γ, IL-10, TGF-β, IL-2 and IL-17 secretion using ELISA according to manufacturer's instructions (R&D duoset). Data presented indicate the mean ± SEM (n = 3) in triplicate. One way Anova with Newman Keuls post-hoc test was used to determine if differences between groups [Groups compared to control (black) and groups compared to OVA alone (red)] were significant (*p<0.05, **p<0.01, ***p<0.001).

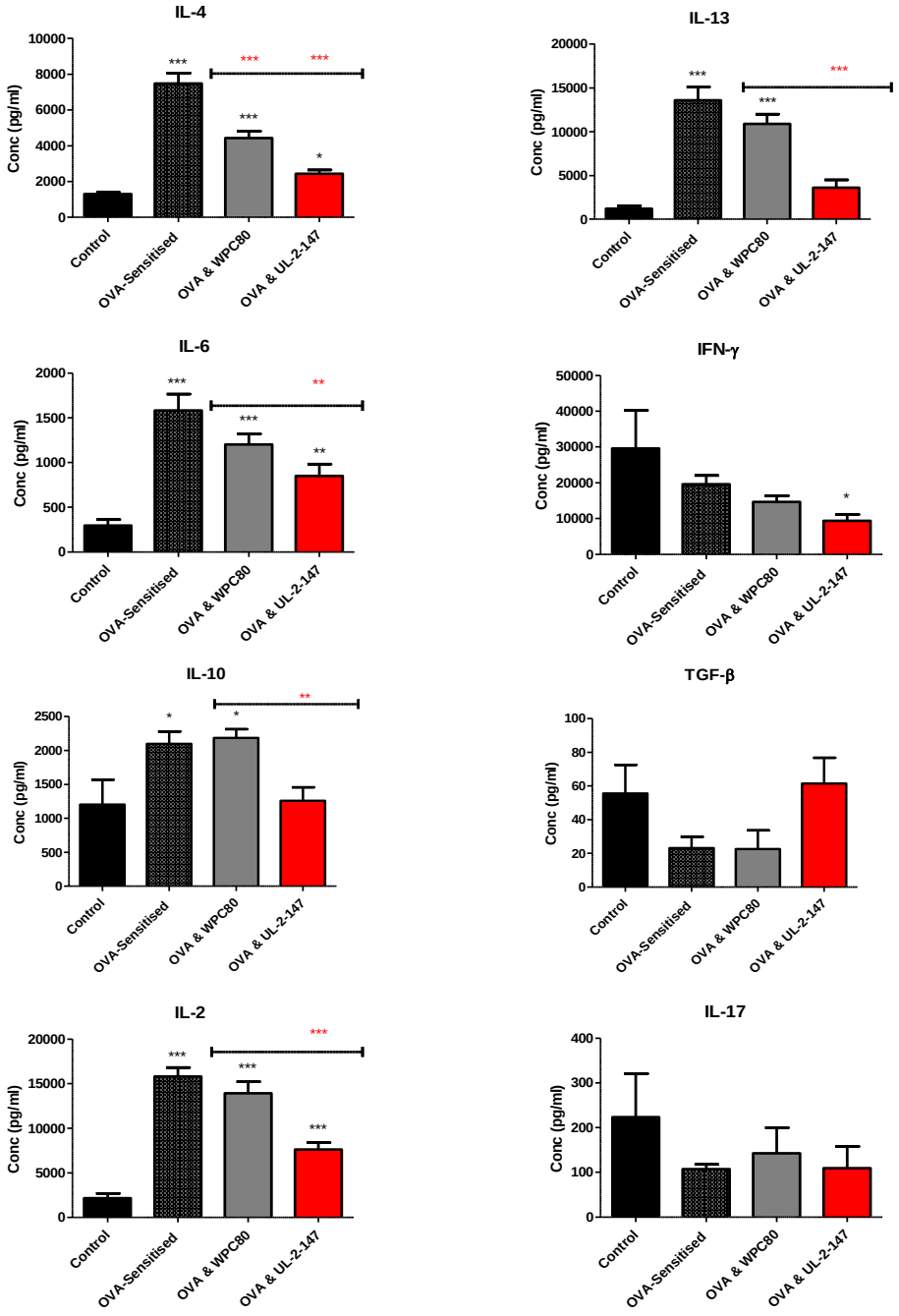


Fig 4.27: UL-2-147 attenuates the Th2 dominant cytokine spike in unstimulated splenocytes from OVA-sensitised mice. 10-12 week old female BALB/c mice were grouped into a non-sensitised control group of 6 mice and 3 test groups (also 6 mice each) with OVA-induced allergy. Two OVA-sensitised groups were treated with WPC80 or UL-2-147 (6 hr). All groups were culled on day 27. Spleens were harvested and splenocytes were plated at $5x10^6$ cells/ml on a 24 well plate and cultured for 72 hrs. Supernatants were collected and measured for IL-4, IL-13, IL-6, IFN-γ, IL-10, TGF-β, IL-2 and IL-17 secretion using ELISA according to manufacturer's instructions (R&D duoset). Data presented indicate the mean ± SEM (n = 3) in triplicate. One way Anova with Newman Keuls post-hoc test was used to determine if differences between groups [Groups compared to control (black) and groups compared to DSS alone (red)] were significant (*p<0.05, **p<0.01, ***p<0.001).

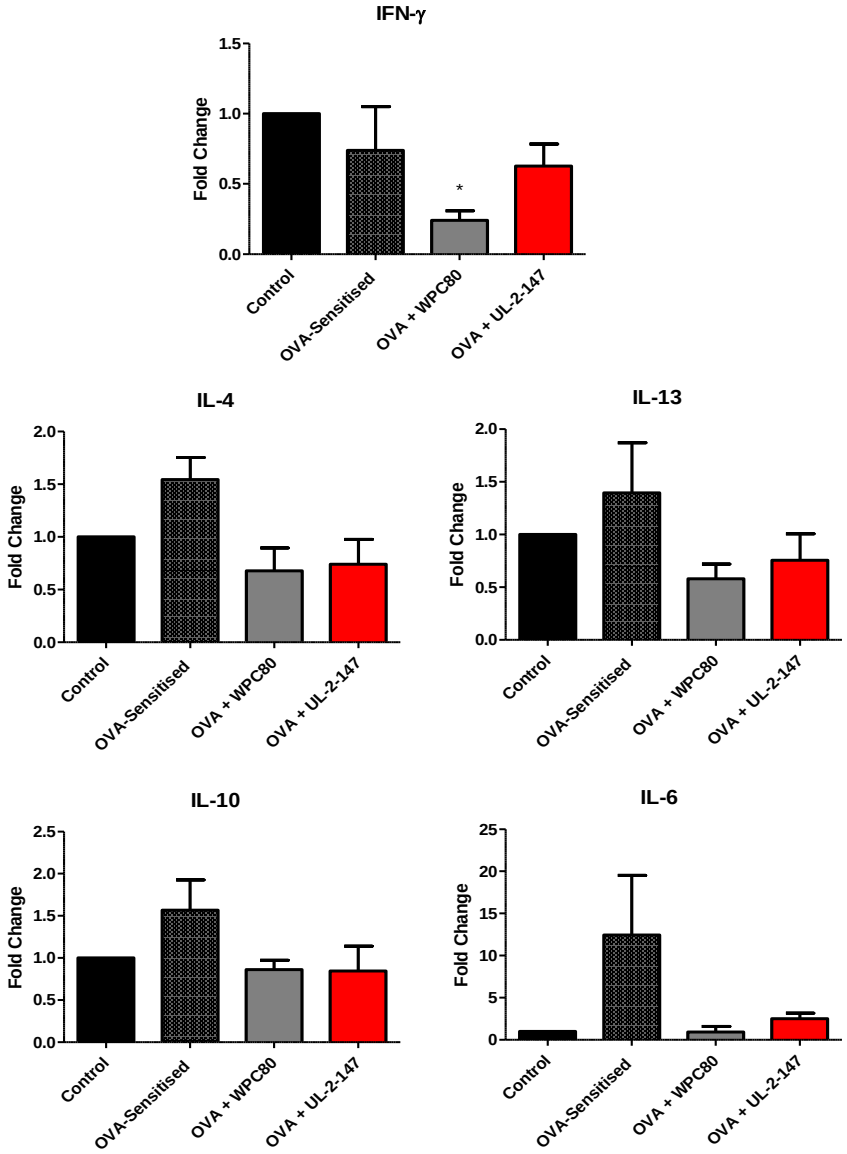


Fig 4.28: UL-2-147 and WPC80 inhibit the mRNA expression of the Th2 dominant cytokine genes in colons of OVA-sensitised mice. 10-12 week old female BALB/c mice were grouped into a non-sensitised control group of 6 mice and 3 test groups (also 6 mice each) with OVA-induced allergy. Two OVA-sensitised groups were treated with WPC80 or UL-2-147 (6 hr). All groups were culled on day 27. Colonic tissue from each sample was weighed and homogenised using the Qiagen TissueLyser LT with stainless steel beads (5 mm). Following homogenisation, RNA was extracted and quantitated on the nanodrop and equalised amounts of RNA were converted to cDNA using the high capacity cDNA mastermix (roche). The cDNA was mixed with primers for IFNγ, IL-4, IL-13, IL-10 and IL-6 (IDT) and Taqman® Gene Expression Mastermix (Applied Biosystems) before analysing samples on the Lightcycler® 96 system (Roche). Groups were compared using relative quantitation; after normalising samples to *GusB*, the control group was normalised to 1.0 and the expression in other groups is shown relative to this value**.** Results are means ±SD of at least 6 mice measured in triplicate. One way Anova with Newman Keuls post-hoc test was used to determine if differences between test groups and control mice were significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

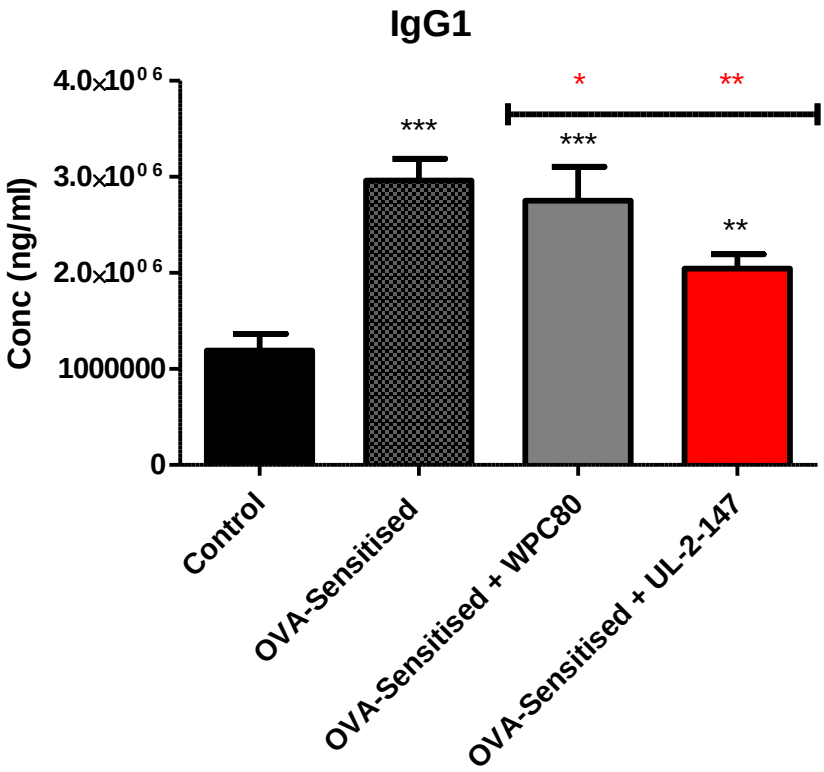


Fig 4.29: IgG1 levels from serum of OVA-sensitised mice treated with UL-2-147. 10-12 week old female BALB/c mice were grouped into a non-sensitised control group of 6 mice and 3 test groups (also 6 mice each) with OVA-induced allergy. Two OVA-sensitised groups were treated with WPC80 or UL-2-147 (6 hr). All groups were culled on day 27. Blood serum was collected and measured for IgG1 secretion using ELISA according to manufacturer's instructions (Invitrogen). OVA-sensitised mice treated with UL-2-147 secrete less IgG1 than untreated and WPC80 treated groups. Data presented indicate the mean ± SEM (n = 6) in triplicate. One way Anova with Newman Keuls post-hoc test was used to determine if differences between groups [Groups compared to control (black) and groups compared to DSS alone (red)] were significant (*p<0.05, **p<0.01, ***p<0.001).

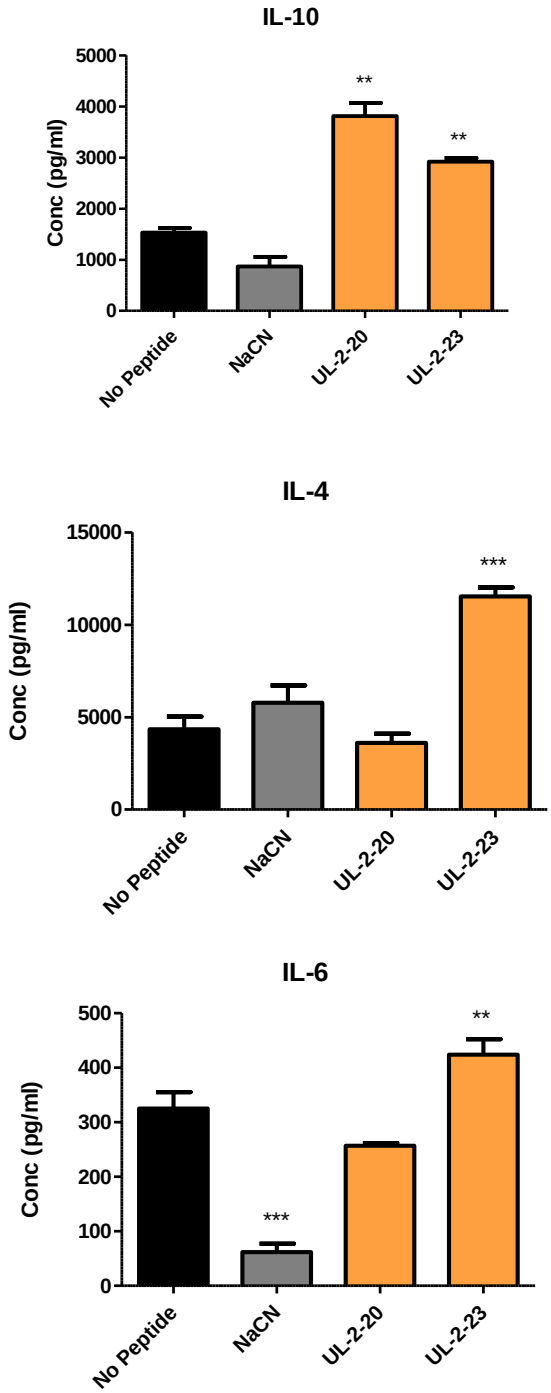


Fig 4.30: First-Round hydrolysates modulate secretion of cytokines associated with Th2 and Treg cells from Undifferentiated T-cells *in vitro*. $CD4^+$ cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19752). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml). Cells were incubated for 72 hrs. Supernatants were collected and IL-10, IL-4 and IL-6 levels were measured using ELISA according to manufacturer's instructions (R&D duoset). One way Anova was used to determine if differences between groups were significant (*p<0.05, **p<0.01, ***p<0.001). Hydrolysates are compared to untreated cells (No Peptide).

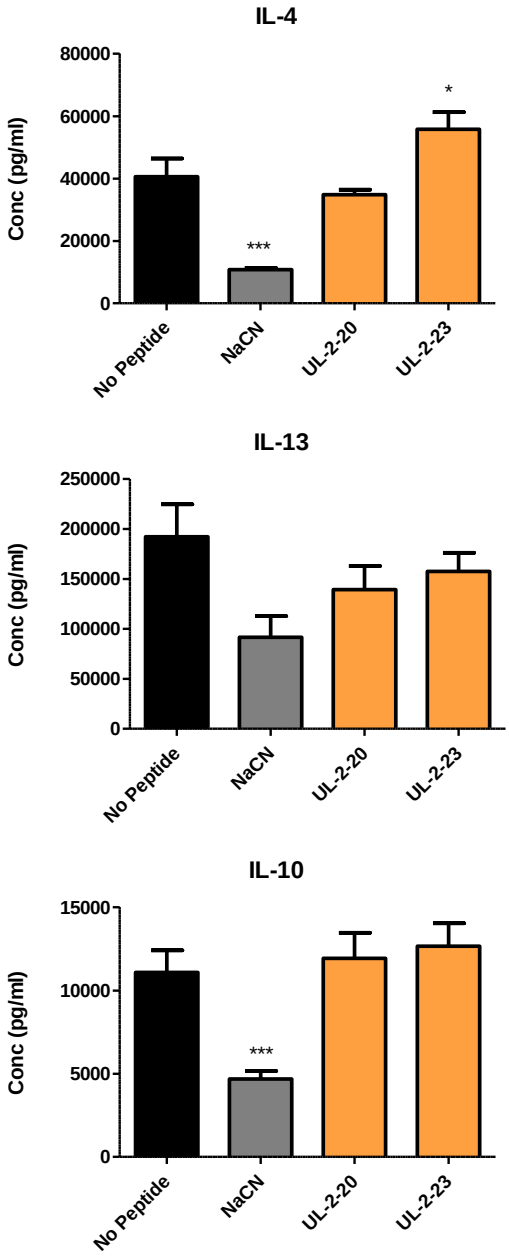


Fig 4.31: First-Round hydrolysates modulate the Allergic Th2 response *in vitro*. $CD4^+$ cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19752). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) in media supplemented with 10 µg/ml neutralising anti-IFN-γ antibody and 10 ng/ml IL-4 for Th2 polarising conditions. Cells were treated with hydrolysates (1 mg/ml). Cells were incubated for 72 hrs and were restimulated in fresh media for a further 24 hrs. Supernatants were collected and IL-4, IL-13 and IL-10 levels were measured using ELISA according to manufacturer's instructions (R&D duoset). One way Anova was used to determine if differences between groups were significant (*p<0.05, **p<0.01, ***p<0.001). Hydrolysates are compared to untreated cells (No Peptide).

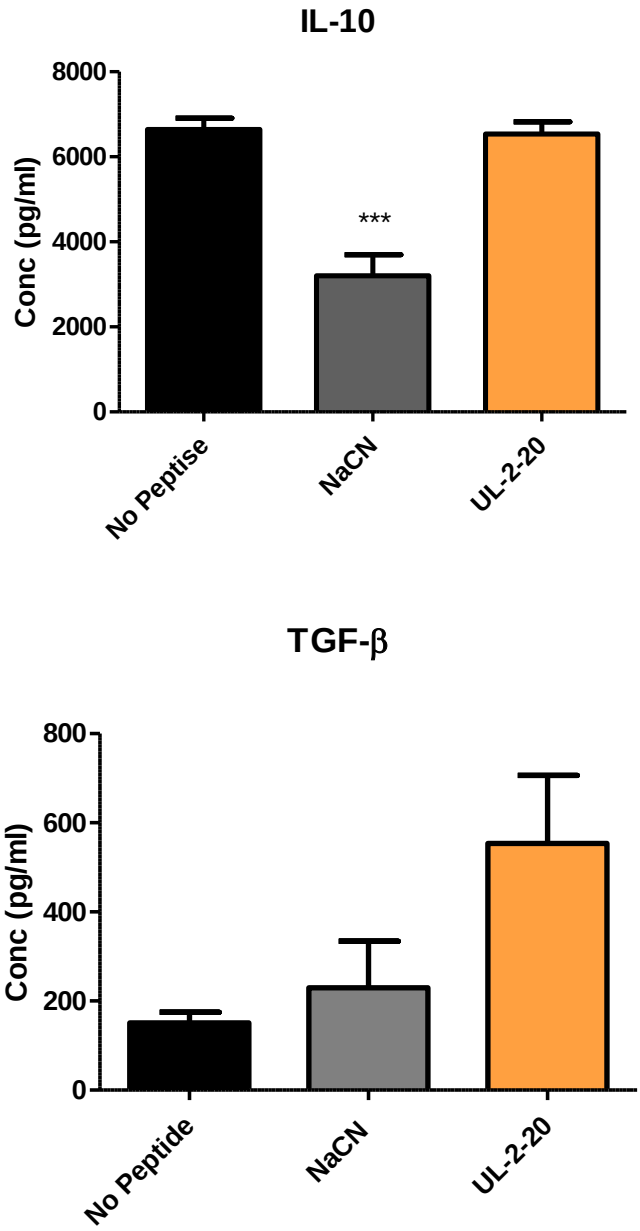


Fig 4.32: First-Round UL-2-20 drives a Treg response *in vitro*. $CD4^+$ Treg cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep™ Mouse CD25 Regulatory T Cell positive selection Kit (Stemcell Cat#18782). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml). Supernatants were collected at 72 hrs and IL-10 and TGF-β levels were measured using ELISA according to manufacturer's instructions (R&D duoset). One way Anova was used to determine if differences between groups were significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Hydrolysates are compared to untreated cells (No Peptide).

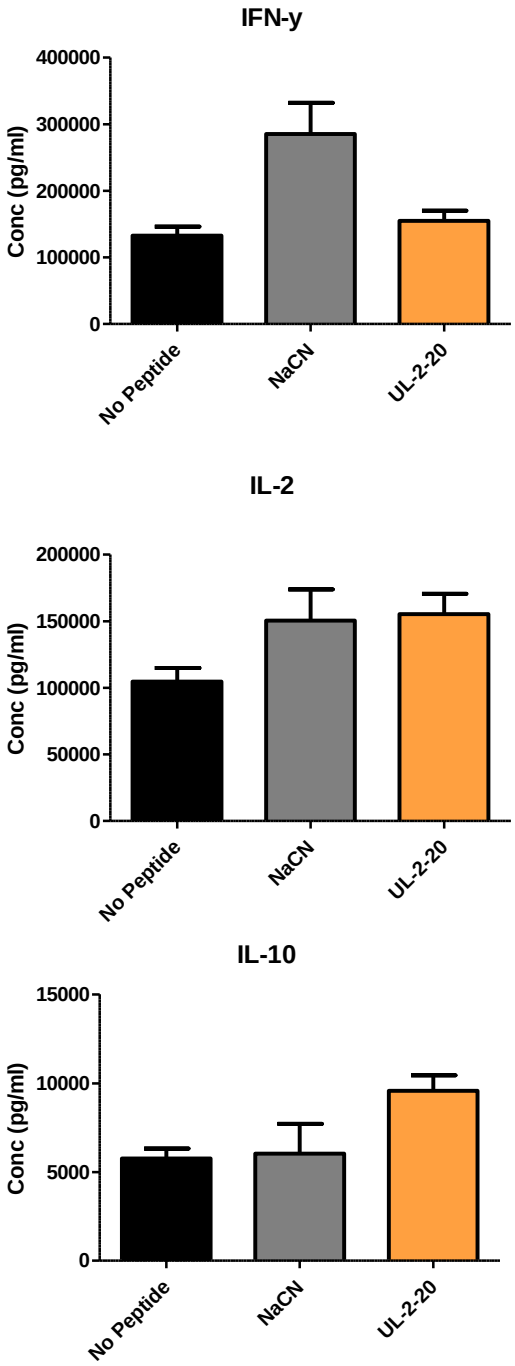


Fig 4.33: First-Round UL-2-20 has no effect on Th1 polarisation *in vitro*. $CD4^+$ T cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19752). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/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 hrs and were restimulated in fresh media for a further 24 hrs. Supernatants were collected and IFN-γ, IL-2 and IL10 levels were measured using ELISA according to manufacturer's instructions (R&D duoset). Hydrolysates are compared to untreated cells (No Peptide).

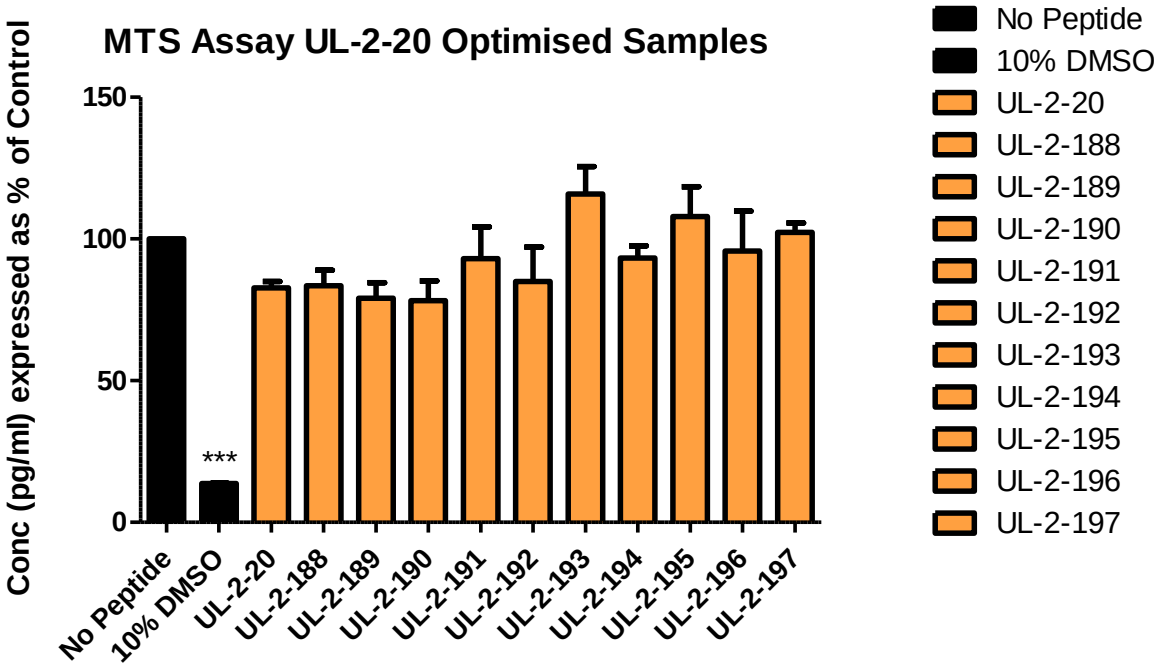


Fig 4.34: The concentrations of optimised protein hydrolysates from UL-2-20 used do not affect the viability of undifferentiated T-cells. $CD4^+$ cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19752). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml) or 10% DMSO (vehicle control). Cells were incubated for 72 hrs. Viability was assessed using an MTS assay (Cell Titer 96 Aqueous One Solution – Promega, WI, USA). One way Anova was used to determine if differences between groups were significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Results are expressed as a percentage of untreated cells.

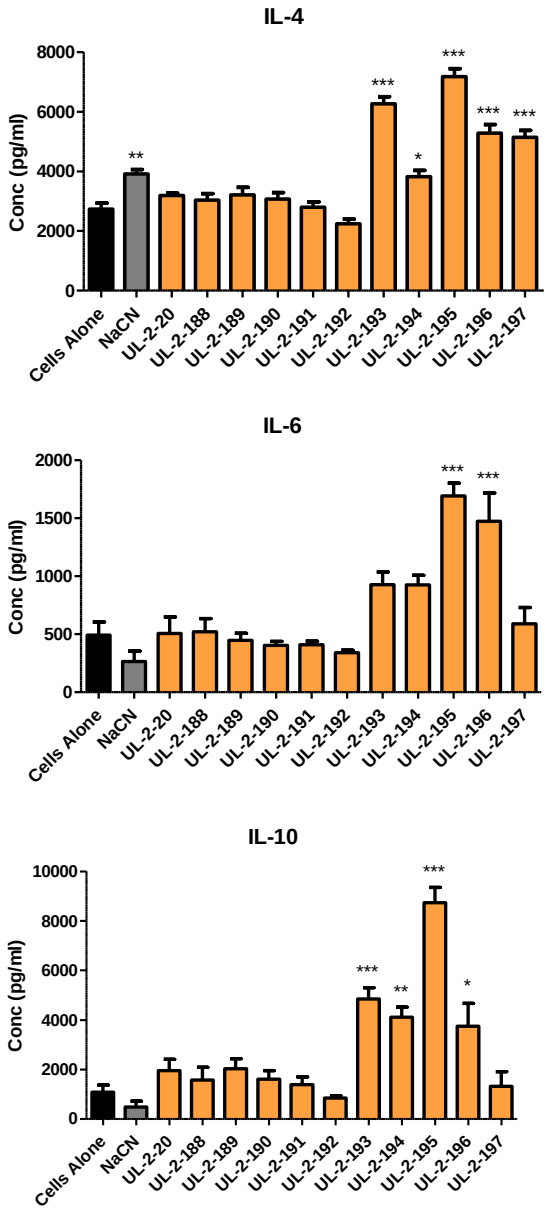


Fig 4.35: Third-Round UL-2-20 hydrolysate, regenerate and optimised hydrolysates modulate cytokine secretion from Undifferentiated T-cells *in vitro*. $CD4^+$ cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19752). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml). Cells were incubated for 72 hrs. Supernatants were collected and IL-4, IL-6 and IL-10 levels measured using ELISA according to manufacturer's instructions (R&D duoset). One way Anova was used to determine if differences between groups were significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Hydrolysates are compared to untreated cells (No Peptide).

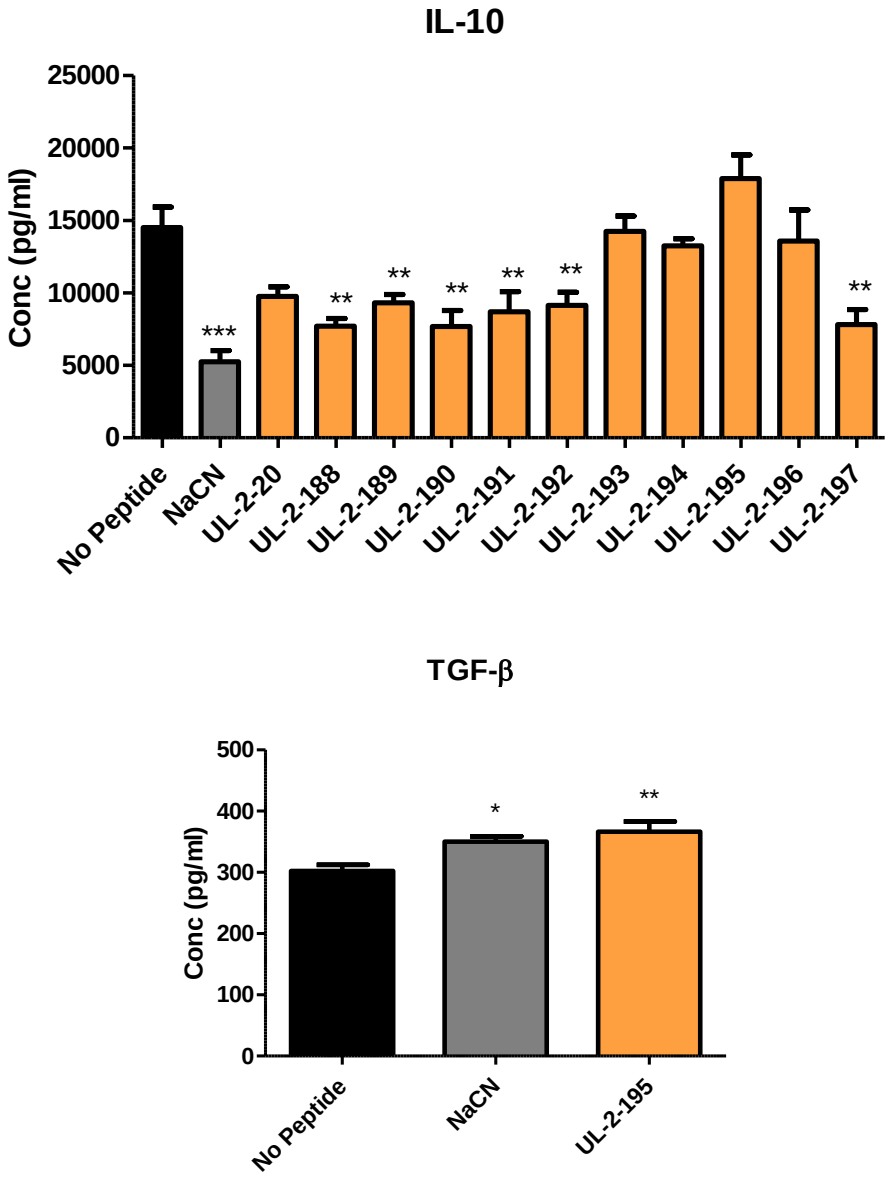


Fig 4.36: Third-Round UL-2-195 induces Treg cells *in vitro*. $CD4^+$ Treg cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep™ Mouse CD25 Regulatory T Cell positive selection Kit (Stemcell Cat#18782). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml). Supernatants were collected at 72 hrs and IL-10 and TGF-β levels were measured using ELISA according to manufacturer's instructions (R&D duoset). One way Anova was used to determine if differences between groups were significant (*p<0.05, **p<0.01, ***p<0.001). Hydrolysates are compared to untreated cells (No Peptide).

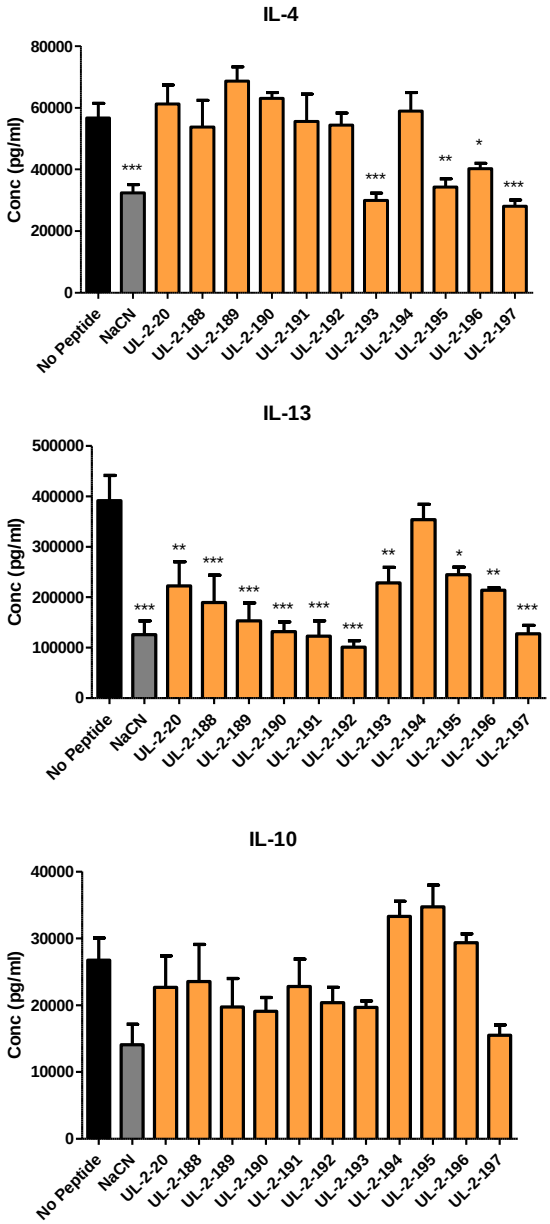


Fig 4.37: The effect of Third-Round UL-2-20 hydrolysate, regenerated and optimised hydrolysates was assessed on Th2 cells *in vitro*. $CD4^+$ cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19752). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) in media supplemented with 10 µg/ml neutralising anti-IFN-γ antibody and 10 ng/ml IL-4 for Th2 polarising conditions. Cells were treated with hydrolysates (1 mg/ml). Cells were incubated for 72 hrs and were restimulated in fresh media for a further 24 hrs. Supernatants were collected and secretion of IL-4, IL-13 and IL-10 was measured using ELISA according to manufacturer's instructions (R&D duoset). One way Anova was used to determine if differences between groups were significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Hydrolysates are compared to untreated cells (No Peptide).

4.3 DISCUSSION

Food allergy incidence has increased substantially in recent times, with a 15-30% incidence rate in the Western world (van den Elsen et al. 2013). However, despite this increased threat, treatment and prevention strategies are severely lacking and remain poor, with avoidance being the main management/approach. Although most infants eventually recover from food allergy, such as CMPA, many retain a skewed immune response and remain atopic. This can lead to further problems associated with atopy occurring in later life, known as the atopic march, such as asthma and atopic dermatitis. It is, therefore, important to target allergy early in healthy infants who are at risk of becoming atopic in order to prevent this sensitisation process from occurring in the first instance. Infants who are unable to breastfeed are highly susceptible to developing food allergy, as they fail to generate immune balance, and are therefore a key target group for the objective of this chapter. It has been well reported that dairy proteins and milk derived peptides have immunomodulatory effects *in vitro.* The immunomodulatory effects that cow's milk (CM) hydrolysates possess, include the ability to suppress cells involved with allergy, drive allergy, suppress inflammation or display multiple functions (Cross ML and Gill HS, 2000). This chapter aims to identify novel CM protein whey and casein hydrolysates that possess positive immunomodulatory properties, which involve priming the immune system of infants to avoid allergy in later life by bringing about immune balance and reducing the time to tolerance. In order to identify possible novel CM hydrolysates that could be used for the management of food allergy and subsequent "retraining" of the T-helper 1/T-helper 2 (Th1/Th2) or Treg/Th2-paradigms, we first examined their effect, *in vitro*, on modulation of cytokine secretion from undifferentiated $CD4^+$ T-cells and T-cell subsets, from which candidate hydrolysates were selected, based on their immune modulating ability, to be assessed on their effectiveness in an *in vivo* murine model of food allergy. The hydrolysate's ability to modulate a plethora of cytokines and biomarkers associated with food allergy was examined.

The first aim of this chapter was to use the clearly defined T-helper cell subsets, as outlined in Chapter 3, to identify candidate hydrolysates that can modulate the immune response away from an a harmful excessive Th2 response *in vitro* and ideally towards a Th1 and/or Treg subtype. Th1 cells mediate a cellular immune response and Th2 cells drive a humoral response. Depending on the type of pathogen encountered, one

subset is dominant at any one time. The cytokines secreted by each subtype have an antagonistic effect on the other subsets, inhibiting their differentiation (Kaiko et al. 2008). As mentioned, there is a degree of plasticity within the T-cell subsets, suggesting that the subsets are capable of co-expressing certain cytokines characteristic of other subsets or may develop into another subset based on the environment they are exposed to (Kidd 2003). Th2 cells, when dysregulated or excessive, play key roles in triggering isotype switching and IgE production by B-cells which facilitate the development of allergic reactions to harmless dietary proteins. The immune response in the gut is regulated by the Th1/Th2 cytokine balance, which favours a Th2 phenotype at birth (Prescott et al. 1998; Vighi et al. 2008). Allergy is characterised by an imbalance between Th2 cells and Treg and/or Th1 cells. Breastfeeding is the optimum method to bring about this natural immune balance. Failure to balance the Th2 and Treg/Th1 paradigm following birth results in excessive production of the Th2 dominant cytokines, IL-4, IL-6, IL-10 and IL-13, which, in turn, dampen the Treg and Th1 responses and thus, expression of TGF-β and IFN-γ, resulting in sensitisation and allergic disease (Prescott, Sly & Holt 1998, Prescott et al. 1998, Prescott et al. 1999, Neaville et al. 2003). The first step of this objective was to determine what effect the hydrolysates have on the polarisation of T-cell subsets by examining cytokine secretion from undifferentiated $CD4^+$ T-cells treated with hydrolysates. Candidates were then chosen and brought forward for testing in T-cell subsets (Th2, Th1 and Treg) based on their potential to modulate the cytokines required to drive each subset. Cytokines play a key role in the pathogenesis and progression of allergic disease and contribute to the overall disease severity and were, therefore, the key focus of our *in vitro* studies (Brandt and Sivaprasad, 2011).

Treatment of undifferentiated $CD4^+$ T-cells with UL-2-40/41/42/43/45/46/47/48 hydrolysates resulted in a reduction of IL-4, IL-6 and IL-10 secretion, the key cytokines required to drive a Th2 response. When examined further in an *in vitro* Th2 assay, it was confirmed that UL-2-40/41/42/43/47 possessed the ability to suppress a Th2 phenotype, as they inhibited secretion of the characteristic Th2 cytokines, IL-4, IL-10 and IL-13. The effect of UL-2-40/41/42/43/47 on Th1 cells was also examined. It was found that they had no suppressive effect and allowed the polarisation of Th1 cells as they did not inhibit the secretion of the key Th1 cytokine, IFN-γ. This was a

desirable effect as it suggests that these hydrolysates may possess the potential to balance the Th1/Th2 paradigm in an allergic *in vivo* state.

The next step was to assess the reproducibility of the bioactivity of UL-2-40/41/42/47. These hydrolysates were regenerated, using identical hydrolysis conditions as the parent compounds, and titled UL-2-77/78/79/151 respectively. The hydrolysis processes for UL-2-43 was considered expensive in comparison to the others and so it was not brought forward for further testing. Regenerated hydrolysates were examined in undifferentiated and Th2 cells alongside the parent hydrolysates. All regenerates behaved in a similar manner to their parent hydrolysate, which confirmed that their bioactivity was reproducible. UL-2-42 and UL-2-47 were chosen for further testing as their effect on IL-4 secretion in Th2 cells was most potent.

The overall aim of this project was to identify novel CM hydrolysates suitable for commercialisation and incorporation into infant milk formula that confer health benefits in infants. It was, therefore, necessary to ensure the candidate hydrolysates were suitable for large scale production and desirable for industry use. The next step was thus, to screen optimised candidate hydrolysates to determine whether UL-2-42 and UL-2-47 could be optimised further while maintaining or enhancing their bioactivity. Optimisation was carried out with the hope to improve a number of factors: to enhance hydrolysis conditions, improve solubility, enhance bioactivity and to lower the cost of production. Both UL-2-42 and UL-2-47 displayed poor solubility during their preparation in sterile water prior to putting on cells. It was hoped that this solubility issue could be improved through the optimisation process in order to make these candidate hydrolysates more consistent and reproducible. Optimised hydrolysates, UL-2-147/148 and UL-2-151/152/154, produced for UL-2-42 and UL-2-47, respectively, were tested in undifferentiated T-cells and Th2 cells *in vitro*. Details of these hydrolysates and the various controls tested can be seen in **Table 4.2** in **Section 4.2**. Optimised hydrolysates, UL-2-147/148, behaved in a similar manner to their parent hydrolysate, UL-2-42, by suppressing the cytokines required to both drive and maintain a Th2 response, IL-4, IL-6, IL-10 and IL-13, from undifferentiated and Th2 cells. The enzyme control (inactive enzyme) was produced using the same hydrolysis process as UL-2-42 but the enzyme was inactivated after hydrolysis. The purpose of this was to determine that the bioactivity observed in the cells was solely

as a result of the hydrolysate treatment and not as a result of the enzyme source. The bioactivity was confirmed to be from the hydrolysates as the inactive enzyme also significantly reduced the Th2 cytokines after the enzyme was removed. Negative hydrolysate controls (UL-2-150 and UL-2-153) were produced in order to determine the importance of the enzyme source used in the hydrolysis process. They underwent the same heat variations as the hydrolysates but were not exposed to enzymes during hydrolysis. A decreased bioactivity compared to the hydrolysates was observed following treatment with UL-2-150, as it suppressed IL-4 but had no effect on suppressing IL-10 and IL-13 secretion from Th2 cells, confirming that the enzyme used for hydrolysis was essential for inducing the beneficial bioactivity identified and linked with these hydrolysates. A concern arose regarding WPC80 treatment, as it also suppressed the Th2 cytokines, to a similar level as the hydrolysates themselves. The *in vitro* Th2 assay we used mimics an activated Th2 immune response which allowed us to do a crude screen and identify certain hydrolysates that may potentially possess the ability to suppress this cell type *in vivo*. This assay is not intended for examining the effect of a hydrolysate treatment on a specific disease state as it is not specific to a certain allergy. We, therefore, hoped that our *in vivo* trials, specific to OVA allergy, would determine differences between the bioactivity of the intact protein and hydrolysate treatments and the capacity of the hydrolysates to modulate an allergic environment. UL-2-47 did not inhibit the secretion of IL-4 from undifferentiated T-cells or Th2 cells as it did in the first round of screening. UL-2-47 regenerate, UL-2-151, and optimised samples, UL-2-152/154 did not have any desirable effects. The whole NaCN and hydrolysate control, UL-2-153, were more effective in suppressing a Th2 phenotype than the hydrolysates themselves. As mentioned, UL-2-47 has very poor solubility which may be responsible for the inconsistent bioactivity observed. UL-2-47 and optimized hydrolysates were not tested any further than this point.

Further desirable *in vitro* properties of UL-2-42/147 treatments included their ability to allow for the polarisation of Treg cells. They did not result in a reduction of TGF-β, the key Treg cytokine, when cultured with Treg cells. Similar to their effect on Th1 cells, this suggests that these hydrolysates may possess the potential to balance the Treg/Th2 paradigm in an allergic *in vivo* state. This would be a beneficial feature of these hydrolysates as Treg activation may induce immune homeostasis between the T-cell subsets in an *in vivo* allergy setting in infants. Although the parent hydrolysate,

UL-2-42, displayed desirable bioactivity in our *in vitro* assays, its commercial value is low due to its poor solubility, as mentioned. For this reason it was not brought forward for further screening. Optimised hydrolysate, UL-2-147, had no solubility issues and was, therefore, the best candidate to be examined further. At this point, a dose response study was carried out on UL-2-147, which confirmed that its suppression of IL-4 and IL-13 in Th2 cells was dose dependent. UL-2-147 was scaled up at industry level in the BFE plant in Teagasc in Moorepark for *in vivo* trials. The activity of the scaled up hydrolysate was compared to the hydrolysates produced at lab scale in Th2 cells. Hydrolysate bioactivity between the small and large scale production correlated, which finalised the *in vitro* work in this chapter.

Although IL-4 is the key cytokine required for the differentiation of a Th2 response by blocking Th1 and Th17 differentiation, other cytokines also play a part in this process. IL-6 is released during the early stages of a Th2 immune response from macrophages, mast cells and DCs (Dodge et al. 2003; Kaiko et al. 2008). IL-6 promotes Th2 polarisation through the upregulation of IL-4, while also inhibiting STAT1, subsequently blocking Th1 differentiation (Diehl and Rincón, 2002). In addition to UL-2-147 treatment inhibiting IL-6 secretion from undifferentiated T-cells, a decrease in IL-6 secretion from DCs was also observed by other members of the DCU research group within the FHI team (not shown). This suggests that inhibition of IL-6 in an *in vivo* atopic scenario, as a result of UL-2-147 treatment, may allow for the development of a Th1 response, balancing the Th1/Th2 ratio that is askew in atopic infants. We hoped that the *in vivo* allergy study would provide a deeper insight into this potential mechanism.

Food allergy is beyond just Th1 and Th2 cells, however. The role of Th17 cells in allergy is still unclear. Kiewiet et al. (2015) states that Th17 cells play a role in maintaining homeostasis in the intestine and aid in the development of tolerance. Dhuban et al. (2013) carried out a study on children with food allergies. Significantly lower IL-17 secretion from $CD4^+$ undifferentiated T-cells was observed in children with food allergies. Furthermore, when exposed to food antigens *in vitro*, IL-17 secretion from undifferentiated T-cells was significantly inhibited in patients with food allergy when compared to healthy control subjects. This study suggests that IL-17 could potentially play a role in the induction of tolerance to food antigens. While our focus was on Th1, Th2 and Treg cells, we examined the effect of UL-2-147 on IL-

17 secretion from undifferentiated T-cells to assess whether it could induce this cytokine **[Appendix B: Figure 8.1]**. WPC80 significantly lowered levels of IL-17 secretion. However, when treated with UL-2-147, IL-17 levels were restored to the basal levels secreted from untreated cells (3 hr UL-2-147) and significantly increased (6 hr UL-2-147). This suggests it could potentially have the same effect *in vivo* and aid in bringing about immune homeostasis in allergic mice. Only a few studies have addressed this mechanism as a potential treatment for CMPA. More evidence supporting this potential therapeutic role for IL-17 can be seen in a recent study on $TSLPR^{-/-}$ mice. Thymic stromal lymphopoietin (TSLP) is known to play a role in the development of allergic reactions. Lymphocytes from the mesenteric lymph nodes (MLNs) of $TSLPR^{-/-}$ mice were examined. Frossard et al. (2016) identified lower levels of IL-4, IL-5 and IL-10 and an increase in IL-17 levels in lymphocytes following an antigen challenge *in vivo*. An increase in IL-12 and IL-17 secretion from MLNs and activated DCs was also observed. While IL-17 could have potential benefits for the treatment of CMPA, it may not have the same effect in adults with food allergies. In this scenario, Th17 cells and IL-17 have been shown to play a role in the development of various allergic diseases, in addition to the Th2 response, such as asthma (Keisuke et al. 2008). Asthma is not developed until later in life. As mentioned previously, an infant's immune system is not fully developed and functions much differently to that of an adult's. For this reason, IL-17 may only play a therapeutic role as a biomarker in the prevention of allergy in infants and not in allergies developed later in life. This suggests that IL-17 could have a role in balancing the immune system in early years, making it a suitable biomarker for this study. As little research exists on targeting IL-17 modulation *in vivo* for the treatment of atopic disease this could enhance our understanding of its role in food allergy, potentially adding to current research in this area.

It is important to note that the physiological relevance of the hydrolysate *in vitro* dose was carefully considered and planned prior to commencing this particular project. *In vitro* dose response studies were carried out in the first phase of FHI to determine an optimal universal hydrolysate treatment concentration between all research groups so that results could be compared between studies. It was found that cow's milk hydrolysates were effective at a concentration of 1 mg/ml throughout all groups and this concentration was therefore used in this project, which was carried out in the

second phase of the FHI project. A crude screening process was used to compare and identify candidate hydrolysates with positive bioactivity in order to get an idea of their potential activity *in vivo*. The 1 mg/ml dose used was, therefore, sufficient to identify hydrolysates with potential bioactivity. Furthermore, many studies have demonstrated that cow's milk hydrolysates have good bioavailability. Many studies have shown that bioactive food peptides are potent and can resist digestion in the stomach and cross the epithelial barrier, as they have shown to exert effects at both intestinal and systemic levels. Bioactive peptides are also being used as biocarriers for nutrients and minerals to increase their bioavailabilty and absorption (Silva and Malcata, 2005).

Cell viability was monitored throughout the *in vitro* screening process to ensure the hydrolysates were not toxic to the cells. It was observed that the viability of cells treated with hydrolysates was often greater than 100%. This is a common issue with the MTS assay. This is possible due to natural variations of cellular metabolism or if slightly more cells were seeded in that well due to small pipetting errors. It is also possible that the hydrolysates may have increased the enzymatic activity of the cells. The MTS assay is based on the conversion of tetrazole to formazan by a mitochondrial reductase enzyme, in metabolically active cells (Riss et al. 2016). The assumption is that the conversion is dependent on the number of viable cells, however, it is also a possibility that treatment of cells with hydrolysates may result in increased enzymatic activity without effecting cell number or cell viability. Positive and negative controls were used in all MTS assays throughout this project. Cells alone acted as the positive control and cells treated with 10% DMSO acted as a negative control. Toxic effects on cell behaviour and viability have been reported in cells treated with 1% DMSO or higher (Singh, McKenzie and Ma, 2017). Some hydrolysate samples reduced viability when compared to cells alone, which was not a concern as they were still significantly more viable than cells treated with 10% DMSO.

Some minor inconcistencies are evident during the early stages of the *in vitro* screening process. These include differences between batch-to-batch cell activity in experiments carried out in the earlier stages in the *in vitro* work. For example, there is an inconsistency in batch-to-batch cytokine secretory levels by undifferentiated and Th2 $CD4^+$ T-cells between the first and second screening rounds. Slight differences in IL-4, IL-6 and IL-10 secretion by undifferentiated T-cells and IL-4, IL-10 and IL-13 secretion by Th2 cells were observed between the two screening rounds. As

mentioned, these experiments were carried out in the early stages of this PhD project. At this premature stage the researcher was learning processes and optimising protocols and thus, consistency of cell activity was not ideal during this time. There were also slight differences in batches and ages of mice used for cell culture which may affect the level of cytokine secretion. Although inconsistencies were observed, the ultimate aim of this section was carried out successfully as it was still possible to determine that the hydolysates were reproducable as both parent and regenerated samples were screened alongside each other in the second screening round. Cells were cultured and treated again in a new experiment with the parent hydrolysates alongside the regenerated samples, allowing direct comparison between both treatments. All *in vitro* experiments that followed these early studies further validated this finding and thus, this inconsistency was not a major concern for the outcome of this project

A second inconsistency was noted in the activity of some of the hydrolysates. Some hydrolysates that significantly reduced cytokine secretion in the first round of screening no longer demonstrated a significant result in the second screening round. As mentioned, there was an issue regarding the solubility of some of the hydrolysates and thus, it was observed that these particular hydrolysates did not always behave in a reproducible manner with regards to significant activity. For example, cytokine secretion from Th2 cells treated with UL-2-40/41/42/47 was significantly reduced in the first screening round (IL-4 and IL-13 were significantly suppressed by UL-2-40/41/42 and IL-10 and IL-13 were significantly reduced by UL-2-47) but the result was no longer significant in the second screening round. These parent hydrolysates displayed poor solubility during sample preparation. Although this was not ideal, it was hoped that this parameter would be improved following the optimisation process by the technology research group in UL. The poor solubility of these parent samples therefore was not a major concern at this point. Furthermore, following the optimisation steps the solubility of these samples improved greatly, hence why the optimised hydrolysate, UL-2-147, was chosen rather than its parent hydrolysate, UL-2-42, for further assessment in *in vivo* trials.

Overall, throughout our *in vitro* screening platform, we successfully identified a candidate whey-derived hydrolysate, UL-2-147, which demonstrated consistent specific bioactivity in targeting and suppressing the Th2 subtype alone. Furthermore UL-2-147 had no effect on the Th1 and Treg subsets and did not inhibit their

polarisation upon stimulation. We were, therefore, interested to explore whether UL-2-147 could induce immune balance by balancing the Th1/Th2 and/or Treg/Th2-paradigms in an *in vivo* setting of allergy. This would be beneficial in priming the infant immune system whilst also avoiding the risk of developing food allergy or preventing further allergy later in life. **Table 4.3** in **Section 4.2** summarises the results from the *in vitro* work carried out by all groups involved in the Allergy and Immunity Infant Nutrition FHI Workpackage. Our collaborators in National University of Maynooth (NUIM), working on humanised mouse models, further demonstrated that UL-2-147 also decreased expression of the Th2 transcription factor, GATA-3 (data not published yet), which further adds to the overall potential allergy-suppressing profile of this hydrolysate. The *in vitro* results from this chapter indicate that UL-2-147 may have beneficial immuno-modulatory effects in food allergy and infant formula products. The next objective of this chapter was to examine the effects of the candidate hydrolysate in an *in vivo* mouse model of food allergy. We examined the bioactivity of UL-2-147 in an OVA allergy mouse model in order to determine its capacity to suppress allergic symptoms and bring about immune balance in allergic mice. The OVA model is associated with disease in the Th2 subset and is a rapid and very reliable food allergy model with literature reports involving the food allergy topics. Aluminium adjuvants are widely understood to be safe stimulators of Th2 immunity (Lindblad, 2004). Thus, sensitisation to OVA using OVA and an aluminium hydroxide (Alum) adjuvant induces a Th2 response in mice, making this an ideal sensitisation method to assess the translation of our *in vitro* results into *in vivo*. OVA-induced experimental allergy was induced in mice and the hydrolysate's ability to modulate the allergic cell environment and suppress symptoms was assessed. We hoped that the results would confirm some of the suggested mechanisms of action for UL-2-147, hypothesised from our *in vitro* studies.

Sensitisation to OVA using OVA and Alum elicited the typical clinical symptoms of food allergy following subsequent oral OVA challenges which included weight loss, hudddled posture and inactive, little response to handling, unkempt rough coat, swollen eyes with discharge, rapid and abdominal breathing, crypt loss, destruction of the epithelial layer, and infiltration of pro-inflammatory cells to the distal colon. These clinical symptoms were efficiently reversed by the treatment of UL-2-147 hydrolysate. Throughout each of the four oral challenges, UL-2-147 treatment significantly reduced

the DAI score compared to PBS and WPC80 treated DSS-induced mice. By the final day of the model WPC80 treatment also significantly reduced the DAI score compared to PBS treated DSS-induced mice. This result is adequate confirmation that the UL-2-147 hydrolysate is largely more effective than the whole protein WPC80 control in improving allergic symptoms. Not only was its effect more potent in reducing clinical symptoms by the final day, but UL-2-147 treatment almost completely abolished any clinical symptoms from occurring as early as the first oral challenge, unlike the WPC80 control. It took three oral challenges with OVA before the therapeutic effects of WPC80 treatment were evident, and even still WPC80 was unable to completely ablate the clinical symptoms on the final oral challenge. UL-2-147 reversed symptoms such as being hudddled and inactive, unkempt rough coat, swollen eyes with discharge, rapid and abdominal breathing which were associated with OVA-sensitised mice within ten minutes of each oral challenge with OVA. This resulted in a significant reduction of DAI in mice treated with UL-2-147. Although treatment with UL-2-147 did not restore body weight of DSS-induced mice back to a baseline control level, they retained a steady weight throughout the course of oral challenges, unlike PBS and WPC80 treated mice who experience a drop in weight in the middle of the challenges. It may be possible that by continuing or prolonging the treatment course or increasing the dose of treatment would continue to improve this parameter. While the weight change is not an indicator of the beneficial effect of UL-2-147 treatment, the overall DAI score is proof of its therapeutic effect. Additionally, UL-2-147 treatment restored histopathology in the gut, unlike PBS and WPC80 treated OVA-sensitised mice, as seen by H&E stained colonic sections.

A major readout of this model involved looking at cytokine patterns in order to assess the ability of UL-2-147 to modulate immune populations. Limited studies have assessed the ability of whey hydrolysates to modulate immune populations in an allergic setting. Most animal studies that have examined the effects of hydrolysates on sensitisation to date have focussed on measuring immunoglobulin levels (Kiewiet et al. 2017). Analysis of cytokine secretion in the spleen and mRNA cytokine expression in the colon, in addition to the clinical assessment, strongly suggested that allergy, in the form of a skewed Th2 response, was present in OVA-sensitised mice. This was characterised by the significant increase in the characteristic Th2 cytokines, IL-4, IL-6, IL-10 and IL-13, secreted from unstimulated splenocytes and stimulated

splenocytes (both with OVA and CD3/CD28 antibodies) and by the increased expression of these cytokines in OVA-sensitised mice compared to healthy control mice. Further evidence that supports the indication of a favoured Th2 response can be observed upon examination of the levels of the key pro-inflammatory Th1 cytokine, IFN-γ. The ability of allergic mice to induce IFN-γ secretion, and thus a Th1 response, was hindered in splenocytes following OVA stimulation and in an unstimulated resting state when compared to healthy control mice. A similar pattern was observed for mRNA expression, as we observed a decrease in IFN-γ expression in the colons of OVA-sensitised mice. This again suggests that the immune response was skewed toward a Th2 response in OVA-sensitised mice. This result led us to question the source of the inflammatory state in the H&E stained colonic sections of allergic mice. As we are aware, food allergy plays an important role in the induction of intestinal inflammation. It was clear from our H&E results that inflammation was indeed present in the colons of OVA-sensitised mice following oral challenge with OVA. As the colonic mRNA and splenocyte cytokine results confirmed that Th1 cells were inhibited in allergic mice, this suggested that this T-cell subtype was not the cause of induced inflammation and pathology in the colon. Li et al (2016) claims that food allergy is a Th2 pattern inflammation in the intestine. They observed an increase in the levels of IL-4 and TNF-α, but not IFN-γ or IL-17, in the colons of OVA-sensitised mice, which correlates well with the results from our model. They demonstrated that the OVA-induced inflammation in the colon was Th2-induced inflammation which seems to be the case in our model. This feature made it an appropriate model for assessing the translation of the *in vitro* effects of UL-2-147 into *in vivo*. Reverting back to cytokine pattern analysis, it is of importance to discuss TGF-β levels, which were reduced in OVA-sensitised mice compared to control mice, following OVA and CD3/CD28 stimulation and in unstimulated splenocytes. Once again this implies a favoured Th2 phenotype as TGF-β is the key Treg cytokine. Collectively these results, observed both in the spleens and in the colons of OVA-sensitised mice, indicate a favoured Th2 pattern, resulting in reduced Th1 and Treg populations.

UL-2-147 demonstrated consistent allergy suppressing capabilities in dampening the excessive Th2 allergic cytokine profile associated with food allergy, in this context OVA allergy, which was evident in the OVA-sensitised mice in our study. We first

examined the effect of UL-2-147 treatment to modulate cytokine secretion patterns in the spleens of OVA-sensitised mice following the successive OVA challenges. Splenocytes were stimulated with OVA or CD3 plus CD28 or left unstimulated in culture. A significant reduction in the OVA-induced spike in the Th2 cytokines (IL-4, IL-6, IL-10 and IL-13) was consistently observed in the UL-2-147 treated OVA-sensitised mice following OVA and CD3/CD28 stimulation and in unstimulated splenocytes. This was not observed for WPC80 treated OVA-sensitised mice. While WPC80 slightly reduced some of the Th2 cytokines, this activity was not consistent in the cytokines it targeted over the various methods of stimulation and was, therefore, not regarded as Th2 suppressive. Furthermore, UL-2-147 treatment restored the spike in expression of allergic cytokines in the colon back to the levels of control mice. The same Th2 dampening effect was observed in WPC80 treated mice, which indicates a therapeutic role for whole whey in food allergy. Van Esch et al. (2011) observed similar results with regard to whole whey treatment. They examined the therapeutic effect of whole whey protein as well as partial and extensive whey hydrolysates in mice prior to oral whey sensitisation. Following oral challenge with whey, both whole whey and partially hydrolysed whey peptide treatments reduced the acute allergic skin response and the number of effector Th2-cells associated with whey-sensitised mice.

UL-2-147 treatment restored the drop in TGF-β levels associated with OVA-sensitisation back to those of healthy control mice in CD3/CD28 stimulated and unstimulated OVA-sensitised mice. Interestingly, TGF-β was even further enhanced in OVA stimulated allergic mice treated with UL-2-147, compared to control mice and was significantly increased compared to PBS treated allergic mice. UL-2-147 has a low degree of hydrolysis (DH) value, making it a partially hydrolysed peptide. A low DH value allows a protein to retain immunogenicity and thus, elicit immunomodulatory properties (Kiewiet et al. 2015). An increase in TGF-β was not observed in WPC80 treated mice in any of the stimulated groups. This result reflects that of the study by Van Esch et al. (2011), where, in addition to its mention above, an increase in Foxp3 expressing Treg cells in the MLN were observed in whey-sensitised mice treated with partially hydrolysed whey peptide, following a whey challenge. An increase in Treg cells was not observed in mice treated with the whole whey. This, coupled with the drop in Th2 associated cytokines, suggests that UL-2-147 treatment induces a Treg response to counteract and dampen Th2 activation that occurred in line

with OVA-sensitised mice when challenged with OVA, in an attempt to generate tolerance to the harmless protein and bring about immune balance. As the overall of aim this chapter was to identify hydrolysates that can bring about immune balance in infants at risk of developing food allergy, the ability of UL-2-147 to drive Treg cytokines in an *in vivo* allergic setting demonstrates its efficacy and confirms its ability to do so.

It is well known that effector $CD4^+$ T-cells produce IL-2 upon activation. IL-2 was significantly increased in WPC80 and PBS treated OVA-sensitised mice compared to healthy control mice in a resting unstimulated state and in OVA stimulated splenocytes. It is possible that IL-2 is high in allergic mice in order to activate and maintain effector T-cell function, in this context a Th2 response. Although IL-2 is required for the activation of Treg cells, Treg cells, themselves, do not secrete IL-2 (Malek and Castro, 2010). While IL-2 is significantly reduced in OVA-sensitised mice treated with UL-2-147 compared to PBS treated allergic mice in unstimulated splenocytes, it is still significantly higher than healthy control mice. It is possible that UL-2-147 treatment reduced IL-2 secretion in allergic mice in an attempt to switch off the excessive Th2 response but retained a raised level of IL-2 in order to activate Tregs to subsequently suppress Th2 response and bring about immune balance. This result may provide further evidence to the possibility that UL-2-147 activates Tregs to bring about immune balance. IL-2 has not been studied as a biomarker in the treatment of allergy and is, therefore, an interesting addition to the effects of UL-2-147. Further in depth studies would be required to understand the exact mechanism by which UL-2-147 targets IL-2 and the knock on effects this causes in Treg cells, if any at all.

It is interesting to note the Th1 pattern, through IFN-γ measurement, in OVA-sensitised groups. IL-6 directly inhibits differentiation of the Th1 subset. We hypothesised that the suppression of IL-6 as a result of UL-2-147 treatment *in vitro*, may promote the polarisation of Th1 cells in an *in vivo* setting, however, this was not the case. IFN-γ was reduced in splenocytes of all OVA-sensitised groups that were stimulated with OVA and those left unstimulated. Splenocytes from all OVA-sensitised mice that were stimulated with CD3 plus CD28 antibodies, however, were able to produce IFN-γ, similar to levels of the healthy control mice. *In vitro* stimulation of T-cells using plate bound anti-CD3 plus anti-CD28 mimics the presentation of pathogenic antigen to the TCR on T-cells and the co-stimulatory signals necessary for

activation, respectively. As Th1 responses are important for protection against viruses and intracellular bacteria, this result suggests that UL-2-147 treatment does not interfere with the host's ability to detect and respond to potential pathogenic microorganisms, which is a desirable feature of this hydrolysate. As mentioned, UL-2-147 treatment did not restore the drop in IFN-γ associated with OVA-sensitisation in OVA stimulated and unstimulated splenocytes. This result implies that UL-2-147 does not target Th1 cell function, suggesting it specifically acts on Treg cells in order to suppress the Th2 response.

As limited research exists on the use of IL-17 as a biomarker for the treatment of atopic disease, we were interested in examining the effect of UL-2-147 on this cytokine in allergic mice. While recent studies suggest that targeting IL-17 could potentially play a role in the induction of tolerance to food antigens, because lower IL-17 secretion from $CD4^+$ undifferentiated T-cells was observed in children with food allergies, it is likely that IL-17 may only play a therapeutic role as a biomarker in the prevention of allergy in infants. IL-17 has been linked to various allergic diseases that can be developed in later life, such as asthma. While our *in vitro* results suggested that increasing IL-17 levels could be another possible therapeutic mechanism of UL-2-147 in suppressing an allergic state, this did not translate into our *in vivo* study. We observed increased IL-17 levels in OVA stimulated OVA-sensitised splenocytes. As we used adult and not newborn mice, IL-17 may not be a suitable biomarker for this particular study.

Finally, IgG1 is a key Th2 cytokine-stimulated antibody which is often interpreted in line with IgE as an "allergic" antibody in murine allergy models (Van Halteren et al. 1997; and Meulenbroek et al. 2013). Although IgG1 serum levels remained higher than those of healthy control mice, UL-2-147 treatment significantly reduced the increased levels that were associated with allergic mice (PBS treated OVA-sensitised mice). A study by Holt et al. (1981) demonstrated that suppression of the allergic IgE and IgG1 responses is associated with the generation of tolerance, as their levels were reduced following intraperitoneal OVA challenges in mice who had achieved airway tolerance to OVA. This suggests an additional therapeutic effect and target of UL-2-147. While Van Esch et al. (2011) reported similar results regarding the activity of a partially hydrolysed whey peptide, it did not prevent the generation of serum-specific IgG1.

In summary, the collective results from our *in vivo* OVA model study suggest that oral administration of UL-2-147 is capable of redirecting an exaggerated Th2 response, associated with allergic mice, towards a beneficial Treg phenotype, and in doing so reduced the clinical symptoms of allergy, including restoring pathology in the gut, and did not inhibit the host's ability to mount a Th1 response to an external harmful stimulus. While WPC80 also elicited some beneficial Th2 suppressing effects, clinical symptoms were still evident in these mice following oral challenges and it had no effect on increasing Treg cell populations. Collectively, the splenocyte cytokine and PCR results confirmed that induction of Th1/Th2 balance is not the mechanism of action through which UL-2-147 elicits its therapeutic activity. These results do, however, suggest that promotion of Treg/Th2 balance could be the possible key modulatory mechanism. We propose that UL-2-147 has the ability to bring about immune balance by targeting Th2 cells (reducing splenic and colonic Th2 cytokine levels and IgG1 serum levels) and Treg cells (inducing TGF-β and modulating IL-2 in spleens), whilst not curtailing the host's ability to mount a Th1 response when required.

On a side note, two hydrolysates, UL-2-20 and UL-2-23, were identified, much later in the screening process, which demonstrated potential ability to drive a Treg response, as they were the only hydrolysates examined in this project that significantly increased IL-10 secretion from undifferentiated T-cells *in vitro.* While high IL-10 levels can lead to the development of Treg cells, as we understand IL-10 secretion can also favour Th2 polarisation depending on the cytokine environment (Kaiko et al. 2008). UL-2-20, unlike UL-2-23, did not enhance IL-4 and IL-6 secretion which are also essential for Th2 differentiation. These hydrolysates were also examined in Th2 and UL-2-20 was confirmed to suppress Th2 cells, however, UL-2-23 drove IL-4 secretion from Th2 cells. This suggests that UL-2-20 may have potential for driving a Treg subset. As mentioned, Treg cells regulate the immune system and inhibit the effector T-cells that drive inflammation and allergy. They have potential to induce tolerance to harmless antigens and drive immune homeostasis. Unlike UL-2-147, UL-2-20 possessed the ability to drive TGF-β secretion from Treg cells *in vitro*. UL-2-20 did not drive or inhibit a Th1 subset, suggesting that its bioactivity is specific to Treg cells. Regenerated and optimised hydrolysates for UL-2-20 were produced and tested. UL-2-195, an optimised hydrolysate, was identified which displayed an even more

enhanced ability to drive TGF-β secretion from Treg cells, whilst also significantly reducing Th2 polarisation. Unfortunately, this hydrolysate was identified too late in the FHI program to allow time for assessment in *in vivo* trials, but it has demonstrated huge potential in its ability to drive Treg cells and inhibit the allergic Th2 response, whilst not effecting Th1 polarisation. It would be interesting to examine the bioactivity of UL-2-195 in combination with UL-2-147 or with the candidate hydrolysate studied in Chapter 5. This possibility is discussed in further detail in Chapter 6, General Discussion.

In conclusion, throughout this chapter we explored the ability of CM hydrolysates to change the function of the key allergy cells, Th2 cells, in *in vitro* and *in vivo* settings. We demonstrated the ability of UL-2-147 to decrease the production of the Th2 priming cytokines, IL-4, IL-6 and IL-10, in undifferentiated T-cells as well as inhibit secretion of the characteristic Th2 cytokines, IL-4 and IL-13, from Th2 cells in a dose dependent manner *in vitro*. We then validated further ability of UL-2-147 to "retrain" the immune system of OVA-sensitised mice *in vivo*, which was characterised by the prevention of the clinical symptoms of allergy and modulation of the immune response away from the favoured Th2 phenotype, associated with allergy, towards a Treg response, following OVA challenge. As mentioned, UL-2-147 is a partially hydrolysed peptide. While this is beneficial, in that it retains immunogenicity to allow activation of Treg cells, the retained immunogenicity can also still potentially induce allergic symptoms. For this reason partially hydrolysed formulas are not advised for feeding infants who already have been diagnosed with food allergy or CMPA. We have, therefore, successfully identified and demonstrated the efficacy of a whey hydrolysate, which would be of benefit incorporated into IMF for the primary prevention of atopic disease in healthy infants who do not obtain all the benefits of exclusive breastfeeding and are, therefore, at risk of atopy. The ability to prime the immune response and bring about immune balance from birth confers many advantages; lowering the incidence of atopy and thus, the problems associated with allergy in later life being the prime advantage in this chapter's objective.

CHAPTER 5

IMMUNOMODULATORY PROPERTIES OF PROTEIN HYDROLYSATES FOR APPLICATION IN INFANT NECROTISING ENTEROCOLITIS

5.1 INTRODUCTION

Newborns are highly susceptible to gut inflammatory disorders. Premature low birth weight infants are, particularly, at risk of developing a severe form of intestinal inflammation, known as necrotizing enterocolitis (NEC). NEC is a leading cause of morbidity and mortality in preterm infants (Chatterton et al. 2013). Immaturity, enteral feeding and formula-feeding are amongst some of the predisposing factors of the disease. Almost 12% of premature infants who weigh less than 1,500 g at birth develop NEC. Onset of the disease typically occurs in the second to third week of life. The overall economic cost of NEC is high, with hospitalisation costs estimating at $0.5–1 billion in the United States per year alone. Mortality rates in newborns with NEC are high and can reach up to a staggering 50%, depending on severity. Death typically results from subsequent sepsis and multi-organ failure (Gephart et al. 2012). It is also common amongst recovered infants for subsequent short and long-term complications to develop, such as malabsorption, poor growth, short gut syndrome and neurodevelopmental impairment (Pacheco, Underwood and Mills, 2015).

A specific aim of this PhD project is to identify novel bovine milk-derived protein hydrolysates which can be incorporated into infant formula for use as a novel and natural refeeding strategy for infants following NEC recovery. In doing so it may help prevent relapse and further problems associated with inflammatory disease. This chapter aims to identify candidate hydrolysates which can modulate the immune response away from the inflammatory type 1 and/or type 17 helper T-cells (Th1 and Th17) dominant phenotypes *in vitro*. This will be carried out using the clearly defined T-helper cell subsets, Th1, Th2, Th17 and Treg, optimised in chapter three, for the screening of cow's milk (CM). The selected candidates will then be brought forward for *in vivo* testing and proof of concept in an inflammatory murine model in order to determine their effectiveness to alleviate inflammatory symptoms and potential as a treatment for NEC.

As mentioned, preterm infants have a higher risk of developing NEC than infants who are born at term. The more premature an infant is at birth, the higher the risk of NEC. Many factors affect this increased threat. Differences exist in the breastmilk of mothers between the two groups. Preterm milk has an immature production of human milk oligosaccharides (HMOs) and contains higher levels of protein when compared to

milk from mothers of at term infants (De Leoz et al. 2012). The intestinal microbiota of preterm infants may therefore, be underdeveloped as a result of the lack of HMOs they receive from their mother's milk. Much lower levels of the healthy *Bifidobacterium* and *Bacteroides* have been observed in premature infants when compared to levels in infants delivered at term. A rise in Proteobacteria levels, such as Enterobacteriaceae, has also been identified in preterm infants (Butel et al. 2007; Arboleya et al. 2012; and Hill and Newburg, 2015). The intestinal mucosa of preterm infants is also both functionally and immunologically immature, leaving it prone to damaging inflammation (Neu and Walker, 2011). This is characterised by poor digestion and absorption, damaged barrier function, a lack of antimicrobial peptides and excessive inflammatory response to gut luminal microbes.

While enterally fed infants are most susceptible to NEC, formula-fed newborns also have an increased risk of developing the disease when compared to breast-fed infants. As mentioned previously, breast milk plays a key role in inducing the maturation of immune function of the gastrointestinal (GI) tract. Differences have been observed in the stool flora between both groups, which once again suggests alterations in the development of their intestinal microbiota, potentially being the trigger for intestinal inflammatory disorders (Chuang et al. 2009; and Yoshioka, Iseki and Fujita, 1983).

Many cytokines and chemokines have been linked to an involvement in NEC progression, triggering a state of necrosis. Imbalance of inflammatory cytokines plays a key role in T-cell dysregulation, resulting in the activation and proliferation of uncontrolled hyperactive and excessive T cell responses. This promotes an imbalance of Treg/Th1, Th2, and Th17 subsets, which eventually contributes to excessive inflammation and the pathogenesis of inflammatory disorders. As the excess or lack of these cytokines has been proven to disrupt the epithelial barrier they are, therefore, desirable therapeutic targets in NEC. Monoclonal antibodies and small molecule inhibitors have been developed that can inhibit the pro-inflammatory effects of these cytokines. Research has focussed on compounds that could target NF-κB activation. Pentoxyphylline has been shown to lower the prevalence of NEC by inhibiting TNF-α and subsequently NF-κB and NEC incidence (Travadi et al. 2006). It is still unclear whether anti-cytokine therapy may inhibit pathways essential in many tissues besides the intestine, potentially promoting harmful side effects or collateral damage in the developing gut. Many of these cytokines also play important roles in developing

infants' immune systems and maturing their gut (Hsueh et al. 1998; and Maheshwari, 2004). Therefore identification of novel natural therapies that do not affect the host's ability to mount an immune response to a potential pathogen is of utmost importance and would be of particular benefit. Another area that NEC research focusses on is strengthening the intestinal barrier in newborn infants. Breast milk and probiotics have a plethora of health benefits for newborns and have been strongly proven to improve barrier function (Lucas and Cole 1990; and Lawrence et al. 1982).

To date, most research has focussed on the nutritional prevention of NEC. Many practices exist nowadays to reduce NEC risk and prevent onset of the disease, breastfeeding and feeding of probiotics have been proven to be effective in this area and reduce the incidence of NEC (Lapillonne et al. 2016). While the prevention of NEC is crucial, little research has been carried out to identify potential novel and improved treatments of the disease. There is also a lack of clarity surrounding the recovery process following NEC. It is recommended to suspend feeding for a period of time depending on the disease severity, however, confusion exists around when to restart feeding and over the type of milk to be used for this process (Gephart et al. 2012; and Parks et al. 2008). Many factors determine the type of milk formula suitable for refeeding: availability of human breast milk/ability to breastfeed, whether the infant was premature, malabsorption and risk of cow's milk protein allergy (CMPA).

Many studies have shown that a link between CMPA and NEC exists, relating the two conditions (Faber et al. 2005). Infants who suffer with NEC, particularly preterm infants, also have an increased risk of sensitisation to intact proteins and developing CMPA. Abdelhamid et al. (2011 and 2013) demonstrated that peripheral blood mononuclear cells (PBMCs) of preterm infants with NEC become sensitised to CM protein *in vitro*. NEC may, therefore, be an indicator of potential sensitisation to CM. Signs and symptoms of CMPA should be closely monitored in infants post NEC. Furthermore, studies have demonstrated that newborns, who underwent GI surgery, have a higher susceptibility to developing CMPA compared to infants with no family history of allergy (El Hassani et al. 2005). Hydrolysed formulas have, therefore, strong potential for lowering this risk of CM sensitisation. As mentioned in the previous chapter, extensive hydrolysis of a protein results in complete destruction of the allergic IgE epitopes, which greatly inhibits the likelihood of sensitisation and onset of clinical symptoms. For this reason, this chapter focuses on the development of extensively

hydrolysed peptides that can treat inflammatory disorders whilst simultaneously protecting the vulnerable infant against sensitisation to CM proteins during recovery.

The overall objective of this chapter was to identify novel natural CM protein hydrolysates that can modulate cytokine secretion from $CD4^+$ T-cells both *in vitro* and *in vivo*. Modulation of the immune response can result in a shift away from an excessive inflammatory response, bringing about immune balance and preventing recurring inflammatory disorders. Hence, the first objective of this chapter was to characterise the anti-inflammatory effects of bovine hydrolysates *in vitro*. The second objective was to confirm these characterised anti-inflammatory effects of the chosen candidates in an *in vivo* DSS murine model of colitis. $CD4^+$ T-cells were chosen for this study because they are the precursors to Th1, Th2, Th17 and Treg cells, the key players in the allergy and inflammatory processes when immune balance is not achieved and dysregulation prevails. They may, therefore, be logical therapeutic targets of the CM hydrolysates. A number of whey and casein bovine milk hydrolysates were prepared by our collaborators, Professor Fitzgerald's research group in the Department of Biological Sciences, University of Limerick (UL) for examination.

The identification of novel hydrolysates with selective anti-inflammatory bioactivity could be useful for incorporation into formula for refeeding NEC infants post recovery who are unable to breastfeed. This may eliminate the potential of relapse and recurring inflammatory disorders and also lower the risk of sensitisation to CM proteins. The study of these hydrolysates and their anti-inflammatory effects may also have potential benefits for the development of alternative natural therapies for other forms of diseases of intestinal inflammation in adults as well as infants.

5.2 RESULTS

5.2.1 ASSESSMENT OF ANTI-INFLAMMATORY ACTIVITY OF FIRST-ROUND HYDROLYSATES *IN VITRO*.

This chapter aims to identify hydrolysates that can modulate the key inflammatory $CD4^+$ T-cell subtypes, Th1 and/or Th17, which could have implications in a murine model of intestinal inflammation and thus in the treatment for infants recovering from NEC. We looked at the ability of a range of CM whey and casein hydrolysates to suppress a Th1 and Th17 subset *in vitro*. A list of these hydrolysates and details about source, enzyme, etc can be found in Section 1 **[Table 1.2].**

5.2.1.1 THE DOSES OF FIRST-ROUND WHEY AND CASEIN PROTEIN HYDROLYSATES USED HAVE NO SIGNIFICANT AFFECT ON CELL VIABILITY.

It was necessary to first determine whether the hydrolysates were toxic to T-cells prior to assessing their bioactivity. Cells were isolated from the spleens of BALB/c mice using the EasySep Mouse $CD4^+$ T Cell Isolation Kit (Stemcell Technologies #19852). $CD4^+$ 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) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml) or 10% DMSO (vehicle control). Cells were incubated for 72 hrs. Viability was assessed using an MTS assay (Cell Titer 96 Aqueous One Solution – Promega, WI, USA) according to the manufacturer's instructions. At 1 mg/ml, hydrolysates did not have any significant cytotoxic effect on undifferentiated T-cells when compared to untreated cells *in vitro* **[Figure 5.1].** It was observed that the viability of cells treated with hydrolysates was often greater than 100%. This concept was explained in **Section 4.3** and was not a concern for this project.

5.2.1.2 FIRST-ROUND HYDROLYSATES SUPPRESS THE CYTOKINES REQUIRED TO DRIVE AN INFLAMMATORY TH1 RESPONSE FROM UNDIFFERENTIATED T-CELLS IN VITRO.

In order to identify whether the hydrolysates possessed anti-inflammatory properties, their ability to modulate the key cytokines required to drive an inflammatory Th1and/or Th17 cell type was examined in undifferentiated T-cells. Cells were isolated from the spleens of BALB/c mice using the EasySep Mouse $CD4^+$ T Cell Isolation Kit (Stemcell Technologies #19852). $CD4^+$ cells were plated in triplicate at a concentration of $1x10^6$ cells/ml on a 96-well plate, 250 µl/well. The cells were then stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml). Cells were incubated for 72 hrs. Supernatants were collected and measured for IFN-γ, IL-6 and IL-17 secretion using ELISA according to manufacturer's instructions (R&D duoset).

Figure 5.2 shows a significant reduction in IFN-γ secretion when treated with UL-2-33/34/35/36/37/38 (UL-2-33/34/35/37/38: $p<0.001$; and UL-2-36: $p<0.01$) from undifferentiated T-cells. This indicates that these hydrolysates may potentially prevent differentiation of a Th1 subset and are, therefore, of interest to examine in Th1 cells *in vitro*.

Additionally, IL-6 and IL-17 secretion was also significantly inhibited when treated with UL-2-43 ($p<0.001$ and $p<0.05$, respectively) and UL-2-47 ($p<0.01$ and $p<0.01$, respectively). UL-2-41 and UL-2-42 also significantly inhibited IL-6 secretion ($p<0.01$) and reduced IL-17 levels, although this reduction was not significant. UL-2-40 resulted in a slight reduction in IL-6 and IL-17 secretion **[Figure 5.3]**. This indicates that these hydrolysates may potentially prevent differentiation of a Th17 subset.

5.2.1.3 FIRST-ROUND HYDROLYSATES CAN PREVENT DIFFERENTIATION OF T-HELPER 1 CELL SUBSET BUT DO NOT SUPPRESS DIFFERENTIATION OF A T-HELPER 17 SUBTYPE IN VITRO.

After examining the cytokine profile secreted from undifferentiated T-cells and establishing which hydrolysates could potentially inhibit a Th1 or Th17 phenotype, it

was necessary to confirm this activity in the Th1 and Th17 subsets *in vitro*. Cells were isolated from the spleens of BALB/c mice using the EasySep Mouse $CD4^+$ T Cell Isolation Kit (Stemcell Technologies #19852). $CD4^+$ cells were plated in triplicate at a concentration of $1x10^6$ cells/ml on a 96-well plate, 250 µl/well. The cells were then stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and 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 hrs and restimulated in fresh media for 24 hrs. Supernatants were collected and measured for IFN-γ and IL-2 or IL-17 secretion using ELISA according to manufacturer's instructions (R&D duoset).

Figure 5.4 shows a significant reduction in IFN-γ secretion from Th1 cells when treated with UL-2-33/34/35/38. These hydrolysates also reduced IL-2 secretion in Th1 cells, although UL-2-35 was the only hydrolysate to show a significant reduction. This indicates that these hydrolysates may have potential in an *in vivo* inflammatory mouse model. UL-2-37 also slightly reduced IFN-γ but also slightly increased IL-2 secretion and was therefore of no further interest. UL-2-40/41/42/43/47 enhanced IL-17 secretion from Th17 cells **[Figure 5.5]**. This indicates that these hydrolysates may not suppress differentiation of a Th17 subset and does not confirm any beneficial activity, therefore, the ability of hydrolysates to modulate this subset was not examined further.

5.2.2 ASSESSMENT OF ANTI-INFLAMMATORY ABILITY OF SECOND-ROUND REGENERATED HYDROLYSATES IN VITRO.

In order to determine whether the bioactivity of the hydrolysates of interest (now referred to parent hydrolysates) is reproducible, UL-2-33/34/35 hydrolysates were regenerated, using identical hydrolysis conditions as parent compounds, and named UL-2-83/84/85 respectively, as seen in **Table 5.1**.

Parent Hydrolysate	*Regenerate Hydrolysate*	*Starting Substrate*	*Activity/Reason brought forward*
UL-2-33	**UL-2-83**	**NaCN**	**Anti-inflammatory ↓ Th1**
UL-2-34	**UL-2-84**	**NaCN**	**Anti-inflammatory ↓ Th1**
UL-2-35	**UL-2-85**	**NaCN**	**Anti-inflammatory ↓ Th1**

Table 5.1: Overview of Parent and Regenerated Hydrolysates in Round 2 Screening.

5.2.2.1 THE DOSES OF SECOND-ROUND WHEY AND CASEIN PROTEIN REGENERATED HYDROLYSATES USED HAVE NO SIGNIFICANT AFFECT ON CELL VIABILITY.

In order to determine whether the regenerated hydrolysates were toxic to T-cells, cells were isolated from the spleens of BALB/c mice using the EasySep Mouse $CD4^+$ T Cell Isolation Kit (Stemcell Technologies #19852). $CD4^+$ 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) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml) or 10% DMSO (vehicle control). Cells were incubated for 72 hrs. Viability was assessed using an MTS assay (Cell Titer 96 Aqueous One Solution – Promega, WI, USA) according to the manufacturer's instructions. At 1 mg/ml, regenerated hydrolysates did not have any significant cytotoxic effect on undifferentiated T-cells when compared to untreated cells *in vitro* **[Figure 5.6].**

5.2.2.2 SECOND-ROUND REGENERATED HYDROLYSATES DISPLAY SIMILAR ACTIVITY AS PARENT SAMPLES IN UNDIFFERENTIATED $CD4^+$ T-CELLS AND TH1 CELLS IN VITRO.

In order to confirm the bioactivity of regenerated hydrolysates UL-2-83/84/85, parent and regenerated hydrolysates were screened on undifferentiated T-cells and cytokine secretion was assessed. Cells were isolated from the spleens of BALB/c mice using the EasySep Mouse $CD4^+$ T Cell Isolation Kit (Stemcell Technologies #19852). $CD4^+$ cells were plated in triplicate at a concentration of $1x10^6$ cells/ml on a 96-well plate, 250 µl/well. The cells were then stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml). The media of Th1 cells

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 hrs. Th1 cells were restimulated in fresh media for a further 24 hrs. Supernatants were collected and measured for IFN-γ and IL-2 secretion using ELISA according to manufacturer's instructions (R&D duoset).

Figure 5.7 (A) shows a slight reduction in IFN-γ secretion from undifferentiated T-cells when treated with UL-2-33/34/35 as well as regenerated hydrolysates UL-2-84/85. UL-2-83 had no effect on IFN-γ secretion from undifferentiated T-cells. These hydrolysates also reduced IFN-γ secretion from Th1 cells **[Figure 5.7 (B)]**, although UL-2-33 was the only hydrolysate to show a significant reduction ($p<0.01$). A slight decrease in IL-2 from Th1 cells was also observed from all hydrolysates. These results confirm that bioactivity of these hydrolysates is reproducible.

5.2.3 ASSESSMENT OF ANTI-INFLAMMATORY ACTIVITY OF THIRD-ROUND OPTIMISED HYDROLYSATES *IN VITRO*.

As mentioned in Chapter 4, it was necessary to enhance the hydrolysis conditions of the proteins. In doing so it was essential to assess if we had retained or enhanced the bioactivity of the candidate hydrolysates. Optimised hydrolysates, for both UL-2-34 and UL-2-35, were produced for further testing **[Table 5.2].** UL-2-33 was also of interest but the enzyme preparation for this hydrolysate was no longer available.

Parent Hydrolysate	*Regenerate Hydrolysate*	*Optimised Hydrolysates*	*Substrate*	*Reason Brought Forward*
UL-2-34	**UL-2-84**	**UL-2-132 UL-2-133 UL-2-134**	**NaCN**	**Anti-Inflammatory ↓ Th1**
UL-2-35	**UL-2-85**	**UL-2-136 UL-2-137 UL-2-138**	**NaCN**	**Anti-Inflammatory ↓ Th1**

Table 5.2: Overview of Parent, Regenerated and Optimised Hydrolysates in Round 3 Screening.

5.2.3.1 THE DOSES OF THIRD-ROUND OPTIMISED UL-2-34 AND UL-2-35 HYDROLYSATES USED HAVE NO SIGNIFICANT AFFECT ON CELL VIABILITY.

In order to determine whether the optimised hydrolysates were toxic to T-cells, cells were isolated from the spleens of BALB/c mice using the EasySep Mouse $CD4^+$ T Cell Isolation Kit (Stemcell Technologies #19852). $CD4^+$ 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) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml) or 10% DMSO (vehicle control). Cells were incubated for 72 hrs. Viability was assessed using an MTS assay (Cell Titer 96 Aqueous One Solution – Promega, WI, USA) according to the manufacturer's instructions. At 1 mg/ml, optimised hydrolysates did not have any significant cytotoxic effect on undifferentiated T-cells when compared to untreated cells *in vitro* **[Figure 5.8].**

5.2.3.2 THIRD-ROUND UL-2-34 & UL-2-35, REGENERATED AND OPTIMISED HYDROLYSATES DISPLAY CONSISTENT ANTI-INFLAMMATORY PROPERTIES IN UNDIFFERENTIATED T CELLS IN VITRO.

In order to determine whether the hydrolysis optimisation of UL-2-34 and UL-2-35 could enhance the function of these hydrolysates, they were first tested in undifferentiated T-cells. Cells were isolated from the spleens of BALB/c mice using the EasySep Mouse $CD4^+$ T Cell Isolation Kit (Stemcell Technologies #19852). $CD4^+$ cells were plated in triplicate at a concentration of $1x10^6$ cells/ml on a 96-well plate, 250 µl/well. The cells were then stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml). Cells were incubated for 72 hrs. Supernatants were collected and measured for IFN-γ secretion using ELISA according to manufacturer's instructions (R&D duoset).

Figure 5.9 (A) shows a significant reduction in IFN-γ secretion from Th1 cells when treated with UL-2-34 ($p<0.05$) and regenerate, UL-2-84, ($p<0.01$). Optimised hydrolysates, UL-2-132/133/134 also reduce IFN-γ secretion, although this result is not significant. UL-2-35, regenerate, UL-2-85, and optimised hydrolysates, Ul-2-136/137/138, all significantly inhibit IFN-γ secretion ($p<0.05$, $p<0.001$, $p<0.01$, $p<0.01$ and $p<0.05$, respectively) **[Figure 5.9 (B)]**.

5.2.3.3 THIRD-ROUND UL-2-34 & UL-2-35, REGENERATED AND OPTIMISED HYDROLYSATES DISPLAY CONSISTENT ANTI-INFLAMMATORY PROPERTIES IN TH1 CELLS IN VITRO.

Optimised hydrolysates of UL-2-34 and UL-2-35 were then examined for bioactivity in a Th1 subset to determine if its activity could be enhanced or remain unchanged during the optimisation process. Cells were isolated from the spleens of BALB/c mice using the EasySep Mouse $CD4^+$ T Cell Isolation Kit (Stemcell Technologies #19852). $CD4^+$ cells were plated in triplicate at a concentration of $1x10^6$ cells/ml on a 96-well plate, 250 µl/well. The cells were then stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and 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 hrs and restimulated in fresh media for 24 hrs. Supernatants were collected and measured for IFN-γ and IL-2 secretion using ELISA according to manufacturer's instructions (R&D duoset).

Figure 5.10 (A) shows a significant reduction ($p<0.001$) in IFN-γ and IL-2 secretion from Th1 cells when treated with UL-2-34, regenerate, UL-2-84, and all optimised hydrolysates. UL-2-35, regenerate, UL-2-85, and all optimised hydrolysates also significantly inhibited production of IFN-γ ($p<0.05$, $p<0.01$, $p<0.05$, $p<0.05$ and $p<0.01$, respectively) [**Figure 5.10 (B)]**. All of these samples also reduced IL-2 secretion, UL-2-85 and UL-2-138 being significant ($p<0.05$).

As the hydrolysis of UL-2-34 and UL-2-132 was carried out using uncontrolled (free-fall) pH conditions, these hydrolysates were chosen for further testing in an *in vivo* model. A summary of results to date for UL-2-34 and UL-2-132 can be seen in **Table 5.3**.

Sample	*Conditions*	*Comments*	**Activity/Reason brought forward**
UL-2-34	Starting pH of 7.0, **pH freefall**,		Anti-Inflammatory
NaCN	2% E:S, 3 hrs & 6 hrs	Original Sample	↓ DC ↓ Th1 ↓ Th17
			No pH Stat
UL-2-132	Starting pH of 7.0, **pH freefall**,		Anti-Inflammatory
NaCN	1% E:S, 3 hrs & 6 hrs	Optimised Sample	↓ DC ↓ Th1↓ Th17
			No pH Stat

Table 5.3: Overview and summary of hydrolysates being brought forward for upscaling into animal trials.

5.2.3.4 UL-2-34 AND UL-2-132 DOSE DEPENDENTLY REDUCE IFN-γ SECRETION FROM TH1 CELLS.

In order to determine whether the hydrolysates activity is dose dependent, a dose response was carried out on Th1 cells and IFN-γ secretion was measured. Cells were isolated from the spleens of BALB/c mice using the EasySep Mouse $CD4^+$ T Cell Isolation Kit (Stemcell Technologies #19852). $CD4^+$ cells were plated in triplicate at a concentration of $1x10^6$ cells/ml on a 96-well plate, 250 µl/well. The cells were then stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and treated with UL-2-34 and UL-2-132 at various doses (10 µg/ml / 100 µg/ml / 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 hrs and restimulated in fresh media for a further 24 hrs. Supernatants were collected and measured for IFN-γ secretion.

IFN-γ was reduced significantly, in a dose dependent manner from Th1 cells treated with increasing doses of protein hydrolysates, UL-2-34 **[Figure 5.11 (A)]** and UL-2-132 **[Figure 5.11 (B)]**, compared to untreated cells (100 µg/ml UL-2-34: $p<0.05$; 1 mg/ml UL-2-34: $p<0.001$ **[Figure 5.11 (A)]**; 10 µg/ml UL-2-132: $p<0.001$; 100 µg/ml UL-2-132: $p<0.01$; and 1 mg/ml UL-2-132: $p<0.001$ **[Figure 5.11 (B)]**).

4.2.3.5 UPSCALED UL-2-34 & UL-2-132 SUPPRESS THE CHARACTERISTIC TH1 CYTOKINE IN VITRO, CONFIRMING BIOACTIVITY PRIOR TO IN VIVO DSS MURINE MODEL.

The final experiment, before examining the activity of UL-2-34/132 *in vivo*, was to confirm that upscaling of the hydrolysates at large scale did not affect its bioactivity. Scale up of UL-2-34 and UL-2-132 and associated protocol transfer took place at the bio-functional food engineering (BFE) (industry-large scale) plant in Teagasc, Moorepark. To date all hydrolysates had only been produced in small quantities in the lab in UCC. Scale- up is carried out firstly to produce larger quantities of hydrolysates for animal and human trials taking place within FHI, and secondly to ensure that the hydrolysis protocol could be transferred successfully and yield hydrolysates with the same bioactivity. UL-2-34 and UL-2-132 were produced in large scale at 3hr (original protocol) and 6 hr. The 6 hr duration was been requested by industry to ensure that they could transfer the protocol in their plant without losing the activity. The scaled up hydrolysates were tested in Th1 cells alongside the original samples. Cells were isolated from the spleens of BALB/c mice using the EasySep Mouse $CD4^+$ T Cell Isolation Kit (Stemcell Technologies #19852). $CD4^+$ cells were plated in triplicate at a concentration of $1x10^6$ cells/ml on a 96-well plate, 250 µl/well. The cells were then stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and treated with UL-2-34 and UL-2-132 (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 hrs and restimulated in fresh media for a further 24 hrs. Supernatants were collected and measured for IFN-γ secretion.

All hydrolysates inhibited IFN-γ secretion. The original UL-2-34/132 (produced at 3 hr) and upscaled UL-2-34/132 (produced at 3 hr) continue to significantly inhibit IFN-γ secretion ($p<0.05$). The 6 hr hydrolysis resulted in the most potent effect, however, as UL-2-34 and UL-2-132 produced at 6 hr both significantly reduced IFN-γ secretion ($p<0.01$ and $p<0.001$, respectively) **[Figure 5.12]**. The next step in the study was to determine the ability of UL-2-34/132 (6 hr) to alleviate symptoms in an inflammatory mouse model of colitis for proof of principle. A summary of *in vitro* results collected from all of research groups within the Allergy and Immunity stream of the Infant Nutrition work package on UL-2-34 and UL-2-132 can be seen in **Table 5.4.**

	Humanised	*Murine*
DCs/Macrophage	**↓IL-12** **↓CD86**	**↑IL-10** **↓IL-12** **↓IL-6** **↓TNF-α**
Undifferentiated $CD4^+$ T-Cells	**↓IFN-γ**	**↓IFN-γ**
Th1 Cells	**↓T-bet**	**↓IFN-γ** **↓IL-2**
Th17 Cells	**↓RORγt**	**No effect**

Table 5.4: Summary of *in vitro* results from Allergy and Immunity Infant Nutrition FHI Workpackage. Humanised and murine immune cells treated with UL-2-34/132 hydrolysates being brought forward for assessment in animal trials**.**

5.2.4 DSS-INDUCED MODEL OF COLITIS

In order to determine the ability of UL-2-34 and UL-2-132 to modulate the immune system and alleviate an excessive inflammatory state *in vivo*, a mouse model of DSS-induced colitis was carried out. A pilot study was first performed to ensure disease progression. DSS is a well studied mouse model and a number of publications have identified that Th1 cells have a role to play in the progression of the disease. The DSS colitis model is, thus, a valuable model to investigate the increase of T-cell associated responses during intestinal inflammation mimicking early IBD. Altogether, T-cells are associated with the onset of acute colitis.

5.2.4.1 CLINICAL ASSESSMENT OF DSS-INDUCED COLITIS

The DSS model was carried out in the Biological Resource Unit (BRU), at Dublin City University, as described in **Section 2.9.1**. To assess the development of the disease, mice were weighed and scored (every day) for daily disease activity index (DDAI) based on body weight loss, stool consistency presence of occult blood, stool composition, fur texture and posture. **Table 5.5** details the scoring system used for the comparative analysis of intestinal bleeding.

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

Table 5.5: Scoring system used to determine intestinal bleeding in the DSS model.

There is a drop in body weight in DSS-treated mice from Day 3 onwards **[Figure 5.13 (A)]**, which reached its maximum, with a significant weight loss ($p<0.001$) in DSS mice, on Day 6 **[Figure 5.13 (B)]**. A steady weight was retained in control mice. The DDAI also showed disease progression. No disease activity was observed in the control mice, while there was an increase in the disease activity scores in DSS treated mice as soon as day 2 **[Figure 5.14 (A)],** which peaked, with a significant increase ($p<0.001$), on day 7 **[Figure 5.14 (B)]**. On the final day (Day 7), colons were removed, measured and weighed. The weight and length of the colon are useful indicators of disease progression as the cell infiltration and inflammation increase the weight of the colon and also shrink the colon length (Okayasu et al. 1990). As we can see in **Figure 5.15 (A)**, colons from the DSS treated mice were shorter than control mice. There was also an increase in colon weight in DSS treated mice **[Figure 5.15 (B)].** However, neither of these results achieved significance. Sections of the distal colon (0.5cm) were removed and rolled for histological examination and stained with haematoxylin and eosin (H&E), as described in **Section 2.9.4**. DSS exposure in mice led to destruction of crypt structure together with visible reduction in goblet cells, a disturbed epithelial layer and massive infiltration of inflammatory cells in the mucosa when compared to the normal morphology of colon tissue **[Figure 5.15 (C)]**. The H&E staining of control colonic tissue shows a healthy colon with crypts and goblet cells present **[Figure 5.15 (C)].**

5.2.4.2 CYTOKINE EXPRESSION IN COLON IN DSS-INDUCED COLITIS

RNA was isolated from colonic tissue from mice, as described in **Section 2.8.1**. Complementary DNA (cDNA) was then generated from RNA using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, USA), as described in materials and methods section 2.9.2. The levels of IL-1β, IL-17, IL-6, TNF-α, IL-10 and IFN-γ mRNA were quantified using qPCR. The expression levels were normalised to Gus-b levels. The levels of gene expression in control mice were normalised to one and the other groups measured were calculated as fold change in expression relative to the control group. Expression of TNF-α and IL-6 are significantly increased ($p<0.05$) in DSS treated colons compared to healthy controls with a 10-15 fold increase observed in TNF-α and a 15 fold increase in IL-6 expression. IL-1β, IFN-γ and IL-17 expression levels were also increased in DSS treated colons with a 3-4 fold increase in IL-1β levels, 7-13 fold increase in IFN-γ and 7 fold increase in IL-17 expression **[Figure 5.16].**

5.2.5 EFFECT OF UL-2-34 & UL-2-132 CASEIN HYDROLYSATE TREATMENTS IN A MOUSE MODEL OF COLITIS.

Collectively these results indicate a successful DSS induced model of colitis in the mice. The next step was to examine the effects of UL-2-34 and UL-2-132 as a treatment for this disease and assess their capacity to treat and alleviate inflammation in a mouse model of intestinal inflammation.

5.2.5.1 CLINICAL ASSESSMENT OF DSS-INDUCED MICE TREATED WITH UL-2-34 & UL-2-132

Mice were weighed and scored (every day) for daily disease activity index (DDAI) based on body weight loss, stool consistency, stool blood, fur texture and posture. DSS (5%) administration was associated with significant clinical changes including weight loss, appearance of occult faecal blood and diarrhoea in DSS-induced mice. There is a drop in body weight in all test groups treated with 5% of DSS from Day 5 onwards **[Figure 5.17 (A)]**, which reached its maximum, as seen by a significant

decrease in all test groups compared to control mice ($p<0.001$), the next day, on Day 6 **[Figure 5.17 (B)]**. A healthy gain in weight for control mice was observed. From Day 8 (Day 1 treatment) onwards, all test groups began to gradually gain weight **[Figure 5.19 (A)]**. On the final day, mice treated with UL-2-132, UL-2-34 and NaCN had a significant improvement ($p<0.001$) in weight gain when compared to the untreated DSS control group, however, this weight loss was still significantly lower ($p<0.001$) than that of control mice **[Figure 5.17 (C)]**. The DAI results reflect this weight loss pattern in DSS-induced mice. The DAI of DSS-induced groups were elevated on day 2 compared to baseline control mice and peaked on day 7 **[Figure 5.18 (B)]**. The decline in weight occurred in parallel with the initiation of bleeding and thus the rise in DAI. The maximum weight loss and DAI of the DSS-induced test groups occurred over a similar time period, on Day 6 and Day 7, respectively. No disease activity was observed in the control mice over the course of the study **[Figure 5.18 (A)]**. Whilst all test groups experienced a similar pattern of DAI drop from Day 8 (Day 1 treatment) and on the final day UL-2-34, UL-2-132 and NaCN treatments significantly reduced ($p<0.001$) the DAI score compared to untreated DSS-induced mice (red) **[Figure 5.18 (C)]**, UL-2-132 treated mice had the most significant DAI improvement. Treatment with UL-2-132 continuously reduced the DAI score from Day 8 (Day 1 treatment) until the end of the study **[Figure 5.18 (A)].** On the final day, colons were removed, measured and weighed to assess disease progression and ability of hydrolysates to alleviate this. Inflammation induced decrease in colon length and increase in colon weight is a classical characteristic of colonic inflammation. As we can see in **Figure 5.19 (A)**, colons from the DSS treated mice were significantly ($p<0.05$) shorter than those of control mice. UL-2-132 treatment in DSS-induced mice almost restored colon length back to that of the control. However, treatment with UL-2-34 had no therapeutic effect on DSS exposure, as colons were significantly ($p<0.05$) shorter than those of control mice. NaCN treatment appeared to aggravate the effects of DSS further, as seen by an even more significant decrease in colon length ($p<0.001$) compared to control mice **[Figure 5.19 (A)].** There was also a significant increase in colon weight ($p<0.05$) in DSS treated mice compared to controls. NaCN treated colons also slightly further increased in weight although the increase was not significant. Colons treated with UL-2-34 and UL-2-132 treatments had no weight gain compared to control **[Figure 5.19 (B)].** Sections of the distal colon (0.5cm) were removed and rolled for histological examination and stained with

haematoxylin and eosin (H&E), as described in Materials and methods **Section 2.9.4**. The H&E staining of control shows a healthy colon with crypts and goblet cells present **[Figure 5.20].** We can see a visible reduction in goblet cells, together with loss of crypts and infiltration of inflammatory cells to the mucosa in the colons of DSS treated mice and a similar pattern in DSS-induced mice treated with NaCN. UL-2-34 and UL-2-132 seemed to resolve superficial inflammation, preserved epithelial layer and crypt structure and restored the overall tissue morphology in colons of DSS treated mice when compared to non-treated DSS-induced mice **[Figure 5.20]**.

5.2.5.2 CYTOKINE EXPRESSION IN COLON IN DSS-INDUCED MICE TREATED WITH UL-2-34 & UL-2-132

RNA was isolated from colonic tissue from mice, as described in **Section 2.8.1**. cDNA was then generated from RNA using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, USA). The levels of IL-1β, IL-17, IL-6, TNF-α, IL-10 and IFN-γ mRNA were quantified using qPCR. The expression levels were normalised to Gus-b levels. The levels of gene expression in control mice were normalised to one and the other groups measured were calculated as fold change in expression relative to the control group.

DSS-induced colitis, on its own, significantly increased the mRNA expression of IL-17 ($p<0.05$) and also increased expression of IL-1β and IL-6 (non-significant) compared to control mice. DSS administration, however, also reduced expression of the pro-inflammatory cytokines TNF-α and IFN-γ ($p<0.05$). Treatment with UL-2-132 reversed this pro-inflammatory effect and induced a similar mRNA cytokine expression pattern observed in the colons of healthy control mice with respect to IL-1β, IL-17, IL-6 and IL-10 expression. However, treatment with UL-2-34, UL-2-132 and NaCN also resulted in similar decreased expression levels as untreated DSS-induced mice for TNF-α and IFN-γ **[Figure 5.21]**.

5.2.5.3 CYTOKINE SECRETION FROM UNSTIMULATED SPLENOCYTES OF DSS-INDUCED MICE TREATED WITH UL-2-34 & UL-2-132

Perše and Cerar, (2012) claim that adaptive immune responses are evident in the spleen in addition to the colon, following administration of DSS from Day 1 until the end of the model (Day 25). For this reason and because our *in vitro* results were based around T-cells in the spleen, cytokine levels were assessed in the spleens of mice. Spleens were collected, red blood cells were lysed and splenocytes were plated at 5 x 10^6 cells/ml on a 24 well plate. Cells were left unstimulated for 72 hr before harvesting. Supernatants were collected and measured for pro-inflammatory cytokines IFN-γ, IL-2, IL-6, IL-1β, TNF-α and IL-17 and the anti-inflammatory cytokine, IL-10 secretion using ELISA according to manufacturer's instructions (R&D duoset).

Secretion of all of the pro-inflammatory cytokines examined were significantly increased in unstimulated splenocytes from untreated DSS-induced mice ($p < 0.001$) compared to control mice **[Figure 5.22]**. The same significant boost in pro-inflammatory cytokines was observed in unstimulated splenocytes of DSS-induced mice treated with NaCN, as seen by a significant increase in IFN-γ, IL-2, IL-6, TNF-α, IL-17 ($p < 0.001$) and IL-1β ($p < 0.05$) compared to control mice. Both treatments with UL-2-34 and UL-2-132 displayed therapeutic anti-inflammatory effects as seen by their ability to reverse the pro-inflammatory cytokine spike in DSS mice, UL-2-132 being most effective. While UL-2-132 treatment significantly increased some inflammatory cytokines in unstimulated splenocytes compared to control mice (black), IL-6 ($p < 0.01$) and IL-1β ($p < 0.05$), its anti-inflammatory therapeutic potential is evident when compared to untreated DSS-induced splenocytes (red). This is demonstrated by its ability to significantly reverse the DSS-associated pro-inflammatory cytokine profile (IFN-γ, IL-6, TNF-α, and IL-17: $p < 0.001$; IL-2: $p < 0.01$; and IL-1β: $p < 0.05$). UL-2-34 treatment demonstrates a similar but less significant/effective pattern. UL-2-34 significantly increased secretion of IFN-γ, IL-6 ($p < 0.001$), IL-2, IL-17 ($p < 0.01$) and IL-1β ($p < 0.05$) from splenocytes when compared to control mice (black). When compared to untreated DSS-induced splenocytes (red), UL-2-34 significantly reduced IL-6 ($p < 0.001$), IFN-γ (($p < 0.01$), TNF-α and IL-17 ($p < 0.05$). Both UL-2-34 and UL-2-132 treatments reduced IL-10 secretion compared to control and DSS mice. A

slight increase in IL-10 secretion was observed in DSS-induced mice compared to control mice. NaCN treatment has no effect on IL-10 secretion.

5.2.5.4 CYTOKINE SECRETION FROM STIMULATED SPLENOCYTES OF DSS-INDUCED MICE TREATED WITH UL-2-34 & UL-2-132

Spleens were collected, red blood cells were lysed 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) for 72 hr before harvesting. Supernatants were collected and measured for pro-inflammatory cytokines IFN-γ, IL-2, IL-6, IL-1β, TNF-α and IL-17 and the anti-inflammatory cytokine, IL-10 secretion using ELISA according to manufacturer's instructions (R&D duoset).

IL-6 and IL-1β secretion was significantly increased in stimulated splenocytes from untreated DSS-induced mice ($p < 0.001$) and mice treated with NaCN ($p < 0.01$ and $p < 0.05$ respectively) compared to control mice, indicating an over-excessive response to a stimulant. This response was reversed in mice treated with UL-2-34 and UL-2-132. UL-2-132 treatment significantly reduced IL-6 and IL-1β secretion ($p < 0.01$ and $p < 0.001$ respectively) in stimulated splenocytes compared to untreated DSS-induced mice (red). In the same context, UL-2-34 slightly reduced IL-6 and significantly reduced IL-1β ($p < 0.001$). Moreover, UL-2-34 and UL-2-132 treated DSS-induced mice secreted almost identical levels of all pro-inflammatory cytokines examined from stimulated splenocytes when compared to control mice (black). UL-2-132 increased IL-10 secretion when compared to both control and DSS-induced mice. UL-2-34, however, reduced IL-10 compared to control and DSS-induced mice, reduction compared to DSS mice was significant ($p < 0.01$). NaCN treatment also significantly increased ($p < 0.05$) IL-10 compared to untreated DSS mice and a slight increase was also observed compared to control mice **[Figure 5.23]**.

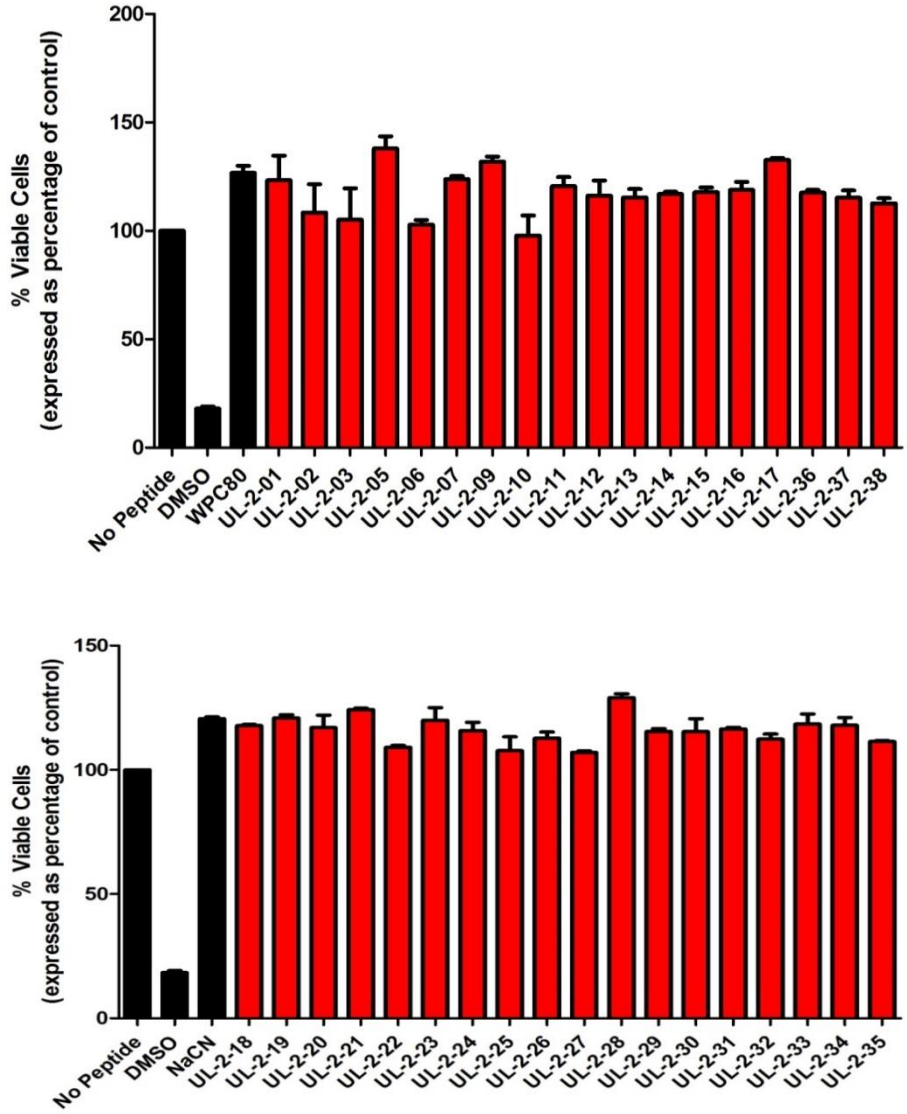


Fig 5.1: The concentrations of protein hydrolysates used do not affect the viability of undifferentiated T-cells. $CD4^+$ cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19852). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml) or 10% DMSO (vehicle control). Cells were incubated for 72 hrs. Viability was assessed using an MTS assay (Cell Titer 96 Aqueous One Solution – Promega, WI, USA). One way Anova with Newman Keuls post-hoc test was used to determine if differences between groups were significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Results are expressed as a percentage of untreated cells.

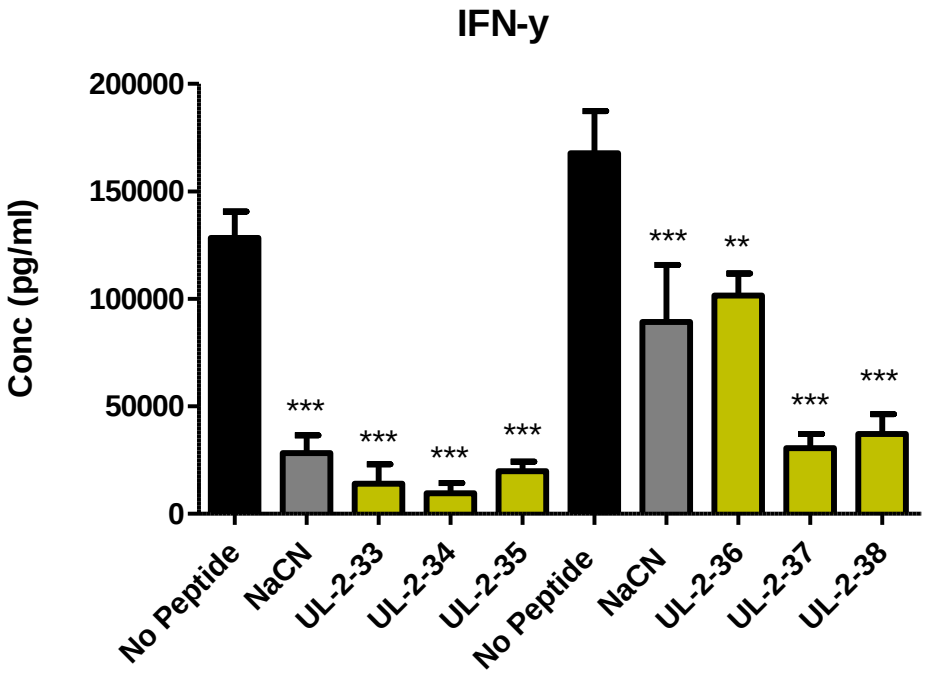


Fig 5.2: First-round hydrolysates suppress the cytokine required to drive an Inflammatory Th1 response from Undifferentiated T-cells *in vitro*. $CD4^+$ cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19852). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml). Cells were incubated for 72 hrs. Supernatants were collected and measured for IFN-γ secretion using ELISA according to manufacturer's instructions (R&D duoset). One way Anova with Newman Keuls post-hoc test was used to determine if differences between groups were significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Hydrolysates are compared to untreated cells (No Peptide).

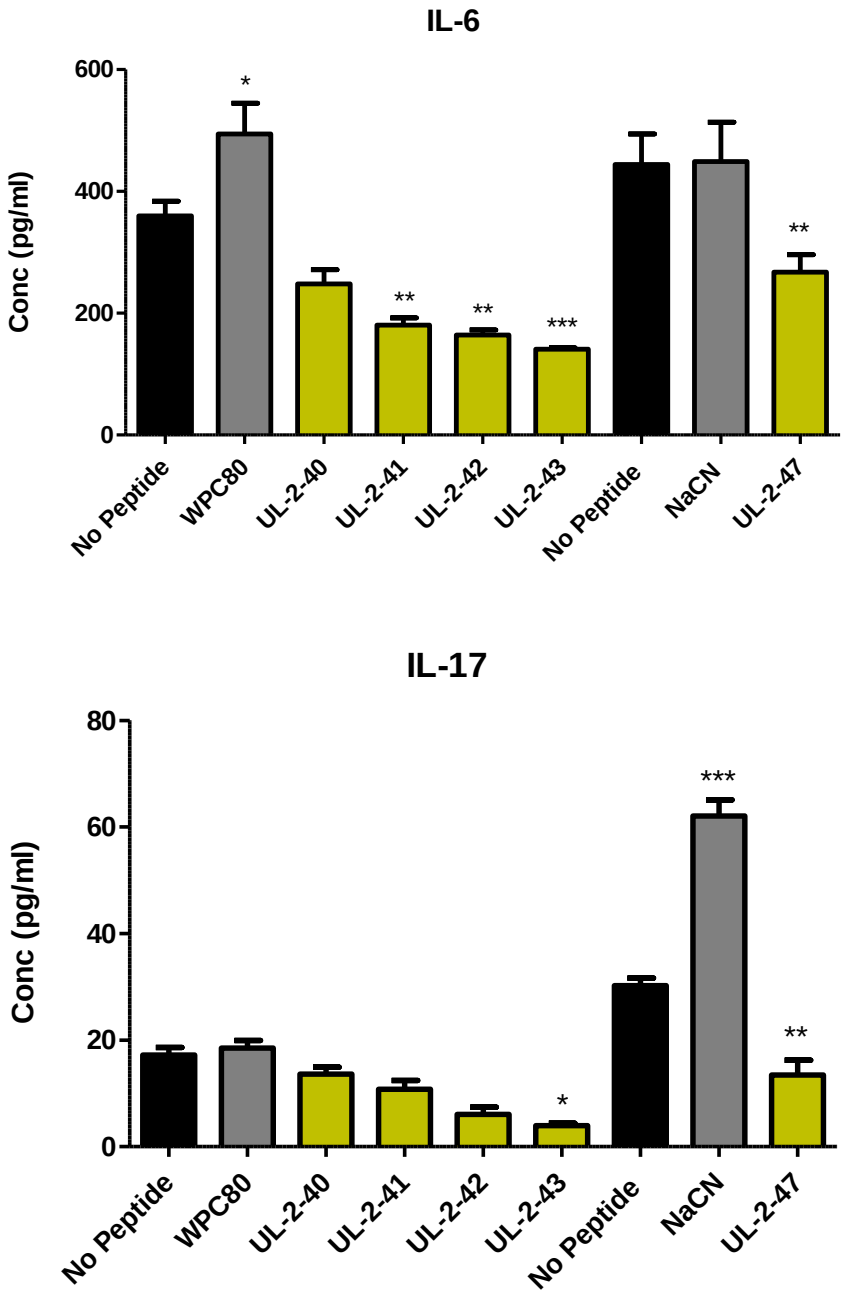


Fig 5.3: First-round hydrolysates suppress the cytokines required to drive an Inflammatory Th17 response from Undifferentiated T-cells *in vitro*. $CD4^+$ cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19852). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml). Cells were incubated for 72 hrs. Supernatants were collected and measured for IFN-γ secretion using ELISA according to manufacturer's instructions (R&D duoset). One way Anova with Newman Keuls post-hoc test was used to determine if differences between groups were significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Hydrolysates are compared to untreated cells (No Peptide).

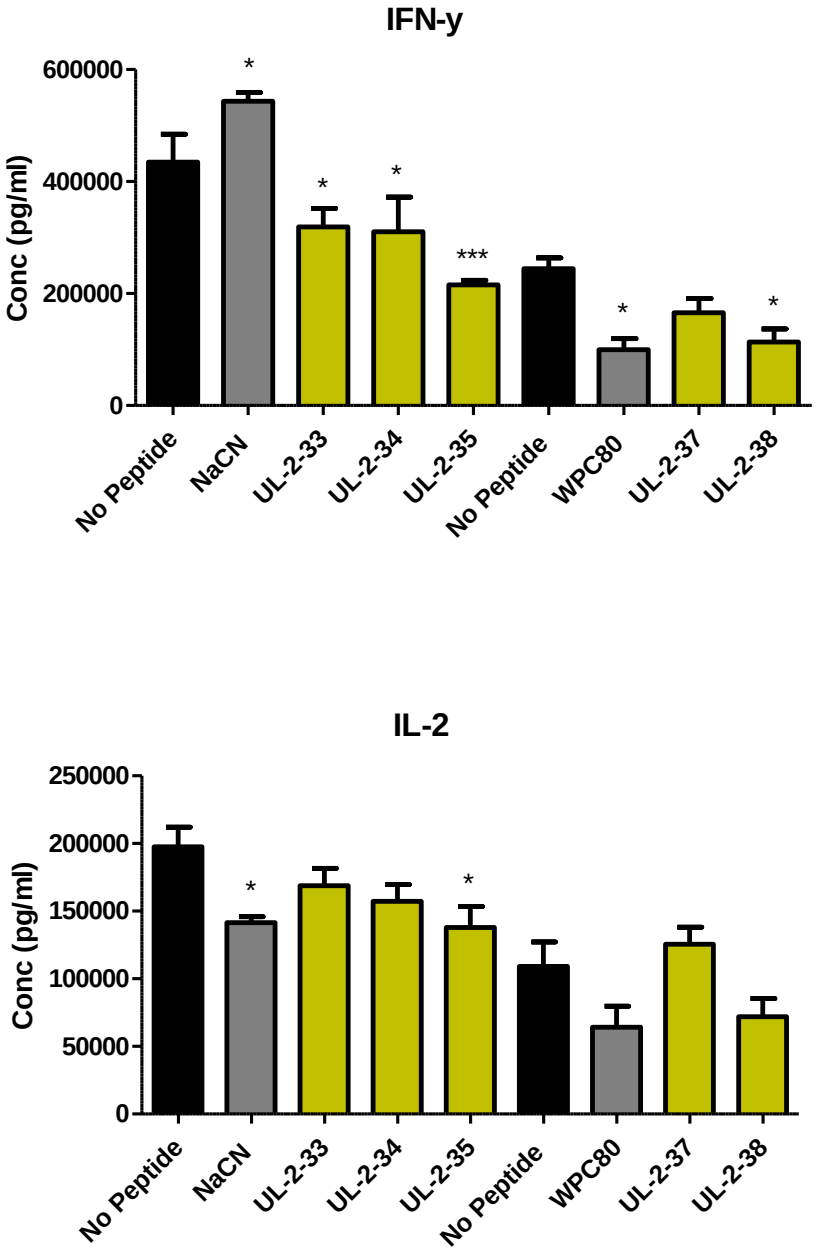


Fig 5.4: First-round hydrolysates can prevent differentiation of Th1 cells *in vitro* by suppressing the characteristic Th1 cytokines. $CD4^+$ cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19852). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and 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 hrs and were restimulated in fresh media for a further 24 hrs. Supernatants were collected and measured for IFN-γ and IL-2 secretion using ELISA according to manufacturer's instructions (R&D duoset). One way Anova with Newman Keuls post-hoc test was used to determine if differences between groups were significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Hydrolysates are compared to untreated cells (No Peptide).

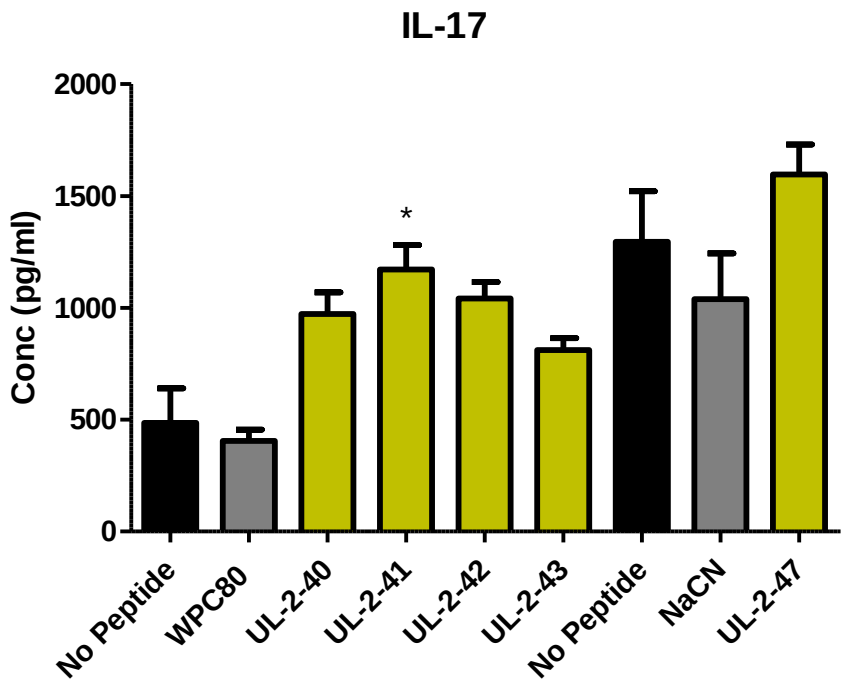


Fig 5.5: First-round hydrolysates promote differentiation of Th17 cells *in vitro* by inducing the characteristic Th17 cytokine. $CD4^+$ cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19852). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml). The media was supplemented with 20 ng/ml IL-6, 20 ng/ml IL-23 and 10 µg/ml neutralising anti-IFN-γ antibody for Th17 polarising conditions. Cells were incubated for 72 hrs and were restimulated in fresh media for a further 24 hrs. Supernatants were collected and measured for IL-17 secretion using ELISA according to manufacturer's instructions (R&D duoset). One way Anova with Newman Keuls post-hoc test was used to determine if differences between groups were significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Hydrolysates are compared to untreated cells (No Peptide).

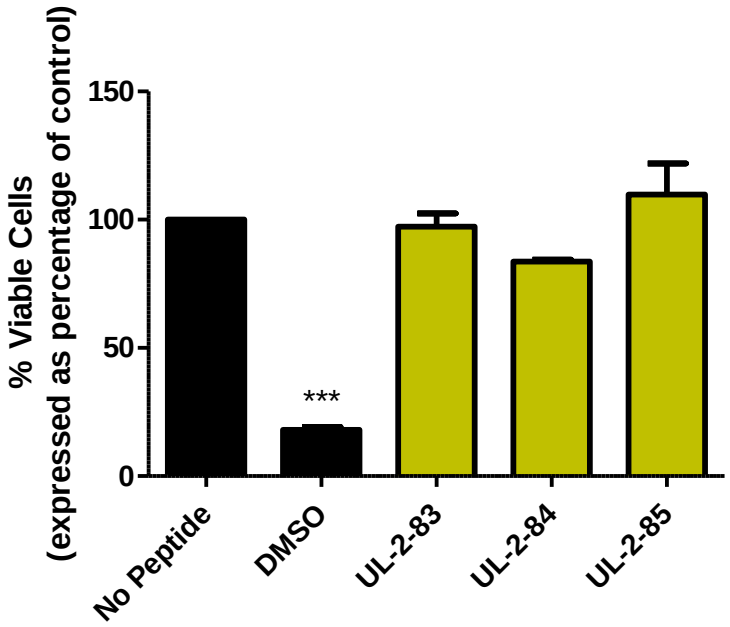


Fig 5.6: The concentrations of protein hydrolysates used do not affect the viability of undifferentiated T-cells. $CD4^+$ cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19852). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml) or 10% DMSO (vehicle control). Cells were incubated for 72 hrs. Viability was assessed using an MTS assay (Cell Titer 96 Aqueous One Solution – Promega, WI, USA). One way Anova with Newman Keuls post-hoc test was used to determine if differences between groups were significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Results are expressed as a percentage of untreated cells.

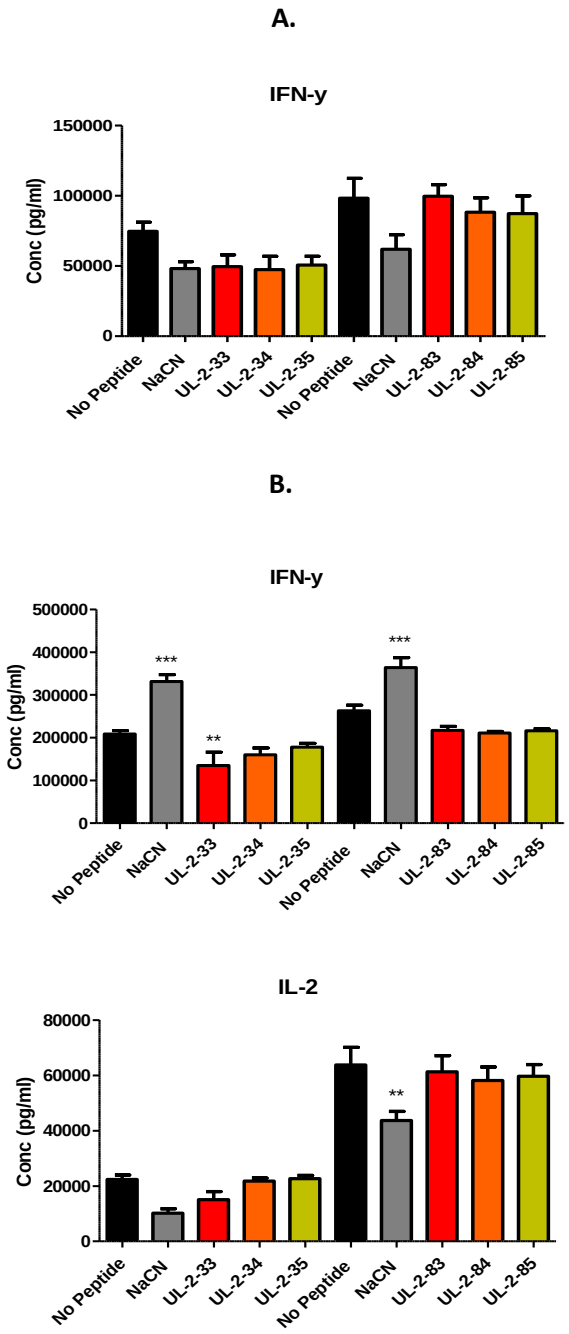


Fig 5.7: Second-round Regenerated hydrolysates suppress the Th1 cytokines from undifferentiated and Th1 cells *in vitro*. $CD4^+$ cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19852). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml). Cells were incubated for 72 hrs. The media of Th1 cells was supplemented with 20 ng/ml IL-2, 20 ng/ml IL-12 and 10 µg/ml anti-IL-4 antibody. Cells were incubated for 72 hrs and were restimulated in fresh media for a further 24 hrs. Supernatants were collected and measured for IFN-γ and IL-2 secretion using ELISA according to manufacturer's instructions (R&D duoset). One way Anova with Newman Keuls post-hoc test was used to determine if differences between groups were significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Hydrolysates are compared to untreated cells (No Peptide).

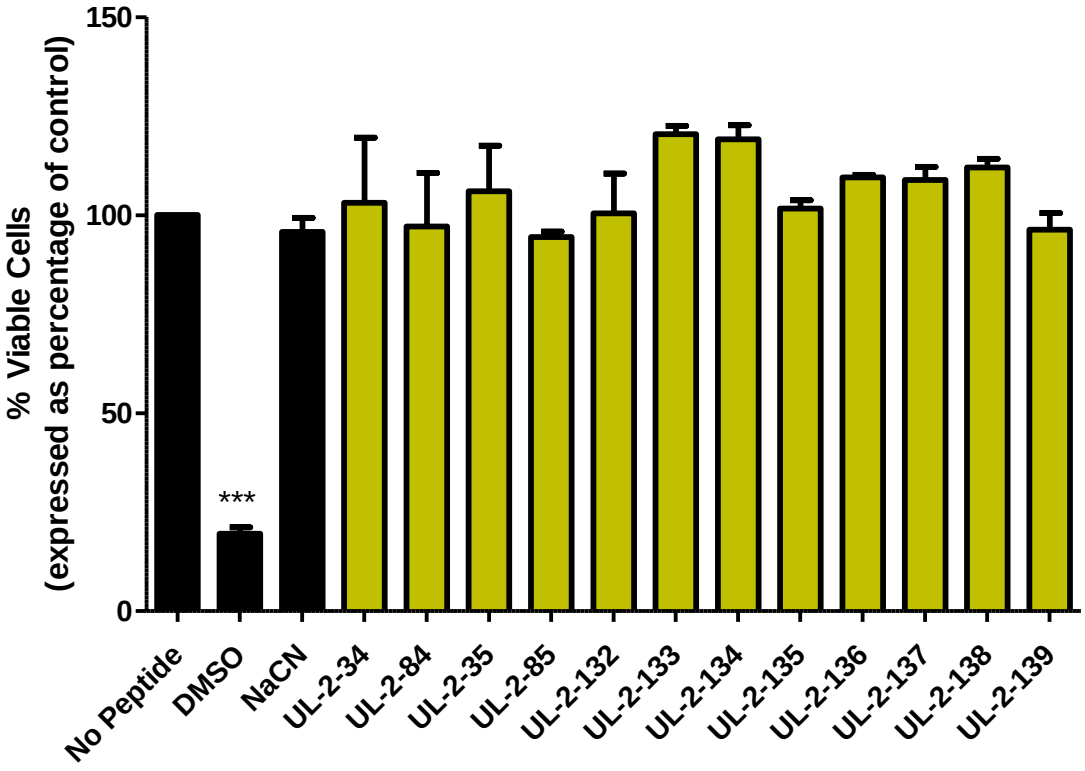


Fig 5.8: The concentrations of optimised protein hydrolysates from UL-2-34 and UL-2-35 used do not affect the viability of undifferentiated T-cells. $CD4^+$ cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19852). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml) or 10% DMSO (vehicle control). Cells were incubated for 72 hrs. Viability was assessed using an MTS assay (Cell Titer 96 Aqueous One Solution – Promega, WI, USA). One way Anova with Newman Keuls post-hoc test was used to determine if differences between groups were significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Results are expressed as a percentage of untreated cells.

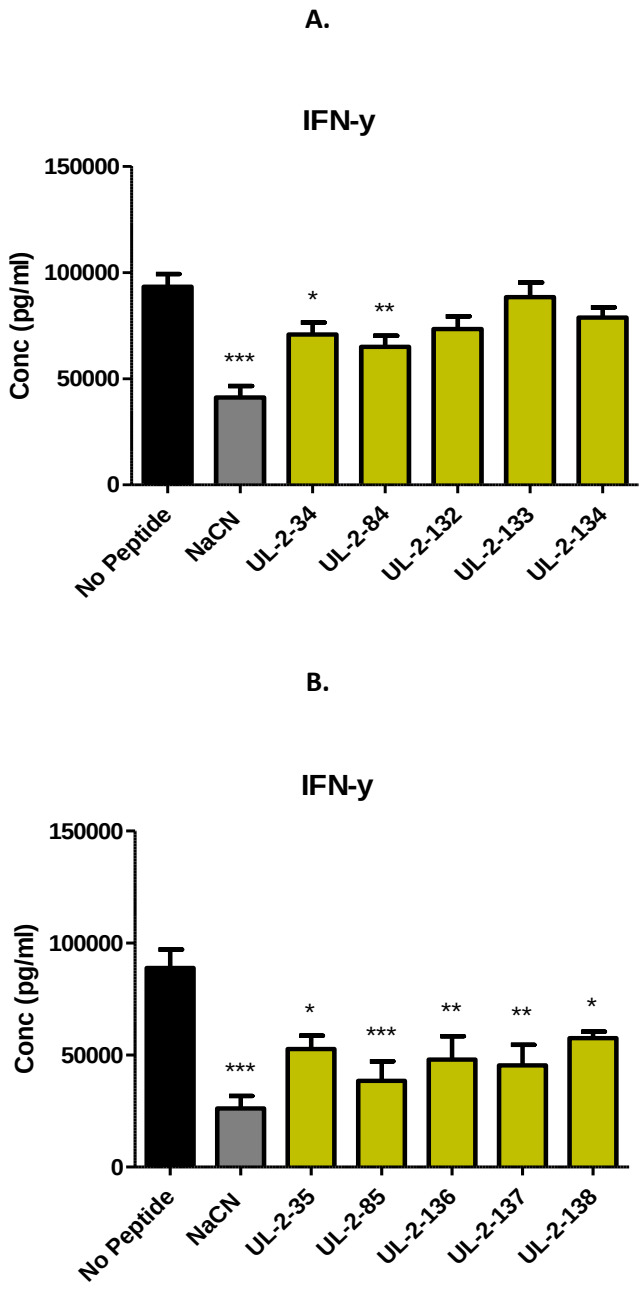


Fig 5.9: Third-round UL-2-34/35, regenerated and optimised hydrolysates suppress the cytokine required to drive an Inflammatory Th1 response from Undifferentiated T-cells *in vitro*. $CD4^+$ cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19852). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and treated with hydrolysates (1 mg/ml). Cells were incubated for 72 hrs. Supernatants were collected and measured for IFN-γ secretion for both UL-2-34 optimised hydrolysates **(A)** and UL-2-35 optimised hydrolysates **(B)**, using ELISA according to manufacturer's instructions (R&D duoset). One way Anova with Newman Keuls post-hoc test was used to determine if differences between groups were significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Hydrolysates are compared to untreated cells (No Peptide).

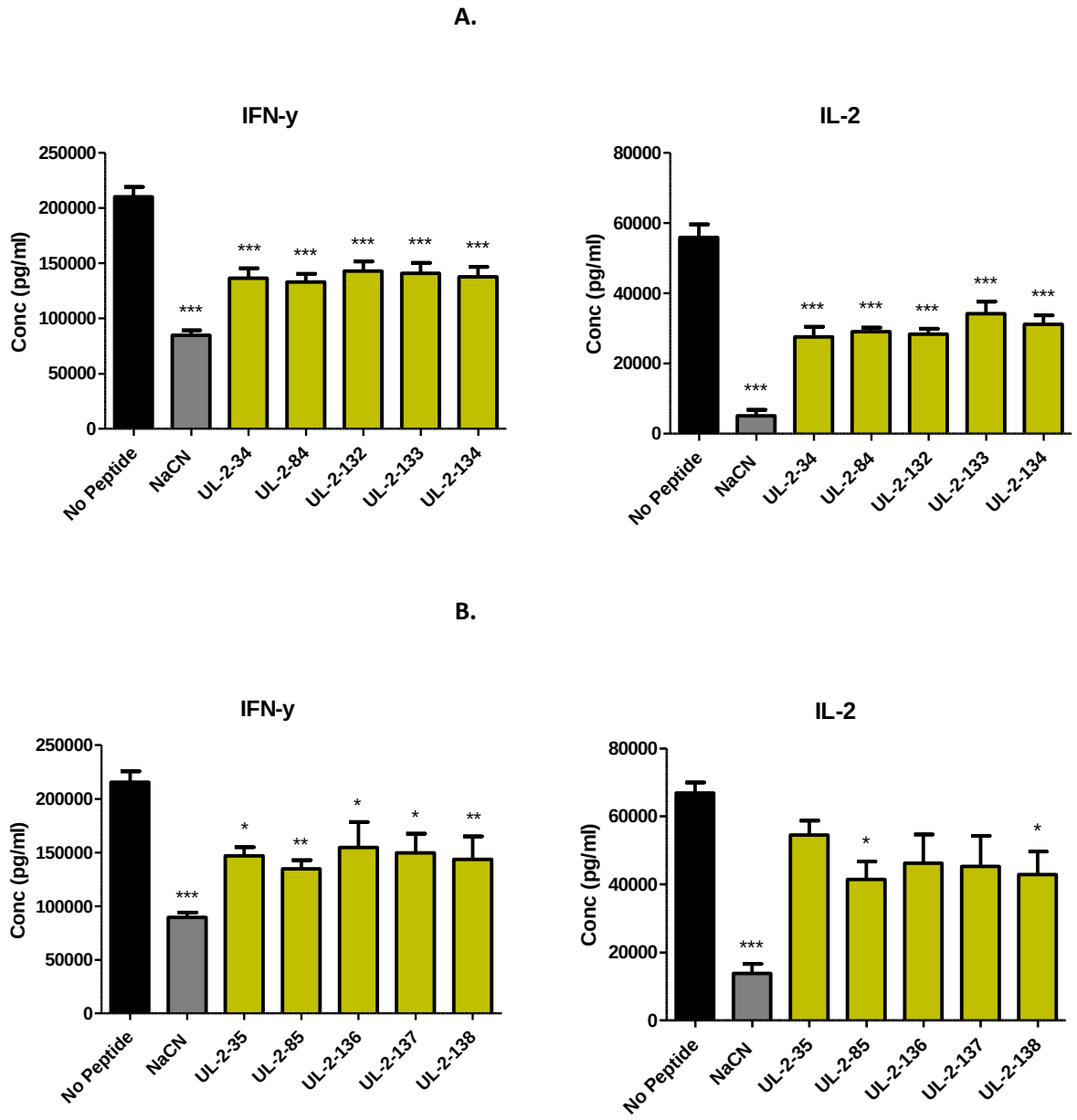


Fig 5.10: Third-round UL-2-34/35, regenerated and optimised hydrolysates prevent differentiation of a Th1 subset *in vitro*. $CD4^+$ cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19852). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and 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 hrs and were restimulated in fresh media for a further 24 hrs. Supernatants were collected and measured for IFN-γ and IL-2 secretion for both UL-2-34 optimised hydrolysates **(A)** and UL-2-35 optimised hydrolysates **(B)**, using ELISA according to manufacturer's instructions (R&D duoset). One way Anova with Newman Keuls post-hoc test was used to determine if differences between groups were significant (*p<0.05, **p<0.01, ***p<0.001). Hydrolysates are compared to untreated cells (No Peptide).

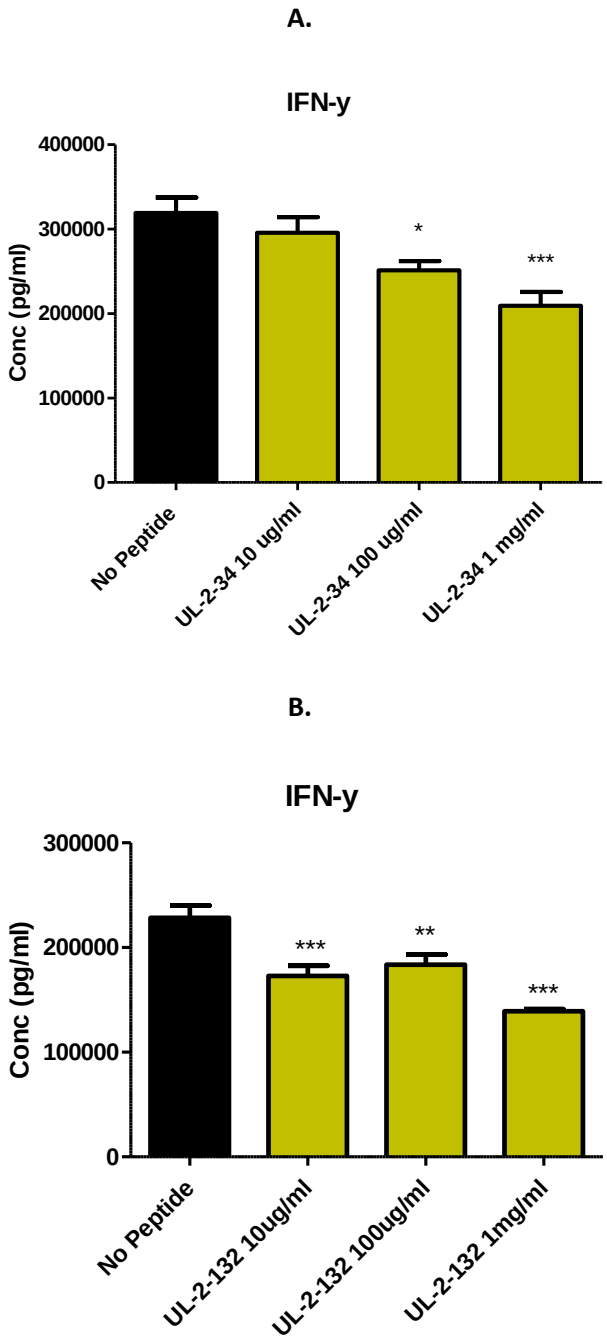


Fig 5.11: UL-2-34 and UL-2-132 Inhibit the Inflammatory Th1 response in dose dependent manner *in vitro* $CD4^+$ cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19852). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and 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 hrs and were restimulated in fresh media for a further 24 hrs. Supernatants were collected and measured for IFN-γ secretion for both parent hydrolysate, UL-2-34, **(A)** and optimised hydrolysate, UL-2-132, **(B)**, using ELISA according to manufacturer's instructions (R&D duoset). One way Anova with Newman Keuls post-hoc test was used to determine if differences between groups were significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Hydrolysates are compared to untreated cells (No Peptide).

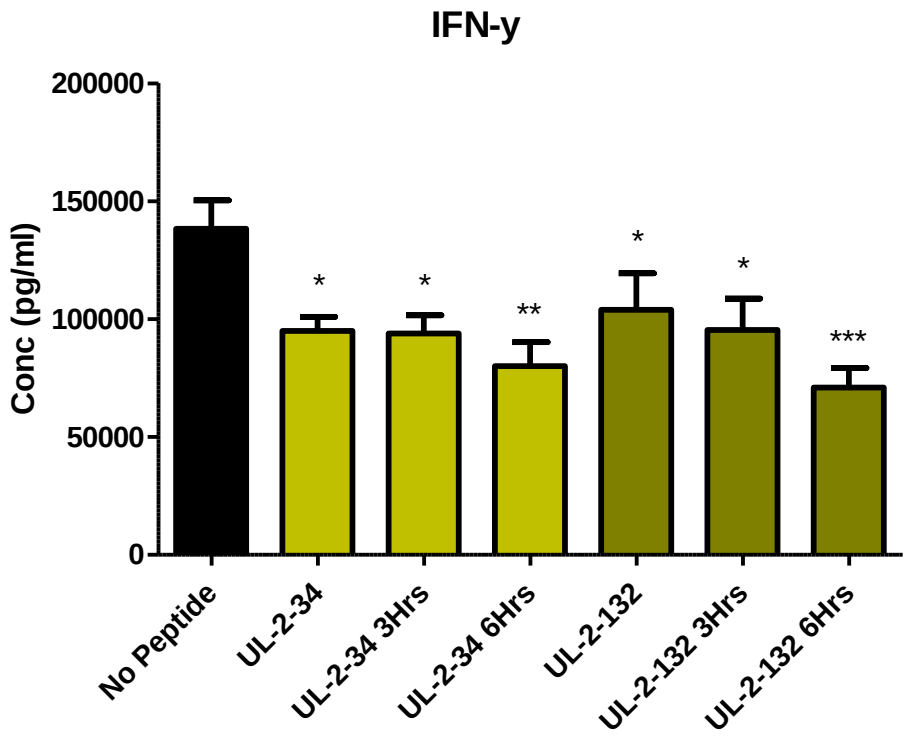
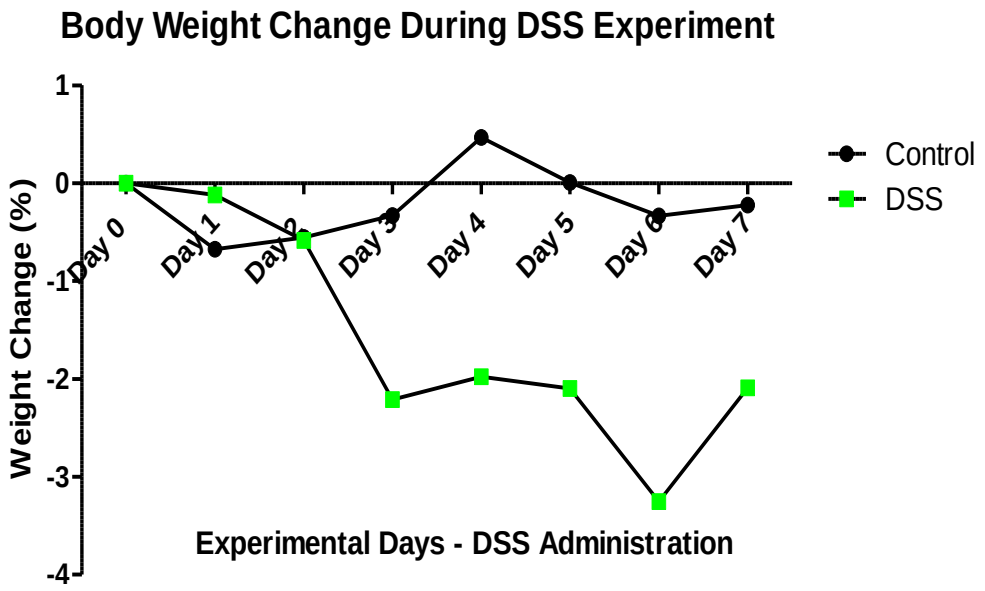


Fig 5.12: Upscaled UL-2-34 and UL-2-132 Continue to Inhibit the Th1 response *in vitro*. $CD4^+$ cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19852). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) plus anti-CD28 (5 µg/ml) and 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 hrs and were restimulated in fresh media for a further 24 hrs. Supernatants were collected and measured for IFN-γ and IL-2 secretion using ELISA according to manufacturer's instructions (R&D duoset). One way Anova with Newman Keuls post-hoc test was used to determine if differences between groups were significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Hydrolysates are compared to untreated cells (No Peptide).

A.



B.

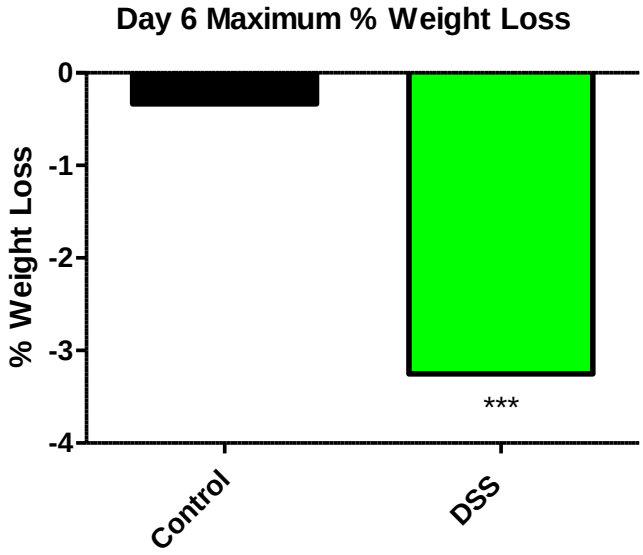
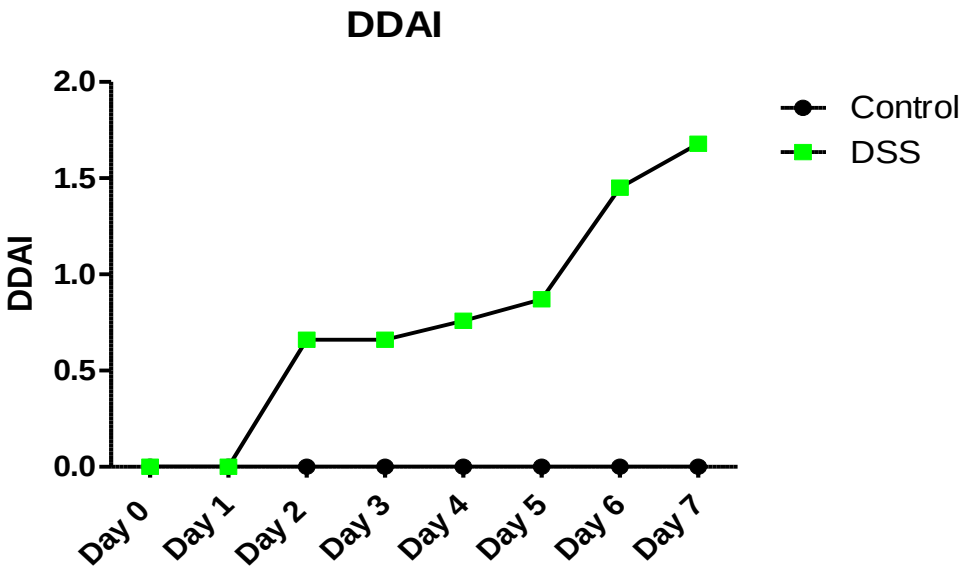


Fig 5.13: Disease associated symptoms in the DSS colitis model. 8-10 week old female BALB/c mice were grouped into a control group of 4 mice and a test group (also 4 mice) of early acute colitis. DSS was administered to mice in the drinking water for 7 days at a final concentration of 5% and mice were culled on day 7. Mice were weighed every day. The average % weight change of the 2 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 **(A)**. Maximum weight loss was recorded on day 6 (**B**). 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, ***p<0.001)

A.



B.

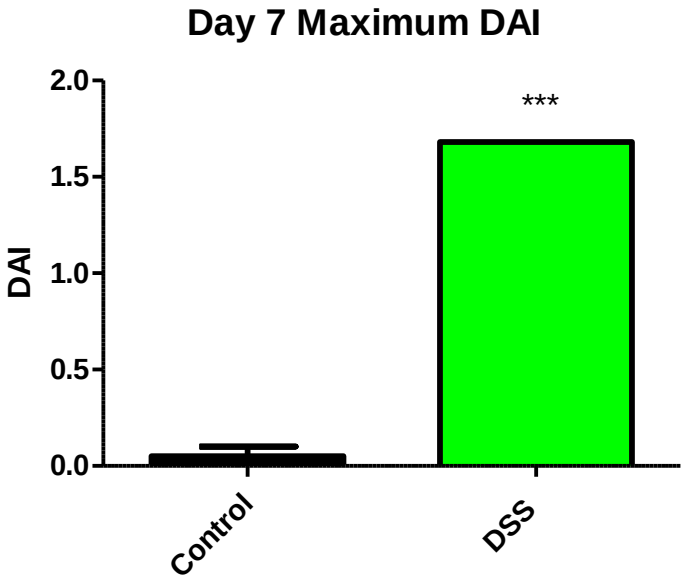


Fig 5.14: Disease associated symptoms in the DSS colitis model. 8-10 week old female BALB/c mice were grouped into a control group of 4 mice and a test group (also 4 mice) of early acute colitis. DSS was administered to mice in the drinking water for 7 days at a final concentration of 5% and mice were culled on day 7. Mice were weighed and disease scored based a composite measure of weight loss, stool consistency and blood in stool for daily disease activity index (DDAI) (**A**). Maximum DAI was recorded on Day 7 (**B**). 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, ***p<0.001).

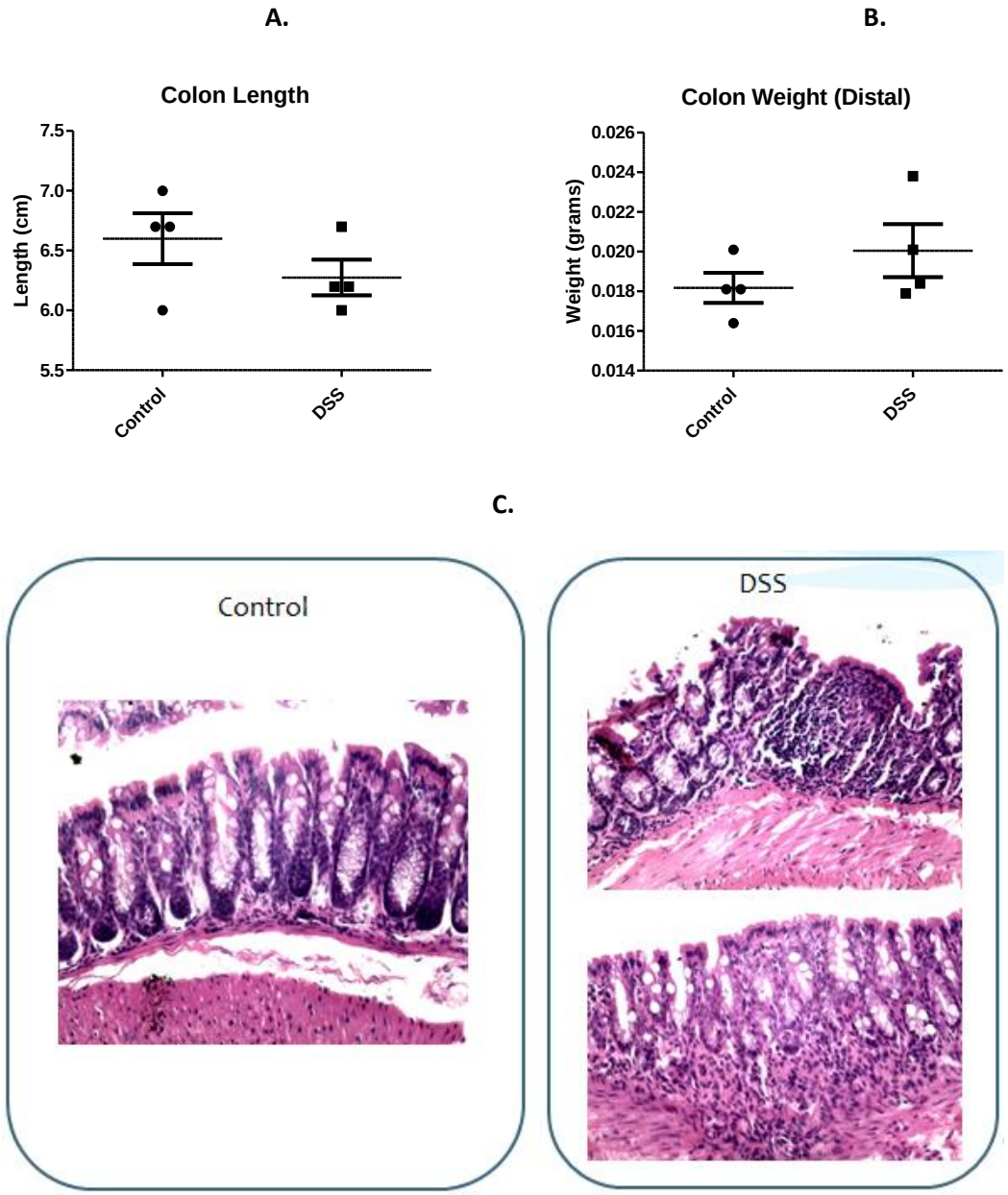


Fig 5.15: Disease associated symptoms in the DSS colitis model Cont. 8-10 week old female BALB/c mice were grouped into a control group of 4 mice and a test group (also 4 mice) of early acute colitis. DSS was administered to mice in the drinking water for 7 days at a final concentration of 5% and mice were culled on day 7. At the end point of each group the length **(A)** and weight **(B)** of each removed and washed colon was measured and used as an indication of inflammation in the colon. 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 acute slides with recovery in the chronic slides as expected **(C)**.

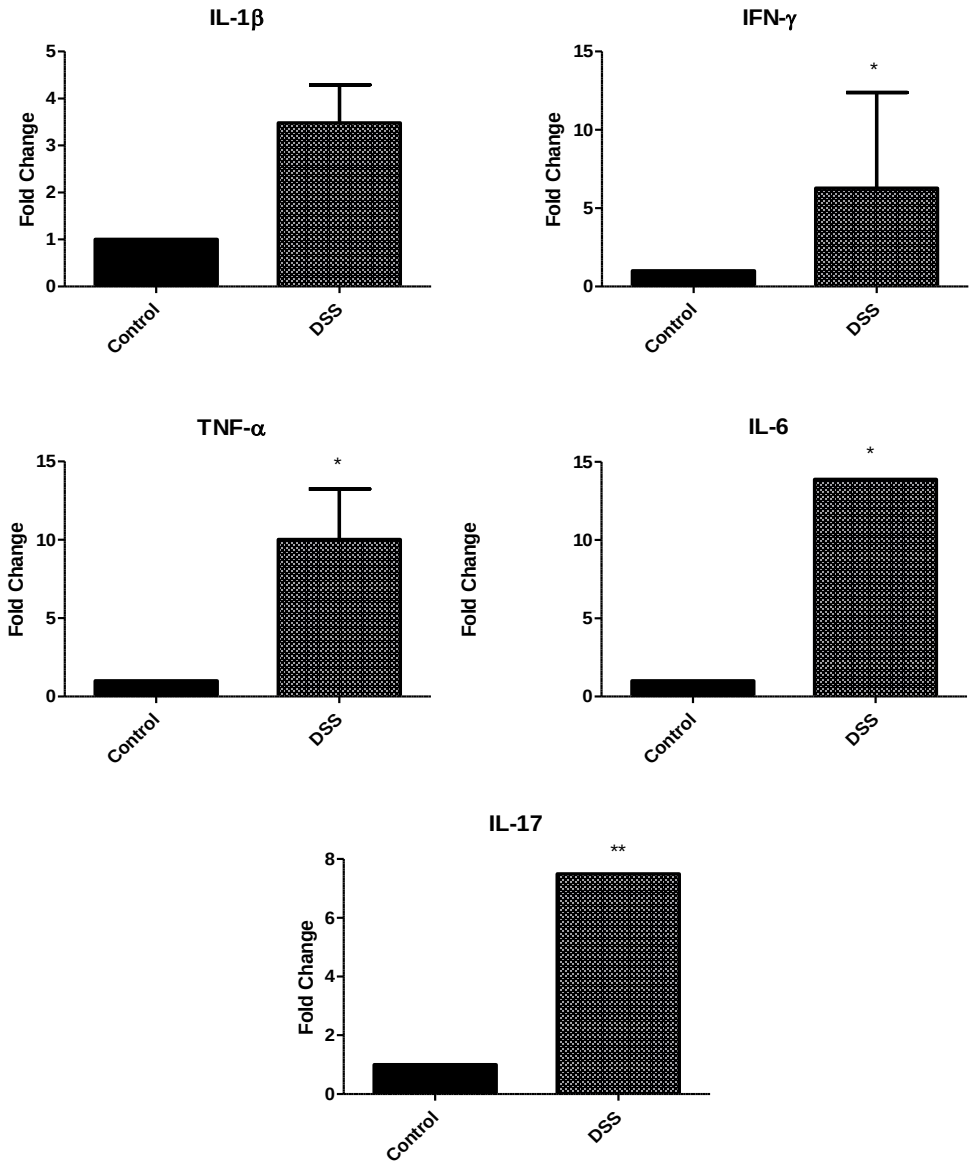


Fig 5.16: Cytokine mRNA expression in a mouse model of colitis. BALB/c mice were grouped into a control group of 4 mice and a test group (also 4 mice) of early acute colitis. DSS was administered to mice in the drinking water for 7 days at a final concentration of 5% and mice were culled on day 7. Colonic tissue from each sample was weighed and homogenised using the Qiagen TissueLyser LT with stainless steel beads (5mm). Following homogenisation, RNA was extracted and quantitated on the nanodrop and equalised amounts of RNA (2 µg) were converted to cDNA using the high capacity cDNA mastermix (Roche). The cDNA was mixed with primers (IDT) for IL-1β, IFN-γ, TNF-α, IL-6 and IL-17 and Taqman® Gene Expression Mastermix (Applied Biosystems) before analysing 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 normalised 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). An unpaired T-test was used to determine if differences between DSS and Control group were significantly different (*p<0.05, **p<0.01, ***p<0.001).

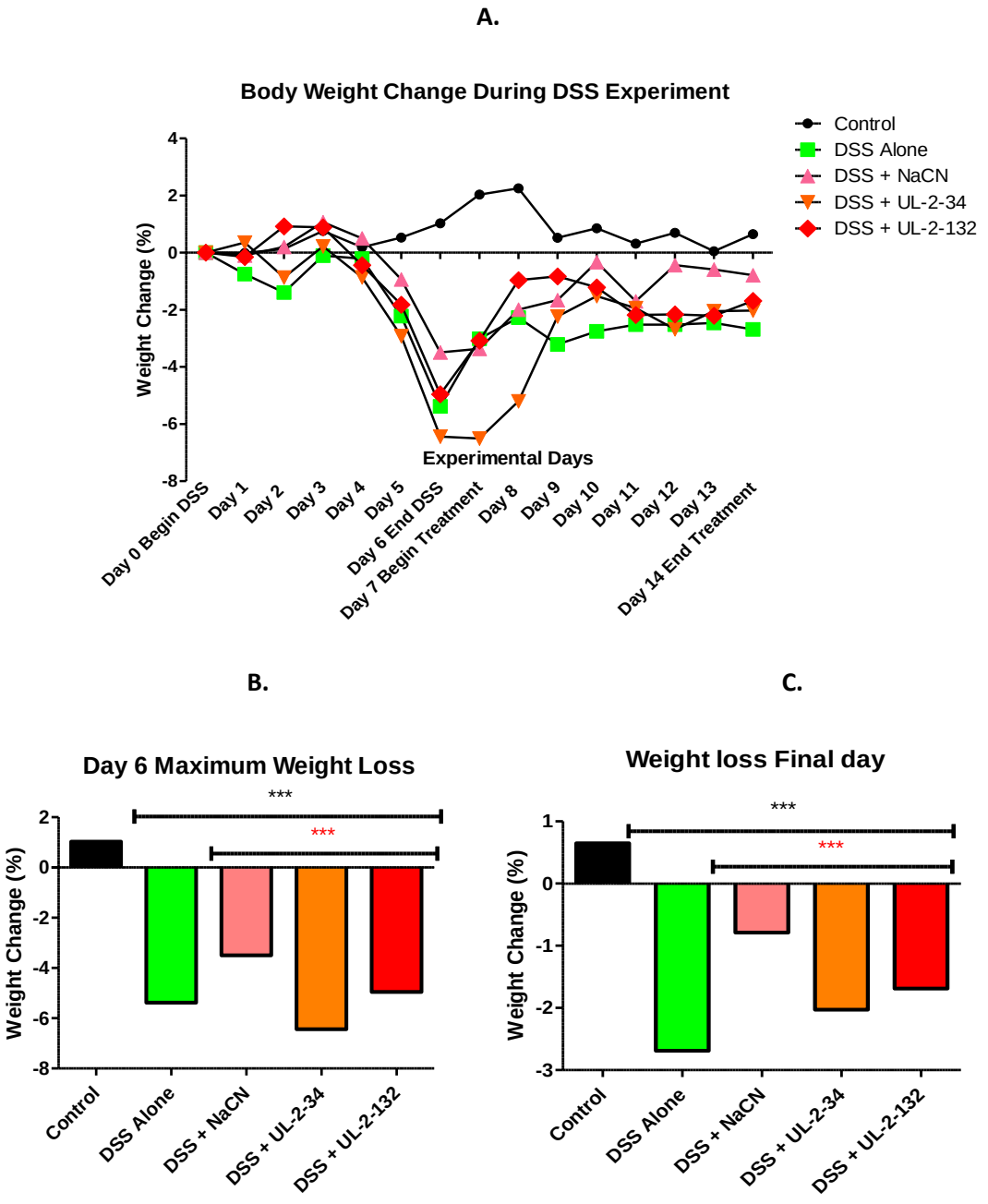


Figure 5.17: Disease associated symptoms (body weight change) in the DSS colitis model treated with UL-2-34 & UL-2-132 hydrolysates: Effect of hydrolysate treatment on the clinical signs of colitis and macroscopic signs of inflammation in colon tissue. DSS was administered to test groups in the drinking water for 7 days at a final concentration of 5%. Mice were treated orally with PBS (control and DSS groups), NaCN, UL-2-34 or UL-2-132 in PBS every day for 7 days after DSS administration. 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 (**A**). Maximum weight loss was recorded on day 6 (**B**). Weight loss values on final day of study (**C**). Data presented indicate the mean ± SEM (n = 6). One way Anova with Newman Keuls post-hoc test was used to determine if differences between groups [Groups compared to control (black) and groups compared to DSS alone (red)] were significant (*p<0.05, **p<0.01, ***p<0.001).

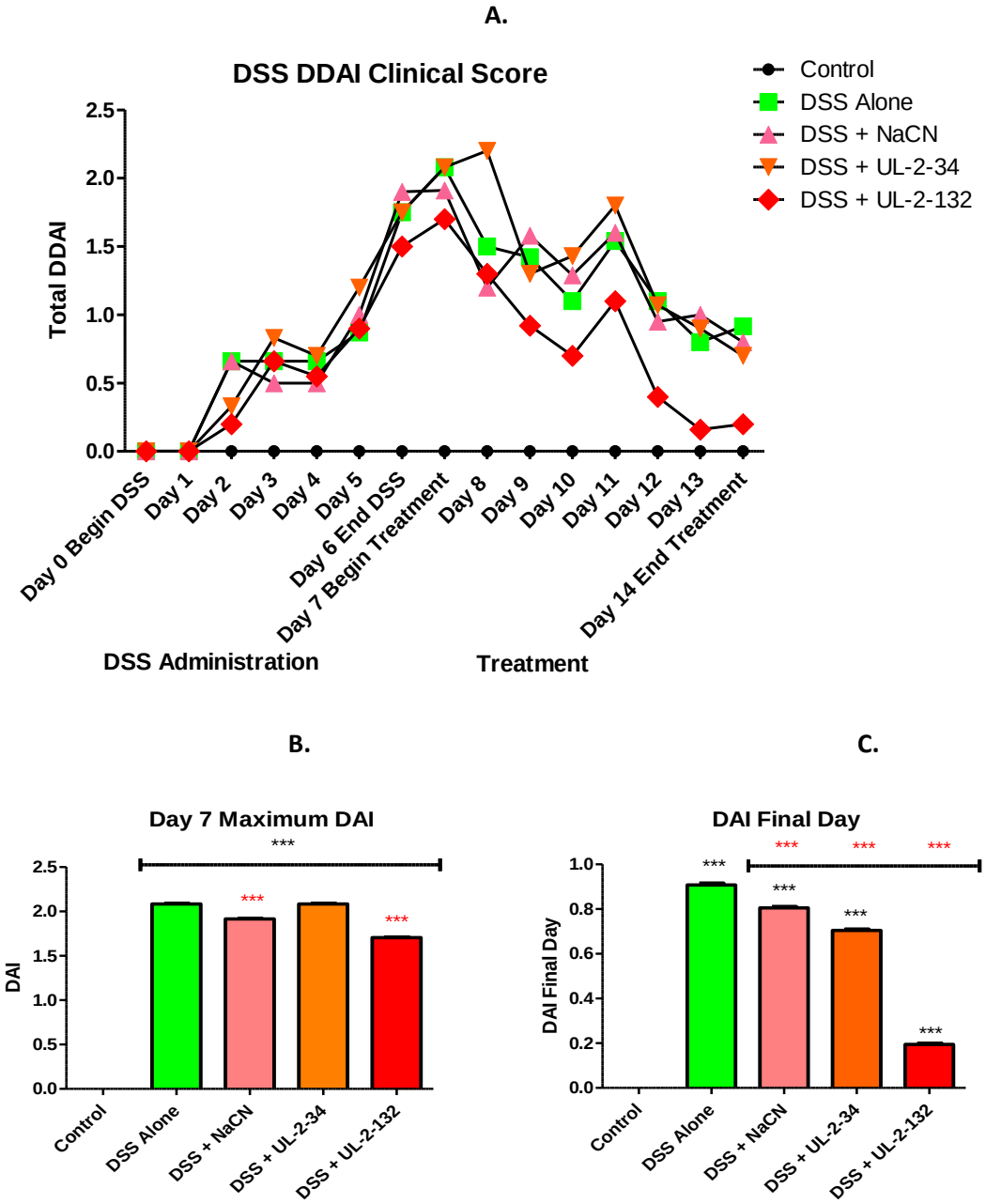


Figure 5.18: Disease associated symptoms (DDAI) in the DSS colitis model treated with UL-2-34 & UL-2-132 hydrolysates: Effect of hydrolysate treatment on the clinical signs of colitis and macroscopic signs of inflammation in colon tissue. DSS was administered to test groups in the drinking water for 7 days at a final concentration of 5%. Mice were treated orally with PBS (control and DSS groups), NaCN, UL-2-34 or UL-2-132 in PBS every day for 7 days after DSS administration. Mice were weighed and disease scored based a composite measure of weight loss, stool consistency and blood in stool for daily disease activity index (DAI) (**A**). Maximum DAI was recorded on Day 7 (**B**). Final day DAI plotted with significance values (**C**). Data presented indicate the mean ± SEM (n = 6). One way Anova with Newman Keuls post-hoc test was used to determine if differences between groups [Groups compared to control (black) and groups compared to DSS alone (red)] were significant (*p<0.05, **p<0.01, ***p<0.001).

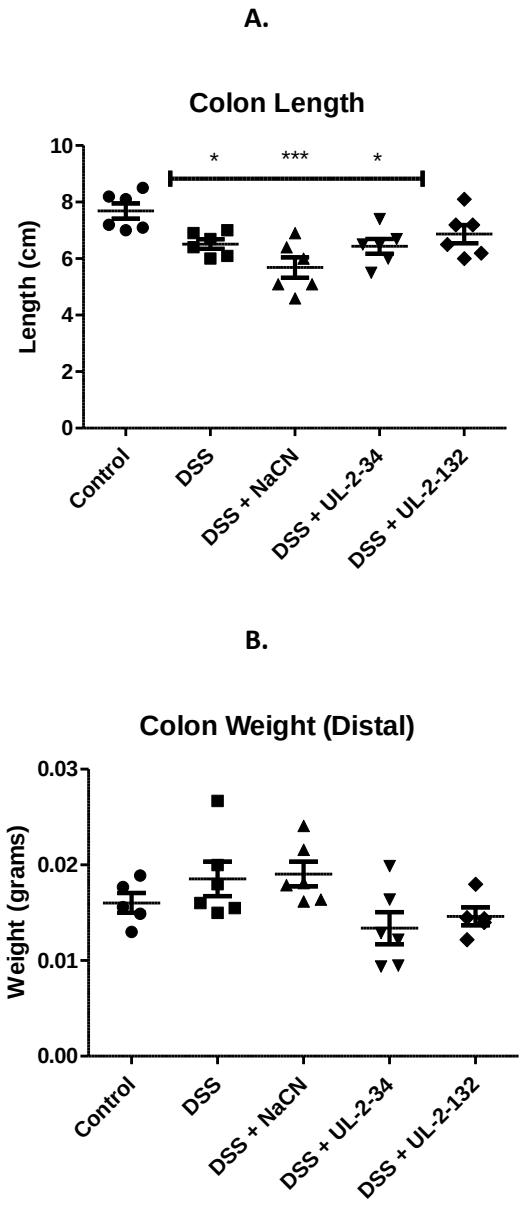


Figure 5.19: Disease associated symptoms (colon length and weight) in the DSS colitis model treated with UL-2-34 & UL-2-132 hydrolysates: Effect of hydrolysate treatment on the clinical signs of colitis and macroscopic signs of inflammation in colon tissue. DSS was administered to test groups in the drinking water for 7 days at a final concentration of 5%. Mice were treated orally with PBS (control and DSS groups), NaCN, UL-2-34 or UL-2-132 in PBS every day for 7 days after DSS administration. At the end point of each group the length **(B)** and weight **(C)** of each removed and washed colon was measured and used as an indication of inflammation in the colon. Data presented indicate the mean ± SEM (n = 6). One way Anova with Newman Keuls post-hoc test was used to determine if differences between test groups and control mice were significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

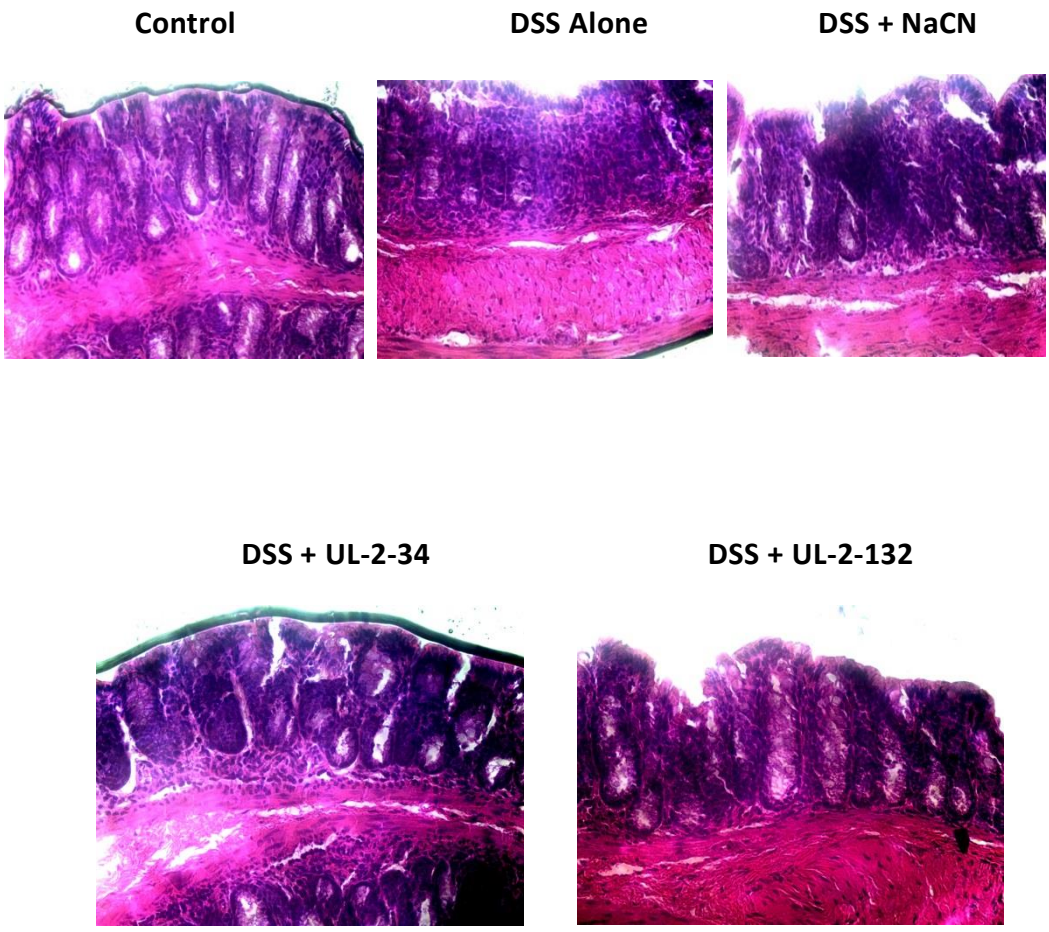


Figure 5.20: Effect of UL-2-34 & UL-2-132 hydrolysates on disease associated symptoms (histopathological changes of the colon tissue) in the DSS colitis model: Effect of hydrolysate treatment on the macroscopic signs of inflammation in colon tissue. DSS was administered to test groups in the drinking water for 7 days at a final concentration of 5%. Mice were treated orally with PBS (control and DSS groups), NaCN, UL-2-34 or UL-2-132 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 H&E staining of colonic tissue from each group are taken at 40x magnification.

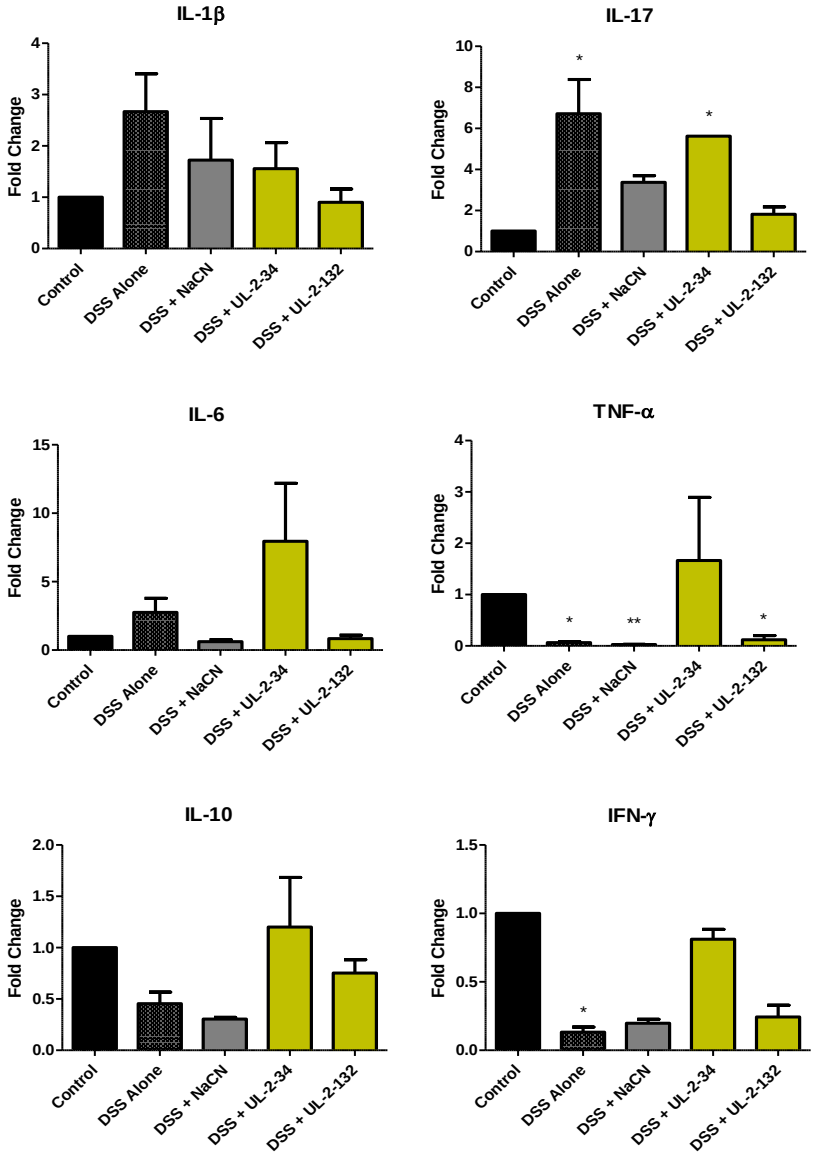


Figure 5.21: Cytokine mRNA expression in a mouse model of colitis treated with UL-2-34 & UL-2-132 Hydrolysates. BALB/c mice were grouped into a control group, a DSS only group, a DSS and NaCN treated group, a DSS and UL-2-34 treated group and a DSS and UL-2-132 treated group. Each group had 6 mice which were culled on Day 14. Colonic tissue from each sample was weighed and homogenised using the Qiagen TissueLyser LT with stainless steel beads (5mm). Following homogenisation, RNA was extracted and quantitated on the nanodrop and equalised amounts of RNA (2 µg) were converted to cDNA using the high capacity cDNA mastermix (roche). The cDNA was mixed with primers (IDT) for IL-1β, IL-17, IL-6, TNF-α, IL-10 and IFN-γ and Taqman® Gene Expression Mastermix (Applied Biosystems) before analysing 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 normalised 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 = 6). One way Anova with Newman Keuls post-hoc test was used to determine if differences between test groups and control mice were significant (*p<0.05, **p<0.01, ***p<0.001).

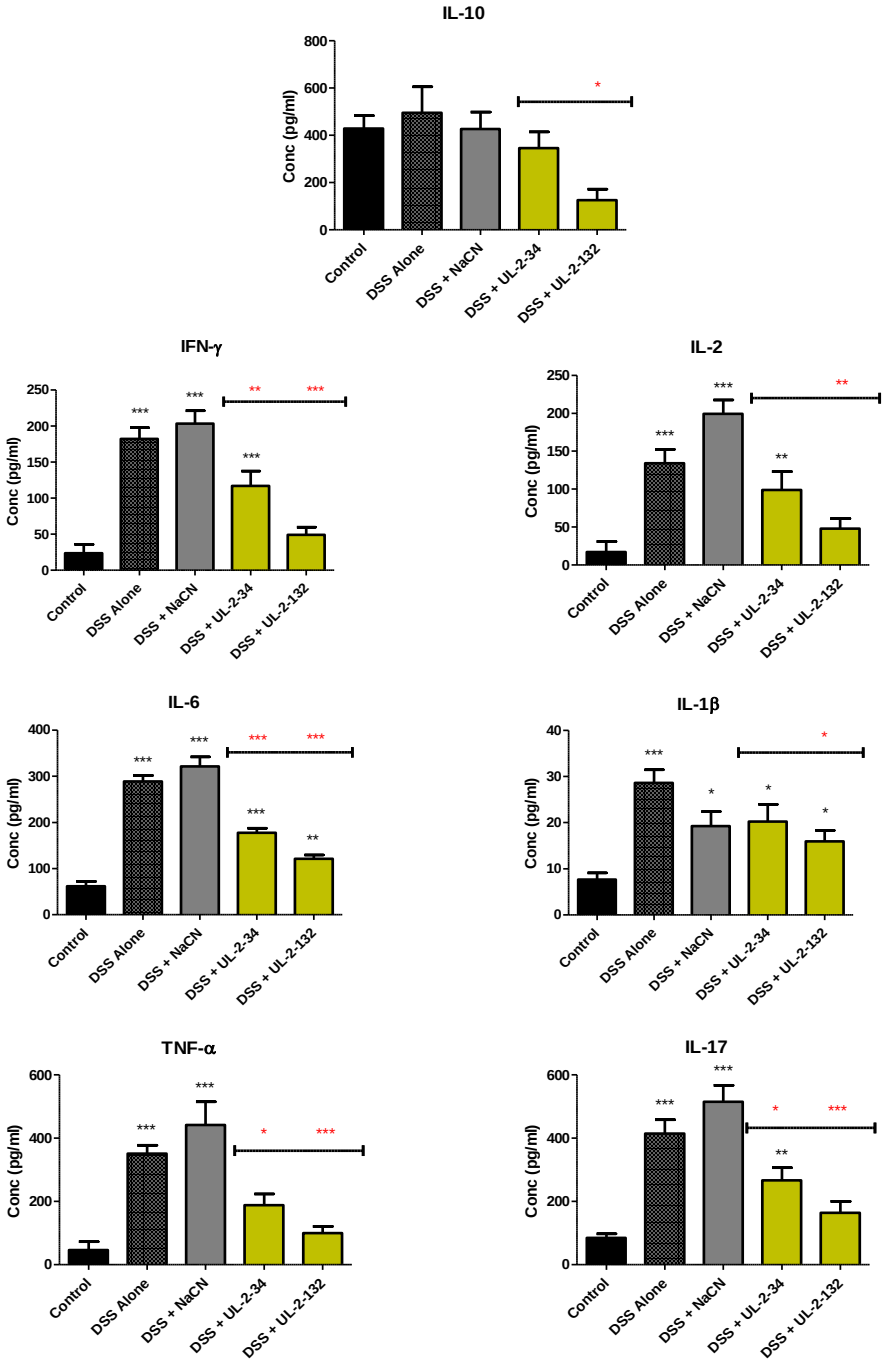


Figure 5.22: Cytokine secretion from unstimulated splenocytes in a mouse model of colitis treated with UL-2-34 & UL-2-132 Hydrolysates. BALB/c mice were grouped into a control group, a DSS only group, a DSS and NaCN treated group, a DSS and UL-2-34 treated group and a DSS and UL-2-132 treated group. Each group had 6 mice which were culled on day 14. Spleens were collected and splenocytes were plated at $5x10^6$ cells/ml on a 24 well plate. Cells were incubated for 72 hrs and Supernatants were collected and measured for IL-10, IFN-γ, IL-2, IL-6, IL-1β, TNF-α and IL-17 secretion using ELISA according to manufacturer's instructions (R&D duoset). Data presented indicate the mean ± SEM (n = 3) in triplicate. One way Anova with Newman Keuls post-hoc test was used to determine if differences between groups [Groups compared to control (black) and groups compared to DSS alone (red)] were significant (*p<0.05, **p<0.01, ***p<0.001).

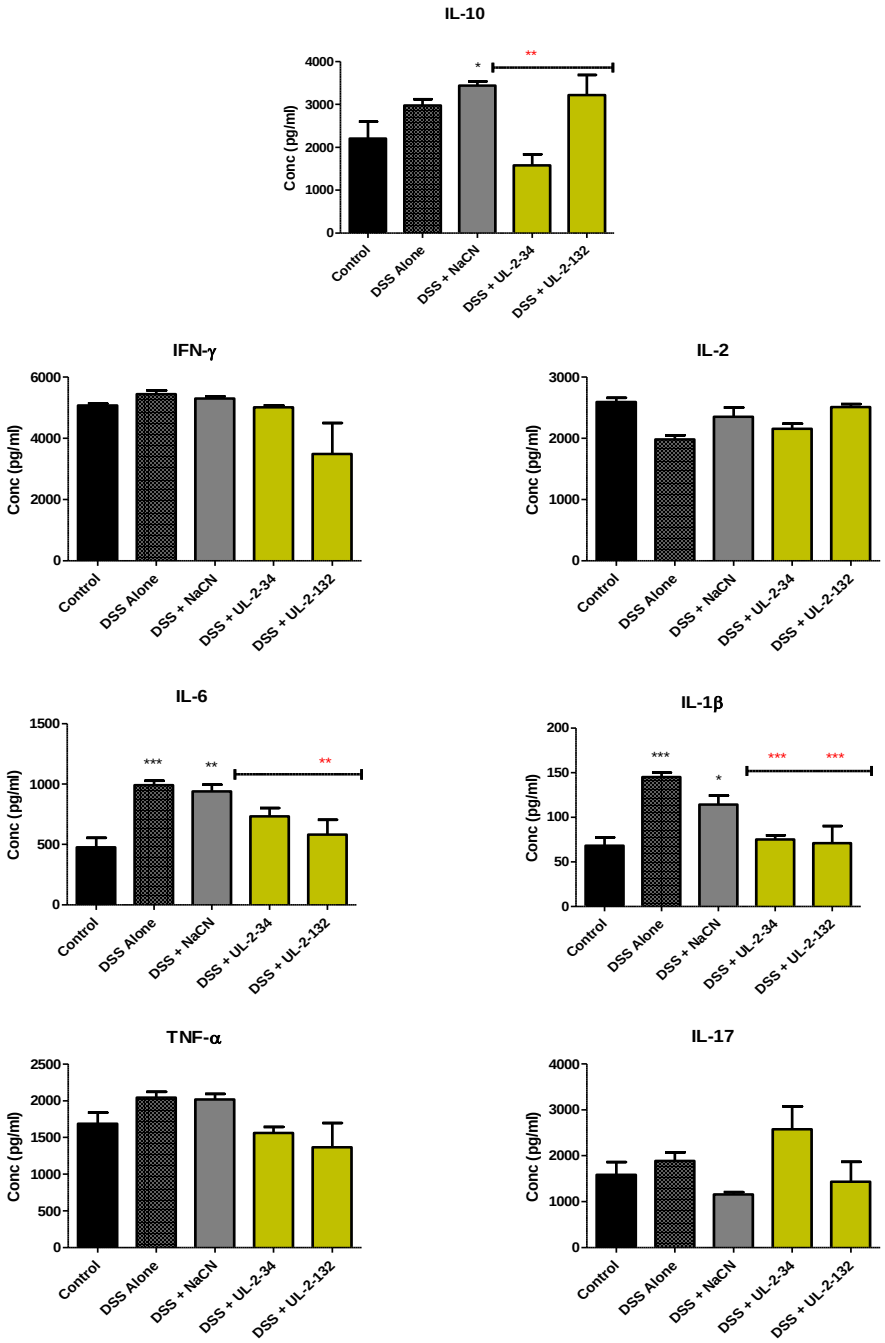


Figure 5.23: Cytokine secretion from stimulated splenocytes in a mouse model of colitis treated with UL-2-34 & UL-2-132 Hydrolysates. BALB/c mice were grouped into a control group, a DSS only group, a DSS and NaCN treated group, a DSS and UL-2-34 treated group and a DSS and UL-2-132 treated group. Each group had 6 mice which were culled on day 14. Spleens were collected and splenocytes were plated at $5x10^6$ 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 hrs and Supernatants were collected and measured for IL-10, IFN-γ, IL-2, IL-6, IL-1β, TNF-α and IL-17 secretion using ELISA according to manufacturer's instructions (R&D duoset). Data presented indicate the mean ± SEM (n = 3) in triplicate. One way Anova with Newman Keuls post-hoc test was used to determine if differences between groups [Groups compared to control (black) and groups compared to DSS alone (red)] were significant (*p<0.05, **p<0.01, ***p<0.001).

5.3 DISCUSSION

NEC is an inflammatory disorder, similar to IBD, which affects the gastrointestinal tract of infants, particularly preterm neonates, worldwide. Mortality rates can be as high as 50% depending on disease severity and relapse is common amongst recovered infants (Gephart et al. 2012; Fine, 2008; and Malaty et al. 2010). Little research has been carried out on the development of novel treatments for NEC as the main focus has been on disease prevention (Lapillonne et al. 2016). It is understood that certain cow's milk (CM) hydrolysates possess a multitude of immunomodulatory effects. Bioactive hydrolysates have been discovered that suppress inflammation, which is well demonstrated in this chapter (Cross ML and Gill HS, 2000). The aim of this chapter was to identify novel CM whey and casein protein hydrolysates that possess positive immunomodulatory properties that could be used to dampen a dysregulated excessive inflammatory response, whilst also retaining or bringing about immune balance, in the treatment of NEC in infants. It is hoped that these hydrolysates will bring a better quality of life to infants and avoid potential relapse and subsequent inflammatory problems in later life. We first examined their ability to modulate cytokine secretion from undifferentiated $CD4^+$ T-cells and T-cell subsets *in vitro*, from which candidate hydrolysates were selected based on their anti-inflammatory activity to be assessed on their effectiveness in an *in vivo* murine model of inflammatory bowel disease (IBD). The hydrolysate's ability to modulate a plethora of cytokines and biomarkers associated with NEC was examined.

The first aim of this chapter was to use the clearly defined T-helper cell subsets, as outlined in Chapter 3, to identify candidate hydrolysates that can modulate the immune response away from a harmful excessive inflammatory T-helper 1 (Th1) and/or T-helper 17 (Th17) response *in vitro* and bring about immune-balance. The immune response in the gut is regulated by the Th1/Th2 cytokine balance, which tends to favour a Th1 phenotype in most human intestinal inflammatory conditions (Monteleone et al 2002; and Hansson et al.1999). IBD is characterised by an imbalance between Th17 and/or Th1 cells and Treg cells. An unwanted boost in Th1/Th17 response results in excessive production of IFN and IL-17, respectively, which dampens the Treg response and expression of IL-10 and TGF-β (Bouma and Strober, 2003). It has recently been shown that Th17 cells express a high level of plasticity in IBD. Such studies have demonstrated that Th17 cells possess the ability to produce

IFN-γ as well as upregulate the Th1 transcription factor, t-bet, resulting in both Th1/Th17 phenotypes working in synergy with an enhanced inflammatory response. This Th1/Th17 synergetic population has been found in the inflamed gut mucosa. Th1 cells, however, are the main subset responsible for the onset of chronic IBD (Lee et al. 2009). Whilst it is evident that T-cells also have a role to play in NEC, very few studies have determined a dominant T-cell phenotype expressed in NEC. A high percentage of $CD4^+$ T cells have recently been shown to be present in the intestine of mice and humans with NEC (Denning et al. 2017; and Egan et al. 2016). Some studies suggest an imbalance of Th17/Treg cell populations contribute to the onset of the disease, as they have observed raised levels of Th17 cells and a reduction of Foxp3-expressing Tregs in the intestine (Weitkamp et al. 2013). Such studies have identified that blocking of IL-17 receptor or STAT3 lowered disease severity, suggesting a role for Th17 cells in promoting intestinal damage. Studies have shown that an excessive Th1 state has also been linked to the progression of NEC. Takeda et al. (1999) observed an increase in the Th1 cytokines, IFN-γ and TNF-α, in mice with chronic enterocolitis. The first step of this objective was to determine what effect the hydrolysates have on the polarisation of T-cell subsets by examining cytokine secretion from undifferentiated $CD4^+$ T-cells treated with hydrolysates. Cytokines play a key role in the pathogenesis and progression of inflammatory disease and contribute to the overall disease severity and therefore were the key focus of our *in vitro* studies (Hsueh et al. 2003). Treatment of undifferentiated CD4+ T-cells with UL-2-33/34/35/36/37/38 hydrolysates resulted in a significant reduction of IFN-γ secretion, the key cytokine required to drive a Th1 response. UL-2-40/41/42/43/47 treatment significantly decreased secretion of the cytokines necessary for Th17 polarisation, IL-6 and IL-17. These candidate hydrolysates were then brought forward for testing in Th1 and Th17 cell subsets. The hydrolysates demonstrated desirable effects in dampening the Th1 response, however, no positive effect on Th17 cells was observed. A significant reduction of IFN-γ secretion and non-significant reduction of IL-2, the two characteristic Th1 cytokines, from Th1 cells was observed following UL-2-33/34/35 treatment, which confirmed their ability to suppress a Th1 response. The hydrolysates were not effective in dampening the Th17 response. UL-2-40/41/42/43/47 enhanced IL-17 secretion from Th17 cells, which suggests they exert pro-inflammatory effects, driving a Th17 response. For this reason these hydrolysates were not examined further in Th17 cells. UL-2-33/34/35 demonstrated the potential to

restore immune-balance by dampening the inflammatory Th1 response whilst also retaining a basal level of secretion of IL-4, IL-6 and IL-17 from undifferentiated T-cells, and were therefore selected for further examination.

The next step was to assess the reproducibility of the bioactivity of UL-2-33/34/35. UL-2-33/34/35 were regenerated and labelled UL-2-83/84/85, respectively. Regenerated hydrolysates were examined in undifferentiated and Th1 cells alongside the parent hydrolysates. All regenerates behaved in a similar manner to their parent hydrolysate, confirming their bioactivity and reproducibility.

The overall aim of this project was to identify novel CM hydrolysates suitable for commercialisation and incorporation into infant milk formula. As mentioned in the previous chapter, it was, therefore, necessary to ensure that candidate hydrolysates were suitable for large scale production and desirable for industry use. The next step was to screen optimised candidate hydrolysates to determine whether the hydrolysates could be optimised further while maintaining or enhancing their bioactivity in order to make them more desirable for industry production and thus increase their potential for commercialisation. Optimised hydrolysates were produced for UL-2-34 and UL-2-35. UL-2-33, which was also of interest, was no longer deemed a viable option going forward at this point as the enzyme preparation used for this hydrolysate was no longer commercially available. Optimised hydrolysates, UL-2-132/133/134 and UL-2-136/137/138, produced from parent hydrolysates UL-2-34 and UL-2-35, respectively, were tested in undifferentiated T-cells and Th1 cells *in vitro*. Details of these hydrolysates can be seen in **Table 5.2** in **Section 5.2**. All optimised samples behaved in a similar manner to the parent and regenerated hydrolysates and it was, therefore, difficult to rank them in terms of their potency or choose the most ideal candidate to bring forward for *in vivo* testing. UL-2-34 and UL-2-132 were chosen to be scaled up for animal trials on the basis that their hydrolysis was carried out using uncontrolled (free-fall) pH conditions. The other optimised hydrolysates were hydrolysed using controlled (pH-stat) conditions. Alkaline or acidic solutions are used to maintain constant pH during pH-stat experiments. UL-2-35 and its optimised hydrolysates could no longer be brought forward as a result of its enzyme preparation being no longer available. At this point, a dose response study was carried out on UL-2-34 and UL-2-132, which confirmed that the suppression of IFN-γ in Th1 cells was dose dependent. UL-2-34 and UL-2-132 were scaled up at industry level in the BFE plant

in Teagasc, Moorepark, Co. Cork for *in vivo* studies. The activity of the scaled up hydrolysates was compared to the hydrolysates produced at lab scale in Th1 cells. Hydrolysate bioactivity between the small and large scale production correlated, which finalised the *in vitro* work in this chapter. Overall, through *in vitro* screening we successfully identified two NaCN hydrolysates, UL-2-34 and UL-2-132, which displayed anti-inflammatory activity. These hydrolysates consistently suppressed an inflammatory Th1 phenotype. Whilst our studies did not identify any hydrolysates that targeted a Th17 phenotype, our collaborators in National University of Maynooth (NUIM), working on humanised mouse models, showed that UL-2-34 and UL-2-132 also decreased expression of the Th17 transcription factor, ROR-γ (data not published yet), which adds to the overall anti-inflammatory profile of these hydrolysates. Interestingly, the anti-inflammatory properties of UL-2-34 and UL-2-132 observed up to this point in this chapter would be desirable and potentially applicable in the case of inflammatory disease when the immune system is skewed in excess towards a Th1 and/or potentially a Th17 response.

The next step was to assess the capacity of UL-2-34 and UL-2-132 to modulate the immune response away from an excessive inflammatory response and alleviate the symptoms associated with intestinal inflammation in an *in vivo* murine model of inflammatory bowel disease (IBD), also known as colitis. NEC and IBD are associated with increased expression of inflammatory cytokines. These cytokines directly disrupt the epithelial barrier and drive intestinal destruction and are, therefore, logical therapeutic targets for developing novel treatments (Laukoetter et al. 2008). This model has been carried out in the research group previously and involved the induction of colitis in mice, using dextran sulfate sodium (DSS). The capacity of the hydrolysates to treat the inflammatory symptoms and reduce disease pathology was assessed. DSS results in the development of colitis similar to what would also develop in humans (Chassaing et al. 2014). DSS compromises the integrity of the intestinal barrier, being toxic to the epithelial layer in the colon, resulting in erosions, altered expression of tight junction proteins and thus, increased epithelial permeability and infiltration of pro-inflammatory cytokines. Intestinal bleeding typically follows DSS administration as DSS is also an anticoagulant. Pathology typically presents in the large intestine, particularly the distal colon. For this reason most of our analysis was focused on tissue from the distal colon of mice. The clinical symptoms, such as weight

loss, diarrhea and bloody stools, and histological features induced by DSS are an accurate representation of human IBD (Chassaing et al. 2014; and Laroui,et al. 2012). While the DSS model is a model of IBD, it also relevant for studying the effect of therapeutics targeting NEC. Ginzel et al. (2017) compared an established LPS induced NEC murine model with a DSS adult murine model of IBD. The lesion size was greater, bacterial growth was more pronounced and luminal erythrocytes number increased in the DSS group compared to the LPS group. The DSS model displayed identical histology to that found in NEC as well as increased humoral and cellular immune responses in the small and large intestine of mice. This suggests that the DSS model is a suitable, if not superior, model for the induction of NEC-like mucosal tissue lesions and was, therefore, the model system of choice to examine the anti-inflammatory effects of the candidate casein hydrolysates in this chapter. In addition to this, the DSS is the most suitable model/was the preferred model as it does not require physical stressors such as hypoxia and/or hypothermia, which are harmful to the mice. DSS-induced colitis can be characterised by a Th1, Th2 or Th17 response depending on the length of DSS exposure. Short-term DSS treatment in mice drives an acute inflammatory cytokine cascade: TNF-α, IL-1β, IL-6, IL-10, IL-12, IL-17 and IFN-γ being the most potently expressed mediators. Exposure to DSS long-term induces a chronic inflammatory state, which is characterised by a strong Th2 cytokine profile particularly IL-4 and IL-5 (Jiminez et al. 2015; and Yan et al. 2009). We carried out an acute model to examine the ability of the hydrolysates to inhibit the pro-inflammatory Th1 and Th17 associated cytokines *in vivo* and to assess the translation of our *in vitro* results.

Administration of 5% DSS to mice for 7 days elicited the typical clinical symptoms of colitis including mild weight loss, diarrhoea, bloody faeces, crypt loss, destruction of the epithelial layer, significant reduction in colon length, increased colon weight and infiltration of pro-inflammatory cells. These clinical symptoms were efficiently reversed by the treatment of UL-2-132 hydrolysate treatment. A positive effect was also observed with UL-2-34 treatment. By the final day of the model UL-2-34, UL-2-132 and NaCN treatments all significantly reduced the DAI score compared to untreated DSS-induced mice, however UL-2-132 was most effective as DAI was almost at the same level as control mice, indicating that treatment with UL-2-132 reversed the symptoms such as appearance of hemoccult and diarrhoea which resulted

in a significant reduction of DAI in these mice. Whilst UL-2-34 and NaCN treatments also reduced the clinical symptoms in DSS-induced mice, their DAI was still quite high on the final day. Although UL-2-34 and UL-2-132 treatments did not restore body weight of DSS-induced mice back to a baseline control level, the significant weight increase compared to untreated DSS-induced mice indicates a protective effect on colitis-induced body weight loss. It may be possible that by continuing or prolonging the treatment course or increasing the dose of treatment would continue to improve this parameter. UL-2-132 reversed inflammation induced reduction in colon length and increase in colon length. UL-2-34 had no effect on the reduction of colon length but reversed the inflammatory increase in colon weight. Additionally, UL-2-132 treatment restored histopathology in the gut, as shown by the H&E stained colonic sections.

UL-2-132 demonstrated consistent anti-inflammatory activity in dampening the pro-inflammatory cytokine profile associated with DSS. We first examined the hydrolysates' effects on mRNA cytokine expression levels in the colon. Treatment with UL-2-132 in DSS treated colons completely reversed the DSS associated spike in IL-1β, IL-17 and IL-6 mRNA gene expression, as levels were identical to those of control mice. IL-1β, IL-17 and IL-6 are all linked to a Th17 phenotype and play harmful roles in NEC. Cho et al. (2016) states that increased IL-1 aggravates tissue damage and contributes to the onset of NEC. They also suggest that IL-1 is responsible for disease severity, as IL-1β is most enhanced in stage III NEC in infants compared with stage I and II infants (Edelson et al. 1999). IL-1β is a member of IL-1 family and plays an important role in the Th17-mediated immune response (Mukhopadhya et al. 2014; and Chung et al. 2009). IL-6 also plays a role in disease severity and with short-term survival in NEC (Harris et al. 1994; and Sharma et al. 2007). Increased risk of neonatal morbidity has been associated with excessive IL-6 levels in fetal blood (Goepfert et al. 2004). IL-6 plays a key role in the polarisation of Th17 cells. IL-17 is the key player responsible for the pro-inflammatory activity of Th17 cells (Cătană et al. 2015; and Bettelli et al. 2007). Egan et al. (2016) found that intestinal IL-17 and IL-17 receptor (IL-17R) are increased in both mouse and human NEC. It was mentioned at the beginning of the discussion, that blocking of IL-17R or STAT3 resulted in improvement in disease severity. Collectively, these studies suggest that a Th17 dominant population may characterise our DSS study and reduction of IL-1β,

IL-17 and IL-6 by UL-2-132 treatment would be beneficial in the case of NEC and IBD. Furthermore, expression of IL-10 was slightly reduced in untreated DSS-induced mice but UL-2-132 treatment almost restored these levels back to those of control mice. Although whole NaCN treatment was as effective as UL-2-132 in reversing IL-6 expression from DSS-induced mice, UL-2-132 was more effective than whole NaCN treatment in lowering and restoring IL-1β and IL-17 levels to those of control mice, which indicates improved activity following its hydrolysis.

Major differences can also be observed between whole NaCN and UL-2-132 treatments upon examination of cytokine secretion patterns from splenocyte culture. NaCN resulted in a further increase in the pro-inflammatory IFN-γ, IL-2, IL-6, TNF-α and IL-17 inflammatory cytokine spike that is associated with DSS-exposure in unstimulated splenocytes. Whereas, a significant reduction in the DSS associated spike in these cytokines as well as IL-17 was observed in both UL-2-34 and UL-2-132 treated groups, UL-2-132 displaying the most effective response by completely reversing the effects of DSS as we observed a similar level of cytokine secretion from mice treated with UL-2-132 compared to control mice. While IFN-γ, IL-2, IL-6 and IL-17 were significantly reduced following UL-2-34 treatment compared to untreated DSS treated splenocytes, they were still significantly increased when compared to control mice. While cytokine secretion levels were not as high as those observed from stimulated cells, all pro-inflammatory cytokines were significantly increased in unstimulated splenocytes from DSS-induced mice compared to control mice. This indicates that inflammatory markers are still present at low levels in the spleen and were targeted by UL-2-34 and UL-2-132 hydrolysates.

Similar to the Th17 inflammatory cytokine expression profile observed in DSS-treated colons, we observed a significant increase in IL-6 and IL-1β secretion in stimulated splenocytes from DSS-induced mice compared to healthy controls, indicating an inflammatory presence in the spleen. IL-6 and IL-1β are both commonly secreted from DCs. However, Melgar et al. (2011), leading researchers in the area of the DSS model, observed a decrease in splenic DCs and subsequent increase in DCs in the colon from day 5 onwards in their DSS study, which suggests the transport of DCs from the spleen into the colon from this point on. It is, therefore, unlikely that a high DC population exists in the spleens of our mice at the end of the study, due to their migration to the colon. IL-1β is also secreted from neutrophils and IL-6 from macrophages. It is

possible that macrophage and neutrophils may explain the excessive levels of IL-6 and IL-1β in the spleen following stimulation. A possible role for IL-6 in this scenario may be to enhance further production of Th17 cells, creating an inflammatory feedback loop, resulting in an excessive inflammatory response. Therefore, UL-2-34 and UL-2-132 may exert their activity by targeting these cell types in the spleen, as their treatment in DSS-induced mice significantly reduced secretion of both of these cytokines in stimulated splenocytes compared to untreated DSS-induced mice. UL-2-132 was most effective as it reversed the DSS associated increase in these cytokines back to the levels of healthy control mice. Few differences were observed between stimulated splenocytes in test groups and control mice, after examination of the remaining cytokines in the profile. As secretion of IFN-γ, IL-2, TNF-α and IL-17 from stimulated splenocytes in DSS-induced mice was similar to those in control mice, and an increase in IL-17 expression was observed in DSS treated colons parallel to this, this indicates a transfer of immune cells, most likely Th17 cells, from the spleen into the colon in DSS-induced mice.

Interestingly, expression of both IFN-γ and TNF-α (characteristic Th1 cytokines) was significantly reduced in DSS treated colons, which indicates a lack of a Th1 response. Many studies observe an increase in systemic and intestinal TNF in NEC patients when compared to healthy controls (Caplan et al. 1990; and Baregamian et al. 2009). Whilst, in our DSS model, we observed a significant increase in TNF-α secretion from unstimulated splenocytes cultured from DSS-induced mice compared to control mice, a significant reduction in TNF-α mRNA from colonic tissue of DSS-induced mice was also observed. Benkoe et al. (2013); Harris et al. (1994); and Morecroft et al. (1994) observed that presence of TNF has no effect on disease severity. Studies using monoclonal anti-TNF antibodies, such as pentoxiphylline, infliximab or etanercept, which have demonstrated their ability to reduce intestinal inflammation and tissue destruction, suggest that TNF contributes to NEC progression, likely with a major role in the early stages of the disease (Travadi et al. 2006; and Yurttutan et al. 2014). TNF-α is also responsible for IL-6 activation, which contributes to the severity of clinical symptoms. TNF-α may, therefore, only be detectable immediately after onset of NEC (Romagnoli et al. 2001). As our model is 14 days into disease state, this may explain our decreased levels of TNF-α mRNA in DSS-induced mice compared to controls. It is possible that TNF played a role in the onset of colitis in mice at the beginning of the

study but as the disease progressed, TNF levels were decreased. Evidence is limited on the use of TNF as a biomarker in NEC. Furthermore, De Plaen (2013) also reports that anti-TNF had no beneficial effect in both a model of acute bowel injury induced by PAF and in a neonatal rat NEC model. An increase in IFN-γ was expected in our DSS-induced mice as a increased IFN-γ is associated with neonatal rats and infants with NEC. However, Simpson et al. (1998) questions the role of IFN-γ in NEC development as they state that mice deficient in IFN-γ still developed colitis when stressed. As our *in vitro* studies suggest that UL-2-34 and UL-2-132 may play a beneficial role in reducing disease associated with an overactive Th1 response, we hoped to examine this further in *in vivo* studies. The lack of Th1 response in DSS mice did not allow for this assessment in the colon. We did, however, observe a significant increase in IFN-γ, IL-2 and TNF-α secretion from DSS treated unstimulated splenocytes which was significantly reduced by UL-2-34 and UL-2-132, in fact UL-2-132 treatment completely reversed this effect.

It is noteworthy that UL-2-34 and UL-2-132 treatments in DSS-induced mice did not curtail the ability of splenocytes to mount an immune response to an external stimulus, as levels of all cytokines examined (IFN-γ, IL-2, IL-6, IL-1β, TNF-α and IL-17) in splenocytes stimulated with plate-bound CD3 plus CD28 from DSS-induced mice treated with UL-2-34 or UL-2-132 were almost identical to those of control mice. Major concerns currently exist around the possible harmful side effects from anti-cytokine and immuno-modulatory therapy in preterm infants because many of these pro-inflammatory cytokines play important developmental roles in the gut mucosa and mucosa-associated immune system. Switching these cytokines off could thus, prevent these important developments in infants during a primal time or leave them susceptible to infections (Maheshwari et al. 2014). As UL-2-34 and UL-2-132 treatments did not inhibit the splenocytes capacity to secrete cytokines in response to an external stimulus, this indicates that this may be a key advantage over anti-cytokine therapy and drugs which result in adverse side effects, leaving the patient open to infection. Furthermore, UL-2-132 treatment reduced IL-10 secretion in an unstimulated state in DSS-induced mice (in unstimulated splenocyte culture), yet demonstrated similar IL-10 secretion levels as healthy control mice and untreated DSS-induced mice after stimulation, suggesting that this may be another mechanism of the hydrolysate in order to prevent susceptibility in the host. IL-10 is known to switch off pro-inflammatory

responses and excessive upregulation of IL-10 could leave the host open to infection. In keeping IL-10 low, UL-2-132 allows pro-inflammatory cytokines to retain their ability to mount an immune response to potential pathogens. The IL-10 secretion pattern also suggests a potential therapeutic mechanism for UL-2-132 treatment. As UL-2-132 treatment reduced IL-10 secretion in an unstimulated state in DSS-induced mice and did not enhance IL-10 levels in these mice following stimulation, it is likely that UL-2-132 does not require IL-10 to exert its anti-inflammatory effects, indicating further that it may possess anti-inflammatory ability itself, being one of its *in vivo* therapeutic effects. Further work is required, however, to fully understand this as a potential mechanism.

UL-2-34 treatment, however, was not as consistent in its effects, displaying a mix of both pro- and anti-inflammatory activity. Whilst UL-2-34 treatment did demonstrate protective effects against some of the pro-inflammatory cytokines in the spleen, in unstimulated splenocytes and a reduction of the DSS-associated increase in IL-6 and IL-1β in stimulated splenocytes, a negative effect of UL-2-34 treatment was observed in the colon. The increase in mRNA expression of TNF-α and IFN-γ following UL-2-34 treatment in DSS treated colons may suggest that the DSS associated inflammatory loop is still active in these mice. Additionally, UL-2-34 treatment also resulted in reduced IL-10 and increased IL-17 levels in DSS-treated stimulated splenocytes when compared to both untreated DSS-induced mice as well as control mice, suggesting involvement of Th17 cells. The DAI of UL-2-34 treated mice was also higher than that of untreated DSS-induced mice and treatment had no effect on reversing the shortening of colon length associated with DSS administration. As all analysis was carried out 7 days after DSS exposure was terminated, in order to assess the effectiveness of our hydrolysate treatments, this may have allowed time for untreated DSS-induced mice to improve slightly. It is possible that UL-2-34 treatment actually allowed the disease to remain active or aggravated these symptoms further. The reduction in IL-10 in stimulated splenocytes and simultaneous increase in IL-10 mRNA in DSS-treated colons associated with UL-2-34 treatment also suggests that the disease is still active in these mice. Benkoe et al. (2013) state that an increase in IL-10 is often associated with human NEC, even more so in patients with advanced NEC. They reported an increase in IL-10 levels from infants with NEC compared to healthy controls. They suggest that the most likely explanation for this is that it is the

immune system's attempt to switch off the excessive inflammation. Other studies have also reported an increase in IL-10 secretion in the serum of newborns with NEC (Harris et al. 2005; Romagnoli et al. 2001; Edelson et al. 1999; and Chan et al.2012). It thus appears likely that, in our study, IL-10 has been upregulated in the colon in an attempt to dampen the inflammation, indicating that UL-2-34 treatment is not effective in doing so itself. The precise role of IL-10 in NEC pathogenesis remains unclear.

Infants who suffer with NEC, particularly preterm infants, have an increased risk of sensitisation to intact proteins and developing CMPA. This link between NEC and CMPA development suggests that extensively hydrolysed formulas (EHF) may be a useful method for refeeding infants following recovery of NEC. Extensive hydrolysis of a protein results in complete destruction of the allergic IgE epitopes, which prevents sensitisation and onset of clinical symptoms. On average 12.1% of infants are using EHFs as refeeding strategy following NEC to prevent sensitisation to milk proteins (Lapillonne et al. 2016). CM EHFs have a high nutritional value and possess many other beneficial effects for infants recovering from NEC. The use of EHF may improve absorption during refeeding due to the significant amount of medium-chain triglycerides they often contain and their lack of lactose. Available mucosal lactase is limited in infants who have NEC and other intestinal disorders. As a result, lactose is poorly tolerated, and thus, a formula free of lactose would be desirable for infants post NEC (Sondheimer, 2006). Although EHF are currently used to prevent the onset of CMPA in infants recovering from NEC, limited studies have assessed the ability of EHF to modulate the immune system to prevent subsequent NEC relapse, making this a novel research area. UL-2-34 and UL-2-132 have high degree of hydrolysis (DH) values of 48.2% and 30.9%, respectively. This high DH value results in extensive hydrolysis and break down of the protein, making them particularly desirable for NEC infants at risk of sensitisation to CM proteins. While it is rare that extensively hydrolysed peptides possess immunomodulatory properties, UL-2-132 seems to be an excellent candidate for NEC refeeding, as it possesses excellent therapeutic anti-inflammatory bioactivity, unique for EHF, whilst also being extensively hydrolysed.

The pro-inflammatory cytokines such as IFN-γ, IL-2, IL-6, IL-1β, TNF-α and IL-17 have a pivotal role in the pathogenesis of inflammatory conditions of the GIT including IBD and NEC. Several studies have also reported that interaction between pro-inflammatory cytokines and the intestinal mucosal immune system can lead to the

disruption of tight junction proteins and affect intestinal homeostasis (Abraham and Medzhitov, 2011). The potential of the CM NaCN hydrolysate, UL-2-132, to suppress inflammatory cytokines has, therefore, particular relevance to IBD and NEC. While therapeutics are currently available for NEC and IBD, relapse and further inflammation is common amongst recovered patients. The identification of natural modulatory bioactives, such as CM hydrolysates, holds strong potential as an alternative form of therapy that may prevent recurring inflammation as they can be taken as part of a balanced diet without the onset of adverse side effects (Khan et al. 2012).

Taken together, our results suggest that UL-2-132 was able to decrease local levels of pro-inflammatory cytokines in the spleen as well as cytokine expression levels in the colon at the mRNA level. UL-2-132 effectively reversed the progression of the DSS induced colitis symptoms in BALB/c mice. For now, we suggest the underlying mechanism of UL-2-132 may potentially be associated with inhibition of two of the key cells involved in IBD etiopathology, Th1 and Th17 cells, simultaneously. *In vitro* results demonstrated that UL-2-132 inhibits a Th1 subset, while *in vivo* results suggest Th17 as an additional potential target of this hydrolysate. It is possible that UL-2-132 targets IL-6 secretion from macrophage and IL-1β from neutrophils in the spleen as well as IL-6 and IL-17 associated with Th17 cells in the colon in an attempt to dampen the DSS-induced inflammatory response and prevent further intestinal injury. It is possible that inhibition of this pro-inflammatory cytokine cascade then leads to inhibition of NF-κB signaling activation, associated with IBD and NEC. Moreover, another possible mechanism we suggest is that UL-2-132 alleviates inflammatory responses, acting as an anti-inflammatory agent directly itself rather than upregulating anti-inflammatory cytokines, such as IL-10, which may decrease or prevent the onset subsequent adverse side effects and host susceptibility to infection. At present we have insufficient evidence to draw a conclusion on the mechanism of action of UL-2-132, we can only make collective suggestions from our *in vitro* and *in vivo* results to date. Further studies are required to develop a deeper understanding of these exact mechanisms, using the results from this chapter as a guideline.

This chapter demonstrates the consistent anti-inflammatory bioactivity of a novel CM NaCN hydrolysate in both *in vitro* and *in vivo* inflammatory models. Collectively, our results suggest that UL-2-132 has the potential to serve an as a novel and effective

anti-NEC/IBD therapy, given the similar pathogenesis of NEC and IBD, and indicate that further analysis of UL-2-132 would be valuable in the exploration of its potential as an anti-inflammatory functional food ingredient (Harpavat et al. 2012). Further, it would be of great interest to investigate therapeutic benefit of UL-2-132 treatment in already established IBD and NEC.

CHAPTER 6

GENERAL DISCUSSION

6.1 GENERAL DISCUSSION

It is widely accepted that diet is a major impact on our health and in the development of certain diseases. Protein has a key role in aiding the development of the immune system, particularly during infancy, boasting both antimicrobial and immuno-modulatory properties (Van Loon, 2007; and Raikos and Dassios, 2014). Cow's milk (CM) is an abundant source of protein and provides infants with the adequate nutrition required for optimal growth and immune development. Protein hydrolysates have become a particular focus for infant development. Proteins that undergo hydrolysis can result in the release of biologically active peptides that are inactive in the precursor protein sequences. The bioactive peptides found in hydrolysates possess additional immunological properties to the whole protein and have been demonstrated to modulate the immune system for benefit. They have been shown to display anti-inflammatory effects and stimulate the intestinal epithelial barrier, which may be useful in the setting of food allergy and intestinal inflammatory conditions (Kiewiet et al. 2015). Our understanding of protein hydrolysate diets is growing and currently much research is focussed on their potential health benefits. Many studies indicate that they may be a beneficial addition to pharmacological therapy to compliment the overall effect, or in some cases they have even been shown to confer a beneficial therapeutic effect on their own. Marks et al. 2002 used a hydrolysed soy-protein diet alone to treat dogs with IBD. Histological improvement was observed in 4 of 6 treated dogs. Swallow, (2017) carried out a study using 18 dogs with chronic enteropathy to compare a hydrolysed diet to a highly digestible diet. They concluded that the hydrolysed diet was much more effective for the long-term management of the chronic small-bowel enteropathy. By tailoring the hydrolysis conditions it is possible to yield novel protein hydrolysates with specific bioactivity. Food for Health Ireland (FHI) employs an intelligent milk mining approach, with a vision to identify novel protein hydrolysates that have scientifically proven health benefits. The aim of this project was to identify novel CM hydrolysates that can modulate the immune system for benefit in the field of infant nutrition. Although breast milk is considered the gold standard for feeding in the first six months of life, in cases where it is not possible we aim to identify hydrolysates that can mimic the modulatory effects of breast milk in order to prevent immune disease. The identification and use of these bioactive peptides

as a functional food incorporated into the diet could prove extremely beneficial in the future for the food industry as well as contribute to the overall health of society.

It is understood that the gastrointestinal tract and immune system of newborns are immature, leaving them vulnerable to infection, as well as prone to autoimmune disorders. Establishment of an appropriate balance between self-tolerance to the commensal gut microbiota and oral tolerance to harmless food antigens, while maintaining the ability to induce a response to a potential pathogen, known as immune homeostasis, can take up to three years to set in/generate (Crimeen-Irwin et al. 2005; Koenig et al., 2011; Weng & Walker, 2013; and Yang et al. 2016). The human gut harbours approximately 70% of the entire immune system (Vighi et al. 2008). The gastrointestinal immune system plays a major role in the development of oral tolerance and immune homeostasis and it is understood that a healthy intestinal microbiome displays beneficial immune-modulatory activity that leads to this development and maintenance of immune tolerance (Pabst and Mowat, 2012). Failure to develop mucosal immunity and establish immune balance in the infant gut is the cause of many immune disorders, such as food allergy and intestinal inflammatory disease (IBD), also known as necrotising enterocolitis (NEC) in infants. For this reason most research focuses on targeting the gastrointestinal tract of infants when developing therapeutics linked to immune dysregulation during infancy (Kiewiet et al. 2015).

$CD4^+$ T-cells are key cells of the adaptive immune response, which recognise peptides presented on major histocompatibility complex (MHC) class II molecules by antigen presenting cells (APCs**)**. Intestinal $CD4^+$ T-cells are mostly located in the lamina propia (LP) of the intestine (Wu and Wu, 2012). Depending on the type of pathogenic exposure, naive $CD4^+$ T-cells can differentiate into one of four T-helper cells - T helper 1 (Th1), Th2, Th17 or regulatory T-cells (Treg). Each $CD4^+$ T-cell subset has a unique function, characterised by distinctive transcription factors and cytokine profiles (Zhu and Paul, 2008). When homeostasis is achieved, each of the effector T-cell subtypes exists in a dynamic balance with Treg cells (Smith and Garrett, 2011). A dysregulation in this process or failure to develop immune homeostasis results in underactive or excessive and uncontrolled T-cell activation, which can lead to deleterious effects and often results in disease (Zhu et al. 2010). Excessive Th1 and Th17 responses have been associated with the onset of organ-specific inflammation, while the Th2 response is strongly associated with the development of allergic disease

(Mosmann et al. 1986). Treg cells are critical for the development and regulation of immune homeostasis and self-tolerance (Sakaguchi, 2004). Failure to induce Treg populations in infants can be pathological, resulting in failure to induce tolerance followed by the development of autoimmune disorders or allergy. The overall objective of this project was to identify CM protein hydrolysates that could bring about immune balance in infants in two different disease settings – allergy (Th2 favoured) and NEC (Th1 and/or Th17 favoured). In order to execute this objective, we first investigated the ability of various CM hydrolysates to modulate cytokine secretion from the effector T-cell subsets *in vitro*, in order to assess whether the hydrolysates could shift this skewed or favoured T-cell response towards a Treg response to bring about immune homeostasis. Candidate hydrolysates that displayed potential bioactivity *in vitro* were then assessed in *in vivo* models of allergy and IBD, in an attempt to confirm their efficacy and demonstrate the capacity of their therapeutic ability in models of gut-associated pathology**.**

Infants are susceptible to developing food allergy. Their poor ability to digest proteins and increased epithelial layer permeability leaves them in danger of larger proteins crossing the gastrointestinal barrier and influencing the development of allergy (Perrier and Corthésy, 2011; and Vickery et al. 2011). The allergic potential of peptides is dependent on the level of exposure and their size, larger peptides being more allergenic and increasing risk of sensitisation to food proteins. Once crossed over the gastrointestinal barrier, these larger peptides can activate and over-express a type 2 helper T-cell (Th2) phenotype. Th2 cells are the key drivers of allergy and secrete IL-4, IL-10, IL-13 and IL-5; cytokines that aid in the persistence of allergic reactions (Kiewiet et al. 2015; Takastu 1997). The contribution of these cytokines to allergy induction and persistence has been well documented, making them valuable disease biomarkers for our study (El-housseiny et al. 2017; Deo et al. 2010; and van Ree et al. 2014). The immune system of newborns naturally favours the Th2 response, which results in dampened Th1 and Treg responses (Prescott et al. 1998; Vighi et al. 2008). Failure to establish a balance of the Th1/Th2-paradigm can result in allergic disease (Kidd 2003). As mentioned, Treg cells are a critical part of the tolerance induction process. TGF-β and IL-10, the characteristic cytokines secreted from Treg cells, promote induction of immune homeostasis and oral tolerance to food antigens and colonising commensal bacteria in the gut (Hori et al. 2003). As CM proteins are often

the first proteins infants are exposed to, cow's milk protein allergy (CMPA) is the most common food allergy amongst infants and children. Current dietary management of CMPA involves complete elimination of CM from the diet, through the use of extensively hydrolysed formula (EHF). However, accidental exposure is common, which can be life threatening. Therefore, there is an urgent requirement for the development of novel treatment or prevention strategies for food allergy in infants.

Using the clearly defined *in vitro* T-helper cell subsets, Th1, Th2 and Treg cells, optimised in Chapter three, we screened an array of whey protein concentrate (WPC80) and sodium caseinate (NaCN) CM hydrolysates. After determining their effects on cytokine secretion levels, we identified a whey hydrolysate, UL-2-147, that specifically modulated the immune response away from a Th2 phenotype without affecting the cells ability to differentiate into Th1 and Treg cells. UL-2-147 inhibited secretion of IL-4, IL10 and IL-13 levels from Th2 cells, whilst having no effect on IFN-γ from Th1 cells and IL-10 and TGF-β from Treg cells. Additionally, our DCU collaborators found that UL-2-147 treatment prevented IgE-mediated degranulation in mast cells. Furthermore, our collaborators in Maynooth University observed a similar response following treatment of humanised Th2 cells with UL-2-147, which was characterised by an inhibition of IL-4 secretion and GATA-3 (the key Th2 transcription factor) expression. Bioactivity of UL-2-147 is reproducible and its production is relatively low costing, following optimisation. This makes it a suitable hydrolysate for commercialisation and incorporation into infant milk formula, which is hoped to be the final outcome in the future of this project.

These *in vitro* results suggested that UL-2-147 may have beneficial immuno-modulatory effects in food allergy and may potentially balance the Th1/Th2 and/or Treg/Th2-paradigms in an *in vivo* setting of allergy, inducing immune homeostasis. We were, therefore, interested to observe these possible mechanisms in an OVA mouse model of gut-associated allergy. It is well reported that the OVA model, when sensitization is carried out using an aluminium hydroxide (alum) adjuvant, induces allergic disease associated with a Th2 phenotype, which was ideal for the objective of this project. It is known to be a rapid and very reliable food allergy model for the study of food allergy therapeutics. Following four successive oral challenges to OVA, UL-2-147 effectively reversed the clinical symptoms of allergy associated with OVA-sensitised mice when compared to PBS and WPC80 treated OVA-sensitised mice.

Symptoms scored included weight loss, hudddled posture and inactive, little response to handling, unkempt rough coat, swollen eyes with discharge, rapid and abdominal breathing. Furthermore, UL-2-147 also restored histopathology in the gut. Destructive features in allergic mice such as crypt loss, destruction of the epithelial layer and infiltration of pro-inflammatory cells were reversed by the hydrolysate treatment, as seen in the H&E stained distal colonic sections.

Few studies have examined the ability of whey hydrolysates to modulate immune populations in an allergic setting. Most animal studies that have examined the effects of hydrolysates on allergic sensitisation to date have focussed on measuring immunoglobulin levels (Kiewiet et al. 2017). We, therefore, next examined the effect of the UL-2-147 treatment on cytokine secretion. By assessing cytokine patterns this allowed us to determine what T-cell subsets were dominant in allergic mice and demonstrate the capacity of UL-2-147 to modulate immune populations and thus, induce immune balance between these cell populations. Cytokines play a key role in the pathogenesis and progression of allergic disease and contribute to the overall disease severity and are, therefore, key biomarkers to assess disease progression as well as assess the translation of our *in vitro* studies into *in vivo* (Brandt and Sivaprasad, 2011). As expected, UL-2-147 dampened the excessive allergic Th2 cytokine profile associated with food allergy in both the colons and spleens of mice. Reduction of IL-4, IL-6, IL-10 and IL-13 secretion from splenocytes as well as expression in colons was observed in OVA-sensitised mice, following UL-2-147 treatment. Splenocytes from mice were stimulated with OVA or CD3 plus CD28 or left unstimulated in culture. A significant reduction in the OVA-induced spike in the Th2 cytokines (IL-4, IL-6, IL-10 and IL-13) was consistently observed in the UL-2-147 treated splenocytes in all culture conditions examined. This was not observed in the WPC80 treated OVA-sensitised mice. Additionally, UL-2-147 treatment restored the allergy-associated spike in expression of Th2 cytokines in the colon back to the levels of control mice. A similar Th2 dampening effect was also observed in WPC80 treated mice, which indicates a therapeutic role for whole whey in food allergy. Van Esch et al. (2011) observed similar results with regard to whole whey treatment. Following oral challenge with whey in whey-sensitised mice, both whole whey and partially hydrolysed whey peptide treatments reduced the number of effector Th2-cells associated with whey-sensitised mice. In order to assess the ability of UL-2-147 to

induce Treg populations, TGF-β secretory levels were measured from splenocytes. UL-2-147 treatment significantly enhanced the levels of TGF-β in OVA stimulated allergic mice, compared to PBS treated allergic mice. Adding further value to the treatment and stronger evidence of its therapeutic role in allergy, UL-2-147 treatment even increased TGF-β levels when compared to healthy control mice. WPC80 was unable to restore TGF-β levels in allergic mice. Similarly, Van Esch et al. (2011) observed an increase in Foxp3 expressing Treg cells in the MLN in whey-sensitised mice treated with partially hydrolysed whey peptide but not in mice treated with the whole whey, following a whey challenge. The ability of UL-2-147 to drive Treg cytokines in an *in vivo* allergic setting indicates that it may possess the potential to bring about immune homeostasis in infants with food allergy. In addition to this, UL-2-147 treatment significantly reduced the increased levels of IgG1, a key Th2 cytokine-stimulated antibody, in allergic mice. Holt et al. (1981) claims that reduction of the IgG1 response is associated with the generation of tolerance, making this a desirable feature of UL-2-147. Another interesting finding from our OVA study was that splenocytes from all OVA-sensitised treatment groups stimulated with CD3 plus CD28 antibodies were able to produce IFN-γ, similar to levels of the healthy control mice. This indicated that UL-2-147 had no effect on Th1 cells in this study and further suggests that it did not dampen the host's ability to mount an immune response to a potential pathogen. In summary, oral administration of UL-2-147 was capable of redirecting an exaggerated Th2 response, associated with allergic mice, towards a beneficial Treg phenotype, and in doing so reduced the clinical symptoms of allergy, including restoring pathology in the gut, and did not inhibit the host's ability to mount a Th1 response to an external harmful stimulus. From these results, we propose that UL-2-147 would be beneficial in priming the infant immune system, by bringing about immune balance.

Necrotizing Enterocolitis (NEC) is a form of severe intestinal inflammation, which generally presents in premature low birth weight infants (Gephart et al. 2012). While understanding of its pathogenesis is poor, it is known that NEC typically affects the terminal ileum, the point at which the small intestine intersects with the large intestine. The disease is characterised by coagulation necrosis, acute and chronic inflammation, bacterial overgrowth, formation of lesions and tissue repair (Claud, 2009; and Ballance et al. 1990). Inflammatory cytokine release is the main cause of chronic

inflammation in this condition (Lu et al. 2014). Many cytokines and chemokines have been linked to NEC progression, triggering a state of necrosis. The pro-inflammatory cytokines, IL-1β, TNF-α, IL-6, IL-17 and IFN-γ have also been shown to play a key role in disease onset and progression, as evidenced by their increased concentrations in serum and intestinal tissue in infants with NEC, and hence they were the main focus of our studies (Claud, 2009; Morecroft et al. 1994; Maheshwari et al. 2014; Edelson et al. 1999; and Ng et al. 2003). As the excess or lack of these cytokines has been proven to disrupt the epithelial barrier, they are, therefore, desirable therapeutic targets in NEC. Studies have also identified a role for T-lymphocytes in NEC development. It is understood that a Th17/Treg and/or Th1/Treg imbalance in the intestinal mucosa is involved in NEC progression (Niño et al. 2016; Weitkamp et al. 2013; and Takeda et al. 1999). To date, most research has focussed on the nutritional prevention of NEC. Many practices exist nowadays to reduce NEC risk and prevent onset of the disease. Breastfeeding and feeding of probiotics have been proven to be effective in this area and reduce the incidence of NEC by improving barrier function (Lucas and Cole 1990; Lawrence et al. 1982; and Lapillonne et al. 2016). While the prevention of NEC is crucial, little research has been carried out to identify potential novel and improved treatments of the disease.

Using the *in vitro* Th1 and Th17 assays optimised in Chapter three, we screened an array of WPC80 and NaCN CM hydrolysates once again, in order to identify hydrolysates that possessed anti-inflammatory activity. We identified two potential NaCN candidate hydrolysates, UL-2-34 and UL-2-132, which modulated and suppressed the pro-inflammatory Th1 cytokines, IFN-γ and IL-2. Similarly, our collaborators in Maynooth observed a suppression of IFN-γ from humanized Th1 cells as well as inhibition of the Th17 transcription factor, ROR-γ, when treated with UL-2-34/132, which added further evidence to the anti-inflammatory profile of these hydrolysates. Similar to UL-2-147, we confirmed the bioactivity of these hydrolysates to be reproducible. Moreover, their production is relatively low costing and they both have a freefall pH. They would both, therefore, be suitable hydrolysates for commercialisation and incorporation into infant milk formula.

These *in vitro* results suggested that UL-2-34 and UL-2-132 may have beneficial immuno-modulatory effects in IBD and NEC and may potentially suppress the overactive Th1 and/or Th17 responses, that characterise NEC, in an *in vivo* setting of

IBD. We next examined the ability of these hydrolysates to modulate the immune response away from an excessive inflammatory response and alleviate the symptoms associated with intestinal inflammation in an *in vivo* murine model of acute IBD, also known as colitis, to assess the translation of our *in vitro* results. Administration of dextran sulfate sodium (DSS) induces colitis in mice similar to that that would also develop in humans (Chassaing et al. 2014). Although the DSS model resembles IBD, many studies have shown that it is also a relevant model for studying the effect of therapeutics targeting NEC (Ginzel et al. 2017). DSS-induced colitis activates the adaptive T-cell response. Short-term DSS treatment in mice drives an acute inflammatory cytokine cascade: TNF-α, IL-1β, IL-6, IL-10, IL-12, IL-17 and IFN-γ being the most potently expressed mediators (Jiminez et al. 2015; and Yan et al. 2009). These cytokines directly disrupt the epithelial barrier and drive intestinal destruction and are, therefore, logical therapeutic targets for developing novel treatments (Laukoetter et al. 2008). The clinical symptoms, such as weight loss, diarrhoea, bloody stools, crypt loss, destruction of the epithelial layer, significant reduction in colon length, increased colon weight and infiltration of pro-inflammatory cells induced by DSS are an accurate representation of human IBD and were observed in DSS-induced mice in our study (Chassaing et al. 2014; and Laroui,et al. 2012). These clinical symptoms were effectively reversed by the UL-2-132 hydrolysate treatment. An improvement in these symptoms was also observed in UL-2-34 and NaCN treated mice by the end of the study, although UL-2-132 treatment was most effective. UL-2-132 reversed the inflammation induced reduction in colon length and increase in colon length. UL-2-34 had no effect on the reduction of colon length but reversed the increase in colon weight. Additionally, UL-2-132 treatment restored histopathology in the gut, as seen by H&E stained colonic sections.

Human NEC and IBD are associated with increased expression of the inflammatory cytokines also induced by DSS (Laukoetter et al. 2008). We, therefore, assessed the ability of UL-2-34 and UL-2-132 to inhibit the pro-inflammatory Th1 and Th17 associated cytokines. UL-2-132 demonstrated a consistent anti-inflammatory capacity in dampening the pro-inflammatory cytokine profile associated with DSS, unlike UL-2-34 and NaCN treatments. As pathology in the DSS model typically presents in the large intestine, particularly the distal colon, we collected sections of distal colon of mice for cytokine examination. A Th17 cytokine population was dominant in our DSS

study. UL-2-132 treatment completely reversed the DSS associated spike in IL-1β, IL-17 and IL-6 inflammatory mRNA gene expression in the colon and significantly reduced their levels in unstimulated DSS-induced splenocytes compared to untreated DSS-induced mice. IL-1β, IL-17 and IL-6 are all linked to a Th17 phenotype and have been well documented to play harmful roles in NEC (Edelson et al. 1999; Mukhopadhya et al. 2014; Goepfert et al. 2004; and Egan et al. 2016). Furthermore, both UL-2-34 and UL-2-132 treatments reduced secretion of IL-6 and IL-1β in stimulated splenocytes of DSS-induced mice; UL-2-132 reversed levels back to those of healthy control mice. We were unable to assess the ability of UL-2-132 to suppress a Th1 response in this model as the Th1 cytokines, IFN-γ and TNF-α, were reduced in all DSS-induced mice. There is much evidence that indicates that TNF-α plays a role in the early stages of NEC and so may only be detectable immediately after onset of NEC (Travadi et al. 2006; and Yurttutan et al. 2014; and Romagnoli et al. 2001). Therefore, in order to assess the suppressive effect of UL-2-132 on this cytokine, it would be a possibility to administer the hydrolysate treatment prior to DSS exposure and collect organs for analysis as soon as clinical symptoms start to show. The role of IFN-γ in NEC development has been debateable. While many studies have demonstrated an increase in IFN-γ secretion in NEC, some studies have observed that mice deficient in IFN-γ still developed colitis when stressed (Simpson et al. 1998). Further studies assessing IFN-γ levels at various time points in the DSS model would be required to confirm the effect of UL-2-132 on its regulation. We did, however, observe a significant increase in IFN-γ, IL-2 and TNF-α secretion from DSS treated unstimulated splenocytes, which was effectively reversed by UL-2-132 treatment, which was evidence that UL-2-132 does target the Th1 phenotype *in vivo*.

An additional desirable feature of UL-2-132 treatment was that it did not curtail the ability of splenocytes of DSS-induced mice to detect and mount an immune response to an external stimulus, as levels of all cytokines examined (IFN-γ, IL-2, IL-6, IL-1β, TNF-α and IL-17) in splenocytes stimulated with plate-bound CD3 plus CD28 from DSS-induced mice treated with UL-2-34 or UL-2-132 were almost identical to those of control mice. Furthermore, the IL-10 secretion pattern suggests a potential therapeutic mechanism as to how the UL-2-132 treatment inhibits the harmful pro-inflammatory cytokine cascade. As UL-2-132 treatment reduced IL-10 secretion in an unstimulated state in DSS-induced mice and did not enhance IL-10

levels in these mice following stimulation, it is likely that UL-2-132 does not require IL-10 to exert its anti-inflammatory effects, indicating further that it may possess anti-inflammatory ability itself, being one of its *in vivo* therapeutic effects. Further work is required, however, to fully understand this as a potential mechanism and if UL-2-132 is in fact effective in preventing susceptibility in the host.

UL-2-132 effectively reversed the DSS induced colitis symptoms as well as the pro-inflammatory cytokines associated with the disease in mice. The pro-inflammatory cytokines such as IFN-γ, IL-2, IL-6, IL-1β, TNF-α and IL-17 have a pivotal role in the pathogenesis of inflammatory conditions of the GIT including IBD and NEC. Several studies have also reported that interaction between pro-inflammatory cytokines and the intestinal mucosal immune system can lead to the disruption of tight junction proteins and affect intestinal homeostasis (Abraham and Medzhitov, 2011). The potential of the CM NaCN hydrolysate, UL-2-132, to suppress inflammatory cytokines has, therefore, particular relevance to IBD and NEC. Although UL-2-34 treatment demonstrated protective effects against some of the pro-inflammatory cytokines in the spleen, it increased mRNA expression of TNF-α and IFN-γ in DSS treated colons. This suggests that the DSS associated inflammatory loop is still active in mice treated with UL-2-34.

Synthetic immuno-suppressants and corticosteroids are the main therapeutics in use for the treatment of chronic inflammatory disorders. Monoclonal antibodies and small molecule inhibitors have been developed that can inhibit the pro-inflammatory effects of the pro-inflammatory cytokines associated with IBD and NEC (Maheshwari et al. 2014). Research has focussed on compounds that could target NF-κB activation. Pentoxyphylline has been shown to lower the prevalence of NEC by inhibiting TNF-α and subsequently NF-κB and NEC incidence (Travadi et al. 2006). Many of these drugs can elicit harmful side effects in the host and have inconsistent activity with respect to controlling symptoms (Rogler, 2010). Newborns are more susceptible than adults to developing these undesirable drug side effects because of their undeveloped and immature responses for handling drugs (Knight, 1994). Furthermore, relapse is common in infants who have recovered from NEC. There is, therefore, an urgent requirement for the development of more natural and improved treatments that could be taken on a regular basis or used longer term (Fine, 2008; and Malaty et al. 2010). The identification of natural modulatory bioactives, such as CM hydrolysates, holds

strong potential as an alternative form of therapy that may prevent reoccurring inflammation as they can be taken as part of a balanced diet without the onset of adverse side effects as hydrolysed bioactive peptides are known to be safer than synthetic drugs (Khan et al. 2012; and Bah et al. 2013).

It is still unclear whether anti-cytokine therapy may inhibit pathways essential in many tissues besides the intestine, potentially promoting harmful side effects or collateral damage in the developing gut. Major concerns currently exist surrounding possible harmful side effects from anti-cytokine and immuno-modulatory therapy in infants. Many of these cytokines also play important roles in developing infants' immune systems and maturing their gut and dampening them could leave them susceptible to infection (Hsueh et al. 1998; and Maheshwari, 2004). Therefore, identification of novel natural therapies that do not affect the host's ability to mount an immune response to a potential pathogen is of utmost importance and would be of particular benefit. As UL-2-147 and UL-2-132 treatments did not inhibit the splenocytes capacity to secrete cytokines in response to an external stimulus, this indicates that this may be a key advantage over anti-cytokine therapy and drugs, which result in adverse side effects leaving the patient open to infection. Hydrolysed diets would, therefore, be much more effective for the long-term management of these conditions in infants.

EHF, consisting of protein peptides derived from CM, have been used for more than 50 years for feeding infants with CMPA or severe inflammatory bowel diseases (Baker et al. 2000). CM EHFs have a high nutritional value and improve nutrient absorption. Available mucosal lactase is limited in infants who suffer with intestinal disorders. As a result, lactose is poorly tolerated. EHFs lack lactose and thus, are desirable feeding solutions for these infants (Sondheimer, 2006). Extensive hydrolysis of a protein results in the complete destruction of the allergic IgE epitopes, which prevents sensitisation and onset of clinical symptoms (Terheggen-Lagro et al. 2002; and Sampson et al. 1991). Although EHFs are currently used to prevent the onset of CMPA in infants, as 90% of infants can safely tolerate extensively hydrolysed peptides, they can still provoke a reaction in a small population of infants and they do not generate tolerance (Baker et al. 2000). Therefore, there is a requirement for the development of more effective treatments and prevention strategies. UL-2-147 is a partially hydrolysed protein, thus it retains immunogenicity, which allows it to bring about

immune balance and potentially induce tolerance. It, therefore, yields high potential as a novel prevention strategy for atopic neonates at risk of developing CMPA, which may reduce the time for allergic patients to generate tolerance and avoid subsequent allergy in later life. Approximately 12.1% of infants currently use EHFs as a refeeding strategy during NEC recovery (Lapillonne et al. 2016). Infants recovering from NEC have an increased risk of sensitisation to intact proteins and subsequently developing CMPA. This is, therefore, the main reason for the use of EHF as they guarantee complete removal of the allergic IgE epitopes. Limited studies, however, have assessed a therapeutic role for EHF in NEC treatment. Little research has been carried out to examine the ability of EHF to modulate the immune system in order to prevent NEC or NEC relapse. We have, therefore, identified a novel CM milk hydrolysate with a potential novel therapeutic application. While it is rare that extensively hydrolysed peptides possess immunomodulatory properties, UL-2-132 seems to be an excellent candidate for NEC refeeding and prevention of relapse, as its efficacy and therapeutic anti-inflammatory bioactivity has been demonstrated *in vivo*, which is unique for EHF, whilst also being extensively hydrolysed.

The prevalence of allergic disease has risen in developing countries, and even more substantially in developed countries. Significant global increases in all allergic diseases, such as atopic dermatitis, asthma, and food allergy, have been observed (Prescott et al. 2013). Food allergy is a major burden on society, impairing the quality of life for patients and their families. The economic impact of food allergy is considerable. Financial costs include direct (treatments, emergency department visits, hospitalisations) and indirect (absence of a parent from work or child from school, reduced productivity and performance) associated healthcare costs (Hendaus et al. 2016). It is estimated that the annual economic cost of food allergy in the United States is 24.8 billion dollars (Gupta et al. 2013). Studies have demonstrated that children with food allergy are two to four times more likely to develop other atopic diseases throughout life, such as asthma, allergic rhinitis, and atopic dermatitis (Henson and Burks, 2012; and Branum and Lukacs, 2008).

NEC is a leading cause of morbidity and mortality in preterm infants (Chatterton et al. 2013). Almost 12% of premature infants who weigh less than 1,500 g at birth develop NEC. Onset of the disease typically occurs in the second to third week of life. The economic cost of NEC is high, with hospitalisation costs estimating at $0.5–1 billion

in the United States per year alone. Mortality rates in newborns with NEC are high and can reach up to a staggering 50%, depending on severity (Gephart et al. 2012). While therapeutics are currently available for NEC and IBD, side effects are often associated with these. Relapse and further inflammation is also common amongst recovered patients. It is also common amongst recovered infants for subsequent short and long-term complications to develop, such as malabsorption, poor growth, short gut syndrome and neurodevelopmental impairment (Pacheco, Underwood and Mills, 2015).

We validated the ability of a novel CM whey protein hydrolysate, UL-2-147, to "retrain" the immune system of OVA-sensitised mice *in vivo*, which was characterised by the prevention of the clinical symptoms of allergy and modulation of the immune response away from the favoured Th2 phenotype, associated with allergy, towards a Treg response, following OVA challenge. We have, therefore, successfully identified and demonstrated the efficacy of a whey hydrolysate, which would be of benefit incorporated into IMF for the primary prevention of atopic disease in healthy infants who do not obtain all the benefits of exclusive breastfeeding and are, therefore, at risk of atopy. Furthermore, we demonstrated the consistent anti-inflammatory bioactivity of a novel CM NaCN protein hydrolysate, UL-2-132, in an *in vitro* setting and an *in vivo* inflammatory gut model. UL-2-132 completely reversed the harmful effects of DSS in mice. Collectively, our results suggest that UL-2-132 has the potential to serve an as a novel and effective anti-NEC/IBD therapy, given the similar pathogenesis of NEC and IBD.

Murine models were chosen for *in vivo* investigation throughout this project due to their many advantages. They can be carried out at low cost, they are ready available, they have a well-characterized immune system with a similar adaptive immune response to humans (in particular their production of T-cells which was important for this project), they share over 90% of their genes with humans, their gut microbiome is similar to human gut microbiota, their gastrointestinal tract is also similar to that of humans and they are thus, the most commonly used animal model for intestinal studies (Jiminez et al. 2015). Although the use of murine models to study aspects of human intestinal disorders employs many advantages, it was also importnant to consider the shortcomings and limitations associated with these models whilst carrying out this project. The lesions produced in mice using chemical agents are not consistently

representative of those found in people with IBD. DSS administration induces chemical injury to the epithelial lining in mice which represents mucosal injury similar to IBD in humans, however, the severity of the lesions are not identical to those found in people with IBD (Low, Nguyen and Mizoguchi, 2013). Another potential drawback that may be applicable to this project is the difference in behavioural patterns between mice and humans. It is understood that mice exhibit a behaviour, known as coprophagy, in which they eat faeces in order to re-absorb nutrients and balance their microbiome. As coprophagy is not a behaviour observed in humans, it was important to note this while studying the impact of diet on the induction or suppression of intestinal inflammation and allergy as this activity could potentially alter microbial populations and overall intestinal health, resulting in inaccurate interpretations (Hedrich, 2004; and Jiminez et al. 2015). Murine models of food allergy stimulate a severe form of the disease. Although they elicit clinical symptoms similar to the human disease, it is unclear how accurately the artificial sensitisation methods used mimic the natural method of sensitisation. This could affect our interpretation of results when examining the effect of our treatment. We still do not have a complete understanding of the disease and the mechanisms of sensitisation. Until our understanding is further developed we do not know precisely how useful these models are in predicting the effectiveness of novel treatments for the disease. For now, murine models remain the most widely used and weas the ideal choice for our research at this time (Santoro and Marsella, 2014).

The first 6 months of life represent a crucial window of opportunity for shaping the development of the gastrointestinal tract and immune system and is the most critical time for establishing immune balance, as once sensitisation to a food protein or inflammatory disease progresses it is difficult to correct. Throughout this project we successfully identified novel CM protein hydrolysates that would be beneficial in the setting of immune gut disorders, such as food allergy and NEC. While our focus was on developing hydrolysates for infant nutrition, the hydrolysates identified in this project also possess potential to be beneficial for other life-threatening food allergies and inflammatory conditions. Patients or consumers are more knowledgeable nowadays and conscious about undesirable side effects of drugs and the association between diet and health. The identification of natural modulatory bioactives, such as CM hydrolysates, holds strong potential as an alternative form of therapy for the treatment or prevention of allergy and inflammatory disease in infants, as they can be

taken as part of a balanced diet without the onset of adverse side effects. Hydrolysed diets would, therefore, be much more effective for the long-term management of these conditions in infants. The ability to prime the immune response and bring about immune balance from birth confers many advantages; lowering the incidence of atopy and inflammatory disorders and thus, the problems associated with these conditions in later life.

Future Work:

- Although the results from this project yield much potential and add a novel insight to this research area, additional pre-clinical studies are required to further explore the immuno-modulatory mechanisms and to develop a deeper understanding of the peptide sequence responsible for the immuno-regulatory functions observed. In order to identify the peptide(s) responsible for the bioactivity observed in this project, fractionation of both UL-2-147 and UL-2-132 hydrolysates will proceed in January 2018. Identifying the specific bioactive peptides will contribute valuable knowledge to this field of study and will smartly inform and guide future studies.
- The influence of the gut microbiota on health and disease through the development and regulation of the immune system and immune balance has become increasingly popular as an area of scientific and clinical importance. It would, therefore, be of interest to examine the effect of the hydrolysates identified in this project on commensal bacteria in future studies. The first bacterial species identified that brought about balance of the Th1/Th2-paradigm was *B. fragilis* (Sears and Pardoll, 2011). As we found that the hydrolysates targeted T-cells in the gut of mice, it is, therefore, possible that they may also have a beneficial effect on and interact with the gut microbiota to enhance immune balance.
- Smith and Garrett (2011) claim that some hydrolysed peptides have similar structures to endogenous peptides, such as cytokines, neurotransmitters and hormones, and can communicate with the same receptors. They have been shown to exert anti-inflammatory properties as well as protective actions on intestinal mucus (Hernández-Ledesma et al. 2014). Peptides can control

physiological actions, regardless of their low bioavailability. This suggests that they do not necessarily need to be absorbed in order to elicit a biological activity. It is, therefore, believed that peptide interaction with receptors found on the gut may be the reason behind the physiological effects observed, as the gastrointestinal tract constantly interacts with a wide range of exogenous food-derived peptides (Hernández-Ledesma et al. 2014). It would, therefore, be interesting to carry out studies to identify which, if any, toll-like receptors (TLRs) the hydrolysates may work through.

- It would be worthwhile to carry out restructured OVA and DSS models to assess a number of factors. It was clear that disease in our DSS model was characterised by a Th17 phenotype. As TNF-α may only be detectable immediately after onset of NEC, by administering the hydrolysate treatment prior to DSS exposure and collection of organs for analysis as soon as clinical symptoms start to show, may represent an opportunity to exam the effect of U-2-132 on this cytokine and its ability to inhibit a Th1 response. It would also be of interest administer the hydrolysate treatment prior to DSS exposure and OVA sensitisation in order to examine the ability of the hydrolysates to prevent the onset of disease and the clinical symptoms.

CHAPTER 7

BIBLIOGRAPHY

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8.1 APPENDICES

Appendix A - Media and Buffers

Complete RPMI 1640 500 Ml

Heat inactivated Foetal Bovine Serum (FBS)	10%
Penicillin/streptomycin/L-glutamine Culture Cocktail	2%
(Gives a final concentration of 2 mM L-glutamine,	
100 μg/ml penicillin and 100 U/ml streptomycin)	
B-Mercaptoethanol	50μM

Phosphate buffered saline (PBS)

$Na_2HPO_4 \times 2H_2O$	8.0 mM
KH_2PO_4	1.5 mM
NaCl	137 mM
KCl	2.7 mM

Dissolve in dH_2O and pH to 7.4

Tris buffered saline (TBS)

NaCl	1.5 M
Trizma Base	0.2 M

Dissolve dH_2O pH to 7.6 247

TBS-Tween

Add 0.05% Tween-20 to 1x TBS

TAE buffer

Tris 40 mM

Acetic acid 20 mM

EDTA 1 mM

Dissolve in dH2O

FACS buffer

FCS 2%

NaN3 0.05%

Dissolve in PBS

Appendix B - Chapter 4 Additional Data

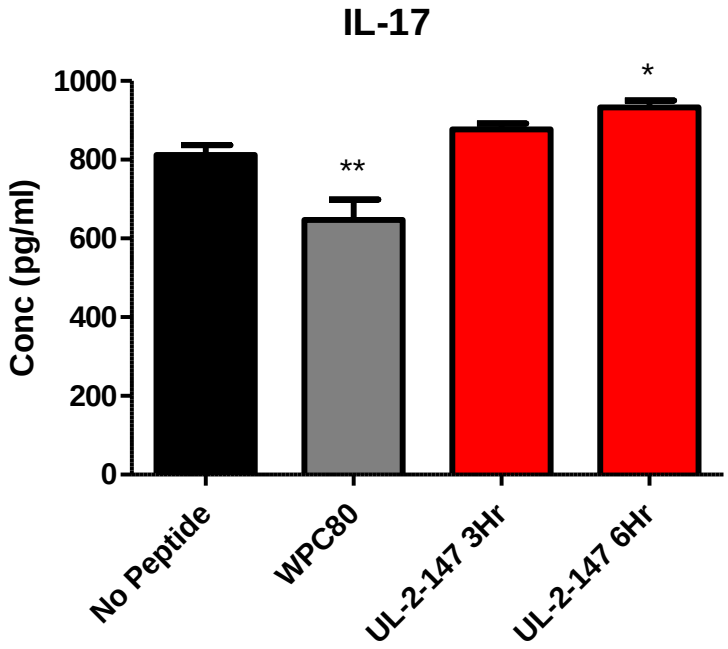

Fig 8.1: Assessment of IL-17 secretion in undifferentiated T-cells treated with UL-2-147. $CD4^+$ cells were isolated from the spleens of female BALB/c mice aged 8-12 weeks using EasySep $CD4^+$ Isolation (Stemcell Cat# 19752). Cells were plated at $1x10^6$/ml on a 96 well plate. Cells were stimulated with plate-bound anti-CD3 (5 µg/mL) plus anti-CD28 (5 µg/mL) and treated with hydrolysates (1 mg/mL). Cells were incubated for 72 hrs. Supernatants were collected and IL-17 secretion was measured using ELISA according to manufacturer's instructions (R&D duoset). One way Anova was used to determine if differences between groups were significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Hydrolysates are compared to untreated cells (No Peptide).

Appendix C - Primer Sequences and PCR Gels

IL-1β

Primer 1 5'-CTC TTG TTG ATG TGC TGC TG-3'

Primer 2 5'-GAC CTG TTC TTT GAA GTT GAC G-3'

IL-6

Primer 1 5’-TCC TTA GCC ACT CCT TCT GT-3’

Primer 2 5’-AGC CAG AGT CCT TCA GAG A-3’

TNF-α

Primer 1 5’-TCT TTG AGA TCC ATG CCG TTG-3’

Primer 2 5’-AGA CCC TCA CAC TCA GAT CA-3’

IFN-γ

Primer 1 5’-TCC ACA TCT ATG CCA CTT GAG-3’

Primer 2 5’-CTG AGA CAA TGA ACG CTA CAC A -3’

IL-17a

Primer 1 5’- GAG CTT CCC AGA TCA CAG AG-3’

Primer 2 5’- AGA CTA CCT CAA CCG TTC CA-3’

IL-10

Primer 1 5'-ATG GCC TTG TAG ACA CCT TG-3'

Primer 2 5'-GTC ATC GAT TTC TCC CCT GTG-3'

IL-4

Primer 1 5'- TCT TTA GGC TTT CCA GGA AGT C-3'

Primer 2 5'- GAG CTG CAG AGA CTC TTT CG-3'

IL-13

Primer 1 5'-GCT TTG TGT AGC TGA GCA GT-3'

Primer 2 5'-GGA TAT TGC ATG GCC TCT GTA-3'

GUSB

Primer 1 5'-AGC AAT GGT ACC GGC AG-3'

Primer 2 5'-ACC ACA CCC AGC CAA TAA AG-3'

PCR Gels

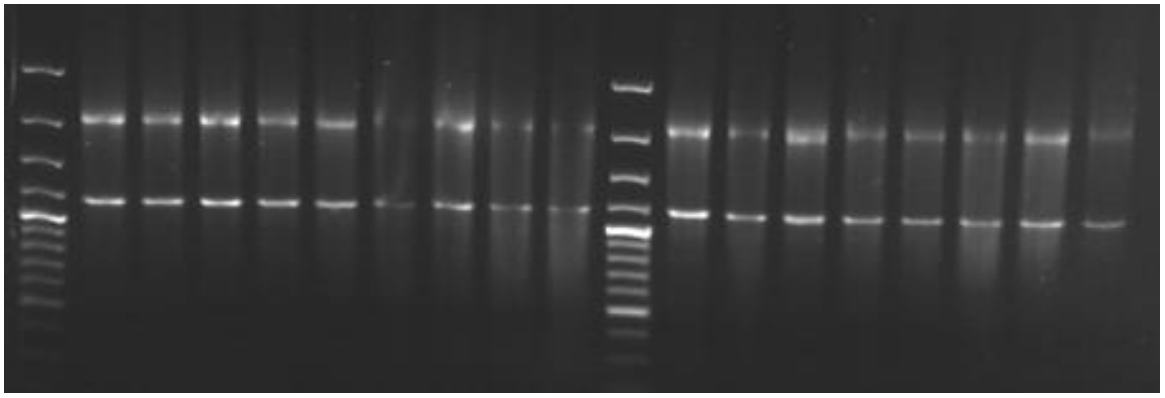

Fig 8.2: RNA product analysis by gel electrophoresis. RNA samples were mixed with loading buffer (Fermentas) and loaded onto the 2% agarose gel, together with GeneRuler 100bp DNA ladder (Termo-Fisher Scientific). Gels were run for 1h in 1x TAE buffer at 100V and visualised using the G-box imaging system (Syngene). The 18S and 28S ribosomal RNA bands are clearly visible in the RNA samples, indicating that the samples are intact. **Lane 1**: marker; **2**: Control 1; **3**: Control 2; **4**: Control 3; **5**: Control 4; **6**: Control 5; **7**: Control 6; **8**: WPC80 1; **9**: WPC80 2; **10**: WPC80 3; **11**: marker; **12**: WPC80 4; **13**: WPC80 5; **14**: WPC80 6; **15**: OVA 1; **16**: OVA 2; **17**: OVA 3; **18**: OVA 4; **19**: OVA 5. All samples visualised on this gel were from the OVA experimental model carried out in **Chapter 4**.

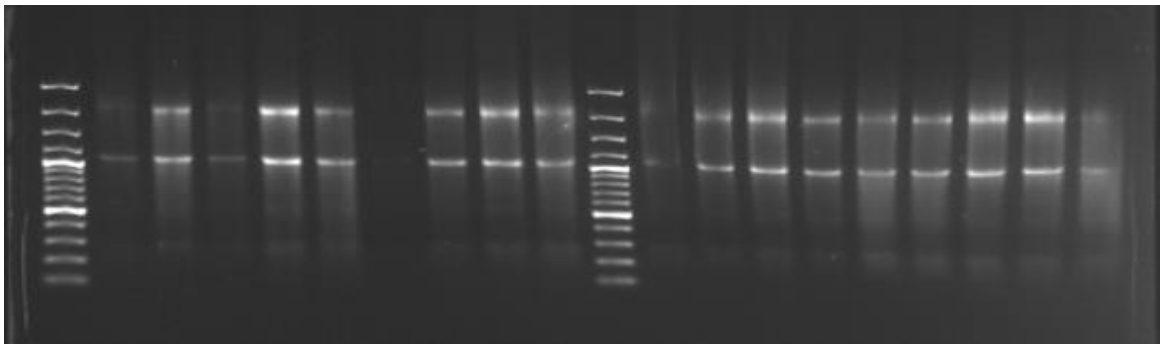

Fig 8.3: RNA product analysis by gel electrophoresis. RNA samples were mixed with loading buffer (Fermentas) and loaded onto the 2% agarose gel, together with GeneRuler 100bp DNA ladder (Termo-Fisher Scientific). Gels were run for 1h in 1x TAE buffer at 100V and visualised using the G-box imaging system (Syngene). The 18S and 28S ribosomal RNA bands are clearly visible in the RNA samples, indicating that the samples are intact. **Lane 1**: marker; **2**: UL-2-147 1; **3**: UL-2-147 2; **4**: UL-2-147 3; **5**: UL-2-147 4; **6**: UL-2-147 5; **7**: UL-2-147 6; **8**: OVA Pilot Control 1; **9**: OVA Pilot Control 2; **10**: OVA Pilot Control 3; **11**: marker; **12**: OVA Pilot Control 4; **13**: OVA Pilot Control 5; **14**: OVA Pilot Control 6; **15**: OVA 1 (Pilot); **16**: OVA 2 (Pilot); **17**: OVA 3 (Pilot); **18**: OVA 4 (Pilot); **19**: OVA 5 (Pilot); **20**: OVA 6 (Pilot). All samples visualised on this gel were from the OVA experimental model and OVA pilot carried out in **Chapter 4**. Samples in **Lanes 2, 4, 7, 12** and **20** were repeated and can be seen in **Figure 8.6**.

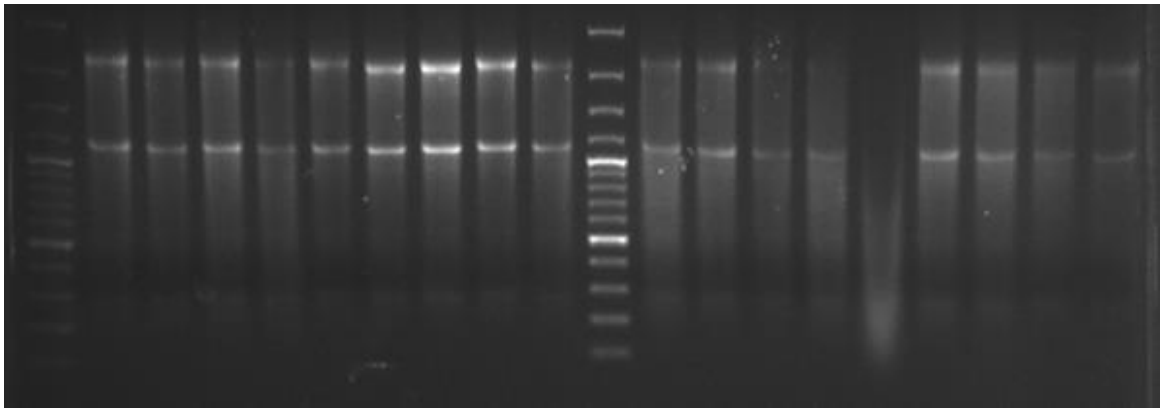

Fig 8.4: RNA product analysis by gel electrophoresis. RNA samples were mixed with loading buffer (Fermentas) and loaded onto the 2% agarose gel, together with GeneRuler 100bp DNA ladder (Termo-Fisher Scientific). Gels were run for 1h in 1x TAE buffer at 100V and visualised using the G-box imaging system (Syngene). The 18S and 28S ribosomal RNA bands are clearly visible in the RNA samples, indicating that the samples are intact. **Lane 1**: marker; **2**: OVA 6; **3**: Control 1; **4**: Control 2; **5**: Control 3; **6**: Control 4; **7**: Control 5; **8**: Control 6; **9**: NaCN 1; **10**: NaCN 2; **11**: marker; **12**: DSS 1; **13**: DSS 2; **14**: DSS 3; **15**: DSS 4; **16**: DSS 5; **17**: DSS 6; **18**: NaCN 3; **19**: NaCN 4; **20**: NaCN 5. All samples from **Lane 3** – **Lane 20** visualised on this gel were from the DSS experimental model carried out in **Chapter 5**. The sample in **Lane 2** (OVA 6) was from the OVA experimental model carried out in **Chapter 4**. Sample in **Lane 16** was repeated and can be seen in **Figure 8.6**.

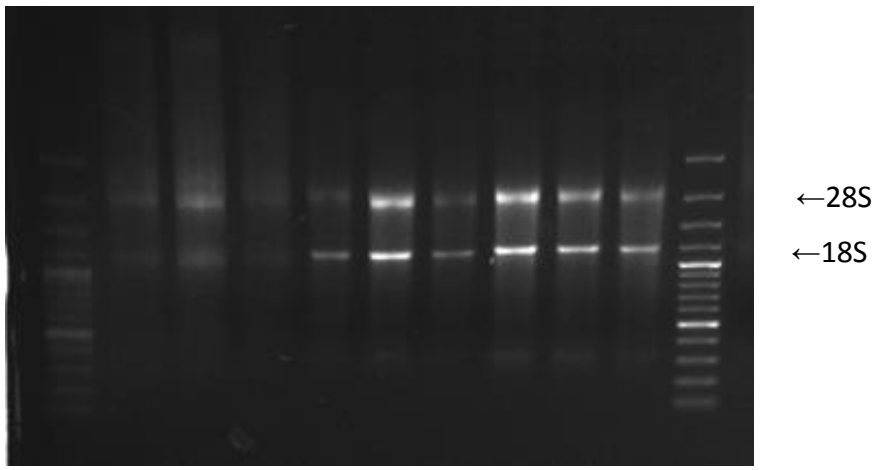

Fig 8.5: RNA product analysis by gel electrophoresis. RNA samples were mixed with loading buffer (Fermentas) and loaded onto the 2% agarose gel, together with GeneRuler 100bp DNA ladder (Termo-Fisher Scientific). Gels were run for 1h in 1x TAE buffer at 100V and visualised using the G-box imaging system (Syngene). The 18S and 28S ribosomal RNA bands are clearly visible in the RNA samples, indicating that the samples are intact. **Lane 5**: UL-2-34 1; **6**: UL-2-34 2; **7**: UL-2-34 3; **8**: UL-2-34 4; **9**: UL-2-34 5; **10**: UL-2-34 6; **11**: marker. All samples visualised on this gel were from the DSS experimental model carried out in **Chapter 5**.

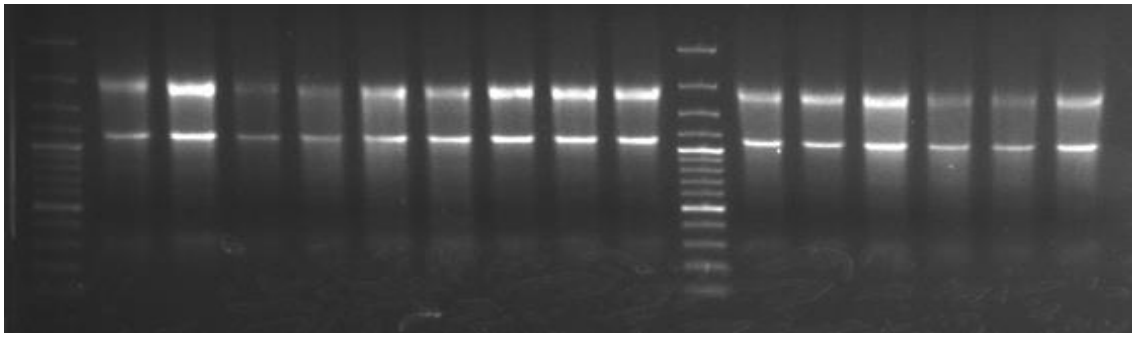

Fig 8.6: RNA product analysis by gel electrophoresis. RNA samples were mixed with loading buffer (Fermentas) and loaded onto the 2% agarose gel, together with GeneRuler 100bp DNA ladder (Termo-Fisher Scientific). Gels were run for 1h in 1x TAE buffer at 100V and visualised using the G-box imaging system (Syngene). The 18S and 28S ribosomal RNA bands are clearly visible in the RNA samples, indicating that the samples are intact. **Lane 1**: marker; **2**: OVA Pilot Control 4; **3**: OVA 6 (Pilot); **4**: UL-2-147 1; **5**: UL-2-147 3; **6**: UL-2-147 6; **7**: DSS Pilot Control 1; **8**: DSS Pilot Control 2; **9**: DSS Pilot Control 3; **10**: DSS Pilot Control 4; **11**: marker; **12**: NaCN 6; **13**: DSS 5; **14**: DSS 1 (Pilot); **15**: DSS 2 (Pilot); **16**: DSS 3 (Pilot); **17**: DSS 4 (Pilot). All samples from **Lane 2** – **Lane 6** visualised were repeated samples from **Figure 8.3**. The samples in **Lanes 12** and **13** were from the DSS experimental model carried out in **Chapter 5** and sample in **Lane 13** was repeated from **Figure 8.4**. All other samples were from the DSS Pilot carried out in **Chapter 5**.

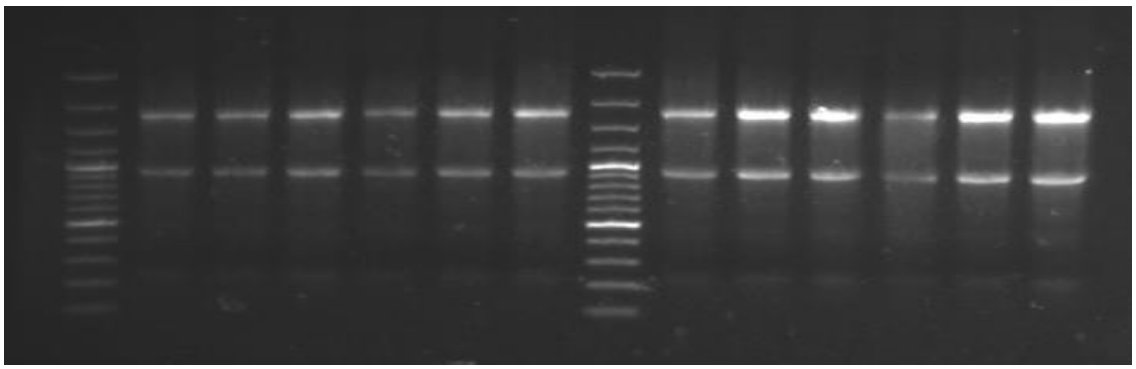

Fig 8.7: RNA product analysis by gel electrophoresis. RNA samples were mixed with loading buffer (Fermentas) and loaded onto the 2% agarose gel, together with GeneRuler 100bp DNA ladder (Termo-Fisher Scientific). Gels were run for 1h in 1x TAE buffer at 100V and visualised using the G-box imaging system (Syngene). The 18S and 28S ribosomal RNA bands are clearly visible in the RNA samples, indicating that the samples are intact. **Lane 1**: marker; **2**: UL-2-132 1; **3**: UL-2-132 2; **4**: UL-2-132 3; **5**: UL-2-132 4; **6**: UL-2-132 5; **7**: UL-2-132 6; **8**: marker; **9**: Control 6 (OVA); **10**: UL-2-34 1; **11**: UL-2-34 3; **12**: DSS 3; **13**: DSS 4; **14**: Control 3 (DSS). Samples in **Lanes 2 – 7** and **10 – 14** were from the DSS experimental model carried out in **Chapter 5**. Samples in **Lanes 10 – 14** were repeated from **Figures 8.4** and **8.5**. Sample in **Lane 9** was from the OVA experimental model carried out in **Chapter 4** and is repeated from **Figure 8.2**.