

This electronic thesis or dissertation has been downloaded from the King's Research Portal at <https://kclpure.kcl.ac.uk/portal/>



**Investigating the expression and function of IL-17A+ and IL-17F+ CD4+ T cells in human health and inflammatory arthritis**

Burns, Lachrissa Anne

*Awarding institution:*  
King's College London

The copyright of this thesis rests with the author and no quotation from it or information derived from it may be published without proper acknowledgement.

**END USER LICENCE AGREEMENT**



**Unless another licence is stated on the immediately following page** this work is licensed

under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International

licence. <https://creativecommons.org/licenses/by-nc-nd/4.0/>

You are free to copy, distribute and transmit the work

Under the following conditions:

- Attribution: You must attribute the work in the manner specified by the author (but not in any way that suggests that they endorse you or your use of the work).
- Non Commercial: You may not use this work for commercial purposes.
- No Derivative Works - You may not alter, transform, or build upon this work.

Any of these conditions can be waived if you receive permission from the author. Your fair dealings and other rights are in no way affected by the above.

**Take down policy**

If you believe that this document breaches copyright please contact [librarypure@kcl.ac.uk](mailto:librarypure@kcl.ac.uk) providing details, and we will remove access to the work immediately and investigate your claim.

**Investigating the expression and function of IL-17A+ and  
IL-17F+ CD4+ T cells in human health and inflammatory  
arthritis**

**Lachrissa Anne Burns**

**PhD Immunology  
King's College London  
2019**

## Abstract

IL-17A is a well-characterised pro-inflammatory cytokine, which has been implicated in the immunopathology of inflammatory arthritis. IL-17F is less well-studied. This thesis describes investigations into the phenotype of IL-17F<sup>+</sup> CD4<sup>+</sup> T cells, what drives IL-17F expression in CD4<sup>+</sup> T cells and how IL-17F may contribute to inflammation.

Healthy donor CD4<sup>+</sup> T cells were cultured with IL-1 $\beta$ , IL-23, anti-CD3 mAb and anti-CD28 mAb, and cytokine expression was analysed by flow cytometry. In comparison to IL-17A<sup>+</sup> IL-17F<sup>-</sup> CD4<sup>+</sup> T cells, IL-17F<sup>+</sup>IL-17A<sup>-</sup> and IL-17A<sup>+</sup>IL-17F<sup>+</sup> CD4<sup>+</sup> T cells contained lower proportions of cells co-expressing IL-10 and higher proportions of cells co-expressing IFN $\gamma$ . Titration of anti-CD28 mAb into healthy donor CD4<sup>+</sup> T cell cultures revealed that strong co-stimulation increased the frequency of IL-17F<sup>+</sup>IL-17A<sup>-</sup> and IL-17A<sup>+</sup>IL-17F<sup>+</sup> CD4<sup>+</sup> T cells, whereas IL-17A<sup>+</sup>IL-17F<sup>-</sup> CD4<sup>+</sup> T cell frequencies decreased. This was shown to be via an IL-2 dependent mechanism. To allow for the further characterisation of different IL-17A<sup>+</sup> and IL-17F<sup>+</sup> CD4<sup>+</sup> T cell populations an IL-17A and IL-17F capture assay was developed in this thesis.

Addition of human recombinant IL-17A, IL-17F and TNF $\alpha$  to synovial fibroblasts from patients with psoriatic arthritis (PsA) and rheumatoid arthritis (RA) resulted in significant production of IL-6. Combined blockade of IL-17A and IL-17F reduced IL-6 production to a larger extent than blockade of IL-17A alone, indicating IL-17F can contribute to inflammation. While IL-17A protein and IL-17A<sup>+</sup> T cells were detected at increased levels in the inflamed joint of RA and PsA patients, little IL-17F protein and few IL-17F<sup>+</sup> T cells were detected.

Collectively, data presented in this thesis indicate that IL-17A and IL-17F are differentially regulated and that strong T cell stimulation induces IL-17F. Additionally, data suggests IL-17A<sup>+</sup>IL-17F<sup>-</sup>, IL-17F<sup>+</sup>IL-17A<sup>-</sup> and IL-17A<sup>+</sup> IL-17F<sup>+</sup> CD4<sup>+</sup> T cells are functionally distinct. Finally, it is shown that in an environment

where IL-17A and IL-17F are present dual blockade of IL-17A and IL-17F is more effective at reducing inflammation.

# Table of Contents

<b>1</b>	<b>Introduction.....</b>	<b>16</b>
1.1	Cytokines.....	16
1.1.1	Interleukins.....	16
1.1.2	Interferons .....	20
1.1.3	Tumour necrosis factor (TNF) family .....	20
1.1.4	Chemokines.....	21
1.1.5	Colony stimulating factors .....	22
1.2	Cytokine signalling.....	22
1.2.1	The cytokine receptor families .....	22
1.2.2	JAK-STAT pathway.....	24
1.2.3	NF-KB pathway.....	25
1.3	A brief history of IL-17 identification .....	28
1.4	Structure of IL-17 cytokines .....	29
1.5	IL-17 receptors and signalling.....	29
1.6	Function of IL-17A, IL-17F and IL-17AF.....	33
1.6.1	Individual function .....	33
1.6.2	Synergy between IL-17A, IL-17F and other pro-inflammatory cytokines.....	34
1.6.3	Mechanism of synergy.....	35
1.7	Function of the remaining IL-17 family cytokines.....	36
1.7.1	IL-17E.....	36
1.7.2	IL-17C, IL-17B and IL-17D.....	37
1.8	T cells.....	38
1.8.1	T cell activation .....	38
1.8.2	Memory T cells.....	40
1.8.3	T helper cell subsets.....	41
1.9	Th17 cells .....	42
1.9.1	Th17 cell differentiation .....	42
1.9.2	Transcriptional control of Th17 cells .....	43

1.9.3	Regulation of Th17 cells .....	44
1.9.4	Phenotypic marker and cytokine profiles of IL-17A+ and IL-17F+ CD4+ T cells.....	46
1.10	Other IL-17A and IL-17F expressing cells.....	48
1.10.1	IL-17A+ CD8+ T cells (Tc17 cells) .....	48
1.10.2	$\gamma\delta$ T cells and iNKT cells .....	49
1.10.3	Innate immune cells .....	49
1.11	Cellular sources of IL-17B, IL-17C, IL-17D and IL-17E .....	50
1.12	Inflammatory arthritis.....	51
1.12.1	Genetics of inflammatory arthritis.....	51
1.12.2	Synovial fibroblasts in inflammatory arthritis .....	54
1.12.3	Immune cells in inflammatory arthritis .....	57
1.12.4	Animal models showing the pathological role of IL-17A in arthritis .	62
1.12.5	The presence of IL-17A and IL-17F in inflammatory arthritis.....	63
1.13	IL-17 receptors in RA and PsA.....	66
1.13.1	The presence of IL-17B, IL-17C, IL-17D and IL-17E in RA and PsA	66
1.14	Biologics in RA and PsA.....	67
1.15	Thesis rationale and thesis outline .....	69
<b>2</b>	<b>Materials and Methods .....</b>	<b>71</b>
2.1	Sample Isolation .....	71
2.1.1	PBMC and SFMC Isolation .....	71
2.1.2	Cryopreservation and thawing of PBMC and SFMC .....	72
2.1.3	Serum and synovial fluid isolation .....	72
2.1.4	Isolation of cells from synovial tissue .....	72
2.1.5	Synovial tissue explant culture.....	73
2.2	Cell separation .....	74
2.2.1	CD14+ monocyte isolation .....	74
2.2.2	CD4+ T cell isolation.....	74
2.2.3	Sorting immune cell populations from PBMC and SFMC .....	75
2.3	Cell culture .....	77
2.3.1	CD4+ T cell/CD14+ monocyte co-cultures .....	77
2.3.2	PBMC cultures .....	77

2.3.3	CD4+ T cell only cultures .....	77
2.3.4	Fibroblasts .....	78
2.4	Flow cytometry.....	81
2.4.1	Compensation controls.....	81
2.4.2	Extracellular staining .....	81
2.4.3	Intracellular cytokine staining .....	82
2.4.4	IL-17A secretion assay .....	84
2.4.5	Development of UCB IL-17A and IL-17F capture assay .....	84
2.5	Mass cytometry (CyTOF) .....	87
2.6	Cytokine analysis.....	89
2.6.1	Enzyme-linked immunosorbent assay (ELISA).....	89
2.6.2	Luminex .....	92
2.7	Quantitative polymerase chain reaction (qPCR) .....	93
2.7.1	RNA Isolation.....	93
2.7.2	DNase treatment and cDNA reverse-transcription.....	93
2.7.3	qPCR protocol and analysis .....	94
2.8	Statistics .....	95
<b>3</b>	<b>Investigating the expression of IL-17F in healthy CD4+ T cells in vitro....</b>	<b>96</b>
3.1	Introduction .....	96
3.2	Results.....	97
3.2.1	Induction of IL-17F expressing CD4+ T cells .....	97
3.2.2	Phenotypic characterisation of IL-17F+ expressing CD4+ T cells ...	128
3.3	Discussion .....	136
<b>4</b>	<b>Investigating the contribution of IL-17F to inflammation.....</b>	<b>144</b>
4.1	Introduction .....	144
4.2	Results.....	146
4.2.1	Examining the IL-17 receptor gene expression profile in RA and PsA synovial fibroblasts. ....	146
4.2.2	Assessing the individual and combinatorial function of IL-17A, IL-17F and IL-17AF on synovial fibroblasts .....	148
4.2.3	IL-17A, IL-17F and IL-17AF synergise with TNF $\alpha$ .....	152

4.2.4	Testing blocking antibodies .....	156
4.2.5	Dual IL-17A and IL-17F blockade in a fibroblast:recombinant cytokine system .....	162
4.2.6	Dual IL-17A and IL-17F blockade in bulk CD4+ T cell supernatant cell cultures.....	165
4.2.7	Blockade of IL-17A and IL-17F in cultures of synovial fibroblasts and sorted CCR6+ CD161+ CD4+ T cells .....	168
4.2.8	Sorting IL-17A+ CD4+ T cells for functional analysis .....	172
4.2.9	Examining the interaction of synovial fibroblasts and sorted IL-17A+ and IL-17A- CD4+ T cells .....	177
4.2.10	Development of an IL-17A and IL-17F CD4+ T cell capture assay .	179
4.2.11	Sorting IL-17A+ IL-17F-, IL-17A+ IL-17F+ and IL-17F+ IL-17A- CD4+ T cells.....	186
4.2.12	Examining the function of the remaining IL-17 cytokines .....	190
4.3	Discussion .....	193
<b>5</b>	<b>Investigating the presence of IL-17F and IL-17A in inflammatory arthritis .....</b>	<b>200</b>
5.1	Introduction .....	200
5.2	Results.....	202
5.2.1	Investigating the presence of IL-17A, IL-17F and IL-17AF in matched serum and SF from RA and PsA patients.....	202
Table 5.2 Demographic and clinical parameters of patients included in ELISA assays (patient cohort 2)..... 206		
5.2.2	The cytokine profile of RA and PsA serum and SF and their effect on synovial fibroblasts .....	209
5.2.3	Examining the presence of IL-17A and IL-17F CD4+ T cells in RA and PsA PBMC and SFMC .....	214
5.2.4	In vitro stimulation induces IL-17A, IL-17F and IL-17AF expressing CD4+ T cells from inflammatory arthritis matched PBMC and SFMC .....	221
5.2.5	Investigating the presence of CD8+ IL-17 producing cells in inflammatory arthritis.....	223
5.2.6	Investigating the RNA expression of IL-17 family members in RA and PsA PBMC and SFMC.....	228
5.2.7	Examining the presence of IL-17A and IL-17F expressing cells in RA or PsA tissue .....	234



5.3	Discussion .....	240
<b>6</b>	<b>Final discussion .....</b>	<b>246</b>
6.1	Summary of Chapter 3 results .....	246
6.2	Summary of Chapter 4 results .....	246
6.3	Summary of Chapter 5 results .....	247
6.4	Outstanding questions and future research .....	247
6.4.1	Are IL-17A and IL-17F differentially regulated in CD4+ T cells? ...	247
6.4.2	Is there a functional difference between IL-17A+ and IL-17F+ CD4+ T cells?	249
6.4.3	Is IL-17F present in inflammatory arthritis? .....	252
6.4.4	Does IL-17F contribute to the pathology of other diseases?.....	252
6.4.5	Investigating the presence of IL-17B, IL-17C, IL-17D and IL-17E in inflammatory arthritis.....	256
6.5	Concluding remarks.....	256
	<b>Acknowledgements .....</b>	<b>259</b>
	<b>References .....</b>	<b>260</b>
<b>7</b>	<b>Appendix.....</b>	<b>284</b>
7.1	Analysis of IL-17A/IL-17F CD4+ T cells via CyTOF .....	284

## List of figures

Figure 1.1 JAK/STAT signalling pathway.....	27
Figure 1.2 Timeline of the key discoveries of the IL-17 family .....	28
Figure 1.3 IL-17 receptors.....	31
Figure 1.4 IL-17RA:IL-17RC signalling pathway .....	32
Figure 2.1 Cell purity.....	76
Figure 2.2 Gating strategies for intracellular staining. ....	83
Figure 2.3 ELISA standard curve. ....	91
Figure 3.1 Representative gating strategy following <i>ex vivo</i> stimulation of CD4+ T cells. ....	98
Figure 3.2 Assessing IL-17A+ and IL-17F+ CD4+ T cell frequencies following <i>ex vivo</i> stimulation.....	99
Figure 3.3 Representative gating strategy for <i>in vitro</i> cultures. ....	102
Figure 3.4 LPS-activated monocytes induce an increase in IL-17A and IL-17F expressing CD4+ T cell frequencies and corresponding cytokine secretion. ....	103
Figure 3.5 Elevated levels of IL-1 $\beta$ , IL-6 and IFN $\gamma$ are detected in CD4+ T cell / LPS-activated CD14+ monocyte co-cultures. ....	104
Figure 3.6 IL-1 $\beta$ and IL-23 enhance the frequency of IL-17A+ and IL-17F+ CD4+ T cells. ....	106
Figure 3.7 Correlation analysis of intracellular flow cytometry staining vs. ELISA. ....	108
Figure 3.8 IL-17AF expressing CD4+ T cells are enhanced by IL-1 $\beta$ and IL-23... ..	111
<b>Figure 3.9 CD28 co-stimulation enhances IL-17F expression by CD4+ T cells.</b> .....	114
Figure 3.10 CD28 co-stimulation increases IL-17F+ CD4+ T cell frequencies in a dose-dependent manner.....	115
Figure 3.11 Assessing the kinetic effect of CD28 co-stimulation on protein expression.....	116
Figure 3.12 Increasing doses of anti-CD3 mAb leads to an increase in IL-17F+ CD4+ T cells and IL-17F secretion. ....	118
Figure 3.13 Titration of anti-CD28 and anti-CD3 mAb leads to a dose-dependent increase in IL-2 and IFN $\gamma$ secretion.....	121
Figure 3.14 Isotype controls do not affect IL-17A+ or IL-17F+ CD4+ T cell frequencies.....	122
Figure 3.15 IL-2 blockade reduces IL-17F+ CD4+ T cell expression.....	123
Figure 3.16 Exploring the effect of IFN $\gamma$ blockade on IL-17A and IL-17F expressing CD4+ T cells.....	124
Figure 3.17 Dual blockade of IL-2 and IFN $\gamma$ has no further effect on IL-17A and IL-17F CD4+ T expressing cells than IL-2 blockade alone.....	125
Figure 3.18 Re-stimulation with PMA and ionomycin has no effect on the frequency of IL-17F expressing CD4+ T cells. ....	127

Figure 3.19 Expression of CCR6 and CD161 on IL-17A and IL-17F CD4+ T cells. ....	130
Figure 3.20 IL-17A and IL-17F expressing CD4+ T cells display different cytokine profiles.....	133
Figure 3.21 IL-17A+ IL-17F+ CD4+ T cells display a lower co-expression of IL-10 in comparison to IL-17A+ IL-17F- CD4+ T cells. ....	134
Figure 3.22 Anti-TNF treatment increases co-expression of IL-10 in IL-17A+ IL-17F- and IL-17A+ IL-17F+ CD4+ T cells. ....	135
Figure 4.1 Gene expression of the IL-17 receptors in healthy control, OA and RA synovial fibroblasts. ....	147
Figure 4.2 IL-17A, IL-17F or IL-17AF addition leads to a dose-dependent increase in IL-6 and IL-8 fibroblast secretion. ....	150
Figure 4.3 No synergistic or additive effect is observed between IL-17A, IL-17F and IL-17AF.....	151
Figure 4.4 IL-17A, IL-17F and IL-17AF synergise with TNF $\alpha$ . ....	154
Figure 4.5 Addition of IL-17F or IL-17AF does not further increase the IL-17A and TNF $\alpha$ mediated upregulation of synovial fibroblast pro-inflammatory cytokine secretion. ....	155
Figure 4.6 Testing blocking antibody concentrations. ....	159
Figure 4.7 IL-17F blocking antibody inhibits IL-17F function.....	160
Figure 4.8 Testing blocking antibody specificity. ....	161
Figure 4.9 A combined blockade of IL-17A and IL-17F reduces pro-inflammatory secretion by fibroblasts to a greater extent than blockade of IL-17A alone. ....	163
Figure 4.10 Similar to bimekizumab, combining single IL-17A and IL-17F blocking antibodies reduced IL-6 and IL-8 fibroblast secretion levels to a greater extent than IL-17A blockade alone.....	164
Figure 4.11 Assessing the effect of dual IL-17A and IL-17F blockade in bulk CD4+ T cell supernatants and synovial fibroblast cultures.....	167
Figure 4.12 Gating strategy for sorting for CCR6+ CD161+ T cells. ....	170
Figure 4.13 Dual IL-17A and IL-17F blockade in cultures of synovial fibroblasts and sorted CCR6+ and CD161+ CD4+ T cells.....	171
Figure 4.14 Gating strategy for sorting IL-17A+ CD4+ T cells.....	174
Figure 4.15 Comparison of sorted IL-17A- vs. IL-17A+ sorted CD4+ T cell supernatants on the induction of IL-6 and IL-8 from synovial fibroblasts. ....	175
Figure 4.16 Assessing the effect of dual IL-17A and IL-17F blockade in Th17 supernatant.....	176
Figure 4.17 Assessing the effect of dual IL-17A and IL-17F blockade in fibroblast and IL-17A+ CD4+ T cell cultures.....	178
Figure 4.18 <i>In vitro</i> cultures induce IL-17F+ IL-17A- CD4+ T cells which are not captured by IL-17A secretion assays. ....	182
Figure 4.19 Schematic of UCB IL-17A/IL-17F capture assay.....	183
Figure 4.20 UCB in-house anti-IL-17A and anti- IL-17F Fab fragments successfully recognise human recombinant IL-17A and IL-17F. ....	184
Figure 4.21 Testing the IL-17A and IL-17F capture assay. ....	185

Figure 4.22 Gating strategy for IL-17A and IL-17F capture assay. ....	188
Figure 4.23 Validation of the IL-17A and IL-17F capture assay. ....	189
Figure 4.24 IL-17B, IL-17C, IL-17D and IL-17E alone do not elicit an IL-6 or IL-8 response from synovial fibroblasts. ....	191
Figure 4.25 IL-17E synergises with TNF $\alpha$ , augmenting IL-6 and IL-8 synovial fibroblast secretion. ....	192
Figure 5.1 Analysing the presence of IL-17A and IL-17F in RA and PsA matched serum and SF samples via Luminex. ....	205
Figure 5.2 Analysing the presence of IL-17A and IL-17F in RA and PsA matched serum and SF samples via ELISA. ....	207
Figure 5.3 Human recombinant IL-17A, IL-17F and IL-17AF spiked in serum and SF samples are easily detectable via ELISA. ....	208
Figure 5.4 RA and PsA SF display increased cytokine levels vs. matched serum. .	211
Figure 5.5 Low levels of IL-17E are detectable in RA PB and SF. ....	212
Figure 5.6 Addition of RA or PsA serum or SF to synovial fibroblasts. ....	213
Figure 5.7 Gating Strategy for matched RA and PsA PBMC and SFMC samples. .	217
Figure 5.8 Examining the presence of IL-17A, IL-17F and IL-17AF expressing CD4+ T cells in inflammatory arthritis. ....	218
Figure 5.9 Stimulating RA and PsA paired PBMC and SFMC with different <i>ex vivo</i> conditions. ....	219
Figure 5.10 Correlation analysis on the presence of IL-17A+ CD4+ T cells vs. IL- 17A protein present in inflammatory arthritis samples and assessing correlation with DAS28 scores. ....	220
Figure 5.11 IL-17A, IL-17F and IL-17AF expressing CD4+ T cells can be induced from inflammatory arthritis PBMC and SFMC upon <i>in vitro</i> stimulation. ....	222
Figure 5.12 Assessing the presence of IL-17A and IL-17F expressing CD8+ T cells in inflammatory arthritis. ....	225
Figure 5.13 Back- gating on RA or PsA PBMC and SFMC IL-17 expressing live CD14- cells. ....	226
Figure 5.14 Back- gating on RA or PsA PBMC and SFMC IL-17 expressing live CD14- cells. ....	227
Figure 5.15 Low levels of <i>IL17A</i> and <i>IL17F</i> mRNA are detected from bulk RA and PsA PBMC and SFMC. ....	230
Figure 5.16 Sorting strategies for immune cell subsets from PB and SF. ....	231
Figure 5.17 <i>IL17A</i> and <i>IL17F</i> mRNA is undetectable in sorted CD4+ and CD8+ T cells from RA PB and SF. ....	232
Figure 5.18 IL-17 gene expression profile of CD4+ CD25- T cells from RA PB and SF. ....	233
Figure 5.19 Gating strategy for CD4+ and CD8+ T cells from RA synovial tissue. .....	237
Figure 5.20 Examining the presence of IL-17A and IL-17F expressing CD4+ and CD8+ T cells from RA synovial tissue. ....	238
Figure 5.21 Back-gating on IL-17A and IL-17F producing cells. ....	239
Figure 6.1 Insights into the expression and function of IL-17F .....	258

Figure 7.1 CyTOF gating strategy. ....	287
Figure 7.2 Comparison of IL-17A+ IL-17F-, IL-17A+ IL-17F+ and IL-17F+ IL-17A- CD4+ T cells by SPADE analysis. ....	288
Figure 7.3 Flow Jo analysis on CyTOF acquired data. ....	289

## List of tables

Table 1.1 The interleukin sub-families .....	18
Table 2.1 Human recombinant cytokines used in cell culture .....	80
Table 2.2 Neutralising antibodies used in cell culture .....	80
Table 2.3 Flow cytometry antibodies used in this thesis .....	86
Table 2.4 Metal-conjugated antibodies from UCB used for CyTOF .....	88
Table 2.5 ELISA kits used in this thesis .....	90
Table 2.6 Primers using in this thesis .....	94
Table 5.1 Demographic and clinical parameters of patients included in luminex assay (patient cohort 1).....	204
Table 5.2 Demographic and clinical parameters of patients included in ELISA assays (patient cohort 2).....	206
Table 5.3 Demographic and clinical parameters of patients included in <i>ex vivo</i> IL-17A and IL-17F flow cytometry analysis (patient cohort 3).....	216
Table 5.4 Demographic and clinical parameters of RA synovial tissue sample donors (patient cohort 4).....	236

## Abbreviations

APC	Allophycocyanin
APC Cy7	Allophycocyanin cyanine 7 tandem complex
APC Vio770	Allophycocyanin cyanine 770 tandem complex
BSA	Bovine serum albumin
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
HLA	Human leukocyte antigen
ICCS	Intracellular cytokine staining
IFN $\gamma$	Interferon gamma
IL	Interleukin
Mab	Monoclonal antibody
MACS	Magnetic-activated cell sorting
mRNA	Messenger ribonucleic acid
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PE Cy7	Phycoerythrin cyanine 7 tandem complex
PerCP Cy5.5	Peridinin-chlorophyll-protein cyanine 5.5 tandem complex
PFA	Paraformaldehyde
PMA	Phorbol-12-myristate 13-acetate
PsA	Psoriatic arthritis
RA	Rheumatoid arthritis
Rh	Recombinant human

RT	Room temperature
STAT	Signal Transducer and Activator of Transcription
TCR	T cell receptor
Th	T helper
Treg	Regulatory T cells
TNF $\alpha$	Tumour necrosis factor alpha
TNFR	Tumour necrosis factor receptor
EAE	Experimental autoimmune encephalomyelitis
CIA	Collagen-induced arthritis
APC	Antigen presenting cell
OA	Osteoarthritis
JAK	Janus tyrosine kinase
RANKL	receptor activator of nuclear factor kappa-B ligand (RANKL)
LPS	Lipopolysaccharide
GM-CSF	Granulocyte colony-stimulating factor
Ig	Immunoglobulin
NFkB	Nuclear factor Kb
MCP	Monocyte chemoattractant protein
NK	Natural killer
SpA	Spondyloarthritis



# 1 Introduction

## 1.1 Cytokines

The term 'cytokines' defines a broad group of proteins that are secreted by cells and elicit diverse and overlapping effects on target cells. Essentially, they act as a vital communication network between cells. Cytokines affect a plethora of biological processes including embryonic development, cognitive function and the immune response. They can regulate non-specific response to infection, specific response to antigen and disease pathogenesis <sup>1</sup>. The cytokine families include interleukins (IL), interferons (IFN), tumour necrosis factors (TNF), chemokines and haemopoietic colony-stimulating factor (CSF). Cytokines can act on nearby cells (paracrine action), distant cells (endocrine action) or the cytokine secreting cell itself (autocrine action). Two or more cytokines targeting the same cell can act synergistically or antagonistically. Finally, cytokines can be pleiotropic or redundant. Pleiotropic refers to the ability of cytokines to act on multiple cell types, whereas redundant refers to different cytokines inducing similar signals <sup>2,3</sup>. Pleiotropic actions can be attributed to the presence of a cytokine receptor on multiple cell lineages and redundancies can in part be attributed to similar cellular distributions of receptors for different cytokines, which share the same signalling pathway. Prior to discussing the IL-17 cytokine family, I will provide an overview of all cytokine families and the function of other cytokines which are important in this project.

### 1.1.1 Interleukins

The ILs are a diverse group of cytokines, which were originally identified to be secreted by leukocytes (T cells, monocytes and macrophages) and act upon leukocytes. However, research has since shown other cell subsets including endothelial cells and fibroblasts can be cellular sources and targets of ILs <sup>4</sup>. ILs can be classified into sub-groups based on sequence homology, signalling receptors or functional properties. The IL sub-families include the common  $\gamma$ -chain, IL-1, IL-6, IL-10 and IL-17 sub-families. These families and their members are displayed in table 1.1. Members of the common- $\gamma$  chain cytokine family signal through a receptor complex sharing a  $\gamma$ -chain subunit. This family plays a crucial role in regulating the

proliferation, differentiation and survival of immune cells <sup>5</sup>. IL-2 is a pleotropic cytokine, which is predominantly produced by activated CD4+ T cells. It plays a critical role in the development and function of T regulatory cells (Tregs) <sup>6</sup>. Accordingly, *IL2R* knock-out mice develop systemic autoimmune disease characteristic of reduced Treg cell numbers <sup>7</sup>. With its pleotropic ability, IL-2 can also induce the expansion of CD4+ and CD8+ T cells <sup>8</sup>, promote the proliferation of B cells <sup>9</sup> and augment cytokine production and cytotoxic activity of natural killer (NK) cells <sup>10</sup>. Moreover, IL-2 can prevent over-stimulation of T cells by inducing Fas-mediated apoptosis in CD4+ T cells <sup>11</sup>.

Of the IL-1 family members, IL-1 $\alpha$  and IL-1 $\beta$  are the two major agonistic molecules. Studies have shown that IL-1 $\alpha$  and IL-1 $\beta$  display differences in their expression and roles. The IL-1 $\alpha$  precursor is constitutively expressed in epithelial cells. It is released upon cell death by necrosis, which occurs in ischemic conditions such as stroke. Subsequently, the IL-1 $\alpha$  pre-cursor becomes active and acts as an ‘alarmin’ rapidly initiating a cascade of inflammatory cytokines and chemokines <sup>12,13</sup>. As such, IL-1 $\alpha$  is important in mediating the early phases of sterile inflammation. In contrast, IL-1 $\beta$  is produced by cells of the innate immune system, including monocytes and macrophages. It is rapidly induced upon activation of pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) by pathogen products or factors released by damaged cells <sup>14</sup>. IL-1 $\beta$  is first produced as an inactive pre-cursor, which is activated and released into the extracellular space upon cleavage by caspase-1. Caspase-1 is abundant in hematopoietic cells, however the pro-enzyme (pro-caspase-1) first requires cleavage by the inflammasome. The protein-nucleotide-binding domain and leucine-rich repeat pyrin containing protein-3 (NLRP3) (also known as cryoprin) is a key component of this macromolecular complex <sup>13</sup>. Other enzymes such as elastase, cathepsin G and proteinase-3 also have the ability to generate active IL-1 $\beta$  <sup>15,16</sup>. Functionally, IL-1 $\beta$  has been shown to elicit a wide-range of responses from T cells, in particular findings have identified a key role for IL-1 $\beta$  in the differentiation of T helper (Th17 cells) <sup>17,18</sup>. IL-1 $\beta$  stimulates T cell proliferation by up-regulating IL-2R expression and activating signalling pathways such as the NF $\kappa$ B pathway that can have anti-apoptotic effects <sup>19</sup>. In addition, IL-1 $\beta$  enables effector T cells to proliferate even in the presence of Treg cells <sup>20</sup>.

<b>Interleukin sub-family</b>	<b>Members</b>	<b>Common Feature</b>
Common- $\gamma$ chain cytokines	IL-2, IL-4, IL-7, IL-9, IL-15, IL-21	Signal through type I receptors that contain the $\gamma$ -chain <sup>5</sup>
Common- $\beta$ chain cytokines	IL-3, IL-5, GM-CSF	Signal through type I receptors that contain the $\beta$ -chain <sup>1</sup>
IL-1	IL-1 $\alpha$ , IL-1 $\beta$ , IL-1 receptor agonist (Ra), IL-18, IL-33, IL-36 $\alpha$ , IL-36 $\beta$ , IL-36 $\gamma$ , IL-36Ra, IL-37 and IL-1Hy2	Signal via the IL-1 receptor family <sup>12</sup>
IL-6	IL-6, IL-11, ciliary neutrophilic factor (CNTF), leukemia inhibitory factor (LIF), oncostatin M (OSM), cardiotropin 1 (CT-1), cardiotrophin-like cytokine (CTL) and IL-27	Receptors contain signal-transducing component gp130 <sup>1</sup>
IL-10	IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IL-28, IL-29	Similarities with structure and location of encoding genes, protein structures and receptor complexes <sup>27</sup>
IL-12	IL-12, IL-23, IL-27, IL-35	Heterodimeric cytokines <sup>1</sup>
IL-17	IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, IL-17F	Share a conserved cysteine motif <sup>31</sup>

**Table 1.1 The interleukin sub-families**

The members and common features and interleukin sub-families.

The IL-6 family is highly pleiotropic. The IL-6 cytokine itself is strongly linked to the acute-phase response, which is a complex early-defence system triggered by different stimuli including trauma, infection, stress, neoplasia and inflammation. The aim of this response is to re-establish homeostasis and promote healing <sup>21</sup>. IL-6 has been shown to rapidly induce a wide range of acute phase proteins from hepatocytes, including C-reactive protein (CRP), serum amyloid A, fibrinogen and haptoglobin <sup>22</sup>. In combination with transforming growth factor (TGF)- $\beta$ , IL-6 plays an important role in promoting differentiation of murine naïve CD4<sup>+</sup> T cells into Th17 cells <sup>23</sup>. However, IL-6 also has the ability to suppress TGF- $\beta$ -induced Treg differentiation <sup>24</sup>. Studies have revealed that IL-6 has the ability to induce the differentiation of activated B cells into antibody-producing plasma cells <sup>25</sup>. IL-6 also plays a role in leukocyte recruitment *in vivo*. A study by Romano *et al.* revealed that *IL6* knock-out mice have impaired leukocyte accumulation in subcutaneous air pouches, associated with a reduction in chemokine production. Subsequently, it was shown IL-6 and its soluble receptor sIL-6R $\alpha$  can act on endothelial cells inducing the secretion of the chemokines IL-8 and monocyte chemoattractant protein (MCP)-1 <sup>26</sup>.

In contrast to IL-6, the IL-10 sub-family is anti-inflammatory. The main sources of IL-10 are T cells, monocytes and macrophages. IL-10 signals through the IL-10 receptor (a class II cytokine receptor family composed of IL-10R1 and IL-10R2) and activates the JAK/STAT pathway in target cells <sup>27</sup>. IL-10 is a potent anti-inflammatory cytokine, which plays an important role in regulating the immune response. The importance of IL-10 in regulating the immune response is evidenced by the *IL10* knock-out mouse phenotype, which displays dysregulated inflammatory responses <sup>28</sup>. One of the mechanisms by which IL-10 exerts anti-inflammatory effects is by potently inhibiting monocyte/macrophage activation. Addition of IL-10 to J774 mouse macrophage cells in the presence of LPS, inhibited macrophage activation by LPS <sup>29</sup>. IL-10 potently inhibits monocyte and macrophage production of TNF $\alpha$  and CC and CXC chemokines, therefore reducing chemokine-driven recruitment of neutrophils and other immune cells to sites of inflammation <sup>30</sup>.

The IL-17 family, IL-17A, IL-17B, IL-17C, IL-17D and IL-17E are grouped together as they share a conserved cysteine motif<sup>31</sup>. IL-17A is the best characterised IL-17 family member which displays pro-inflammatory characteristics. The cellular sources, receptors and functions of the IL-17 members are extensively discussed later.

### **1.1.2 Interferons**

The IFN family are important for fighting viral infections and regulation of the immune system. They can bind to surface receptors on infected and neighbouring cells triggering a cascade of events, which results in the rapid transcription of several host proteins (including oligoadenylate synthetase) that can suppress viral infection<sup>32</sup>. Three classes of IFNs exist: Type I IFN, Type II IFN and Type III IFN. Type I IFN includes IFN $\alpha$  and IFN $\beta$ , which are secreted by innate immune cells such as macrophages and dendritic cells<sup>33</sup>. As well as their anti-viral properties, these cytokines can activate CD8+ T cells and NK cells that kill infected targets<sup>34</sup>. The type II includes IFN $\gamma$ , which is secreted by T cells, NK cells, monocytes, macrophages and dendritic cells<sup>35</sup>. IFN $\gamma$  has been shown to induce a T helper (Th) 1 immune response and activate macrophages and NK cells. Furthermore, it has the ability to up-regulate class II major-histocompatibility complex (MHC), which in turn promotes peptide-specific activation of CD4+ T cells<sup>35</sup>. Finally, Type III comprises of IFN $\lambda$ 1, IFN $\lambda$ 2 and IFN $\lambda$ 3, which can be produced by plasmacytoid dendritic cells<sup>36</sup>. Studies have also shown that the type III IFNs have the ability to increase MHC I and MHC II expression on DCs<sup>36</sup>.

### **1.1.3 Tumour necrosis factor (TNF) family**

Early studies demonstrated that tumours contaminated by a bacterial infection could regress and disappear<sup>37</sup>. At this point, it was hypothesised that bacteria were releasing a factor which induced the necrosis of specific tumours, this factor was termed tumour necrosis factor (TNF). Later studies revealed that antigens, such as lipopolysaccharide (LPS), present in the outer membrane of gram-negative bacteria elicited the production of TNF from immune cells<sup>38</sup>. Since then the TNF family has expanded to comprise 19 members, including TNF $\alpha$  (also referred to as TNF), TNF $\beta$  (lymphotoxin- $\alpha$ ) and other ligands such as receptor activator of nuclear factor kappa-B ligand (RANKL)

and TNF-related apoptosis-inducing ligand (TRAIL). TNF $\alpha$  is mainly produced by activated macrophages and monocytes. It is expressed as a transmembrane protein that can be cleaved by the metalloprotease TNF $\alpha$  converting enzyme (TACE) to release a soluble TNF $\alpha$  form<sup>39</sup>. Through the activation of the NF $\kappa$ B pathway, TNF $\alpha$  has a powerful pro-inflammatory capacity. TNF $\alpha$  can promote monocyte/macrophage differentiation<sup>40</sup>, enhance activated B cell proliferation<sup>41</sup> and promote the proliferation of fibroblasts<sup>42</sup>. Depending on the target cell, TNF $\alpha$  is a potent inducer of IL-6<sup>43</sup> and the further production of TNF $\alpha$  itself<sup>44</sup>. In concordance, a report demonstrated that in healthy humans infused with recombinant TNF $\alpha$ , circulating IL-6 is significantly elevated<sup>45</sup>. TNF $\alpha$  alongside other TNF family members (TRAIL) demonstrate a potent apoptotic ability. LPS has been shown to induce apoptosis in macrophages via the autocrine production of TNF $\alpha$ <sup>46</sup>.

#### **1.1.4 Chemokines**

Chemokines are characterised by the presence of three to four conserved cysteine residues and can be sub-divided based on the positioning of the N-terminal cysteine residues. The majority of known chemokines are from the C-X-C or C-C subfamily. The C-X-C subfamily is characterised by a variable amino acid separating the first two cysteines, while the cysteine residues are adjacent to one another in the C-C family<sup>47</sup>. Chemokines are essential for the directional migration of leukocytes during normal and inflammatory processes. Once the chemokine is induced, the directed migration of cells expressing the appropriate chemokine receptors occurs along a chemical ligand gradient known as the chemokine gradient. Subsequently, cells move towards an environment of high local concentrations of chemokines. The best studied members of the C-X-C and C-C chemokine subfamilies are CXCL8 (also known as IL-8) and MCP-1 (also known as CCL2), respectively<sup>48,49</sup>. IL-8 is a critical inflammatory mediator, which is predominantly known for its ability to recruit neutrophils to sites of inflammation. However, it has also been identified in the chemoattraction of monocytes, lymphocytes, basophils and eosinophils<sup>50</sup>. MCP-1 was initially reported to recruit monocytes. In an *in vitro* chemotaxis assay, approximately 30% of input monocytes responded and migrated towards MCP-1<sup>51</sup>. However, further studies have suggested MCP-1 also plays an important role in the migration, generation and survival of memory CD8+ T cells<sup>52</sup>.

### **1.1.5 Colony stimulating factors**

Granulocytes and macrophages play an important role in the innate immune system, protecting the body against bacterial, viral and fungal infections. The colony-stimulating factors are master regulators of granulocyte and macrophage populations. Multiple tissue types produce them locally at the site of inflammation or systemically. The distinct types of CSF include granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF) and multipotential colony-stimulating factor (commonly referred to as IL-3)<sup>53</sup>. A common set of pluripotent stem cells, residing mostly in the bone marrow, give rise to large numbers of neutrophils, basophils, eosinophils, monocytes and lymphocytes circulating in the periphery. As many of these cells are short-lived they must be continually replenished. In health, numbers of these cells are constant, however during infection the production of granulocytes and macrophages can be greatly and rapidly increased. CSFs are crucial for co-ordinating and controlling this cell production. G-CSF and M-CSF support the growth and proliferation of neutrophils and macrophages, respectively. Whereas, GM-CSF supports the growth of neutrophils, macrophages, eosinophils and other cell types<sup>53,54</sup>.

Collectively, a plethora of evidence demonstrates the diverse functions of cytokines mediated via a variety of different receptors, signalling pathways and molecules. Cytokines are interlinked and play a crucial role in regulating the immune and inflammatory response.

## **1.2 Cytokine signalling**

### **1.2.1 The cytokine receptor families**

Receptors for cytokines are structurally diverse. However, the majority of cytokines signal through one of five families of receptor proteins – type I or type II cytokine receptor family, tumour necrosis factor receptor (TNFR) family, IL-1 receptor family or the chemokine receptor family. A significant number of cytokines signal through the type I cytokine receptor family (hematopoietin receptor subfamily) which includes

homodimeric and heterodimeric receptors. Heterodimeric receptors are subdivided based on the presence of receptor signalling components ( $\beta$ -chain,  $\gamma$ -chain and gp130). The conserved extracellular domain of type I cytokine receptors contains four conserved cysteine residues in the amino-terminal region and a conserved Trp-Ser-X-Trp-Ser (WSXWS) motif located proximal to the transmembrane domain <sup>55</sup>. Type II cytokine receptors are similar to type I receptors but lack the conserved motif. They are multimeric receptors composed of heterologous subunits. Type II cytokine receptors are predominantly receptors for IFNs and the IL-10 family <sup>56</sup>. Type I and II cytokine receptors signal through the Janus tyrosine kinase (JAK)/ signal transducer and activator of transcription (STAT) pathway.

The IL-1 receptor family is characterised by extracellular immunoglobulin (Ig)-like domains and Toll-IL-1-receptor (TIR) domains. Each IL-1 ligand (IL-1 $\alpha$ , IL-1 $\beta$  etc.) binds specifically to an extracellular ligand binding chain containing three Ig-like regions. In the case of IL-1 $\alpha$ , IL-1 $\beta$  this is type I IL-1 receptor (IL-1RI). A second accessory chain is recruited, forming a heterotrimeric complex with the ligand binding chain and ligand. This initiates signal transduction. The accessory chain is not present upon binding of the IL-1 and IL-36 receptor agonists. Each ligand binding or accessory chain bears a TIR domain. This domain is also shared by the Toll-like receptors (TLR), which bind a broad spectrum of microbial products <sup>57</sup>. Signal transduction rapidly assembles two signalling proteins, myeloid differentiation primary response gene88 (MyD88) and interleukin-1 receptor-activated protein kinase (IRAK)4. Autophosphorylation of IRAK4 leads to the phosphorylation of IRAK1 and IRAK2 which subsequently elicits the recruitment and oligomerization of tumour necrosis-associated factor (TRAF)6. TRAF6 subsequently induces NF- $\kappa$ B-induced transcription of inflammatory genes <sup>58</sup>.

The tumour necrosis factor receptor (TNFR) family bears a repeated cysteine rich extracellular sequence homology. To date, there are at least 27 members of the TNFR family including LT receptor, Fas, CD40, TRAIL receptors, RANK and death or decoy receptors. However, the main TNF ligands mediate their functional effects by binding to either TNFR1 or TNFR2 receptor sub-types <sup>39</sup>. Activated TNFR1 recruits



TNFR-associated death domain (TRADD) which in turn recruits TNFR-associated factors (TRAFs) to activate the NF $\kappa$ B pathway. TNFR1 also stimulates the formation of a TRADD complex which contains FAS-associated death domain (FADD) and procaspase 8. This leads to the activation of caspase 8 and the initiation of an apoptotic signalling cascade <sup>59,60</sup>.

Chemokine receptors are composed of 7-transmembrane domains and coupled to G-proteins. The chemokine receptors CXCR1 to CXCR5 selectively bind certain CXC chemokines, whereas the CC receptors CCR1 to CCR9 bind CC chemokines <sup>61</sup>. Intracellular signalling by chemokine receptors depend on the coupled-G proteins. G-proteins are inactive when guanosine diphosphate (GDP) is bound to the G-protein sub-unit, however they become active when GDP is exchanged for guanosine triphosphate (GTP). During ligand binding, chemokine receptors associate with the G-proteins allowing for the exchange of GDP to GTP. Subsequently, this activates a series of co-ordinated signalling events that lead to cellular responses <sup>61</sup>.

### **1.2.2 JAK-STAT pathway**

The JAK/ STAT pathway is the predominant signalling cascade that mediates the effects of cytokines, as well as growth factors. It directly translates an extracellular signal into a transcriptional response. The JAK family are non-receptor protein tyrosine kinases consisting of JAK1, JAK2, JAK3 and non-receptor protein kinase 2 (TYK2). The STAT proteins comprise of STAT1 to STAT6. STAT4 exists in two forms, STAT4 $\alpha$  and STAT4 $\beta$ . Additionally, there are two isoforms of STAT5, STAT5a and STAT5b, which are encoded by separate genes <sup>62</sup>. STATs are latent cytoplasmic transcription factors that are activated following receptor phosphorylation. Activation of the JAK/STAT pathway occurs when ligand binding induces the multimerization of receptor subunits. Upon ligand-mediated receptor multimerization, two JAKs are brought into close proximity, allowing trans-phosphorylation. Activated JAKs phosphorylate their associated receptors on specific tyrosine residues. Subsequently, STATs bind to the phosphorylated receptors and are themselves phosphorylated by the JAKs. Through interaction with a conserved Src-homology 2 (SH2) domain, STATs form dimers. Phosphorylated STAT dimers can

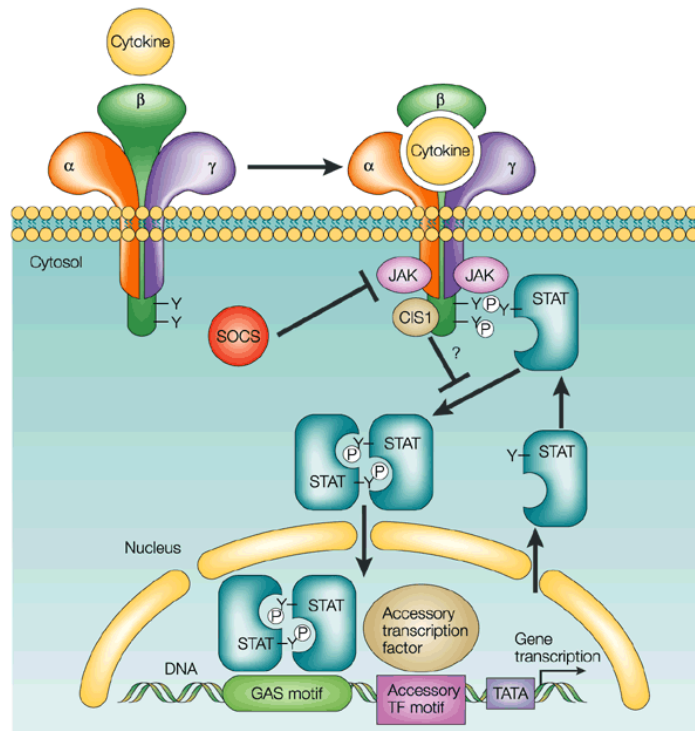
then enter the cell nucleus by a mechanism that is dependent on importin  $\alpha$ -5 and the Ran nuclear import pathway. Once in the nucleus, dimerized STATs bind specific regulatory sequences to activate or repress transcription of target genes<sup>63-65</sup>. In addition to the principal components of the JAK/STAT pathway, effector proteins and negative regulators can affect the signalling events. Effector proteins include signal-transducing adapter molecules (STAMAS), which can facilitate the transcription of specific target genes. Classes of negative regulators include suppressors of cytokine signalling (SOCS), protein inhibitors of activated STATs (PIAS) and protein tyrosine phosphatases (PTPs)<sup>63</sup>.

Different cytokine receptors recruit a different combination of JAKs, which enables a diversity of signalling responses. However, JAKs are not an absolute determinant in cytokine signalling specificity as the same JAKs can be activated by different cytokines. The specificity for a particular STAT phosphorylation appears to be dictated by the STAT docking sites present in the receptors and not the activated JAKS. This is evidenced by the fact that different receptors can activate a common STAT even though they activate distinctively different JAKs. Moreover, it has been shown that chimeric receptors that bear different JAK binding sites can activate the same STAT protein<sup>62</sup>. The phenotypes of STAT knock-out mice have revealed distinctive functions for the different STAT molecules. For example, *STAT1* deficient mice display a severe defect in IFN $\gamma$ -dependent immune responses against viruses. As the mice still retain the ability to other cytokines, it demonstrates the importance of STAT1 for mediating IFN $\gamma$ -dependent pathways<sup>66,67</sup>.

### **1.2.3 NF- $\kappa$ B pathway**

The nuclear factor- $\kappa$ B (NF- $\kappa$ B) pathway serves as a pivotal mediator of inflammatory responses. NF- $\kappa$ B comprises a family of inducible transcription factors, including NF- $\kappa$ B1 (p50), NF- $\kappa$ B2 (p52), RELA (p65), RELB and C-Rel. NF- $\kappa$ B proteins normally exist as inactive cytoplasmic complexes bound by members of the inhibitor of  $\kappa$ B (I $\kappa$ B) family, including I $\kappa$ B $\alpha$ . NF- $\kappa$ B activation occurs via the canonical and the non-canonical NF- $\kappa$ B signalling pathways<sup>68,69</sup>. The canonical pathway is activated by various cytokines including TNF $\alpha$ , IL-1 and IL-17 cytokine family members, as well

as T-cell receptor and B-cell receptor activation. The primary mechanism for the canonical NF- $\kappa$ B activation involves activation of TGF $\beta$ -activated kinase (TAK1) which in turn activates a trimeric I $\kappa$ B (IKK) complex. IKK is composed of catalytic (IKK $\alpha$  and IKK $\beta$ ) and regulatory IKK $\gamma$ , (also known as NEMO) subunits. Upon activation, IKK phosphorylates I $\kappa$ B $\alpha$ , triggering ubiquitin-dependent I $\kappa$ B $\alpha$  degradation in the proteasome. Subsequently, nuclear translocation of the canonical NF- $\kappa$ B family members, predominantly the p50/RelA and p50/c-Rel dimers are released and translocated to the nucleus <sup>69</sup>.



Nature Reviews | Immunology

### Figure 1.1 JAK/STAT signalling pathway

Binding of a type I or type II cytokine receptor to cytokine can induce the activation of JAKs. Subsequently, cellular substrates including one of the receptor chains is phosphorylated. This allows the recruitment of STAT proteins to the phosphorylated receptors and are themselves phosphorylated by the JAKs. Through STAT SH2 domains, they can dimerize, translocate to the nucleus and bind DNA. STAT-dependent responses can be differentially induced by accessory transcription factors. Suppressor of cytokine signalling (SOCS) and cytokine-inducible SH2- containing protein 1 (CIS1) can negative regulate cytokine signalling. Printed with permission from Leonard, W. (2001) Nature <sup>65</sup>.

### 1.3 A brief history of IL-17 identification

Having discussed cytokines in general, I will now focus on the IL-17 family, beginning with the discovery of each IL-17 family member. IL-17A, then referred to as cytotoxic T lymphocyte antigen 8 (CTLA8) was first discovered in a subtractive hybridization screen of a rodent T cell library <sup>70</sup>. Subsequent work detected *CTLA8* mRNA in a human CD4+ T cell clone and revealed it encodes a protein which displays cytokine like characteristics, including activation of NFκB and induction of IL-6 from fibroblasts, thus giving rise to its new term IL-17 <sup>71</sup>. Proteomic and genome database searches led to the discovery of human proteins with similar amino acid sequences to IL-17A, including IL-17B (29% homology), IL-17C (23% homology) and IL-17E (17%) <sup>72-74</sup>. Using nested rapid amplification of cDNA ends (RACE) polymerase chain reaction (PCR), IL-17F was cloned, bearing 50% homology to IL-17A. Expression of IL-17F was observed in activated CD4+ T cells and it was shown to induce IL-2 and TGF-β expression in endothelial cells <sup>75</sup>. Completing the IL-17 family, Starnes *et al.* cloned IL-17D (25% homology to IL-17A) <sup>74,76</sup>. IL-17A and IL-17F exist as homodimers. Due to the sequence homology of IL-17A and IL-17F and previous reports that cysteine knot family members can exist as heterodimers, it was hypothesised that IL-17A and IL-17F could form a heterodimer. Indeed, both mouse and human studies from 2005 onwards identified an IL-17AF heterodimer secreted by CD4+ T cells <sup>77, 78</sup>. A timeline of the IL-17 family history is presented in Figure 1.2.

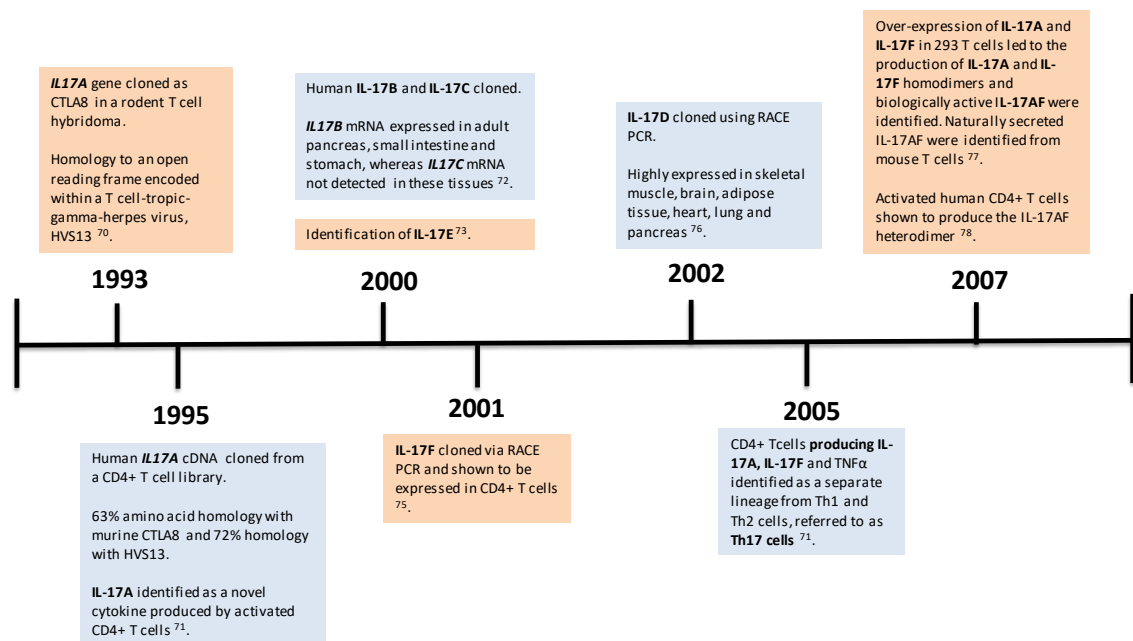


Figure 1.2 Timeline of the key discoveries of the IL-17 family

#### 1.4 Structure of IL-17 cytokines

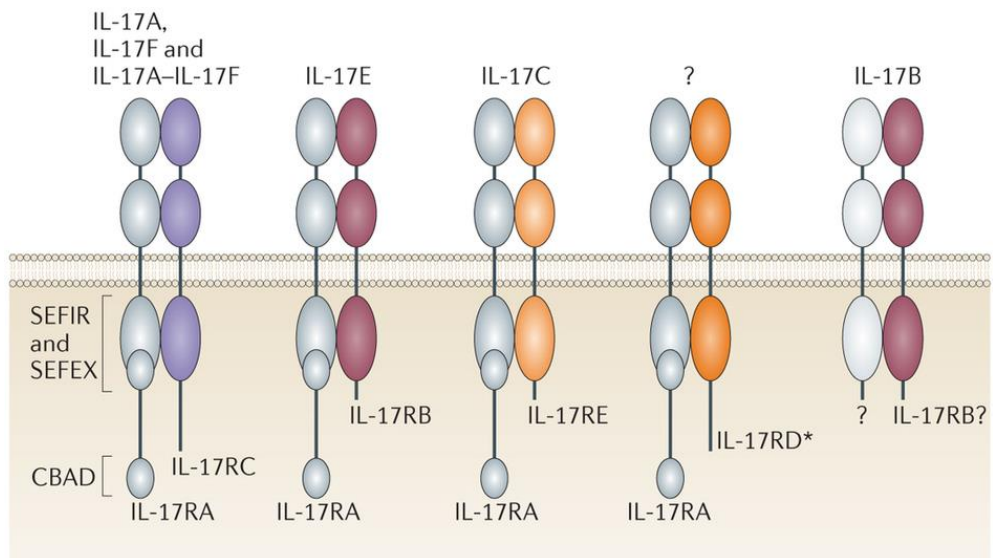
IL-17 family members share limited homology in their primary sequence, however elements of their secondary structure are preserved, especially in the four  $\beta$ -strands in the C-terminal region. Specifically, there are four cysteine and two serine residues highly conserved among IL-17 members, which are critical to forming a cysteine knot fold <sup>79</sup>. To date, only three crystal structures have been resolved including IL-17A with its neutralising antibody <sup>80</sup>, IL-17F <sup>79</sup> and IL-17F with its receptor IL-17RA <sup>81</sup>. The crystal structure of IL-17F demonstrated it is a disulphide-linked homodimeric protein with a cysteine-knot fold, which is also found in the TGF $\beta$ , bone morphogenetic protein and nerve growth factor superfamilies <sup>79,82</sup>. IL-17A and IL-17E are also disulphide-linked homodimeric proteins, however, their precise subunit interactions are not fully understood <sup>83,84</sup>. Homodimeric proteins are formed post-transcriptionally, in the case of the IL-17AF heterodimer it is believed that when a T cell produces high levels of IL-17A and IL-17F monomeric proteins, IL-17AF heterodimers can form. In concordance, no *IL17AF* mRNA can be detected <sup>77,85</sup>. While the majority of genes encoding the IL-17 family members are on different chromosomes, the *IL17F* gene is located adjacent to *IL17A* on chromosome 6p12 <sup>74</sup>.

#### 1.5 IL-17 receptors and signalling

The biological functions of the IL-17 cytokines are mediated via surface receptors on target cells. There are 5 members of the IL-17 receptor family, IL-17RA, RB, RC, RD and RE. It is thought that functional receptors exist as heterodimers, with IL-17RA as a common subunit. The IL-17RA and IL-17RC heterodimer has been reported as the receptor for IL-17A, IL-17F and IL-17AF <sup>86</sup>. While IL-17RA is ubiquitously expressed in haematopoietic cell types, IL-17RC is preferentially expressed by non-haematopoietic cells. Since IL-17A and IL-17F signalling requires IL-17RA and IL-17RC, these cytokines mostly act on parenchymal cells such as fibroblasts, epithelial cells and endothelial cells <sup>31</sup>. In addition research has shown IL-17F binds to IL-17RA with 100-1000 fold lower affinity than IL-17A <sup>79</sup>. The heterodimeric receptor for IL-17E consists of IL-17RA and IL-17RB. IL-17RB is expressed on Th2 cells, various endocrine tissues and the kidney <sup>87</sup>. The receptor for IL-17B includes IL-17RB, although it is unknown if this receptor forms a complex with IL-17RA. Finally, the

receptor for IL-17C comprises an IL-17RE/ IL-17RA complex<sup>31,88</sup>. Although an IL-17RD/IL-17RA receptor complex has been identified the ligand is unknown. Similarly, the receptor for the IL-17D cytokine is unknown<sup>31</sup> (Figure 1.3).

At the C-terminus of the IL-17 receptor is a conserved region known as the SEFIR (similar expression of fibroblast growth factor genes and IL-17Rs) domain. Following IL-17 stimulation, the cytosolic protein NF $\kappa$ B activator 1 (Act 1) is recruited to the IL-17 complex through homotypic interactions of the SEFIR domain. Act1 recruits TNFR-associated factor 6 (TRAF6) proteins, and acts as an E3 ligase, mediating the ubiquitination of TRAF6. This leads to the activation of the canonical nuclear factor- $\kappa$ B (NF $\kappa$ B) pathway and mitogen-activated protein kinase (MAPK) pathways. A unique TRAF6 independent signalling pathway includes Act1 recruitment of TRAF2 and TRAF5, which promotes the activation of an mRNA stability pathway by sequestering ASF/SF2 and recruiting the stabilizing factor HuR<sup>89-92</sup> (Figure 1.4).

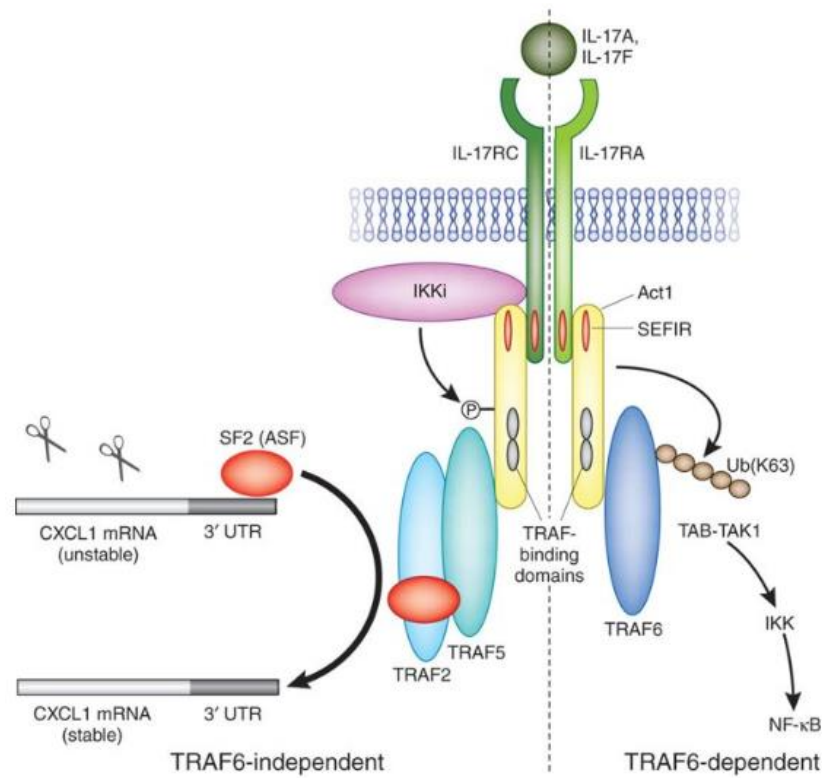


Nature Reviews | Immunology

**Figure 1.3 IL-17 receptors.**

Schematic diagram showing the receptors identified for the IL-17 cytokine family. The majority of IL-17 receptors exist in the form of a heterodimer with IL-17RA as a common sub-unit. The IL-17RA and IL-17RC heterodimer is the receptor for IL-17A, IL-17F and IL-17AF. The IL-17RA sub-unit also couples with IL-17RB to form the receptor for IL-17E, IL-17RE to form the receptor for IL-17C. IL-17B signals via IL-17RB it is unknown if this couples with another receptor sub-unit. An IL-17D/IL-17RA complex has been identified, however the ligand is unknown. Printed with permission from Gaffen (2014) Nature Reviews Immunology<sup>93</sup>.





**Figure 1.4 IL-17RA:IL-17RC signalling pathway**

After stimulation with IL-17 cytokines Act1 and IL-17R interact via their respective SEFIR domains. This leads to the recruitment and ubiquitination of Act1 in a nondegradative way through Lys63-linked ubiquitination. Subsequently, TAK1 and its binding partners TAB2 and TAB3 (TAB) are recruited and activate the IKK complex and NF-κB pathway. An alternative pathway involves phosphorylation of Act1 at Ser311 by IKKi. This generates a docking site which recruits TRAF2 and TRAF6. In the absence of IL-17 cytokines SF2 (ASF) binds to the 3' UTR of CXCL1, causing its instability. After IL-17 stimulation SF2 associates with the TRAF2-TRAF5-Act1 complex, preventing the repression of CXCL1 mRNA. Printed with permission from May Nature Immunology (2011)<sup>92</sup>.

## **1.6 Function of IL-17A, IL-17F and IL-17AF**

### **1.6.1 Individual function**

IL-17A is defined as a pro-inflammatory cytokine and its individual function has been extensively studied. Activation of NF $\kappa$ B and MAPK pathways in target cells by IL-17A results in the transcription of pro-inflammatory genes. This leads to the secretion of pro-inflammatory cytokines (IL-6, TNF $\alpha$ , IL-1) <sup>71</sup>, T cell and myeloid cell-attracting chemokines (CCL20, CCL2 and CCL7) <sup>94,95</sup> and many neutrophilic granulocyte-attracting chemokines (CXCL1, CXCL2, CXCL5 and CXCL8) <sup>71,96</sup> from target cells including fibroblasts, epithelial cells and synoviocytes. In addition, IL-17A can strengthen granulopoiesis by up-regulating the expression of G-CSF and GM-CSF in tissue cells, macrophages and T cells <sup>97</sup> and enhancing the secretion of antimicrobial peptides (defensins and S100 proteins) in target cells <sup>98</sup>. Pathologically, IL-17A can contribute to joint degradation. IL-17A can elicit the production of matrix metalloproteinases (MMP1, MMP9 and MMP13) from target cells, which in turn drives the degradation of extracellular matrix within the joint <sup>99,100</sup>. Osteoclastogenesis is a process that can ultimately lead to bone destruction. Bone-resorbing osteoclasts derive from the haemopoietic cells of the monocyte-macrophage lineage under the control of bone-forming osteoblasts. In the presence of M-CSF, the TNF family member RANKL (expressed on osteoblasts) and its receptor RANK (expressed on osteoblast precursor cells) are vital for inducing osteoclast differentiation <sup>101</sup>. The osteoprotegerin (OPG) ligand is produced by a variety of cells including osteoblasts and acts as a soluble decoy receptor for RANK. Therefore, it inhibits osteoclastogenesis by competing against RANK <sup>102</sup>. Reports have shown IL-17A increases RANKL expression and decreases OPG expression levels in osteoblasts and other target cells, which results in osteoclastogenesis and subsequent bone destruction <sup>103-105</sup>. Collectively, while IL-17A plays an important role in inflammation and host protection against specific pathogen, excessive activation of this pathway can contribute to inflammatory diseases.

The function of IL-17F has not been as extensively studied as IL-17A. However, numerous reports have shown IL-17F to function similarly to IL-17A, albeit less potently than IL-17A. Literature describes a hierarchy in inflammatory potential with

IL-17A eliciting the strongest inflammatory response, followed by IL-17AF and IL-17F <sup>77</sup>. IL-17 can upregulate pro-inflammatory mediators including IL-6, CXCL1, CXCL8, GM-CSF, CCL2, CCL7 and MMP13 from fibroblasts and epithelial cells <sup>106,107</sup>. Additionally, IL-17F has been shown to induce the production of IL-8 in normal epidermal keratinocytes. Mouse skin intradermally injected with IL-17F expressed high *IL8* mRNA levels and histological examination of the mouse skin revealed marked neutrophilia in the dermis <sup>108</sup>. In concordance, Sorbello *et al.* demonstrated that IL-17F was present in nasal biopsies from asthma patients and positively correlated with the neutrophil count <sup>109</sup>. IL-17F has been shown to induce the expression of MMP-9 from epithelial cells, therefore it has the potential to contribute to bone destruction <sup>110</sup>. Very limited studies which examine the function of the IL-17AF heterodimer exist. However, it has been shown that IL-17AF induces IL-6 and CXCL1 secretion from mouse embryonic fibroblasts <sup>77</sup>.

### **1.6.2 Synergy between IL-17A, IL-17F and other pro-inflammatory cytokines**

One of the best-studied cytokines that synergises with IL-17A is TNF $\alpha$ . Studies have demonstrated that IL-17A can synergise with TNF $\alpha$  to induce increased levels of pro-inflammatory mediators such as IL-6, IL-8 and CCL20 production from RA synoviocytes <sup>83,111,112</sup>. Additionally, IL-17A can synergistically enhance the levels of GM-CSF from RA synoviocytes <sup>100</sup>, G-CSF from human epithelial cells <sup>113</sup> and MCP-1 and macrophage inflammatory protein-2 (MIP-2) from mouse mesangial cells <sup>114</sup>. One report showed that IL-17A and TNF $\alpha$  enhanced MMP-2 and CXCR4 expression by RA synoviocytes, leading to an increase in cell invasion as examined by transwell Matrigel invasion chambers <sup>115</sup>. With regards to joint destruction, a study of TNF transgenic mice demonstrated that combined blockade of IL-17A and TNF $\alpha$  was more effective at providing bone protection from bone resorption than blockade of IL-17A or TNF $\alpha$  alone <sup>116</sup>. This was associated with decreased RANKL expression on osteoblasts, increased osteoblast numbers and decreased osteoclast numbers <sup>116</sup>. The suppression of collagen degradation in an RA *ex vivo* bone explant model was shown to be further enhanced with a triple combined blockade of TNF $\alpha$ , IL-1 and IL-17A than blockade of either cytokine alone <sup>117</sup>. In addition to TNF $\alpha$ , synergy between IL-17A and the pro-inflammatory cytokines IL-1 $\beta$  and IFN $\gamma$  has been reported. The

combination of IL-17A and IL-1 $\beta$  was shown to enhance IL-6 production by RA synoviocytes <sup>118</sup> and increase CCL20 production by fibroblast-like synovial cells <sup>119</sup>. Finally, an early study showed IL-17A and IFN- $\gamma$  synergistically upregulated IL-6 and IL-8 production by keratinocytes and led to a subtle increase in expression of ICAM-1, a ligand that binds LFA-1 on T cells to cause T cell adhesion to keratinocytes <sup>91,120</sup>.

Studies have demonstrated that IL-17F can significantly amplify its inflammatory potential by synergising with TNF $\alpha$  and IL-1 $\beta$ . Zrioual *et al.* revealed IL-17F synergises with TNF $\alpha$  to increase *IL6*, *IL8* and *CXCL5* mRNA levels from RA synoviocytes. While the combination of IL-17A and TNF $\alpha$  induces a higher fold increase in pro-inflammatory mRNA levels than IL-17F and TNF $\alpha$ , the effect of IL-17F and TNF $\alpha$  synergy still remained potent <sup>112</sup>. Similar to IL-17A, the combination of IL-17F and TNF $\alpha$  can synergistically elevate levels of G-CSF from human epithelial cells <sup>113</sup>. Additionally, IL-17F can synergise with both TNF $\alpha$  and IL-1 $\beta$ , enhancing the expression of MCP-1 and MIP-2 from mouse mesangial cells <sup>114</sup>. Finally, it has been reported that IL-17AF and TNF $\alpha$  synergistically enhance the production of CXCL1 from macrophages <sup>77</sup>. Literature has suggested that IL-17F is redundant to IL-17A due to the similar functional roles and lower potency of IL-17F <sup>31</sup>. However, a recent study by Glatt *et al.* demonstrated that dual neutralization of IL-17A and IL-17F in Th17 supernatant reduced the pro-inflammatory signature of target cells to a greater extent than neutralization of IL-17A alone <sup>121</sup>. This suggests that dual blockade of IL-17A and IL-17F is more effective at reducing inflammation than the blockade of IL-17A alone.

### **1.6.3 Mechanism of synergy**

Mechanistically, the synergy of IL-17A and IL-17F with other cytokines is not fully understood. However, reports suggest it may occur through the ability of IL-17 cytokines to stabilise mRNA transcripts. One report showed that IL-17A has the ability to extend the half-life of the unstable TNF $\alpha$  -induced *IL8* mRNA, thereby leading to a synergistic increase in IL-8 protein expression and gene expression. It is suggested that IL-17A mRNA stabilisation is via a p38 MAPK-dependent pathway, evidenced by the fact that cells pre-treated with a p38 MAPK inhibitor displayed a

faster *IL8* mRNA decay rate following IL-17A/ TNF $\alpha$  stimulation <sup>122</sup>. In concordance, a study by Hartupee *et al.* demonstrated that IL-17A elicited a synergistic increase in *CXCL1* mRNA expression by extending the half-life of the unstable TNF $\alpha$ -induced *CXCL1* mRNA. Additionally, as Act1 over-expression resulted in *CXCL1* mRNA stabilization, it is proposed that Act1 may be a common mechanism by which IL-17A enhances gene expression <sup>96</sup>. The half-life of other mRNA transcripts including *MIP2* and *GMCSF* is also extended in response to IL-17A, implicating this as a common mechanism for IL-17A synergy <sup>96</sup>. A recent report demonstrated that inhibition of phospholipase D enzymes in cultures of RA fibroblasts stimulated with IL-17A and TNF $\alpha$  led to decreased IL-6, IL-8 and CCL20 production, thereby suggesting these enzymes play a role in the synergistic effect of TNF $\alpha$  and IL-17A <sup>123</sup>. Currently, the mechanism of IL-17F synergy and whether it has the ability to stabilize mRNA has not been investigated.

## **1.7 Function of the remaining IL-17 family cytokines**

### **1.7.1 IL-17E**

In contrast to the pro-inflammatory role of IL-17A and IL-17F, IL-17E is associated with Th2 responses. It promotes the production of type 2 cytokines such as IL-4, IL-5 and IL-13 for eosinophil recruitment and contributes to host defense against helminth, parasitic infections and allergic disease development <sup>84,124</sup>. Interestingly, IL-17E treatment protects mice from Experimental autoimmune encephalomyelitis (EAE) by suppressing IL-17A producing T cells in an IL-13 dependent manner <sup>125</sup>. EAE is an animal model of brain inflammation, although it is mostly used as a model for multiple sclerosis, it is also a prototype for T cell-mediated autoimmune disease <sup>126</sup>. This suggests IL-17E supports a cytokine environment that limits chronic inflammatory responses, an opposite role to IL-17A. The opposing roles of IL-17E and IL-17A are further supported by a report from Xu *et al.* When stimulated with dsRNA and IL-17E, primary human nasal epithelial cells (HNECs) from patients with allergic rhinitis (AR) produced elevated levels of thymic stromal lymphopoietin (TSLP). TSLP is associated with asthma and its overexpression has been shown to correlate with disease severity. In contrast, IL-17A significantly inhibited double-stranded (ds)

RNA-induced TSLP production. Moreover, IL-17E was dominant to the inhibitory effect of IL-17A on TSLP regulation<sup>127</sup>.

### **1.7.2 IL-17C, IL-17B and IL-17D**

Numerous studies have revealed a role for IL-17C in regulating epithelial immune responses<sup>128</sup>, maintaining mucosal barrier maintenance<sup>129</sup> and promoting Th17 responses<sup>130</sup>. Additionally, IL-17C has been implicated in lesional psoriasis skin formation<sup>131,132</sup>. The biological function of IL-17B and IL-17D are less extensively studied. Bie *et al.* have recently reviewed current knowledge of IL-17B<sup>133</sup>. Reports have identified a role for IL-17B in inflammation, upregulating the expression of TNF $\alpha$  and IL-1b from THP-1 cells and synergising with TNF $\alpha$  to enhance the production of G-CSF and IL-6 from fibroblasts<sup>72,134</sup>. Additionally, a more recent study has linked IL-17B to germinal centre B cell migration<sup>135</sup>. IL-17B has been detected in the arthritic paws of CIA mice and in human rheumatoid synovial tissue, suggesting it may play a role in inflammatory disease<sup>134,136</sup>. IL-17D is the least studied IL-17 family member. Initial reports showed IL-17D upregulated the expression of IL-6, IL-8 and GM-CSF from endothelial cells<sup>76</sup>. Interestingly, more recent studies have implicated IL-17D in tumor rejection. These studies have shown IL-17D to be highly expressed in certain tumors and induce MCP-1 production from tumor endothelial cells, leading to the recruitment of natural killer (NK) cells, which in turn promotes M1 macrophage development and anti-tumor responses<sup>137-139</sup>.

## 1.8 T cells

One of the main cellular sources of IL-17A and IL-17F is CD4<sup>+</sup> T cells. Before discussing IL-17 expressing T cells, an overview of T cells is provided. T cells play a central role in the adaptive immune system, orchestrating many aspects of the inflammatory response. T cells can be divided into two categories classically referred to as; cytotoxic CD8<sup>+</sup> T cells and CD4<sup>+</sup> T helper (Th) cells. CD8<sup>+</sup> T cells can act directly to kill virally infected or transformed cells <sup>140</sup> and CD4<sup>+</sup> T cells can provide signals that modulate the behaviour and function of other immune cells <sup>141</sup>. Both CD4<sup>+</sup> and CD8<sup>+</sup> T cells derive from the common lymphoid progenitor cell in the bone marrow and mature and undergo thymic selection in the thymus. This process involves positive and negative selection based on interactions with major histocompatibility complex (MHC) and peptides derived from self-antigens. Subsequently, auto-reactive T cells are deleted and the T cell repertoire contains a diverse T cell receptor (TCR) expression, which promotes the protection against a vast number of foreign antigens <sup>142</sup>. The TCR expressed on T cells is a highly variable disulphide-linked membrane bound heterodimeric protein. In humans, the majority (95%) of T cells express a TCR formed of the highly variable  $\alpha$  and  $\beta$  chains expressed as part of a complex with invariant CD3 chain molecules, these are referred to as  $\alpha\beta$  T cells. However, a minority of T cells express an alternate receptor composed of variant  $\gamma$  and  $\delta$  chains ( $\gamma\delta$  T cells) <sup>143</sup>. The TCR confers antigen specificity to the cell, via recognition of antigen presented in the context of the major histocompatibility complex (MHC) by antigen-presenting cells. The co-receptors CD4 (for T helper cells) and CD8 (for cytotoxic T cells) bind to invariant parts of MHC class II or I, respectively <sup>144</sup>.

### 1.8.1 T cell activation

Following maturation in the thymus antigen-specific 'naïve' T cells exit into the periphery. They circulate via the secondary lymphoid organs and tissues including the spleen and lymph nodes. Here, they may encounter antigen presented by antigen presenting cells (APCs), which can include dendritic cells (DCs), monocytes, macrophages or B cells. APCs have the ability to collect antigens from the periphery and migrate to lymphoid tissues to display the antigen to lymphocytes. For full T cell activation a specific T cell firstly binds via its TCR to an antigen presented in the peptide groove of an MHC molecule on the APC. Antigens derived from the cytosol

of the cell are presented in the context of MHC class I molecules to CD8<sup>+</sup> T cells, whilst antigens derived from exogenous proteins are presented in the context of MHC class II molecules to CD4<sup>+</sup> T cells. However, it is important to note that the process of cross-presentation can occur, whereby some APCs can present exogenous antigens into the MHC class I pathway for presentation to CD8<sup>+</sup> T cells<sup>145</sup>. Ligation of co-stimulatory molecules, such as CD28 which interacts with CD80 (B7.1) and CD86 (B7.2) on the surface of APCs, provides a second signal which is necessary for efficient T cell activation<sup>144</sup>. Another co-stimulation signal involves interaction between CD40L expressed on T cells and CD40 expressed on APCs<sup>146</sup>. CD28<sup>-/-</sup> naïve T cells have been shown to be deficient in IL-2 secretion and proliferation when stimulated with a wide range of antigen concentrations and APC numbers, whereas CD40L<sup>-/-</sup> T cells respond normally in the presence of high antigen or high APC to T cell ratios and show defects only when stimulated with low antigen concentration or antigen concentration<sup>146</sup>. This suggests that the predominant role of the CD40L/CD40 interaction is to amplify an existing co-stimulation signal. Indeed, CD40L engagement of CD40 on APCs increases the APC expression of B7 molecules<sup>147</sup>. Studies have shown that signalling via the TCR in the absence of co-stimulation can lead to the induction of anergy in the T cell<sup>148</sup>. A CD28 homolog called cytotoxic T lymphocyte-associated antigen 4 (CTLA4) exists. Similar to CD28 it binds to B7 molecules, however it binds with higher affinity and does not produce a stimulatory signal. As such, this competitive binding can prevent the CD28/B7 co-stimulatory signal<sup>149,150</sup>.

Following the recognition of the cognate antigen and MHC by a specific TCR, the T cell and APC form an immunological synapse and undergo actin-mediated membrane re-organisation<sup>151</sup>. The TCR, co-stimulatory and adhesion molecules are grouped together at the site of the TCR-MHC interaction and form a large multimolecular structure referred to as the supramolecular activation complex (SMAC). The spatial segregation of the SMAC complex was first described by Monk *et al.* using deconvolution microscopy and has since been confirmed by others. They describe the SMAC complex comprising of a focal point of signalling molecules (cSMAC) surrounded by a ring of adhesion molecules including leukocyte function-associated molecule-1 (LFA-1) (pSMAC)<sup>152,153</sup>. This arrangement supports strong and prolonged intracellular interactions<sup>154</sup>. Arrangement of the TCR/peptide/MHC within the



cSMAC results in the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) located on CD3 by the Src family kinases Lck and Fyn. This in turn leads to the recruitment and activation of  $\zeta$ -chain-associated protein 70 (ZAP70)<sup>155,156</sup>, which phosphorylates the scaffold proteins linker for activation of T cells (LAT)<sup>157</sup> and the cytosolic adapter protein leukocyte phosphorylation of 76kDa (SLP-76)<sup>158</sup>. The adaptor protein GADS bring SLP-76 and LAT together<sup>159</sup>. The GADS:SLP-76: LAT complex recruits phospholipidase-C- $\gamma$ 1 (PLC $\gamma$ 1), which is a key signalling molecule activated by the phosphorylation of Itk. Activated PLC $\gamma$ 1 hydrolyses the membrane lipid (phosphatidylinositol 4,5 bisphosphate) (PI(4,5)P<sub>2</sub>) forming the second messengers IP<sub>3</sub> and DAG. These messengers are important for mediating the remaining down-stream signals which lead to T cell activation, including the activation of NF $\kappa$ B, NFAT and AP-1 to induce specific gene transcription, leading to cell proliferation, differentiation and cytokine production<sup>160</sup>. Finally, the last signal involved in T cell activation involves cytokines. Cytokines drive the differentiation of T cells into specific subsets<sup>144</sup>. The CD4+ T helper subsets will be discussed in more detail in section 1.8.3.

### 1.8.2 Memory T cells

Following APC presentation of a pathogenic antigen, naïve T cells become activated, proliferate and differentiate into T effector cells which can adopt functional roles aimed at clearing a host of infectious agent. These T effector cells are short-lived and their cell number rapidly declines once the primary response is over and the infection is cleared. However, a small pool of T cells with ‘memory’ for the pathogen remains<sup>161,162</sup>. Both CD4+ and CD8+ T cells have memory populations. Memory T cells have a potential of long-term survival and can rapidly respond should re-infection occur. They can be located in the secondary lymphoid organs (central memory (T<sub>CM</sub>) cells) or the recently infected tissues (effector memory (T<sub>EM</sub>) cells). Compared with their naïve counter-parts, memory T cells demonstrate less strict requirements for activation via antigen and co-stimulation signals<sup>162</sup>. In addition, memory T cells can elicit effector responses more rapidly than primary responding cells<sup>163</sup>. In humans, naïve and memory T cells are distinguished by the CD45 isoform, with naïve T cells expressing CD45RA and memory cells expressing CD45RO<sup>164</sup>.

### 1.8.3 T helper cell subsets

Seminal work from Mosmann and Coffman first identified the existence of distinctive CD4<sup>+</sup> Th subsets; Th1 and Th2<sup>165</sup>. Th1 cells produce IFN $\gamma$ , IL-2 and tumour necrosis factor- $\alpha$  TNF $\alpha$ , which promotes a cellular immune response resulting in T cell proliferation and the classical activation of macrophages<sup>166</sup>. This subset of cells also has the ability to promote complement-fixing and opsonising antibodies<sup>141</sup>. Naïve CD4<sup>+</sup> T cells are driven towards a Th1 subset by IFN $\gamma$  and IL-12, which signal via STAT1 and STAT4, respectively<sup>167,168</sup>. T-bet is also a critical transcription factor for Th1 differentiation, inducing the production of IFN $\gamma$ <sup>169</sup>. In contrast, Th2 cells produce IL-4, IL-5 and IL-13 this promotes alternative macrophage activation and drives mast cell and eosinophil proliferation and activation. Moreover, Th2 cells contribute towards immunity to extracellular pathogens such as helminths and primarily promote a humoral immune response<sup>170</sup>. Th2 differentiation is dependent on IL-4, which activates STAT6 and GATA3<sup>171</sup>. IFN $\gamma$  and IL-4 have the ability to antagonize one another. For example, IFN $\gamma$  can activate STAT1 leading to the expression of T-bet and studies have shown that T-bet is a repressor of Th2 differentiation. T-bet transduced into polarised Th2 cells can modulate their phenotype towards Th1, as evidenced by induction of IFN $\gamma$  and repression of IL-4<sup>172</sup>. The central paradigm of the Th subsets has since been expanded to include additional subsets such as Treg cells, Th17 cells, Th9, Th22, Th3 and T follicular helper cells (Tfh).

Treg cells are a unique subset of Th cells, which are essential for the maintenance of immunological tolerance and the prevention of autoimmunity and chronic inflammation. Their suppressive function can be elicited via the production of the anti-inflammatory cytokine IL-10 and their constitutive expression of the inhibitory co-stimulation marker CTLA4<sup>173</sup>. The cytokines IL-2 and TGF- $\beta$  drive Treg differentiation by inducing the expression of the forkhead box P3 (FOXP3) transcription factor<sup>174,175</sup>. FOXP3 is critical for the development, maintenance and function of Treg cells<sup>176,177</sup>. Another regulatory lineage identified includes Th3 cells. Th3 cells were originally identified in murine models following the induction of oral tolerance to myelin basic protein (MBP). These cells suppressed MBP-specific Th1

effector cells in a TGF- $\beta$  dependent, antigen non-specific manner<sup>178</sup>. In the presence of TGF- $\beta$ , naïve CD4<sup>+</sup> T cells can differentiate into Th9 cells and also produce IL-4 and IL-10<sup>179</sup>.

While early studies implicated that IL-9 is produced by Th2 cells, reports emerged demonstrating that IL-9 is also produced exclusively by a Th9 lineage<sup>180,181</sup>. Th9 differentiation is dependent on the cytokines IL-4 and TGF- $\beta$  and the transcription factors PU.1 and interferon-regulatory factor 4 (IRF4)<sup>182-184</sup>. Th22 cells are characterised by their production of IL-22 without IFN $\gamma$ , IL-4 and IL-17<sup>185</sup>. Naïve CD4<sup>+</sup> T cells can be driven towards the IL-22 lineage by IL-6 and TNF $\alpha$ <sup>186</sup> and the transcription factor aryl hydrocarbon receptor (AHR)<sup>187</sup>. With their expression of the skin homing marker CCR4 and CCR10, Th22 cells play a critical role in skin diseases<sup>186</sup>. Tfh cells are located in secondary lymphoid organs including the lymph nodes, spleen and tonsil and they specifically provide help to B cells. They play an important role in the development of antibodies and memory B cells, germinal centre formation and affinity maturation<sup>188</sup>.

## **1.9 Th17 cells**

### **1.9.1 Th17 cell differentiation**

The most studied source of IL-17A is the CD4<sup>+</sup> T helper type (Th17) subset. Following the discovery of IL-17A and the identification of activated human CD4<sup>+</sup> T cells as IL-17A producers, a plethora of studies quickly defined the Th17 paradigm<sup>70,71</sup>. Studies have shown a combination of TGF- $\beta$  and IL-6 can differentiate naïve mouse CD4<sup>+</sup> T cells into the Th17 lineage<sup>23,24,189</sup>. However, in the absence of IL-6, TGF- $\beta$  inhibits Th17 differentiation and induces Foxp3 expression and Treg induction<sup>190</sup>. It has also been shown that the IL-2 cytokine family member, IL-21 co-operates with TGF- $\beta$  to induce Th17 cells from naïve murine CD4<sup>+</sup> T cells<sup>191</sup>. In humans, studies have shown that TGF- $\beta$  and IL-6 alone are not sufficient to induce Th17 cells<sup>192-194</sup>. Additional pro-inflammatory cytokines such as IL-1 $\beta$  or IL-21 are required for human Th17 differentiation, while IL-23 is important for lineage maintenance and survival<sup>193,195</sup>. In addition to the generation of Th17 cells from naïve CD4<sup>+</sup> T cells,

Th17 differentiation can also be induced from effector memory CD4<sup>+</sup> T cells <sup>192,196,197</sup>. Our lab shown that significant numbers of human IL-17A<sup>+</sup> CD4<sup>+</sup> T cells were induced by stimulating CD4<sup>+</sup> CD45RO<sup>+</sup> T cells through the TCR in the presence of LPS-activated monocytes <sup>197</sup>. Moreover, we have shown that periodontal pathogens promote an IL-17A<sup>+</sup> CD4<sup>+</sup> T cell response which is dependent, in part on IL-1 $\beta$ , IL-23 and TLR2/ TLR4 signalling from CD14<sup>+</sup> monocytes <sup>198</sup>. Finally, a study demonstrated that upon sensing bacterial nucleotide oligomerization domain 2 (NOD2)-ligand muramyl dipeptide (MDP), dendritic cells promoted IL-17 production from memory CD4<sup>+</sup> T cells via enhanced expression of IL-1 $\beta$  and IL-23 <sup>199</sup>. Collectively, these studies show that ligation of TLRs such as TLR2 and TLR4 leads to an up-regulation of Th17-polarizing conditions, which subsequently induces Th17 differentiation.

Th17 cells also produce IL-17F. Studies frequently report that IL-17F is co-expressed with IL-17A, however different IL-17 producing populations exist including IL-17A<sup>+</sup> IL-17F<sup>-</sup>, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells <sup>106,193</sup>. It is important to note that in general Th17 cells are determined by their ability to produce IL-17A and not IL-17F. Moreover, the majority of studies which investigate the differentiation of Th17 cells examine IL-17A<sup>+</sup> CD4<sup>+</sup> T cells and not IL-17F<sup>+</sup> CD4<sup>+</sup> T cells. One study by Wilson *et al.* has demonstrated that like IL-17A, human IL-17F<sup>+</sup> CD4<sup>+</sup> T cells are enhanced in the presence of IL-1 $\beta$  and IL-23 <sup>193</sup>.

### **1.9.2 Transcriptional control of Th17 cells**

The main regulator of Th17 cells is the transcription factor retinoic acid (RA)- related orphan nuclear receptor  $\gamma$  (ROR $\gamma$ t), which is encoded by the *RORC* gene <sup>200</sup>. However, ROR $\gamma$ t deficiency does not completely abolish Th17 cytokine expression <sup>106</sup>. RA-related orphan receptor  $\alpha$  has also been acknowledged as a transcription factor critical for Th17 differentiation <sup>201</sup>. STAT3 has been shown to be critical for Th17 differentiation. ChIP-seq analysis has demonstrated STAT3 directly regulates the *IL17A*, *IL17F* and *RORC* as well as multiple other genes associated with Th17 polarisation and survival <sup>202</sup>. Mice that lack STAT3 in T cells have an impaired ability to generate Th17 cells <sup>203</sup>. Other transcription factors which contribute in driving Th17

differentiation include basic leucine zipper transcription factor ATF-like (BATF) and interferon regulatory factor 4 (IRF4). BATF is a member of the activator protein 1 (AP-1) transcription factor family that is regulated in Th cells following TCR activation. While BATF<sup>-/-</sup> mice displayed normal Th1 and Th2 differentiation, Th17 differentiation was defective<sup>204</sup>. *IRF4* deficient mice displayed a defective Th17 differentiation as a result of decreased ROR $\gamma$ t expression<sup>205</sup>. Studies have suggested that BATF and IRF4 co-operatively bind and govern chromatin accessibility, which enables ROR $\gamma$ t binding to Th17 associated genes<sup>89,206,207</sup>. Finally, hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) has also been linked to Th17 differentiation. HIF-1 $\alpha$  is induced upon TCR activation and its expression is increased under hypoxic conditions associated with tissue inflammation. It has been shown that HIF-1 $\alpha$  directly binds and drives the transcription of ROR $\gamma$ t and p300 (a histone acetyltransferase) to drive IL-17A expression<sup>208</sup>.

### 1.9.3 Regulation of Th17 cells

Cytokines have emerged as key negative regulators of Th17 cells. Reports have shown the Th1 and Th2 cytokines, IFN $\gamma$  and IL-4 respectively, inhibit Th17 differentiation<sup>209,210</sup>. Harrington *et al.* showed that the development of mouse Th17 cells from naïve CD4<sup>+</sup> T cells was potently inhibited by IFN $\gamma$  and IL-4, whereas committed Th17 cells were resistant to IFN $\gamma$  and IL-4 mediated-suppression<sup>209</sup>. Titration of IFN $\gamma$  led to a dose-dependent decrease in *IL17A* and *IL17F* mRNA levels present in mouse CD4<sup>+</sup> T cells<sup>211</sup>. *In vivo*, IFN $\gamma$  deficient mice display increased frequencies of IL-17A<sup>+</sup> CD4<sup>+</sup> T cells and IL-17A protein<sup>210</sup>. In a human study, it has been shown that memory CD4<sup>+</sup> T cells cultured with monocytes and LPS in the absence or presence of Th1 polarising conditions (recombinant IL-12 and IL-4 blocking antibody) leads to an up-regulation of IFN $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells and IFN $\gamma$  secreted protein, while IL-17A<sup>+</sup> CD4<sup>+</sup> T cells and IL-17A secretion is significantly decreased<sup>192</sup>. Similarly, blockade of IFN $\gamma$  in memory CD4<sup>+</sup> T cell cultures stimulated with anti-CD3 and anti-CD28 leads to an enhanced frequency of IL-17A<sup>+</sup> CD4<sup>+</sup> T cells<sup>192</sup>. Mechanistically, reports have shown the IFN $\gamma$ -mediated suppression of Th17 cells can be via STAT1 and T-bet dependent mechanisms<sup>211,212</sup>. IFN $\gamma$  propagates a JAK/STAT signalling cascade leading to the robust activation of STAT1<sup>213</sup>. Additionally it drives the induction of the transcription factor T-bet<sup>169</sup>. In concordance, gain-of-function mutations in *STAT1*

in patients suffering with chronic mucocutaneous candidiasis inhibited the development of IL-17A<sup>+</sup> CD4<sup>+</sup> T cells<sup>214</sup> and in *Tbet* knock-out mice (*Tbx21*<sup>-/-</sup>), T cells produce higher levels of IL-17A in response to TCR stimulation when compared to wild-type mice<sup>215</sup>. Similar to IFN $\gamma$ , other STAT1 activating cytokines can regulate Th17 responses including IL-27, a member of the IL-12 family of cytokines. *In vitro* studies have demonstrated IL-27 suppresses IL-6 and TGF- $\beta$  induced Th17 differentiation in a STAT1-dependent manner<sup>216,217</sup>.

Reports have also suggested that IL-2 has the ability to suppress Th17 development. Laurence *et al.* showed that genetic deletion or antibody blockade of IL-2 in mice promoted differentiation of IL-17A<sup>+</sup> CD4<sup>+</sup> T cells<sup>218</sup>. Additionally, a characteristic of autoimmune disease associated with IL-2 deficiency is the over-production of IL-17A *in vivo*<sup>218</sup>. High doses of IL-2 in combination with TGF- $\beta$  and retinoic acid have been shown to inhibit IL-17A expression via the induction of FOXP3 expression and skewing CD4<sup>+</sup> T cell differentiation into Tregs<sup>219,220</sup>. However, it has also been reported that IL-2 can also inhibit IL-17A production independently of FOXP3<sup>221,222</sup>. Studies have shown the effect of IL-2 appear to be largely associated with its downstream target STAT5<sup>223</sup>. Deficiency in STAT5 displays similar aspects to IL-2 deficiency. Mice with deletion of *STAT5a* and *STAT5b* exhibit widespread autoimmune disease linked with IL-17A over-production<sup>218,223</sup>. The underlying mechanism has been attributed to the ability of STAT5 to directly compete with IL-17A-inducing STAT3 to enhancers within the *IL17A* gene locus<sup>222</sup>. Using chromatin immunoprecipitation (ChIP) sequencing it was shown that the presence of IL-2 led to increased binding of STAT5 across the *IL17* locus and at all the sites in the *IL17A* locus this was correlated with significantly less binding of STAT3<sup>222</sup>. In concordance, a recent mouse study demonstrated that phosphate and tensin homologue (PTEN) supports Th17 differentiation by suppressing IL-2 production. *Pten* deficient mice displayed an up-regulation of IL-2 and phosphorylation of STAT5, while STAT3 phosphorylation was decreased. Collectively, this led to suppression of IL-17A<sup>+</sup> CD4<sup>+</sup> T cells<sup>224</sup>. It is important to note that all of these reports investigate the role of IL-2 in mice. How IL-2 affects the frequency of IL-17A<sup>+</sup> CD4<sup>+</sup> T cells in humans is currently unclear. Few reports examine the effect of IL-2 on IL-17F<sup>+</sup> CD4<sup>+</sup> T cell frequencies. As literature often describes IL-17F to be co-expressed with IL-17A it

could be assumed that IL-17F expression is similarly suppressed by IL-2. However, it has been shown using mouse cells that in the presence of high levels of IL-6 while addition of IL-2 decreases the percentage of IL-17A+ CD4+ T cells it has no effect on the frequency of IL-17F+ CD4+ T cells <sup>222</sup>.

CD28 co-stimulation is known to induce the expression of IL-2 and IFN $\gamma$ . A study using mouse cells has shown that anti-CD28 mAb suppressed the differentiation of IL-17A+ CD4+ T cells in an IL-2 and IFN $\gamma$  dependent mechanism <sup>221</sup>. Moreover, Purvis *et al.* stimulated human CD4+ T cells with either high or low-strength stimulation via anti-CD3/CD28 beads or dendritic cells pulsed with superantigen in the presence of IL-1 $\beta$ , TGF- $\beta$  and IL-23. They found that low-strength stimulation significantly enhanced IL-17A expression, whereas IL-17A expression was reduced when receiving a high-strength stimulation <sup>225</sup>. Collectively, these reports demonstrate that IL-17A+ CD4+ T cells are induced upon low-strength T cell activation. As these studies examine the expression of IL-17A only, it is not known how high-strength T cell activation effects the expression of IL-17F from CD4+ T cells. In light of the study which suggests IL-2 may differentially effect IL-17A and IL-17F expression <sup>222</sup>, how T cell signalling strength impacts IL-17F would be an interesting area to pursue.

#### **1.9.4 Phenotypic marker and cytokine profiles of IL-17A+ and IL-17F+ CD4+ T cells**

Studies have characterised Th17 cells by their expression IL-23 receptor (IL-23R), the chemokine receptor CCR6 and the lectin receptor CD161 <sup>226,227</sup>. Annunziato and colleagues showed that Th17 clones exhibit higher levels of the chemokine receptors CXCR4, CXCR6, CCR4 and CCR5 when compared to other clone types including Th1 and Th2. They also demonstrated that Th17 cells selectively expressed CCR6 <sup>226</sup>. CCR6 expression had been identified on B cells, dendritic cells (DCs) and memory T cells, however, the CCR6 ligand, CCL20 only exerts chemotactic activity on memory T cells <sup>228</sup>. It has been shown that CCR6 expression in memory T cells is lost following prolonged TCR triggering <sup>229</sup>. This suggests that the selective expression of CCR6 on

Th17 cells could be a result of the ability of Th17 cells to continue to express CCR6 following prolonged antigen activation, thereby maintaining the ability to recruit in response to CCL20<sup>226</sup>. CD161 is a C-type lectin-like receptor expressed in the majority of natural killer (NK) cells<sup>230</sup>. Cosmi *et al.* demonstrated that the *CD161* gene is up-regulated in Th17 clones and in human peripheral blood (PB) and inflamed tissues all IL-17A<sup>+</sup> RORC<sup>+</sup> cells are contained within the CD161<sup>+</sup> fraction of CD4<sup>+</sup> T cells. However, not all CD161<sup>+</sup> T cells produce IL-17A<sup>231</sup>. It was also shown that IL-17A<sup>+</sup> cells originate from the CD161<sup>+</sup> (and not the CD161<sup>-</sup>) naïve CD4<sup>+</sup> T cells of umbilical blood in response to IL-1 $\beta$  and IL-23<sup>231</sup>. The exact function of CD161 is unknown, however, it has been suggested to play a role in transendothelial migration as CD161<sup>+</sup> T cells migrate across endothelial cell monolayers to a greater extent than CD161<sup>-</sup> T cells<sup>232</sup>. It has been implied that expression of CD161 is RORC-dependent. Naïve CD4<sup>+</sup> CD161<sup>-</sup> T cells transduced with *RORC* produce IL-17A and up-regulate CD161 expression. In contrast, inhibition of RORC down-regulates IL-17A and CD161 expression on Th17 clones<sup>233</sup>.

IL-17A<sup>+</sup> CD4<sup>+</sup> T cells have been shown to co-express IL-17F, IFN $\gamma$ , TNF $\alpha$ , GM-CSF, IL-21, IL-22, IL-9 and IL-10. The presence and expression level of these cytokines depends on the cytokine milieu present upon Th17 differentiation<sup>234-236</sup>. As previously mentioned, different IL-17 producing subsets exist including IL-17A<sup>+</sup> IL-17F<sup>-</sup>, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells. In the current literature, there is a lack of characterisation of IL-17F<sup>+</sup> CD4<sup>+</sup> T cells and comparison of these cells vs. IL-17A<sup>+</sup> CD4<sup>+</sup> T cells. It is not known whether marker expression on IL-17A<sup>+</sup> vs. IL-17F<sup>+</sup> CD4<sup>+</sup> T cells may differ or whether one subset demonstrates a more inflammatory cytokine profile. Reports have previously demonstrated Th17 cells are a heterogeneous population, including ‘non-pathogenic’ and ‘pathogenic’ subtypes. McGeachy *et al.* shown that TGF- $\beta$  and IL-6 promote the generation of non-pathogenic Th17 cells, characterised by the production of IL-10<sup>237</sup>. However, exposure to IL-23 can result in the conversion of non-pathogenic Th17 cells to pathogenic Th17 cells<sup>238</sup>. Prostaglandin E2 and the Notch signalling molecule, RPB1 are also associated with driving Th17 pathogenicity<sup>239,240</sup>. Pathogenic Th17 cells are associated with a lack of IL-10 production and enhanced GM-CSF production<sup>241</sup>. A study comparing the gene expression profiles of *in vitro* Th17 cells polarised by



cytokine combinations which induce non-pathogenic or pathogenic Th17 cells revealed 233 differentially expressed genes<sup>242</sup>. Pathogenic Th17 cells display a higher gene expression level of pro-inflammatory chemokines and cytokines including *CXCL3*, *CCL4*, *CCL5*, *IL3* and *IL22*. In contrast, non-pathogenic Th17 cells exhibit enhanced expression of genes encoding the immune suppressive IL-10 and the transcription factor IKZF3<sup>242,243</sup>.

## **1.10 Other IL-17A and IL-17F expressing cells**

### **1.10.1 IL-17A+ CD8+ T cells (Tc17 cells)**

Although Th17 cells are the best-characterised cellular source of IL-17A and IL-17F, various reports have demonstrated that other immune cells can produce these cytokines, one group of which are IL-17A+ CD8+ cytotoxic T (Tc17) cells. CD8+ T cells play a vital role in immune defence against intracellular pathogens and tumour surveillance. They primarily secrete TNF $\alpha$  and IFN $\gamma$  and release cytotoxic granules including perforin and granzymes. Moreover, they can kill infected cells via Fas/FasL interactions. Similar to CD4+ T cells, different subpopulations of cytotoxic CD8+ T cells exist including Tc1, Tc2, Tc9, Tc17 and CD8+ Treg cells. IL-17A+ CD8+ T cells were first identified by Teunissen *et al.*, whereby *IL17A* mRNA was detected in CD8+ T cells derived from psoriatic lesional skin<sup>120</sup>. Subsequently, flow cytometry analysis revealed the presence Tc17 cells in psoriatic skin plaques<sup>18</sup>. The requirements for differentiation and/or polarisation of Tc17 cells are not as well defined as Th17 cells, particularly in humans<sup>244</sup>. One report demonstrated the induction of human IL-17A+ CD8+ T cells upon culture of naïve CD8+ T cells with recombinant TGF- $\beta$ , IL-6, IL-1 $\beta$  and IL-23<sup>245</sup>. Moreover, mouse models have shown the importance of the transcription factors STAT3, IRF3 and IRF4 in the induction of Tc17 cells. Phenotypic profiling of IL-17A+ CD8+ T cells has revealed these cells can co-express IFN $\gamma$ , TNF $\alpha$ , IL-21, IL-22 and GM-CSF<sup>91</sup>. Although a IL-17A+ IL-17F+ CD8+ T cell population has not currently been reported, a recent study has revealed the presence of a mouse IL-17F+ IL-17A- CD8+ T cell population<sup>246</sup>. A proportion of IL-17A+ CD8+ T cell subsets belong to a subset of innate T cells named mucosal-associated invariant T (MAIT) cells. MAIT cells express an invariant T cell receptor- $\alpha$  chain

(V $\alpha$ 7.2) that is restricted by the non-classical MHC class I molecule MHC-related protein (MR1)<sup>247</sup>.

### **1.10.2 $\gamma\delta$ T cells and iNKT cells**

With regards to other T cells, a proportion of  $\gamma\delta$  T cells have been shown to produce IL-17A<sup>248</sup> and IL-17F<sup>106</sup> and share characteristic features of Th17 cells including CCR6 and ROR $\gamma$ t expression<sup>248</sup>. Invariant natural killer T (iNKT) cells characterised by their expression of NK1.1+ TCR $\alpha\beta$  and invariant V $\alpha$ 14 chain expression have also been shown to produce IL-17A<sup>249</sup>.

### **1.10.3 Innate immune cells**

Immunohistological studies have identified IL-17A positive mast cells in human tissues including SpA synovial tissue<sup>250</sup>, RA synovium<sup>251</sup> and psoriatic lesions<sup>252</sup>. Interestingly, a study revealed that mast cells can capture and store IL-17A in intracellular granules, through a process of receptor-mediated endocytosis. Mast cells are then able to release bioactive IL-17A, although it remains to be fully explored what drives the IL-17A release<sup>253</sup>. With regards to IL-17F, a recent study has shown that in oral tongue squamous cell carcinoma mast cells are a major source of IL-17F<sup>254</sup>. However, it is currently not reported whether mast cells take up and store IL-17F. The role of neutrophils as a source of IL-17A is controversial. Neutrophils have been identified as a source of IL-17A in numerous mouse models of infection and autoimmune inflammation<sup>255-257</sup>. Moreover, flow cytometry and immunofluorescence staining has determined the presence of IL-17A positive neutrophils in human psoriatic lesions<sup>252</sup>. However, other mouse and human studies have failed to detect IL-17A+ neutrophils<sup>258,259</sup>. Similar to mast cells, debates are ongoing as to whether IL-17A is transcribed in neutrophils or neutrophils capture IL-17A from the environment. Another source of IL-17A and IL-17F includes innate lymphoid cells type 3 (ILC3)<sup>260,261</sup>. ILCs are a family of lymphoid-lineage cells that lack T and B cell receptors and display innate immune effector functions. In particular, ILC3s require ROR $\gamma$ t, respond to IL-1 $\beta$  and IL-23 and also produce IL-22<sup>262</sup>. Other non T-cells reported to express IL-17A includes paneth cells<sup>263</sup>.

### **1.11 Cellular sources of IL-17B, IL-17C, IL-17D and IL-17E**

Human and mouse studies have reported the presence of *IL17E* mRNA in Th2-polarised T cells <sup>124</sup>. Moreover, in human studies *IL17E* transcripts have been identified in peripheral blood eosinophils and basophils. IL-5, GM-CSF and IL-3 were shown to induce IL-17E protein secretion by eosinophils. Moreover, IgE cross-linking triggered strong IL-25 production by basophils <sup>264</sup>. IL-17E is also produced by mast cells upon IgE cross-linking <sup>265</sup>. Finally, *IL17E* mRNA has been detected in mouse intestinal epithelial cells<sup>266</sup> and rat macrophages <sup>267</sup>. The cellular source of the remaining IL-17 cytokines is less well studied. Immunohistochemical analysis of the human spinal cord demonstrated that IL-17B protein is primarily localised to neurons <sup>268</sup>. IL-17C has been shown to be distinctly produced by epithelial cells <sup>128</sup>. Finally, *IL17D* mRNA has been detected in resting CD4+ T cells and resting CD19+ B cells <sup>76</sup>. In summary, while IL-17A and IL-17F are detected by a similar profile of cell subsets, the remaining IL-17 cytokines display distinctly different cellular sources.

Taken together the inflammatory function of IL-17A and IL-17F and their expression from immune cells, it became of significant interest to target these cytokines in immune-mediated diseases including inflammatory arthritis. Next, I will review inflammatory arthritis and the presence and role of the IL-17 family members in this disease.

## **1.12 Inflammatory arthritis**

Inflammatory arthritis is a group of diseases characterised by inflammation of the joints and often other tissues. These diseases include rheumatoid arthritis (RA) and spondyloarthritis (SpA). RA is the most common chronic inflammatory and destructive arthritis. It is characterised by persistent synovitis, systemic inflammation and the presence of autoantibodies including rheumatoid factor (which targets the Fc region of immunoglobulin G) and anti-citrullinated protein antibodies (APCA) (which are directed against citrullinated proteins). However, not all patients are positive for rheumatoid factor and APCA (seronegative RA) <sup>269</sup>. SpA refers to psoriatic arthritis (PsA), axial SpA and non-radiographic axial SpA, reactive arthritis, enteropathic arthritis and undifferentiated SpA, all of which share clinical and immunological features. SpA subtypes are negative for autoantibodies and can include inflammation of the joint (peripheral and axial); skin, gut and eye <sup>91</sup>. This project focuses on PsA. PsA is an asymmetric arthropathy but can develop as polyarticular and symmetrical <sup>270</sup>. Psoriasis is a chronic inflammatory disease of the skin and 10-20% of these patients have associated PsA. In contrast to RA and PsA, osteoarthritis (OA) is not an autoimmune disease but a degenerative joint disorder. Clinically, the condition is characterised by break-down of cartilage, which allows bones to rub against each other. This causes joint pain, tenderness, stiffness, limited movement and variable degrees of local inflammation. A combination of structural, mechanical and biological pathways contributes to OA pathology <sup>271</sup>. Studies often compare RA and PsA to OA.

### **1.12.1 Genetics of inflammatory arthritis**

#### **1.12.1.1 RA**

RA is a complex disease. It can involve factors such as bacteria, viruses and autoantigens targeting heavy chain binding proteins and cyclic citrullinated peptides, which trigger disease in genetically susceptible individuals <sup>269</sup>. Moreover, environmental factors such as obesity, smoking and caffeine intake can increase the likelihood of disease <sup>272</sup>. Concordance rates for RA were found between 12.3% and 15.4% for monozygotic and 3.5% for dizygotic twins, suggesting that genetic factors

can impact susceptibility to disease, <sup>273</sup>. In concordance, a genome-wide association study revealed 49 single nucleotide polymorphisms (SNPs) associated with RA <sup>274</sup>.

The HLA class II alleles are strongly associated with RA and contribute at least 30% of the total genetic effect <sup>275</sup>. As HLA class II molecules play an important role in antigen presentation to CD4+ T cells, this suggests that predisposing CD4+ T cell repertoire selection, antigen presentation or alterations in peptide affinity might have a role in promoting autoreactive responses in RA. The HLA-DRB1 alleles \*0101, \*0102, \*0401, \*0408, \*1001 and \*1402 share a consensus sequence known as the 'shared epitope' (SE). The conserved amino acid sequence can include QKRAA, QRRAA or RRRAA on the third hyper-variable region of the DRβ1 chain <sup>276,277</sup>. Extensive evidence exists associating *HLADRB1* alleles encoding the shared epitope to RA susceptibility and severity <sup>278,279</sup>. The shared epitope hypothesis proposes that the shared epitope itself is directly involved in the pathogenesis of RA by allowing the presentation of an arithrogenic peptide to CD4+ T cells. It has also been proposed that *HLA-SE* alleles can influence the T cell repertoire to permit escape from tolerance or survival of auto-reactive clones, or *HLA-SE* alleles molecules can act as targets themselves for auto-reactive T cells <sup>280</sup>. Interestingly, the association between SE-encoding *HLADRB1* alleles and RA has only been observed for ACPA-positive disease <sup>281</sup>. In addition to *HLADRB1* alleles that confer susceptibility to RA, other *HLADRB1* alleles that protect against RA have been identified. A study by Carrer *et al.* demonstrated that the *HLADRB\*0402* allele, which contains a DERAA sequence was protective in RA. The presence of a DERAA sequence was a strong independent predictor of a better RA prognosis <sup>282</sup>.

Other non-HLA genetic associations have been implicated CD4+ T cells in the pathology of RA. The intra-cellular phosphatase protein tyrosine phosphatase non-receptor type 22 (PTPN22) plays an important role in T and B cell signalling. Studies have shown the C1885T polymorphism of the *PTPN22* gene confers risk for RA as well as other diseases such as lupus, type I diabetes and others <sup>283</sup>. Implicating the IL-23/IL-17 axis in RA pathology, genetic studies have shown polymorphisms in the *IL23R* gene are associated with RA susceptibility <sup>284</sup>. Implicating Th17 cells in the

pathogenesis of RA, a genome-wide association study of RA revealed that a polymorphism in the *CCR6* gene was associated with RA susceptibility. In concordance with the fact that *CCR6* is a characteristic marker of Th17 cells, the RA *CCR6* genotype displayed increased IL-17A protein in sera <sup>285</sup>.

### 1.12.1.2 PsA

The strongest genetic susceptibility to SpA diseases, including PsA lies within the MHC class I region, particularly the HLA-B27 region <sup>286</sup>. Other variants including HLA-cw6, HLA-B38 and HLA-B08 are associated with PsA <sup>287,288</sup>. The association of a variety of HLA-B loci with PsA implies that genetic associations alter immunological processes. Considerable debate is ongoing on how variants in the HLA-B27 region can affect immunological mechanisms. The first and most extensively studied hypothesis is driven by the fact that MHC class I molecules present peptides to CD8+ T cells. It proposes that PsA and other SpA types including AS results from the ability of HLA-B27 to bind and present unique arthritogenic peptides to CD8+ cytotoxic T cells. In support of this theory, a studied demonstrated the presence of HLA-B27-restricted cytotoxic CD8+ T cells that could recognize both bacterially infected and uninfected target cells in the PB of reactive arthritis and AS <sup>289</sup>. Other studies have suggested that variants within the HLA-B27 locus can contribute to PsA/SpA pathology by promoting HLA-B27 homodimerization instead of heterodimerization with  $\beta$ 2-microglobulin. Under normal physiological conditions HLA-B27 heavy chains form heterotrimer complexes with  $\beta$ 2-microglobulin and intracellular peptides derived from viruses, bacteria or self-proteins. These heterotrimeric complexes (which form HLA-B27) are presented to the cell surface for antigen-recognition <sup>290</sup>. However, HLA-B27 heavy chains can also form  $\beta$ 2-microglobulin-free disulphide-bonded heavy chain homodimers <sup>291</sup>. Following endosomal recycling, HLA-B27 homodimers can be expressed at the cell surface and studies have shown they can be recognized by several innate immune receptors on T cells, NK cells and other APCs <sup>292</sup>. Therefore, it has been proposed that interaction of HLA-B27 homodimers with T cells and NK cells can result in altered cell signalling and promote a pro-inflammatory response. One of the receptors which recognizes HLA-B27 homodimers includes the killer cell immunoglobulin-like receptor 3DL2

(KIR3DL2). Interestingly, IL-17A+ CD4+ T cells, which express KIR3DL2 have been identified in the blood and SF of AS patients<sup>293</sup>. Importantly, co-culture of KIR3DL2+ IL-17A+ CD4+ T cells with HLA-B27 homodimer expressing APCs result in enhanced survival and proliferation of KIR3DL2+ IL-17A+ CD4+ T cells<sup>293</sup>. These data implicate a link between MHC class I and the IL-23 – IL-17 axis.

The IL-23-IL-17 axis has been highlighted by genetic association studies in PsA and psoriasis. Variants in the *IL12B* and *IL23A* region which encodes the IL-12p40 sub-unit shared between IL-12 and IL-23 and the IL-23p19 sub-unit, respectively are associated with PsA and psoriasis<sup>294,295</sup>. Additionally, variation within *IL23R*, encoding the IL-23 receptor is associated with PsA/psoriasis<sup>295</sup>. Genome-wide association studies demonstrated that the *IL23R R381Q* gene variant is protective against psoriasis<sup>296</sup> and Di Meglio *et al.* showed that *IL23R R381Q* exerts its protective effects through selective attenuation of IL-23-induced Th17 cell effector function<sup>297</sup>. These data demonstrated a critical role for IL-23/IL-23R signalling in generating a pathogenic Th17 response. Further supporting the IL-23-IL-17 axis in the genetic susceptibility of PsA is the identification of susceptibility variants in genes encoding key IL-23 and IL-17 signalling molecules including STAT3<sup>298</sup>, TYK2 (required for IL-23 signalling)<sup>299</sup> and TRAF3IP2 (encodes the IL-17 signalling molecule, Act1)<sup>299</sup>.

### **1.12.2 Synovial fibroblasts in inflammatory arthritis**

Although most of the research focuses on the role of immune cells in inflammatory diseases, the pathological role of stromal cells is increasingly recognised<sup>300-304</sup>. Stromal cells are connective tissue cells, which build the structural framework of organ or tissues. The main type of stromal cell is fibroblasts. Fibroblasts play a key role in supporting normal wound healing<sup>305</sup>. They are recruited to wound sites via chemo-attractants such as platelet-derived growth factor (PDGF), IL-1 $\beta$  and TNF $\alpha$ , which are produced by platelets and macrophages as part of the inflammatory response. Once recruited to the wound site they produce MMPs that degrade the fibrin clot and replace it with extracellular matrix components such as collagen I-IV, XVIII, glycoproteins,

thrombospondin, lamin and hyaluronic acid. The secreted fibroblast matrix supports and regulates the migration and activity of fibroblasts and co-ordinates angiogenesis, granulation-tissue generation and epithelisation<sup>305</sup>. In the healthy joint, synovial fibroblasts (also referred to as synovial-fibroblast-like cells) are located in the synovial sub-lining and lining layer and are one or two layers thick interspersed with tissue-resident macrophages. Here, they produce extracellular matrix components of the synovial fluid (SF), which is vital for cartilage integrity and lubrication of the joint. In RA, the synovial sub-lining thickens and is infiltrated by immune cells. Synovial fibroblasts proliferate and become chronically activated<sup>300</sup>.

Strong evidence exists suggesting that synovial fibroblasts are aggressive drivers in the destructive process of RA<sup>306-308</sup>. In response to inflammatory mediators (including IL-17A, IL-17F and TNF $\alpha$ ) synovial fibroblasts can secrete a variety of pro-inflammatory cytokines and chemokines including IL-6, IL-8, CCL2, CCL3, CCL4 and CXCL10. Subsequently, this can lead to the recruitment of neutrophils, monocytes and T cells which can perpetuate inflammation. Synovial fibroblasts can also contribute to the pathology of RA by acting as innate immune cells. It has been demonstrated that synovial fibroblasts express TLR3 and TLR7 (which bind double-stranded and single-stranded RNA, respectively) and the cytosolic pattern recognition receptors RIG-1 and MDA-5 (which recognise double-stranded RNA)<sup>309</sup>. A study by Bretano *et al.* demonstrated that necrotic synovial fluid cells activate synovial fibroblasts via TLR3, which can lead to the production of high levels of IL-6, CXCL10, CCL5 and IFN $\beta$ <sup>306</sup>. Studies have shown that interaction between synovial fibroblasts and T cells can elicit many different effects. Co-cultures of activated T cells with synovial fibroblasts led to increased IL-6 and IL-8 production by fibroblasts and in turn, increased production of IL-17A and IFN $\gamma$  by T cells<sup>307,310</sup>. Studies have also suggested that synovial fibroblasts can extend T cell survival, leading to persistence in inflammation in the RA joint. RA synovial T cells were rescued from spontaneous apoptosis *in vitro* by interaction with synovial fibroblasts<sup>308</sup>.

A characteristic feature of RA synovial fibroblasts is their ability to invade and destroy cartilage via the production of a disintegrin and metalloproteinase with



thrombospondin motifs (ADAMTS), MMPs and cathepsins. Pro-inflammatory cytokines such as IL-1 $\beta$  and TNF $\alpha$  present in the inflamed joint induce the production of these proteinases by fibroblasts<sup>311</sup>. Interestingly, it has been shown that epigenetic changes maintain high production of MMPs in RA synovial fibroblasts<sup>312</sup>. Evidence in the TNF transgenic (hTNFtg) mice suggests that loss of cartilage proteoglycan is required for the attachment and invasion of synovial fibroblasts to cartilage<sup>313</sup>. Interestingly, a study in the severe combined immunodeficiency (SCID) mouse model demonstrated that RA synovial fibroblasts have the ability to migrate to cartilage at sites distant from their origin. These data support the hypothesis that destructive arthritis spreading between joints is mediated, in part, by the migration of synovial fibroblasts<sup>301</sup>.

It was originally thought that fibroblasts are a homogenous population, however data are emerging demonstrating that several different phenotypes of fibroblasts exist<sup>302-304</sup>. Work from Bertonecelj *et al.* identified that synovial fibroblasts from different anatomical locations exhibit significant differences in gene expression pattern, epigenetic marks and function<sup>304</sup>. A significant number of differentially expressed transcripts between synovial fibroblasts of different locations were encoded in the homeobox (HOX) loci. Interestingly, it was shown that cluster analysis of RNA sequencing data using transcripts exclusively encoded in the HOX loci can cluster synovial fibroblasts according to joint location. In particular, synovial fibroblasts from proximal joints can be separated from synovial fibroblasts of distal joints. Functionally, while RA synovial tissues from hand joints expressed significantly higher levels of MMP13 than synovial tissues from the knees of RA patients, knee RA synovial fibroblasts induced higher levels of MMP1 in response to TNF $\alpha$  than that of upper extremity synovial fibroblasts. In summary, these data suggest that RA might manifest differently at different joint locations<sup>304</sup>. A recent study has characterised CD90 (also known as THY1)- podoplanin (PDPN)+ and CD90+ PDPN+ fibroblast populations which are either CD34+ or CD34-. An increase in the PDPN+ CD90+ CD34- subset was observed around the sub-lining of RA synovial tissue. This was identified as a dominant change in the RA synovium, not observed in OA samples<sup>303</sup>. While the majority of synovial fibroblast studies focus on RA, it is likely similar

results can be observed in synovial fibroblasts derived from the inflamed joint of PsA patients.

### **1.12.3 Immune cells in inflammatory arthritis**

#### **1.12.3.1 Innate immunity in RA**

In the synovial membrane and SF of RA patients, innate immune cells have been identified, including monocytes, macrophages, mast cells, natural killer cells and neutrophils. Moreover, these cells have been shown to contribute to the pathology of these diseases<sup>314</sup>. Monocytes and macrophages are present in abundance in the rheumatoid joint. Studies have shown these cells have an activated phenotype with increased expression of HLA-DR<sup>315</sup>, co-stimulatory molecules (e.g. CD80, CD86 and CD40), adhesion molecules (e.g. CD44 and CD45)<sup>316</sup> and chemokine receptors (e.g. CCR3 and CCR5)<sup>317</sup>. Many monocyte/ macrophage functions can contribute to the immunopathology of RA. These include their potent ability to secrete pro-inflammatory cytokines including TNF $\alpha$ , IL-6, IL-1 $\beta$  and MMPs into the SF which can lead to endothelial cell activation, acute phase reactions and cartilage damage<sup>318</sup>. Moreover, monocytes can drive Th1 and Th17 responses by their ability to produce IL-12 and IL-1 $\beta$ /IL-6/IL-23, respectively. A report from our lab has shown that *in vivo* activated CD14<sup>+</sup> monocytes derived from the inflamed joints of RA patients spontaneously and specifically promoted Th17 responses, compared with resting monocytes from the blood. This monocyte-induced Th17 differentiation was shown to be a TNF $\alpha$  and IL-1 $\beta$ -dependent mechanism<sup>197</sup>. Reports have also demonstrated that activated monocytes or macrophages can positively or negatively influence the function of CD4<sup>+</sup> Tregs via production of soluble mediators<sup>318</sup>. For example, TNF $\alpha$  can decrease FOXP3 expression and reduce Treg cell function<sup>319</sup>. Finally, monocytes/macrophages can contribute to the pathology of RA by recruiting or maintaining CD4<sup>+</sup> T cells in the arthritic joint. Elevated expression of CXCL16 in the RA synovium either due to an enhanced influx of monocytes or an increased expression of CXCL16 by macrophages promotes recruitment of CXCR6<sup>+</sup> T cells, which can exacerbate the inflammatory response in the synovium<sup>320,321</sup>. Reports have demonstrated a potential role for monocytes in influencing the CCL20-CCR6 axis in

RA. Synovial monocytes can display enhanced expression of CCL20, which could subsequently lead to the recruitment of CCR6+ cells including Th17 cells <sup>322,323</sup>.

### **1.12.3.2 Adaptive immunity in RA**

The adaptive immune system plays a central role in the immunopathology of RA, as evidenced by the presence of auto-antibodies and the genetics of RA. Studies have showed that central and peripheral B cell tolerance checkpoints are defective in RA. Subsequently, there is an accumulation of auto-reactive B cells in the mature naïve B cell compartment <sup>324</sup>. The pathogenic role of B cells in RA can occur via several mechanisms including autoantibody production, T cell activation and cytokine synthesis. Auto-reactive B cells present in RA can produce a wide range of auto-antibodies, of which the most widely studied are rheumatoid factor and ACPA. Immune complexes consisting of immunoglobulins bound to their cognate antigens can be characteristic of RA <sup>325</sup>. A significant proportion of immune complexes present in the RA joint consists of rheumatoid factor bound to the Fc portion of IgG <sup>326</sup>. These immune complexes trigger the complement system, releasing chemotactic factors such as C5a, which leads to the recruitment of inflammatory cells via C5a receptors. As such, local inflammation and tissue damage is induced <sup>327</sup>. B cells can also contribute to the pathology of RA by acting as efficient antigen presenting cells. In contrast to conventional APCs, which display no binding specificity for antigens, B cells capture antigens via antigen-specific surface immunoglobulin receptors. Following antigen internalisation and processing by specific B cells, antigens are presented to CD4+ T cells in an MHC- restricted manner, leading to CD4+ T cell activation <sup>328</sup>. It has been suggested that T cell responses in RA synovitis are dependent on B cells. Treatment with a monoclonal anti-CD20 antibody (which depletes B cells) in SCID mice xenotransplanted with RA synovial tissue enriched in B cells led to the impairment of T cell activation and T-cell derived cytokines <sup>329</sup>. Finally, it has also been demonstrated that B cells can contribute to the pathology of RA via the secretion of RANKL, which can trigger osteoclastogenesis <sup>330</sup>.

Early studies reported that T cells are the dominant cell population in RA synovial membranes <sup>331,332</sup>. Moreover, T cells are abundant in the synovial membrane of RA

patients when compared with the synovial membrane of OA or healthy joints <sup>332</sup>. Reports have shown that RA SF T cells predominantly bear the memory CD45 'RO' phenotype, rather than the naïve 'RA' isoform. These cells have also been shown to express CD69 suggesting that they have recently been activated <sup>333</sup>. Animal models have also provided evidence for the role of T cells in inflammatory diseases. Adjuvant arthritis is similar to RA; it is an inflammatory disease displaying extensive cartilage and bone destruction. This disease can be induced in rats by subcutaneous injection of *Mycobacterium tuberculosis*. A study showed that adjuvant arthritis is T cell dependent, with T cell clones capable of transferring disease to naïve recipients <sup>334</sup>. Another mouse model study demonstrated that chronic autoimmune arthritis, similar to RA, developed as a result of mutations in ZAP70, which is a key signal transduction molecule in T cells. From this report, it was suggested that ZAP70 mutations may alter the thresholds of T cells to thymic selection, allowing positive selection of otherwise negatively selected self-reactive T cells. Therefore, thymic production of arthritogenic T cells due to a genetic shift of the T cell repertoire towards high self-reactivity may play a fundamental role in the immunopathology of RA <sup>335</sup>.

It was originally thought that Th1 cells were the main drivers of RA. In the rheumatoid synovium considerable amounts of IFN $\gamma$  were present, whilst IL-4 was absent <sup>336,337</sup> and the Th1 polarising cytokine IL-12 (formed of IL-23p35 and IL-12p40 sub-units) was detectable in RA SF <sup>338</sup>. In experimental models the presence of Th1 cells also appeared to correlate to autoimmune disease. Collagen-induced arthritis (CIA) is the most commonly studied autoimmune model of RA, which is elicited in genetically susceptible strains of mice by immunisation with type II collagen emulsified in complete Freund's adjuvant <sup>339</sup>. In both CIA and EAE, blockade of IL-12p40 reduced the disease severity and inflammation <sup>340,341</sup>. Additionally, it was also shown that in a myelin basic protein induced form of EAE, IL-12p40 knock-out mice were resistant to disease <sup>342</sup>.

However, opposing the role of Th1 cells in driving RA, it was reported that loss of IFN $\gamma$  signalling increased CIA severity <sup>343,344</sup>. Furthermore, mice lacking IL-12p35 were shown to exacerbate CIA <sup>345</sup>. This indicated that IL-12p40 contributes to arthritis

in an IL-12 independent manner. IL-12p40 also combines with the IL-23p19 sub-unit, forming the IL-23 cytokine. Therefore, IL-12p40 deficient mice are deficient in both IL-12 and IL-23. Studies which compared IL-23p19 deficient mice and IL-12p35 deficient mice soon revealed that many of the inflammatory functions which had been attributed to IL-12 were in fact due to the biological activities of IL-23. Cua *et al.* reported that IL-23 deficient (*IL-23P19*<sup>-/-</sup>) mice are resistant to EAE and Murphy *et al.* demonstrated that IL-23p19-deficient mice are resistant to the development of joint destruction in CIA<sup>345,346</sup>. Linking the IL-23/IL-17 axis to autoimmune disease, Langrish *et al.* demonstrated that EAE-resistant IL-23a<sup>-/-</sup> mice displayed similar numbers of central nervous system- infiltrating IFN $\gamma$  but not IL-17A expressing T cells<sup>347</sup>.

### **1.12.3.3 Involvement of the immune system in PsA**

In comparison to RA, PsA synovial tissue contains less cellular infiltrate and displays a more vascular phenotype<sup>348,349</sup>. Histological changes are characterised by endothelial swelling and vessel wall thickening<sup>349</sup>. The role of T cells in the PsA joint has been highlighted by the beneficial effects of T-cell therapies such as ciclosporin and abatacept on the treatment of PsA<sup>350,351</sup>. Cyclosporin inhibits the calcineurin-dependent nuclear translocation of the transcription cytosolic proteins, nuclear factor of activated T cells, pre-existing (NFATp) and nuclear factor of activated T cells, cytoplasm (NFATc). Subsequently, this leads to the blockade of early T cell activation<sup>351</sup>. Abatacept is a soluble fusion protein consisting of the extra-cellular domain of human CTLA4 and a fragment of the Fc portion of human IgG1, which blocks the CD28/B7 pathway causing a near-complete inhibition of T cell proliferation<sup>352</sup>. The association of PsA with MHC class I alleles provide evidence for a critical role in CD8<sup>+</sup> T cells and suggest that the disease is driven by presentation of self-peptides by susceptibility MHC class I molecules to auto-reactive antigen-specific CD8<sup>+</sup> T cells<sup>286</sup>. Unexpectedly, observations made in HIV also indicated the role of CD8<sup>+</sup> T cells in PsA. In the advanced setting of HIV disease, whereby CD4<sup>+</sup> T cells are depleted, PsA still occurred<sup>353</sup>. CD8<sup>+</sup> T cells derived from PsA SF have been shown to have an activated (HLA-DR<sup>+</sup>), memory (CD45RO<sup>+</sup>) phenotype with low CD25 expression levels, indicating a non-classical activation pattern<sup>354</sup>.

Studies have investigated the mechanism of accumulation of T cells in the sites of inflammation. It has been of interest to investigate whether the joint T cells recognise a single or several antigens or whether it reflects secondary non-antigen-specific T cell recruitment mediated by chemokines released by the local joint inflammation. It has been suggested that recruitment of T cells into the PsA synovial compartment may be mediated by chemokines such as MCP-1. In the plasma of PsA patients, MCP-1 levels were increased in comparison to healthy individuals and a positive correlation was identified between SF-derived T cells and SF MCP-1 levels<sup>355</sup>. If indeed recruitment of T cells was non-antigen specific, the T cells present at the site of inflammation would lack clonal expansions specific to the joint and primarily display a polyclonal repertoire similar to that of the blood. In contrast, a super-antigen present in the joint could elicit polyclonal expansion of both CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets. Finally, stimulation of T cell clones by a specific antigen would induce oligoclonal T cell expansions characterised by related sequence motifs in the CDR3 region<sup>356</sup>. A study by Costello *et al.* investigated the TCR $\beta$  chain repertoire in paired samples of PB and SF from PsA patients. The study demonstrated that the  $\alpha\beta$  T cell repertoire of PsA SF CD8<sup>+</sup> T cells contained an average of 32 major oligoclonal expansions in many variable genes of the TCR $\beta$  chain families, as evidenced by  $\beta$ -chain CDR3 length analysis. The majority of oligoclonal expansions were not present in the PB counterpart, highlighting the immunologic specificity of the clonal events for the inflamed joint. The TCR repertoire of the CD4<sup>+</sup> T cells derived from PsA SF contained fewer and smaller oligoclonal expansion predominantly restricted to the joint. Collectively, these data suggest that the accumulation of CD8<sup>+</sup> T cells in the PsA joint is driven by antigen (likely auto-antigen) driven clonal expansion. Moreover, as clones are preferentially expressed in the joint, it is suggested that the inciting peptide antigen is selectively expressed in the joint or related tissues. The study demonstrated that CD4<sup>+</sup> T cell oligoclonal expansion was also present in PsA SF, however, they were smaller in size and number compared with CD8 expansions<sup>356</sup>.

#### 1.12.4 Animal models showing the pathological role of IL-17A in arthritis

Murine models of inflammatory arthritis have unequivocally demonstrated the pathogenic role of IL-17A in inflammatory arthritis<sup>357</sup>. Seminal research blocked IL-17A in the CIA mouse model (during the initial phase of arthritis) with a soluble IL-17 receptor fusion protein, which resulted in the suppression of arthritis development and joint damage. In contrast, overexpression of IL-17A in CIA led to accelerated development and enhanced severity of synovial inflammation and joint damage<sup>358</sup>. In concordance, another study demonstrated that blocking IL-17RA signalling provided complete protection against the development of CIA<sup>359</sup>. As well as playing a role in the early stages of arthritis, it has been shown that IL-17A contributes to the effector phase of arthritis. Neutralising IL-17A after the onset of CIA significantly reduced disease severity, as evidenced by the suppression of joint damage in the knee and ankle joints. Suppressed joint damage was attributed to the significantly reduced systemic levels of IL-6 and decrease in IL-1 $\beta$  and RANKL+ cells<sup>360</sup>. Another study by Lubberts *et al.* demonstrated that a lack of IL-17R signaling prevented full progression and persistence of chronic synovitis. This study involved a mouse model which begins as a macrophage-driven process but becomes T cell dependent with time. This involved repeated injections of gram-positive bacterial cell wall fragments (SCW) directly into the knee joint. While no difference in the acute phase of arthritis was observed in IL-17R<sup>-/-</sup> mice vs. wild-type mice, IL-17R<sup>-/-</sup> prevented progression to chronic destructive synovitis. This was associated with a significant down-regulation of chemokines, selectins, cytokines and collagenase-3 in the synovium of IL-17R<sup>-/-</sup> mice<sup>361</sup>.

One of the early mouse-model studies investigating the role of IL-17F showed that in dextran sulfate sodium (DSS) induced colitis, *IL17A* knock-out mice exhibited severe disease symptoms, whereas *IL17F* knock-out mice were protective against disease<sup>106</sup>. In contrast, in the EAE model *IL17F* knock-out mice had no affect on disease severity<sup>106</sup>. Similarly, blockade of IL-17F in CIA did not reduce the arthritis score<sup>362</sup>. A combined blockade of IL-17A and IL-17F significantly reduced disease severity, however, no clear difference was observed between the blockade of IL-17A alone vs. combined IL-17A and IL-17F blockade. In contrast, a study in the experimental colitis mouse model showed that neutralization of both IL-17A and IL-17F ameliorated

colitis, whereas neutralization of IL-17A or IL-17F alone had a limited effect <sup>363</sup>. This suggests that in certain environments dual IL-17A and IL-17F blockade may be more effective at reducing inflammation than blockade of IL-17A alone.

### **1.12.5 The presence of IL-17A and IL-17F in inflammatory arthritis**

#### **1.12.5.1 RA**

The link between IL-17A and RA pathology was established in 1999 by Chabaud *et al*, whereby *IL17A* mRNA expression was detected in RA synovial tissue and not OA tissue. Additionally, functional IL-17A was shown to be spontaneously produced by RA synovial explants and IL-17A producing cells were identified in T cell-rich areas of the synovium <sup>364</sup>. Since then immunohistochemistry analysis has identified increased expression of IL-17A protein in RA vs. OA synovial tissue <sup>111,365</sup>. Studies have also shown increased IL-17A production or frequency of IL-17A+ CD4+ T cells in the serum or SF of RA patients. RA patients showed significantly higher levels of IL-17A in the serum of RA patients vs. healthy controls <sup>366</sup> and levels of IL-17A in SF were significantly higher in RA patients than OA patients <sup>103</sup>. Gullick *et al*. demonstrated that following *ex vivo* stimulation with PMA and ionomycin for 3 hours, IL-17A+ CD4+ T cells were significantly elevated in the PB from RA patients vs. healthy control. IL-17A+ CD4+ T cells were further enriched in the RA SF compared to RA PB, demonstrating that IL-17A is preferentially enhanced at the site of inflammation rather than systemically <sup>367</sup>. Other studies have also observed increased frequencies of IL-17A+ CD4+ T cells in the PB and SF of RA patients vs. controls <sup>368,369</sup>. Studies have linked levels of IL-17A protein and IL-17A+ CD4+ T cells to RA severity. In comparison to patients in remission, patients with active disease had a higher percentage of IL-17A+ CD4+ T cells in RA synovial tissue. Moreover, the frequency of IL-17A+ CD4+ T cells, but not Th1 cells in SF positively correlated with CRP and local power doppler ultrasound-defined synovitis <sup>367</sup>.

Limited studies examining the presence of IL-17F in RA exist. A study by Zrioual *et al*. performed immunohistochemistry analysis on RA synovial tissue and identified the selective expression of IL-17F protein when compared with OA samples.



Additionally, they observed a more sustained staining for IL-17F compared with IL-17A. IL-17F secreting cells were described to display a plasma cell-like morphology, similar to IL-17A expressing cells <sup>112</sup>. Similar experiments performed by van Baarsen *et al.* also demonstrated the presence of IL-17F protein in RA synovial tissue, however similar IL-17F protein levels were also observed in healthy control and OA synovial tissue samples <sup>365</sup>.

While the presence of IL-17 cytokines in serum/ plasma samples has been undetectable in some cases <sup>362</sup>, other studies have detected IL-17F <sup>370,371</sup>. One study analysed plasma samples from RA and OA patients and revealed significantly increased levels of IL-17F in RA vs. healthy control samples <sup>370</sup>. With regards to SF analysis, IL-17F levels were identified at significantly increased levels in RA and OA patients vs. healthy controls. However, no significant difference was observed between RA vs. OA SF <sup>370</sup>. It is important to note that in this study, in contrast to robust data provided in other reports, IL-17A protein was identified at very low levels with no difference between RA vs OA or healthy control SF samples. A study by Sarkar *et al.* stimulated RA and OA PBMC for twenty-four hours with PMA and ionomycin. Similar to IL-17A, they identified IL-17F protein at increased levels in RA vs. OA PBMC supernatants. However, no IL-17A or IL-17F expressing cells were detectable via flow cytometry. With regards to this study it is important to question the physiological relevance and damage to cells following a twenty-four hour PMA/ionomycin stimulation period. The study also showed that no IL-17F protein was detectable in the supernatant of PMA/ionomycin stimulated RA SFMC or RA PB and SF CD3+ T cells <sup>362</sup>. Collectively, it is clear the current literature lacks robust evidence demonstrating the presence of IL-17F in RA. The same holds true for IL-17AF. The only reports for IL-17AF have demonstrated that very low levels of IL-17AF are present in the SF of RA patients <sup>370</sup> and twenty-four hour PMA and ionomycin stimulated CD3+ T cells from RA PB display increased IL-17AF levels compared with CD3+ T cells derived from OA blood <sup>362</sup>.

### 1.12.5.2 PsA

*IL17A* mRNA and IL-17A protein has been detected in the SF and synovial tissue of PsA patients. Out of fourteen PsA synovial tissue samples, *IL17A* mRNA was detectable in 9 samples<sup>121</sup> and IL-17A protein was detected at higher levels in PsA SF vs. PB<sup>368</sup>. Circulating IL-17A+ CD4+ T cells are increased in the peripheral blood of patients with PsA<sup>372</sup>. Additionally, it has been shown that there is a significant increase in the number of IL-17A+ CD4+ and IL-17A+ CD8+ T cells in the synovial fluid of PsA patients when compared with matched PB or osteoarthritis SF<sup>373,374</sup>. A report from our lab demonstrated that the frequency of IL-17A+ CD4- T cells present in PsA SF positively correlated with the CRP level, ESR, DAS28 score and was increased in patients with erosive disease, suggesting a pathological role for IL-17A+ CD8+ T cells in RA<sup>374</sup>. Within the IL-17A+ CD8+ T cell population in PsA SF, low frequencies of CD161+ V $\alpha$ 7.2 MAIT cells have been reported<sup>374</sup>. Other cellular sources of IL-17A in PsA PB and SF include  $\gamma\delta$  T cells and ILC3s<sup>375-377</sup>. *IL-17RA* expression has been shown to be increased in PsA vs. OA synoviocytes, suggesting that PsA synoviocytes are more responsive to levels of IL-17A (as well as IL-17F and IL-17AF) present in the inflamed joint<sup>373</sup>.

IL-17A+ T cells have been implicated as a key contributor to the pro-inflammatory state of psoriasis<sup>378,379</sup>. Expression of *IL17A* mRNA and IL-17A protein has been shown to be increased in psoriatic lesions when compared with non-lesional skin<sup>380,381</sup>. A study by Kolbinger *et al.* detected high IL-17A protein levels in lesional skin from psoriasis patients, whereas no IL-17A was detectable in the skin of healthy volunteers. However, they detected low but detectable levels of IL-17A protein in the non-lesional skin of PsA patients, suggesting that there is an on-going IL-17A pathway activation in PsA non-lesional skin<sup>382</sup>. Di Meglio *et al.* showed that there was an accumulation of IL-17A+ CD8+ T cells in human psoriatic lesions and blockade of CD8+ T cells via a neutralising antibody prevented the development of psoriasis in a xenotransplantation mouse model<sup>383</sup>.

Similar to RA, current literature lacks robust data confirming the presence of IL-17F in PsA. Among the limited studies investigating IL-17F in PsA, Glatt *et al.* observed

detectable *IL17F* mRNA levels in six out of fourteen PsA synovial tissue samples <sup>121</sup>. Van baarsen *et al.* observed increased IL-17F expression as measured by immunohistochemistry staining in PsA synovial tissue compared with OA synovial tissue <sup>365</sup>. With regards to psoriasis, a study by Watanabe *et al.* performed western blotting analysis to demonstrate the presence of IL-17F protein in psoriatic lesions at higher levels than non-lesional controls. Moreover, similar results were observed by ELISA <sup>108</sup>. A more recent study detected elevated expression of *IL17F* mRNA in psoriatic lesional skin compared with healthy skin <sup>382</sup>. To the best of my knowledge, no studies have investigated the presence of IL-17AF in PsA.

### **1.13 IL-17 receptors in RA and PsA**

As previously discussed, IL-17A, IL-17F and IL-17AF signal through the IL-17RA/IL-17RC heterodimer receptor complex. These receptors are critical in mediating cellular responsiveness to IL-17A, IL-17F and IL-17AF <sup>31</sup>. In rheumatic diseases, IL-17RA and IL-17C have been reported to be upregulated systemically and at the site of inflammation, thus, having the ability to exacerbate the effect of IL-17A, IL-17F and IL-17AF. Microarray analysis revealed significant upregulation of both *IL17RA* and *IL17RC* mRNA expression in RA PBMC when compared with healthy volunteers <sup>111</sup>. At the site of inflammation, immunohistochemical analysis has shown IL-17RA and IL-17RC receptors to be broadly expressed within the synovium of RA patients, located on stromal cells and infiltrating immune cells <sup>111</sup>. Moreover, a study that compared IL-17RA expression in RA, PsA and OA synovocytes via flow cytometry and western blot observed IL-17RA expression to be significantly higher in RA and PsA when compared with OA <sup>373</sup>.

#### **1.13.1 The presence of IL-17B, IL-17C, IL-17D and IL-17E in RA and PsA**

Studies have highlighted a possible role of the remaining IL-17 family members in inflammatory arthritis. Immunohistochemistry and qPCR analysis revealed that neutrophils contained significant levels of IL-17B in the RA synovium <sup>134</sup>. Moreover, a study by Yamaguchi *et al.* identified *IL17B* and *IL17C* mRNA expression in the CIA arthritic paw. Adoptive transfer of IL-17B- and IL-17C-transduced CD4+ T cells

exacerbated arthritis. Finally, in CIA mice neutralisation of IL-17B inhibited arthritis development and bone destruction <sup>136</sup>. IL-17C is strongly linked to skin, being produced and acting upon epithelial cells. Substantial evidence implicates IL-17C in lesional psoriasis skin formation. Johnston *et al.* shown IL-17C protein to be abundantly expressed in human lesional psoriasis skin and co-localise with keratinocytes and epithelial cells. In concordance, mice engineered to overexpress IL-17C keratinocytes develop psoriasis-like dermatitis <sup>132</sup>. Little evidence exists on the presence of IL-17D in inflammatory arthritis. Interestingly, a recent report has suggested a pro-inflammatory role of keratinocyte-derived IL-17E in psoriasis <sup>384</sup>.

#### **1.14 Biologics in RA and PsA**

There are three main groups of treatment for inflammatory diseases: non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids and disease-modifying anti-rheumatic drugs (DMARDs). DMARDs are grouped into conventional DMARDs and biologic therapies. Conventional DMARDs include methotrexate, hydroxychloroquine, leflunomide and sulphasalazine and are often first-line therapy <sup>385</sup>. They can be used individually or in combination with other DMARDs or biologic therapies. A study showed that the combination of methotrexate in combination with sulfasalazine and hydroxychloroquine demonstrated greater efficacy in early RA and patients with an inadequate response to methotrexate (non-responders), however, caution must be taken regarding toxicity <sup>386</sup>. Extensive research into understanding the pathology and involvement of pro-inflammatory cytokine production in RA and PsA has allowed for the development of biologic therapies. Biologic therapies have revolutionised the management of inflammatory arthritis, directly targeting molecules and cells involved in the pathogenesis. The TNF $\alpha$  inhibitor, infliximab was the first biologic therapy to be licensed. Infliximab is a chimeric anti-TNF monoclonal antibody with murine variable regions and a human IgG1 constant region <sup>387</sup>, which demonstrated efficacy in RA and PsA <sup>388,389</sup>. This was closely followed by other TNF $\alpha$  inhibitors including etanercept, adalimumab, golimumab and certolizumab, all of which have shown to be effective in the treatment of RA and PsA. Although, TNF inhibitors have shown great success, not all patients respond. Clinical improvement after TNF inhibition is observed in approximately in 60% - 70% of patients who previously failed conventional DMARDs. At present, it is not fully understood why

30% to 40% of patients do not respond <sup>390</sup>. Current studies are aiming to identify features which can predict anti-TNF responders vs. non-responders.

With strong evidence linking the IL-23/IL-17 axis in the immunopathology of RA and PsA, biological therapies targeting this pathway have been developed including ustekinumab, a human immunoglobulin G1 $\kappa$  monoclonal antibody that binds to the p40-subunit shared by IL-12 and IL-23 <sup>391,392</sup>. Ustekinumab has demonstrated efficacy in PsA and psoriasis. While ustekinumab displayed less efficacy in PsA than TNF inhibitors <sup>393</sup>, it was the first therapy to be more effective than TNF inhibitors in the treatment of psoriasis <sup>394</sup>. In contrast, ustekinumab has not been shown to reduce the signs and symptoms of RA <sup>395</sup>. Anti-IL-17A directed therapies including secukinumab (anti-IL-17A mAb) and ixekizumab (anti-IL-17A mAb) have demonstrated efficacy in PsA and psoriasis. These biologics are now FDA approved and have shown success in patients who failed to respond to TNF inhibitor treatment <sup>396-399</sup>. In RA however, secukinumab and ixekizumab have failed to show consistent results <sup>400-402</sup>. The biologic brodalumab is directed at IL-17RA and therefore inhibits the signalling of both IL-17A and IL-17F as well as IL-17C and IL-17E. Blockade of IL-17RA has shown good efficacy in PsA and psoriasis <sup>403,404</sup>, however in patients with active RA and an inadequate methotrexate response, no clinical response to brodalumab were observed <sup>405</sup>. As IL-17E has demonstrated anti-inflammatory potential <sup>125</sup>, it is important to consider that blockade of IL-17E signalling by brodalumab may be a disadvantage, dampening brodalumab's ability to reduce inflammation. Bimekizumab is a humanized monoclonal IgG1 antibody that selectively neutralizes IL-17A, IL-17F and IL-17AF. This agent has demonstrated good efficacy in a proof-of-concept study in 39 patients with PsA <sup>121</sup>.

### 1.15 Thesis rationale and thesis outline

As evidenced by the literature, IL-17A<sup>+</sup> IL-17F<sup>-</sup>, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cell populations exist; however, it is unknown whether there are factors that can preferentially skew Th17 cells to be IL-17F producing. Moreover, IL-17F expressing CD4<sup>+</sup> T cells have not been extensively characterised and it is unknown whether there are distinct phenotypic or functional differences between IL-17A and IL-17F CD4<sup>+</sup> T cells. Reports have shown that IL-17F is less potent at inducing inflammation when compared with IL-17A. However, studies have suggested dual IL-17A and IL-17F blockade is more effective at reducing inflammation than blockade of IL-17A alone<sup>121,363</sup>. Finally, while strong evidence implicates IL-17A in the immunopathological role of inflammatory arthritis, robust evidence for IL-17F is lacking. This literature research provided us with the rationale to further characterise IL-17F and IL-17F<sup>+</sup> CD4<sup>+</sup> T cells.

In summary the main aims of this thesis were to:

1. Investigate what drives IL-17F expression from CD4<sup>+</sup> T cells and perform phenotypic characterisation of IL-17F<sup>+</sup> CD4<sup>+</sup> T cells. Here I hypothesise that IL-17A<sup>+</sup> and IL-17F<sup>+</sup> CD4<sup>+</sup> T cells are differentially regulated.
2. In the context of RA and PsA, investigate the functional role of IL-17F alongside IL-17A and investigate the hypothesis that dual IL-17A and IL-17F blockade is more effective at reducing inflammation than IL-17A.
3. Investigate the hypothesis that IL-17F is present and plays a role in RA and PsA.

In the first chapter, *in vitro* healthy CD4<sup>+</sup> T cell cultures were set up to explore what drives IL-17F expression. This section begins by expanding upon previous work of the lab, assessing the effect on LPS-activated monocytes in driving IL-17A and IL-17F expression. Moreover, the effect of anti-CD3 and anti-CD28 titration on induction of IL-17A and IL-17F expressing CD4<sup>+</sup> T cells was investigated. Mass cytometry (CyTOF) was performed to provide an initial insight into whether IL-17A and IL-17F CD4<sup>+</sup> T cells display different cytokine profiles.

Chapter 4 explores the individual and combinatorial role of IL-17A, IL-17F, IL-17AF and TNF $\alpha$  in synovial fibroblast assays. Moreover, the effect of single and dual IL-17A and IL-17F blockade on the inflammatory response of fibroblasts was assessed across multiple culture systems. Due to an increasing need to separate IL-17A+ and IL-17F+ CD4+ T cells for functional assays and further characterisation, an IL-17A and IL-17F secretion assay was developed in collaboration with UCB. Finally, the function of IL-17B, IL-17C, IL-17D and IL-17E on synovial fibroblasts was examined.

The final results chapter (Chapter 5) predominantly aims to identify the presence of IL-17F in inflammatory arthritis. Using Luminex and ELISA techniques the presence of IL-17A, IL-17F, IL-17AF and other pro-inflammatory cytokines in matched serum and SF samples from RA and PsA patients was assessed. Additionally, we examined the presence of IL-17A and IL-17F producing CD4+ T cells and other immune cell subsets in RA and PsA PB and SF. This was further examined at a molecular level. Finally, the presence of IL-17 producing cells in RA synovial tissue was assessed.

## **2 Materials and Methods**

### **2.1 Sample Isolation**

Ethical approval for the use of peripheral blood from healthy donors and peripheral blood, synovial fluid and synovial tissue from RA, PsA and OA patients was obtained from the Bromley Research Ethics Committee (06/Q0705/20). Healthy control subjects were recruited amongst university staff. Patients with RA, PsA and OA were recruited from the Guy's and St Thomas' Foundation NHS Trust Rheumatology outpatient clinic. Informed consent was obtained for each sample taken from both healthy controls and patients. At the time of sample isolation patients were assessed using the Disease Activity Score in 28 joints (DAS28). This score is calculated using a number of assessment criteria including the number of tender and swollen joints, the erythrocyte sedimentation rate (ESR) and the C-reactive protein (CRP) level.

#### **2.1.1 PBMC and SFMC Isolation**

Peripheral blood mononuclear cells (PBMC) were isolated from healthy individuals or RA/ PsA patients using density gradient centrifugation. Blood was diluted 1:1 with PBS (Gibco, Invitrogen, UK) and carefully pipetted over a layer of 15mls lymphoprep (2:1 ratio) (Alere, UK) in a 50ml falcon centrifuge tube. The tubes were then spun in a centrifuge at 1600rpm for 20 minutes and no brake. Once centrifuged, the PBMC interphase layer was carefully removed with a Pasteur pipette. Cells were washed twice in 50mls PBS and counted with Trypan Blue (Gibco, Invitrogen, UK).

Synovial fluid (SF) from RA/PsA joints was removed by needle aspiration by the clinicians and collected into sterile tubes. Depending on the sample viscosity, SF was diluted between 1:1 – 1:10 with PBS. As described with blood, SF was layered over lymphoprep, centrifuged and the synovial fluid mononuclear cells (SFMC) interphase layer was collected with a Pasteur pipette. SFMC were washed twice in 50mls PBS and any synovial debris was removed by passing the SFMC suspension through 70µm cell strainers. Cells were counted with trypan Blue.



### **2.1.2 Cryopreservation and thawing of PBMC and SFMC**

In some cases, healthy PBMC or RA/PsA PBMC and SFMC were cryopreserved and stored in liquid nitrogen in cryovials containing 1ml freezing media, made up of foetal calf serum (FCS) (Gibco, Invitrogen, UK) and 10% Dimethyl sulfoxide (DMSO) (Sigma-Aldrich). When required, frozen vials were quickly thawed in a water bath at 37°C. The cell suspension was then added to pre-warmed media, washed and counted.

### **2.1.3 Serum and synovial fluid isolation**

For serum samples, blood from healthy volunteers or RA/PsA/OA patients was collected in a no additive serum tube (Fisher Scientific, UK) and stored at 4°C for 1-2 hours. The tube was centrifuged (1600rpm, 10 minutes) and the serum was decanted into a new falcon tube. This process was repeated until no red blood cells were seen. For cell-free SF samples, 1.5mls of SF was centrifuged at 1200rpm for 10 minutes and the supernatant was removed from the cell pellet. Final serum or SF samples were stored at -80°C before being analysed by luminex or ELISA.

### **2.1.4 Isolation of cells from synovial tissue**

Synovial membrane tissue samples were collected via arthroscopic biopsy from the joints of RA patients. Samples were transferred on a damp gauze to the laboratory tissue culture hood and immediately cut into small pieces (1-2mm), discarding any fat. Tissue samples were kept moist with MACS buffer (PBS supplemented with 0.5% BSA (Sigma-Aldrich) and 2mM EDTA (Sigma-Aldrich)). Next, 500mg – 3g of cut tissue was placed into a C-tube (Miltenyi Biotec) and an enzyme mastermix was added according to manufacturer's instructions (Miltenyi Biotec, multi-tissue dissociation kit 1). C-tubes were then loaded on to a gentleMACS dissociator (Miltenyi Biotec). The programme used comprised of 29 seconds mechanical digestion, 45 – 60 minutes incubation at 37°C with gentle mixing and 27 seconds mechanical digestion. Once the programme had finished, the digestion reaction was quenched by adding the same volume of culture media: Roswell park memorial institute media (RPMI) 1640 (Gibco, Invitrogen, UK) supplemented with 10% FCS (Gibco, Invitrogen, UK), 100 units/ml penicillin, 100µg of streptomycin/ml (Gibco, Invitrogen, UK) and 1% glutamine (Gibco, Invitrogen, UK) and 20µl/ml ethylenediaminetetraacetic acid (EDTA). To

remove tissue debris, the cell suspension was first passed through a 100µm cell strainer. MACS buffer was used to help pass the cell suspension through the strainer. This cell suspension was then passed through a 70µm cell strainer. Finally, cells were centrifuged (1600rpm for 8 minutes) and counted with trypan blue.

### **2.1.5 Synovial tissue explant culture**

In-house fibroblast cell lines were obtained from RA or PsA synovial membrane tissue samples. Synovial membrane tissue samples were collected via arthroscopic biopsy from the joints of RA or PsA patients. Samples were transferred to the laboratory on a damp gauze and then immediately cut into small pieces. Whilst tissue pieces were left wet in culture media (Dulbecco Modified Eagle Medium (DMEM) (Gibco, Invitrogen, UK) supplemented with 20% foetal calf serum (FCS) (Gibco, Invitrogen, UK), 100 units/ml penicillin, 100µg of streptomycin/ml) (Gibco, Invitrogen, UK), 2% glutamine (Gibco, Invitrogen, UK) and 1mg/ml Fungizone (Gibco, Invitrogen, UK), explant plates were prepared. Wells of a 6 well plate were evenly coated with 1ml sterile 0.1% bovine gelatin (Sigma, UK) in distilled water and allowed to set for 30 minutes at RT. Any excess gelatin was carefully removed with a Pasteur pipette, leaving a thin film of set gelatin remaining on the well surface. A piece of tissue (approximately 1mm<sup>3</sup>) was placed in the centre of each well and 1ml of supplemented DMEM was carefully added. Tissue explants were incubated at 37°C for 5% CO<sub>2</sub> and supplemented DMEM media was replenished every 3 days. When fibroblasts had grown out of the synovial tissue and expanded to 80% confluency on the well surface, they were transferred to a T175 flask. This involved removing and discarding the synovial tissue and detaching the adherent fibroblasts by adding 300µl Trypsin-EDTA (Thermo Fisher Scientific, UK) for 5 minutes at 37°C. When the fibroblasts were detached, Trypsin was neutralised with the addition of 700µl supplemented DMEM. The 1ml fibroblast suspension was then added to a T175 flask to a total volume of 20ml supplemented DMEM.

## **2.2 Cell separation**

### **2.2.1 CD14+ monocyte isolation**

Isolation of CD14+ monocytes was performed using CD14 Microbeads (Miltenyi Biotec, UK). The number of PBMCs required for separation was calculated and resuspended in 90µl per 10<sup>7</sup> cells MACS buffer. Next, 10µl of CD14+ microbeads were added per 10<sup>7</sup> cells, the suspension was thoroughly mixed and incubated at 4°C for 15 minutes. After incubation, the suspension was diluted with MACS buffer with 10-20x the labelling volume and centrifuged at 1200rpm for 10 minutes (4°C). For CD14+ selection an LS column (Miltenyi Biotec) was placed in a magnet and pre-wet with 3mls of MACS buffer. The cell pellet was resuspended in 1ml MACS buffer and applied to the column. Once the cell suspension passed through the column another 3mls of MACS buffer was applied, this wash step was repeated three times. The purified CD14+ monocytes captured within the column were then eluted. This was done by removing the column from the magnet and plunging 5mls of MACS buffer through. Cells were then counted using Trypan Blue and stained for purity with CD14 (Miltenyi Biotec, UK), CD3, CD4, CD8 and CD19 (Biolegend, UK). In general, the purity was >95% of all cells within the live gate. A representative dot plot is shown in Figure 2.1.

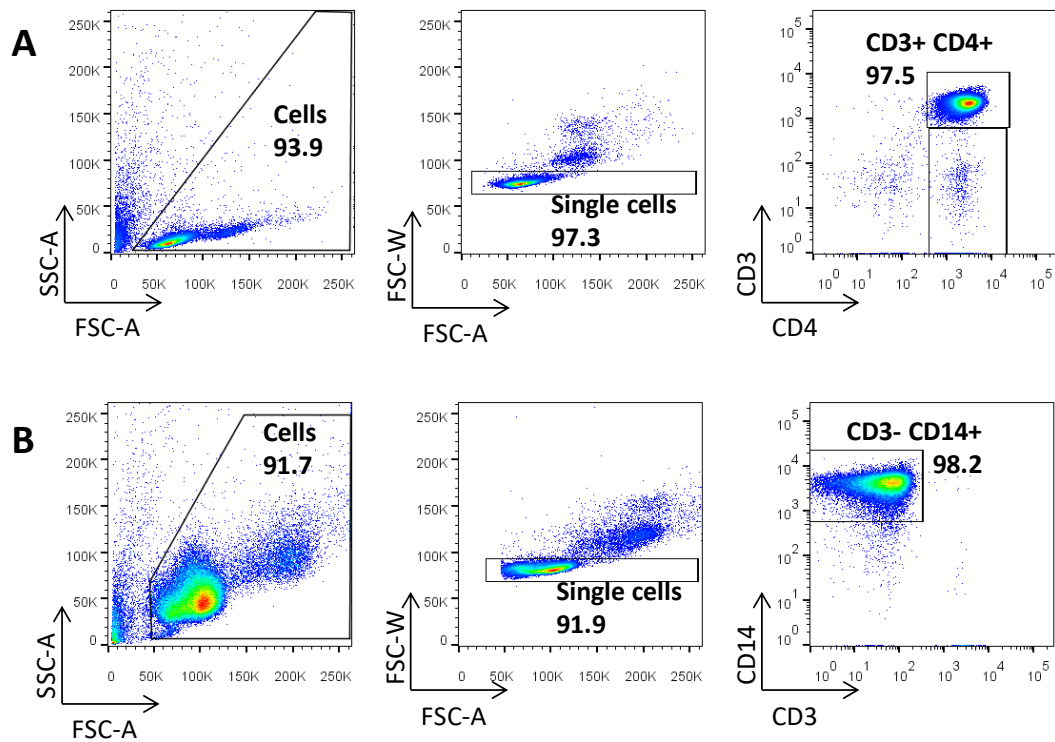
### **2.2.2 CD4+ T cell isolation**

Isolation of CD4+ T cells was performed using a CD4+ T cell isolation kit (Miltenyi Biotec). The number of PBMCs required for separation was calculated and resuspended in 40µl MACS buffer per 10<sup>7</sup> cells. The CD4+ T cell population was negatively isolated via magnetic separation. In brief, the cells were incubated with a cocktail of biotin-conjugated monoclonal antibodies (mAb) against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCRγ/δ and glycophorin (10µl/10<sup>7</sup> cells) for 10 minutes at 4°C. After 10 minutes beads coated with an anti-biotin antibody were added (10µl/10<sup>7</sup> cells), mixed and incubated at 4°C for 15 minutes. After incubation the suspension was diluted with MACS buffer with 10-20x the labelling volume and centrifuged for 10 minutes at 1200rpm. For CD4+ T cell isolation an LS column (Miltenyi Biotec) was placed on a magnet and pre-wet with 3mls MACS buffer. The suspension was applied to the column, followed by 3ml of MACS buffer which had

been used to rinse the suspension tube. The CD4<sup>+</sup> T cells collected were counted using Trypan Blue and stained for purity with CD3, CD4, CD8 (Biolegend) and CD14 (Miltenyi Biotec, UK). In general, the purity for CD4<sup>+</sup> T cells was >97 % (Figure 2.1).

### **2.2.3 Sorting immune cell populations from PBMC and SFMC**

To sort immune cell subsets from PBMC and SFMC for RNA analysis, PBMC and SFMC isolation was performed as described in section 2.1.1 and approximately 5-20 x10<sup>6</sup> cells were incubated simultaneously with viability dye eFluor 506 (1:1000) (eBioscience, UK), CD4 PerCp-Cy5.5, CD3 PE-Cy7, CD19 PE, CD8 PB (Biolegend, UK) and CD14 APC-Vio 770 (Miltenyi Biotec, UK) in PBS for 30mins at 4 °C. Cells were then washed and resuspended in MACS buffer. Based on the expression of extracellular markers, CD3<sup>+</sup> CD4<sup>+</sup> CD8<sup>-</sup> T cells were sorted using a FACSAria II (BD Biosciences). Sorted immune cell populations were stored in TRIzol (Invitrogen, UK) for analysis of RNA expression. An example of the sorting gating strategy is shown in Figure 2.2.



**Figure 2.1 Cell purity.**

PBMCs from healthy donors were isolated using density gradient centrifugation, CD14<sup>+</sup> monocytes were isolated via positive selection and the remaining CD14<sup>-</sup> cells were enriched for CD4<sup>+</sup> T cells. To assess cell purity, small aliquots of isolated CD4<sup>+</sup> T cells and CD14<sup>+</sup> monocytes were stained with PE-Cy7-CD3, PerCP-Cy5.5-CD4, APC-Cy7-CD14, PB-CD8 and APC-CD19. One representative example is shown for (A) purified CD4<sup>+</sup> T cells and (B) purified CD14<sup>+</sup> monocytes. Purity of CD4<sup>+</sup> and CD14<sup>+</sup> was consistently over 97% and 95%, respectively.

### **2.3 Cell culture**

Human PBMC or SFMC were cultured in culture medium: Roswell park memorial institute media (RPMI) 1640 (Gibco, Invitrogen, UK) supplemented with 10% foetal calf serum (FCS) (Gibco, Invitrogen, UK), 100 units/ml penicillin, 100µg of streptomycin/ml (Gibco, Invitrogen, UK) and 1% glutamine (Gibco, Invitrogen, UK). RA and PsA fibroblasts were cultured with DMEM (Gibco, Invitrogen, UK) supplemented with either 10% or 20% foetal calf serum (FCS) (Gibco, Invitrogen, UK), 100 units/ml penicillin, 100µg of streptomycin/ml (Gibco, Invitrogen, UK), 2% glutamine and fungizone (Gibco, Invitrogen, UK).

#### **2.3.1 CD4+ T cell/CD14+ monocyte co-cultures**

$0.5 \times 10^6$  bulk CD4+ T cells were cultured with  $0.5 \times 10^6$  autologous CD14+ monocytes in a 48 well plate in a total volume of 1ml culture medium. Soluble anti-CD3 mAb (Janssen Cilag, UK or Biolegend, UK) was added at 100ng/ml in the absence or presence of 100ng/ml LPS (Invitrogen, Sigma, UK), in some experiments 1µg/ml adalimumab (ADA) (Abbvie, UK) was added. Co-cultures were incubated for 3 days at 37 °C, 5% CO<sub>2</sub>.

#### **2.3.2 PBMC cultures**

$1 \times 10^6$  PBMC were cultured with soluble anti-CD3 in the absence or presence of human recombinant (hr) IL-1β (10ng/ml) (Peprotech, UK) and hrIL-23 (20ng/ml) (R&D, UK) and the absence or presence of soluble anti-CD28 (BD, UK) in a total volume of 1ml of culture medium. In some experiments, plate-bound anti-CD3 was used. To prepare plate-bound anti-CD3 plates, anti-CD3 was coated in each plate well at 1.25µg/ml in PBS for a minimum of 2 hours at 37°C. Wells were washed three times with PBS before cells were plated.

#### **2.3.3 CD4+ T cell only cultures**

In all CD4+ T cell cultures, plate-bound anti-CD3 was used. In some experiments,  $1 \times 10^6$  bulk CD4+ T cells were cultured in the absence or presence of hrIL-1β (10ng/ml) and hrIL-23 (20ng/ml) and the absence or presence of soluble anti-CD28 in

a total volume of 1ml of culture medium. Other experiments involved titration of soluble anti-CD28 (0-2µg/ml) and plate-bound anti-CD3 (0-2 µg/ml) in cultures of  $0.2 \times 10^6$  bulk CD4<sup>+</sup> T cells in a 96 well plate with a total volume of 200µl in culture medium with IL-1β (10ng/ml) and IL-23 (R&D, UK). Finally, in some experiments CD4<sup>+</sup> T cells were incubated with anti-IL-2 (1-5µg/ml) (R&D, UK) and/ or anti-IFNγ (1-5µg/ml) (Biolegend, UK). Cultures were incubated at 37 °C at 5% CO<sub>2</sub> for 3 days, unless otherwise stated.

### **2.3.4 Fibroblasts**

#### **2.3.4.1 General fibroblast cell-line maintenance**

RA and PsA fibroblast cell lines were either generated in-house (as described in section 2.1.3) or received as a kind gift from the Anca Catrina lab, Karolinska Institute. Fibroblast cell lines were frequently set up from a cryopreserved stock aliquot, however, in some cases, they were immediately grown and used from a tissue explant culture. To set up a fibroblast cell line from cryopreserved stock, fibroblast cryovials were sat in a water bath at 37°C and when completely thawed, the cell suspension was immediately transferred to a 15ml falcon tube containing 9ml of pre-warmed culture media; DMEM + 20% FCS +100 units/ml penicillin + 100µg of streptomycin/ml + 2% glutamine + 1mg/ml fungizone. Fibroblasts were then centrifuged at 1200rpm for 10 minutes. After resuspending the cell pellet in 1ml of culture media, fibroblasts were transferred into a T175 flask to a total volume of 20ml culture media. Fibroblasts were kept at 37 °C at 5% CO<sub>2</sub> and culture media was replenished every 3 days. After the first 3 days of setting up a cell line from a cryopreserved stock, the FCS in DMEM media was reduced to 10%. When fibroblasts reached approximately 80% confluency, the T175 flask was washed twice with 10ml PBS and incubated for 5 minutes at 37 °C with 2.5ml Trypsin-EDTA. Once all the fibroblasts were detached, 7.5ml culture media was added to the flask, the cell suspension was transferred to a 50ml falcon tube and cells were counted using Trypan blue. Whilst some fibroblasts were set up for a functional experiment (as described in the next section), others were transferred back into a T175 flask and allowed to grow in the new passage. Fibroblast cell lines were kept and used between passage 2 and 8.

#### **2.3.4.2 Fibroblast assays**

10x10<sup>3</sup> fibroblasts were seeded per well in a 96-well flat-bottom plate in total volume of 200µl DMEM culture media and incubated for 24 hours at 37°C with 5% CO<sub>2</sub>. Taking care not to disturb the adhered fibroblasts, the supernatant was removed and discarded. Human recombinant cytokines (IL-17A, IL-17F, IL-17AF, TNF $\alpha$ , IL-17B, IL-17C, IL-17D, IL-17E) were added either individually or in combination to the fibroblasts at the indicated concentrations. In some experiments, supernatants from CD4<sup>+</sup> T cells cultured *in vitro* were added to the fibroblasts at indicated percentages. Additionally, stimulated CD4<sup>+</sup> T cells were cultured with fibroblasts at a variety of ratios. In some of the experiments described, blocking antibodies were added at the indicated concentrations. Table 2.1 and 2.2 lists the human recombinant cytokines and blocking antibodies used.

All fibroblast cultures had a final volume of 200µl and were incubated for 24 hours at 37°C with 5% CO<sub>2</sub>. Fibroblast supernatants were collected and stored at -80°C before being analysed for IL-6 and IL-8 protein levels by ELISA.



**Table 2.1 Human recombinant cytokines used in cell culture**

	<b>Cytokine</b>	<b>Manufacturer</b>
<b>Primary cell culture</b>	IL-1 $\beta$	R&D
	IL-23	R&D
<b>Fibroblast cell culture</b>	IL-17A	Peprotech
	IL-17F	R&D
	IL-17AF	Miltenyi Biotec
	TNF $\alpha$	Miltenyi Biotec
	IL-17B	R&D
	IL-17C	Miltenyi Biotec
	IL-17D	R&D
	IL-17E	R&D

**Table 2.2 Neutralising antibodies used in cell culture**

	<b>Antibody target</b>	<b>Isotype</b>	<b>Manufacturer</b>
<b>Primary cell culture</b>	IL-2	Goat IgG	R&D
	IFN $\gamma$	Mouse (m) IgG1	Biologend
<b>Fibroblast cell culture</b>	Anti-IL-17A	Human (h) IgG1	UCB In-house
	Anti- IL-17F	hIgG1	UCB In-house
	Combined anti-IL-17A and IL-17F (Bimekizumab)	hIgG1	UCB In-house
	Anti-TNF $\alpha$ (Adalimumab)	hIgG1	Abbvie (purchased from pharmacy)

## 2.4 Flow cytometry

### 2.4.1 Compensation controls

In all flow cytometric settings, single-stained compensation controls were used to optimise fluorescence compensation settings. To prepare these controls, BD CompBeads (BD Biosciences, UK) were stained with fluorescently conjugated antibodies, according to manufacturer's instructions. For viability dye compensation controls, a mixture of live and heat-killed (1 minute, >65°C on heat block cells were used).

### 2.4.2 Extracellular staining

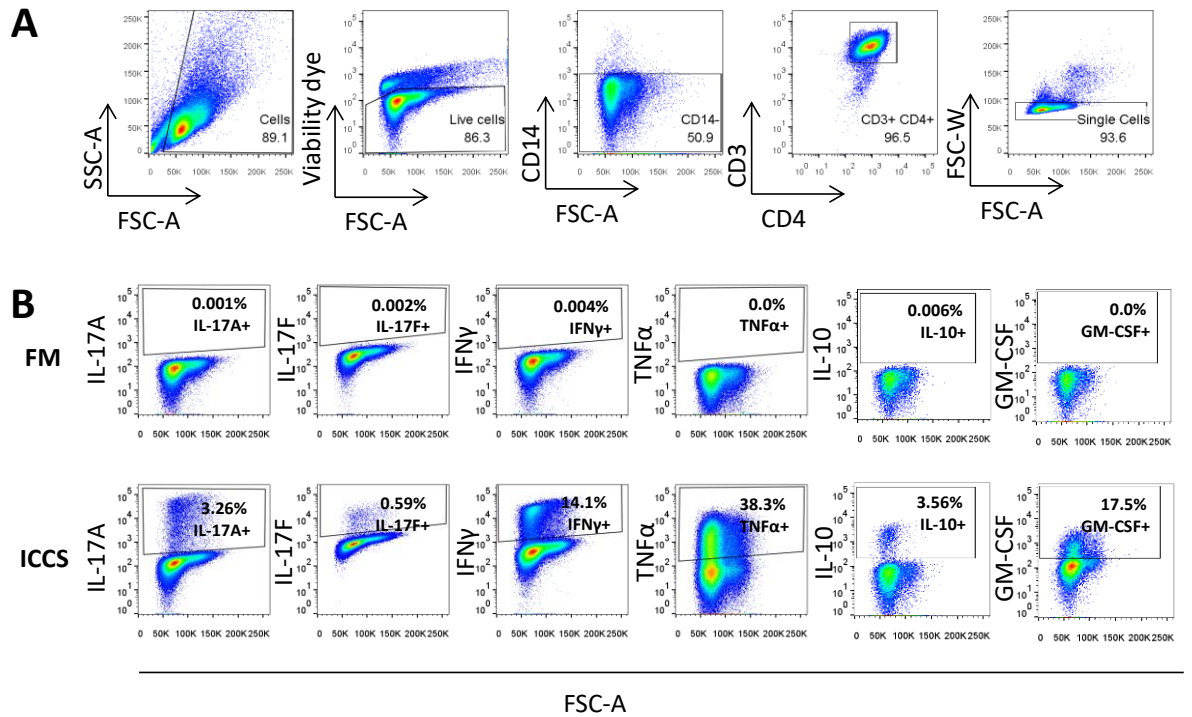
In some experiments, extracellular marker and cytokine expression was analysed from fresh or cryopreserved cells (PBMC, SFMC or CD4<sup>+</sup> T cells) following a 3 hour *ex vivo* stimulation with phorbol 12-myristate 13-acetate (PMA) (50ng/ml) (Sigma-Aldrich, UK), ionomycin (750ng/ml) (Sigma-Aldrich, UK) and Golgi stop (monensin) (BD, UK) or Golgi plug (brefeldin A) (BD, UK) (following manufacturer's guidelines). PMA and ionomycin stimulation is one of the most widely applied methods for activation of T cells. PMA activates protein kinase C, while ionomycin is a calcium ionophore, which synergises with PMA to enhance PKC activation <sup>1</sup>. Monensin and Brefeldin A prevent cytokine secretion. Monensin prevents protein secretion by interacting with the Golgi transmembrane Na<sup>++</sup>/ H<sup>+</sup> transport, while brefeldin A redistributes intracellularly produced proteins from the cis/medial Golgi complex to the endoplasmic reticulum <sup>2</sup>. *In vitro* PBMC/ SFMC or CD4<sup>+</sup> T cell cultures, described in section 2.3, were also re-stimulated with PMA, ionomycin and monensin or brefeldin, Both *ex vivo* and *in vitro* cultures were stimulated for 3 hours at 37°C with 5% CO<sub>2</sub>.

Following stimulation, cells were transferred into 1.5ml eppendorff tubes and washed in PBS. Next, cells were simultaneously stained with the relevant extracellular markers: CD14 (Miltenyi Biotec, UK) and CD8 (Biolegend, UK) and fixable viability dye (either eFluor450 (1:1000) or eFluor780 (1:2000) (eBioscience, UK)) in PBS and incubated for 30 minutes at 4°C. After incubation, cells were

washed in 500µl of FACS buffer (PBS supplemented with 0.5% BSA and 0.001% sodium azide (Sigma-Aldrich). Next, cells were fixed with 2% paraformaldehyde (PFA) for 15 minutes at 4°C. Following incubation cells were washed in 500µl of FACS buffer.

### **2.4.3 Intracellular cytokine staining**

To perform intracellular cytokine staining (ICCS), cells were washed in 500µl of permeabilisation (SAP) buffer (0.5% Saponin (Sigma-Aldrich) in FACS buffer) and centrifuged for 3.5 minutes at 8,000rpm. Cells were then incubated at 4°C in the presence of the chosen antibodies (all antibodies listed in Table 2.3) in a total volume of 100µl SAP buffer for 30 minutes. As CD4 is internalised upon PMA/ionomycin stimulation and  $\alpha$ CD3 binds to the same epitope as the CD3 flow cytometry antibody, both CD4 and CD3 were stained intracellularly. To distinguish cytokine-producing populations, fluorescence minus (FM) controls were used. Following incubation, cells were washed twice with 500µl of SAP buffer and once with 500µl FACS buffer. The final cell pellet was resuspended in 200µl of FACS buffer and analysed using the BD FACS Canto II flow cytometer, LSR Fortessa or BD FACSymphony. Figure 2.3 illustrates the gating strategy used for intracellular staining as well as example flow cytometric plots for FM control and positive cytokine staining.



**Figure 2.2 Gating strategies for intracellular staining.**

(A) Gates were first set around lymphocytes based on SSC-A vs FSC-A properties. The lymphocyte gate was further analysed for uptake of the viability dye, either eFluor506 or eFluor780 to determine live vs. dead cells. Next, CD14<sup>+</sup> monocytes were excluded by gating on CD14<sup>-</sup> cells. In some experiments, the viability dye was stained in the same fluorescent channel as CD14, allowing monocytes and dead cells to be simultaneously excluded in a ‘dump’ channel. Within live CD14<sup>-</sup> cells, CD3<sup>+</sup> CD4<sup>+</sup> T cells were selected and a FSC-W/FSC-A gate was applied allowing the exclusion of doublet CD4<sup>+</sup> T cells. Gated within single CD4<sup>+</sup> T cells, (B) shows representative dot plots of FM4 controls vs. ICCS for the indicated cytokines.

#### **2.4.4 IL-17A secretion assay**

To isolate IL-17A<sup>+</sup> CD4<sup>+</sup> T cells, CD4<sup>+</sup> T cells were cultured for 3 days with IL-1 $\beta$ , IL-23, anti-CD3 mAb and anti-CD28 mAb. After 3 days, cells were re-stimulated with PMA (50ng/ml) and ionomycin (750ng/ml) for 1.5 hours. This was followed by an IL-17A secretion assay (Miltenyi Biotec) according to manufacturer's instructions. Using a BD FACS ARIA sorter, IL-17A<sup>+</sup> CD4<sup>+</sup> T cells or IL-17A<sup>-</sup> CD4<sup>+</sup> T cells were sorted and collected. Sorted cells were either cultured directly with synovial fibroblasts (as described in section 2.4.3.2) or incubated for 24 hours in RPMI media (200x10<sup>3</sup> cells in 200 $\mu$ l).

#### **2.4.5 Development of UCB IL-17A and IL-17F capture assay**

To isolate IL-17A<sup>+</sup> IL-17F<sup>-</sup>, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells a capture assay was developed. A number of optimisation experiments were performed, however the following section describes the final optimised method. Due to UCB proprietary, description of the method remains brief.

UCB developed a bespoke anti-CD45 Fab Y fragment as well as Fab X fragments which recognise IL-17A (both the IL-17A homodimer and the IL-17AF heterodimer) and IL-17F (IL-17F homodimer only). To form IL-17A and IL-17F capture complexes, Fab X anti-IL-17A and Fab X anti-IL-17F were individually incubated with Fab Y CD45 at a 1.5 (Fab X):1 (Fab Y) ratio in PBS and incubated for 1 hour at 37°C. CD4<sup>+</sup> T cells were stimulated *in vitro* with IL-1 $\beta$  and IL-23 and then re-stimulated with PMA and ionomycin (described in section 2.3.2). Stimulated cells were then incubated simultaneously with Fab complex A and Fab complex F in cold X-vivo media on ice for 15 minutes. In a 50ml falcon tube, cells were washed with 50ml of warm X-vivo media and centrifuged for 5 minutes at 400xg. After discarding the supernatant, cells were resuspended in 50ml warm X-vivo media. To prevent non-specific binding, CD4<sup>+</sup> T cells were split into multiple 50ml falcon tubes (maximum of 6x10<sup>6</sup> CD4<sup>+</sup> T cells in each 50ml falcon tube). Cells were then incubated for 45 minutes at 37 °C, whilst rotating on a MACS mix rotator (Biolegend, UK). After

incubation, 0.5M EDTA was added directly into each cell suspension and inverted. This was to prevent cell sticking and cell loss. Cells were then centrifuged for 5 minutes at 400xg. All supernatants were discarded and multiple cell pellets were pooled into one falcon tube. Cells were washed with 50ml ice cold PBS + EDTA and centrifuged for 5 minutes, 400xg at 4°C. The supernatant was discarded and the wash step repeated. In X-vivo media, cells were incubated with CD4 PerCP Cy5.5 (Biolegend, UK), CD3 FITC (Biolegend, UK), CD8 PE Cy7 (Biolegend, UK), IL-17A APC (eBioscience, UK) and IL-17F PE (eBioscience, UK) for 30 minutes on ice. Cells were washed twice in 50ml ice cold PBS + EDTA. Prior to sorting, the cell pellet was resuspended in 1ml PBS+ EDTA and stained with DAPI viability. IL-17A+ IL-17F-, IL-17A+ IL-17F+ and IL-17F+ IL-17A- CD4+ T cells were sorted using a FACSAria II (BD Biosciences). A gating strategy is shown in Figure 3.4. Sorted immune populations were stored in RNA lysis buffer (Qiagen) for analysis of IL-17A and IL-17F mRNA expression.

**Table 2.3 Flow cytometry antibodies used in this thesis**

<b>mAb</b>	<b>Conjugate</b>	<b>Isotype</b>	<b>Clone</b>	<b>Per 100µl stain</b>	<b>Manufacturer</b>
CD3	PE-Cy7	mIgG1k	UCHT1	1µl	Biolegend
CD3	BUV395	mIgG1	SK7	1µl	BD
CD4	PerCP-Cy5.5	mIgG1	SK3	1µl	Biolegend
CD8	PB	mIgG1	RPA-T4	1µl	Biolegend
CD14	APC Vio 770	mIgG2a	TUK4	5µl	Miltenyi
CD19	PE	mIgG1	3G8	1µl	Biolegend
CD45RO	PB	mIgG2a	UCHL1	1µl	Biolegend
CD25	PE	mIgG1	M-A251	1µl	Biolegend
CD127	FITC	mIgG1	A019D5	1µl	Biolegend
CD161	BV605	mIgG1	HP-3G10	3µl	Biolegend
CCR6	APC	mIgG1	11A9	5µl	BD
IL-17A	PE	mIgG1	BL168	1µl	Biolegend
IL-17A	APC	RatIgG2a	eBio17B7	1µl	eBioscience
IL-17F	AF488	Goat IgG	Poly5166	5µl	Biolegend
IL-17F	PE	Rat IgG1	SHLR17	1µl	eBioscience
IL-17AF	PB	mIgG1	20LJS09	5µl	eBioscience
IFN-γ	PerCp Cy5.5	mIgG1k	4S.B4	1µl	Biolegend
IFN-γ	AF488	mIgG1	4S.B3	1µl	eBioscience
TNFα	APC	mIgG1k	MAB11	1µl	Biolegend
TNFα	EF450	mIgG1	Mab11	1µl	eBioscience
IL-10	AF647	RatIgG1	JES3-9D7	1µl	Biolegend
GM-CSF	PB	RatIgG2a	BVD2-21C11	1µl	Biolegend
IL-2	FITC	RatIgG2a	MQ1-17H12	5µl	Biolegend

## 2.5 Mass cytometry (CyTOF)

CyTOF is a variation of flow cytometry which replaces fluochrome conjugated antibodies with antibodies labelled with heavy metal ion tags. This allows for a larger antibody panel in single samples, without significant spillover between channels. Readout is by time-of-flight mass spectrometry<sup>406</sup>.

*Ex vivo*. stimulated RA/PsA PBMC and SFMC ( $1-8 \times 10^6$  cells) were washed once with PBS in eppendorf tubes. After centrifugation for 5 minutes at 8000rpm, cell pellets were resuspended in 5mM Cell-ID Cisplatin (Fluidigm) in PBS and incubated for 5 minutes at RT. Cisplatin allows for the discrimination of live and dead cells by its ability to enter the compromised cell membranes of dead cells and bind to protein nucleophiles. The reaction was quenched with MaxPar cell staining buffer, using 5 times the volume of the cell suspension. Cells were then centrifuged for 5 minutes at 8000rpm and washed once with PBS. Next, cells were fixed in 500 $\mu$ l PFA for 5 minutes at RT. After one wash in PBS, cells were washed again in permeabilisation buffer and then stained intracellularly with all metal conjugated antibodies (Table 2.4) in permeabilisation buffer for 1 hour at RT. Samples were washed once with permeabilisation buffer and twice with PBS. Until acquisition, cells were stored at 4°C in 500 $\mu$ l 2% PFA. On the day of acquisition, cells were washed with PBS and then incubated for 20 minutes at RT with Cell-ID Intercalator-Ir (Fluidigm) to intercalate DNA. Before acquisition on CyTOF Helios, cells were washed twice with PBS and twice with water (MilliQ). Data was analysed and SPADE plots were generated using Cytobank.



**Table 2.4 Metal-conjugated antibodies from UCB used for CyTOF**

<b>mAb</b>	<b>Metal</b>	<b>Isotype</b>	<b>Per 100µl stain</b>
CD8	146Nd	RPA-T8	2µl
CD4	145Nd	RPA-T4	2µl
CD11c	159Tb	3.9	2µl
CD123	151Eu	6H6	2µl
CD16	148Nd	3G8	2µl
CD14	160Gd	61D3	2µl
CD56	173Yb	CMSSB	2µl
CD45	154Sm	HI30	2µl
CD19	142Nd	HIB19	2µl
CD3	170Er	UCHT1	2µl
HLA-DR	174Yb	L243	2µl
IL-17A	156Gd	eBio64CAP17	2µl
IL-17F	153Eu	SHLR17	2µl
IL-22	166Er	IL22JOP	2µl
GM-CSF	164Dy	BVD2-21C11	2µl
IL-10	158Gd	JES3-9D7	2µl
TNFα	155Gd	Mab1	2µl
IFNγ	163Dy	45-15	2µl
IL-4	150Nd	MP4-25D2	2µl
IL-13	175Lu	JES10-5E2	2µl

## 2.6 Cytokine analysis

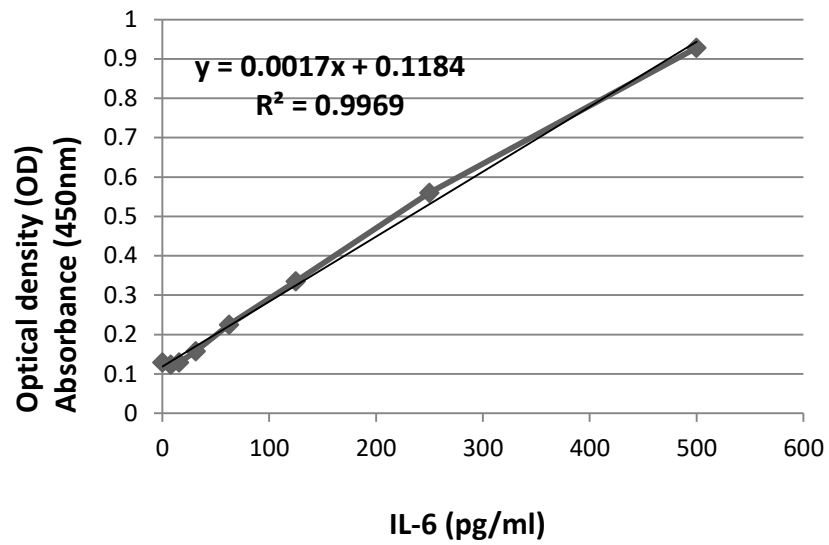
### 2.6.1 Enzyme-linked immunosorbent assay (ELISA)

A variety of cytokines including IL-17A, IL-17F, IL-17AF, IL-6 and IL-8 were detected in samples using ELISA. A full list of ELISA kits used is shown in Table 2.2. The ELISAs were performed according to manufacturer's instructions; generally, different manufacturer kits followed the same protocol.

Ninety-six-well Nunc MaxiSorp microplates (Thermo Fisher Scientific) were coated overnight at 4°C with capture antibody diluted in coating buffer. After washing 4 times with washing buffer (PBS + 0.05% Tween-20 (Thermo Fisher Scientific, UK), non-specific binding was prevented by blocking plates with assay diluent (PBS + 10% BSA (Sigma Aldrich, UK) for 1 hour at RT. Standards were prepared by performing seven two-fold dilutions of the top standard (provided with kit) with assay diluent and samples were diluted appropriately in assay diluent. Plates were washed 4 times with assay wash buffer and 100µl of diluted standards (added in duplicate) and samples were added and incubated for 2 hours at RT. Plates were washed 4 times again, before incubated with detection antibody diluted in assay diluent for 1 hour at RT. Following another 4 washes and a final 1-minute soak, 100µl 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution was added. When a gradual colour change was observed amongst the standards, the reaction was stopped with 50µl 1M sulphuric acid (Sigma-Aldrich, UK). Using either a Wallac 1420 microplate reader (Perkin Elmer, Waltham, MA, USA) or A SPARK 10M plate reader (Tecan, UK), microwell absorbance was read at 450nm. The optical density (OD) values of all standards were used to produce a standard curve, from which the analyte concentration was determined.

**Table 2.5 ELISA kits used in this thesis**

<b>Cytokine</b>	<b>Manufacturer</b>	<b>Top standard</b>
IL-17A	<ul style="list-style-type: none"><li>• Biolegend (detects IL-17A homodimer and IL-17AF heterodimer)</li><li>• eBioscience (detects IL-17A homodimer only)</li></ul>	<ul style="list-style-type: none"><li>• 500pg/ml</li><li>• 500pg/ml</li></ul>
IL-17F	eBioscience (detects IL-17F homodimer only)	2000pg/ml
IL-17AF	eBioscience (detects IL-17AF heterodimer only)	4000pg/ml
IL-2	Biolegend	500pg/ml
IFN $\gamma$	Biolegend	500pg/ml
IL-6	Biolegend	500pg/ml
IL-8	Biolegend	250pg/ml



**Figure 2.3 ELISA standard curve.**

For each analyte a two-fold dilution of standard was performed, providing a standard curve. Assay diluent served as a blank (0 pg/ml) and a standard curve was included on each ELISA plate. Standard absorbance values were used to generate a trendline and  $y=X$  equation, where X is the unknown sample concentration. In general, the  $R^2$  value was not lower than 0.99. After calculating unknown sample concentrations, any sample dilutions made were taken into account by multiplying the concentration value with the dilution factor.

### 2.6.2 Luminex

The principle of Luminex is similar to ELISA, however, luminex offers the benefit of detecting multiple cytokines and proteins in a sample simultaneously. Luminex technology is based on polystyrene or magnetic beads that are internally dyed with red and infrared fluorophores of differing intensities. Each dyed bead is given a unique bead region or number allowing for the discrimination of beads. Individual bead sets are coated with a capture antibody specific for a certain analyte. Multiple analyte-specific beads can then be incubated with a sample in a well of a 96 well plate, allowing for the detection of a variety of analytes simultaneously.

Cytokine levels in serum and synovial fluid samples were measured using the Bio-Plex Pro™ Human Th17 Cytokine Panel 15-Plex (Bio-Rad, UK) according to the manufacturer's instructions. In brief, the diluted bead cocktail was added to the wells of a 96-well plate, followed by the addition of standards (in duplicate) and appropriately diluted samples. After securely covering with a foil plate sealer, plates were incubated for 2 hours at RT on a plate shaker set at 800 +/-rpm. After adding wash buffer to each well, the plate was secured on a magnetic separator block and allowed to sit for 3 minutes. This was to ensure the magnetic beads are held in place by the magnetic separator block. Whilst the luminex plate was on the magnetic separator block, the wash buffer was discarded. The luminex plate was removed from the magnetic separator block and the wash procedure was performed for an additional 2 times. Diluted biotin detection antibody was added to the plate and incubated for 1 hour at RT on the plate shaker (800+/-500 rpm). After performing the wash step 3 times, streptavidin-PE was added to the plate and incubated for 30 minutes at RT on the plate shaker (800+/-500 rpm). Luminex plates were acquired on a FLEXMAP 3D instrument (Luminex, US).

## **2.7 Quantitative polymerase chain reaction (qPCR)**

### **2.7.1 RNA Isolation**

In some experiments, total RNA was isolated with a micro-RNA kit (Qiagen, UK) according to the manufacturer's protocol. For samples which had been lysed in trizol, a chloroform extraction was performed. This involved adding 200 $\mu$ l of chloroform to thawed trizol cell lysates and shaking vigorously for 15 seconds. After incubating at room temperature for 3 minutes, samples were centrifuged for 20 minutes, maximum speed at 4°C. The upper translucent aqueous layer was then transferred to a new RNase free eppendorf. To assist with RNA precipitation and visualisation, 1 $\mu$ l of glycoblue was added to each sample. Next, 500 $\mu$ l of ice cold propanol was added to each sample and vigorously shaken for 15 seconds. Samples were incubated at -20°C for either 30 minutes or overnight. Samples were centrifuged for 30 minutes, maximum speed at 4°C. At this stage, the RNA formed a visible blue pellet. After discarding the supernatant, 1ml of ice cold 70% ethanol was added to each sample and centrifuged for 10 minutes, maximum speed at 4°C. The supernatant was discarded and the RNA pellet was resuspended in 250 $\mu$ l of ice cold 70% ethanol and centrifuged for 10 minutes, maximum speed at 4°C. To evaporate any residual ethanol remaining after removal of the supernatant, the RNA pellet was air-dried for 5 minutes. The RNA pellet was then resuspended in RNase free water. RNA concentration and quality was measured using the Nanodrop 2000 spectrophotometer (Thermo Scientific). The ratio of absorbance at 260nm and 280nm and at 260nm and 230nm was used to assess RNA purity. Pure RNA will exhibit a 260/280 ratio of >2.0, however, accepted values are in the range of 1.7-2.1. The 260/230 ratio is an indication of contamination, while expected values are in the range of 1.8-2.2, a lower ratio indicates guanidine thiocyanate contamination. RNA that met the quality criteria was then DNase treated and reverse transcribed.

### **2.7.2 DNase treatment and cDNA reverse-transcription**

To remove any residual genomic DNA that might be contained in RNA samples, DNase treatment was performed. DNase I is an endonuclease that non-specifically cleaves DNA to release di-, tri- and oligonucleotide products. It acts on single and double stranded DNA, chromatin and RNA: DNA hybrids. To each RNA sample

(maximum concentration 1µg), 1µl of 10x reaction buffer with MgCl<sub>2</sub> and 1µl of DNase I was added, mixed well and incubated on a heat block for 30 minutes at 37°C. To inactivate the DNase I, 1µl of EDTA was added to the RNA sample and incubated at 65°C for 10 minutes. EDTA chelates ions in the digestion buffer, that are required for DNase I activity. Thus, DNase I can be heat-inactivated without loss of RNA. RNA concentration levels were analysed by nanodrop.

Reverse transcription was performed using the high capacity cDNA reverse transcription kit (Applied Biosystem, Carlsbad, CA, USA) according to manufacturer's instructions. Until qPCR was performed, cDNA was stored at -80°C.

### 2.7.3 qPCR protocol and analysis

qPCR reactions were performed using primers for human IL-17A and IL-17F (Integrated DNA technologies IDT, Haverlee, Belgium) and the SensiMix SYBR No-ROX kit according to the manufacturer's protocol. Reactions were performed on a 7900HT Fast Real-Time PCR system (Applied Biosystems). Thermocycler conditions included an initial holding at 95 °C for 10 minutes, which was followed by a cycling program of 95°C for 15 seconds, 60°C for 30 seconds and 72°C for 30 seconds. Data were analysed using the Applied Biosystems RQ manager software version 1.2.2. To normalise for differences in the amount of total RNA in each sample, the *ACTB* gene was used as an endogenous control. For each sample, the mean cycle threshold (CT) for replicates was calculated. Gene expression was calculated as the difference ( $\Delta$ CT) between the mean CT value of the sample for the target gene and the mean CT value of the same sample for the endogenous control (*ACTB*). Relative gene expression was expressed as  $2^{-\Delta$ CT.

**Table 2.6 Primers using in this thesis**

Target	Forward	Reverse
Human IL-17A	TCC CAC GAA ATC CAG GAT GC	GCA TGT TCA GGT TGA CCA TCA
Human IL-17F	GCT GTC GAT ATT GGG TTC AT	GCT GTC GAT ATT GGG GCT TG

## **2.8 Statistics**

Statistical differences were calculated with Prism 7.0 software (GraphPad, San Diego, CA). The D'Agostino and Pearson omnibus normality test was used to test the normality of data sets. This was followed by statistical significance testing using the appropriate tests as described in figure legends. Data sets with n values <8 were tested non-parametrically. P values < 0.05 were considered statistically significant.



### 3 Investigating the expression of IL-17F in healthy CD4+ T cells *in vitro*

#### 3.1 Introduction

The best-studied source of IL-17A and IL-17F is a specific subset of CD4+ T cells, named Th17 cells. As previously discussed in the introduction Th17 differentiation is dependent on signals from IL-6, transforming growth factor  $\beta$  (TGF $\beta$ ), IL-1 $\beta$  and IL-21, while IL-23 is important for lineage maintenance<sup>189,238,407</sup>. Reports from our lab have shown that *in vitro* LPS-activated monocytes induce Th17 responses in co-cultured CD4+ T cells in a TNF $\alpha$  and IL-1 $\beta$  dependent manner<sup>192,198</sup>. Stimulation of T cells with LPS-triggered monocytes resulted in both an increased percentage of IL-17A+ CD4+ T cells and an increase in IL-17A secretion<sup>4</sup>. Studies have also shown that low-strength T cell activation enhances Th17 development<sup>5-6</sup>. The majority of these studies, however, analysed the expression of IL-17A and not IL-17F. Few reports investigate what drives IL-17F expressing CD4+ T cells. In literature, IL-17F is generally described as a cytokine co-expressed with IL-17A. The overall aim of the first part of this chapter was to investigate whether IL-17A and IL-17F are induced in a similar manner or whether there are elements of differential regulation. To achieve this, the following questions were addressed:

- What drives IL-17F expression in CD4+ T cells?
- Does the strength of T cell activation affect IL-17F expression?

The second part of this chapter characterises IL-17F+ CD4+ T cells. Human IL-17A+ CD4+ T cells have been extensively characterised. They have been shown to express the markers CCR6 and CD161 and co-express other cytokines including TNF $\alpha$ , IFN $\gamma$ , GM-CSF and IL-10. However, there remains a lack in literature of an in-depth phenotypic characterisation of IL-17F+ CD4+ T cells. This includes phenotypic comparison of IL-17A+ vs. IL-17F+ CD4+ T cells. In this section, the following question was addressed:

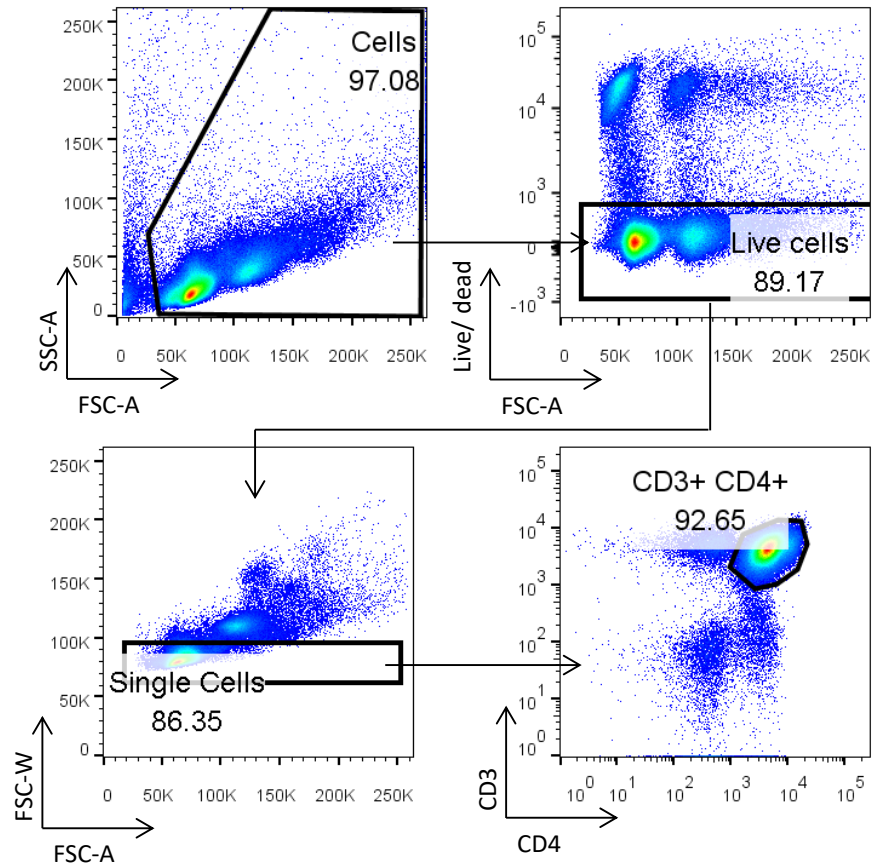
- Do IL-17F+ cells display different cytokine profiles to IL-17A+ CD4+ T cells?

## 3.2 Results

### 3.2.1 Induction of IL-17F expressing CD4<sup>+</sup> T cells

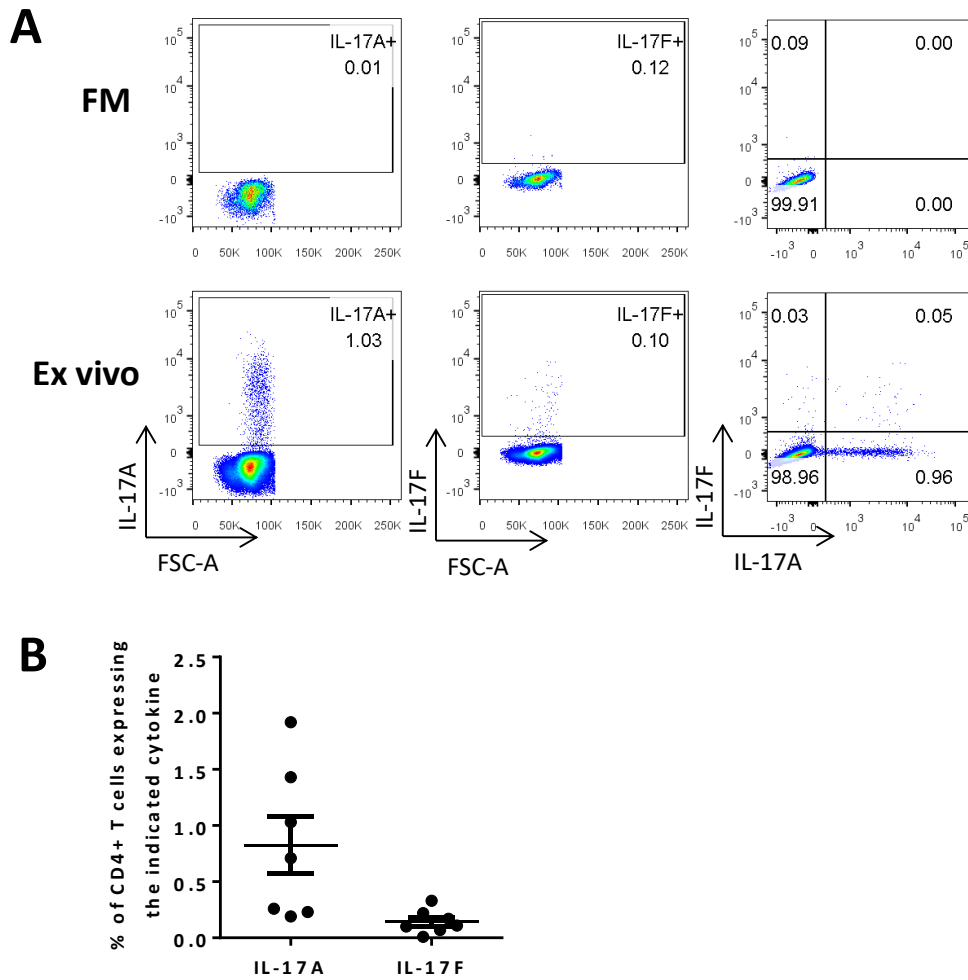
#### 3.2.1.1 *Ex vivo* analysis of IL-17A and IL-17F expressing CD4<sup>+</sup> T cells

I first investigated whether IL-17F<sup>+</sup> CD4<sup>+</sup> T cells could be detected following an *ex vivo* stimulation of healthy donor bulk CD4<sup>+</sup> T cells. Bulk CD4<sup>+</sup> T cells were incubated for 3 hours with PMA, ionomycin and GolgiStop then cells were stained to assess intracellular cytokine expression and analysed by flow cytometry. Figure 3.1 is a representative plot for the gating strategy applied. As shown in Figure 3.2, fluorescence minus (FM) controls were used to set gates to depict positive cytokine expression. In 6 donors, a range of IL-17A<sup>+</sup> CD4<sup>+</sup> T cell frequencies was detected following *ex vivo* stimulation (from 0.2 – 1.9%). In contrast, all donors showed very low IL-17F<sup>+</sup> CD4<sup>+</sup> T cell frequencies (approximately 0.1%).



**Figure 3.1 Representative gating strategy following *ex vivo* stimulation of CD4+ T cells.**

Healthy donor bulk CD4+ T cells were stimulated for 3 hours with PMA, ionomycin and GolgiStop. Cells were stained with fluorescently-conjugated antibodies against extracellular markers and intracellular cytokines. In this sample gating, cells were first gated for lymphocytes (SSC-A vs FSC-A). The lymphocyte gate was further analysed for uptake of the Live/Dead eFluor780 stain to determine live vs. dead cells. Within live cells, a FSC-W/FSC-A gate was applied allowing the exclusion of doublet CD4+ T cells. CD3+ CD4+ T cells were then selected.



**Figure 3.2 Assessing IL-17A+ and IL-17F+ CD4+ T cell frequencies following *ex vivo* stimulation.**

Healthy donor bulk CD4+ T cells were stimulated for 3 hours with PMA, ionomycin and GolgiStop and then assessed for intracellular IL-17A and IL-17F cytokine expression. To set the gates for positive cytokine expression, fluorescence minus controls were used. **(A)** Representative dot plots showing FM controls and the frequencies of IL-17A and IL-17F expressing cells within the total CD4+ T cell population in *ex vivo*-stimulated samples. **(B)** Cumulative data for the frequency of IL-17A (n=6) and IL-17F (n=6) CD4+ T cell frequencies.

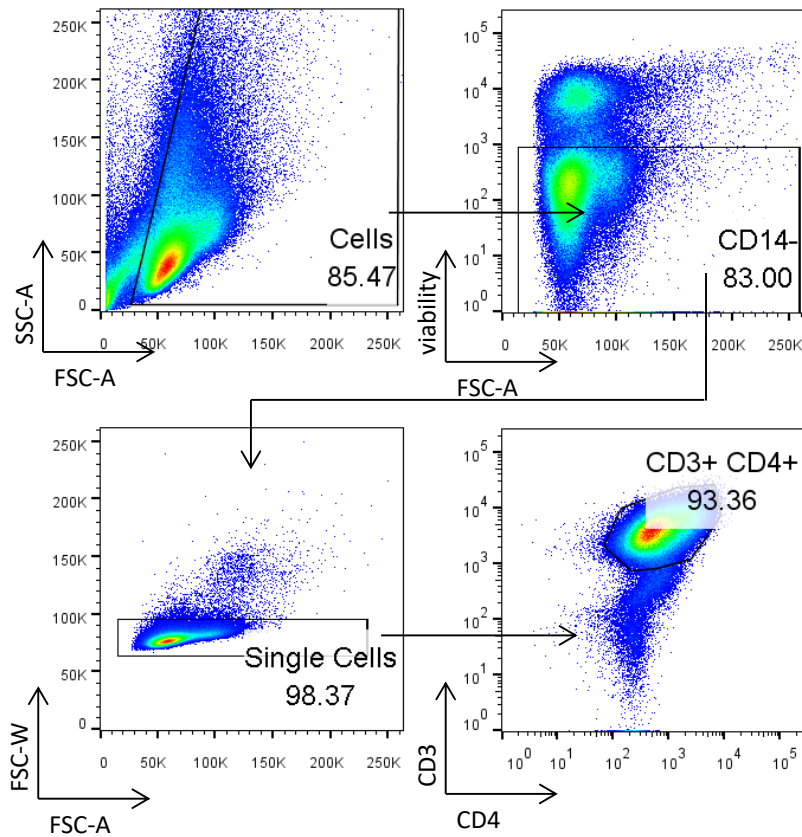
### **3.2.1.2 *In vitro* activated monocytes increase both IL-17A+ CD4+ T cell and IL-17F+ CD4+ T cell frequencies and IL-17A and IL-17F secretion**

Next, we examined factors which could induce IL-17F expressing CD4+ T cells and IL-17F secretion. I first expanded on previous work published by the lab which assessed the effect of LPS-activated monocytes on IL-17A induction<sup>4,22</sup>. Healthy CD4+ T cells were co-cultured with autologous CD14+ monocytes and stimulated with anti-CD3 mAb in the absence or presence of LPS for three days. After incubation, supernatants were collected and stored at -80°C before being tested by ELISA. The remaining cells were then re-stimulated and an intracellular cytokine stain (ICCS) was performed. CD4+ T cell cytokine expression was then analysed by flow cytometry. Figure 3.3 displays a representative gating strategy. Similar gating strategies were used for all other *in vitro* cultures.

When compared to medium control conditions, addition of LPS to T cell/monocyte co-cultures led to a 1.6-fold increase in the frequency of IL-17A+ CD4+ T cells and a 3.2-fold increase in IL-17A secretion (Figure 3.4). This correlates with previous results from our lab<sup>4</sup>. LPS stimulation also resulted in a 3.4-fold increase in IL-17F+ CD4+ T cell frequencies and a 1.8-fold increase in IL-17F secretion (Figure 3.4). Flow cytometric analysis showed the frequency of IL-17A+ CD4+ T cells was higher than IL-17F+ CD4+ T cells in both the control (approximately 3.16 vs. 0.40%, respectively) and LPS conditions (approximately 5.05 vs. 1.34%). Flow cytometric analysis indicated all IL-17F+ CD4+ T cells highly co-expressed IL-17A. No single positive IL-17F+ CD4+ T cells were identified. Therefore, in this T cell/monocyte co-culture system we observed two distinct populations, IL-17A+ IL-17F- CD4+ T cells and double positive IL-17A+ IL-17F+ CD4+ T cells.

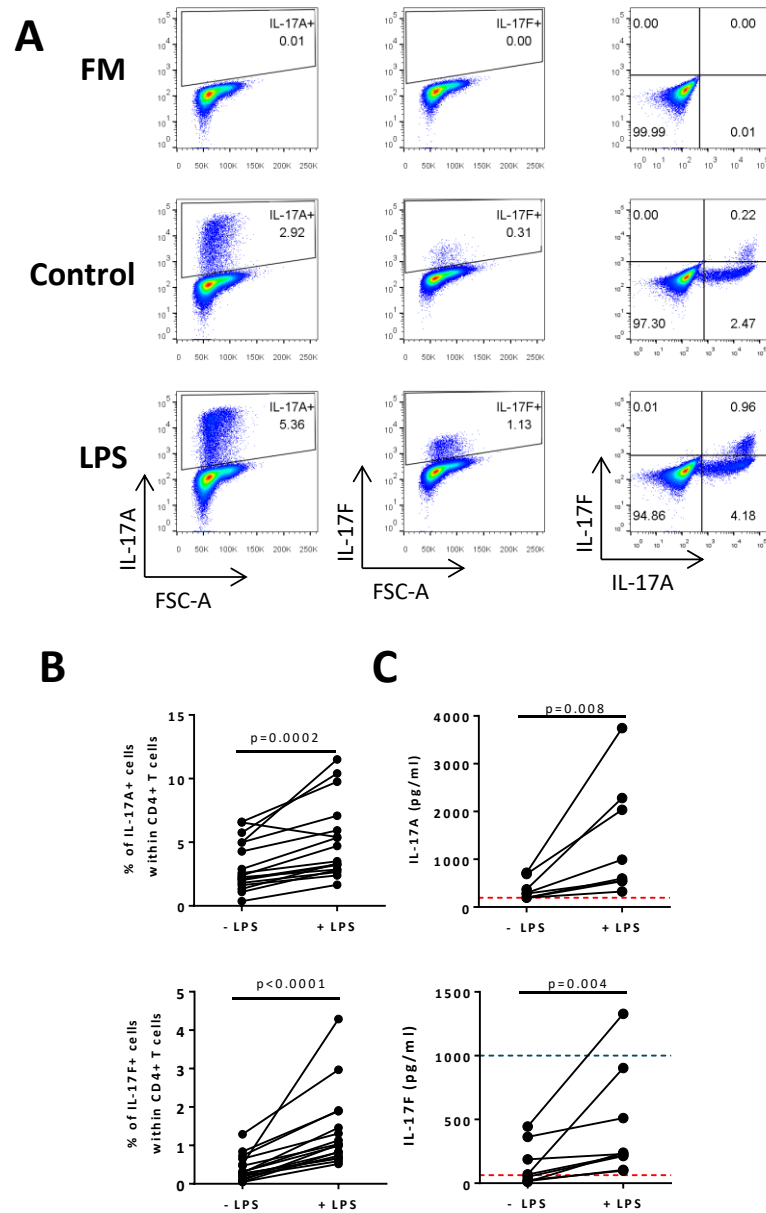
To assess the effect of addition of LPS to co-cultures on other cytokines, supernatants were analysed by luminex (Figure 3.5). We observed an increase in IL-1 $\beta$ , IL-6 and IFN $\gamma$  protein levels in LPS treated conditions. Additionally, we observed an increase in IL-23 levels, however statistical significance was not

achieved. TNF $\alpha$  was detected in both the control and LPS conditions but levels were above the assay detection limit (data not shown).



**Figure 3.3 Representative gating strategy for *in vitro* cultures.**

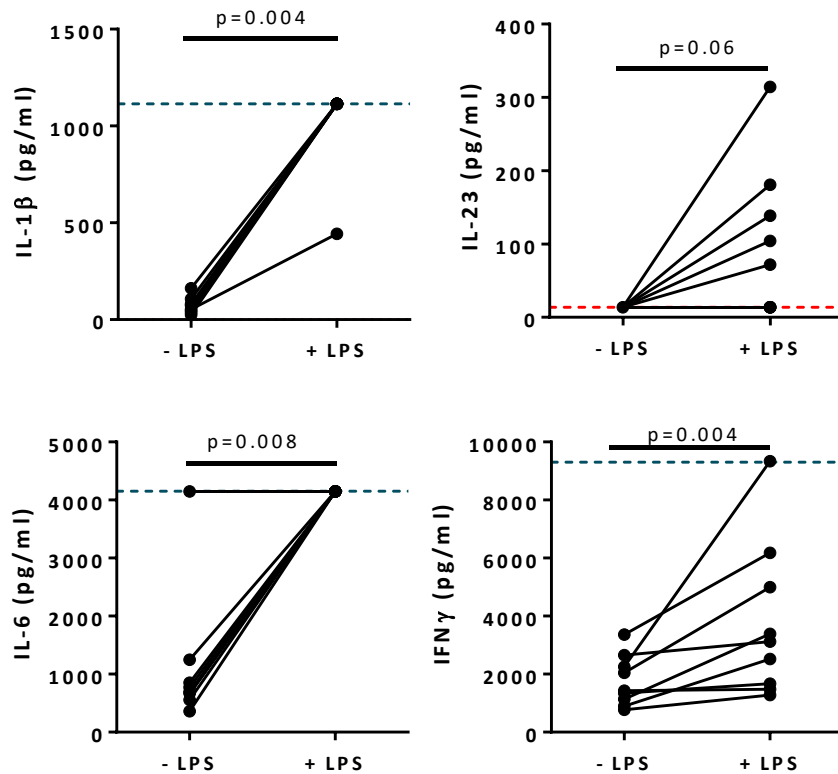
In this sample gating,  $0.5 \times 10^6$  bulk CD4+ T cells were co-cultured with monocytes at a 1:1 ratio in the presence of anti-CD3 mAb (100ng/ml) and LPS (100ng/ml). After three days cells were stimulated with PMA/ionomycin and Golgi stop. Cells were stained with antibodies against extracellular markers and cytokines of interest. In this representative plot, cells were first gated for lymphocytes (SSC-A vs FSC-A). A ‘dump’ gate was then applied, whereby the viability dye and CD14 antibody were on the same fluorochrome, allowing for the simultaneous exclusion of dead and CD14+ cells. Next, a FSC-W/FSC-A gate was applied allowing the exclusion of doublet CD4+ T cells. CD3+ CD4+ T cells were then selected.



**Figure 3.4 LPS-activated monocytes induce an increase in IL-17A and IL-17F expressing CD4+ T cell frequencies and corresponding cytokine secretion.**

Bulk CD4+ T cells ( $0.5 \times 10^6$ ) were co-cultured 1:1 with autologous monocytes and stimulated with anti-CD3 mAb (100ng/ml) in the absence (control) or presence (LPS) of LPS (100ng/ml). After 3 days cells were re-stimulated and assessed for intracellular cytokine expression. **(A)** Representative dot plots and **(B)** cumulative data show frequencies of IL-17A (n=17) and IL-17F (n=17) within the total CD4+ T cell population. **(C)** Levels of IL-17A (n=7) and IL-17F (n=7) expressing cells detected in culture supernatant (collected prior re-stimulation) via ELISA. Upper blue and red dashed lines indicate the upper and lower detection limits, respectively. Data were analysed using matched-pairs signed rank test.





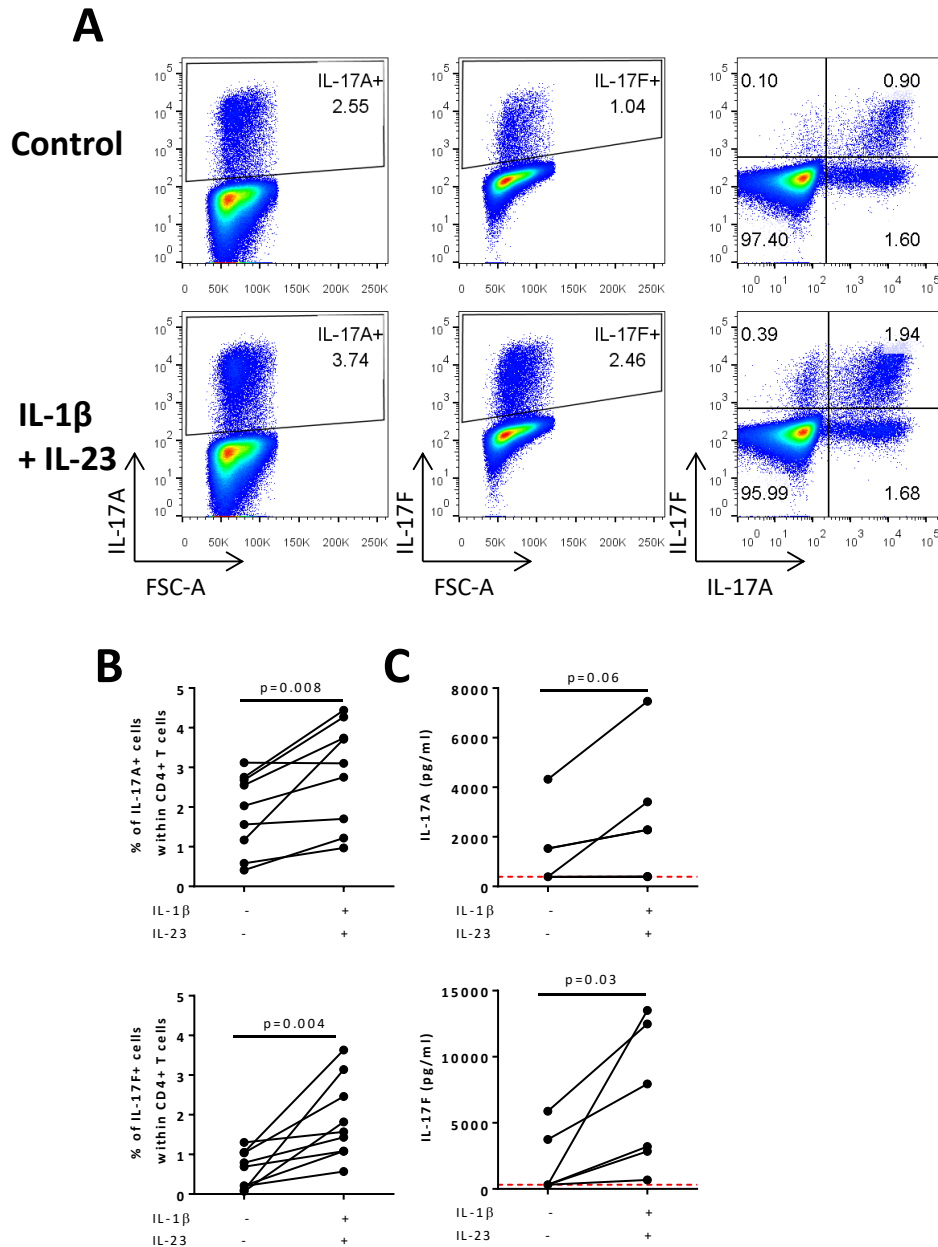
**Figure 3.5 Elevated levels of IL-1 $\beta$ , IL-6 and IFN $\gamma$  are detected in CD4+ T cell / LPS-activated CD14+ monocyte co-cultures.**

Bulk CD4+ T cells were co-cultured 1:1 with autologous monocytes and stimulated with anti-CD3 mAb (100ng/ml) in the absence (control) or presence (LPS) of LPS (100ng/ml). Supernatants were collected and IL-1 $\beta$  (n=9), IL-23 (n=9), IL-6 (n=9) and IFN $\gamma$  (n=9) levels were detected by luminex. Upper blue and red dashed lines indicate the upper and lower detection limits, respectively. Data were analysed using matched-pairs signed rank test.

### **3.2.1.3 CD3 and CD28 stimulation in the presence of IL-1 $\beta$ and IL-23 induces IL-17A and IL-17F CD4+ T cell frequencies and corresponding cytokine secretion**

IL-1 $\beta$  and IL-23 are well-characterised Th17 polarising cytokines, which have been previously described to induce IL-17F<sup>7</sup>. Our LPS-activated monocyte/CD4+ T cell co-culture system, which induced IL-17F+ CD4+ T cells, also showed increased protein levels of IL-1 $\beta$  and IL-23 in comparison to control conditions. With this in mind, I investigated the role of IL-1 $\beta$  and IL-23 in inducing IL-17A and IL-17F.

Bulk CD4+ T cells were cultured with soluble anti-CD28 mAb, replacing the co-stimulation signal provided by the CD14+ monocytes, and plate-bound anti-CD3 mAb. Cultures were set up in the absence or presence of the human recombinant cytokines, IL-1 $\beta$  (10ng/ml) and IL-23 (20ng/ml). Following 3 days incubation, cell culture supernatants were removed for ELISA analysis. Cells were then re-stimulated and ICCS was performed analysing cytokine expression. Addition of IL-1 $\beta$  and IL-23 led to an increase in the frequency of IL-17A (1.7-fold) and IL-17F (3.7-fold) expressing CD4+ T cells (Figure 3.6A & B). In this culture system, three distinct IL-17 populations were observed; IL-17A+ IL-17F-, IL-17A+ IL-17F+ and IL-17F+ IL-17A- CD4+ T cells. In line with this, ELISA analysis showed an increase in IL-17A and IL-17F protein present in IL-1 $\beta$  and IL-23 stimulated conditions (Figure 3.6C). However, in three out of the six samples, IL-17A was below the detection limit. Therefore, for a more accurate analysis these samples should be re-analysed at a lower dilution.



**Figure 3.6 IL-1 $\beta$  and IL-23 enhance the frequency of IL-17A+ and IL-17F+ CD4+ T cells.**

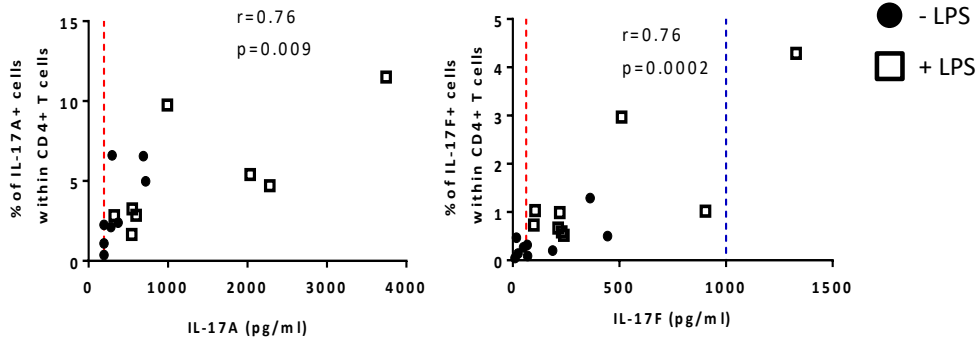
Bulk CD4+ T cells were cultured with platebound anti-CD3 mAb (1.25 $\mu$ g/ml) and soluble anti-CD28 mAb either in the absence or presence of IL-1 $\beta$  (10ng/ml) and IL-23 (20ng/ml) for 3 days. Cells were re-stimulated and assessed for intracellular cytokine expression. **(A)** Representative dot plots and **(B)** cumulative data show frequencies of IL-17A (n=9) and IL-17F (n=9) expressing cells within the total CD4+ T cell population. **(C)** Levels of IL-17A (n=6) and IL-17F (n=6) detected in culture supernatant (prior re-stimulation) via ELISA. Red dashed lines indicate the lower detection limit. Data were analysed using matched-pairs signed rank test.

#### **3.2.1.4 Assessing the correlation between flow cytometry cytokine expression and protein levels**

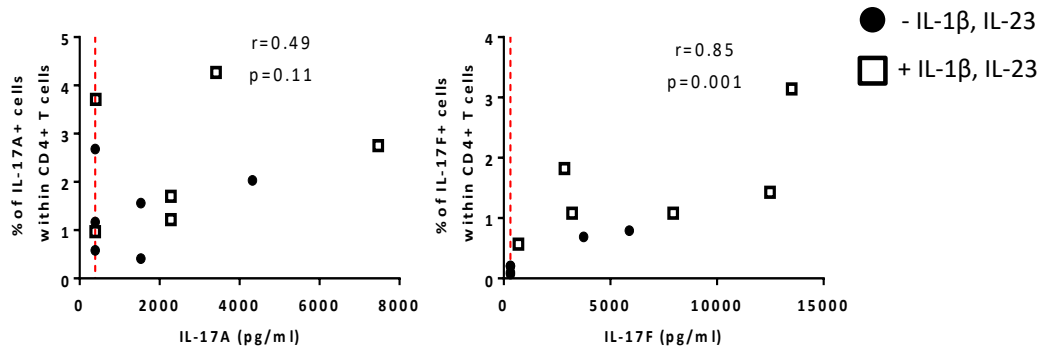
PMA together with ionomycin is one of the most widely applied methods for activation of T cells. However, as PMA and ionomycin stimulation is very strong, researchers question whether it truly reflects what is happening physiologically in the cell. In our CD4<sup>+</sup> T cell/monocyte co-cultures and CD4<sup>+</sup> T cell only cultures, supernatant was collected prior to PMA and ionomycin stimulation and cells were then stimulated with PMA and ionomycin for flow cytometry analysis. To investigate whether cells still reflect the physiological events after a PMA and ionomycin stimulation, IL-17A<sup>+</sup> or IL-17F<sup>+</sup> CD4<sup>+</sup> T cell frequencies (measured post PMA/iono re-stimulation) in each culture system were correlated with the secretion of IL-17A and IL-17F in the culture supernatant (measured prior to PMA/ionomycin re-stimulation).

As shown in Figure 3.7, in all culture conditions, a positive correlation was observed between the percentage of IL-17A<sup>+</sup> CD4<sup>+</sup> T cells and the secretion of IL-17A as well as between the percentage of IL-17F<sup>+</sup> CD4<sup>+</sup> T cells and IL-17F secretion (Figure 3.7). These results indicate that cells still display their physiological profile following a PMA and ionomycin re-stimulation.

### A CD4+ T cell/ CD14+ monocyte co-cultures -/+ LPS



### B CD4+ T cell cultures -/+ IL-1 $\beta$ and IL-23



**Figure 3.7 Correlation analysis of intracellular flow cytometry staining vs. ELISA.**

(A)  $0.5 \times 10^6$  of bulk CD4+ T cells were cultured with anti-CD3 mAb and  $0.5 \times 10^6$  monocytes in the absence (-LPS) or presence (+LPS) of LPS for three days. (B) Bulk CD4+ T cells were cultured with platebound anti-CD3 mAb ( $1.25\mu\text{g/ml}$ ) and soluble anti-CD28 either in the absence or presence of IL-1 $\beta$  ( $10\text{ng/ml}$ ) and IL-23 ( $20\text{ng/ml}$ ) for 3 days. In both experiments, culture supernatant was analysed by ELISA and cells were re-stimulated and analysed by ICCS and flow cytometry. Results show the percentage of IL-17A+ cells plotted against the concentration of IL-17A (pg/ml) and the frequency of IL-17F+ cells plotted against the concentration of IL-17F (pg/ml). Upper blue and red dashed lines indicate the upper and lower ELISA detection limits, respectively. Statistical analysis was performed using Spearman's correlation coefficients for nonparametric data.

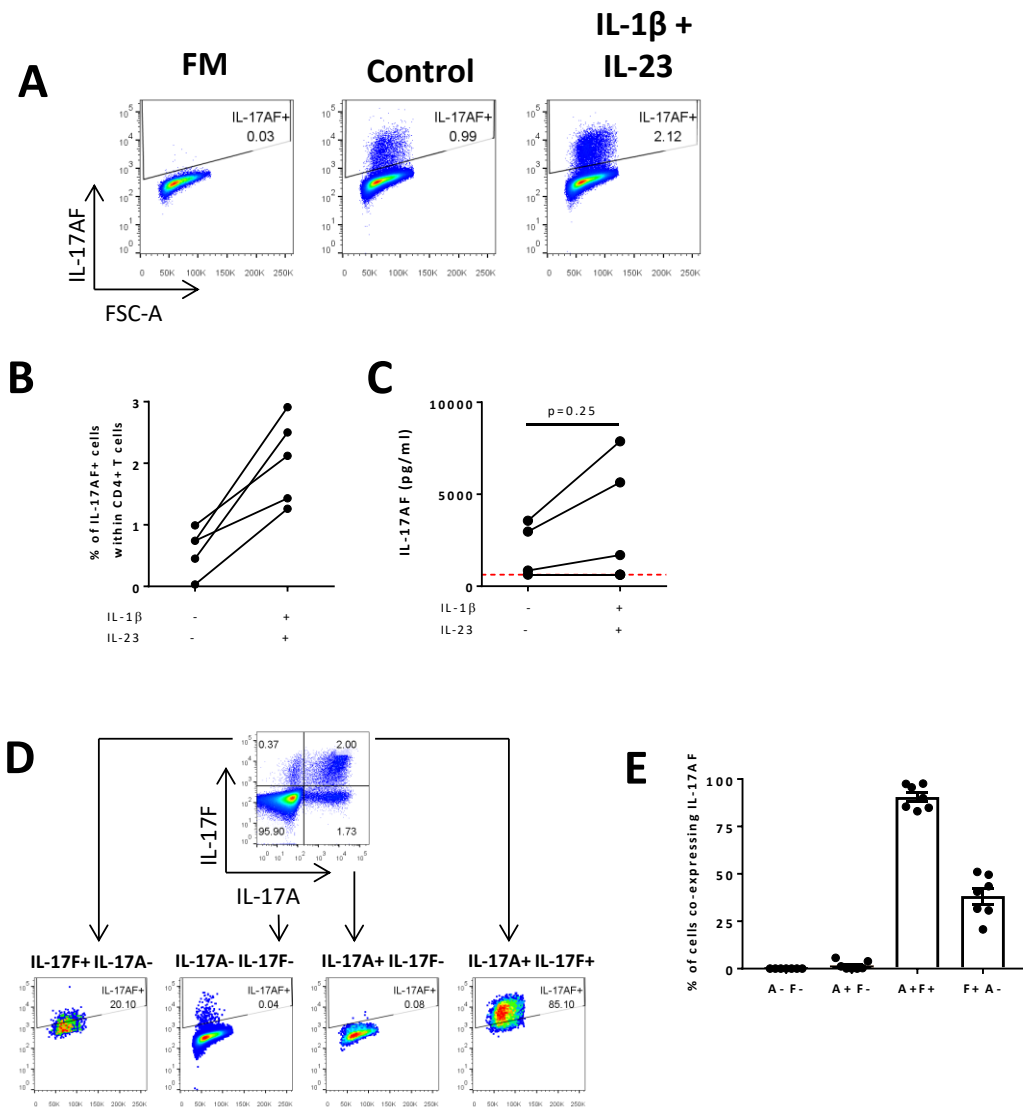
### **3.2.1.5 IL-17AF heterodimer expressing CD4<sup>+</sup> T cell frequencies and IL-17AF secretion are enhanced in IL-1 $\beta$ /IL-23 stimulated cultures**

As discussed in the introduction, in addition to the IL-17A and IL-17F homodimer, an IL-17AF heterodimer exists. I sought to determine whether the IL-17AF heterodimer was present in the CD4<sup>+</sup> T cell -/+ IL-1 $\beta$ / IL-23 culture. Manufacturers describe both IL-17A and IL-17F flow cytometry antibodies used in this project to have cross-reactivity towards the IL-17AF heterodimer. However, the IL-17AF heterodimer flow cytometry antibody used here has no reported cross-reactivity for either the IL-17A or IL-17F homodimer. Preliminary experiments (data not shown) showed that the IL-17AF heterodimer flow cytometry antibody can be simultaneously used with the IL-17A and IL-17F homodimer antibodies with no effect on cytokine detection.

As shown in Figure 3.8, flow cytometry analysis revealed IL-17AF expressing CD4<sup>+</sup> T cells to be present in the control conditions and further enhanced in the presence of IL-1 $\beta$  and IL-23 (2.9-fold) (Figure 3.8A & B). In accordance, using an ELISA specific for the IL-17AF heterodimer only, IL-17AF protein was detected at elevated levels in IL-1 $\beta$  and IL-23 cultures vs. control conditions (Figure 3.8C).

Taking into account the reported cross-reactivity of both IL-17A and IL-17F flow cytometry antibodies for the IL-17AF heterodimer, it was assumed any IL-17AF CD4<sup>+</sup> T cells would be detected within the IL-17A<sup>+</sup> IL-17F<sup>+</sup> double positive CD4<sup>+</sup> T cell population. With this in mind, I wanted to assess how many IL-17A<sup>+</sup> IL-17F<sup>+</sup> CD4<sup>+</sup> T cells co-expressed the IL-17AF heterodimer. As shown in Figure 3.8 D & E, the majority of IL-17A<sup>+</sup> IL-17F<sup>+</sup> CD4<sup>+</sup> T cells co-expressed the IL-17AF heterodimer (approximately 89%). We hypothesise that this population produces both the IL-17A and IL-17F homodimers and the IL-17AF heterodimer. IL-17AF was not detected in IL-17A<sup>-</sup> IL-17F<sup>-</sup> and IL-17A<sup>+</sup> IL-17F<sup>-</sup> CD4<sup>+</sup> T cell populations. Unexpectedly, IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells were shown to co-express levels of IL-17AF (approximately 20%). This is unexpected as IL-17AF<sup>+</sup>

cells should also be detected by the IL-17A flow cytometry antibody. This may be due to the IL-17AF antibody cross-reacting with IL-17F+ CD4+ T cells.



**Figure 3.8 IL-17AF expressing CD4+ T cells are enhanced by IL-1 $\beta$  and IL-23.**

Bulk CD4<sup>+</sup> T cells were cultured with platebound anti-CD3 mAb (1.25 $\mu$ g/ml) and soluble anti-CD28 either in the absence or presence of IL-1 $\beta$  (10ng/ml) and IL-23 (20ng/ml) for 3 days. Supernatants were collected for IL-17AF protein analysis by ELISA and cells were re-stimulated and assessed for intracellular cytokine expression. **(A)** Representative dot plots showing the frequencies of IL-17AF<sup>+</sup> cells within total CD4<sup>+</sup> T cells (n=5) **(B)** cumulative ICCS data (n=5). **(C)** Levels of IL-17AF detected in culture supernatant (prior re-stimulation) via ELISA (n=6). Red dashed lines indicate the lower detection limit. **(D)** Representative gating strategy analysing percentage of IL-17A<sup>+</sup> IL-17F<sup>-</sup>, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells co-expressing IL-17AF and **(E)** cumulative ICCS data (n=7).



### **3.2.1.6 CD28 co-stimulation promotes the frequency of IL-17F expressing CD4+ T cells**

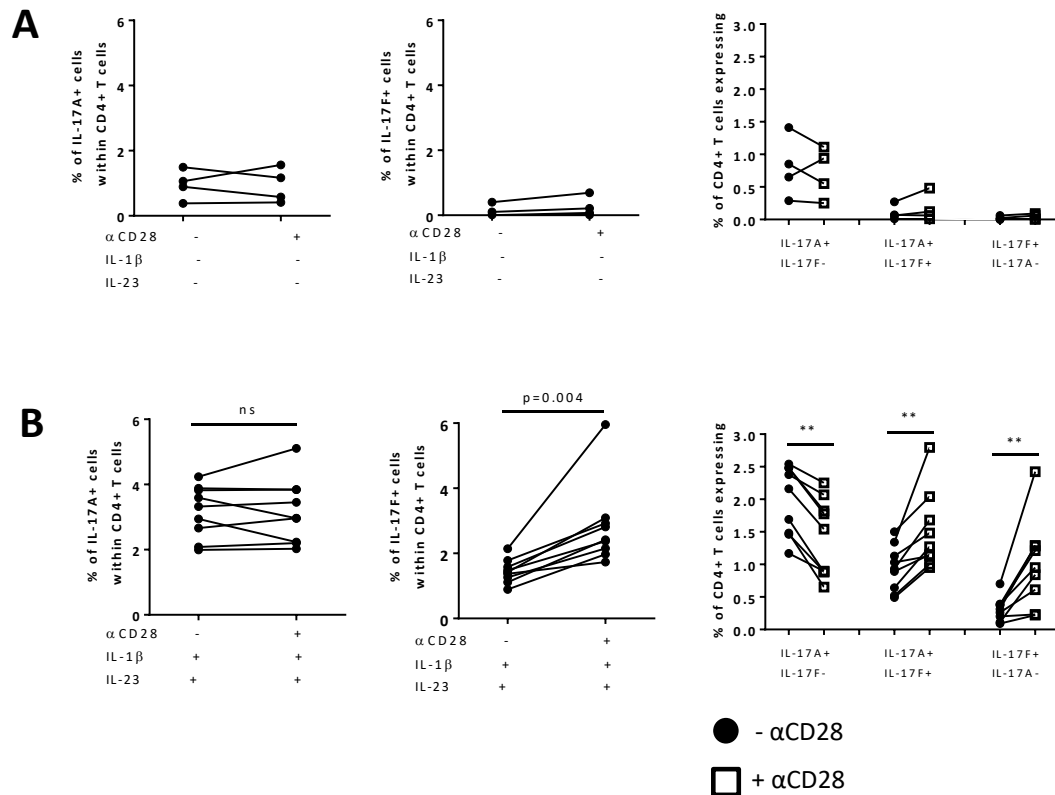
Reports have shown that a strong CD28 co-stimulation signal down regulates IL-17A+ CD4+ T cell frequency<sup>6</sup>. I was interested to explore the effect of CD28 co-stimulation on the frequency of IL-17F expressing CD4+ T cells. To investigate this, bulk CD4+ T cells from healthy donors were cultured with plate-bound anti-CD3 mAb in the absence or presence of anti-CD28 mAb for 3 days. At first, this was performed in the absence of IL-1 $\beta$  and IL-23. As displayed in Figure 3.9A, addition of anti-CD28 did not lead to a significant change in the frequency of total IL-17A+ CD4+ T cell frequencies. Although a small increase in total IL-17F+ CD4+ T cell frequencies was observed, proportions were very small. Addition of IL-1 $\beta$  and IL-23 (Figure 3.9B) led to higher detectable levels of IL-17A+ and IL-17F+ CD4+ T cell frequencies. In the presence of IL-1 $\beta$  and IL-23, addition of anti-CD28 mAb showed no effect on the frequency of total IL-17A+ CD4+ T cell frequencies. In contrast, addition of anti-CD28 mAb led to a significant increase in the percentage of total IL-17F expressing CD4+ T cells. When examining the distinct subsets, it was observed that CD28 co-stimulation signal down-regulates the frequency of IL-17A+ IL-17F- CD4+ T cells, while enhancing the frequency of IL-17A+ IL-17F+ and IL-17F+ IL-17A- CD4+ T cells (3.9B, right panel).

To further investigate the effect of CD28 co-stimulation bulk CD4+ T cells were cultured with platebound anti-CD3 mAb, IL-1 $\beta$  and IL-23 in the presence of increasing doses of soluble anti-CD28 mAb (0-2 $\mu$ g/ml) for 3 days. Following re-stimulation with PMA and ionomycin, cells were assessed for IL-17A and IL-17F cytokine expression. In line with our previous results, titration of anti-CD28 mAb led to a dose-dependent decrease in the percentage of IL-17A+ IL-17F- CD4+ T cells, whilst increasing IL-17A+ IL-17F+ and IL-17F+ IL-17A- CD4+ T cell frequencies (Figure 3.10 A & B). Culture supernatants, taken prior to PMA and ionomycin re-stimulation, were analysed by ELISA (Figure 3.10 C). As expected, titration of anti-CD28 led to a dose-dependent increase in IL-17F homodimer and IL-17AF heterodimer protein secretion. Based on our flow cytometry data, we predicted IL-17A homodimer protein secretion would have remained unchanged with the addition of anti-CD28. However, it unexpectedly led to a dose-dependent

increase in IL-17A homodimer secretion. Although different ELISA antibody affinities make it difficult to draw comparisons between levels of different cytokines, we did detect significantly higher levels of IL-17F vs. IL-17A and IL-17AF.

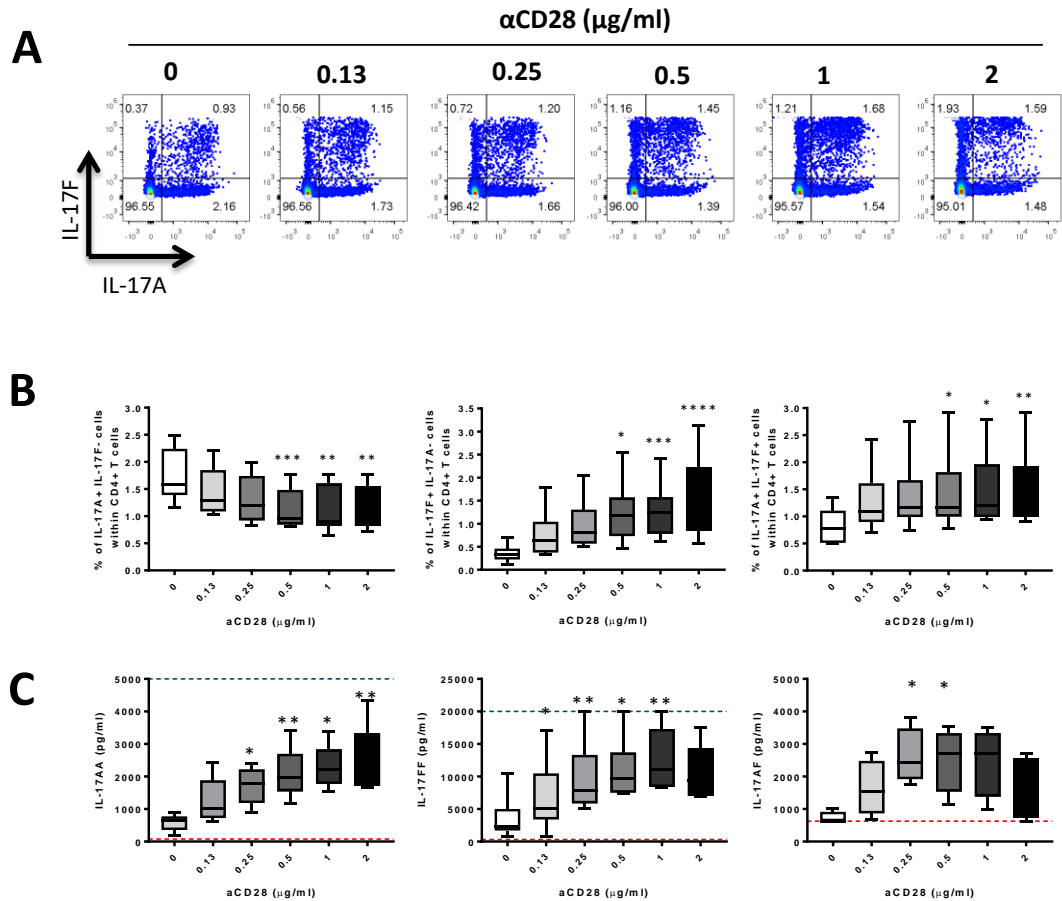
It was speculated that the increase in IL-17A protein secretion in response to the presence of anti-CD28 was due to the kinetics of the assay. To address this, bulk CD4<sup>+</sup> T cells were cultured with platebound anti-CD3 mAb, IL-1 $\beta$  and IL-23 in the presence or absence of anti-CD28 mAb for 1, 2, 3 or 4 days. After the selected time points, cells were re-stimulated and assessed for IL-17A and IL-17F cytokine expression (Figure 3.11). We observed that there were higher percentages of total IL-17A<sup>+</sup> and IL-17F<sup>+</sup> CD4<sup>+</sup> T cells in both control and anti-CD28 conditions at day 4 vs. day 1. At day 1, there was no difference in the frequency of IL-17A<sup>+</sup> CD4<sup>+</sup> T cells between anti-CD28 mAb treated and untreated conditions. However, at day 2, conditions stimulated with anti-CD28 mAb displayed a higher percentage of IL-17A expressing CD4<sup>+</sup> T cells. In agreement with our previous day 3 data, there was no difference in frequencies of IL-17A<sup>+</sup> cells in the absence or presence of anti-CD28 mAb. The frequency of IL-17A<sup>+</sup> CD4<sup>+</sup> T cells was decreased in the presence of anti-CD28 mAb at day 4. Based on this result, we propose that the increase in IL-17A protein secretion identified at day 3 was an accumulation of anti-CD28 mAb driven IL-17A observed by flow cytometry at day 2.

Throughout the time course, frequencies of IL-17F<sup>+</sup> CD4<sup>+</sup> T cells remained higher in the presence vs. absence of anti-CD28 mAb (Figure 3.11).



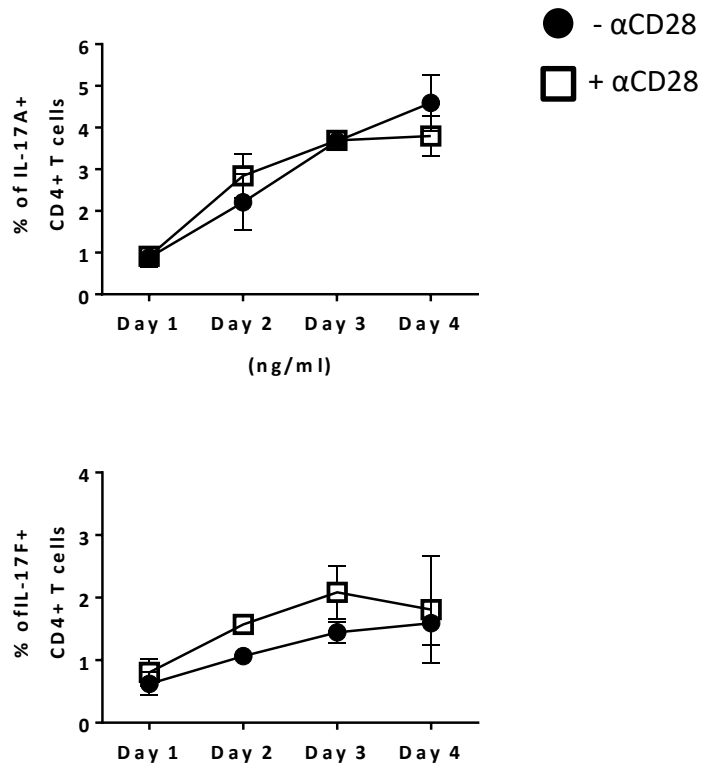
**Figure 3.9 CD28 co-stimulation enhances IL-17F expression by CD4+ T cells.**

(A) Bulk CD4+ T cells were cultured with platebound anti-CD3 mAb either in the absence or presence of soluble anti-CD28 mAb for 3 days (n=4). (B) Bulk CD4+ T cells were cultured with platebound anti-CD3 mAb, soluble anti-CD28 either in the absence or presence of IL-1β and IL-23 for 3 days (n=9). In both experiments, cells were re-stimulated and assessed for IL-17A and IL-17F cytokine expression. Results show the cumulative frequencies of total IL-17A+ and IL-17F+ CD4+ T cells and IL-17A+ IL-17F-, IL-17A+ IL-17F+ and IL-17F- IL-17A+ CD4+ T cell populations. Data were analysed using matched-pairs signed rank test. P values are represented as follows: \*\* P < 0.01.



**Figure 3.10 CD28 co-stimulation increases IL-17F+ CD4+ T cell frequencies in a dose-dependent manner.**

Bulk CD4<sup>+</sup> T cells ( $0.2 \times 10^6$ ) were cultured with platebound anti-CD3 mAb, IL-1 $\beta$  and IL-23 in the presence of increasing doses of soluble anti-CD28 mAb (0-2 $\mu$ g/ml). After 3 days cells were re-stimulated and assessed for intracellular cytokine expression. **(A)** Representative dot plots show the percentages of IL-17A<sup>+</sup> IL-17F<sup>-</sup>, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> cells within total CD4<sup>+</sup> T cells and **(B)** box-whisker plots represent data from n=6 individual donors. Prior to re-stimulation cell culture supernatants were collected and analysed for IL-17A, IL-17F and IL-17AF protein via ELISA, **(C)** displays cumulative ELISA data (n=6). Upper blue and red dashed lines indicate the upper and lower ELISA detection limits, respectively. Statistical analysis was performed using Friedman test with comparison to control (0 $\mu$ g/ml) by Dunn's Multiple Comparisons test. P values are represented as follows: \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001, \*\*\*\* P < 0.0001.



**Figure 3.11 Assessing the kinetic effect of CD28 co-stimulation on protein expression.**

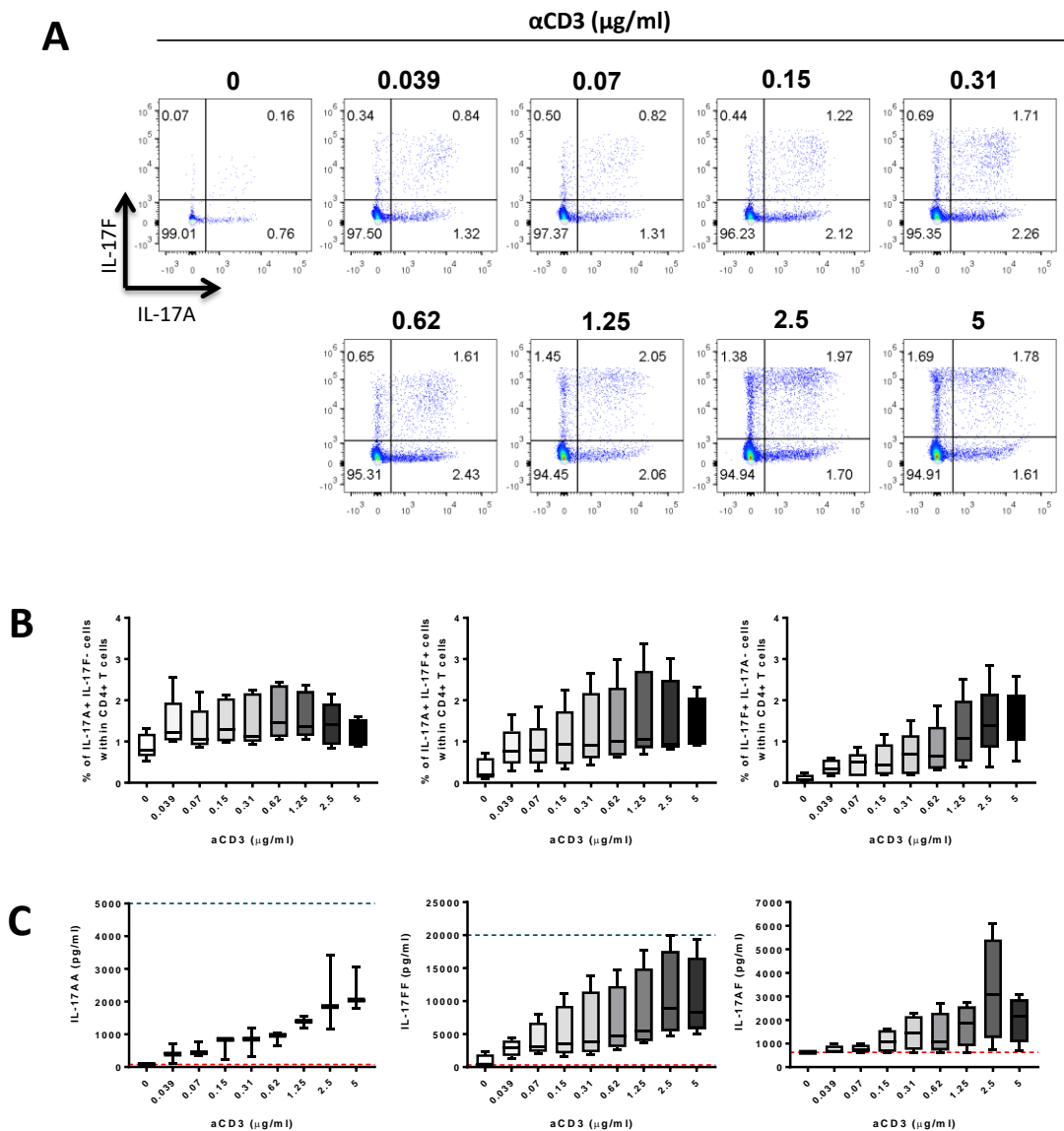
Bulk CD4+ T cells ( $1 \times 10^6$ ) were cultured with platebound anti-CD3 mAb, IL-1 $\beta$  and IL-23 in the absence or presence of anti-CD28 mAb for 1, 2, 3 or 4 days. Cells were re-stimulated and assessed for intracellular cytokine expression. Results show the frequencies of total IL-17A+ and IL-17F+ cells within total CD4+ T cells (n=3 mean  $\pm$  SEM).

### **3.2.1.7 Investigating the effect of CD3 stimulation on IL-17A and IL-17F expression**

Next, the effect of CD3 stimulation on the frequency of IL-17A and IL-17F expressing CD4<sup>+</sup> T cells was assessed. Titration of plate-bound anti-CD3 mAb in 3 day cultures with IL-1 $\beta$ , IL-23 and anti-CD28 mAb increased the frequency of IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells in a dose-dependent manner (Figure 3.12A &B). In the case of IL-17A<sup>+</sup> IL-17F<sup>-</sup> CD4<sup>+</sup> T cells, while there was an initial increase in the frequency of cells upon the addition of anti-CD3 mAb (0.039 $\mu$ g/ml), increasing the anti-CD3 concentration had no further effect on the population frequency.

Following the 3 day culture, supernatants were collected and analysed for IL-17A and IL-17F homodimer and IL-17AF heterodimer protein levels. As depicted in Figure 3.12C, titration of anti-CD3 mAb led to a dose-dependent increase in IL-17A, IL-17F and IL-17AF.

Similar to our CD28 co-stimulation data, further investigation is required to fully understand the effect of CD3 mAb stimulation on IL-17A. However, our data clearly show increased concentrations of anti-CD3 mAb lead to both enhanced frequencies of IL-17F expressing CD4<sup>+</sup> T cells and IL-17F protein secretion. Taken together with our CD28 co-stimulation data we postulate that strong T cell stimulation drives an IL-17F + CD4<sup>+</sup> T cell response.



**Figure 3.12 Increasing doses of anti-CD3 mAb leads to an increase in IL-17F+ CD4+ T cells and IL-17F secretion.**

Bulk CD4+ T cells ( $0.2 \times 10^6$ ) were cultured with soluble anti-CD28 mAb, IL-1 $\beta$  and IL-23 and in the presence of increasing doses of platebound anti-CD3 mAb (0-5 $\mu$ g/ml). After 3 days cells were re-stimulated and assessed for intracellular cytokine expression. **(A)** Representative dot plots show the percentages of IL-17A+ IL-17F-, IL-17A+ IL-17F+ and IL-17F+ IL-17A- cells within total CD4+ T cells and **(B)** box-whisker plots represent data from n=5 individual donors. **(B)** Levels of IL-17A (n=3), IL-17F (n=4) and IL-17AF (n=4) detected in culture supernatant (prior re-stimulation) via ELISA.

### **3.2.1.8 Investigating the role of IL-2 in regulating IL-17A and IL-17F expression**

CD28 signalling is reported to enhance cytokine production from CD4<sup>+</sup> T cells, including IL-2 and IFN $\gamma$ <sup>8</sup>. Moreover, it has been shown that neutralisation of IL-2 and IFN $\gamma$  leads to increased IL-17A production from CD4<sup>+</sup> T cells<sup>6</sup>. Although a decrease in total IL-17A<sup>+</sup> CD4<sup>+</sup> T cells with increased CD28 co-stimulation was not observed, it was of interest to examine the effect of IL-2 and IFN $\gamma$  on IL-17A and IL-17F CD4<sup>+</sup> T cell frequencies. We hypothesised that the CD28-driven enhancement of IL-17F<sup>+</sup> CD4<sup>+</sup> T cell frequency is mediated by IL-2 and/ or IFN $\gamma$ . To begin, protein levels of IL-2 and IFN $\gamma$  in cultures of stimulated CD4<sup>+</sup> T cells with increasing doses of anti-CD28 and anti-CD3 mAb were assessed. As depicted in Figure 3.13A, titration of anti-CD28 and anti-CD3 led to a dose-dependent increase in IL-2 supernatant protein levels. Similar results were observed for IFN $\gamma$  supernatant protein levels, Figure 3.13 B.

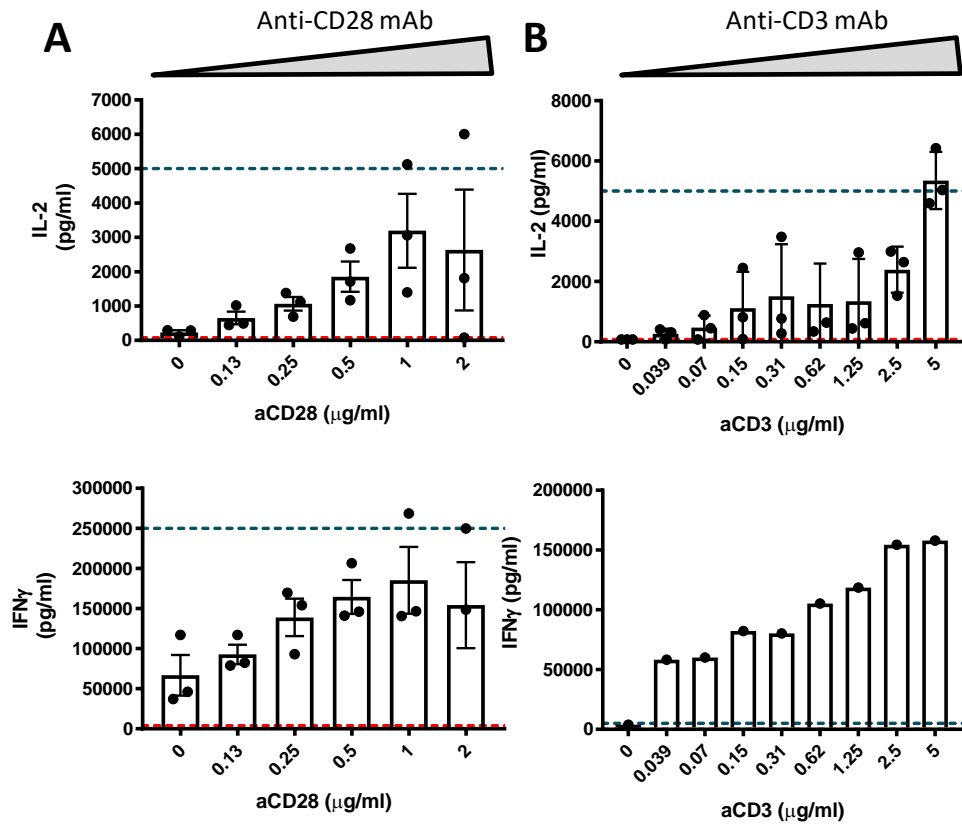
In the next experiments, I focused on the role of IL-2 and IFN $\gamma$  in the CD28-mediated IL-17F response. IL-2 and IFN $\gamma$  were blocked in CD4<sup>+</sup> T cultures stimulated with anti-CD28 mAb, anti-CD3 mAb, IL-1 $\beta$  and IL-23. Prior to this, optimal blocking antibody concentrations and the effect of isotype controls were assessed (Figure 3.14). Results showed 1 $\mu$ g/ml of both anti-IL-2 mAb and IFN $\gamma$  mAb resulted in efficient blockade of IL-2 and IFN $\gamma$ , respectively. Furthermore, addition of an isotype control for anti-IL-2 mAb (goat IgG1) and anti-IFN $\gamma$  mAb (mouse IgG1) did not consistently affect the percentage of CD4<sup>+</sup> T cells expressing IL-17A or IL-17F.

No significant changes in total IL-17A<sup>+</sup> CD4<sup>+</sup> T cell frequencies were observed in the absence or presence of anti-CD28 mAb or in cultures supplemented with anti-CD28 mAb and IL-2 blocking antibody (Figure 3.15 A & B). However, while addition of anti-CD28 mAb promoted total IL-17F<sup>+</sup> CD4<sup>+</sup> T cell frequency (approximately 1% vs. 1.7% in the absence vs. presence of anti-CD28 mAb), this effect was abrogated by blockade of IL-2 (approximately 1%). Similarly, blockade of IL-2 suppressed the anti-CD28 mAb mediated inhibition of IL-17A<sup>+</sup> IL-17F-



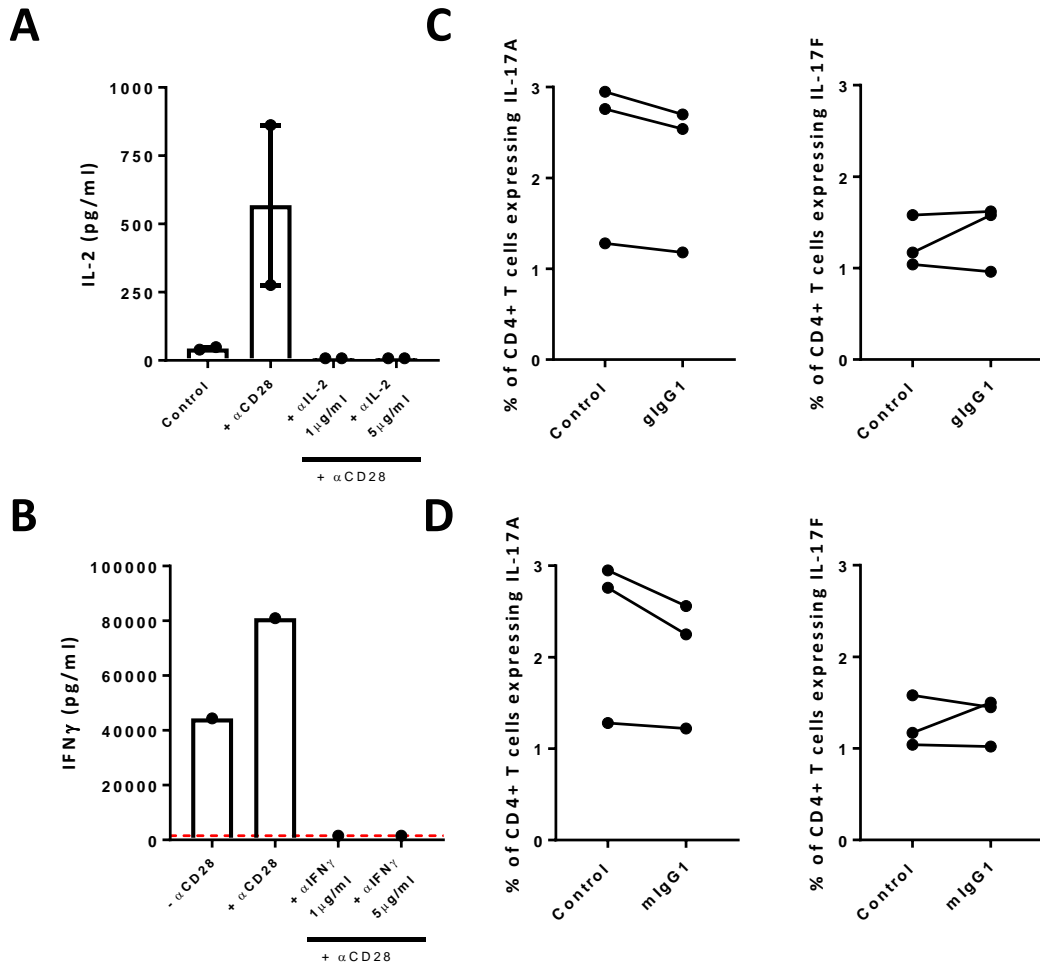
CD4<sup>+</sup> T cells and the anti-CD28 mAb mediated promotion of IL-17F<sup>+</sup> IL-17A<sup>-</sup> and in part IL-17A<sup>+</sup> IL-17F<sup>+</sup> expressing CD4<sup>+</sup> T cells. These data suggest that the anti-CD28 mAb driven increase in IL-17F is mediated by IL-2.

As depicted in Figure 3.16 A & B, addition of anti-IFN $\gamma$  mAb blocking antibody in conditions supplemented with IL-1 $\beta$ , IL-23, anti-CD28 mAb and anti-CD3 mAb did not significantly alter the frequency of total IL-17A expressing CD4<sup>+</sup> T cells. Furthermore, in comparison to anti-CD28 mAb alone, a combination of anti-CD28 mAb and anti-IFN $\gamma$  mAb only resulted in a slight reduction in IL-17F expressing CD4<sup>+</sup> T cells (1.73 vs 1.4%, respectively). Further flow cytometric analysis revealed that addition of anti-IFN $\gamma$  mAb suppressed the anti-CD28 mediated inhibition of IL-17A<sup>+</sup> IL-17F<sup>-</sup> CD4<sup>+</sup> T cells (Figure 3.16 C). Additionally, to some extent IFN $\gamma$  blockade reduced the anti-CD28 mAb mediated enhancement of IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells. These data suggest IFN $\gamma$  may play a role in skewing IL-17A/ IL-17F sub-populations. As shown in Figure 3.17, a combined blockade of IL-2 and IFN $\gamma$  had no further effect on IL-17A and IL-17F CD4<sup>+</sup> T cell expression compared to IL-2 blockade alone.



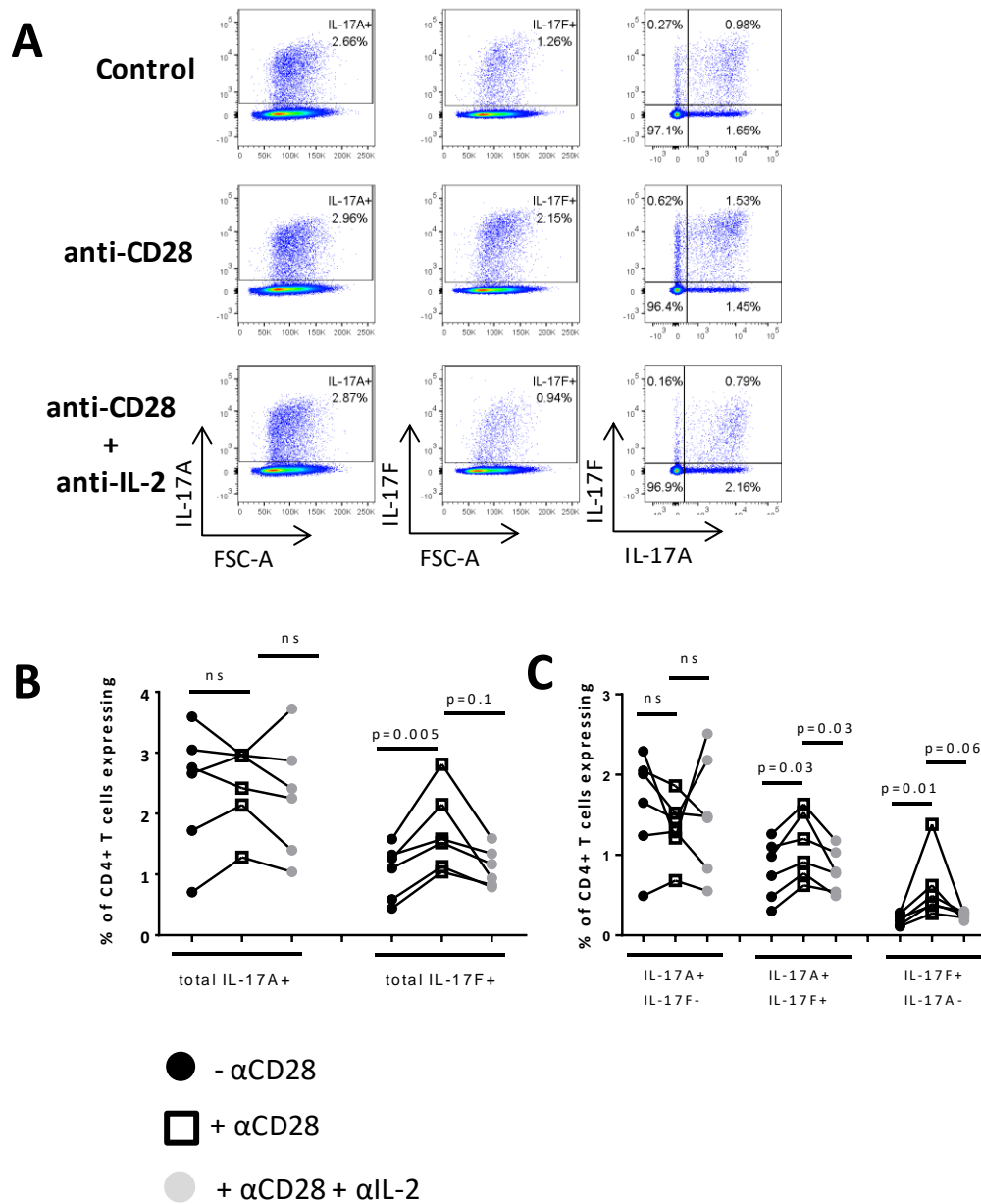
**Figure 3.13 Titration of anti-CD28 and anti-CD3 mAb leads to a dose-dependent increase in IL-2 and IFN $\gamma$  secretion.**

Bulk CD4<sup>+</sup> T cells ( $0.2 \times 10^6$ ) were cultured with (A) platebound anti-CD3 mAb, IL-1 $\beta$  and IL-23 in the presence of increasing doses of soluble anti-CD28 mAb (0-2 $\mu\text{g/ml}$ ). After 3 days culture supernatant was removed and measured for IL-2 (n=3) and IFN $\gamma$  (n=3). (B)  $0.2 \times 10^6$  bulk CD4<sup>+</sup> T cells were cultured with soluble anti-CD28 mAb, IL-1 $\beta$  and IL-23 in the presence of increasing doses of platebound anti-CD3 mAb (0-5 $\mu\text{g/ml}$ ). After 3 days culture supernatant was removed and measured for IL-2 (n=3) and IFN $\gamma$  (n=1). Upper blue and red dashed lines indicate the upper and lower ELISA detection limits, respectively.



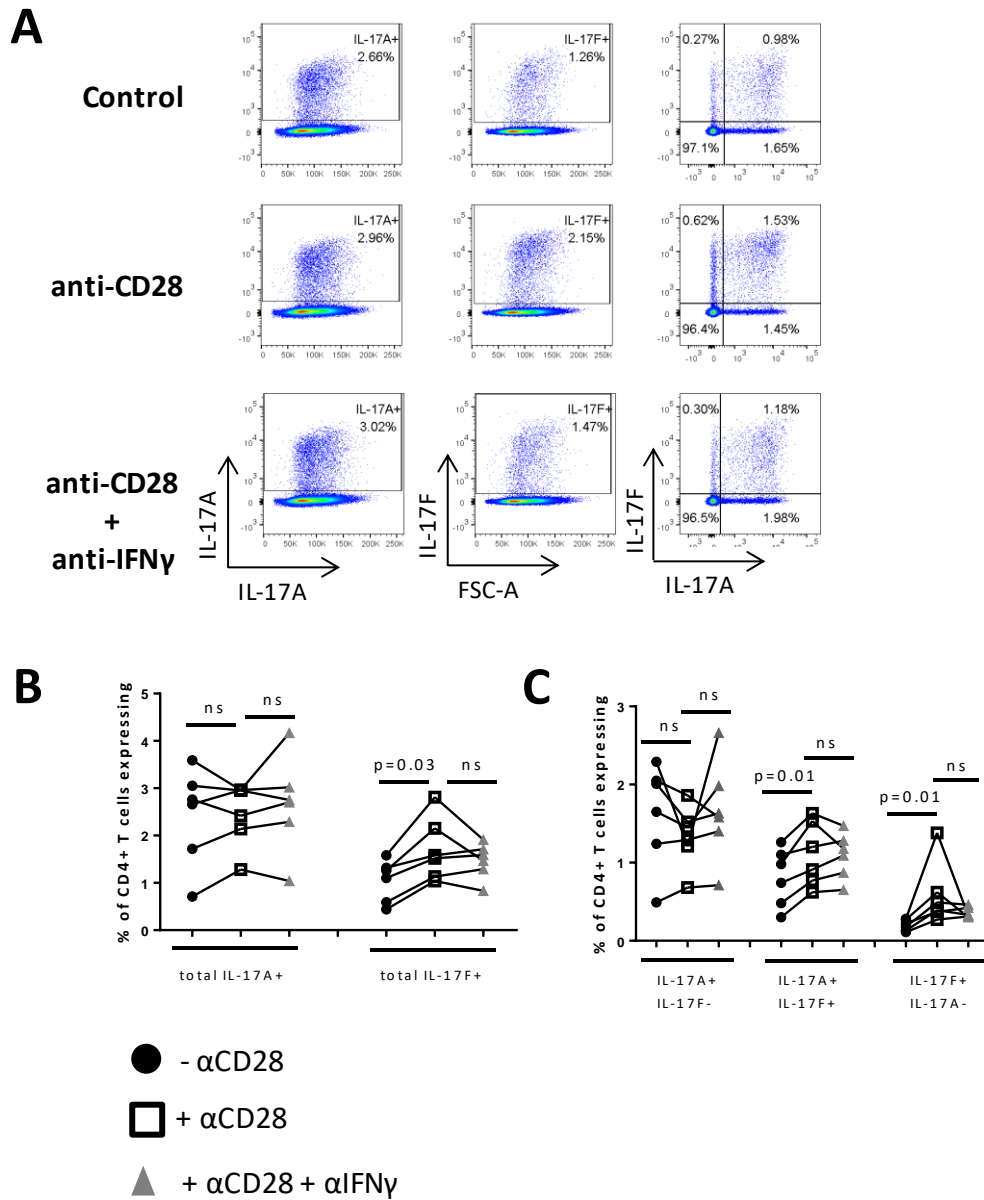
**Figure 3.14 Isotype controls do not affect IL-17A+ or IL-17F+ CD4+ T cell frequencies.**

Bulk CD4+ T cells were cultured with anti-CD3 mAb, IL-23 and IL-1β in the absence or presence of anti-CD28 mAb. In the presence of anti-CD28 mAb, (A) 1 or 5 μg/ml of anti-IL-2 blocking antibody or (B) 1 or 5 μg/ml of anti-IFNγ blocking antibody was added. Following a 3 day incubation, supernatants were collected and IL-2 (n=2) or IFNγ (n=1) protein levels were analysed by ELISA. Bulk CD4+ T cells were cultured with anti-CD3 mAb, IL-23 and IL-1β in the absence (control) or presence of (C) IL-2 blocking antibody isotype (goat IgG1, 1 μg/ml) or (D) IFNγ blocking antibody isotype, (mouse IgG1, 1 μg/ml). After 3 days cells were re-stimulated and assessed for intracellular cytokine expression. Cumulative data shows the frequencies of total IL-17A+ and IL-17F+ CD4+ T cells (n=3).



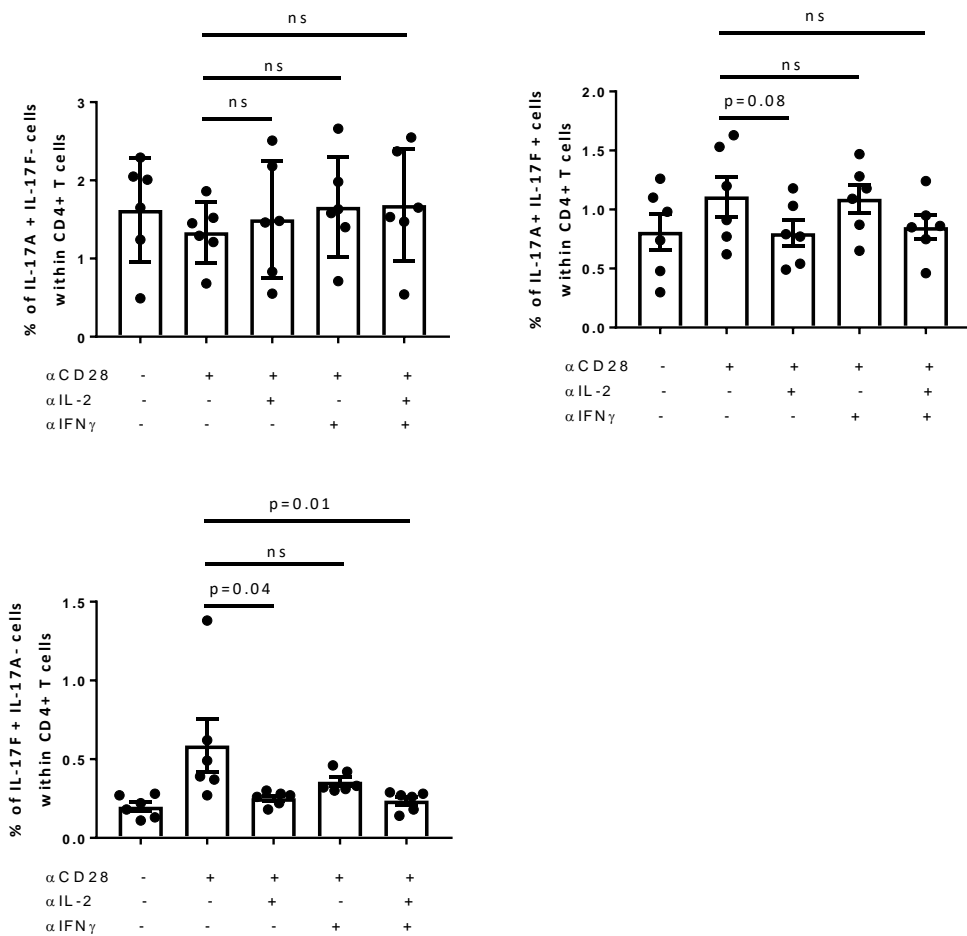
**Figure 3.15 IL-2 blockade reduces IL-17F+ CD4+ T cell expression.**

Bulk CD4+ T cells were cultured with anti-CD3 mAb, IL-23 and IL-1 $\beta$  in the absence or presence of anti-CD28 mAb and anti-IL-2 mAb (1 $\mu$ g/ml). After 3 days cells were re-stimulated and assessed for intracellular cytokine expression. (A) Representative dot plots and cumulative data showing the frequencies of (B) total IL-17A+ and IL-17F+ CD4+ T cells (n=6) and (C) IL-17A+ IL-17F-, IL-17A+ IL-17F+ and IL-17F+ IL-17A- CD4+ T cells (n=6). Statistical analysis was performed using Friedman test with comparison to all samples by Dunn's Multiple Comparisons test.



**Figure 3.16 Exploring the effect of IFN $\gamma$  blockade on IL-17A and IL-17F expressing CD4 $^+$  T cells.**

Bulk CD4 $^+$  T cells were cultured with anti-CD3 mAb, IL-23 and IL-1 $\beta$  in the absence or presence of anti-CD28 mAb and anti-IFN $\gamma$  mAb (1 $\mu$ g/ml). After 3 days cells were re-stimulated and assessed for intracellular cytokine expression. (A) Representative dot plots and cumulative data showing the frequencies of (B) total IL-17A $^+$  and IL-17F $^+$  CD4 $^+$  T cells (n=6) and (C) IL-17A $^+$  IL-17F $^-$ , IL-17A $^+$  IL-17F $^+$  and IL-17F $^+$  IL-17A $^-$  CD4 $^+$  T cells (n=6). Statistical analysis was performed using Friedman test with comparison to all samples by Dunn's Multiple Comparisons test.



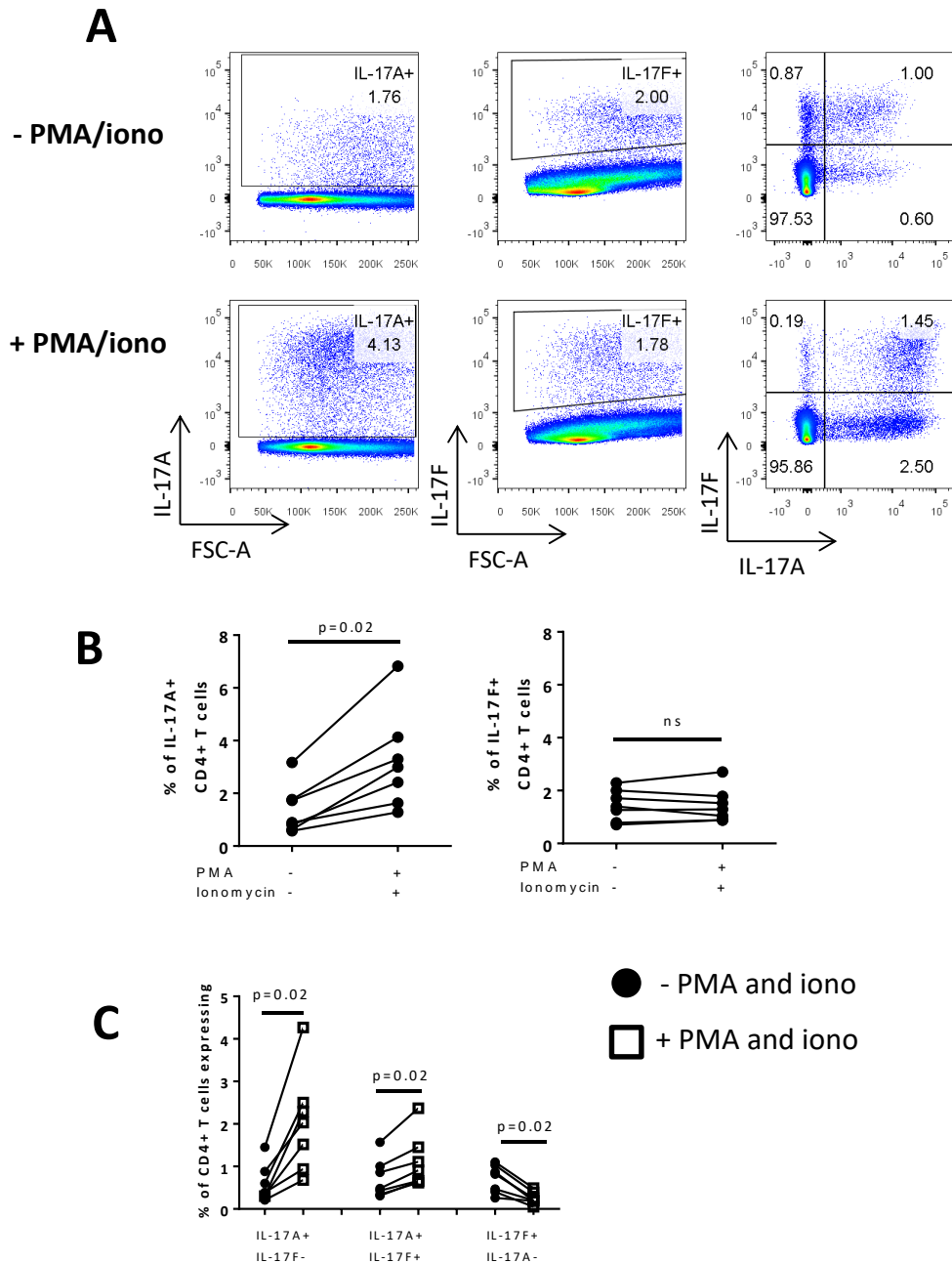
**Figure 3.17 Dual blockade of IL-2 and IFN $\gamma$  has no further effect on IL-17A and IL-17F CD4+ T expressing cells than IL-2 blockade alone.**

Bulk CD4+ T cells were cultured with anti-CD3 mAb, IL-23 and IL-1 $\beta$  in the absence or presence of anti-CD28 mAb, anti-IL-2 mAb (1 $\mu$ g/ml) and anti-IFN $\gamma$  mAb (1 $\mu$ g/ml). After 3 days cells were re-stimulated and assessed for intracellular cytokine expression. Cumulative data show the frequencies of IL-17A+ IL-17F-, IL-17A+ IL-17F+ and IL-17F+ IL-17A- CD4+ T cells (n=6). Statistical analysis was performed using Friedman test with comparison to control (+ anti-CD28  $\mu$ g/ml) by Dunn's Multiple Comparisons test.

### **3.2.1.9 PMA and ionomycin re-stimulation does not affect the frequency of total IL-17F+ CD4+ T cells**

Unpublished data from UCB suggests that PMA and ionomycin re-stimulation may affect IL-17A+ and IL-17F+ CD4+ T cell frequencies differently. I sought to investigate the effect of PMA and ionomycin on IL-17A and IL-17F expressing cells in our culture system.

CD4+ T cells were cultured for 3 days with anti-CD28, anti-CD3, IL-1 $\beta$  and IL-23. Cells were then re-stimulated for 3 hours with golgi stop in the absence or presence of PMA and ionomycin. Cells were stained and intracellular cytokine expression was assessed. Firstly, as depicted in Figure 3.18, the data revealed that in this culture system, IL-17A and IL-17F can be detected at significant frequencies without the addition of PMA and ionomycin. Addition of PMA and ionomycin enhanced the percentage of IL-17A+ CD4+ T cells (2.2-fold). However, the frequency of total IL-17F+ CD4+ T cells remained unchanged. Furthermore, whilst PMA and ionomycin increased IL-17A+ IL-17F- and IL-17A+ IL-17F+ CD4+ T cell frequencies, it led to a small reduction in IL-17F+ IL-17A- CD4+ expressing CD4+ T cells. These data further indicate that there are aspects of differential regulation between IL-17A+ and IL-17F+ CD4+ T cells.



**Figure 3.18 Re-stimulation with PMA and ionomycin has no effect on the frequency of IL-17F expressing CD4+ T cells.**

Isolated CD4+ T cells were cultured with anti-CD3 mAb,  $\alpha$ CD28 mAb, IL-23 and IL-1 $\beta$ . After 3 days, cells were re-stimulated with golgi stop either in the presence or absence of PMA and ionomycin. Cells were stained and intracellular cytokine expression was assessed. **(A)** Representative dot plots and **(B)** cumulative data show frequencies of IL-17A (n=6) and IL-17F (n=4) within the total CD4+ T cell population. **(C)** Results show the cumulative frequencies of IL-17A+ IL-17F-, IL-17A+ IL-17F+ and IL-17F- IL-17A+ CD4+ T cell populations (n=6).



### **3.2.2 Phenotypic characterisation of IL-17F+ expressing CD4+ T cells**

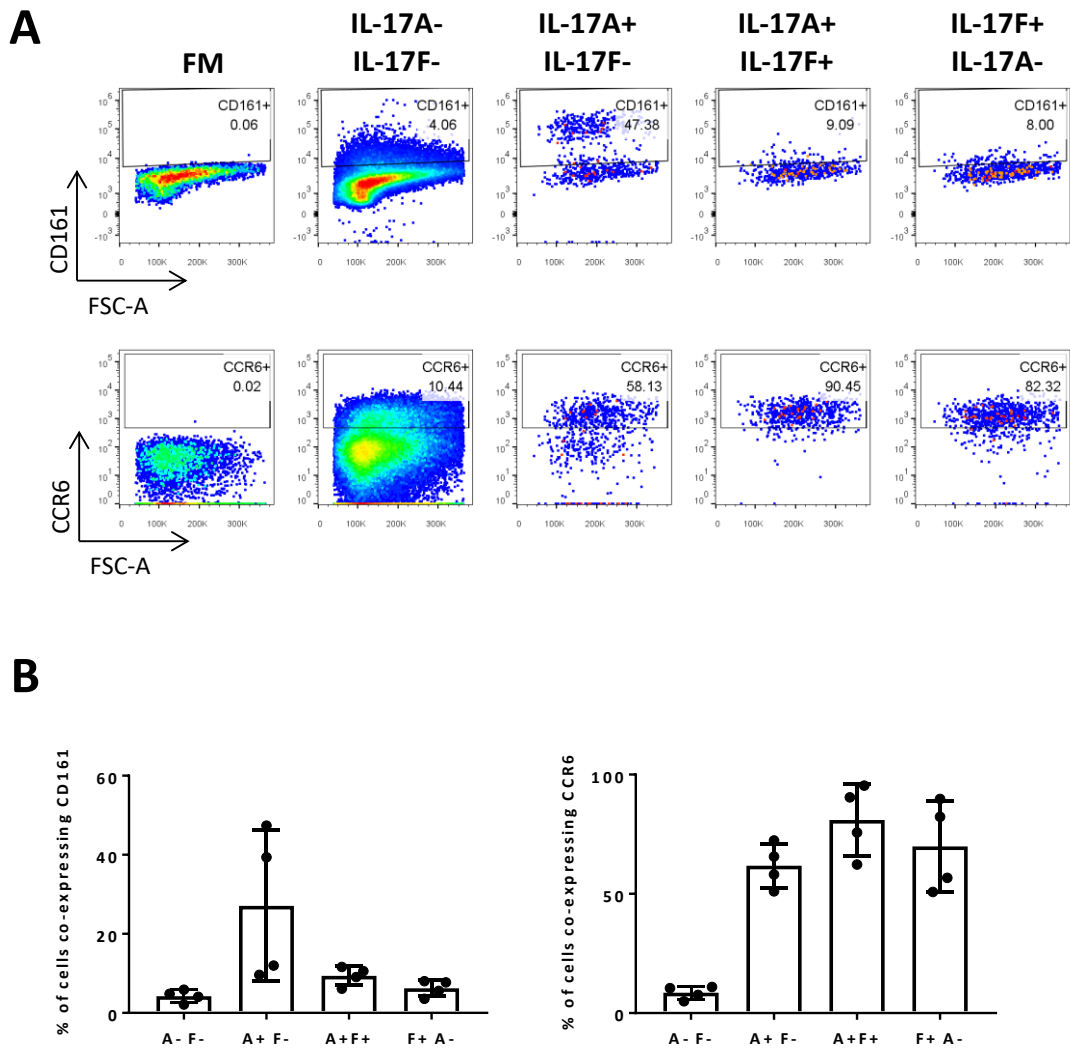
A lack in current literature of the phenotypic characterization of IL-17F+ CD4+ T cells and their comparison with IL-17A+ CD4+ T cells provided us with the rationale to perform these experiments. The second part of this chapter investigates the marker expression and cytokine profile of IL-17A+ IL-17F-, IL-17A+ IL-17F+, IL-17F- IL-17A- CD4+ T cells.

#### **3.2.2.1 CCR6 and CD161 marker expression on IL-17A+ and IL-17F+ CD4+ T cells**

IL-17A+ CD4+ T cells have previously been reported to be CD45RO+ memory cells<sup>4</sup>. By performing an extracellular CD45RO stain on CD4+ T cells cultured with IL-1 $\beta$  and IL-23, it was observed that all IL-17 populations, IL-17A+ IL-17F-, IL-17A+ IL-17F+ and IL-17F+ IL-17A- CD4+ T cells were CD45RO+ memory cells (n=2, data not shown). To further characterise IL-17F+ CD4+ T cells and draw comparisons with IL-17A+ CD4+ T cells, their surface marker expression was analysed, including the Th17 associated markers CCR6 and CD161.

Experiments showed that a three day *in vitro* culture and PMA and ionomycin re-stimulation down-regulates CD161 and CCR6 CD4+ T cell surface marker expression (data not shown, n=2). As a three day culture with IL-1 $\beta$  and IL-23 is necessary to induce IL-17F+ CD4+ T cell populations, CD161 and CCR6 marker expression was analysed following a 3 day culture without a PMA and ionomycin re-stimulation. As illustrated in Figure 3.19, in two out of four donors, IL-17A+ IL-17F- CD4+ T cells displayed a higher expression level of CD161 in comparison to levels observed on IL-17A+ IL-17F+ and IL-17F+ IL-17A- CD4+ T cells. However, in the remaining two donors, expression levels of CD161 were only slightly higher than observed with IL-17A- IL-17F- CD4+ T cells. Similarly, the expression levels of CD161 was not enriched on IL-17A+ IL-17F+ and IL-17F+ IL-17A- CD4+ T cells when compared with IL-17A- IL-17F- CD4+ T cells. Expression levels of CCR6 were enriched on all IL-17 producing CD4+ T cells

and few variations were observed between the IL-17A+ IL-17F-, IL-17A+ IL-17F+ and IL-17F+ IL-17A- CD4+ T cell populations.



**Figure 3.19 Expression of CCR6 and CD161 on IL-17A and IL-17F CD4+ T cells.**

Bulk CD4+ T cells were incubated with golgistop either in the presence of PMA and ionomycin at day 0 or day 3 following culture with anti-CD3 mAb,  $\alpha$ CD28 mAb, IL-23 and IL-1 $\beta$ . Cells were assessed for intracellular cytokine expression. **(A)** Representative dot plots showing the percentage of CD161+ and CCR6+ cells within total CD4+ T cells. **(B)** Gating within IL-17A+ IL-17F-, IL-17A+ IL-17F+ and IL-17F+ IL-17A- CD4+ T cells the co-expression of CD161 (n=4) and CCR6 (n=4) was analysed.

### **3.2.2.2 IL-17A and IL-17F expressing CD4<sup>+</sup> T cells display different cytokine profiles**

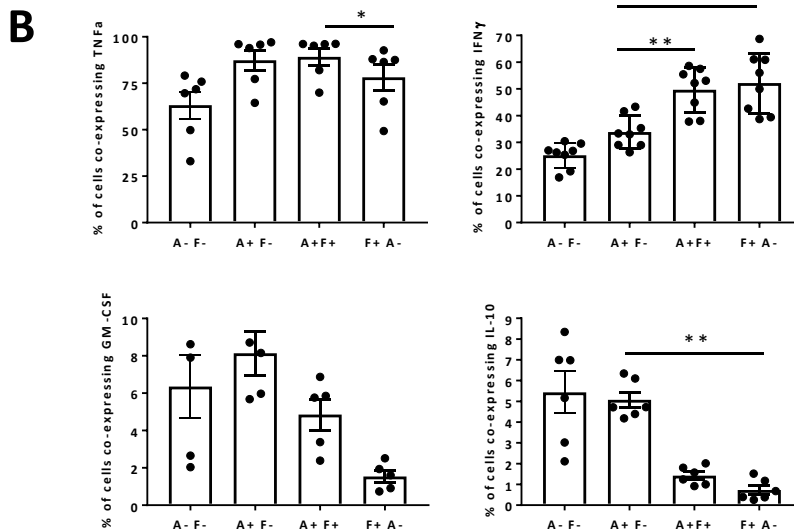
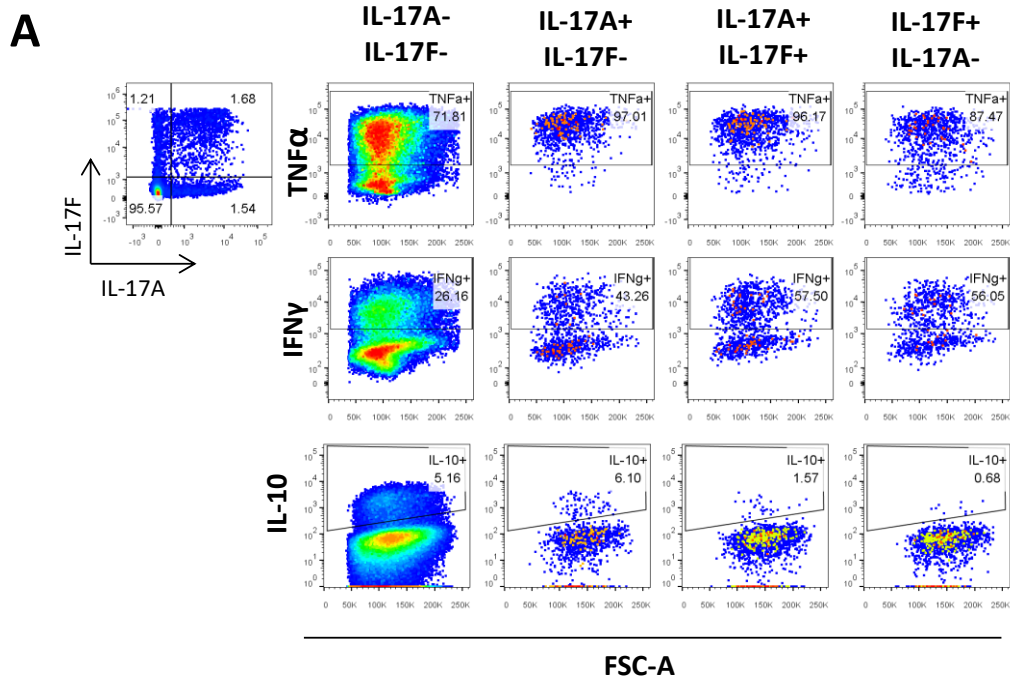
The next aim was to compare the cytokine profiles of IL-17A and IL-17F CD4<sup>+</sup> T cell populations. An initial experiment included performing mass cytometry (CyTOF) to provide a broad overview of the co-expression of cytokines in IL-17A and IL-17F CD4<sup>+</sup> T cell populations. These data are displayed and discussed in Appendix 7.1.

The co-expression of IFN $\gamma$ , TNF $\alpha$ , GM-CSF and IL-10 was assessed by flow cytometry in IL-17A and IL-17F populations induced in bulk CD4<sup>+</sup> T cell cultures stimulated for 3 days with IL-1 $\beta$  and IL-23. Co-expression analysis was only performed when there were over three hundred events in the IL-17 population. IL-17A<sup>+</sup> IL-17F<sup>-</sup> and IL-17A<sup>+</sup> IL-17F<sup>+</sup> CD4<sup>+</sup> T cells displayed similar high co-expression levels of TNF $\alpha$ , while IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells exhibited slightly lower co-expression levels of TNF $\alpha$  (Figure 3.20). Moreover, co-expression levels of IFN $\gamma$  were significantly higher in IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> vs. IL-17A<sup>+</sup> IL-17F<sup>-</sup> CD4<sup>+</sup> T cells. However, IL-17A<sup>+</sup> IL-17F<sup>-</sup> CD4<sup>+</sup> T cells displayed the highest percentage of cells co-expressing GM-CSF vs. IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells. Interestingly, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells exhibited lower co-expression levels of IL-10 when compared with IL-17A<sup>+</sup> IL-17F<sup>+</sup> CD4<sup>+</sup> T cells.

Our next aim was to investigate the cytokine profile of IL-17A and IL-17F CD4<sup>+</sup> T cell populations in a different culture system. Therefore, the co-expression of pro-inflammatory cytokines IFN $\gamma$ , TNF $\alpha$ , GM-CSF and IL-10 were assessed in IL-17A<sup>+</sup> IL-17F<sup>-</sup> and IL-17A<sup>+</sup> IL-17F<sup>+</sup> CD4<sup>+</sup> T cells from LPS-activated monocyte/ CD4<sup>+</sup> T cell co-cultures. It is important to note, in this culture system, only two IL-17 populations are present – IL-17A<sup>+</sup> IL-17F<sup>-</sup> and IL-17A<sup>+</sup> IL-17F<sup>+</sup> CD4<sup>+</sup> T cells. Flow cytometry showed that similar percentages of IL-17A<sup>+</sup>IL-17F<sup>-</sup> and IL-17A<sup>+</sup>IL-17F<sup>+</sup> cells co-expressed TNF $\alpha$  (78 $\pm$ 4% vs. 75 $\pm$ 5%, respectively), IFN $\gamma$  (25 $\pm$ 4% vs. 30 $\pm$ 2%) and GM-CSF (39 $\pm$ 6% vs. 40 $\pm$ 5%). However, similar to the IL-1 $\beta$  and IL-23 CD4<sup>+</sup> T cell cultures, IL-10 co-

expression was significantly reduced in IL-17A+IL-17F+ vs. IL-17A+IL-17F- CD4+ T-cells ( $1\pm 0.2\%$  vs.  $3\pm 0.6\%$ ) (Figure 3.21).

Our lab has previously demonstrated that TNF inhibitor (TNFi) stimulate a significant proportion of IL-17A+ CD4+ T cells to co-express IL-10<sup>9</sup>. Therefore, it was of interest to investigate the effect of anti-TNF drugs on the frequency of IL-17A+ IL-17F+ CD4+ T cells and if this population was resistant to a TNFi-mediated increase in IL-10. Using the same published culture conditions, CD4+ T cells were cultured with CD14+ monocytes, anti-CD3 mAb in the absence or presence of anti-TNF drug, adalimumab (ADA). Flow cytometric analysis indicated that in comparison to medium controls, co-cultures treated with ADA displayed increased frequencies of IL-10+ cells in both IL-17A+ IL-17F- (4 vs. 18 %, respectively) and IL-17A+ IL-17F+ CD4+ T cells (2 vs. 16 %, respectively) (Figure 3.22). It is important to highlight in 2 independent experiments, treatment with ADA did not induce a sufficient amount of IL-17A+IL-17F+ CD4+ T cells for co-expression analysis (< 300 cells). No difference in IFN $\gamma$  co-expression was observed between control vs. ADA conditions in IL-17A+ IL-17F and IL-17A+ IL-17F+ CD4+ T cells.



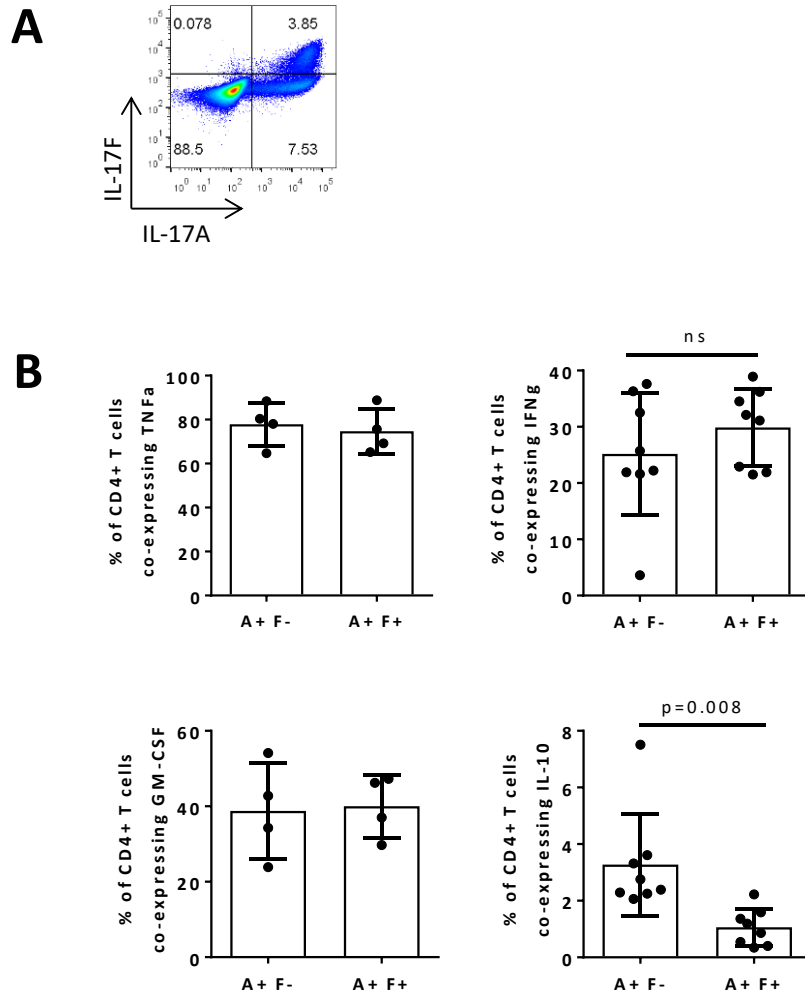
**Figure 3.20 IL-17A and IL-17F expressing CD4<sup>+</sup> T cells display different cytokine profiles.**

Bulk CD4<sup>+</sup> T cells were cultured with anti-CD3 mAb,  $\alpha$ CD28 mAb, IL-23 and IL-1 $\beta$ . After 3 days, cells were re-stimulated and assessed for intracellular cytokine expression. **(A)**

Representative dot plot of IL-17A and IL-17F CD4<sup>+</sup> T cell populations. Following gating within IL-17A- IL-17F-, IL-17A+ IL-17F-, IL-17A+ IL-17F+ and IL-17F+ IL-17A- CD4<sup>+</sup> T cells, the co-expression of other cytokines was analysed. **(B)**

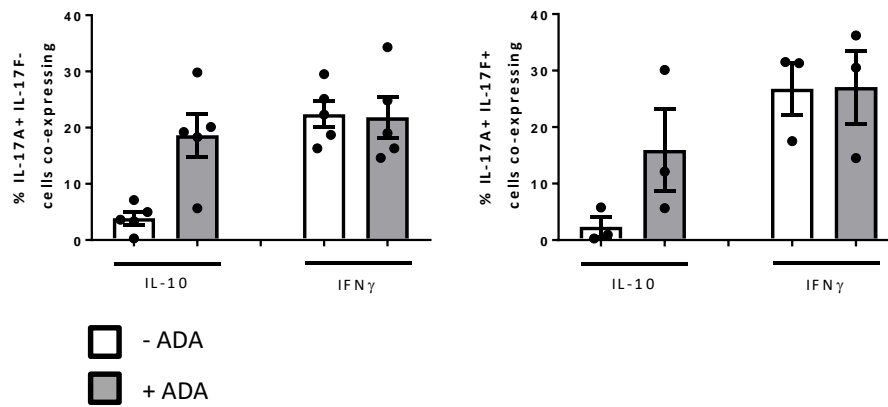
Results show cumulative data for the co-expression of TNF $\alpha$  (n=6), IFN $\gamma$  (n=8), GM-CSF (n=5) and IL-10 (n=6). Data were analysed using Friedman test with Dunn's Multiple Comparisons test. P values are represented as follows:

\* P < 0.05, \*\* P < 0.01.



**Figure 3.21 IL-17A+ IL-17F+ CD4+ T cells display a lower co-expression of IL-10 in comparison to IL-17A+ IL-17F- CD4+ T cells.**

Bulk CD4+ T cells were co-cultured 1:1 with autologous monocytes and stimulated with anti-CD3 mAb (100ng/ml) and LPS (100ng/ml). After 3 days cells were re-stimulated and assessed for intracellular cytokine expression. Gating within IL-17A+ IL-17F- and IL-17A+ IL-17F+ CD4+ T cells the co-expression of other cytokines was analysed. **(A)** Representative dot plots and **(B)** cumulative data showing the percentage of IL-17A+ IL-17F- and IL-17A+ IL-17F+ CD4+ T cells which co-express TNF $\alpha$  (n=4), IFN $\gamma$  (n=8), GM-CSF (n=4) and IL-10 (n=8). Data were analysed using the Wilcoxon matched-pairs signed rank test.



**Figure 3.22 Anti-TNF treatment increases co-expression of IL-10 in IL-17A+ IL-17F- and IL-17A+ IL-17F+ CD4+ T cells.**

Bulk CD4+ T cells were co-cultured 1:1 with autologous monocytes and stimulated with anti-CD3 mAb (100ng/ml) and anti-TNF (ADA) (1 $\mu$ g/ml). After 3 days cells were re-stimulated and assessed for intracellular cytokine expression. **(A)** Cumulative data showing the percentage of total IL-17A and IL-17F cells within total CD4+ T cells. Gating within IL-17A+ IL-17F-, IL-17A+ IL-17F+ and IL-17F+ IL-17A- CD4+ T cells the co-expression of IL-10 and IFN $\gamma$  was analysed. **(B)** Representative dot plots and **(C)** cumulative data for IL-10 and IFN $\gamma$  in IL-17A+ IL-17F- (n=5) and IL-17A+ IL-17F+ CD4+ T cells (n=3), respectively.



### 3.3 Discussion

Data presented in this chapter confirms that IL-1 $\beta$  and IL-23 are important for driving IL-17F expression. Expanding upon this, the data demonstrate for the first time that in the presence of IL-1 $\beta$  and IL-23, titration of anti-CD28 mAb in CD4<sup>+</sup> T cell cultures leads to an increase in IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells, while frequencies of IL-17A<sup>+</sup> IL-17F<sup>-</sup> CD4<sup>+</sup> T cells are reduced. This anti-CD28 mAb effect was shown to be mediated by IL-2. Novel data demonstrate that IL-17A and IL-17F CD4<sup>+</sup> T cells display different cytokine profiles. Most interestingly, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells display a significantly lower IL-10 co-expression level and significantly higher IFN $\gamma$  co-expression levels when compared to IL-17A<sup>+</sup> IL-17F<sup>-</sup> CD4<sup>+</sup> T cells. Overall, the data presented in this chapter suggest IL-17A and IL-17F may be differentially regulated and IL-17A<sup>+</sup> and IL-17F<sup>+</sup> CD4<sup>+</sup> T cells may have distinct functional differences.

As discussed in the introduction, our lab has shown that LPS-activated monocytes can enhance the frequency of IL-17A<sup>+</sup> CD4<sup>+</sup> T cells<sup>192</sup>. Additionally, studies have shown IL-1 $\beta$  can drive human IL-17A<sup>+</sup> CD4<sup>+</sup> T cell differentiation, whilst IL-23 is important for lineage maintenance. IL-17F is often described to be co-expressed with IL-17A, however, limited studies investigating the induction of IL-17A include IL-17F. One study from Wilson *et al.* demonstrated that IL-1 $\beta$  and IL-23 drives the expression of IL-17F from human CD4<sup>+</sup> T cells<sup>193</sup>. Data presented in this chapter expand upon the work of the lab and that of Wilson *et al.* and show that addition of LPS to CD4<sup>+</sup> T cell/ CD14<sup>+</sup> monocyte co-cultures leads to an increase in the percentage of both IL-17A<sup>+</sup> and IL-17F<sup>+</sup> CD4<sup>+</sup> T cells and an increase in IL-17A and IL-17F secretion. Moreover, two IL-17 populations were observed, IL-17A<sup>+</sup> IL-17F<sup>-</sup> and IL-17A<sup>+</sup> IL-17F<sup>+</sup> CD4<sup>+</sup> T cells. Luminex analysis of the co-culture supernatant revealed increased protein levels of IL-1 $\beta$ , IL-23, IL-6 and IFN $\gamma$  in LPS-treated conditions vs. controls. In this study, the importance of IL-1 $\beta$  and IL-23 in inducing IL-17F was further investigated by moving to a different culture system whereby human recombinant IL-1 $\beta$  and IL-23 cytokines were added. Future research to expand on how LPS-activated monocytes induce IL-17F could include blockade of IL-1 $\beta$ , IL-23 and other cytokines in the LPS culture system. Moreover, the expression of co-stimulation markers (for

example CD80) could be analysed, as it may be possible LPS drives the up-regulation of these markers and henceforth T cell activation and IL-17 production.

Addition of IL-1 $\beta$  and IL-23 human recombinant cytokines to CD4<sup>+</sup> T cell stimulated with anti-CD28 mAb and anti-CD3 mAb significantly enhanced the frequency of IL-17A<sup>+</sup> and IL-17F<sup>+</sup> CD4<sup>+</sup> T cell and IL-17A and IL-17F secretion. This confirms that IL-1 $\beta$  and IL-23 are important drivers in both IL-17A and IL-17F expression from CD4<sup>+</sup> T cells. Interestingly, in this culture system three IL-17 populations are observed IL-17A<sup>+</sup> IL-17F<sup>-</sup>, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells. An IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cell population was not present in the CD4<sup>+</sup> T cell/CD14<sup>+</sup> monocyte co-culture. It is possible the anti-CD28 mAb elicited a stronger co-stimulation signal than the LPS-activated monocytes which drove the induction of IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells. Another explanation could be that the higher concentrations of IL-1 $\beta$  and IL-23 induced IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells. The results presented also demonstrate that addition of IL-1 $\beta$  and IL-23 recombinant cytokines to CD4<sup>+</sup> T cell cultures leads to an increase in IL-17AF<sup>+</sup> CD4<sup>+</sup> T cell frequencies and IL-17AF protein secretion. Preliminary CyTOF results indicate the IL-17AF heterodimer is highly expressed by the IL-17A<sup>+</sup> IL-17F<sup>+</sup> CD4<sup>+</sup> T cell population (Appendix 7.1). It is hypothesized that the IL-17A<sup>+</sup> IL-17F<sup>+</sup> CD4<sup>+</sup> T cells produce IL-17A, IL-17F and IL-17AF, although the lack of an IL-17A homodimer specific antibody prevents this being confirmed. In this study we focused on the role of IL-1 $\beta$  and IL-23 in inducing IL-17F and IL-17AF expression. Future work could involve assessing the role of other previously described Th17 cytokines (IL-6, TGF- $\beta$  and IL-21) in IL-17F and IL-17AF induction.

Reports in the literature have demonstrated that IL-17A<sup>+</sup> CD4<sup>+</sup> T cells are promoted with low-strength T cell activation<sup>225,221</sup>. In contrast to the literature, addition of anti-CD28 mAb did not lead to a consistent decrease in the frequency of total IL-17A<sup>+</sup> CD4<sup>+</sup> T cells. Instead, no consistent effect was observed. This difference may be attributed to different cell culture conditions or human vs. mouse cells. A discrepancy between the IL-17A ICS and ELISA data was observed. While there was no difference in the frequency of IL-17A<sup>+</sup> CD4<sup>+</sup> T cells at day 3 in the absence or presence of anti-

CD28, IL-17A protein secretion was increased in the presence of anti-CD28. We have attributed this result to the kinetics of the assay. Our results suggested that there is an increase in the frequency of IL-17A<sup>+</sup> CD4<sup>+</sup> T cells in the presence vs. absence of anti-CD28 during early time-points of the 3 day cultures, which can account for the increased accumulation of IL-17A protein detected at day 3. How T cell activation affects IL-17F expression has not been previously reported. Data in this thesis demonstrate that addition of anti-CD28 mAb to CD4<sup>+</sup> T cell cultures significantly increases the frequency of total IL-17F<sup>+</sup> CD4<sup>+</sup> T cells and IL-17F and IL-17AF protein secretion. Interestingly, titration of anti-CD28 mAb increased the frequency of IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells, while decreasing the frequency of IL-17A<sup>+</sup> IL-17F<sup>+</sup> CD4<sup>+</sup> T cells.

Similar results were observed with anti-CD3 mAb. Titration of anti-CD3 mAb in CD4<sup>+</sup> T cell cultures led a dose dependent increase in the frequency of IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells. However, in the case of IL-17A<sup>+</sup> IL-17F<sup>-</sup> CD4<sup>+</sup> T cells, while there was an initial increase with a small concentration of anti-CD3 mAb, further increasing the concentration had little effect on IL-17A<sup>+</sup> IL-17F<sup>-</sup> CD4<sup>+</sup> T cell frequency. In light of this data, I hypothesise that high-strength T cell stimulation induces IL-17F. Of course, with the current experiments we cannot confirm an increase in anti-CD28 or anti-CD3 mAb led to a stronger T cell activation. This could be confirmed by analysing the up-regulation of the activation marker CD69 or by investigating the phosphorylation of T cell activation components, ZAP 70 or LAT by western blot. An increase in CD69 marker expression or phosphorylation of ZAP 70 or LAT could then be correlated with IL-17F<sup>+</sup> CD4<sup>+</sup> T cell frequency.

To investigate the mechanism by which high-strength T cell activation can drive IL-17F expression, IL-2 and IFN $\gamma$  was blocked in CD4<sup>+</sup> T cell cultures stimulated with IL-1 $\beta$ , IL-23 and anti-CD28. Firstly it is important to note that in contrast to the literature<sup>218,221</sup>, blockade of IL-2 led to no consistent differences in the frequency of IL-17A<sup>+</sup> CD4<sup>+</sup> T cells. One explanation for the lack of response to IL-2 blockade could be that the levels of IL-2 present in the culture were too low. To investigate this, future work could involve examining the effect of adding titrated amounts of human

recombinant IL-2 to stimulated CD4<sup>+</sup> T cells. If indeed IL-2 down-regulates the IL-17A response in humans, we would expect to observe the frequency of IL-17A<sup>+</sup> CD4<sup>+</sup> T cells dose-dependently decreasing upon the addition of IL-2. As the majority of the reports which implicate IL-2 in down-regulating IL-17A<sup>+</sup> CD4<sup>+</sup> T cells are in mice <sup>218,221,222</sup>, another explanation for these results could be that the role of IL-2 in humans may differ. Indeed, in contrast to the role of IL-2 in mice Amadi-Obi *et al.* demonstrated that in human PBMC isolated from the blood of active uveitis patients, IL-2 enhanced IL-17A production by memory CD4<sup>+</sup> T cells.

In contrast to the results of IL-17A, our results demonstrated that blockade of IL-2 reduced IL-17F<sup>+</sup> CD4<sup>+</sup> T cell frequencies to similar levels observed in the absence of anti-CD28. This demonstrated that the CD28-mediated increase in IL-17F<sup>+</sup> CD4<sup>+</sup> T cell expression was mediated by IL-2. Collectively these data indicate that IL-17A and IL-17F are differentially regulated. To confirm the role of IL-2 in IL-17F induction, it can be investigated if the reverse result (a dose-dependent increase in IL-17F<sup>+</sup> CD4<sup>+</sup> T cells) is observed upon addition of human recombinant IL-2. IL-2 has been shown to induce the production of other cytokines from T cells such as TNF $\alpha$  <sup>408</sup>. Therefore, it may of interest to demonstrate whether the IL-2-mediated induction of IL-17F expression is a direct or indirect mechanism. This could include performing a Luminex on the supernatants of CD4<sup>+</sup> T cells cultured in the absence or presence of anti-CD28 and identifying cytokines other than IL-17F and IL-2 which are upregulated in the presence of anti-CD28. If for example TNF $\alpha$  is elevated in the presence of anti-CD28, the effect of blocking TNF $\alpha$  on the frequency of IL-17F<sup>+</sup> CD4<sup>+</sup> T cells can be assessed.

Evidence from mouse <sup>209-211</sup> and human <sup>192</sup> studies have shown that IFN $\gamma$  can decrease the frequency of IL-17A<sup>+</sup> CD4<sup>+</sup> T cells and IL-17A protein secretion. Despite detectable levels of IFN $\gamma$  observed in CD4<sup>+</sup> T cell cultures and efficient blockade of IFN $\gamma$  following addition of a neutralising antibody, the data presented in this thesis showed no difference in the frequency of IL-17A<sup>+</sup> CD4<sup>+</sup> T cells in CD4<sup>+</sup> T cell cultures stimulated with IL-1 $\beta$ , IL-23, anti-CD28 and anti-CD3 in the absence or presence of anti-IFN $\gamma$ . These conflicting results may reflect differences in animal vs.

human Th17 differentiation as the strongest evidence for the role of IFN $\gamma$  in regulating the IL-17A response derive from murine studies. However, it is important to also consider that the majority of mouse reports examine the effect of IFN $\gamma$  on naïve CD4<sup>+</sup> T cells<sup>210</sup> and the human study by Evans *et al.* investigates the role of IFN $\gamma$  on memory CD4<sup>+</sup> T cells<sup>192</sup>. In this study bulk CD4<sup>+</sup> T cells were used. Therefore, future studies could involve performing the same experiment on separate cultures of isolated naïve CD45RA<sup>+</sup> and memory CD45RO<sup>+</sup> CD4<sup>+</sup> T cells. Similar to IL-17A, blockade of IFN $\gamma$  did not show a consistent effect on the frequency of IL-17F<sup>+</sup> CD4<sup>+</sup> T cells.

Data presented in this chapter show that while re-stimulation with PMA and ionomycin increases the frequency of total IL-17A<sup>+</sup> CD4<sup>+</sup> T cells, it has no effect on the frequency of total IL-17F<sup>+</sup> CD4<sup>+</sup> T cells. More specifically, PMA and ionomycin enhances IL-17A<sup>+</sup> IL-17F<sup>-</sup> and IL-17A<sup>+</sup> IL-17F<sup>+</sup> CD4<sup>+</sup> T cell frequencies and decreases IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells. The reason why PMA and ionomycin increases the frequency of IL-17A and not IL-17F remains unclear. One explanation may be that the signalling pathways stimulated with PMA and ionomycin does not elicit an IL-17F response. These data, alongside observations made from titration of anti-CD3 mAb and anti-CD28 mAb, clearly demonstrate that IL-17A and IL-17F expression from CD4<sup>+</sup> T cells are not always enhanced simultaneously. Moreover, the data presented indicate IL-17A and IL-17F may be differentially regulated.

Melton *et al.* have previously reported differential regulation of IL-17A and IL-17F. In their study primary human B cell and peripheral blood mononuclear cell co-cultures were stimulated with anti-IgM and superantigens to promote a Th17 response, as evidenced by IL-17A and IL-17F secretion. Co-cultures were incubated with pharmacologic modulators, which inhibit multiple pathways. Results revealed IL-17A production from CD4<sup>+</sup> T cells was decreased following inhibition of the PI3K/ mTOR pathway, while prostaglandin EP receptor agonists, including PGE2, increased levels of IL-17A. In contrast, PGE2 inhibited IL-17F production. However, TLR2 and TLR5 agonists selectively increased levels of IL-17F<sup>409</sup>. Differential regulation of IL-17A and IL-17F is also supported in a study by Gomez-Rodrigues *et al.*, whereby CD4<sup>+</sup> T cells deficient in the TCR signalling kinase I $\kappa$ k led to a decreased IL-17A expression

whilst exerting no effect on the expression of IL-17F<sup>410</sup>. Finally, a very recent report implicated that IL-17A and IL-17F are frequently unequally regulated, evidenced by the fact that IL-1 $\beta$  preferentially drives a mouse IL-17A<sup>+</sup> CD4<sup>+</sup> T cell profile, whereby TGF- $\beta$  predominantly drives an IL-17F profile<sup>246</sup>. In the final discussion chapter, I discuss how future work can investigate whether IL-17A and IL-17F are differentially regulated.

The second focus of this chapter was to compare the marker and cytokine profile of IL-17A<sup>+</sup> and IL-17F<sup>+</sup> CD4<sup>+</sup> T cells to identify whether there were any key differences between these populations. In line with literature<sup>196</sup>, we observed that CCR6 marker expression was enriched on IL-17A and IL-17F expressing cells, however no consistent difference was observed between the IL-17 populations themselves. In two out of four donors the IL-17A<sup>+</sup> IL-17F<sup>-</sup> CD4<sup>+</sup> T cell population displayed higher levels of CD161 when compared with IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cell populations. To confirm these results, this experiment should be repeated additional times. Identifying differences in IL-17A<sup>+</sup> and IL-17F<sup>+</sup> CD4<sup>+</sup> T cell populations may enable these populations to be separated without the need of a secretion assay. In this study only a limited number of markers was explored, however future work could examine the expression of chemokine receptors previously associated with Th17 cells including CXCR4, CXCR6, CCR4 and CCR5.

Literature describes Th17 cells to produce an array of cytokines alongside IL-17A, including IL-17F, TNF $\alpha$ , IFN $\gamma$ , GM-CSF, IL-21, IL-22, IL-9 and IL-10. Cytokine presence and expression levels are dependent on the cytokine milieu present upon Th17 differentiation<sup>234-236</sup>. Novel data in this chapter show that IL-17A<sup>+</sup> IL-17F<sup>-</sup>, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>+</sup> CD4<sup>+</sup> T cells display different cytokine profiles. In CD4<sup>+</sup> T cell cultures with IL-1 $\beta$  and IL-23, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> display significantly lower frequencies of IL-10 and higher frequencies of IFN $\gamma$  when compared with IL-17A<sup>+</sup> IL-17F<sup>-</sup> CD4<sup>+</sup> T cells. Moreover, IL-17A<sup>+</sup> IL-17F<sup>-</sup> CD4<sup>+</sup> T cells display a higher frequency of GM-CSF when compared with IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells. A lower frequency of IL-10 was also observed in IL-17A<sup>+</sup> IL-17F<sup>+</sup> CD4<sup>+</sup> T cells vs. IL-17A<sup>+</sup> IL-17F<sup>-</sup> CD4<sup>+</sup> T cells in CD4<sup>+</sup> T cell/ CD14<sup>+</sup>

monocyte cultures stimulated with LPS, demonstrating this result it not only observed in IL-1 $\beta$  and IL-23 conditions. However, no differences in IFN $\gamma$  or GM-CSF cytokine co-expression levels were observed. Different cytokine profile results observed in CD4<sup>+</sup> T cell + IL-1 $\beta$  and IL-23 vs. CD4<sup>+</sup> T cell/ CD14<sup>+</sup> monocyte + LPS cultures may be attributed to the different IL-17 cytokine profiles. Firstly, no IL-17A<sup>+</sup> IL-17F<sup>-</sup> CD4<sup>+</sup> T cells are present in the CD4<sup>+</sup> T cell/ CD14<sup>+</sup> monocyte + LPS culture. Moreover, the IL-17A<sup>+</sup> IL-17F<sup>+</sup> CD4<sup>+</sup> T cells in this culture are all highly expressing IL-17A, whereas in the CD4<sup>+</sup> T cell + IL-1 $\beta$  and IL-23 cultures, there is more variation in IL-17A expression levels.

These results suggest IL-17A<sup>+</sup> IL-17F<sup>-</sup>, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells may have different functional properties. An interesting finding is that IL-17F<sup>+</sup> CD4<sup>+</sup> T cells display a lower frequency of IL-10 suggesting these cells are a more inflammatory Th17 population. Indeed, the ‘pathogenic’ Th17 population are characterised by a lack of IL-10. However, they are also characterised by their expression of GM-CSF, of which, IL-17A<sup>+</sup> IL-17F<sup>-</sup> CD4<sup>+</sup> T cells display a higher level of GM-CSF in comparison to IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells. Nevertheless, IL-17F<sup>+</sup> CD4<sup>+</sup> T cells also demonstrated higher levels of IFN $\gamma$  when compared with IL-17A<sup>+</sup> IL-17F<sup>-</sup> CD4<sup>+</sup> T cells. To the best of our knowledge, the cytokine profile of the different IL-17A/IL-17F CD4<sup>+</sup> T cell populations has not been characterised previously. A recent mouse study demonstrated that TGF- $\beta$  induced higher frequencies of IL-17F<sup>+</sup> CD4<sup>+</sup> T cells, suggesting that expression of IL-17F from CD4<sup>+</sup> T cells is associated with non-pathogenic Th17 cells<sup>246</sup>. However, the study did not characterise the cytokine profile of IL-17F<sup>+</sup> CD4<sup>+</sup> T cells. A recent study by Aschenbrenner *et al.* performed RNA-seq on human Th17-IL-10 and Th17-IL-10<sup>+</sup> clones. While several genes associated with the ‘non-pathogenic’ Th17 phenotype were highly expressed in Th17-IL-10<sup>+</sup> cells, genes associated with the ‘pathogenic’ Th17 phenotype were highly expressed in Th17-IL-10<sup>-</sup> cells. Interestingly, in line with our data *IL17F* was highly expressed in Th17-IL-10<sup>-</sup> cells when compared to Th17-IL-10<sup>+</sup> cells. In contrast, *IL17A* was preferentially expressed in Th17-IL-10<sup>+</sup> cells. Th17-IL-10<sup>-</sup> cells were also linked with high expression levels of *IFNG*<sup>411</sup>. The study also demonstrated that Th17-IL-10<sup>-</sup> cells have the ability to polarise monocytes into an inflammatory M1 phenotype, whilst Th17-IL-10<sup>+</sup> cells

induced an M2 anti-inflammatory phenotype <sup>411</sup>. In summary, work from this thesis and other studies <sup>246,411</sup> highlight that performing molecular and functional characterisation of isolated IL-17A/IL-17F CD4<sup>+</sup> T cell sub-populations would be interesting area of research to pursue. This is further discussed in the final discussion chapter.

The next chapter explores the individual and combinatorial function of IL-17A, IL-17F and IL-17AF. Moreover, with the increasing need to separate IL-17A<sup>+</sup> IL-17F<sup>-</sup>, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>-</sup> IL-17A<sup>+</sup> CD4<sup>+</sup> T cells, I describe the development of an IL-17F secretion assay.



## 4 Investigating the contribution of IL-17F to inflammation

### 4.1 Introduction

The IL-17 cytokines mediate their biological function via surface receptors on target cells. The IL-17 receptor family consists of IL-17RA, IL-17RB, IL-17RC, RD and RE. Functional IL-17 receptors exist as heterodimers, with IL-17RA as a common subunit. IL-17A, IL-17F and IL-17AF share the same IL-17RA and IL-17RC heterodimer receptor. These cytokines typically act on fibroblasts, epithelial cells and endothelial cells <sup>31</sup>.

The activation of NF $\kappa$ B and MAPK pathways in target cells by IL-17A, IL-17F and IL-17AF results in the transcription of pro-inflammatory genes. IL-17A and IL-17F have been shown to induce the production of the pro-inflammatory cytokines (IL-6, TNF $\alpha$  and IL-1) <sup>71</sup>, T cell and myeloid cell-attracting chemokines (CCL20, CCL2 and CCL7) <sup>94,95</sup> and many neutrophilic granulocyte-attracting chemokines (CXCL1, CXCL2, CXCL5 and CXCL8) <sup>71,96</sup> from target cells. IL-17A, IL-17F and IL-17AF can exert potent synergistic effects in the presence of other cytokines (including TNF $\alpha$ ) to augment pro-inflammatory responses <sup>112</sup>.

Literature describes a hierarchy in inflammatory potential with IL-17A homodimers eliciting the strongest inflammatory response, followed by IL-17AF heterodimers, and then IL-17F homodimers <sup>78</sup>. In fact, studies have suggested that IL-17F is largely redundant to IL-17A. However, it has been shown that neutralization of both IL-17A and IL-17F significantly reduced T cell-mediated colitis development, whereas neutralisation of IL-17A or IL-17F alone was inefficient at reducing disease severity, indicating a potential role for IL-17F in inflammatory disease pathology <sup>363</sup>. Moreover, a recent study demonstrated that dual neutralization of IL-17A and IL-17F leads to a greater reduction on Th17 cell culture supernatant induced IL-6 and IL-8 secretion by PsA fibroblasts <sup>121</sup>. The overall aim of this chapter was to assess the function of IL-17F and whether it has the potential to contribute to the immunopathology of

inflammatory arthritis, specifically RA and PsA. This aim has been addressed in the following ways:

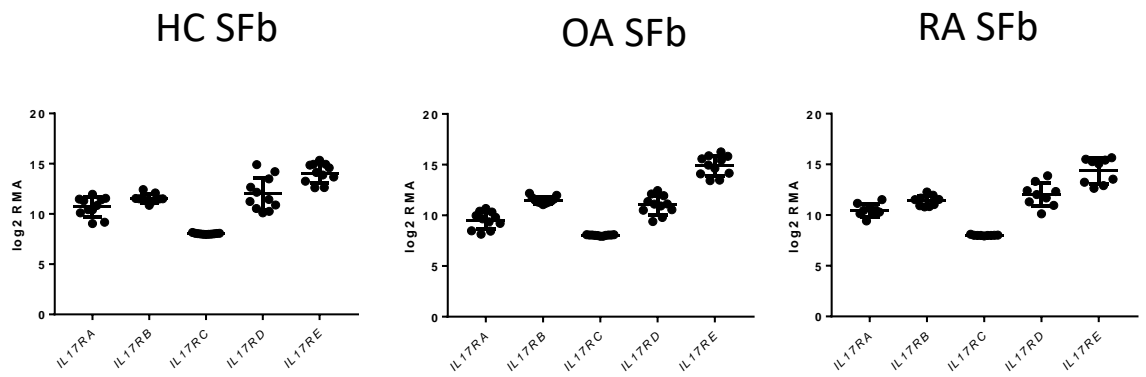
- Assessment of the individual and combinatorial role of IL-17A, IL-17F, IL-17AF and TNF $\alpha$  in synovial fibroblast assays.
- Investigating the effect of single and dual IL-17A and IL-17F blockade on the inflammatory response in a variety of culture systems.
- Developing an IL-17F secretion assay to allow further analysis of the functional role of IL-17F producing cells.

This chapter predominantly explores the inflammatory capacity of IL-17A, IL-17F and IL-17AF. However, it is concluded with a brief investigation of the function of the remaining IL-17 cytokines, IL-17B, IL-17C, IL-17D and IL-17E.

## 4.2 Results

### 4.2.1 Examining the IL-17 receptor gene expression profile in RA and PsA synovial fibroblasts.

To begin work on this chapter, a publicly available dataset (GEO accession number: GSE29746) was mined to investigate the gene expression of all IL-17 receptors (*IL17RA*, *IL17RB*, *IL17RC*, *IL17RD* and *IL17RE*) on synovial fibroblasts derived from RA. Moreover, RA IL-17R expression was compared to that of OA or healthy controls. Figure 4.1A displays data whereby microarray expression profiling was performed in human synovial fibroblasts from healthy, OA and RA synovial tissues. In this data set, all IL-17 receptors genes were present in healthy, OA and RA synovial tissues and they displayed a very similar gene expression profile. Gene expression levels of *IL17RC* were the lowest and demonstrated the least variability between donors, whilst mRNA expression of *IL17RE* was the highest.



**Figure 4.1 Gene expression of the IL-17 receptors in healthy control, OA and RA synovial fibroblasts.**

This figure displays a publicly available dataset (GEO accession number: GSE29746), whereby synovial fibroblasts were obtained from patients with RA (RA SF) (n=9) and sex and age matched adult healthy donors (HC SF) (n=11) and OA donors (n=11). Synovial fibroblasts were collected under similar sub-confluent conditions 24 hours after serum addition. Microarray analysis was then performed on isolated RNA.

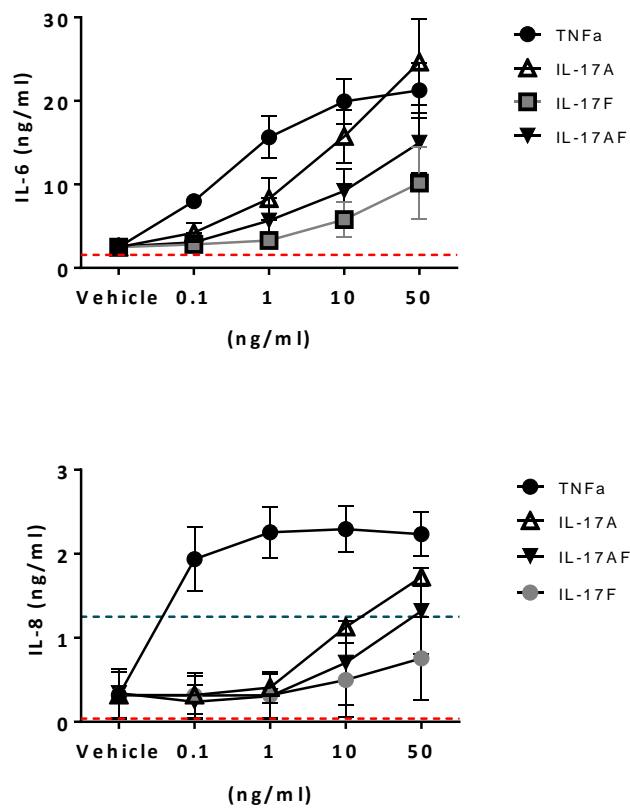
#### **4.2.2 Assessing the individual and combinatorial function of IL-17A, IL-17F and IL-17AF on synovial fibroblasts**

The next aim was to investigate the individual roles of IL-17A, IL-17F, IL-17AF and TNF $\alpha$  in RA and PsA synovial fibroblast cultures. RA and PsA synovial fibroblasts were seeded in a 96 well plate and allowed to rest for 24 hours. Next, fibroblasts were incubated with human recombinant IL-17A, IL-17F, IL-17AF, TNF $\alpha$  and vehicle controls at the indicated concentrations. Following 24 hours incubation, the supernatant was collected and analysed by ELISA for IL-6 and IL-8 protein levels. In total 3 different RA fibroblast cell lines (i.e originating from different donors) and 4 different PsA fibroblast cell lines were used in this thesis. Not all cell lines were used in each experiment. At a steady state, no consistent differences were observed between IL-6 or IL-8 secretion in RA vs. PsA fibroblasts. In general, there were modest variations in IL-6 and IL-8 secretion across all cell lines. Moreover, in some cases fibroblasts derived from the same cell line secreted different protein levels at a different passage. These variations may be attributed to the health of the cells at the time of seeding and the extent of inflammation in the joint from which the fibroblasts were derived.

As shown in Figure 4.2, IL-17A, IL-17F, IL-17AF and TNF $\alpha$  all induced a dose-dependent increase in IL-6 secretion by fibroblasts. In line with literature, a hierarchy in inflammatory potency of the IL-17 cytokines was observed: IL-17A elicited higher levels of IL-6 in comparison to IL-17F, while IL-17AF induced intermediate IL-6 levels. TNF $\alpha$  induced the highest levels of IL-6. However, when recombinant cytokines were added at 50ng/ml, IL-17A elicited higher IL-6 levels than TNF $\alpha$ . Similar results were observed when analysing the levels of IL-8 in the fibroblast supernatant. All cytokines stimulated IL-8 secretion from fibroblasts in a hierarchical fashion. However, in this case TNF $\alpha$  was more potent in driving IL-8 fibroblast secretion compared to the IL-17 cytokines. TNF $\alpha$  at a concentration of 0.1ng/ml stimulated IL-8 protein levels above the ELISA detection limit.

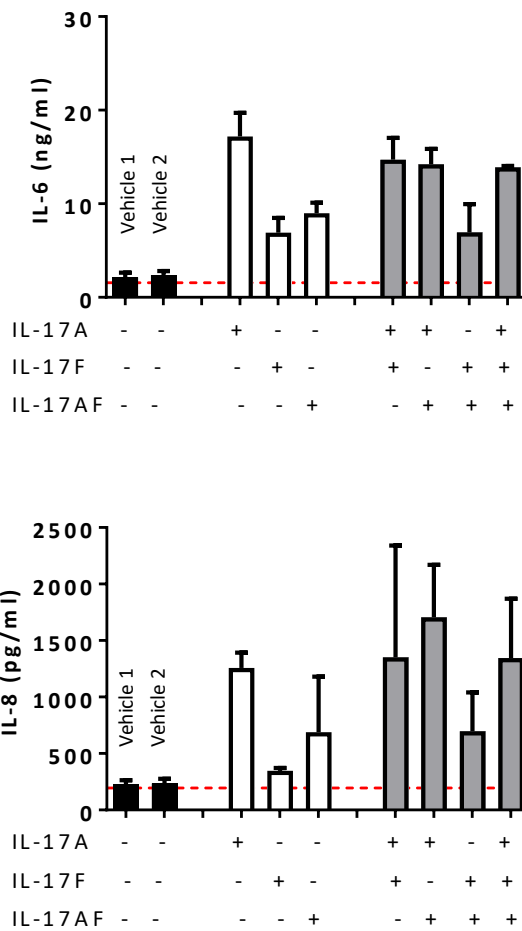
Next, we sought to investigate whether there were any synergistic or additive effects between IL-17A, IL-17F or IL-17AF themselves. To assess this, combinations of the

IL-17 cytokines at 10ng/ml were added to synovial fibroblasts for 24 hours and IL-6 and IL-8 protein levels were assessed by ELISA. As depicted in Figure 4.3, combining IL-17A with IL-17F, IL-17AF or both did not lead to increased IL-6 or IL-8 secretion levels when compared with IL-17A alone, showing there is no synergistic or additive effect between IL-17A and IL-17F or IL-17AF.



**Figure 4.2 IL-17A, IL-17F or IL-17AF addition leads to a dose-dependent increase in IL-6 and IL-8 fibroblast secretion.**

RA and PsA synovial fibroblasts ( $10 \times 10^3$ ) were cultured with indicated concentrations of human recombinant IL-17A, IL-17F, IL-17AF or TNF $\alpha$  for 24 hours. Additionally fibroblasts were cultured with vehicle controls for IL-17A, IL-17F and TNF $\alpha$  (0.1% BSA) and IL-17AF (0.1% BSA and 4mM HCL). Culture supernatants were collected and IL-6 (RA n=3, PsA n=1) and IL-8 (RA n=2) protein levels were measured by ELISA. Dashed red and blue lines indicate lower detection limit and upper detection limit, respectively.



**Figure 4.3 No synergistic or additive effect is observed between IL-17A, IL-17F and IL-17AF.**

RA and PsA synovial fibroblasts ( $10 \times 10^3$ ) were cultured with combinations of IL-17A, IL-17F or IL-17AF at 10ng/ml for 24 hours. Additionally, fibroblasts were cultured with vehicle controls for IL-17A, IL-17F (0.1% BSA, Vehicle 1) and IL-17AF (0.1% BSA and 4 $\mu$ m HCL, Vehicle 2). Culture supernatants were collected and IL-6 (RA n=3, PsA n=1) and IL-8 (PsA n=2) protein levels were measured by ELISA. Dashed red and blue lines indicate lower detection limit and upper detection limit, respectively.



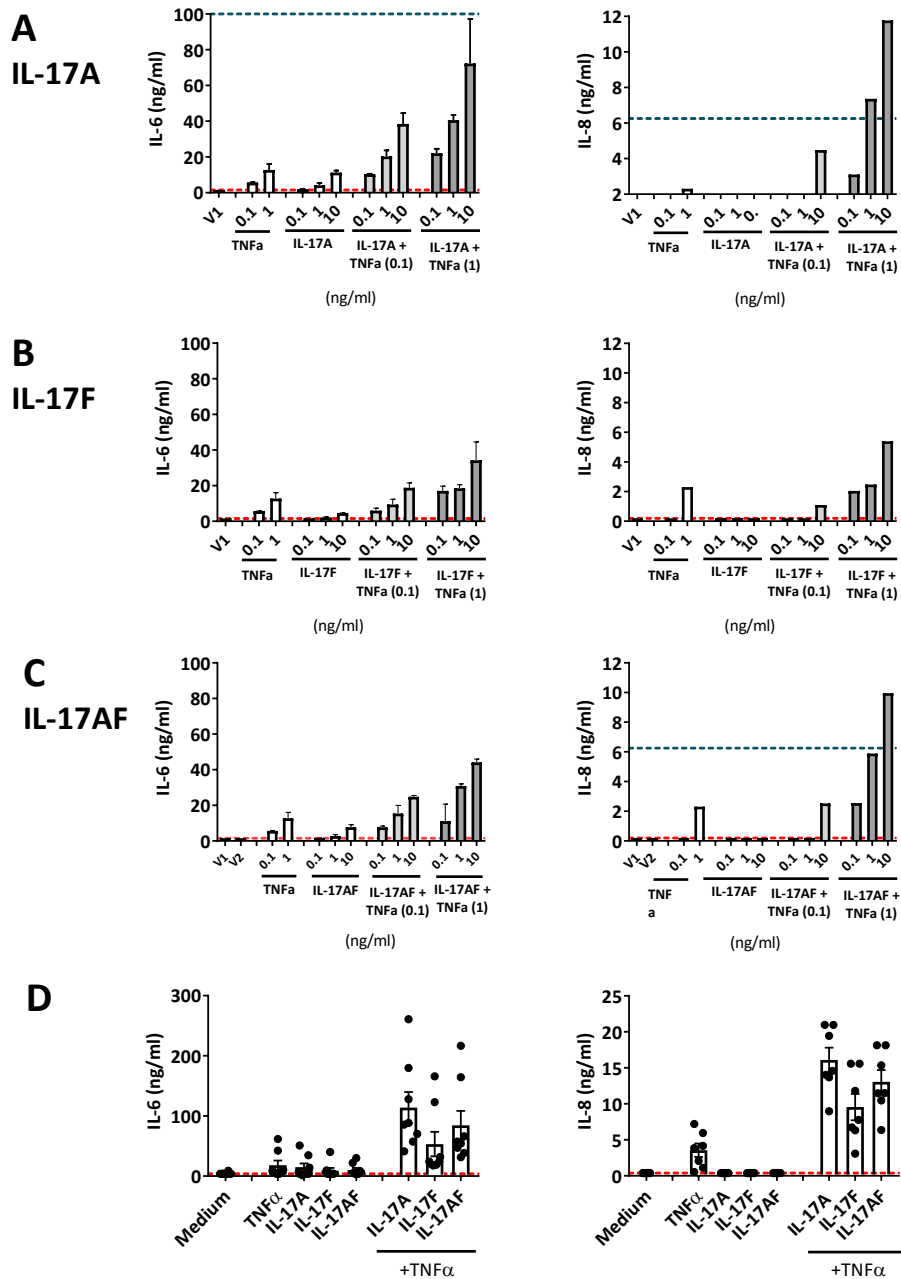
### 4.2.3 IL-17A, IL-17F and IL-17AF synergise with TNF $\alpha$

IL-17A, IL-17F and IL-17AF have the ability to exacerbate their inflammatory potential by synergising with other cytokines. In these experiments, we focused on the synergy between the IL-17 cytokines and TNF $\alpha$ . We investigated to what extent the pro-inflammatory ability of IL-17A, IL-17F and IL-17AF increased with TNF $\alpha$ . In this experiment a range of concentrations was used to determine the sub-optimal/minimum concentration of cytokine required to see a synergistic effect.

As shown in Figure 4.4A, a synergistic increase in IL-6 secretion by fibroblasts was observed with IL-17A and TNF $\alpha$  at concentrations as low as 1 and 0.1ng/ml, respectively. A combination of 10ng/ml IL-17A and 1ng/ml TNF $\alpha$  resulted in a profound 6.5-fold increase in IL-6 fibroblast secretion when compared with 10ng/ml IL-17A alone. Moreover, a 6-fold increase was observed when compared with 1ng/ml TNF $\alpha$  alone. Similarly, synergistic responses were observed in the secretion of IL-8 from concentrations as low as 1ng/ml IL-17A and 0.1ng/ml TNF $\alpha$ . In the case of IL-17F (Figure 4.4B), the lowest concentrations of recombinant cytokines required to drive a synergistic IL-6 response with TNF $\alpha$  were 10ng/ml IL-17F and 0.1ng/ml TNF $\alpha$ . The strongest synergistic effect was observed when 1ng/ml of TNF $\alpha$  was added to 10ng/ml of IL-17F. This led to a 7.5-fold and 2.8-fold increase in IL-6 levels when compared to addition of IL-17F or TNF $\alpha$ , respectively. Similarly, addition of 0.1 or 1ng/ml of TNF $\alpha$  to 10ng/ml IL-17F led to a synergistic increase in IL-8 protein secreted from fibroblasts. Results of the IL-17AF synergy experiments demonstrated that the heterodimer can also synergise with TNF $\alpha$  to induce elevated levels of IL-6 and IL-8 from fibroblasts. Figure 4.4D compares the levels of IL-6 and IL-8 induced with 10ng/ml IL-17A, IL-17F and/ or IL-17AF all combined with 1ng/ml TNF $\alpha$ . The combination of IL-17A and TNF $\alpha$  elicited the highest production of IL-6 and IL-8 from target cells, followed by IL-17AF and IL-17F. These data show that the inflammatory potential of IL-17A, IL-17F and IL-17AF is significantly enhanced in the presence of TNF $\alpha$ .

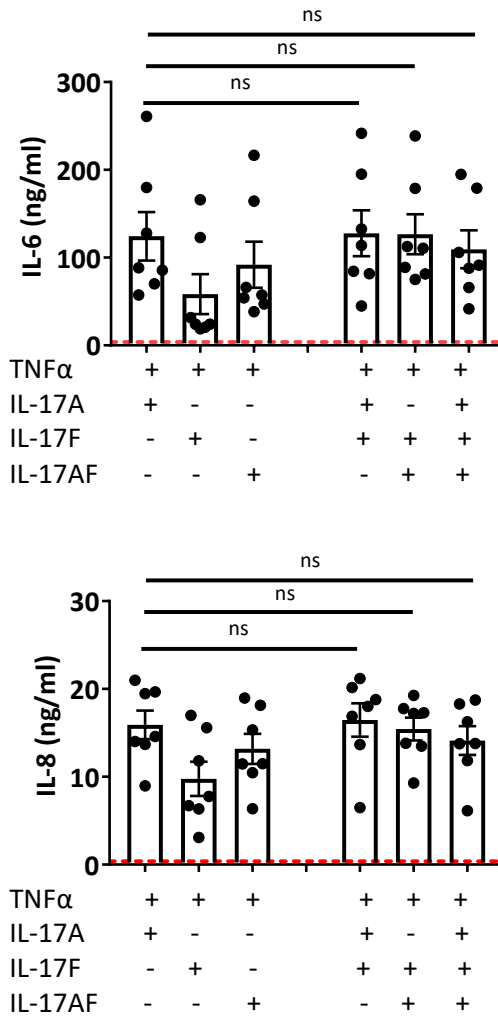
We next sought to assess whether addition of IL-17F or IL-17AF to IL-17A and TNF $\alpha$  further enhances secretion of IL-6 or IL-8. As depicted in Figure 4.5, addition

of IL-17F and/ or IL-17AF to IL-17A and TNF $\alpha$  in the fibroblast cultures did not lead to a further increase in IL-6 or IL-8 secretion by fibroblasts.



**Figure 4.4 IL-17A, IL-17F and IL-17AF synergise with TNF $\alpha$ .**

Synovial RA and PsA fibroblasts ( $10 \times 10^3$ ) were cultured with combinations of IL-17A, IL-17F, IL-17AF and TNF $\alpha$  (at indicated concentrations) or with vehicle controls for IL-17A, IL-17F and TNF $\alpha$  (0.1% BSA, V1) and IL-17AF (0.1% BSA and  $4 \mu\text{m}$  HCL, V2). Culture supernatants were collected and IL-6 and IL-8 protein levels were measured by ELISA. Data show the synergistic effect of TNF $\alpha$  and (A) IL-17A, (B) IL-17F and (C) IL-17AF on IL-6 (RA  $n=1$ , PsA  $n=1$ ) and IL-8 (PsA  $n=1$ ) secretion. (D) Displays cumulative IL-6 (RA  $n=3$ , PsA  $n=5$ ) and IL-8 (RA  $n=2$ , PsA  $n=5$ ) data with the IL-17 cytokines at 10ng/ml and TNF $\alpha$  at 1ng/ml. Dashed red and blue lines indicate lower detection limit and upper detection limit, respectively.



**Figure 4.5 Addition of IL-17F or IL-17AF does not further increase the IL-17A and TNF $\alpha$  mediated upregulation of synovial fibroblast pro-inflammatory cytokine secretion.**

Synovial RA and PsA fibroblasts ( $10 \times 10^3$ ) were cultured with combinations of IL-17A, IL-17F, IL-17AF at 10ng/ml and TNF $\alpha$  at 1ng/ml were for 24 hours. Culture supernatants were collected and IL-6 (RA n=3, PsA n=5) and IL-8 (RA n=2, PsA n=5) protein levels were measured by ELISA. Dashed red and blue lines indicate lower detection limit and upper detection limit, respectively. Statistical analysis was performed using Friedman test with comparison to IL-17A and TNF $\alpha$  by Dunn's Multiple Comparisons test.

#### 4.2.4 Testing blocking antibodies

Assessing the role of IL-17F via addition of recombinant cytokines to fibroblast assays demonstrated that while the potency of IL-17F in inducing inflammatory cytokine production from synovial fibroblasts was enhanced in the presence of TNF $\alpha$ , it was not as potent as the combination of IL-17A and TNF $\alpha$ . Furthermore, addition of IL-17F to IL-17A and TNF $\alpha$  cultures had no further effect on cytokine secretion by fibroblasts. This potentially suggests IL-17F is redundant when in the presence of IL-17A. However, studies have shown dual blockade of IL-17A and IL-17F is more efficient in reducing inflammatory cytokine production than blockade of IL-17A alone<sup>121,363</sup>. I therefore sought to examine the role of IL-17F using blocking antibodies.

To perform these experiments, our pharmaceutical collaborator, UCB, provided IL-17A and IL-17F blocking antibodies. This included a UCB in-house IL-17A blocking antibody which neutralizes both the IL-17A homodimer and IL-17AF heterodimer and a UCB in-house IL-17F blocking antibody which neutralizes IL-17F with no cross reactivity to the other IL-17 cytokines. These experiments also included bimekizumab, which is a novel, humanized monoclonal IgG1 antibody that selectively neutralizes IL-17A, IL-17F and IL-17AF. The affinities of these blocking antibodies have recently been published<sup>121</sup>. In summary, biomolecular interaction analysis performed using a Biacore 3000 demonstrated that bimekizumab shares a similar affinity to the anti-IL-17A antibody. However, bimekizumab has a higher affinity for IL-17F than the anti-IL-17F antibody. Therefore, it was important to determine efficient blocking antibody concentrations which completely block IL-17 cytokine function.

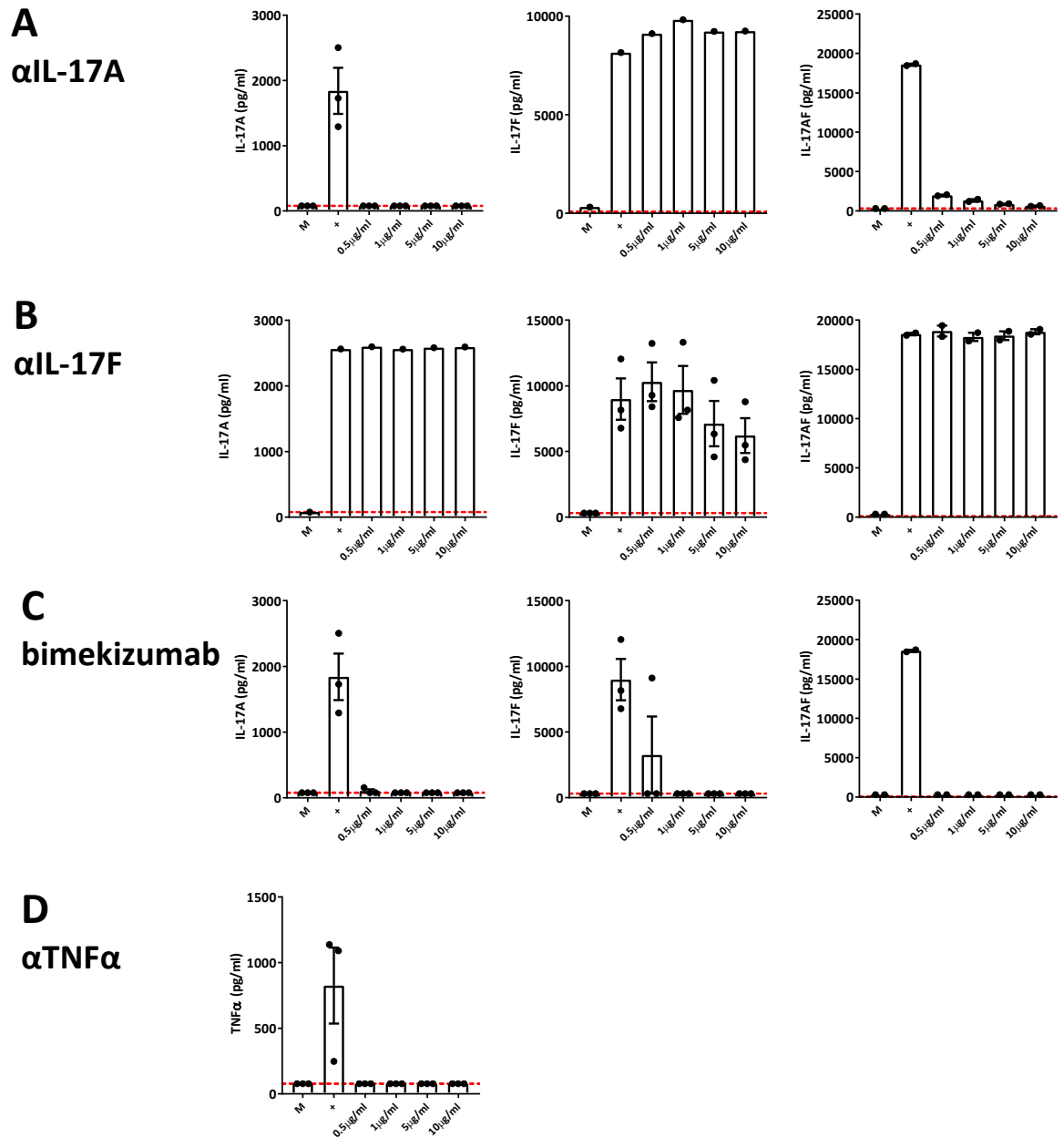
A series of preliminary experiments were performed to ensure efficient blockade of IL-17 and TNF $\alpha$  cytokines and to ensure minimal cross-reactivity. To begin the experiments, a cocktail of IL-17A, IL-17F, IL-17AF and TNF $\alpha$ , all at 10ng/ml in DMEM media were added to fibroblast cultures alongside increasing doses of blocking antibodies. Following a 24 hour incubation, supernatants were collected and IL-17A, IL-17F, IL-17AF and TNF $\alpha$  protein levels were analysed by ELISA. While IL-17A protein was detected in the positive control condition, no IL-17A protein was detected with the addition of 0.5 $\mu$ g/ml or more of IL-17A blocking antibody,

indicating efficient blockade of IL-17A (Figure 4.6A). Similar results were observed for IL-17AF. As expected, IL-17F protein was still detectable following the addition of IL-17A blocking antibody. As depicted in Figure 4.6B, protein levels of IL-17A and IL-17AF were all detectable following the addition of IL-17F blocking antibody. Unexpectedly, while blockade of IL-17F at the highest dose (10 $\mu$ g/ml) reduced IL-17F protein levels to some extent, IL-17F was still clearly detectable. This suggested that either the blocking antibody was inefficient at blocking IL-17F, a higher concentration of anti-IL-17F was required or the blocking antibody is working and inhibits the function of IL-17F but the protein-antibody complex is still recognized by the IL-17F ELISA. Bimekizumab completely blocked IL-17A, IL-17F and IL-17AF from a concentration of 1 $\mu$ g/ml onwards (Figure 4C). Finally, anti-TNF $\alpha$  effectively blocked TNF $\alpha$  protein (Figure 4D).

To investigate why IL-17F protein might still be detectable following addition of 10 $\mu$ g/ml of anti-IL-17F, fibroblasts were cultured with human recombinant IL-17F (10ng/ml) and titrated doses of anti-IL-17F mAb for 24 hours. Although titration of anti-IL-17F led to a dose-dependent decrease in IL-17F protein, IL-17F was not completely blocked even with a high concentration of 100 $\mu$ g/ml anti-IL-17F blocking antibody (Figure 4.7A). To investigate the possibility that the protein-blocking antibody complex is recognized by the IL-17F ELISA but the blocking antibody is still affecting the function of IL-17F, we measured the same supernatant used in Figure 4.7A, for IL-6 and IL-8 protein levels. Supernatant from fibroblasts cultured with recombinant IL-17F displayed an increase in IL-6 protein levels in comparison to medium-only controls. However, an increase in IL-6 protein levels was not observed in supernatants from fibroblasts cultured with recombinant IL-17F and anti-IL-17F blocking antibody. This indicates that although IL-17F protein is detectable following the addition of anti-IL-17F blocking antibody, the IL-17F protein function is neutralised. In this set of experiments IL-8 was below the ELISA detection limit in all conditions (data not shown). IL-17F alone is a weak inducer of IL-6 and IL-8 by fibroblasts. To further validate the efficiency of the IL-17F blocking antibody in a system with higher IL-6 levels and detectable IL-8 levels, I performed the same experiment, this time adding anti-IL-17F to a combination of IL-17F and TNF $\alpha$  recombinant cytokines. As depicted in Figure 4.7C, the combination of IL-17F and

TNF $\alpha$  resulted in a synergistic increase in IL-6 and IL-8 secretion. Addition of IL-17F blocking antibodies reduced IL-6 and IL-8 levels back to levels similar to that of TNF $\alpha$  alone. This further validates that the anti-IL-17F antibody is functional.

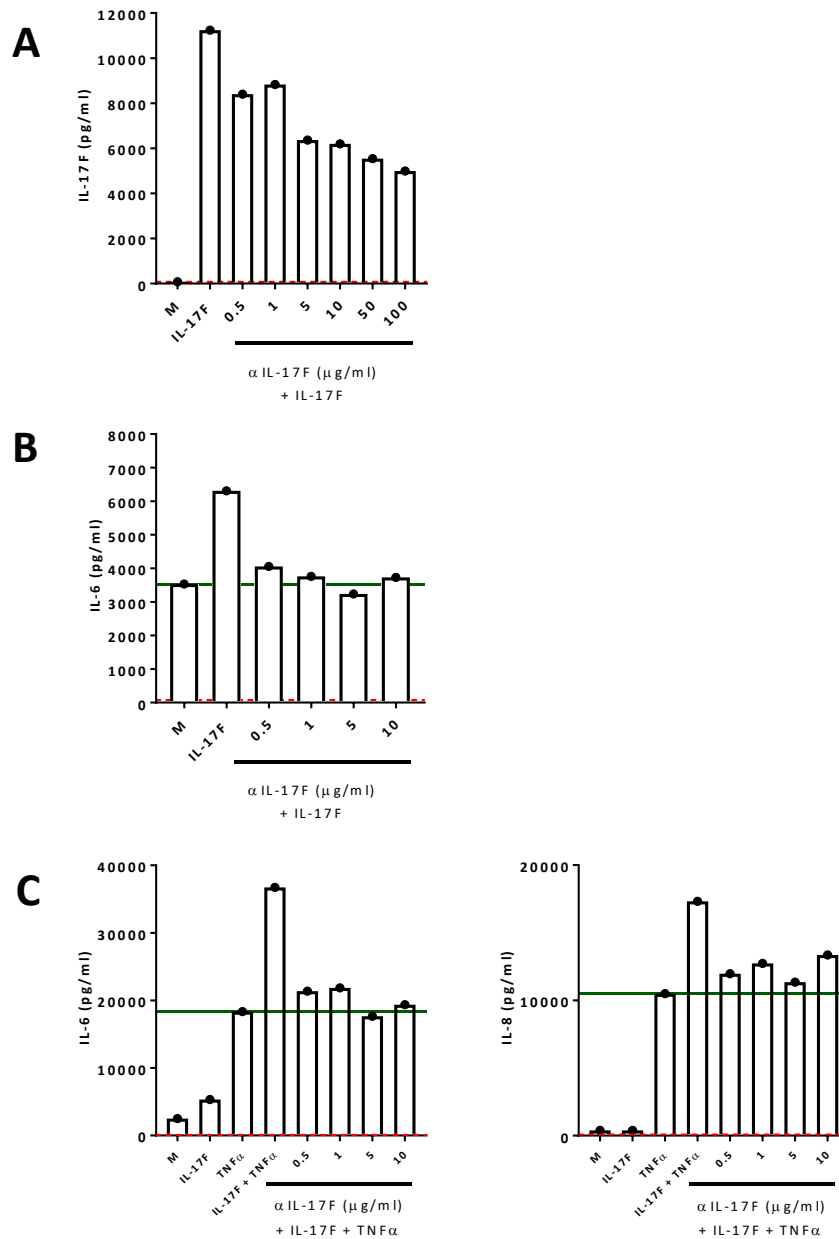
The aim of the final experiment in this section was to validate the blocking specificity of anti-IL-17A, anti-IL-17F, bimekizumab and anti-TNF $\alpha$ . It was previously demonstrated in Figure 4.6 that anti-IL-17A does not recognize the IL-17F protein; similarly, anti-IL-17F does not recognize IL-17A and IL-17AF protein. I sought to extend this analysis to demonstrate that the blocking antibodies do not non-specifically reduce IL-6 secretion by fibroblasts. IL-17A, IL-17F, IL-17AF and TNF $\alpha$  were individually added to fibroblast cultures in the absence or presence of blocking antibodies at 10 $\mu$ g/ml. Cultures were incubated for 24 hours and IL-6 protein levels were measured by ELISA. As depicted in Figure 4.8, IL-17A-induced IL-6 secretion was abrogated by the addition of anti-IL-17A and bimekizumab only. Similar results were observed for the IL-17AF heterodimer. Anti-IL-17F and bimekizumab inhibited IL-17F-induced IL-6 secretion. Anti-IL-17A and anti-TNF $\alpha$  blocking antibodies showed no effect on IL-17F induced cytokine secretion. TNF $\alpha$  induced IL-6 levels were inhibited with the addition of anti-TNF $\alpha$  only. Similar results were observed for IL-8; however levels of IL-17F-induced IL-8 were too low for reliable analysis.



**Figure 4.6 Testing blocking antibody concentrations.**

Synovial RA and PsA fibroblasts ( $10 \times 10^3$ ) were cultured with a cocktail of human recombinant IL-17A, IL-17F, IL-17AF and TNF $\alpha$  (all at 10ng/ml) (+) and indicated neutralising antibodies at the indicated concentrations for 24 hours (n=1-3). Cell supernatants were collected and protein levels of the indicated cytokines were determined by ELISA. Dashed red and blue lines indicate lower detection limit and upper detection limit, respectively.

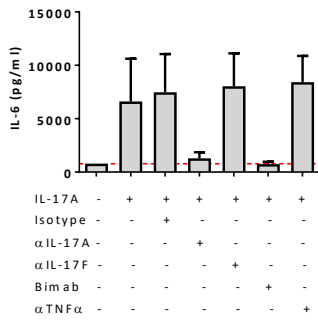




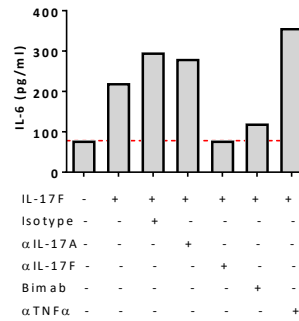
**Figure 4.7 IL-17F blocking antibody inhibits IL-17F function.**

Synovial PsA fibroblasts ( $10 \times 10^3$ ) were cultured with human recombinant IL-17F (10ng/ml) and indicated concentrations of anti-IL-17F blocking antibody for 24 hours. Cell supernatants were collected and protein levels of **(A)** IL-17F ( $n=1$ ) and **(B)** IL-6 ( $n=1$ ) were determined by ELISA. **(C)** Synovial PsA fibroblasts ( $10 \times 10^3$ ) were cultured with IL-17A and TNF $\alpha$  with titrated doses of anti-IL-17F blocking antibody for 24 hours. Fibroblast culture supernatants were measured for IL-6 and IL-8 by ELISA ( $n=1$ ). Dashed red lines indicate the lower detection limit. The solid green line highlights the control protein level.

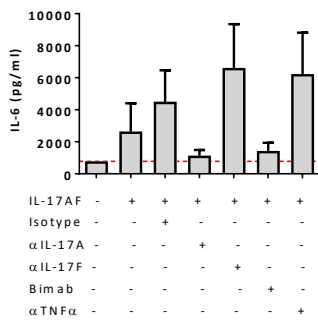
### A IL-17A



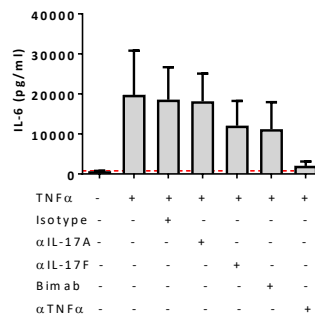
### B IL-17F



### C IL-17AF



### D TNFα



**Figure 4.8 Testing blocking antibody specificity.**

Synovial RA and/or PsA fibroblasts ( $10 \times 10^3$ ) were cultured with 10ng/ml of **(A)** IL-17A, **(B)** IL-17F, **(C)** IL-17AF and **(D)** TNFα in the presence of blocking antibodies at a concentration of 10μg/ml. Following 24 hours incubation, cell supernatants were collected and IL-6 protein levels were determined by ELISA (n=1-2). Dashed red and blue lines indicate lower detection limit and upper detection limit, respectively.

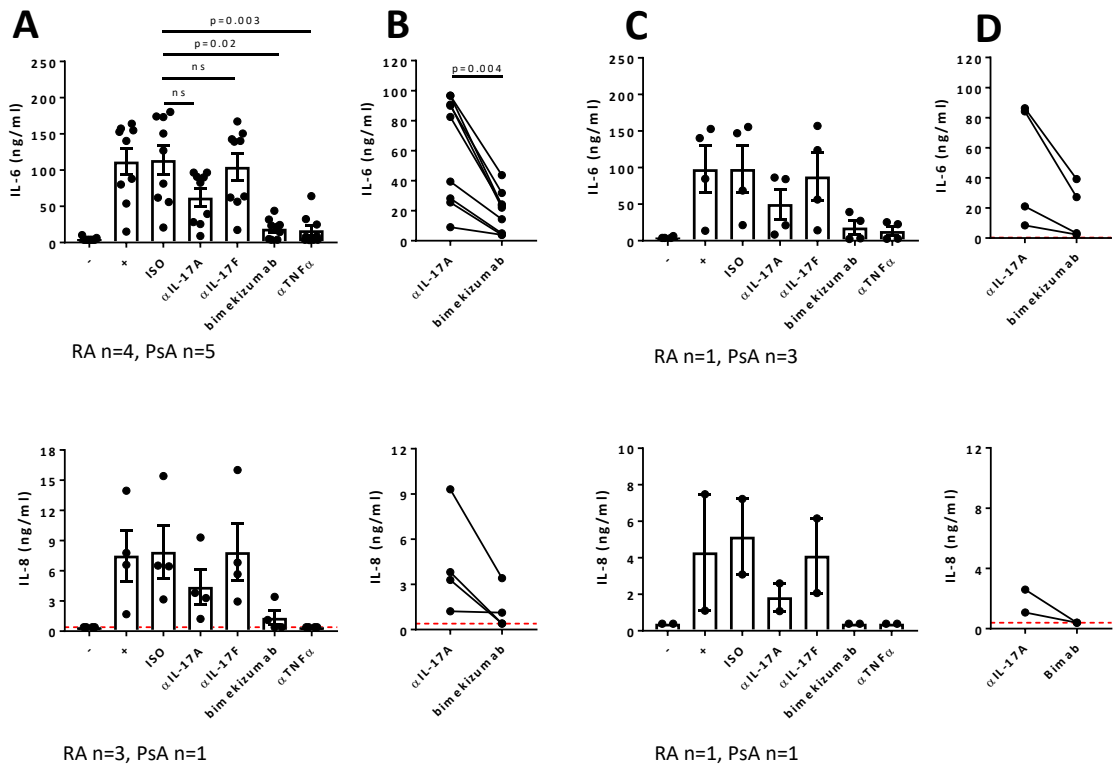
#### **4.2.5 Dual IL-17A and IL-17F blockade in a fibroblast:recombinant cytokine system**

Moving forward from successful validation of the IL-17 blocking antibodies, the next aim was to assess the effect of dual IL-17A and IL-17F blockade in a reductionist system. To achieve this, a cocktail of recombinant cytokine IL-17A, IL-17F, IL-17AF and TNF $\alpha$  were added to synovial fibroblast cultures in the presence of ‘low’ or ‘high’ (1 vs.10 $\mu$ g/ml) concentrations of IL-17 blocking antibody and anti-TNF $\alpha$ . These experiments included a human IgG1 isotype control. As illustrated in Figure 4.9, addition of the cytokine cocktail led to an increase in IL-6 fibroblast cytokine production in comparison to the medium alone condition. Addition of isotype control or anti-IL-17F blocking antibody had no effect on IL-6 production. However, IL-6 secretion was reduced upon addition of IL-17A blocking antibody. Interestingly, IL-6 production was further reduced with a combined blockade of IL-17A and IL-17F by bimekizumab. Blockade of TNF $\alpha$  reduced IL-6 levels similar to that of bimekizumab. Similar results were observed for IL-8. Moreover, similar results were observed when using blocking antibodies at both 1 and 10 $\mu$ g/ml. Collectively, these data suggest that a combined blockade of IL-17A, IL-17F and IL-17AF is more effective at reducing inflammation than blockade of IL-17A alone. In this experiment, the role of IL-17AF cannot be assessed as both bimekizumab and the anti-IL-17A blocking antibody both inhibit IL-17AF.

Next, we sought to demonstrate that the greater effect of bimekizumab was a result of a dual IL-17A and IL-17F blockade and not a result of a potent blockade of IL-17A. To achieve this, a cocktail of IL-17A, IL-17F, IL-17AF and TNF $\alpha$  was added to fibroblast assays and the effect of bimekizumab and a combination of both anti-IL-17A and anti-IL-17F single blocking antibodies was compared. As depicted in Figure 4.10 bimekizumab and a combination of single IL-17A and IL-17F blocking antibodies reduced fibroblast IL-6 and IL-8 secretion to comparable levels. This suggests that the superior result of bimekizumab can be attributed to dual IL-17A and IL-17F blockade.

All blocking antibodies at 1µg/ml

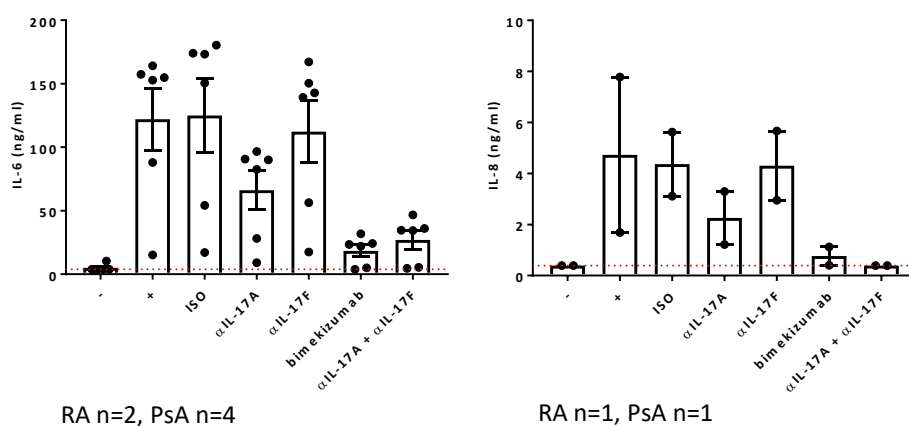
All blocking antibodies at 10µg/ml



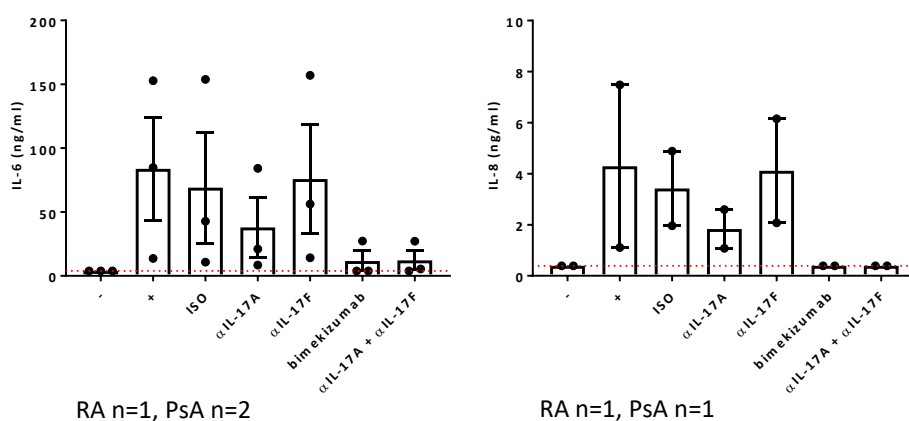
**Figure 4.9 A combined blockade of IL-17A and IL-17F reduces pro-inflammatory secretion by fibroblasts to a greater extent than blockade of IL-17A alone.**

RA and PsA synovial fibroblasts ( $10 \times 10^3$ ) were cultured with a cocktail of human recombinant IL-17A, IL-17F, IL-17AF and TNF $\alpha$  (+) in the absence or presence of blocking antibodies (at the indicated concentrations) for 24 hours. Cell supernatants were collected and IL-6 and IL-8 protein levels were determined by ELISA. (A) displays the results of all blocking antibodies at 1µg/ml while (B) displays the data for anti-IL-17A and bimekizumab in a different format. (C-D) shows the results of the blocking antibodies at 10µg/ml. Dashed red and blue lines indicate lower detection limit and upper detection limit, respectively (n=2-9).

### A All blocking antibodies at 1µg/ml



### B All blocking antibodies at 10µg/ml



**Figure 4.10 Similar to bimekizumab, combining single IL-17A and IL-17F blocking antibodies reduced IL-6 and IL-8 fibroblast secretion levels to a greater extent than IL-17A blockade alone.**

RA and PsA synovial fibroblasts ( $10 \times 10^3$ ) were cultured with a cocktail of human recombinant IL-17A, IL-17F, IL-17AF and  $\text{TNF}\alpha$  (+) in the absence or presence of blocking antibodies for 24 hours. Cell supernatants were collected and IL-6 and IL-8 protein levels were determined by ELISA. **(A)** displays the results of isotype control at 2µg/ml and the remaining blocking antibodies at 1µg/ml. Anti-IL-17A and anti-IL-17F blocking antibodies were added in combination at 1µg/ml each. **(B)** displays the results of isotype control at 20µg/ml and the remaining blocking antibodies at 10µg/ml. Anti-IL-17A and anti-IL-17F blocking antibodies were added in combination at 10µg/ml each. (n=2-6).

#### **4.2.6 Dual IL-17A and IL-17F blockade in bulk CD4+ T cell supernatant cell cultures**

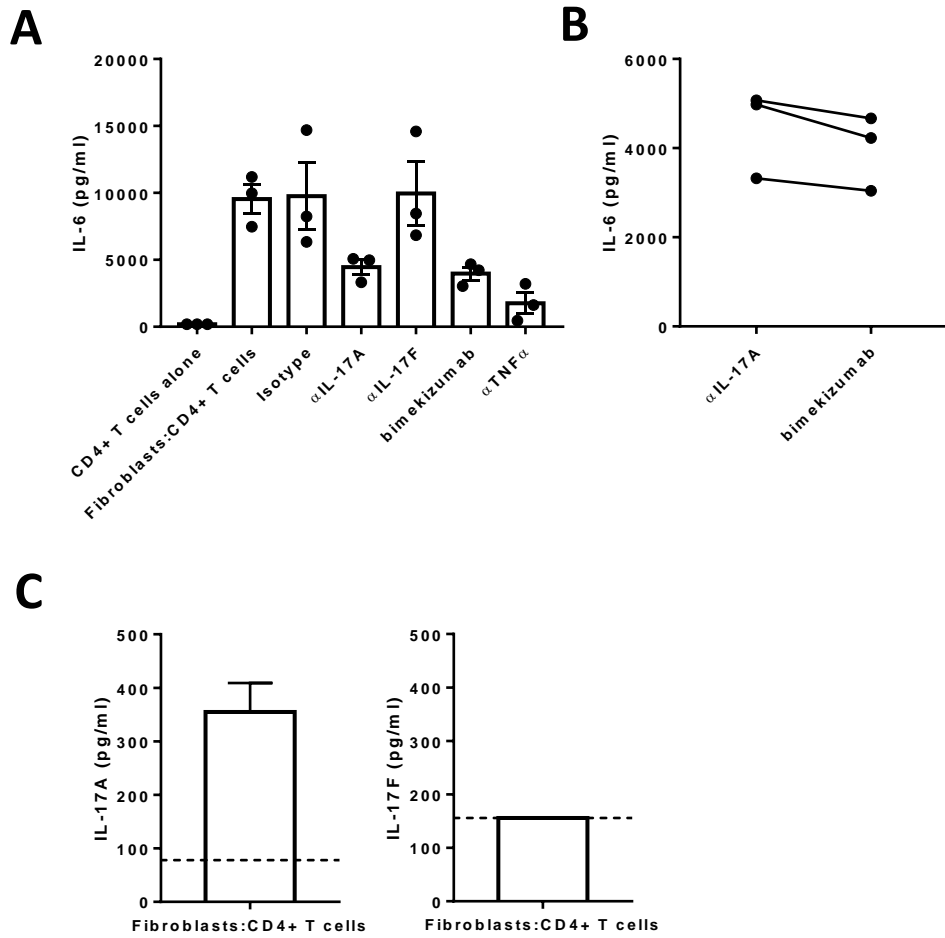
Our next aim was to investigate in a physiological system whether dual IL-17A and IL-17F blockade is more effective at reducing secretion of pro-inflammatory mediators from synovial fibroblasts than blockade of IL-17A alone.

To generate a physiological system, we sought to block IL-17A and IL-17F in supernatants from bulk CD4+ T cell cultures. I previously demonstrated in Chapter 3 that culturing CD4+ T cells with IL-1 $\beta$ , IL-23,  $\alpha$ CD28 and  $\alpha$ CD3 induced IL-17A, IL-17F and IL-17AF protein secretion. My initial approach was to add this supernatant to the fibroblasts. However, results revealed that the IL-1 $\beta$  and/or IL-23 recombinant cytokines used to stimulate the CD4+ T cells elicited a strong IL-6 and IL-8 fibroblast response alone and thus was not suitable for the blocking experiments (data not shown). The next approach involved generating supernatants from CD4+ T cells cultured with  $\alpha$ CD28 and  $\alpha$ CD3 only. However, this method did not induce sufficient levels of the IL-17 cytokines (data not shown).

Next, CD4+ T cells were cultured for 3 days with IL-1 $\beta$ , IL-23,  $\alpha$ CD28 and  $\alpha$ CD3 followed by re-stimulating cells with PMA and ionomycin for either 0, 1.5 or 3 hours. Cells were then washed to ensure removal of any residual IL-1 $\beta$ , IL-23 or PMA and ionomycin and replated ( $40 \times 10^3$  cells in 200 $\mu$ l) for 24 hours incubation. Cell supernatants were then collected and first analysed for IL-17A and IL-17F protein expression via ELISA. No IL-17 protein was detected in CD4+ T cell cultures which were not stimulated with PMA and ionomycin (data not shown). Moreover, levels of IL-17A and IL-17F were low in CD4+ T cell cultures re-stimulated for 1.5 or 3 hours with PMA and ionomycin. Therefore, blocking experiments were not performed using this supernatant (data not shown).

Cell to cell interactions are described to be important in the RA and PsA synovium. My next approach was to explore the effect of culturing *in vitro* stimulated CD4+ T cells (i.e not their supernatant) with synovial fibroblasts in the absence or presence of

blocking antibodies. To achieve this, CD4<sup>+</sup> T cells were cultured for 3 days with IL-1 $\beta$ , IL-23,  $\alpha$ CD28 and  $\alpha$ CD3 followed by re-stimulation with PMA and ionomycin for 3 hours. Following a wash step, stimulated CD4<sup>+</sup> T cells were cultured with synovial fibroblasts were then cultured with the at a 2.5:1 ratio in the absence or presence of blocking antibodies for 24 hours. Supernatants were then collected and levels of IL-6 were measured by ELISA. As shown in Figure 4.11, addition of stimulated bulk CD4<sup>+</sup> T cells to fibroblasts led to an increase in IL-6 secretion. Levels of IL-6 were reduced upon addition of anti-IL-17A blocking antibody, bimekizumab and TNF $\alpha$  inhibitor, while blockade of IL-17F had little effect. In all three experiments levels of IL-6 were lower with the addition of bimekizumab vs. anti-IL-17A blocking antibody, however, the differences were minimal. It was considered that the bimekizumab vs. anti-IL-17A blockade results were not striking because the IL-17 protein levels present in the bulk CD4<sup>+</sup> T cell supernatant were too low. Indeed, as shown in Figure 4.11B levels of IL-17F present in the supernatant of fibroblasts cultured with CD4<sup>+</sup> T cells were below the detection limit and levels of IL-17A were also low. Therefore, the next aim was to generate CD4<sup>+</sup> T cell supernatants, which were enriched for the IL-17 cytokines.



**Figure 4.11 Assessing the effect of dual IL-17A and IL-17F blockade in bulk CD4+ T cell supernatants and synovial fibroblast cultures.**

Bulk CD4+ T cells were cultured with  $\alpha$ CD3,  $\alpha$ CD28, IL-1 $\beta$  and IL-23. Following 3 days incubation, cells were stimulated with PMA and ionomycin for 3 hours.  $10 \times 10^3$  RA and PsA synovial fibroblasts were cultured with  $25 \times 10^3$  activated CD4+ T cells and in the presence of neutralising antibodies (all at 1 $\mu$ g/ml) for 24 hours. **(A)** Cell supernatants were collected and IL-6 (n=3) protein levels were determined by ELISA. **(B)** displays the anti-IL-17A vs. bimekizumab in a different format. Individual donors (n=2) were incubated with RA and/or PsA fibroblasts. **(C)** displays the levels of IL-17A and IL-17F present in cultures of RA fibroblasts cultured with activated CD4+ T cells (n=2 individual donors).



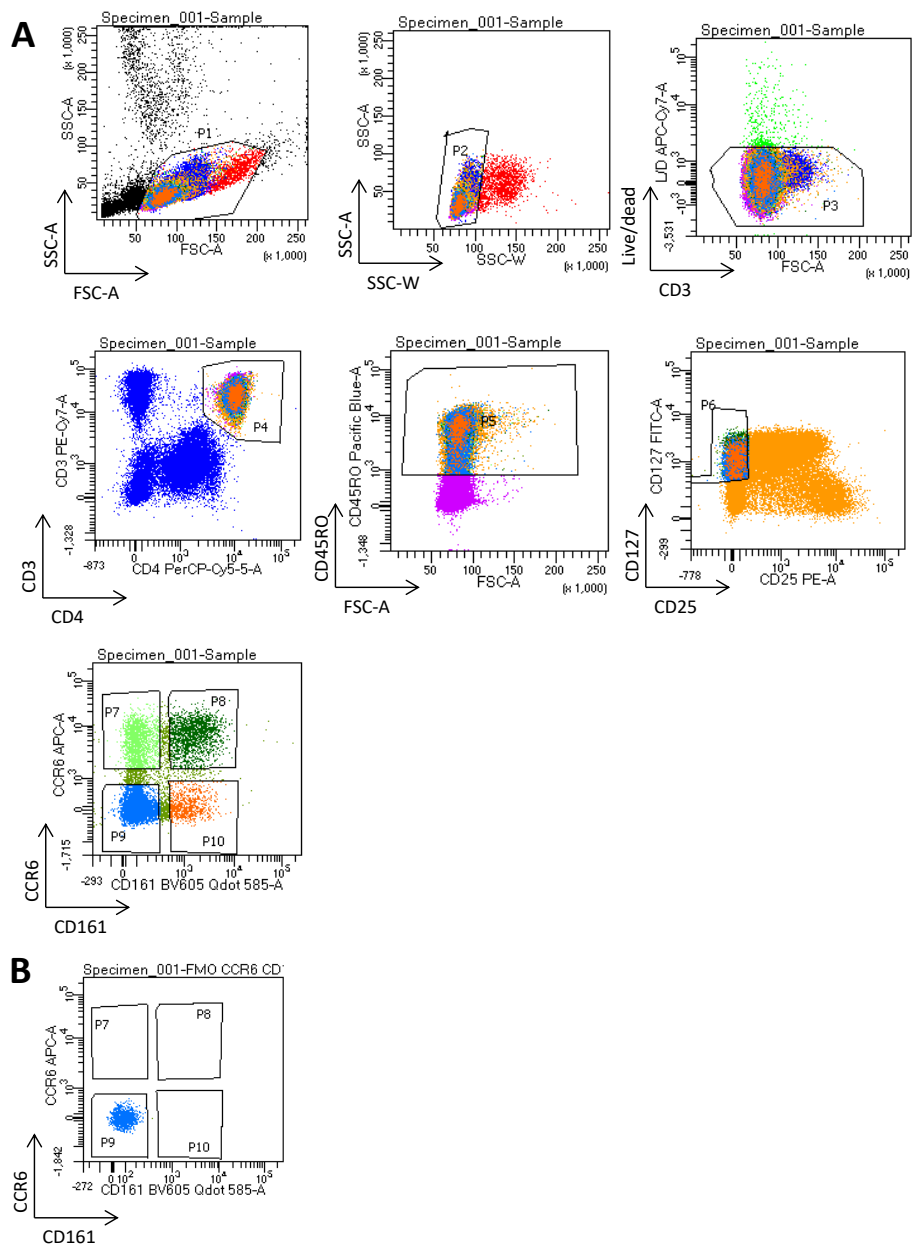
#### **4.2.7 Blockade of IL-17A and IL-17F in cultures of synovial fibroblasts and sorted CCR6+ CD161+ CD4+ T cells**

Our next aim was to block IL-17A and IL-17F in CD4+ T cell supernatants enriched for the IL-17 cytokines. An ideal method would involve generating supernatant from sorted IL-17A and IL-17F CD4+ T cells. However, as previously mentioned, while an IL-17A secretion assay is commercially available, there are no secretion kits available for IL-17F.

To enrich for the IL-17 cytokines, one approach involved sorting CD45RO+ CD25- CCR6+ CD161+ CD3+ CD4+ T cells. As previously discussed in chapter 3, section 3.2.2.1, CCR6 and CD161 have been described as markers of Th17 cells. However, the expression of CCR6 and CD161 on CD4+ T cells is down-regulated following a 3 day *in vitro* culture and re-stimulation with PMA and ionomycin. To overcome this, CCR6+ and CD161+ cells were sorted (using FACS Aria sorter) from freshly isolated CD4+ T cells. Cells were sorted into four populations CCR6- CD161-, CCR6+ CD161-, CCR6+ CD161+ or CCR6- CD161+. This gating strategy is shown in figure 4.12. Sorted cells were cultured for three days with IL-1 $\beta$ , IL-23, anti-CD28 mAb and anti-CD3 mAb and re-stimulated for 3 hours with PMA and ionomycin. Cells were then washed and cultured with synovial fibroblasts at a 2.5:1 ratio in the absence or presence of blocking antibodies. As a control, stimulated bulk CD4+ T cells were also cultured with synovial fibroblasts. After 24 hours, cell culture supernatants were collected.

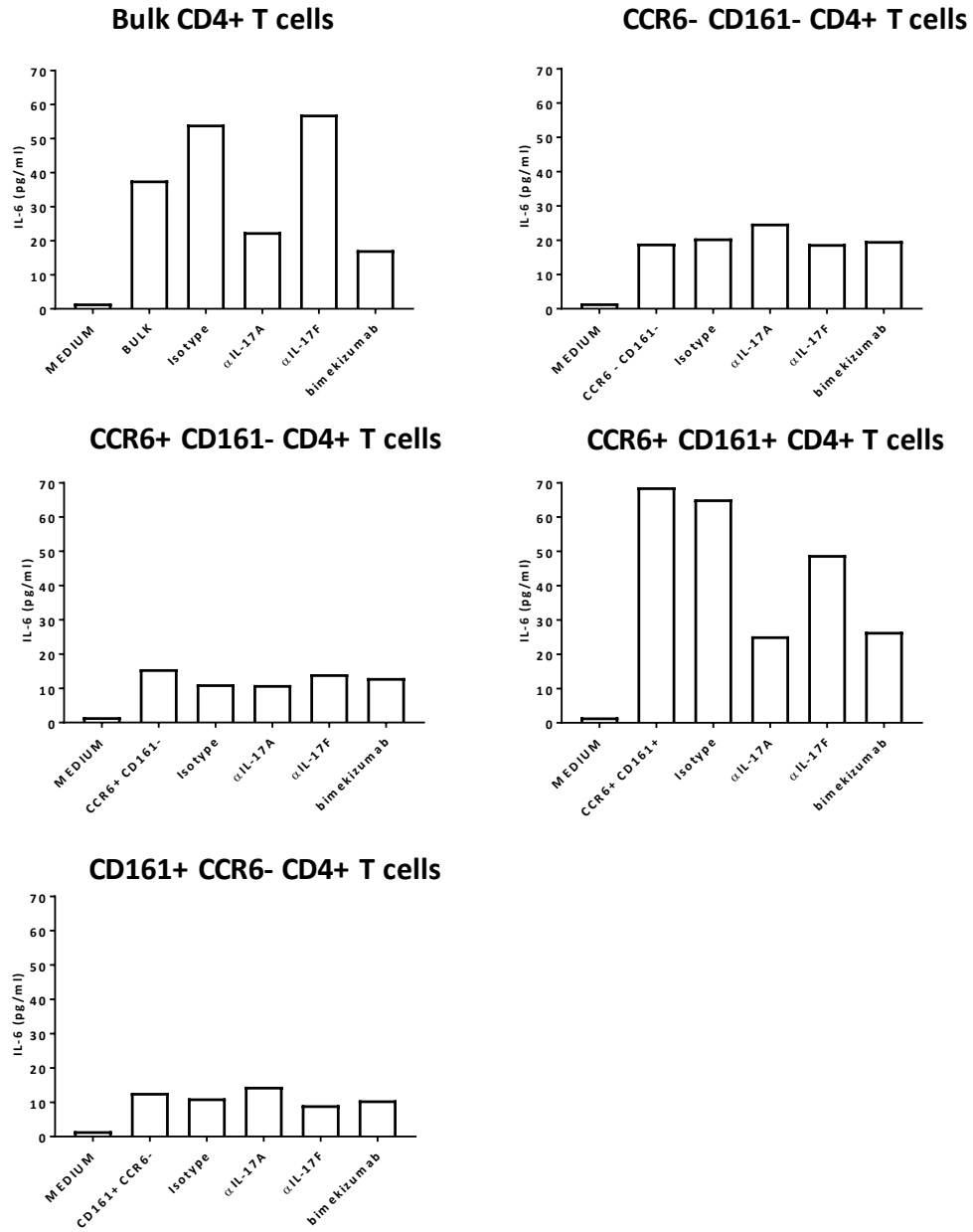
ELISA analysis showed that in comparison to bulk CD4+ T cells and sorted CCR6+ CD161+ CD4+ T cells, sorted CCR6- CD161-, CCR6+ CD161- and CCR6- CD161+ elicited low levels of IL-6 secretion by fibroblasts (Figure 4.13). Sorted CCR6+ CD161+ CD4+ T cells elicited a slightly higher level of IL-6 secretion when compared with bulk CD4+ T cells, suggesting a modest enrichment of the IL-17 cytokines. Unfortunately, there was not enough culture supernatant present to examine the presence of IL-17A and IL-17F. In the CCR6+ CD161+ CD4+ T cell/fibroblast culture

system, while IL-17A blockade reduced levels of IL-6, no superior effect was observed with bimekizumab. A limitation to this experiment is that not all total CCR6+ and/or CD161+ CD4+ T cells express IL-17A and IL-17F. Furthermore, not all IL-17A+ and IL-17F+ CD4+ T cells express CCR6 and/or CD161. Therefore we sought a different method to enrich for IL-17A and IL-17F expressing CD4+ T cells.



**Figure 4.12 Gating strategy for sorting for CCR6+ CD161+ T cells.**

Based on forward scatter/side scatter (FSC/SSC) properties, viable lymphocytes were gated. A FSC-W/FSC-A gate was applied allowing the exclusion of doublet cells. Dead cells were excluded based on their uptake of eFluor 780. Within live, single cells CD3+ CD4+ T cells were gated followed by selection of CD45RO+ memory T cells. Next, CD127+ CD25- cells were gated allowing the exclusion of Tregs. Finally, populations of CCR6- CD161-, CCR6+ CD161-, CCR6+ CD161+ and CD161+ CCR6- T cells were gated and collected. **(B)** To assess non-specific binding, an FM control was acquired.



**Figure 4.13 Dual IL-17A and IL-17F blockade in cultures of synovial fibroblasts and sorted CCR6+ and CD161+ CD4+ T cells.**

From fresh CD4+ T cells CD45RO+ CD127+ CD25- CD4+ T cells were sorted into CCR6- CD161-, CCR6+ CD161-, CCR6+ CD161+ and CCR6- CD161+ populations. Alongside bulk CD4+ T cells, sorted cell populations were cultured with αCD3, αCD28, IL-1β and IL-23 for 3 days. Following a three hour PMA and ionomycin re-stimulation, cells were washed and cultured with PsA synovial fibroblasts at a 2.5:1 ratio in the absence or presence of blocking antibodies (all at 1μg/ml).

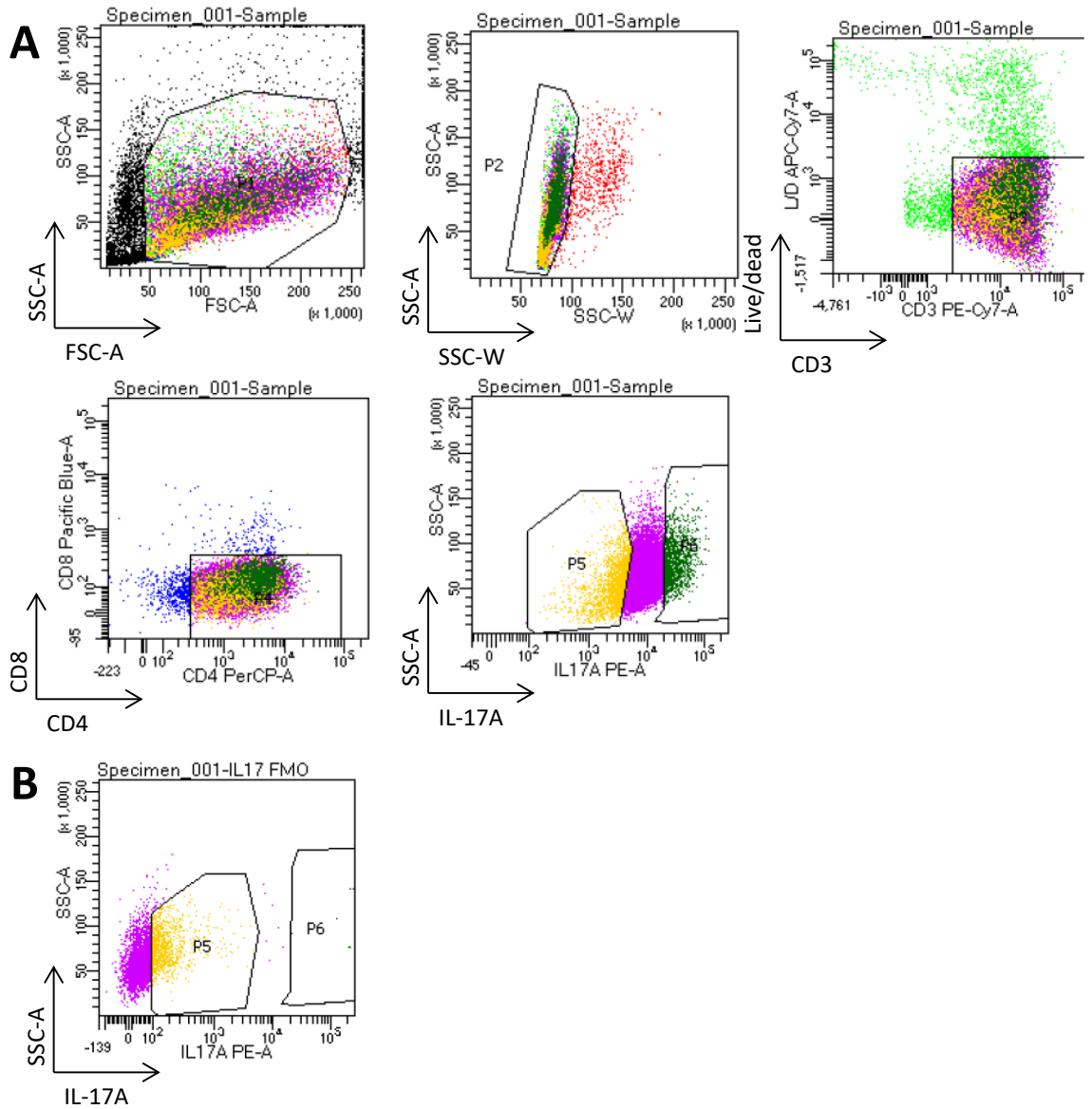
#### 4.2.8 Sorting IL-17A+ CD4+ T cells for functional analysis

As demonstrated in chapter 3, section 3.2.1.3, CD4+ T cells that co-express IL-17A and IL-17F are present in CD4+ T cell cultures stimulated for 3 days with IL-1 $\beta$ , IL-23, anti-CD28 mAb and anti-CD3 mAb. Therefore, I decided to sort IL-17A+ CD4+ T cells and assess the effect of dual IL-17A and IL-17F blockade in this supernatant. It is important to note that in this method IL-17F+ IL-17A- CD4+ T cells are not captured.

To sort IL-17A+ CD4+ T cells, bulk CD4+ T cells were first cultured with IL-1 $\beta$ , IL-23,  $\alpha$ CD28 and  $\alpha$ CD3 for 3 days. Cells were then re-stimulated with PMA and ionomycin for 1.5 hours and a IL-17A secretion assay was performed. Using a FACS Aria sorter both IL-17A+ and IL-17A- CD4+ T cells were sorted. A gating strategy of the sort is illustrated in Figure 4.14. Sorted IL-17A+ and IL-17A- CD4+ T cells were cultured in media for 24 hours. The supernatant was then collected for further analysis and functional fibroblast assays. To validate the IL-17A secretion assay method, the IL-17A- and IL-17A+ CD4+ T cell supernatant generated was analysed for the presence of IL-17A, IL-17F and IL-17AF protein. Detectable levels of IL-17A, IL-17F and IL-17AF in the IL-17A+ CD4+ T cells were observed, while no IL-17 cytokines were detectable from the IL-17A- CD4+ T cell supernatants (Figure 4.15A). This confirms the successful enrichment of IL-17A+ CD4+ T cells. Next, the effect of IL-17A- and IL-17A+ CD4+ T cell supernatant on the IL-6 and IL-8 production from synovial fibroblasts was compared. Addition of IL-17A+ CD4+ T cell supernatant led to a strong dose-dependent induction of IL-6 and IL-8 fibroblast secretion. These data also indicated that addition of 20% IL-17A+ CD4+ T cell supernatant elicits robust IL-6 and IL-8 detection levels, suitable to progress to blocking experiments (Figure 4.14B). In contrast, addition of the IL-17A- CD4+ T cell supernatants did not induce any IL-8 secretion from the synovial fibroblasts and only a limited amount of IL-6 protein was observed upon addition of the maximum 20% concentration. It is important to note that from these data we cannot conclude definitively that IL-17A- CD4+ T cell supernatants do not elicit an IL-6 or IL-8 fibroblast response. This is

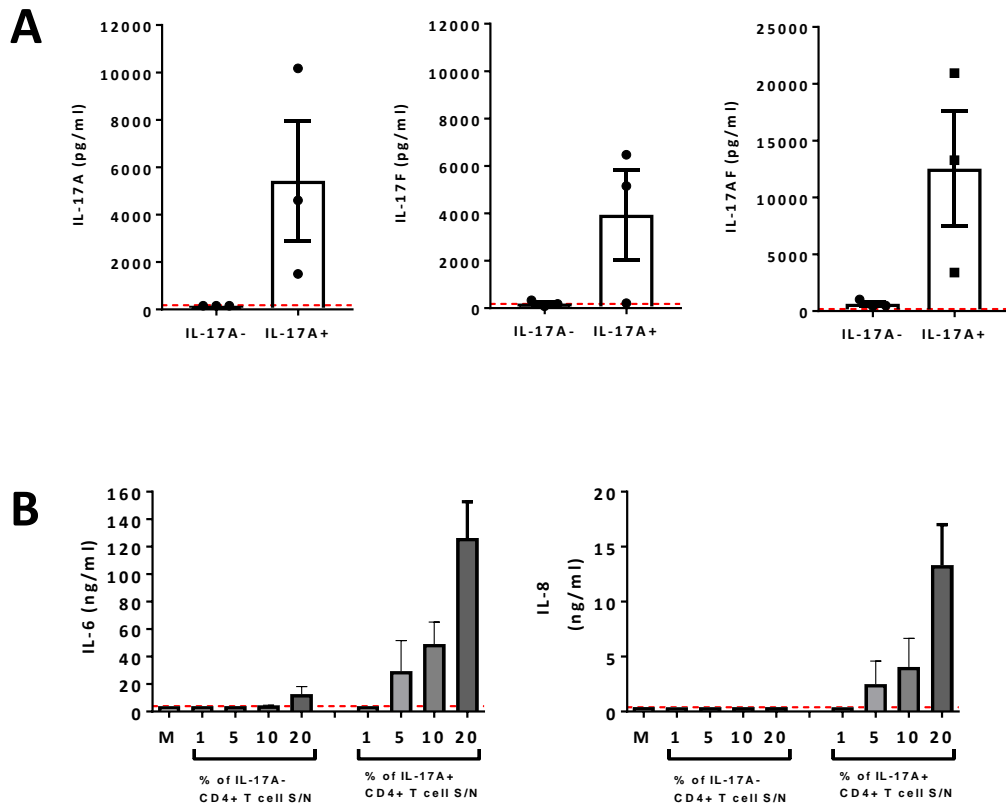
because there may have been a proportion of IL-17A<sup>-</sup> CD4<sup>+</sup> T cells, which were not within the IL-17A<sup>-</sup> CD4<sup>+</sup> T cell-sorting gate, shown in Figure 4.14.

Synovial fibroblasts were cultured with 20% of IL-17A<sup>+</sup> CD4<sup>+</sup> T cell supernatants and the effect of IL-17A and IL-17F dual blockade was assessed. As depicted in Figure 4.15, addition of IL-17A<sup>+</sup> CD4<sup>+</sup> T cell supernatants to fibroblasts led to an increase in IL-6 secretion. Blocking IL-17A reduced IL-6 secretion, whereby IL-17F blockade had no effect. Addition of bimekizumab did not show significantly reduced IL-6 protein when compared to anti-IL-17A. Figure 4.16B examines the anti-IL-17A vs. bimekizumab data in more detail. In four out of five experiments bimekizumab reduced IL-6 secretion by fibroblasts to a greater extent than IL-17A blockade alone. However, in two of these experiments the decrease was minimal. In one out of five experiments, IL-6 was slightly increased upon culture with bimekizumab. Therefore no consistent results were observed. I sought to determine whether the experiments which exhibited a significant decrease in IL-6 levels in bimekizumab vs. anti-IL-17A correlated with high IL-17F protein levels present in the matched neat IL-17A<sup>+</sup> CD4<sup>+</sup> T cell supernatants. Indeed, the sorted IL-17A<sup>+</sup> CD4<sup>+</sup> T cell supernatant which displayed the highest levels of IL-17F protein matched with the greatest decrease in IL-6 levels in bimekizumab vs. anti-IL-17A (Figure 4.16C). In general a positive correlation was observed across the cumulative data. While IL-8 secretion by fibroblasts was increased upon addition of IL-17A<sup>+</sup> CD4<sup>+</sup> T cell supernatant, addition of anti-IL-17A and bimekizumab reduced IL-8 to undetectable levels (Figure 4.16C).



**Figure 4.14 Gating strategy for sorting IL-17A+ CD4+ T cells.**

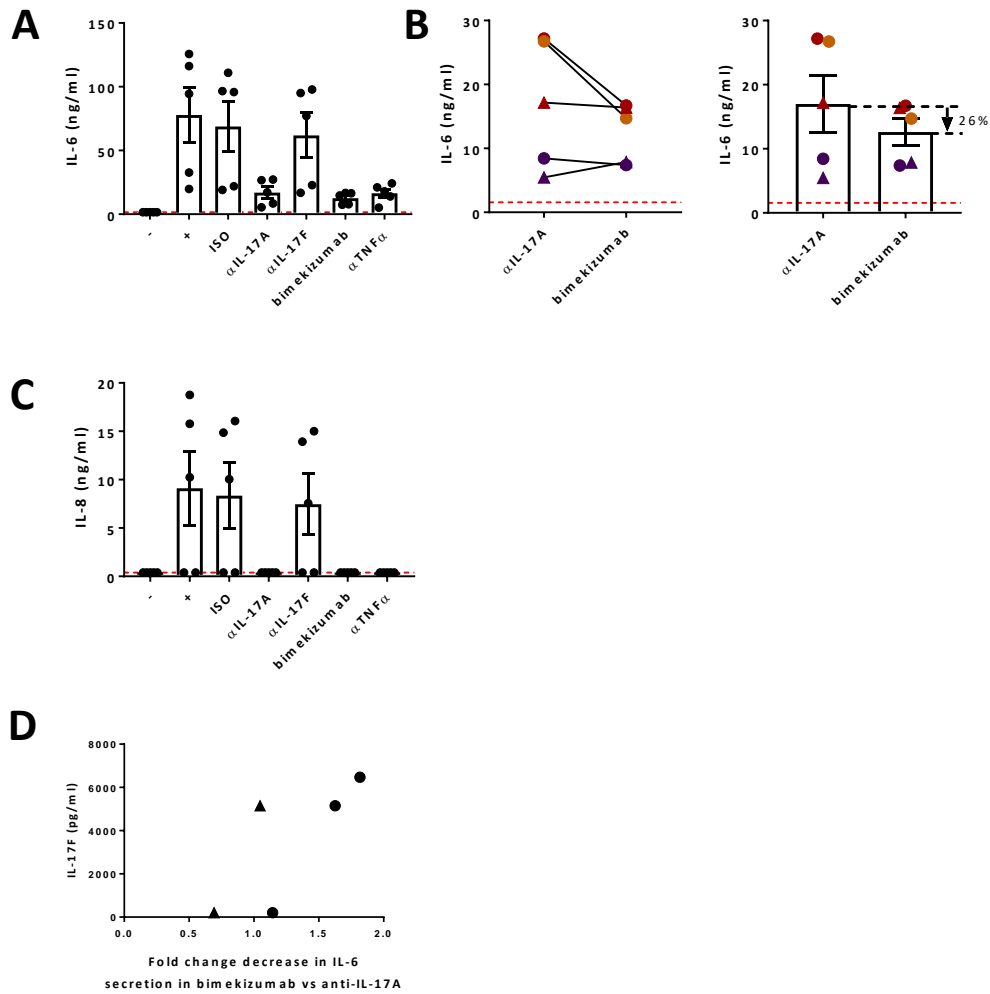
After performing an IL-17A secretion assay (described in the materials and methods), viable lymphocytes were gated based on their SSC-W/FSC-A properties. Using a SSC-A vs. SSC-W plot doublet cells were excluded and dead cells were excluded based on their uptake of the viability dye, eFluor 780. Within CD3+ CD4+ CD8- T cells, expression of IL-17A was analysed. IL-17A+ and IL-17A- gates were set and cells were sorted and collected. **(B)** To assess non-specific binding, a negative control was acquired. The negative control consisted of stimulated cells which had been stained with viability dye, CD3, CD4, CD8 and the secondary IL-17A detection antibodies but had not been incubated with the IL-17A capture reagent.



**Figure 4.15 Comparison of sorted IL-17A- vs. IL-17A+ sorted CD4+ T cell supernatants on the induction of IL-6 and IL-8 from synovial fibroblasts.**

Bulk CD4+ T cells were cultured with  $\alpha$ CD3,  $\alpha$ CD28, IL-1 $\beta$  and IL-23 for 3 days. Following a 1.5 hour PMA and ionomycin re-stimulation an IL-17A secretion assay was performed. A FACS Aria sorter was used to sort IL-17A+ and IL-17A- CD4+ T cells. Sorted IL-17A+ and IL-17A- CD4+ T cells were cultured in RPMI media for 24 hours. **(A)** Cell supernatants were collected and IL-17A, IL-17F and IL-17AF protein levels were analysed by ELISA. Dashed red lines indicate lower detection limit. Results show analysis from three individual donors. **(B)** Supernatants were collected and added to  $10 \times 10^3$  PsA and RA synovial fibroblasts diluted in different ratios of DMEM media (CD4+ T cell donor n=1 added to fibroblasts RA n=1, PsA n=1).





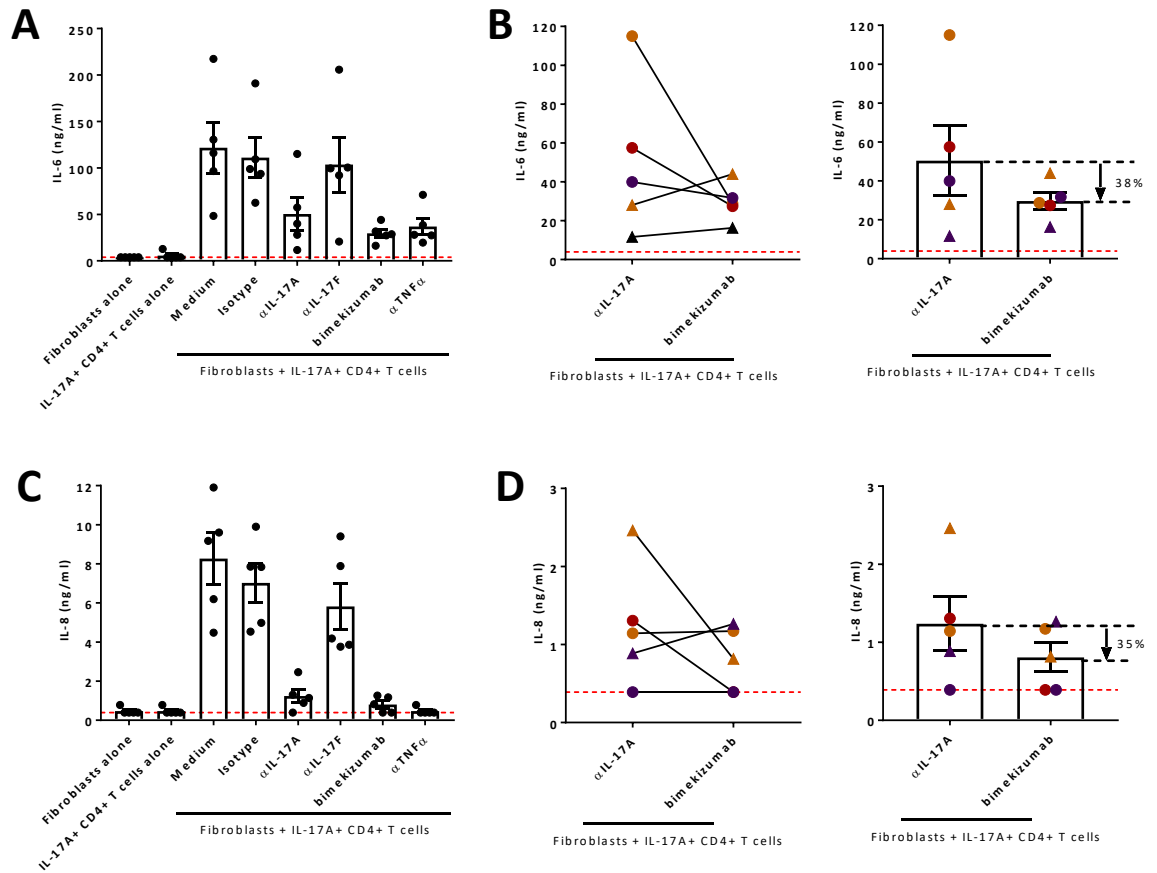
**Figure 4.16 Assessing the effect of dual IL-17A and IL-17F blockade in Th17 supernatant.**

Bulk CD4<sup>+</sup> T cells were cultured with  $\alpha$ CD3,  $\alpha$ CD28, IL-1 $\beta$  and IL-23 for 3 days. Following a 1.5 hour PMA and ionomycin re-stimulation an IL-17A secretion assay was performed. A FACS Aria sorter was used to sort IL-17A<sup>+</sup> CD4<sup>+</sup> T cells. Sorted IL-17A<sup>+</sup> CD4<sup>+</sup> T cells were cultured in RPMI media for 24 hours. Supernatants were collected, diluted at a ratio of 1:5 in media and added to 10 $\times$ 10<sup>3</sup> PsA and RA synovial in the absence or presence of blocking antibodies (10 $\mu$ g/ml). Supernatants were collected and analysed for IL-6 and IL-8 protein levels via ELISA. **(A)** displays the results of all blocking antibodies while **(B)** displays the data for anti-IL-17A and bimekizumab in a different format. Circle symbols represent RA synovial fibroblasts and triangle symbols represent PsA synovial fibroblasts. Each colour represents sorted IL-17A<sup>+</sup> CD4<sup>+</sup> T cell supernatant from an individual healthy donor. **(C)** displays IL-8 ELISA results. **(D)** The fold change decrease in IL-6 secretion in bimekizumab vs. anti-IL-17A conditions was correlated to IL-17F protein levels present in the matching neat sorted IL-17A<sup>+</sup> CD4<sup>+</sup> T cell supernatant. (n=5: IL-17A<sup>+</sup> CD4<sup>+</sup> supernatant individual donors n=3 cultured with RA and/or PsA fibroblasts).

#### **4.2.9 Examining the interaction of synovial fibroblasts and sorted IL-17A+ and IL-17A- CD4+ T cells**

Next, synovial fibroblasts were cultured with sorted IL-17A- and IL-17A+ CD4+ T cells at a 1:2.5 ratio for 24 hours. Supernatants were then collected and IL-6 and IL-8 protein levels were measured by ELISA. Similar to our results observed with IL-17A sorted cell supernatant, IL-17A+ CD4+ T cells elicited an IL-6 and IL-8 fibroblast response, whereas only low levels of IL-6 were observed with IL-17A- CD4+ T cells (data not shown). As previously mentioned, it is important to note that from these data we cannot conclude that IL-17A- CD4+ T cells do not elicit an IL-6 or IL-8 fibroblast response. This is because there may have been a proportion of IL-17A- CD4+ T cells, which were not within the IL-17A- CD4+ T cell-sorting gate

The effect of IL-17 and TNF $\alpha$  blockade was assessed in cultures of synovial fibroblasts and sorted IL-17A+ CD4+ T cells. As depicted in Figure 4.16, in comparison to the isotype control, addition of anti-IL-17A blocking antibody decreased IL-6 secretion by fibroblasts, while IL-17F blockade had no effect. In 3 out of 5 experiments bimekizumab reduced IL-6 levels to a greater extent than IL-17A blockade alone, however in the remaining two experiments elevated levels of IL-6 was observed in bimekizumab conditions vs anti-IL-17A. Therefore, no consistent result was observed.



**Figure 4.17 Assessing the effect of dual IL-17A and IL-17F blockade in fibroblast and IL-17A+ CD4+ T cell cultures.**

Bulk CD4+ T cells were cultured with  $\alpha$ CD3,  $\alpha$ CD28, IL-1 $\beta$  and IL-23 for 3 days. Following a 1.5 hour PMA and ionomycin re-stimulation an IL-17A secretion assay was performed. A FACS Aria sorter was used to sort IL-17A+ CD4+ T cells. IL-17A+ CD4+ T cells were cultured with RA and PsA synovial fibroblasts at a 2.5:1 ratio in the absence or presence of blocking antibodies (all at 1 $\mu$ g/ml). (A) displays the results of all blocking antibodies while (B) displays the data for anti-IL-17A and bimekizumab in a different format. Circle symbols represent RA synovial fibroblasts and triangle symbols represent PsA synovial fibroblasts. Each colour represents sorted IL-17A+ CD4+ T cell supernatant from an individual healthy donor. (C-D) displays IL-8 ELISA results. (n=5: IL-17A+ CD4+ T cell individual donors n=3 cultured with RA and/or PsA fibroblasts).

#### 4.2.10 Development of an IL-17A and IL-17F CD4<sup>+</sup> T cell capture assay

The focus of the work described in this chapter was to investigate whether IL-17F has the functional potential to contribute to the immunopathology of inflammatory arthritis. Currently we have shown that IL-17F synergises with TNF $\alpha$ , enhancing its inflammatory potential. Moreover, we have demonstrated using recombinant cytokines that dual blockade of IL-17A and IL-17F is more effective than blockade of IL-17A alone. The superior effect of dual IL-17A and IL-17F blockade was demonstrated in fibroblast cultures with IL-17A<sup>+</sup> CD4<sup>+</sup> T cells and IL-17A<sup>+</sup> CD4<sup>+</sup> T cell supernatant, however, the effect was not as significant as observed with the recombinant cytokine cultures. This could be due to the limitations of using IL-17A<sup>+</sup> CD4<sup>+</sup> T cells. With no IL-17F secretion assay commercially available, only IL-17A<sup>+</sup> CD4<sup>+</sup> T cells could be sorted. While this method captured IL-17A<sup>+</sup> IL-17F<sup>-</sup> and IL-17A<sup>+</sup> IL-17F<sup>+</sup> CD4<sup>+</sup> T cells, IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells were not captured.

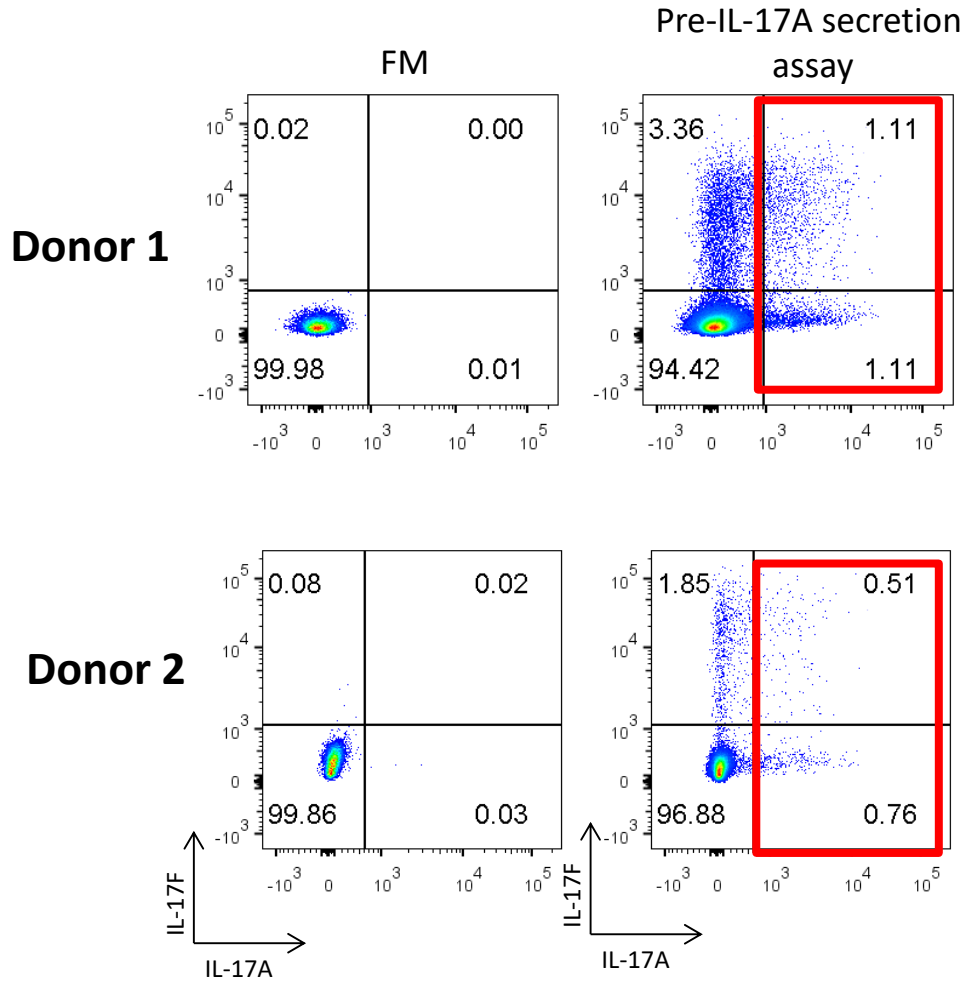
Figure 4.18 illustrates the extent to which IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells may be lost during an IL-17A secretion assay. Here, bulk CD4<sup>+</sup> T cells were cultured for 3 days with IL-1 $\beta$ , IL-23,  $\alpha$ CD28 mAb and  $\alpha$ CD3 mAb. Cells were pooled and while the majority were stimulated for 1.5 hours with PMA and ionomycin and followed by an IL-17A secretion assay, a small number of cells were stimulated for 1.5 hours with PMA, ionomycin and golgistop. This was followed by an intracellular cytokine stain. Figure 4.18 shows the flow cytometric results of two individual donors. The results demonstrate that a significant number of IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells were present in the pre-IL-17A sorted samples which would not be captured by the IL-17A<sup>+</sup> CD4<sup>+</sup> T cell cytokine secretion assay. This suggests that performing the blocking experiment using sorted IL-17A<sup>+</sup> CD4<sup>+</sup> T cells may not reveal the superior extent of dual IL-17A and IL-17F blockade.

These data highlighted the need for an IL-17F secretion assay. Moreover, having the tools to separate IL-17A and IL-17F expressing CD4<sup>+</sup> T cells would allow for further extensive characterisation and comparison of the cells, following on from data shown in Chapter 3. This provided us with the rationale to develop an IL-17F secretion assay.

As illustrated in Figure 4.19, the aim of this series of experiments was to develop an assay which successfully separated IL-17A<sup>+</sup> IL-17F<sup>-</sup>, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells. UCB developed a bespoke anti-CD45 Fab Y fragment as well as Fab X fragments which recognize IL-17A (both the IL-17A homodimer and the IL-17AF heterodimer) and IL-17F (IL-17F homodimer only). These Fab fragments were combined to form an anti-IL-17A - anti-CD45 complex and anti-IL-17F - anti-CD45 complex. While an extensive series of optimisation experiments were performed to set up the capture assay protocol, here I present a selection of key experiments. To begin, we wanted to test the ability of the Fab complexes to recognize CD45, IL-17A and IL-17F. To achieve this, fresh PBMC were cultured simultaneously with the anti-IL-17A – anti-CD45 and anti- IL-17F – anti-CD45 Fab complex. During this culture the Fab complexes should recognize and bind to CD45 expressed on all PBMC. Next, cells were cultured in the absence or presence of high concentrations of either human recombinant cytokine IL-17A or IL-17F. Cells were then extracellularly stained for IL-17A and IL-17F and analysed by flow cytometry. As expected, no IL-17A or IL-17F cells were observed in the absence of recombinant cytokines (Figure 4.20). However, in the presence of recombinant cytokines, over 90% of total cells were IL-17A or IL-17F positive. This indicated that the PBMC CD45 marker and the IL-17A and IL-17F human recombinant cytokines were all successfully recognized by the Fab complexes.

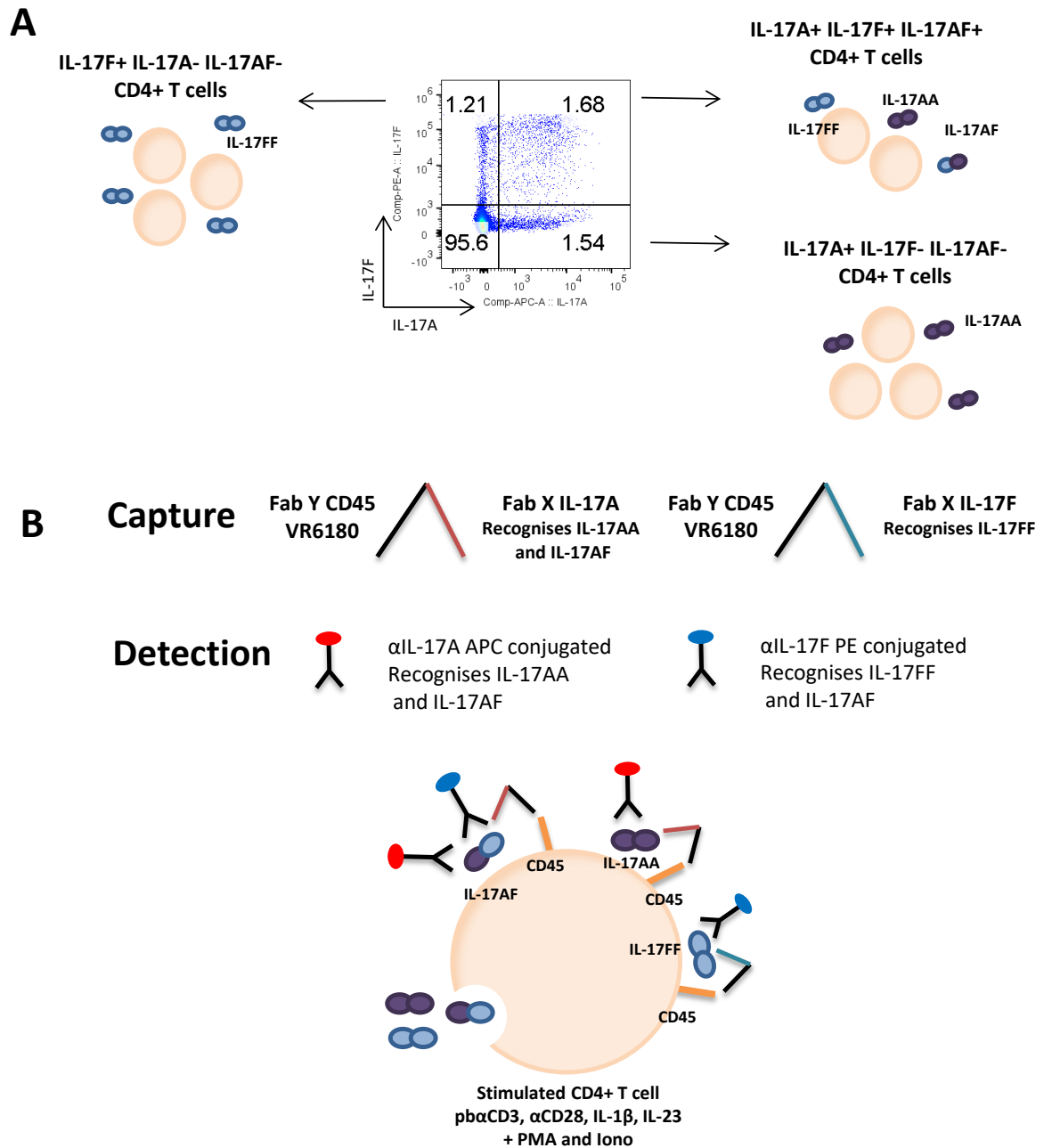
Next, we tested the ability of the Fab complexes to recognize and distinguish IL-17A<sup>+</sup> IL-17F<sup>-</sup>, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells from stimulated PBMC. In this experiment, PBMC were stimulated for 4 days with IL-1 $\beta$ , IL-23,  $\alpha$ CD28 and  $\alpha$ CD3 and re-stimulated for 3 hours with PMA and ionomycin. A detailed summary of the final optimized IL-17A and IL-17F secretion assay protocol is provided in section 2.5.5. In this case, cells were incubated in the absence or presence of either anti-IL-17A – anti-CD45 Fab Complex or anti-IL-17F – anti-CD45 Fab complex or both simultaneously. An extracellular cytokine stain was then performed on the cells assessing the expression of IL-17A and IL-17F. No IL-17A or IL-17F expressing cells were observed in the condition with no Fab complexes (Figure 4.21).

However, IL-17A<sup>+</sup> CD4<sup>+</sup> T cells were detectable in cultures incubated with the IL-17A Fab complex. Moreover, this condition detected IL-17F<sup>+</sup> CD4<sup>+</sup> T cells. As the IL-17F detection antibody recognizes both the IL-17F homodimer and the IL-17AF heterodimer, we predict the IL-17F detection antibody is recognizing IL-17AF heterodimer cytokine which has been captured to IL-17A Fab complexes bound to the CD4<sup>+</sup> T cell. Flow cytometric analysis of PBMC cultures incubated with the IL-17F Fab complex revealed IL-17F<sup>+</sup> CD4<sup>+</sup> T cells. In concordance with the IL-17F Fab complex specificity, no IL-17A<sup>+</sup> CD4<sup>+</sup> T cells were observed. Finally, combining the IL-17A and IL-17F Fab complexes allowed for the IL-17A<sup>+</sup> IL-17F<sup>-</sup>, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells to be distinguished. These data confirmed that the UCB capture reagents and optimized protocol were successful in capturing the IL-17 populations.



**Figure 4.18** *In vitro* cultures induce IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells which are not captured by IL-17A secretion assays.

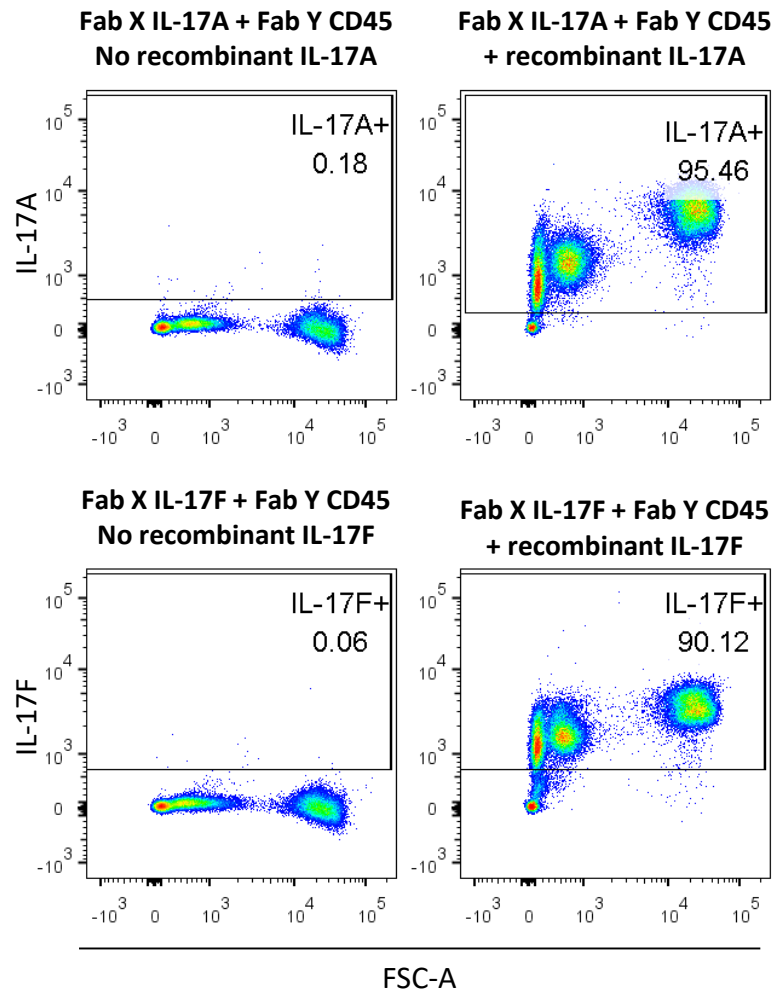
Bulk CD4<sup>+</sup> T cells were cultured with  $\alpha$ CD3,  $\alpha$ CD28, IL-1 $\beta$  and IL-23 for 3 days. While an IL-17A secretion assay protocol was performed on the majority of cells, a small number of cells were stimulated for 1.5 hours with PMA, ionomycin and golgi stop. These cells were stained intracellularly to analyse the expression of IL-17A and IL-17F. Results show the flow cytometric plots from two individual donors. The cells which would have been captured by the IL-17A secretion assay are highlighted in the red box.



**Figure 4.19 Schematic of UCB IL-17A/IL-17F capture assay.**

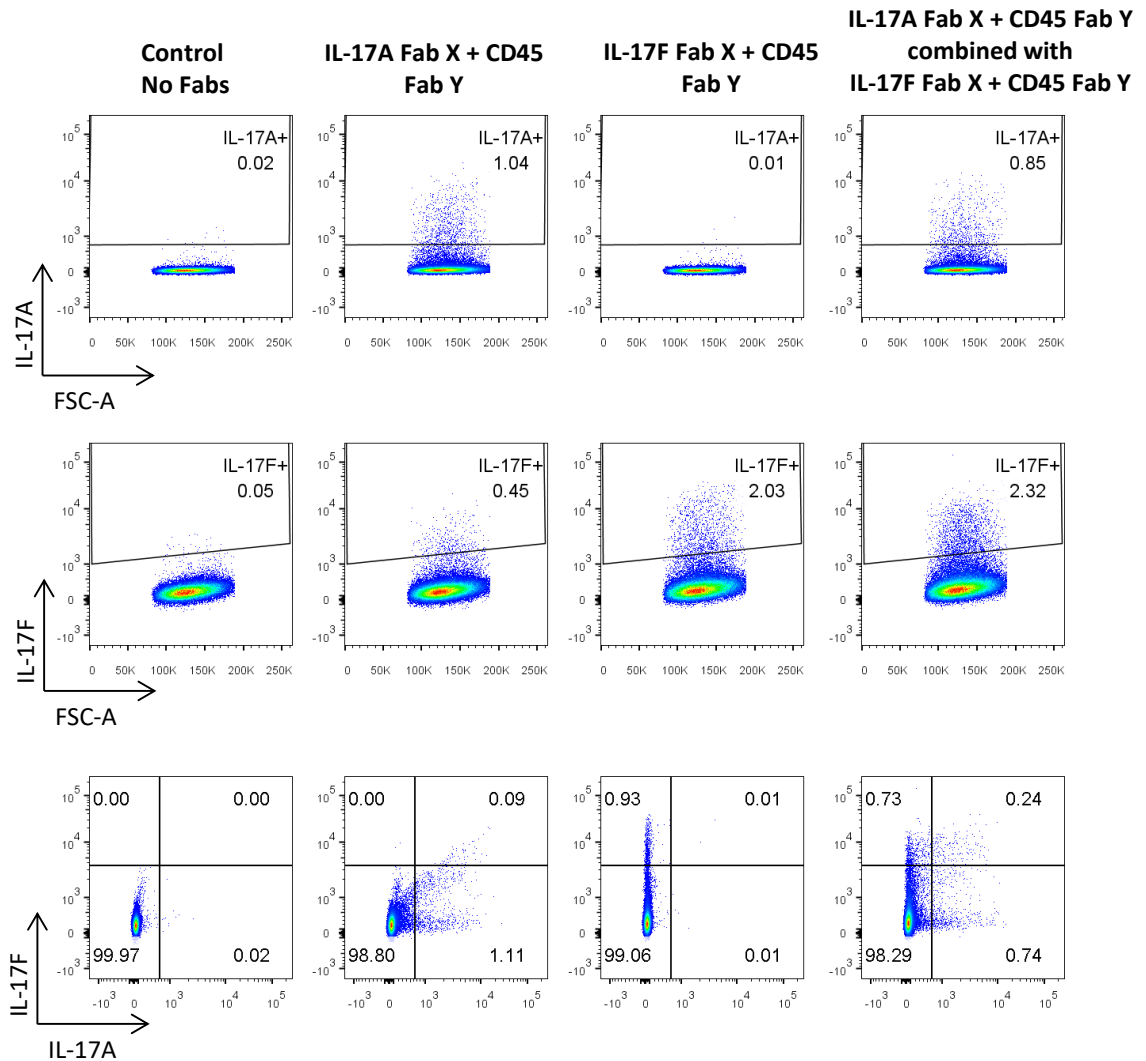
As shown in (A), the aim of the IL-17A/IL-17F capture assay is to separate the three different IL-17A/IL-17F CD4<sup>+</sup> T cell populations – IL-17A<sup>+</sup> IL-17F<sup>-</sup>, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> for functional and molecular characterisation. (B) The capture assay consisted of 2 Fab complexes, Fab Y CD45 – Fab X IL-17A and Fab Y CD45 – Fab X IL-17F. Stimulated CD4<sup>+</sup> T cells were incubated with both Fab complexes, simultaneously. Bound to CD45 expressed on CD4<sup>+</sup> T cells, the Fab complexes captured any IL-17A/IL-17F/IL-17AF cytokines secreted from a CD4<sup>+</sup> T cell. To detect IL-17A/IL-17F CD4<sup>+</sup> T cells, secondary detection antibodies were used, which recognised different epitopes of the IL-17A/IL-17F/IL-17AF cytokines.





**Figure 4.20 UCB in-house anti-IL-17A and anti- IL-17F Fab fragments successfully recognise human recombinant IL-17A and IL-17F.**

Fresh PBMC were incubated with either UCB in-house anti-IL-17A – anti-CD45 Fab complex or anti-IL-17F – anti-CD45 Fab complex. Cells were then incubated in the absence or presence of IL-17A or IL-17F human recombinant cytokine (10µg/ml). An extracellular cytokine stain was then performed assessing IL-17A and IL-17F expression. Flow cytometric plots are gated within all cells and represent one experiment.



**Figure 4.21 Testing the IL-17A and IL-17F capture assay.**

PBMCs were cultured with  $\alpha$ CD3,  $\alpha$ CD28, IL-1 $\beta$  and IL-23 for 4 days and re-stimulated with PMA and ionomycin for 3 hours. Cells were incubated in the absence or presence of either anti-IL-17A – anti-CD45 Fab Complex, anti-IL-17F – anti-CD45 Fab complex or both simultaneously. To allow for cytokine capture, cells were cultured for 45 minutes in X-vivo media, rotating. Intracellular cytokine expression of IL-17A and IL-17F was then assessed. Flow cytometric data shows the cytokine expression within live CD3+ CD4+ T cells (n=1).

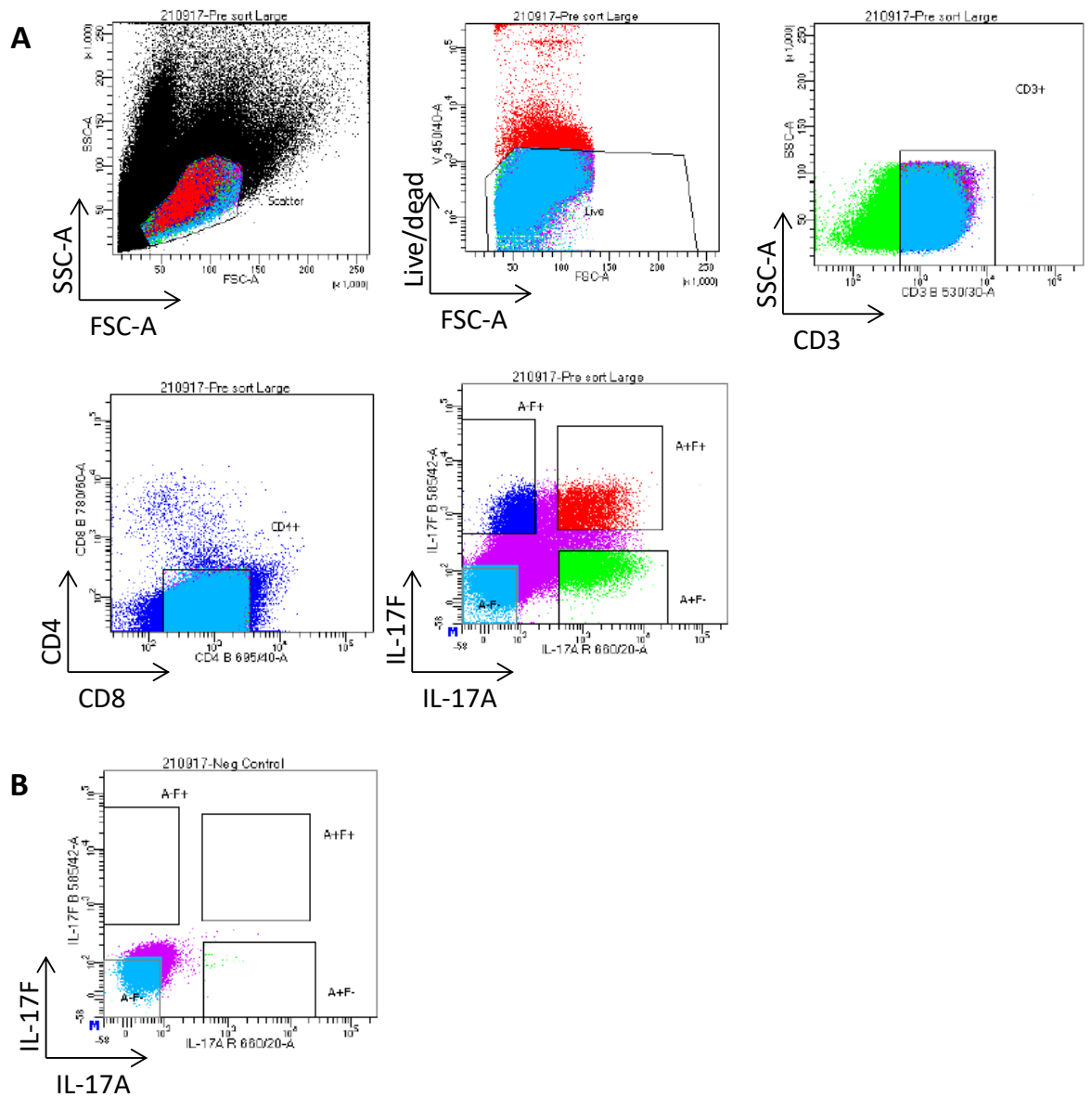
#### **4.2.11 Sorting IL-17A<sup>+</sup> IL-17F<sup>-</sup>, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells**

Having established an IL-17A and IL-17F CD4<sup>+</sup> T cell capture assay protocol, we proceeded to sorting the captured IL-17A<sup>+</sup> IL-17F<sup>-</sup>, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cell population and verifying successful isolation via qPCR. Bulk CD4<sup>+</sup> T cells were cultured with  $\alpha$ CD3,  $\alpha$ CD28, IL-1 $\beta$  and IL-23 for 3 days and re-stimulated for 3 hours with PMA and ionomycin. Cells were then incubated simultaneously with the IL-17A and IL-17F Fab complexes. As a negative control, to assess non-specific binding, a small number of cells were not incubated with the Fab complexes. To allow for cytokine capture, cells were then incubated for 45 mins, rotating. Cells were stained with DAPI viability dye, extracellular markers and IL-17A and IL-17F detection antibody. Using a FACS Aria sorter, the different IL-17-expressing CD4<sup>+</sup> T cell populations were sorted. Figure 4.22 illustrates the gating strategy used. This experiment was performed on two individual donors.

The total RNA from sorted IL-17A<sup>+</sup> IL-17F<sup>-</sup>, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cell populations was isolated using a micro-RNA kit. Reverse transcription was then performed, converting the RNA into cDNA. qPCR reactions were performed using primers for CD4 and human IL-17A and IL-17F. The *ACTB* gene was used as an endogenous control. Figure 4.23 displays the qPCR results from two individual donors. In all sorted populations from the two healthy donors, CD4 mRNA was detected. IL-17A mRNA was detected in sorted IL-17A<sup>+</sup> IL-17F<sup>-</sup> and IL-17A<sup>+</sup> IL-17F<sup>+</sup> CD4<sup>+</sup> T cell populations, whereas, the relative mRNA IL-17A expression was low/ not detectable in the sorted IL-17A<sup>-</sup> IL-17F<sup>-</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cell populations. This result was observed in both sorts. Similarly, IL-17F mRNA was only at high expression levels observed in the sorted IL-17F<sup>+</sup> IL-17A<sup>-</sup> and IL-17A<sup>+</sup> IL-17F<sup>+</sup> CD4<sup>+</sup> T cell populations.

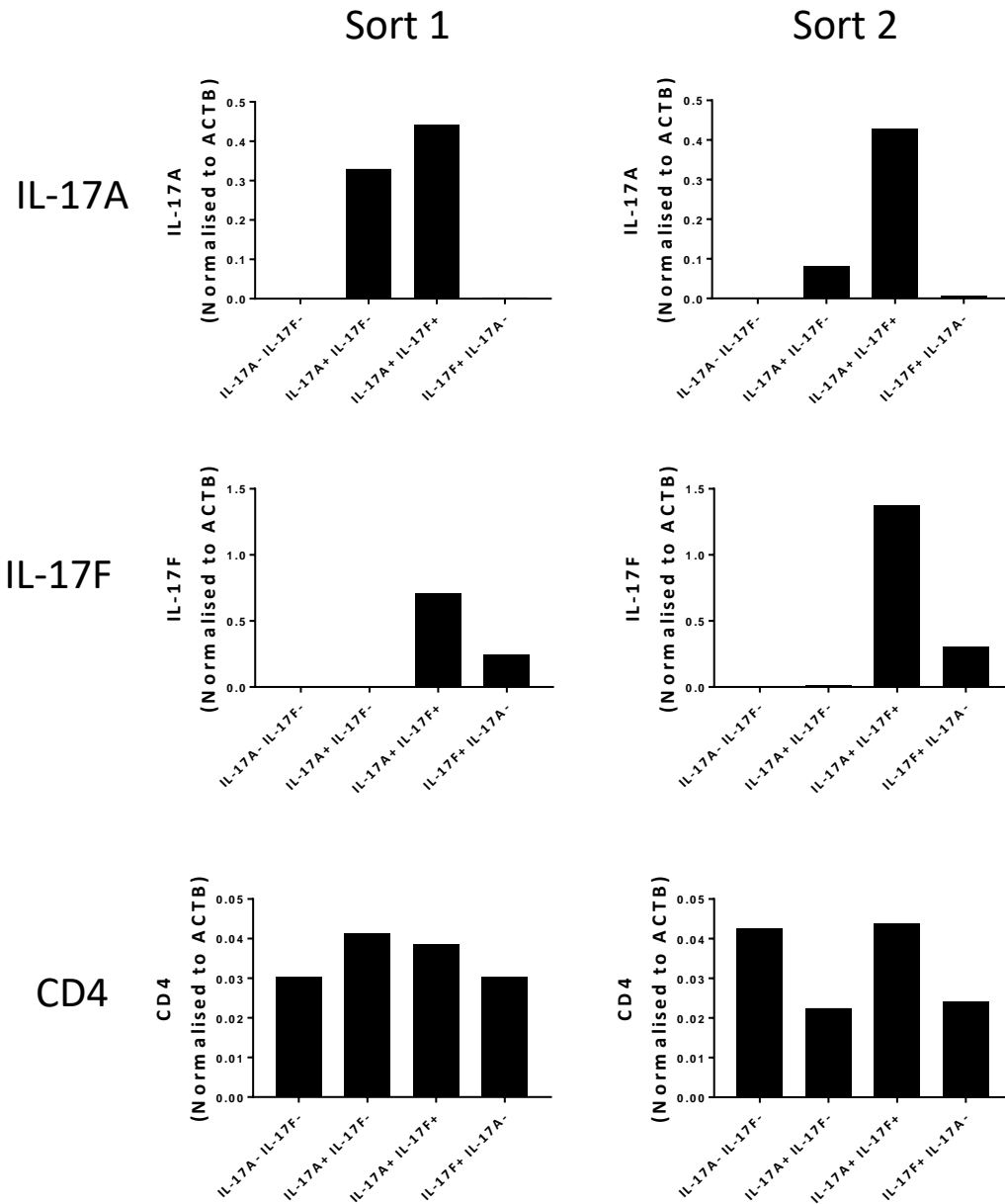
These data suggest that the IL-17A and IL-17F capture assay protocol has successfully isolated and separated three different IL-17 populations – IL-17A<sup>+</sup> IL-17F<sup>-</sup>, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells. However, it is important to note that the raw Ct qPCR values indicate that the sorted populations may not be 100% pure. For

example, IL-17A<sup>+</sup> IL-17F<sup>-</sup> CD4<sup>+</sup> T cells captured in the first sort shown a Ct value of 24 for IL-17F expression. Generally, a Ct value of 30 or higher indicates the absence of the mRNA of interest. Therefore, IL-17F mRNA may be present within the IL-17A<sup>+</sup> IL-17F<sup>-</sup> CD4<sup>+</sup> T cell population. Further validation of the IL-17A and IL-17F capture assay is currently being carried out by UCB. Once validation is complete, the capture assay will be a great tool to fully elucidate the functional potential of IL-17F.



**Figure 4.22 Gating strategy for IL-17A and IL-17F capture assay.**

Following the IL-17A/IL-17F capture assay protocol (described in the materials and methods), viable lymphocytes were gated based on their FSC-W/FSC-A properties. Dead cells were excluded based on their uptake of the viability dye, DAPI. Within CD3<sup>+</sup> CD4<sup>+</sup> T cells, expression of IL-17A and IL-17F was analysed. Gates were applied to separate and sort IL-17A<sup>+</sup> IL-17F<sup>-</sup>, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells. **(B)** To assess non-specific binding, a negative control was acquired. The negative control consisted of stimulated cells which had been stained with CD3, CD4 and the secondary detection antibodies – IL-17A and IL-17F but had not been incubated with the IL-17A and IL-17F Fab complexes.



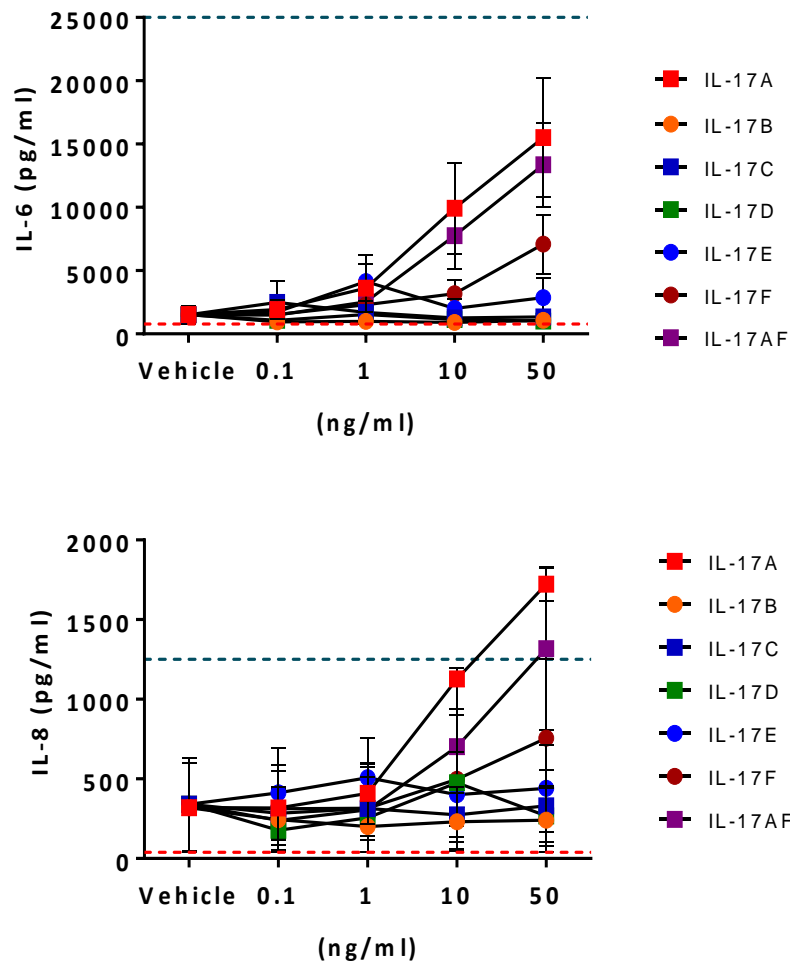
**Figure 4.23 Validation of the IL-17A and IL-17F capture assay.**

Bulk CD4<sup>+</sup> T cells were cultured with  $\alpha$ CD3,  $\alpha$ CD28, IL-1 $\beta$  and IL-23 for 3 days and re-stimulated with PMA and ionomycin for 3 hours. Cells were incubated simultaneously with UCB in-house anti-IL-17A – anti-CD45 Fab Complex, anti-IL-17F – anti-CD45 Fab complex or both simultaneously. To allow for cytokine capture, cells were cultured for 45 minutes in X-vivo media, rotating. Cells were stained with DAPI viability dye, extracellular markers and IL-17A and IL-17F detection antibodies. Using a FACS Aria sorter, IL-17A<sup>-</sup> IL-17F<sup>-</sup>, IL-17A<sup>+</sup> IL-17F<sup>-</sup>, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells were sorted. Total RNA was isolated from all sorted populations and converted into cDNA via reverse transcription. QPCR was then performed to assess the mRNA expression levels of *IL17A*, *IL17F* and *CD4* (n=2 independent experiments).

#### **4.2.12 Examining the function of the remaining IL-17 cytokines**

The function of the remaining IL-17 cytokines - IL-17B, IL-17C, IL-17D and IL-17E is less well studied. In this final section we explored the effect of the remaining IL-17 family members on PsA and RA synovial fibroblasts. Similar to the experiments described in the beginning of this chapter, human recombinant IL-17B, IL-17C, IL-17D and IL-17E were added to PsA and RA synovial fibroblasts at the indicated concentrations. As a positive control, IL-17A, IL-17F, IL-17AF and TNF $\alpha$  were included in the assays. Following 24 hours incubation, the supernatant was collected and stored at -80 before being tested by ELISA for IL-6 and IL-8 protein levels. IL-17A, IL-17F, IL-17AF and TNF $\alpha$  elicited an IL-6 and IL-8 fibroblast response, as previously demonstrated (Figure 4.24). However, IL-17B, IL-17C, IL-17D and IL-17E did not induce IL-6 and IL-8 fibroblast secretion.

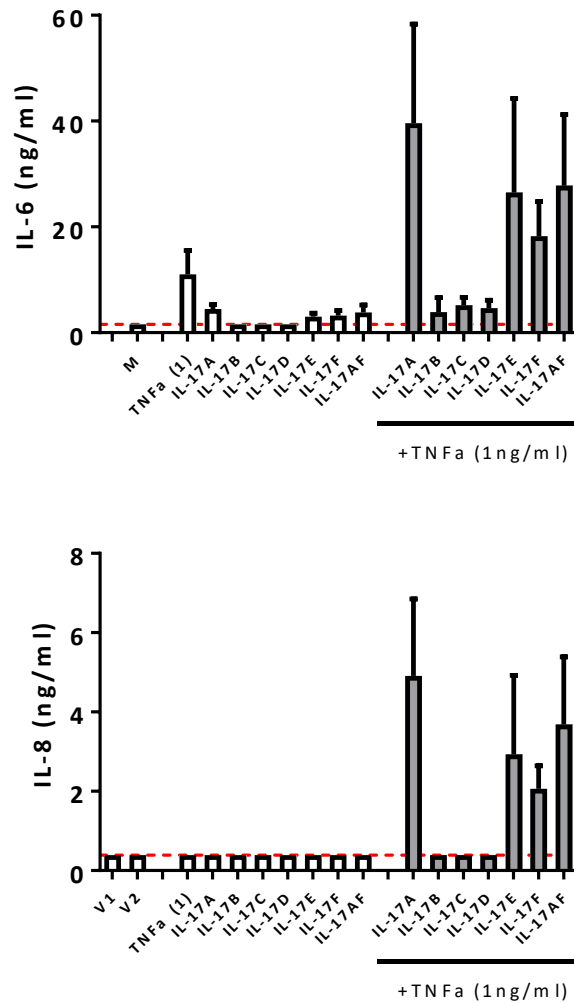
Next, I examined whether there was any synergistic effect between IL-17B, IL-17C, IL-17D or IL-17E and TNF $\alpha$ . Unexpectedly, a strong synergistic effect was observed between IL-17E and TNF $\alpha$  (Figure 4.25).



**Figure 4.24 IL-17B, IL-17C, IL-17D and IL-17E alone do not elicit an IL-6 or IL-8 response from synovial fibroblasts.**

RA and PsA synovial fibroblasts ( $10 \times 10^3$ ) were cultured with indicated concentrations of human recombinant IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, IL-17F, IL-17AF and  $\text{TNF}\alpha$  for 24 hours. Culture supernatants were collected and IL-6 and IL-8 (PsA  $n=1$ , RA  $n=1$ ) protein levels were measured by ELISA. Dashed red and blue lines indicate lower detection limit and upper detection limit, respectively.





**Figure 4.25 IL-17E synergises with TNF $\alpha$ , augmenting IL-6 and IL-8 synovial fibroblast secretion.**

Synovial RA and PsA fibroblasts ( $10 \times 10^3$ ) were cultured with human recombinant IL-17A, IL-17B, IL-17C, IL-17D, IL-17E and IL-17AF (10ng/ml) in the absence or presence of TNF $\alpha$  (1ng/ml) with for 24 hours. Culture supernatants were collected and IL-6 and IL-8 protein levels were measured by ELISA. Dashed red and blue lines indicate lower detection limit and upper detection limit, respectively (RA n=1, PsA n=1).

### 4.3 Discussion

Data presented in this chapter show that the inflammatory potential of IL-17A and IL-17F is synergistically enhanced with TNF $\alpha$ . However, in the presence of IL-17A, the effect of IL-17F on inflammation is limited. Nevertheless, this chapter suggests that in an environment whereby IL-17A and IL-17F are present, dual blockade of these cytokines is more effective at reducing inflammation. Using a reductionist system, whereby synovial fibroblasts are cultured with a cocktail of recombinant cytokines, results show dual blockade of IL-17A and IL-17F is more effective at reducing IL-6 and IL-8 secretion by fibroblasts than blockade of IL-17A alone. This chapter explores the effect of dual IL-17A and IL-17F blockade in a variety of different physiological systems. The superior effect of dual IL-17A and IL-17F blockade was not always consistent across the physiological systems. This is likely due to the levels of IL-17F protein present. These data highlight the need for an IL-17F secretion assay. In collaboration with UCB, an IL-17A and IL-17F secretion assay was developed, which following further validation will be a useful tool to further characterise IL-17A and IL-17F producing cells. Finally, data presented in this chapter demonstrate that IL-17E also synergises with TNF $\alpha$  enhancing IL-6 and IL-8 secretion by fibroblasts.

Experimental evidence has implicated IL-17A in the immunopathology of inflammatory arthritis. Seminal research blocked IL-17A in the CIA mouse model with a soluble IL-17 receptor fusion protein; this resulted in suppressed arthritis development and joint damage. Moreover, overexpression of IL-17A in CIA led to accelerated development and enhanced severity of synovial inflammation and joint damage <sup>358</sup>. In contrast, the role of IL-17F in inflammation remains questioned. Reports often describe IL-17F to be redundant to IL-17A. In the EAE mouse model, *IL17F* knock-out did not lead to a reduction in disease severity <sup>106</sup>.

IL-17A, IL-17F and IL-17AF all signal through the same IL-17RA and IL-17RC receptor complex. Literature has previously shown that IL-17A, IL-17F and IL-17AF elicit an inflammatory response in a hierarchical fashion, with IL-17A the most potent cytokine followed by IL-17AF and then IL-17F <sup>78</sup>. This is supported by data in this

chapter, whereby human recombinant IL-17A induced the highest levels of IL-6 and IL-8 secretion by synovial fibroblasts, IL-17AF induced intermediary levels and IL-17F induced the lowest levels. Reports attribute the difference in potency to the cytokine binding affinities to the IL-17RA receptor. IL-17A and IL-17F bind with equally high affinity to IL-17RC, however, in comparison to IL-17A, IL-17F binds with at least 100-fold lower affinity to IL-17RA <sup>85</sup>.

Data presented in this chapter show that IL-17A, IL-17F and IL-17AF all synergise with TNF $\alpha$  leading to an enhancement in IL-6 and IL-8 secretion by fibroblasts. Once again, a hierarchical result is observed with the combination of TNF $\alpha$  and IL-17A eliciting the highest levels of IL-6 and IL-8, followed by TNF $\alpha$ /IL-17AF and TNF $\alpha$ /IL-17F. This is supported by studies present in the current literature <sup>106</sup>. Nevertheless, these data importantly show that the low potency of IL-17F alone is significantly enhanced in the presence of TNF $\alpha$ . Therefore, it is important to consider the roles of IL-17F in concert with other molecules. Expanding upon data in the literature, the results presented show there is no synergistic or additive effect between the IL-17 cytokines themselves. Moreover, it is shown that addition of IL-17F and/ or IL-17AF does not further enhance IL-6 or IL-8 levels secreted by fibroblasts which are cultured with IL-17A and TNF $\alpha$ . This indicates that in the presence of IL-17A, IL-17F may be redundant.

However, blocking experiments performed in cultures of synovial fibroblasts and recombinant cytokines (a cocktail of IL-17A, IL-17F, IL-17AF and TNF $\alpha$ ) revealed that dual IL-17A and IL-17F blockade by bimekizumab is more effective at reducing IL-6 and IL-8 secretion by fibroblasts vs. blockade of IL-17A alone. Blockade of IL-17F alone had no effect on fibroblast secretion. Blockade of TNF $\alpha$  reduced IL-6 and IL-8 secretion levels similar to that of dual IL-17A and IL-17F blockade. These data show that in the presence of IL-17A, IL-17F has a limited effect on fibroblasts. However, in the absence of IL-17A, IL-17F can synergise with TNF $\alpha$  and elicit a significant inflammatory response. Therefore in an environment where IL-17A and IL-17F are present dual IL-17A and IL-17F will reduce inflammation to a greater extent.

The superior effect of dual IL-17A and IL-17F has recently been reported by Glatt *et al.* In this study, target cells were stimulated with Th17 supernatant in the absence or presence of IL-17 specific blocking antibodies <sup>121</sup>. To generate the Th17 supernatant blood CD4<sup>+</sup> CD45RO<sup>+</sup> CCR6<sup>+</sup> CXCR3<sup>-</sup> Th17 cells were sorted from healthy donors and stimulated with anti-CD3 mAb and anti-CD28 mAb for 96 hours. In PsA synoviocyte cultures, dual IL-17A and IL-17F neutralization by bimekizumab resulted in a greater downregulation of IL-8. Moreover, transcriptional analysis in Th17 stimulated normal synoviocytes confirmed that dual IL-17A and IL-17F led to further decrease in the inflammatory profile vs. blockade of IL-17A alone.

Prior and following the Glatt *et al.* publication, experiments performed in this chapter aimed to show in a physiological *in vitro* system (i.e blockade of IL-17 cytokines in CD4<sup>+</sup> T cell supernatant) that dual IL-17A and IL-17F blockade is more effective in reducing inflammation than IL-17A alone. No superior effect of a combined IL-17A and IL-17F blockade was observed in synovial fibroblast cultures stimulated with *in vitro* bulk CD4<sup>+</sup> T cell supernatants. This is likely due to the low IL-17 protein levels observed in the generated supernatant. To generate CD4<sup>+</sup> T cell supernatant enriched for IL-17 protein levels two approaches were used, sorting CCR6<sup>+</sup> CD161<sup>+</sup> CD4<sup>+</sup> T cells or sorting IL-17A<sup>+</sup> CD4<sup>+</sup> T cells. Although culturing sorted CCR6<sup>+</sup> CD161<sup>+</sup> CD4<sup>+</sup> T cells with synovial fibroblasts elicited a higher level of IL-6 secretion when compared with cultures of bulk CD4<sup>+</sup> T cells and fibroblasts, addition of bimekizumab did not lead to a greater reduction of IL-6 secretion than blockade of IL-17A alone. However, this experiment has only been performed once and it may be worthwhile to repeat. A limitation to this experiment is that not all total CCR6<sup>+</sup> and/or CD161<sup>+</sup> CD4<sup>+</sup> T cells express IL-17A and IL-17F. Furthermore, not all IL-17A<sup>+</sup> and IL-17F<sup>+</sup> CD4<sup>+</sup> T cells express CCR6 and/or CD161.

As previously discussed, while an IL-17A secretion assay is commercially available, there is not one for IL-17F. Therefore, it is not possible to enrich for both IL-17A<sup>+</sup> and IL-17F<sup>+</sup> CD4<sup>+</sup> T cells. However, there is a population of CD4<sup>+</sup> T cells that co-

express IL-17A and IL-17F present in CD4<sup>+</sup> T cell cultures stimulated for 3 days with IL-1 $\beta$ , IL-23, anti-CD28 mAb and anti-CD3 mAb. Therefore, it was decided to sort IL-17A<sup>+</sup> CD4<sup>+</sup> T cells and assess the effect of dual IL-17A and IL-17F blockade in this supernatant. Synovial fibroblasts cultured with sorted IL-17A<sup>+</sup> CD4<sup>+</sup> T cells or sorted IL-17A<sup>+</sup> CD4<sup>+</sup> T cell supernatants in the absence or presence of IL-17 specific blocking antibodies led to varying results. Correlation analysis demonstrated that in general, the greatest effect of dual IL-17A and IL-17F blockade was observed when higher levels of IL-17F protein was present, which varied from donor to donor. Certainly, as this experiment was performed in 3 donors, performing additional experiments may provide stronger evidence for the role of dual IL-17A and IL-17F blockade in reducing inflammation using more physiological supernatants. A limitation to using sorted IL-17A<sup>+</sup> CD4<sup>+</sup> T cell supernatant for dual IL-17A and IL-17F blockade was the fact that IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells are not captured. Therefore, the full effect of dual blockade is not observed. An ideal experiment would involve capturing IL-17A<sup>+</sup> IL-17F<sup>-</sup>, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells, pooling these together and incubating for 24 hours. The generated supernatant could then be added to synovial fibroblast cultures in the absence or presence of IL-17 specific blocking antibodies.

There was thus an increasing need for a combined IL-17A and IL-17F secretion assay. Such an assay would be a great tool for sorting of IL-17A<sup>+</sup> IL-17F<sup>-</sup>, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cell populations. This would allow for the functional potential and characterisation of IL-17F expressing CD4<sup>+</sup> T cells (discussed in chapter 3) to be elucidated. Fab fragments directed against IL-17A, IL-17F and CD45 were designed by UCB. In collaboration with UCB, a protocol was developed to effectively sort IL-17A and IL-17F producing cells using the Fab fragments. Data in this chapter show the effective sorting of IL-17A<sup>+</sup> IL-17F<sup>-</sup>, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells, as evidenced by RNA analysis. However, RNA analysis did indicate the sorted IL-17 populations may not be 100% pure. Therefore, further validation work is required to confirm successful sorting of the different IL-17 populations. Another validation approach could involve culturing the sorted IL-17 populations for 24 hours and then measuring the supernatant for IL-17A, IL-17F and IL-17AF protein levels via ELISA. It could then be confirmed that IL-17A<sup>+</sup> IL-17F<sup>-</sup>

CD4<sup>+</sup> T cells only produce IL-17A protein, IL-17A<sup>+</sup> IL-17F<sup>+</sup> CD4<sup>+</sup> T cells secrete IL-17A, IL-17F and IL-17AF and so on. It is unknown whether the different IL-17 populations arise from one another. For example, under specific stimulatory conditions do IL-17A<sup>+</sup> IL-17F<sup>-</sup> CD4<sup>+</sup> T cell populations skew to IL-17A<sup>+</sup> IL-17F<sup>+</sup> and then IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells? If so, it may be possible sorted IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells over time revert back to IL-17A<sup>+</sup> IL-17F<sup>+</sup> or IL-17A<sup>+</sup> IL-17F<sup>-</sup> CD4<sup>+</sup> T cells and alter the IL-17 cytokines secreted. Therefore, it would be of great interest to analyse the supernatant of the sorted IL-17 expressing CD4<sup>+</sup> T cell populations across multiple time-points.

Blockade of IL-17 cytokines in *in vivo* would be the best way to determine demonstrate whether IL-17A and IL-17F blockade can further help alleviate inflammatory arthritis. It was shown in the CIA mouse model that combined IL-17A and IL-17F blockade was not significantly different when compared to blockade of IL-17A alone. However, the study did demonstrate that in this model levels of IL-17F were low <sup>362</sup>. In contrast, an investigation which involved treating colitic mice with IL-17A and IL-17F neutralising antibodies demonstrated that neutralisation of both IL-17A and IL-17F significantly reduced T cell-mediated colitis development, whereas neutralisation of IL-17A or IL-17F alone was inefficient at reducing disease severity <sup>363</sup>. In humans, brodalumab which blocks the IL-17RA receptor thereby preventing the function of IL-17A and IL-17F has been shown alleviate inflammation in psoriasis and PsA <sup>403,404</sup>. However, with no head-to-head comparisons between the effectiveness of brodalumab and therapies which target IL-17A alone, it is difficult to understand the contribution of IL-17F to disease pathology. A recent report from Sawyer *et al.* performed a systematic literature review and network meta-analysis to examine the relative efficacy of brodalumab compared with other approved biologics in moderate-to-severe psoriasis <sup>412</sup>. The primary analysis assessed the proportion of patients achieving psoriasis area severity index (PASI) 50, 75, 90 or 100. This refers to the percentage of disease reduction compared to base-line. For example, a PASI 50 score represents a 50% improvement from base-line and is acknowledged as a clinically significant end-point <sup>413</sup>. In this study it was suggested that consistent with PASI 50, 75 and 90, brodalumab displayed superior efficacy to the anti-IL-17A antibody secukinumab, however, its efficacy was similar to the other IL-17A inhibitor,

ixekizumab. While this meta-analysis study may provide insights into whether dual IL-17A and IL-17F blockade may be beneficial in reducing inflammation in psoriasis, it is difficult to interpret without a head-to-head study. Additionally, it is difficult to delineate whether possible superior effects of brodalumab are attributed to IL-17F or other mechanisms by IL-17RA blockade. As previously discussed, bimekizumab is currently in clinical trial and demonstrated efficacy in PsA and psoriasis<sup>121,414</sup>. However, at this point it is difficult to assess whether it has a superior effect than blockade of IL-17A alone.

Of course, the success of dual IL-17A and IL-17F in inflammatory arthritis depends on the presence of these cytokines. While extensive literature has confirmed the presence of IL-17A in RA and PsA, there is a lack of robust evidence confirming the presence of IL-17F. This is further explored in the final chapter of this thesis. It is also important to note that as IL-17A and IL-17F play an important role in providing protection against extracellular pathogens and fungal infections, dual blockade of IL-17A and IL-17F in inflammatory disease patients may lead to increased risk of infections. One of these adverse reactions could include chronic mucocutaneous candidiasis (CMC), which is characterised by recurrent or persistent infection of nails, skin and mucosae predominantly by *Candida albicans*. This is evidenced by the fact that autosomal recessive IL-17RA and autosomal dominant IL-17F deficiencies are associated with CMC<sup>415</sup>.

The final section in this chapter explores the effect of the remaining IL-17 cytokines (IL-17B, IL-17C, IL-17D and IL-17E) on synovial fibroblasts. None of these cytokines induced an IL-6 or IL-8 response from synovial fibroblasts. However, IL-17E combined with TNF $\alpha$  elicited a potent IL-6 and IL-8 response. To the best of my knowledge synergy between IL-17E and TNF $\alpha$  has not been previously reported. Studies have described IL-17E to have anti-inflammatory<sup>125</sup> and inflammatory properties<sup>384</sup>. The anti-inflammatory effect of IL-17E has been attributed to the ability of IL-17E to suppress IL-17A expressing cells<sup>125</sup>. In contrast, IL-17E has been shown to activate macrophages leading to an inflammatory response<sup>384</sup>. It would be of interest to investigate whether synergy between IL-17E and TNF $\alpha$  could be observed

in a physiological system (i.e. blockade of IL-17E in a supernatant whereby IL-17E and TNF $\alpha$  are present at significant levels). One challenge to this experiment is that IL-17E producing cells, for example Th2 cells do not reportedly co-express TNF $\alpha$ . For this reason, in the next chapter we were interested to determine the presence of IL-17E protein in RA and PsA synovial fluid.

Although microarray analysis revealed the presence of all IL-17 receptors on RA and PsA synovial fibroblasts, IL-17B, IL-17C or IL-17D did not elicit an inflammatory response from synovial fibroblasts. The receptor expression on the cell lines used was not analysed, therefore it could be that the receptor expression on these cell lines were low. Future experiments could assess the effect of these cytokines on other target cells.

While the function of the IL-17 cytokines is covered in this chapter, the final chapter explores the presence of these cytokines in inflammatory arthritis.



## 5 Investigating the presence of IL-17F and IL-17A in inflammatory arthritis

### 5.1 Introduction

In this final results chapter, I explored the presence of IL-17A and IL-17F in inflammatory arthritis. Inflammatory arthritis encompasses rheumatoid arthritis (RA), psoriatic arthritis (PsA), ankylosing spondylitis and juvenile idiopathic arthritis among others. In this project we focus on RA and PsA.

The link between IL-17A and RA pathology was established in 1999 when rheumatoid synovial explants were shown to produce functional IL-17A and IL-17A producing cells were identified in T cell-rich areas of the synovium<sup>83</sup>. Seminal research blocked IL-17A in the collagen-induced arthritis (CIA) mouse model with a soluble IL-17 receptor fusion protein; this resulted in suppressed arthritis development and joint damage<sup>358</sup>. Studies have revealed levels of IL-17A are elevated in the serum and synovial fluid of RA patients and correlate with the disease activity and severity<sup>366,367</sup>. Moreover, it has been shown that there is a significant increase in the frequencies of IL-17A+ CD4+ and IL-17A+ CD8+ T cells in the synovial fluid of PsA patients when compared to the peripheral blood of the same patient<sup>374</sup>.

While the literature provides strong evidence for the presence and immunopathological role of IL-17A in RA and PsA, robust evidence is lacking for IL-17F. The limited available studies both support and refute the presence of IL-17F in RA and PsA. One study which stimulated RA SFMC for 24 hours with PMA and ionomycin detected IL-17A and IL-17AF protein in the culture supernatant, whereas IL-17F was undetectable<sup>7</sup>. However, immunohistochemistry staining has revealed significantly increased IL-17F expression in PsA synovial tissue compared to OA<sup>112</sup>. Moreover, a recent study has observed *IL17F* mRNA expression in 6 out of 14 PsA synovial tissue samples<sup>121</sup>. The main aim of this final chapter was to investigate whether IL-17F is easily detectable alongside IL-17A, in inflammatory arthritis.

Specific aims for this chapter were as follows:

- Investigate the presence of IL-17A, IL-17F, IL-17AF, IL-17E and other pro-inflammatory cytokines in matched serum and SF samples from RA and PsA patients.
- Assess the effect of RA and PsA serum and SF on synovial fibroblasts.
- Investigate the presence of IL-17A and/or IL-17F producing CD4+ T cells and other immune cell subsets in RA and PsA PB and SF.
- Examine the mRNA levels of *IL17A* and *IL17F* and the remaining IL-17 cytokine members in cells from RA and PsA PB and SF.
- Examine the presence of IL-17 producing cells in synovial tissue.

## 5.2 Results

### 5.2.1 Investigating the presence of IL-17A, IL-17F and IL-17AF in matched serum and SF from RA and PsA patients

To begin, I investigated the presence of IL-17A, IL-17F and IL-17AF in healthy control cell-free serum and RA and PsA matched cell-free serum and synovial fluid by Luminex. Information on this patient cohort is displayed in Table 5.1 (patient cohort 1). At the time of sample collection, patients were receiving no treatment, methotrexate or anti-TNF. Due to the limited numbers of patient samples, comparisons were not drawn between different treatment groups. Figure 5.1 illustrates the Luminex results of IL-17A and IL-17F. In 7 out of 8 RA donors, IL-17A protein was detected at higher levels in the RA SF vs. serum. Statistical significance was not achieved, presumably due to one donor displaying high levels of IL-17A in the serum vs. SF. Similar results were observed for IL-17F. IL-17F protein was detectable and present at higher levels in RA SF vs. serum, approximately 74 vs. 15 pg/ml, respectively. We also observed higher levels of IL-17A and IL-17F in PsA SF vs. serum, although statistical significance was not achieved.

Since data in the current literature both support and refute the presence of IL-17F protein in the synovial fluid, my next aim was to validate these findings. Serum from healthy controls and paired serum and SF from RA and PsA were analysed for IL-17A and IL-17F protein levels via ELISA (eBioscience, Platinum ELISA). For this analysis matched serum and SF from OA patients was also included. This provided an additional negative control. Information on this patient cohort is displayed in Table 5.2 (patient cohort 2). Moreover, samples were analysed with an IL-17AF heterodimer ELISA (eBioscience, Platinum ELISA). In this instance, the IL-17A homodimer, IL-17F homodimer and IL-17AF heterodimer ELISA had no or limited cross-reactivity with one another. In line with the previous findings, IL-17A protein was detectable in both RA and PsA synovial fluid (Figure 5.2). No IL-17A was detectable in the matched RA or PsA serum, healthy control serum or paired OA serum and SF. In contrast with the Luminex results, little IL-17F protein was detected. No clear increase of IL-17F protein in RA or PsA SF vs. serum was observed. Finally, IL-17AF protein was not detectable in any samples. The same result was observed with samples diluted

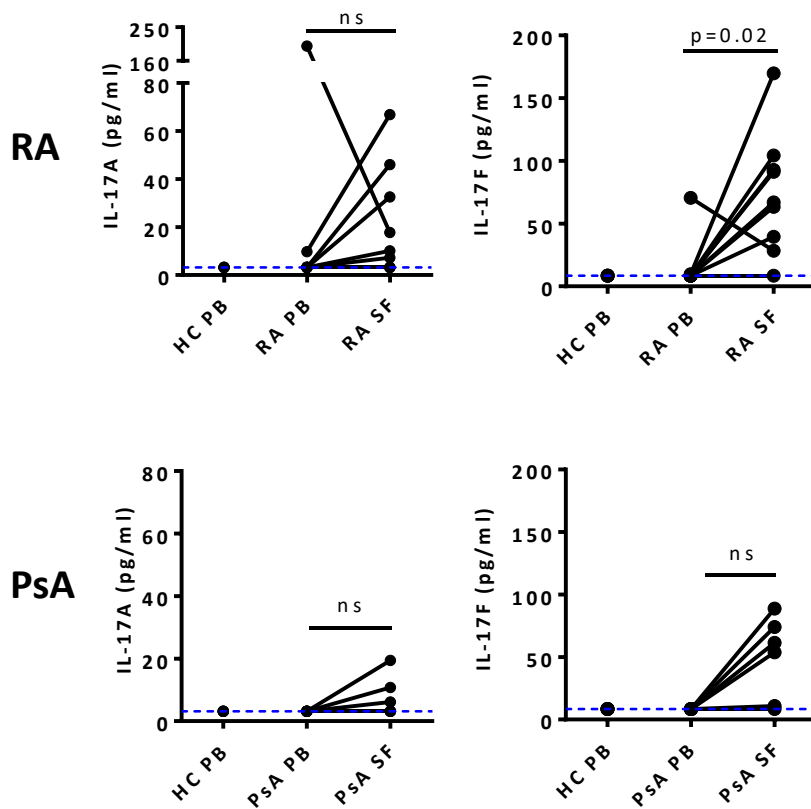
at different ratios including neat and 1:5 (data show IL-17A samples diluted 1:5 and IL-17F and IL-17AF samples analysed neat).

While Luminex analysis detected IL-17F in RA and PsA SF, our ELISA results did not. It is important to note the lower detection limit for IL-17F varied between the Luminex and ELISA (2 vs. 15.6pg/ml, respectively). However, I still expected to detect IL-17F if it was present in SF with the ELISA. With these contradicting results, we sought to address whether the SF composition prevents detection of IL-17F using this particular ELISA kit. To achieve this, assay diluent or OA/RA/PsA serum or SF were spiked with either human recombinant IL-17A, IL-17F or IL-17AF. Samples were then analysed with the corresponding IL-17 ELISA. As displayed in Figure 5.3, IL-17F protein was detected in assay diluent and paired serum and SF samples spiked with human recombinant IL-17F. Similar results were observed for IL-17A and IL-17AF. These data suggest that the SF composition does not block the recognition of IL-17F or IL-17AF protein via ELISA. These results indicate that the serum and SF ELISA analysis were reliable and IL-17F and IL-17AF were not present in the patient samples.

With these data, the presence of IL-17F protein in RA and PsA serum and SF remains inconclusive. Why the luminex analysis generated clear positive IL-17F results and the ELISA analysis did not remains unknown. One speculation is that the positive IL-17F luminex result was due to non-specific binding. These results highlight the variability in detecting IL-17F in patient samples using different methods. This, in part, could explain the contradicting studies present in the current literature. To move forward it was decided to investigate the presence of IL-17F using different methods including IL-17F blockade in serum and SF and flow cytometry analysis of IL-17F+ cells. These results are shown later in this chapter.

**Table 5.1 Demographic and clinical parameters of patients included in luminex assay (patient cohort 1)**

	<b>RA Paired PB/SF (n = 9)</b>	<b>PsA Paired PB/SF (n = 6)</b>	<b>Healthy control PB (n = 4)</b>
<b>Male, no.</b>	3	3	2
<b>Female, no.</b>	6	3	2
<b>Age, mean ± SEM years</b>	61.0 ± 3.8	44.2 ± 4.8	39.50 ± 8.0
<b>Disease duration ± SEM years</b>	13.8 ± 4.1	3.0 ± 0.7	-
<b>Treatment, no.</b>			
No treatment	3	3	-
MTX	3	3	-
Anti-TNF	3	-	-
<b>DAS28</b>			
Mean ± SEM	4.8 ± 0.46	4.6 ± 0.64	-
Range	3.47 – 6.3	2.25 – 5.86	-



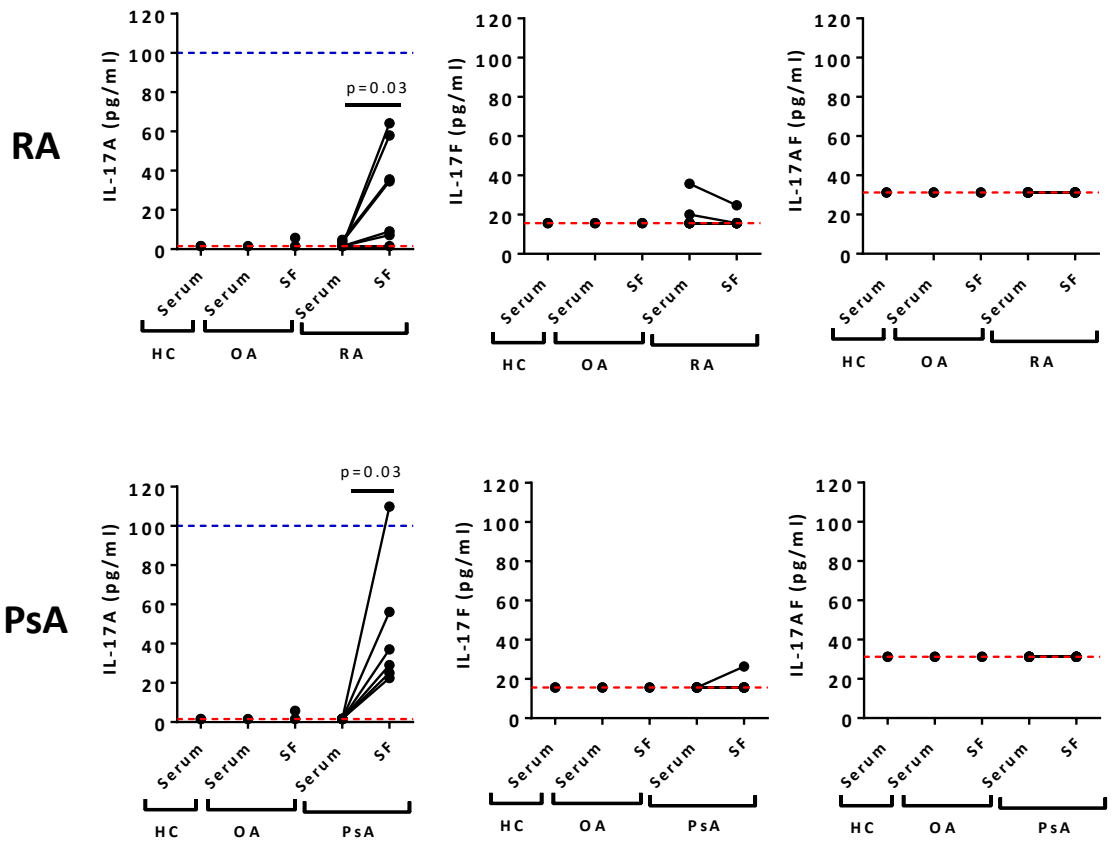
**Figure 5.1 Analysing the presence of IL-17A and IL-17F in RA and PsA matched serum and SF samples via Luminex.**

IL-17A and IL-17F protein levels in paired cell-free serum and synovial fluid from RA (n=7) and PsA (n=6) patients were analysed by luminex. Dashed blue lines represent the lower detection limit. Statistical significance was calculated using a Wilcoxon matched pairs test. Patient cohort information displayed in Table 5.1 (Patient cohort 1).

**Table 5.2 Demographic and clinical parameters of patients included in ELISA assays (patient cohort 2)**

	<b>RA Paired PB/SF (n = 7)</b>	<b>PsA Paired PB/SF (n = 6)</b>	<b>OA Paired PB/SF (n = 2) *</b>	<b>Healthy Control PB (n=2)</b>
<b>Male, no.</b>	2	5	-	1
<b>Female, no.</b>	5	1	1	1
<b>Age, mean ± SEM years</b>	47.0 ± 0.3	36.5 ± 2.9	72	39.50 ± 8.02
<b>Treatment, no:</b>				-
No treatment	2	2	-	
MTX	1	2		
Anti-TNF	2	2	-	-
Abatacept	2	-		
<b>DAS28</b>				
Mean ± SEM	4.6 ± 0.3	4.6 ± 0.5	-	-
Range	3.5 – 5.43	1.89 – 5.78	-	-

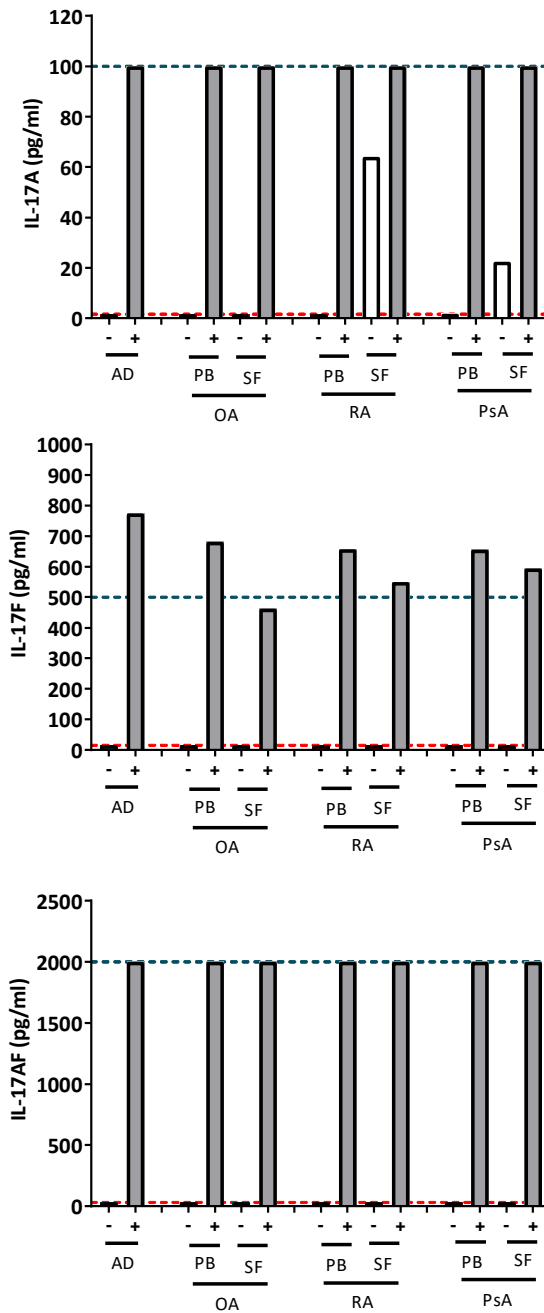
\* Gender and age not available for one OA patient.



**Figure 5.2 Analysing the presence of IL-17A and IL-17F in RA and PsA matched serum and SF samples via ELISA.**

Serum from healthy controls (n=3) and paired serum and synovial fluid from OA patients (n=3), RA patients (n=7) and PsA patients (n=6) were analysed by ELISA for protein levels of IL-17A, IL-17F and IL-17AF. Dashed red and blue lines represent the lower and upper detection limit respectively. Statistical significance was calculated using a Wilcoxon matched pairs test. Patient cohort information displayed in Table 5.2 (Patient cohort 2).





**Figure 5.3 Human recombinant IL-17A, IL-17F and IL-17AF spiked in serum and SF samples are easily detectable via ELISA.**

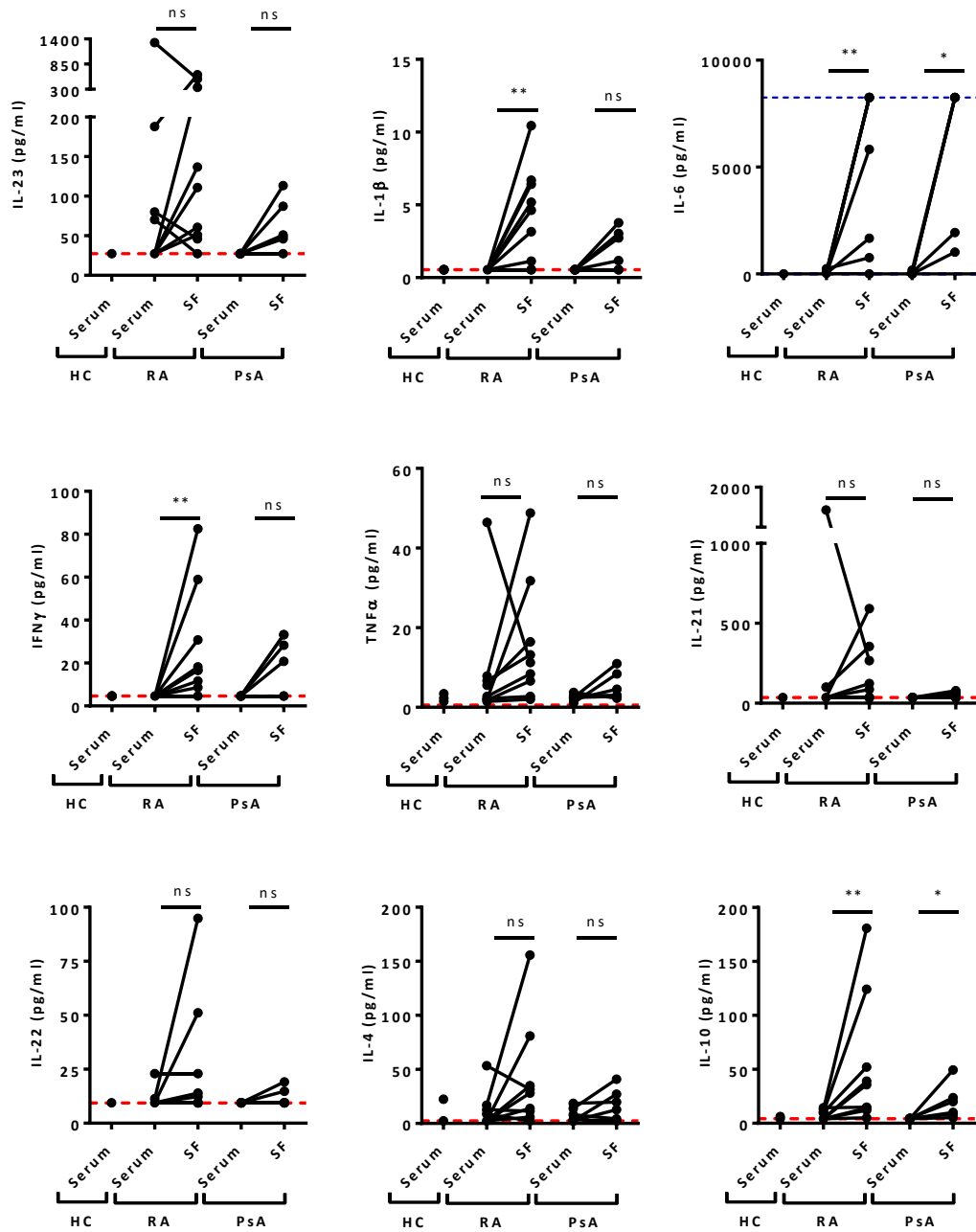
Human recombinant IL-17A (100pg/ml), IL-17F (500pg/ml) and IL-17AF (500pg/ml) were individually spiked in either assay diluent (AD), or matched serum or SF from OA, RA or PsA patients. Samples were then analysed by the corresponding IL-17 cytokine ELISA (n=1). Dashed red and blue lines represent the lower and upper detection limit respectively.

### 5.2.2 The cytokine profile of RA and PsA serum and SF and their effect on synovial fibroblasts

In addition to IL-17A and IL-17F, the luminex assay also analysed the presence of other cytokines – including IL-23, IL-1 $\beta$ , IL-6, IFN $\gamma$ , TNF $\alpha$ , IL-10, IL-4, IL-21, IL-22 and IL-17E. These results are shown in Figure 5.4 and Figure 5.5. Focusing on the Th17 polarising cytokines - IL-1 $\beta$  and IL-23, luminex analysis revealed elevated levels of IL-1 $\beta$  in RA and PsA SF vs. matched serum, although levels of IL-1 $\beta$  in SF are low. IL-23 was present in RA and PsA SF. IL-23 was also detected in 4 out of 9 RA serum samples and at higher levels than the matched SF. No IL-23 was detected in the healthy control serum samples. Levels of the pro-inflammatory cytokines – IL-6, IFN $\gamma$  and TNF $\alpha$  were detected at higher levels in RA and PsA SF vs serum samples. IL-21 and IL-22 were detectable in RA SF, however levels were very low in PsA SF. Interestingly, the anti-inflammatory cytokine, IL-10, was detected at elevated levels in both RA and PsA SF. Similar results were observed for the Th2 polarising cytokine, IL-4. Overall, these data show the cytokine profile of SF is different to matching serum in both RA and PsA samples. Finally, following our results in chapter 4, whereby IL-17E synergised with TNF $\alpha$  to induce elevated levels of IL-6 and IL-8 from synovial fibroblasts, we were interested to investigate whether IL-17E was present in inflammatory arthritis. As shown in Figure 5.5, IL-17E protein was detected in one out of four HC serum samples and some RA and PsA serum and SF samples, however levels were low.

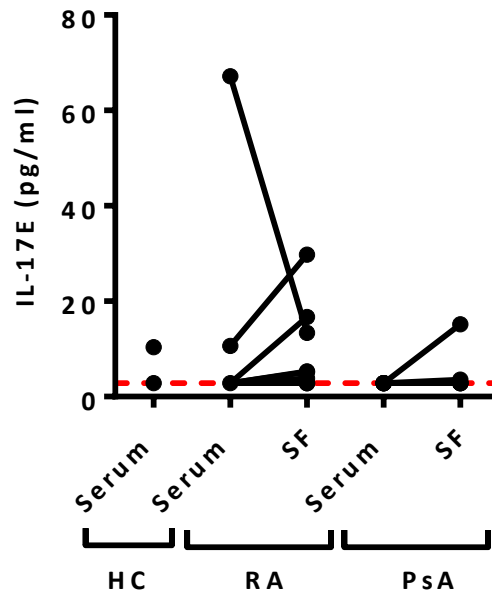
Next, we assessed the effect of RA and PsA serum and SF on synovial fibroblasts. PsA synovial fibroblasts were cultured with increasing percentage concentration of matched PsA serum and SF. Following a 24 hour culture, supernatants were collected and IL-6 and IL-8 protein levels were analysed by ELISA. As shown in Figure 5.6A, addition of 0.5% PsA serum led to an increased IL-6 secretion. Similar IL-6 protein levels were observed upon addition of 1 and 2% serum, however levels significantly dropped with 5% serum. In contrast, addition of 0.5% PsA SF to PsA fibroblasts led to a slight increase in IL-6 secretion, however levels were low. Moreover, IL-6 was undetectable following addition of 1, 2 or 5% SF. The lack of IL-6 induction by SF may be attributed to the toxicity of the sample. For example, SF contains hyaluronic acid, which in a compact *in vitro* culture system may be toxic to fibroblasts.

As IL-17A and TNF $\alpha$  have been shown to be present in RA and PsA SF, blocking experiments were performed to assess their role in SF-mediated IL-6 and IL-8 fibroblast secretion. Moreover, as the presence of IL-17F in RA and PsA remained inconclusive, it was of interest to examine the effect of IL-17F blockade. As 0.5% serum or SF elicited the highest levels of IL-6 secretion, this concentration was added to fibroblasts in the absence or presence of blocking antibodies. As illustrated in figure 5.6B, in this experiment addition of 0.5% serum or SF did not result in an increase in IL-6 secretion. In concordance, addition of the blocking antibodies has no effect.



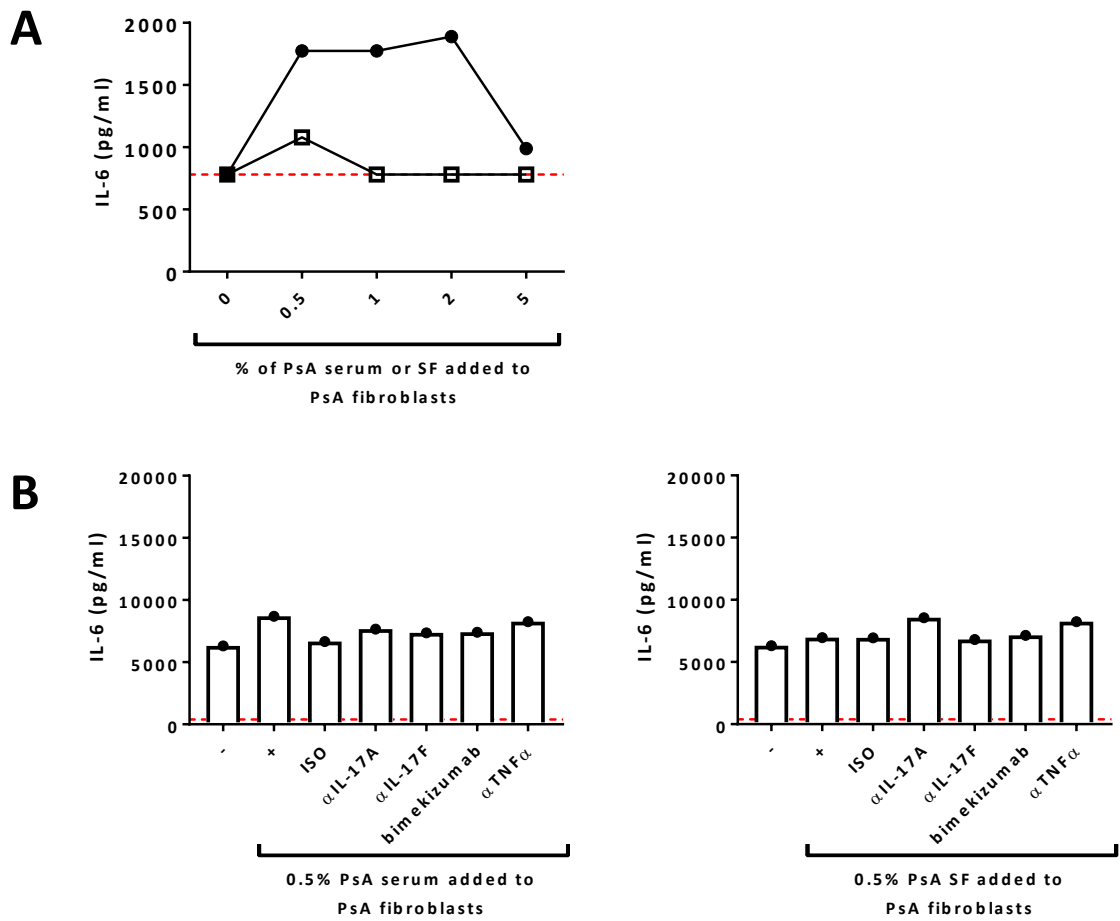
**Figure 5.4 RA and PsA SF display increased cytokine levels vs. matched serum.**

Paired cell-free serum and synovial fluid from RA (n=7) and PsA (n=6) patients were analysed by luminex. Dashed blue lines represent the lower detection limit. Statistical significance was calculated using a Wilcoxon matched pairs test.



**Figure 5.5 Low levels of IL-17E are detectable in RA PB and SF.**

IL-17E protein levels in paired cell-free serum and synovial fluid from RA (n=7) and PsA (n=6) patients were analysed by luminex. Dashed red lines represent the lower detection limit. Statistical significance was calculated using a Wilcoxon matched pairs test. Patient cohort information displayed in Table 5.1 (Patient cohort 1).



**Figure 5.6 Addition of RA or PsA serum or SF to synovial fibroblasts.**

(A)  $10 \times 10^3$  PsA fibroblasts were cultured with increasing doses of PsA serum or SF ( $n=1$ ). (B)  $10 \times 10^3$  PsA fibroblasts were 0.5% of PsA serum or SF in the absence or presence of blocking antibodies ( $n=1$ ). After 24 hours incubation, culture supernatant was removed and analysed for IL-6 protein levels via ELISA.

### 5.2.3 Examining the presence of IL-17A and IL-17F CD4+ T cells in RA and PsA PBMC and SFMC

While the presence of IL-17A+ CD4+ T cells in the inflamed arthritic knee has been widely reported <sup>10</sup>, little is known regarding the presence of IL-17F+ CD4+ T cells. With this in mind and the inconclusive IL-17F protein detection results in RA and PsA SF, it was of interest to explore the presence of IL-17 expressing CD4+ T cells in RA and PsA PBMC and SFMC.

Paired PBMC and SFMC from RA or PsA patients were stimulated for 3 hours with PMA, ionomycin and Golgistop. Patient cohort information is provided in Table 5.3 (Patient cohort 3). Cells were stained to assess intracellular cytokine expression and analysed by flow cytometry. Figure 5.7 shows a representative plot of the gating strategy applied to PBMC and SFMC samples. Within CD3+ CD4+ T cells, IL-17A expressing cells were increased in the SF compared to the PB of patients with RA (2.7 vs. 0.9%, respectively) and PsA (2.5 vs. 1%, respectively) (Figure 5.8). However, statistical significance was only achieved with the PsA cohort. Flow cytometry analysis revealed very few IL-17F expressing CD4+ T cells in matched PBMC and SFMC from RA and PsA samples. Similarly, the frequency of IL-17AF+ CD4+ T cells detected in inflammatory arthritis PB and SF was low.

We next examined whether different *ex vivo* stimulation conditions would reveal the presence of IL-17F+ CD4+ T cells. First we compared the 3 hour PMA and ionomycin stimulation with Golgistop (monensin), GolgiPlug (Brefeldin) or a combination of both Golgistop and Golgiplug. Our results revealed no difference in the percentage of IL-17F+ CD4+ T cells detected with the different protein transport inhibitors (data not shown). Our next approach involved stimulating PBMC and SFMC with either PMA and ionomycin,  $\alpha$ CD3 and  $\alpha$ CD28 or Golgistop alone for 3, 5 or 8 hours. This was performed with one RA and one PsA donor. As shown in Figure 5.9, IL-17A expressing CD4+ T cells were increased in the SF compared to the PB only in the RA sample following 3 hours PMA and ionomycin stimulation. However, IL-17A+ CD4+ T cells were detected at higher frequencies in the SF vs. PB in both donors following a 3 hour stimulation with  $\alpha$ CD3 and  $\alpha$ CD28. Very few IL-17A+ CD4+ T cells were

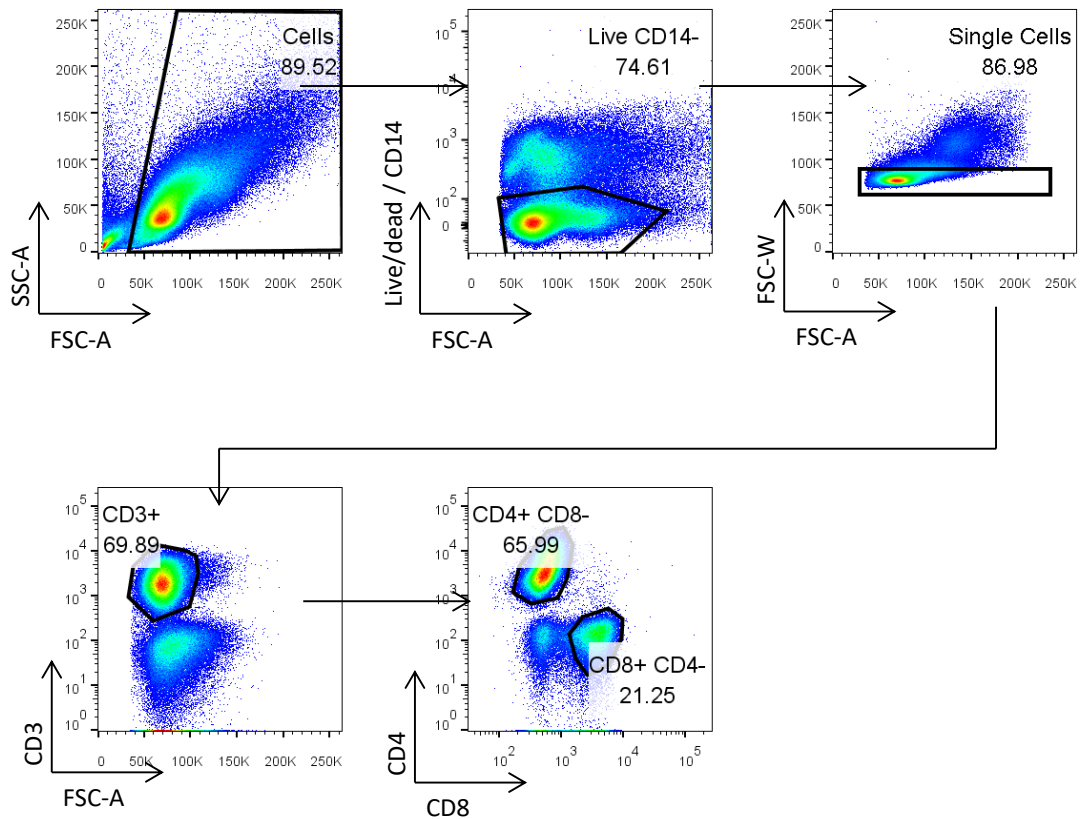
observed following incubation with Golgistop only. Only a small increase in the percentage of IL-17A+ CD4+ T cells was observed in the RA SFMC with 8 hours incubation with Golgistop. None of these conditions revealed the presence of IL-17F+ CD4+ T cells in the RA and PsA paired PB and SF sample. The IL-17AF heterodimer stain was not included in this experiment.

IL-17A protein and IL-17A+ CD4+ T cells were robustly detected in RA and PsA SF. To conclude this section, correlation analyses were performed. Firstly, correlation analysis was performed on matched ICCS and ELISA patient samples. As shown in Figure 5.10 A, the frequency of IL-17A+ CD4+ T cells detected in RA/PsA PB or SF demonstrated a positive correlation to the levels of IL-17A protein detected in the serum or SF counterpart. Next, we correlated the frequency of IL-17A+ CD4+ T cells present in RA or PsA SF to the patient's disease activity score in 28 joints (DAS28). This score is calculated at the time of sample collection using a number of assessment criteria including the number of tender and swollen joints, the erythrocyte sedimentation rate (ESR) and the C-reactive protein (CRP) level. No correlation was observed in either RA or PsA samples (Figure 5.10 B). Moreover, no correlation was observed between the concentration of IL-17A present in RA or PsA SF and the patient DAS28 score (Figure 5.10C).



**Table 5.3 Demographic and clinical parameters of patients included in *ex vivo* IL-17A and IL-17F flow cytometry analysis (patient cohort 3)**

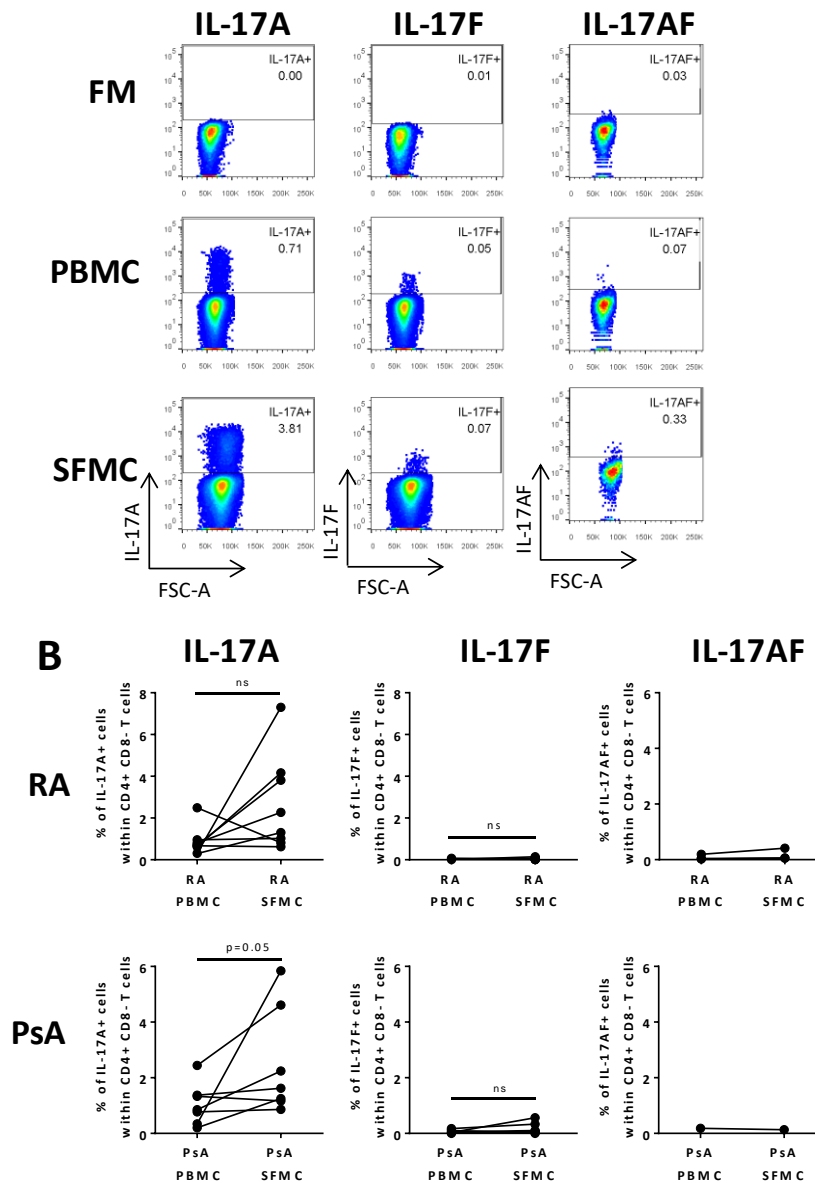
	<b>RA Paired PB/SF (n = 8)</b>	<b>PsA Paired PB/SF (n = 7)</b>
<b>Male, no.</b>	0	5
<b>Female, no.</b>	8	2
<b>Age, mean ± SEM years</b>	54.9 ± 7.0	41.7 ± 4.2
<b>Treatment, no.</b>		
No treatment	2	2
MTX	5	3
Anti-TNF	1	1
Other (Abatacept, Ustekinumab)	-	1
<b>DAS28</b>		
Mean ± SEM	5.2 ± 0.4	4.5 ± 0.5
Range	3.8 – 7.02	1.89 – 6.0
Seronegative	2	7

**A**

**Figure 5.7 Gating Strategy for matched RA and PsA PBMC and SFMC samples.**

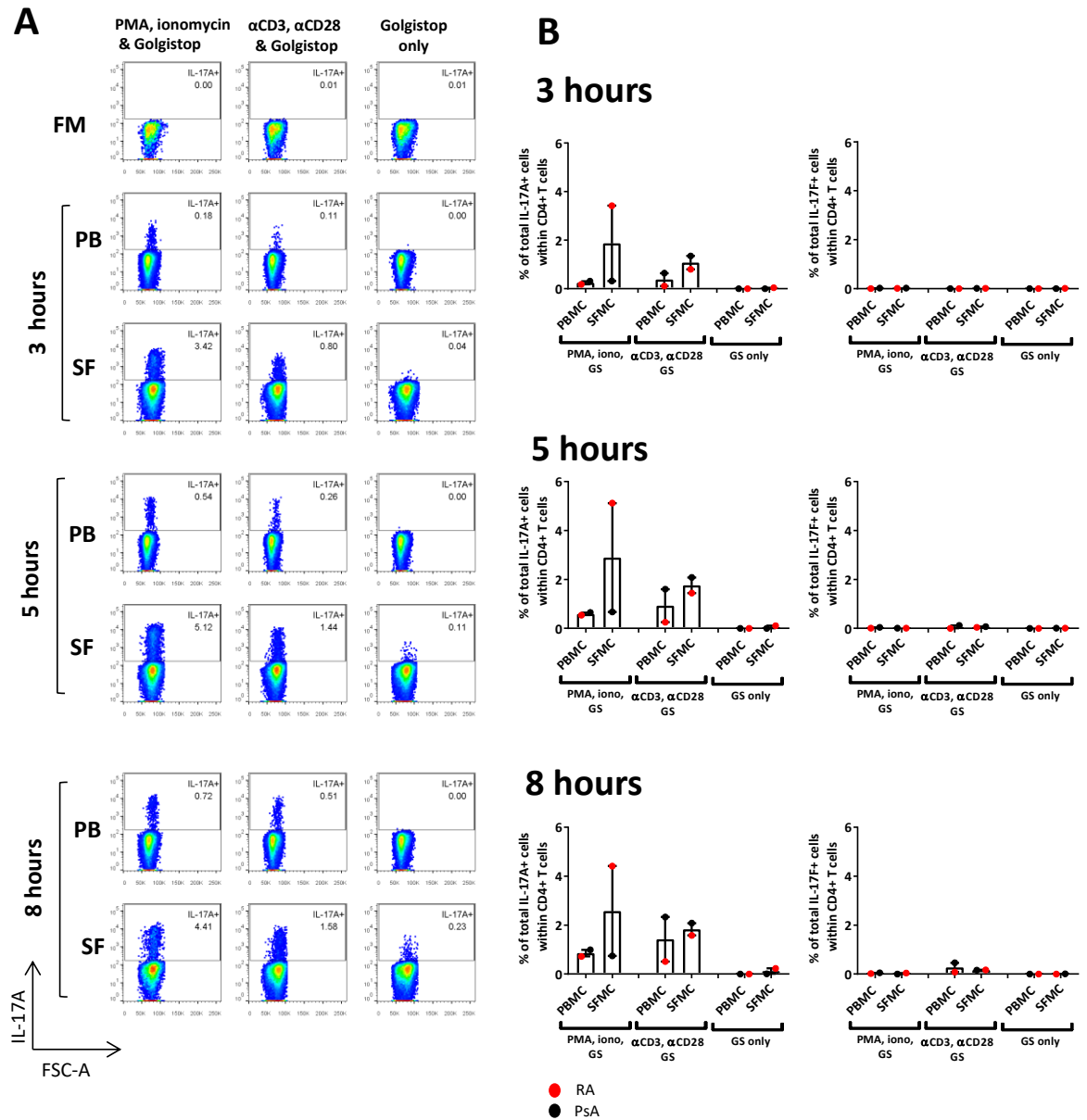
RA or PsA PBMC and SFMC were stimulated for 3 hours with PMA, ionomycin and GolgiStop. Cells were then stained with fluorescently-conjugated antibodies against extracellular markers and intracellular cytokines. In this representative SFMC gating, cells were first gated for lymphocytes (SSC-A vs. FSC-A). The lymphocyte gate was further analysed for uptake of the Live/Dead eFlour 780 stain to determine live vs. dead cells. Within live cells, a FSC-W/FSC-A gate was applied allowing exclusion of doublet cells. Next, within CD3+ cells, CD4+ CD8- and CD8+ CD4- T cells were selected for analysis of intracellular cytokines.

## A Within CD3+ CD4+ CD8- T cells...



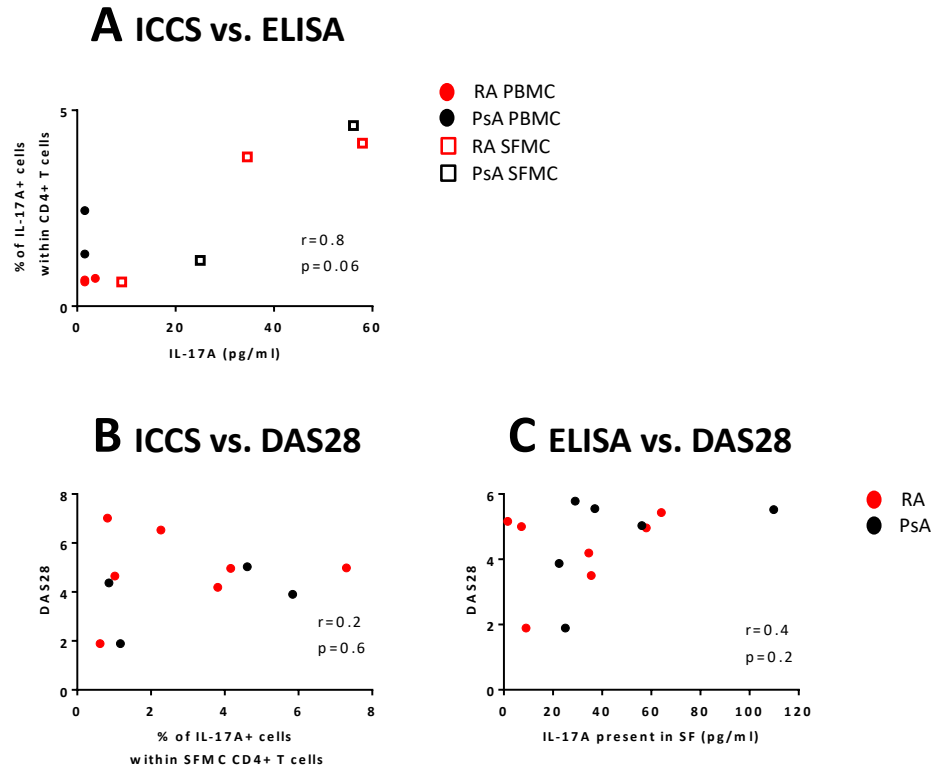
**Figure 5.8 Examining the presence of IL-17A, IL-17F and IL-17AF expressing CD4+ T cells in inflammatory arthritis.**

Matched PBMC and SFMC from RA and PsA donors were stimulated for 3 hours with PMA, ionomycin and Golgistop and then assessed for intracellular IL-17A, IL-17F and IL-17AF cytokine expression. (A) Representative dot plot whereby fluorescence minus (FM) controls were used to gates to depict positive cytokine expression. (B) Cumulative data show frequencies of IL-17A and IL-17F expressing cells within the total CD4+ T cell population of matched PBMC and SFMC from RA (n=8) and PsA (n=7) samples. Similarly, the frequencies of IL-17AF expressing CD4+ T cells were analysed in RA (n=3) and PsA (n=1) samples. Statistical significance was calculated using a Wilcoxon matched pairs test.



**Figure 5.9 Stimulating RA and PsA paired PBMC and SFMC with different *ex vivo* conditions.**

Matched PBMC and SFMC from one RA and one PsA donor were stimulated with PMA, ionomycin and Golgistop,  $\alpha$ CD3,  $\alpha$ CD28 and Golgistop or Golgistop alone for 3, 5 or 8 hours. Cells were then stained for analysis of IL-17A and IL-17F cytokine expression by flow cytometry. Gated within live CD3+ CD4+ T cells (A) displays representative dot plots of IL-17A cytokine expression observed in RA PBMC and SFMC. (B) Cumulative flow cytometric data show frequencies of IL-17A and IL-17F within the PBMC and SFMC CD4+ T cell population from RA (n=1) (red) and PsA (n=1) (black) donors.



**Figure 5.10 Correlation analysis on the presence of IL-17A+ CD4+ T cells vs. IL-17A protein present in inflammatory arthritis samples and assessing correlation with DAS28 scores.**

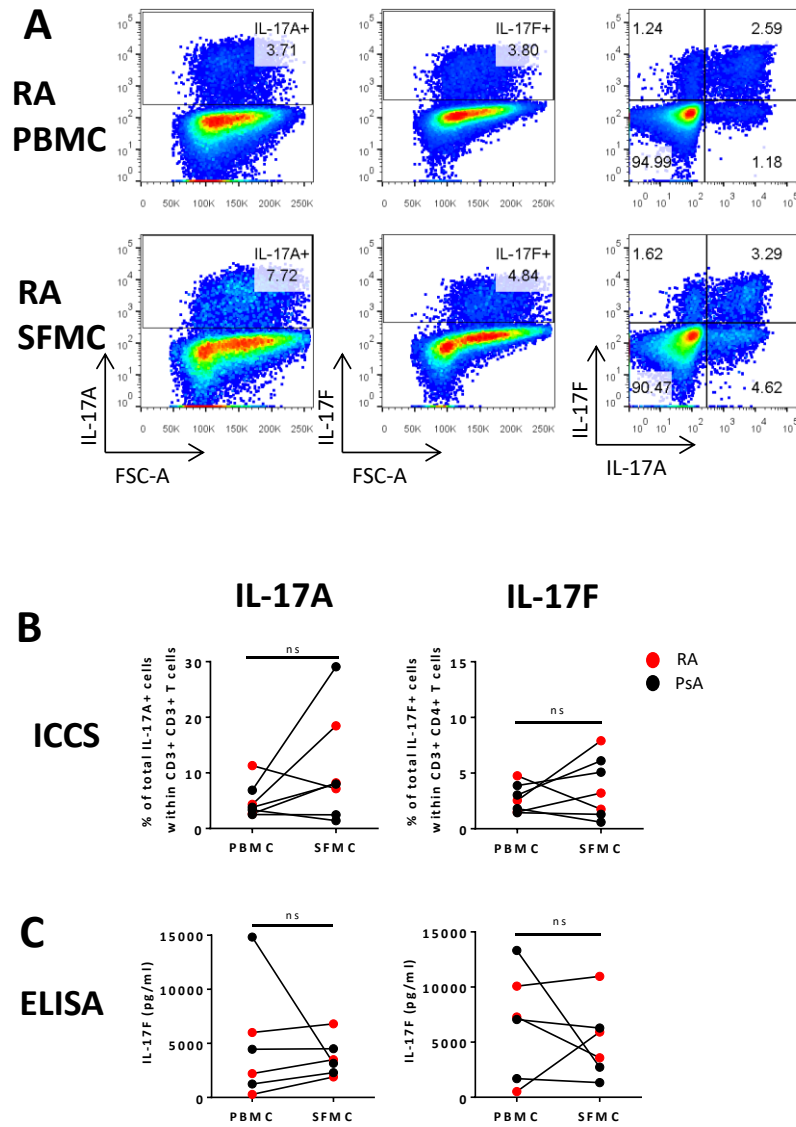
(A) The frequency of IL-17A CD4+ T cells detected in RA (n=3) and PsA (n=2) PB and SF following an *ex vivo* stimulation were correlated with the levels of IL-17A detected in the matched serum or SF via ELISA. (B) The frequency of SF IL-17A+ CD4+ T cells detected in RA (n=7) (red symbols) or PsA (n=4) (black symbols) were compared against the corresponding patient DAS28 score. (C) The levels of IL-17A protein present in RA (n=7) or PsA (n=6) were correlated with patient DAS28 scores. Symbols represent individual donors. Data was analysed using Spearman non-parametric correlation analysis.

#### **5.2.4 *In vitro* stimulation induces IL-17A, IL-17F and IL-17AF expressing CD4+ T cells from inflammatory arthritis matched PBMC and SFMC**

We hypothesised that culturing inflammatory arthritis PBMC and SFMC *in vitro* with IL-1 $\beta$ , IL-23,  $\alpha$ CD3 and  $\alpha$ CD28 would induce IL-17A, IL-17F and IL-17AF expressing CD4+ T cells. Moreover, we speculated that due to the inflammatory origin of SFMC, this stimulation would induce a higher frequency of IL-17A+, IL-17F+ and IL-17AF+ CD4+ T cells in SFMC vs. PBMC.

As shown in Figure 5.11, culturing inflammatory arthritis PBMC and SFMC *in vitro* induced IL-17A and IL-17F expressing CD4+ T cells. However, there was no consistent enhancement of IL-17 expressing CD4+ T cells in the SF vs. PB. It is important to note the viability of the SFMC samples following *in vitro* culture varied, with some samples displaying a significant percentage of cell death. It may be SFMCs are fragile and more prone to cell death following a culture period. Similarly, while ELISA analysis revealed the presence of IL-17A and IL-17F in inflammatory arthritis PBMC and SFMC *in vitro* cultures, no consistent differences were observed between PBMC and SFMC (Figure 5.11B).

**Within CD3+ CD4+ CD8- T cells...**



**Figure 5.11 IL-17A, IL-17F and IL-17AF expressing CD4+ T cells can be induced from inflammatory arthritis PBMC and SFMC upon *in vitro* stimulation.**

RA or PsA matched PBMC and SFMC ( $1 \times 10^6$ ) were cultured with platebound anti-CD3 mAb, anti-CD28 mAb, IL-1 $\beta$  (10ng/ml) and IL-23 (20ng/ml). After 3 days incubation, cells were re-stimulated for 3 hours with PMA, ionomycin and Golgistop. Cells were then stained with fluorescently-conjugated antibodies against extracellular markers (CD3, CD4 and CD8) and intracellular IL-17 cytokines. (A) Representative dot plots and (B) cumulative data show frequencies of IL-17A and IL-17F expressing cells within the total CD4+ T cells of paired PBMC and SFMC from RA (n=3) (red symbols) and PsA (n=3) (black symbols) patients, respectively. (C) Prior to re-stimulation, cell supernatant was analysed for IL-17A and IL-17F supernatant via ELISA (RA (n=3) and PsA (n=3)).

### 5.2.5 Investigating the presence of CD8+ IL-17 producing cells in inflammatory arthritis

Although this project focused on IL-17 producing CD4+ T cells, we were keen to explore whether other immune cells producing IL-17F are present in inflammatory arthritis. Our lab has previously reported the increased presence of IL-17A+ CD8+ T cells in the inflamed joint of PsA patients <sup>6</sup>. Currently, IL-17F+ CD8+ T cells have not been described in PsA. This provided me with the rationale to examine the presence of IL-17F+ CD8+ T cells in inflammatory arthritis. The cytokine expression of CD8+ T cells present in RA and PsA was analysed using the same PMA and ionomycin *ex vivo* stimulated RA/PsA PBMC and SFMC data set generated to analyse CD4+ T cells (patient cohort 3). Using the gating strategy shown in Figure 5.5, we gated within CD8+ CD4- T cells and analysed the cytokine expression of IL-17A and IL-17F. In line with the lab's previous findings, we observed a significant enrichment of IL-17A+ CD8+ T cells in PsA SF vs. matched PB (approximately 3.5 vs. 0.2 % of IL-17A+ cells within CD8+ T cells) (Figure 5.12). While one out of seven PsA samples showed an enhanced frequency of IL-17F+ CD8+ T cells in the SF vs. PB (0.66 vs. 0.03%, respectively), the remaining six PsA samples displayed low percentages (0.08 vs. 0.03%, respectively). Therefore, although this data set generated statistical significance, IL-17F expressing CD8+ T cells were only robustly detected in one sample. A small percentage of IL-17A+ CD8+ T cells was observed in RA SFMC, however this did not reach significance. Very few IL-17F+ CD8+ T cells were observed in RA PBMC or SFMC.

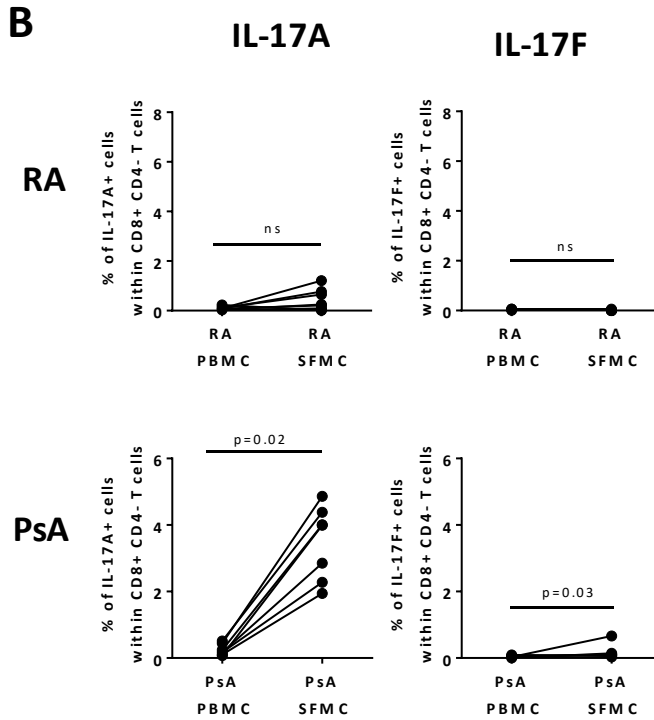
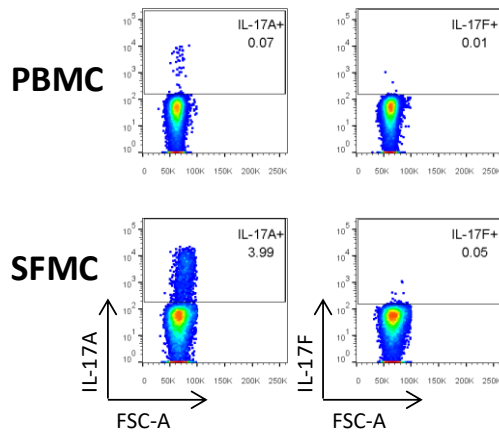
Next, we sought to investigate whether any CD4- CD8- immune cell subsets present in RA or PsA PB or SF expressed IL-17A or IL-17F. In the majority of patient *ex vivo* experiments, the live/dead and CD14 stain were combined to generate a 'dump' gate. However, in some experiments, the cytokine profile of CD14+ monocytes could be analysed. In these experiments, IL-17A or IL-17F expressing CD14+ monocytes were not observed in RA or PsA PB or SF (data not shown). To finalise the analysis of the *ex vivo* patient data set, backgating analysis was performed on all live CD14- IL-17 expressing cells. This was to potentially reveal any IL-17A or IL-17F producing cells which were CD3+ CD4- CD8- or CD3-. Double negative (DN) T cells (CD3+ CD4- CD8-) constitute approximately 1-5% of T cells in humans. Studies have shown they



can function similarly Treg cells or produce cytokines including IL-17A, IFN $\gamma$  and TNF $\alpha$  <sup>10</sup>. CD14<sup>-</sup> CD3<sup>-</sup> cells present in PBMC and SFMC can include CD19<sup>+</sup> B cells, CD56<sup>+</sup> NK cells and innate lymphoid cells (ILC), of which NK cells and ILC3 have been identified as an IL-17A producing population <sup>11</sup>.

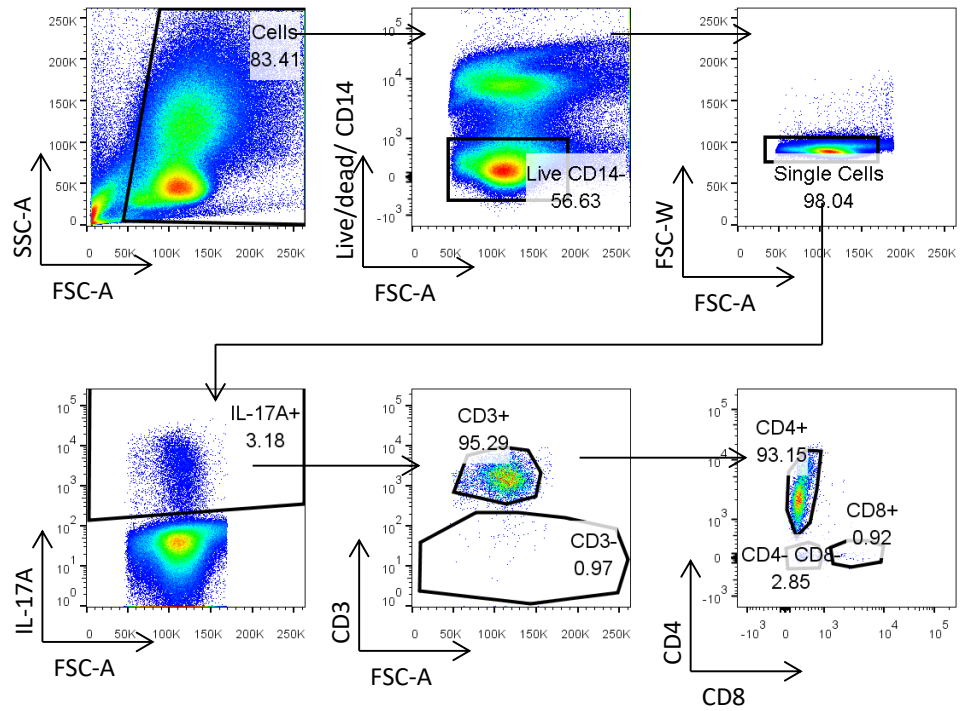
As demonstrated in Figure 5.13, live CD14<sup>-</sup> IL-17<sup>+</sup> cells were gated and then surface marker expression was used to determine the cytokine producing cell. Back gating was only performed on samples with 300 or more IL-17A or IL-17F expressing cell events. The majority of IL-17A producing cells in both RA and PsA PBMC and SFMC were CD3<sup>+</sup> cells (approximately 94%) (Figure 5.14). In the RA PBMC samples, the majority of CD3<sup>+</sup> IL-17A<sup>+</sup> cells were CD4<sup>+</sup> CD8<sup>-</sup> T cells (approximately 93%). In 5 out of 8 RA SFMC samples approximately 95% of CD3<sup>+</sup> IL-17A<sup>+</sup> cells were CD4<sup>+</sup> CD8<sup>-</sup> T cells. However, in three RA donors 53%, 64% and 61% respectively of SF CD3<sup>+</sup> IL-17A<sup>+</sup> cells were CD4<sup>+</sup> CD8<sup>-</sup> T cells. Moreover, in these three donors 31%, 29% and 29% respectively of CD3<sup>+</sup> IL-17A<sup>+</sup> cells were CD8<sup>+</sup> CD4<sup>-</sup> T cells. This is in contrast to the low frequencies of IL-17A<sup>+</sup> CD8<sup>+</sup> CD4<sup>-</sup> T cells present in the remaining RA SF samples (approximately 0.9%). Two of the RA patients with enhanced frequencies of IL-17A<sup>+</sup> CD8<sup>+</sup> CD4<sup>-</sup> T cells in the SF was seronegative, whilst the other was seropositive. In 7 PsA PB donors approximately 85% of IL-17A<sup>+</sup> cells were CD4<sup>+</sup> CD8<sup>-</sup> T cells and 7% were CD8<sup>+</sup> CD4<sup>-</sup> T cells. In contrast, the PsA SF was enriched with IL-17A<sup>+</sup> CD8<sup>+</sup> CD4<sup>-</sup> T cells. Approximately 36% of IL-17A<sup>+</sup> cells were CD8<sup>+</sup> CD4<sup>-</sup> T cells, whereas 45% were CD4<sup>+</sup> CD8<sup>-</sup> T cells. Across all RA and PsA samples, only one PsA SF sample had over 300 events of detectable IL-17F expressing cells. Back-gating analysis revealed all IL-17F expressing cells were CD3<sup>+</sup> cells, of which 0.7% were CD4<sup>-</sup> CD8<sup>-</sup>, 35% were CD4<sup>+</sup> CD8<sup>-</sup> and 27% were CD8<sup>+</sup> CD4<sup>-</sup>.

### A Within CD3+ CD8+ CD4- T cells...



**Figure 5.12 Assessing the presence of IL-17A and IL-17F expressing CD8+ T cells in inflammatory arthritis.**

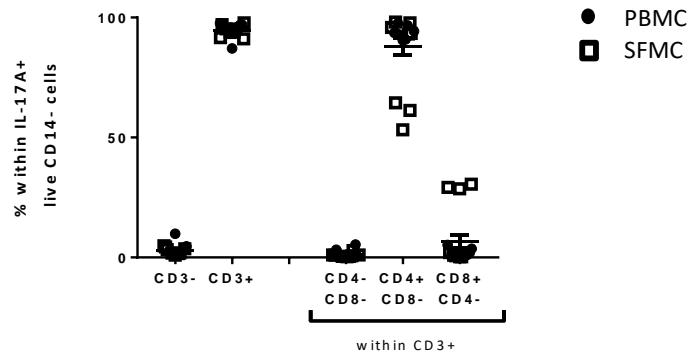
Matched PBMC and SFMC from RA and PsA donors were stimulated for 3 hours with PMA, ionomycin and Golgistop and then assessed for intracellular IL-17A, IL-17F and IL-17AF cytokine expression via flow cytometry. Gating within live CD8+ CD4- T cells (**A**) displays representative dot plots of a patient with PsA and (**B**) shows cumulative data of IL-17A and IL-17F frequencies within the total CD8+ T cell population of matched PBMC and SFMC from RA (n=8) and PsA (n=7) samples. Statistical significance was calculated using a Wilcoxon matched pairs test.



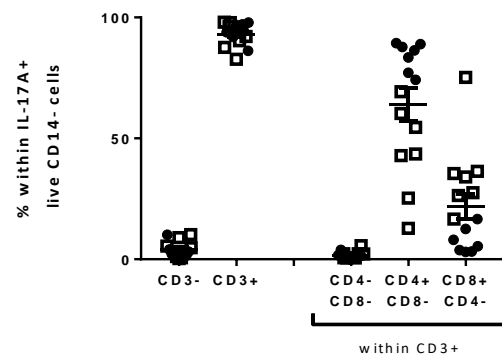
**Figure 5.13 Back- gating on RA or PsA PBMC and SFMC IL-17 expressing live CD14- cells.**

Matched PBMC and SFMC from RA and PsA donors were stimulated for 3 hours with PMA, ionomycin and Golgistop and then assessed for intracellular IL-17A and IL-17F cytokine expression via flow cytometry. Gating within live CD8+ CD4- T cells

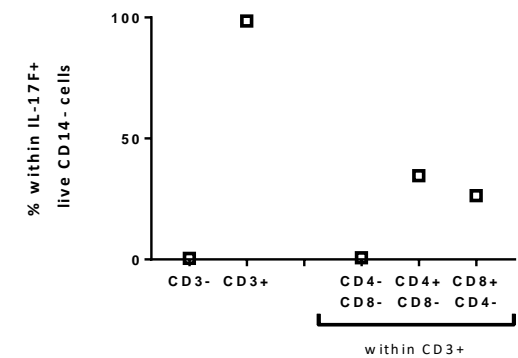
### A RA PB and SF IL-17A+ cells



### B PsA PB and SF IL-17A+ cells



### C PsA SF IL-17F+ cells



**Figure 5.14 Back-gating on RA or PsA PBMC and SFMC IL-17 expressing live CD14- cells.**

Matched PBMC and SFMC from RA and PsA donors were stimulated for 3 hours with PMA, ionomycin and Golgistop and then assessed for intracellular IL-17A and IL-17F cytokine expression via flow cytometry. Live CD14- IL-17A+ or IL-17F+ cells were gated upon. Back gating analysis was performed on samples with 300 or more IL-17+ events to determine the cytokine producing cells. Cumulative data showing cell surface marker expression on IL-17A+ cells present in (A) RA PB and SF (n=8) and (B) PsA PB and SF (n=7). (C) Marker expression on IL-17F+ cells present in PsA SF (n=1).

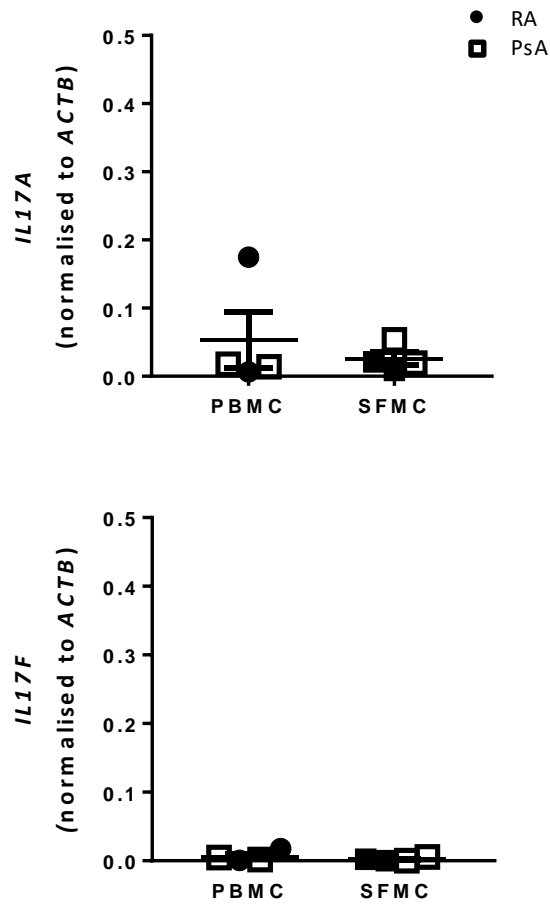
### 5.2.6 Investigating the RNA expression of IL-17 family members in RA and PsA PBMC and SFMC

My next aim was to investigate whether *IL17A* and *IL17F* mRNA could be detected in cells from inflammatory arthritis serum or SF. It is important to note that IL-17AF is formed post-transcriptionally and cannot be analysed at an RNA level. Bulk cells were isolated from fresh RA or PsA serum or SF samples and approximately  $1-2 \times 10^6$  cells were lysed in trizol. Cells were not stimulated with PMA or ionomycin. Using the chloroform immunoprecipitation method, RNA was extracted and reverse transcription was performed. QPCR reactions were performed using primers for human IL-17A and IL-17F; moreover, the *ACTB* gene was used as an endogenous control. In all RA and PsA PBMC and SFMC samples, *IL17A* and *IL17F* mRNA expression levels were not reliably detected (Figure 5.15) with a CT value of 30 or above and similar to no reverse transcription control samples. Unfortunately, in this experiment a positive control (i.e RNA isolated from stimulated PBMC known to contain *IL17A* and *IL17F* mRNA) was not used. ELISA and ICCS results have clearly demonstrated the presence of IL-17A in the SF. Therefore, it was proposed the *IL17A* mRNA was not detected in bulk SFMC because the IL-17 producing cells are only a small proportion of the total cells in the SF.

To investigate this hypothesis, the presence of *IL17A* and *IL17F* mRNA in sorted immune subsets from RA PB and SF was examined. Unfortunately, due to limited patient sample availability this experiment was only performed in RA samples (n=3). Fresh PBMC or SFMC were stained, without stimulation for extracellular markers (CD3, CD4, CD8, CD19 and CD14). Immune cell subsets were then sorted on a BD FACS ARIA. This gating strategy is shown in Figure 5.16. Sorted cell subsets were lysed in trizol and total RNA was extracted. Following this, reverse transcription was performed. As a positive control, RNA isolated from healthy PBMC stimulated for 3 days with IL-1 $\beta$ , IL-23, anti-CD3 and anti-CD28 was also included in the reverse transcription stage, adding in the same total of RNA as the sorted T cell samples. QPCR reactions were then performed using primers for IL-17A and IL-17F and the *ACTB* gene was used as an endogenous control. As displayed in Figure 5.17, *IL17A* and *IL17F* mRNA was detectable in the positive control sample, indicating the reverse transcription step and qPCR reactions were successful. In contrast, no *IL17A* and

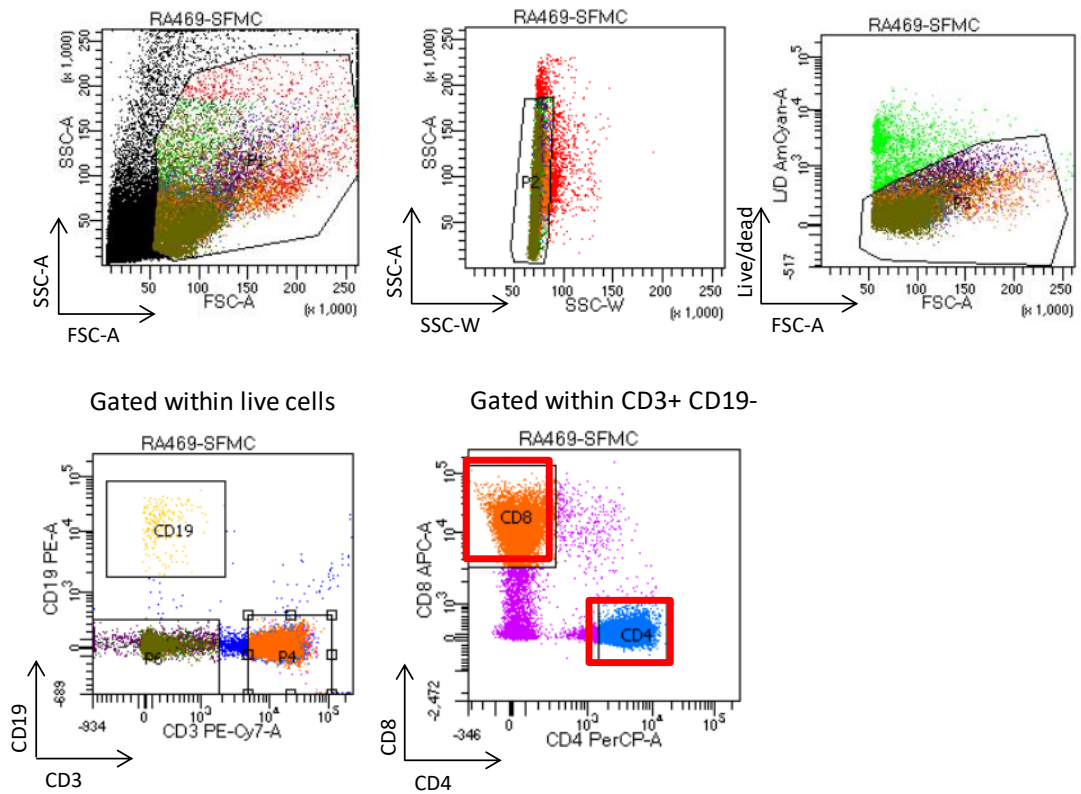
*IL17F* mRNA was detectable in CD4+ or CD8+ T cell subsets sorted from HC PB or RA PB and SF. All CT values were above 30 and similar to no reverse transcription controls.

Next, I investigated whether similar results were observed in data previously generated in our lab which analysed the gene expression profile of sorted CD25- CD4+ T cells from RA PB and SF using an affymetrix microarray. Affymetrix data analysis uses robust multi-array average (RMA) values, whereby the raw intensity values are background corrected, log2 transformed and quantile normalised. I analysed this dataset to assess the gene expression levels of all IL-17 cytokines members in RA CD25- CD4+ T cells. As a positive control the expression of CD4 was analysed. Additionally, as a significant proportion of CD4+ T cells express TNF $\alpha$  and IFN $\gamma$ , the expression levels of these genes were analysed. To achieve this, RMA values were transformed ( $2^Y$ ) and plotted as the probe intensities. In general, a gene with a probe intensity of below 100 is considered not detectably expressed. As shown in Figure 5.18, *CD4* gene expression was detected at moderate levels and with minimal differences between the PB and SF subset. Gene expression of *TNFA* was detectable at various levels across the samples, with no consistent difference between PB and SF. Where *IFNG* gene expression levels were moderate and in 4 out of 5 samples, the gene expression level was enhanced in the SF CD25- CD4+ subsets. In contrast, all IL-17 family members exhibited low probe intensity values. In fact, *IL17A* displayed the lowest probe intensity values compared with *IL17B* and *IL17C* which are cytokines that have not been reported to be produced by CD4+ T cells. Therefore, it can be concluded that there are no detectable gene expression levels of the IL-17 cytokines in CD25- CD4+ T cells from RA PB and SF samples.



**Figure 5.15 Low levels of *IL17A* and *IL17F* mRNA are detected from bulk RA and PsA PBMC and SFMC.**

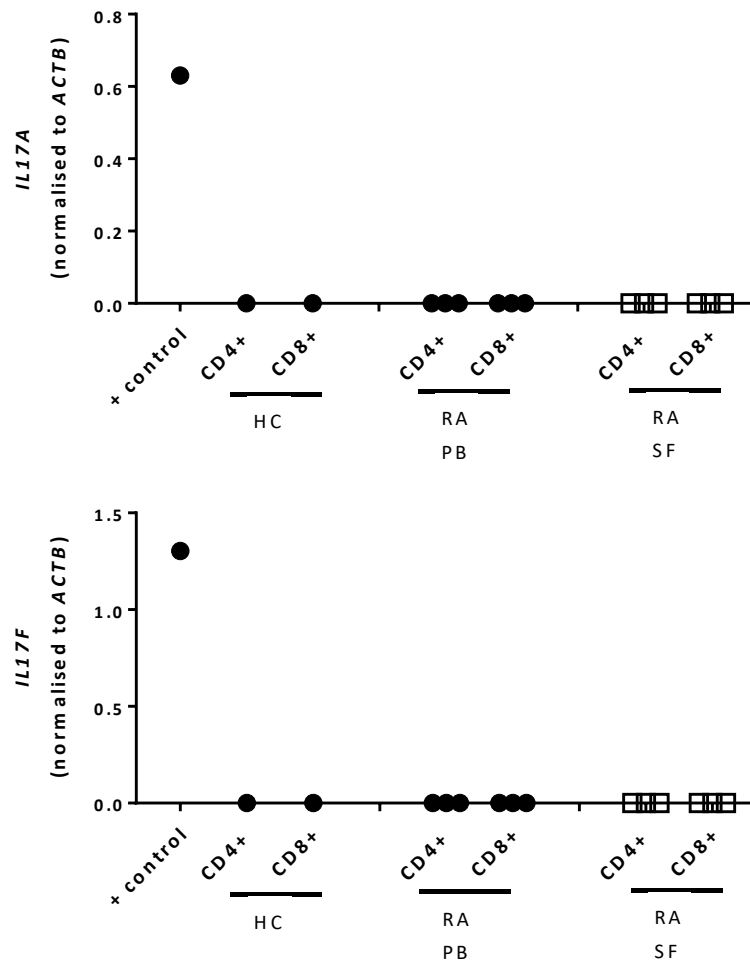
Approximately  $1-2 \times 10^6$  RA (n=2) (red symbol) or PsA (n=2) (black symbol) PBMC and SFMC were lysed in trizol. Following RNA extraction, reverse transcription was performed. QPCR was performed using primers for *IL17A* and *IL17F* and *ACTB* was used as an endogenous control. Each data point is an individual donor.



**Figure 5.16** Sorting strategies for immune cell subsets from PB and SF.

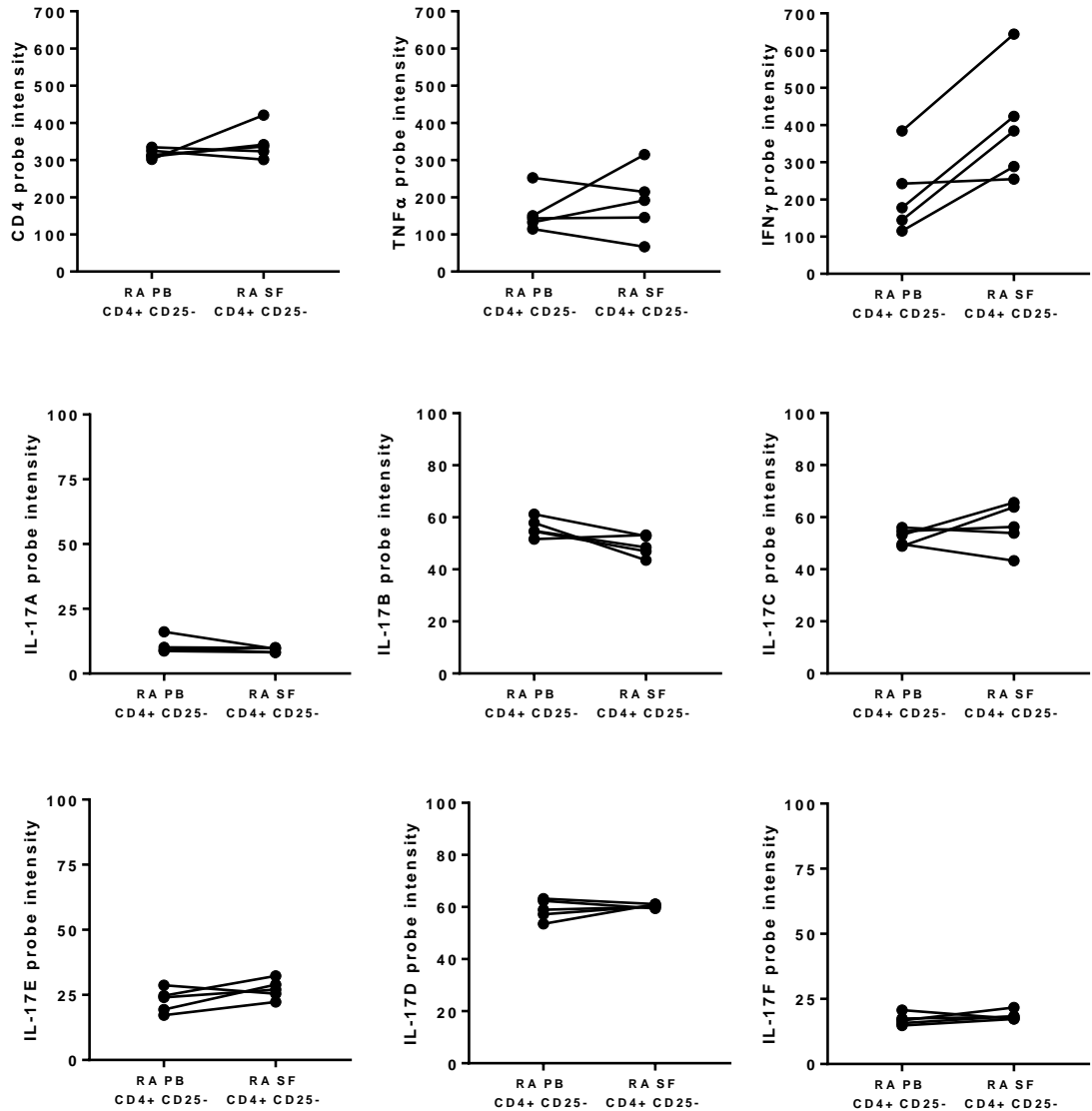
Based on forward scatter/side scatter (FSC/SSC) properties, viable lymphocytes were gated. A FSC-W/FSC-A gate was applied allowing the exclusion of doublet cells. Dead cells were excluded based on their uptake of the viability dye, eFluor506. Within live, single cells CD19+ CD3- B cells were gated and collected. CD3+ cells were further analysed for their expression of CD4+ and CD8+ and CD4+CD8- and CD8+CD4- cells were gated and collected. Gating within CD3- cells. Red boxes indicate the sorted populations.





**Figure 5.17 *IL17A* and *IL17F* mRNA is undetectable in sorted CD4+ and CD8+ T cells from RA PB and SF.**

Mononuclear cells were isolated from the blood of a healthy donor (HC) (n=1) and the PB and SF from RA patients (n=3). Cells were stained with extracellular surface markers and using a FACS Aria sorter CD3+ CD4+ CD8- and CD3+ CD8+ CD4- T cells were sorted. Total RNA was isolated from all sorted populations and converted into cDNA via reverse transcription. QPCR was then performed to assess the mRNA expression levels of *IL17A* and *IL17F*.



**Figure 5.18 IL-17 gene expression profile of CD4+ CD25- T cells from RA PB and SF.**

Cell sorting, RNA isolation and microarray analysis was performed by a previous member of the lab, Veerle Fleskens. Data mining for the indicated genes was performed by myself. CD25- CD4+ T cells from RA matched PB and SF were sorted on a BD FACS Aria (n=5). Total RNA was isolated from all sorted populations and analysed by an affymetrix microarray. The RMA values of *CD4*, *TNFA*, *IFNG* and IL-17 family members were transformed ( $2^Y$ ) and plotted as probe intensity. Each line is an individual donor.

### **5.2.7 Examining the presence of IL-17A and IL-17F expressing cells in RA or PsA tissue**

The synovium of patients with active RA and PsA is infiltrated with a variety of mononuclear cells, including T cells. While T cells can be detected in the SF of RA or PsA, some T cells remain embedded in the synovial tissue. To conclude the investigation of IL-17F present in inflammatory arthritis, we examined the presence of IL-17F and IL-17A cells present in RA synovial tissue.

The following protocol was optimised by another member of our lab, Lucy Durham. Moreover, these experiments were performed in collaboration with Lucy Durham, with Lucy acquiring all samples. Synovial tissue samples were collected from two RA patients at the time of elective knee replacement surgery. Synovial tissue was cut into 1-2 mm pieces and digested by incubating with an enzyme mastermix (Miltenyi) on a gentleMACS dissociator. Isolated cells were stimulated for 3 hours with PMA and ionomycin. Cells were then stained for extracellular markers and intracellular cytokines and analysed by flow cytometry. Figure 5.19 illustrates the gating strategies applied to cells isolated from both RA synovial tissue samples. In donor one, only a small proportion of these cells were lymphocytes, with the remaining being either debris or synovial fibroblasts, whereas donor two displayed a higher frequency of lymphocytes.

In the RA synovial tissue from donor 1, flow cytometry results shown that 2.7% of total CD4<sup>+</sup> T cells expressed IL-17A. However only three events were captured within the IL-17A<sup>+</sup> gate, therefore this result cannot be interpreted accurately (Figure 5.20). However, in donor two, 2.3% of total CD4<sup>+</sup> T cells expressed IL-17A and a substantial number of events were captured within the IL-17A<sup>+</sup> gate. No IL-17F<sup>+</sup> CD4<sup>+</sup> T cells were detected in donor one and although 0.3% IL-17F<sup>+</sup> CD4<sup>+</sup> T cells were detected in donor two, the event number is too low for accurate interpretation. Donor one and donor two displayed 53% and 55% of IFN $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells, respectively. With regards to CD8<sup>+</sup> T cells, donor one displayed 21.4% of IL-17A<sup>+</sup> CD8<sup>+</sup> T cells, whereas no IL-17A<sup>+</sup> CD8<sup>+</sup> T cells were detectable in donor 2. In both donors, event numbers were too low to draw conclusions on IL-17F<sup>+</sup> CD8<sup>+</sup> T cells. In donor one, 41% of

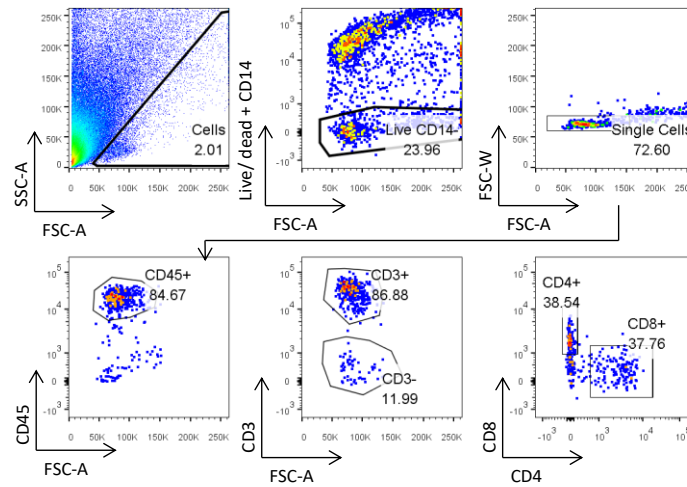
total CD8<sup>+</sup> T cells were IFN $\gamma$ <sup>+</sup>, whereas donor two displayed 93% IFN $\gamma$ <sup>+</sup> CD8<sup>+</sup> T cells. In summary, IL-17A<sup>+</sup> CD4<sup>+</sup> T cells were present in one donor and IL-17A<sup>+</sup> CD8<sup>+</sup> T cells were present in the other. Due to low event numbers, no IL-17F<sup>+</sup> CD4<sup>+</sup> or CD8<sup>+</sup> T cells were definitively detected.

Our next aim was investigate the presence of any IL-17 expressing CD4<sup>-</sup> or CD8<sup>-</sup> immune cell subsets present in RA synovial tissue. To achieve this, back-gating analysis was performed on IL-17A and IL-17F expressing live CD14<sup>-</sup> cells (Figure 5.21). In both donors the majority of IL-17A<sup>+</sup> cells were CD45<sup>+</sup> and CD3<sup>+</sup>, although a CD45<sup>+</sup> CD3<sup>-</sup> population was observed in donor one. The majority of IL-17A<sup>+</sup> CD3<sup>+</sup> cells in donor one were CD4<sup>-</sup>CD8<sup>-</sup> and CD8<sup>+</sup>CD4<sup>-</sup> T cells, however for donor two the predominant subsets were CD4<sup>+</sup>CD8<sup>-</sup> and CD4<sup>-</sup>CD8<sup>-</sup> T cells. Caution must be taken when interpreting the CD4<sup>+</sup>CD8<sup>-</sup> and CD4<sup>-</sup>CD8<sup>-</sup> T cell populations as results may be due to poor staining of the CD4 marker. Too few IL-17F<sup>+</sup> cells are present in the donor one tissue sample to accurately interpret. However back-gating analysis performed on the donor 2 sample revealed that approximately 70% of IL-17F<sup>+</sup> cells are CD45<sup>-</sup>. Within CD45<sup>+</sup> cells approximately 57% and 43% are CD3<sup>+</sup> cells and CD3<sup>-</sup> cells, respectively. Finally, the CD45<sup>+</sup> CD3<sup>+</sup> cells appear to be both CD4<sup>+</sup>CD8<sup>-</sup> and CD4<sup>-</sup>CD8<sup>-</sup> subsets.

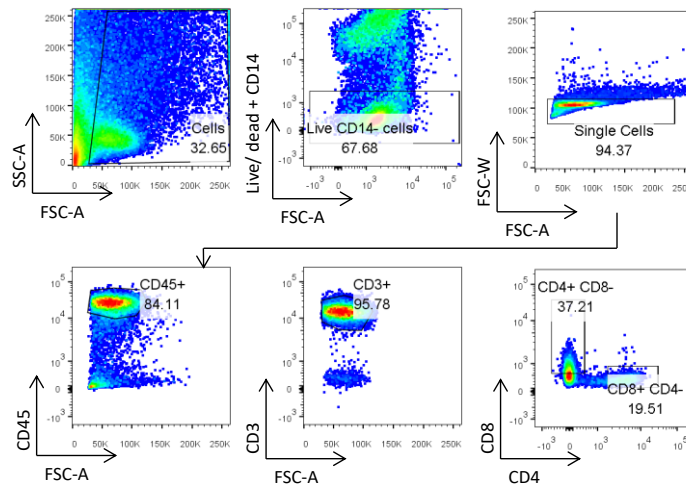
**Table 5.4 Demographic and clinical parameters of RA synovial tissue sample donors (patient cohort 4)**

<b>RA synovial tissue sample</b>	<b>Sex</b>	<b>Age</b>	<b>Treatment</b>	<b>Seropositive/negative</b>
<b>Donor 1</b>	F	32	Anti-TNF, Prednisone	Seronegative
<b>Donor 2</b>	F	57	Anti-TNF and MTX	Not known

### A Donor 1



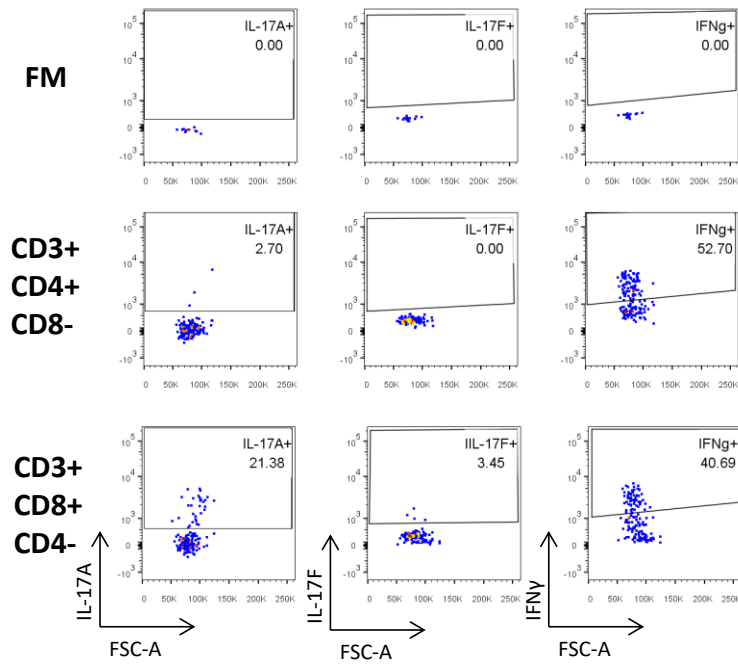
### B Donor 2



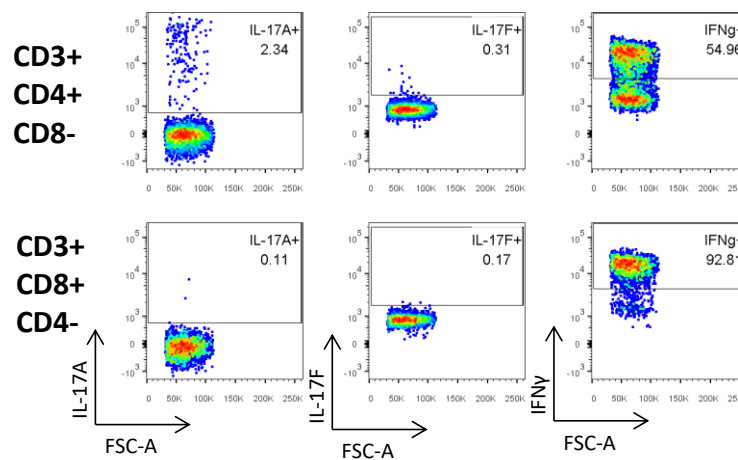
**Figure 5.19 Gating strategy for CD4+ and CD8+ T cells from RA synovial tissue.**

Synovial tissue was digested by incubation with a Miltenyi enzyme mastermix on a gentleMACS dissociator. Isolated cells were stimulated *ex vivo* for 3 hours with PMA and ionomycin. Cells were then stained with fluorescently-conjugated antibodies against extracellular markers and intracellular cytokines. Flow cytometric plots show the gating strategy for (A) donor 1 and (B) donor 2. Cells were first gated for lymphocytes (SSC-A vs. FSC-A), excluding any synovial tissue debris or fibroblasts. The lymphocyte gate was further analysed for uptake of the Live/Dead eFlour 780 stain to determine live vs. dead cells. Within live cells, a FSC-W/FSC-A gate was applied allowing exclusion of doublet cells. Next, within CD3+ cells, CD4+ CD8- and CD8+ CD4- T cells were selected for analysis of intracellular cytokines.

## A Donor 1

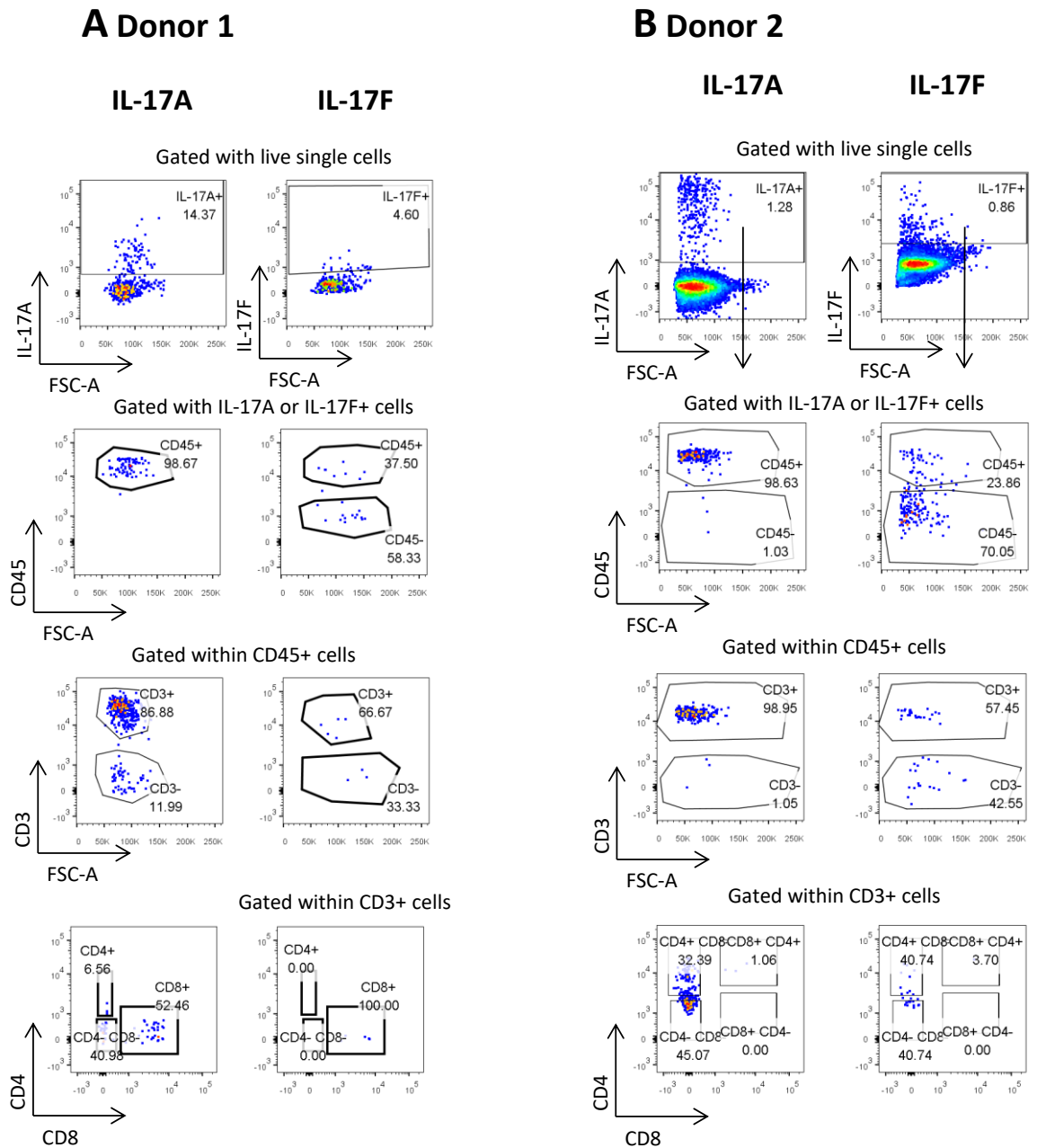


## B Donor 2



**Figure 5.20 Examining the presence of IL-17A and IL-17F expressing CD4+ and CD8+ T cells from RA synovial tissue.**

Cells isolated from RA synovial tissue were stimulated for 3 hours with PMA, ionomycin and Golgistop and then assessed for intracellular IL-17A, IL-17F and IFN $\gamma$  cytokine expression via flow cytometry. Gating within live CD4+ T cells or CD8+ T cells the frequency of IL-17A, IL-17F and IFN $\gamma$  expressing cells was assessed. Flow cytometric plots show the results of (A) donor 1 and (B) donor 2.



**Figure 5.21 Back-gating on IL-17A and IL-17F producing cells.**

Cells isolated from RA synovial tissue were stimulated for 3 hours with PMA, ionomycin and Golgistop and then assessed for intracellular IL-17A, IL-17F cytokine expression via flow cytometry. Live CD14-IL-17A+ or IL-17F+ cells were gated upon. Flow cytometric plots show the results of (A) donor 1 and (B) donor 2.



### 5.3 Discussion

The data presented in this chapter clearly demonstrate that the presence of IL-17A protein and IL-17A+ T cells is increased in the SF vs. PB of RA and PsA patients. In contrast, the data suggests IL-17F and IL-17F+ CD4+ T cells is not consistently present in inflammatory arthritis. The presence of IL-17F in RA and PsA SF remained inconclusive with inconsistent Luminex and ELISA and results and *ex vivo* analysis showed that IL-17F+ CD4+ T cells were either undetectable or present at very low frequencies. In one out of seven PsA patients, IL-17F+ CD8+ T cells frequencies were observed in the SF but this was not seen in the other six patients. Analysis of *IL17F* gene expression in RA T cells provided no further clarification on the presence of IL-17F. Similarly, few IL-17F+ expressing CD4+ T cells were present in RA synovial tissue. Collectively, this chapter suggests that while IL-17A+ T cells are consistently present in RA and PsA SF, only a small percentage of RA or PsA patients may exhibit low frequencies of IL-17F+ T cells.

In concordance with previous studies <sup>103,251</sup>, Luminex and ELISA analysis confirmed the presence of IL-17A protein at elevated levels in RA and PsA synovial fluid vs. serum. This shows IL-17A protein is elevated in the inflamed joints rather than systemically. While Luminex analysis revealed the presence of IL-17F in the SF of RA and PsA patients, these results were not confirmed by ELISA. ELISA results showed IL-17F to be undetectable or present at very low levels in both serum and SF samples. Moreover, IL-17AF protein was undetectable in RA and PsA serum and SF samples. Recombinant IL-17F spiked-in serum and SF samples was detected by ELISA, demonstrating the composition of the samples was not preventing the detection of IL-17F. These data highlight the difficulties in generating reliable results for detecting IL-17F in SF samples. Moreover, they highlight how investigating a hypothesis using different methods may lead to different results. In concordance with these results, there are inconsistencies across the literature of the levels of IL-17A detected in SF. For example, a publication by Ziolkowska *et al.* detected high IL-17A protein levels (approximately 1000pg/ml) in RA SF using ELISA <sup>416</sup>. In contrast, a different report analysed the SF from RA and healthy control donors using a highly sensitive Erenna immunoassay system and observed very low levels of IL-17A with

no difference between RA and healthy control SF (0.07 vs. 0.07pg/ml)<sup>370</sup>. Moreover, this report identified IL-17F protein at higher levels in RA vs. healthy control samples (130 vs. 1.6pg/ml).

These inconsistencies in IL-17 protein levels present in SF may arise from true variation in patient groups, differences in sampling handling or differences in the specific assay method used. Moreover, it is important to take into consideration the possibility of interference from heterophilic antibodies. Heterophilic antibodies can include autoantibodies such as rheumatic factor or polyspecific antibodies that consist of poorly defined antibodies with multiple binding specificities<sup>417</sup>. They can cause false negative results but more frequently, they cause false positive results. Moreover, the extent of heterophilic antibody interference may vary across assays as it is often unclear what measures, if any, manufacturers have taken to prevent interference. In our experiments, no steps were taken to prevent heterophilic interference. Therefore, it may be possible the IL-17F luminex results are false-positive due to heterophilic interference. Future repeat experiments should include measures to prevent heterophilic antibody interference. This can include diluting serum or SF samples in buffer containing IgG from the same species as the capture antibody. Moreover, samples could be preadsorbed on human  $\gamma$ -globulin-coated polystyrene beads. To conclude this section it is important to also highlight a study by Soderstrom *et al.*, which raises the issue that outside of cultured cell environments, cytokines (in particular IL-17A) are often difficult to study. This is because cytokines act locally and often in low abundance. Their report suggests that analysis of IL-17 cytokines in serum or SF samples should be carried out using a highly sensitive single molecule (SMC) immunoassay<sup>418</sup>. Although having an assay that can effectively detect low levels of cytokine is beneficial, it is important to consider whether the cytokine can elicit much of a functional effect at the detected concentration. For example, as shown in chapter 4, IL-17F has a low inflammatory potency and requires substantial levels to elicit an IL-6 fibroblast response.

The composition of SF is complex and strongly influences the microenvironment of the joint. Both the literature and our data have shown that RA and PsA SF contains

pro-inflammatory cytokines including IL-17A and TNF $\alpha$ . As SF and synovial fibroblasts play pivotal roles in both the initiation and perpetuation of inflammatory arthritis. Preliminary experiments sought to assess the effect of SF derived from the inflamed arthritic joint on *in vitro* synovial fibroblasts. This would recreate a physiopathological microenvironment, typical of RA or PsA. However, unfortunately these preliminary experiments were unsuccessful. Addition of 0.5% PsA SF led to a small increase in IL-6 secretion, however, undetectable levels of IL-6 were observed with higher concentrations of SF. Higher concentrations of SF are likely to be required to assess the effect of blockade of IL-17 cytokines and TNF $\alpha$ . As an early study has reported that 30% of RA SF elicited an increase in IL-6 levels from baseline <sup>419</sup>, further work should be continued. This should also involve assessing the viability of fibroblasts upon culture with SF, as there may be components which are toxic to the cell.

In concordance with previous studies <sup>367,368</sup> and the Luminex and ELISA data generated in this thesis, flow cytometry analysis demonstrated the presence of IL-17A+ CD4+ T cells at elevated frequencies in the synovial fluid vs. blood in both RA and PsA patients. Moreover, we observed a positive correlation between the levels of IL-17A protein present in the SF vs. the frequency of IL-17A+ CD4+ T cells detected. The levels of IL-17A protein or frequencies of IL-17A+ CD4+ T cells did not correlate with the patient DAS28 scores. However, further n numbers are required to confirm this. Elevated frequencies of IL-17A+ CD4+ T cells were also detected in the SF vs. PB of an RA and PsA patient upon a three hour stimulation with  $\alpha$ CD3 and  $\alpha$ CD28. This demonstrates that PMA and ionomycin is not required to detect these cells. However, no IL-17A+ CD4+ T cells were observed in SFMC samples which were not re-stimulated and cultured with Golgi-Stop alone. It is possible that as soon as the cells are removed from the inflamed joint or the inflammatory microenvironment of the SF, their ability to produce IL-17A is limited. Therefore, SF CD4+ T cells require a small stimulation to re-elicite IL-17A expression and demonstrate their enhanced potential to produce IL-17A vs. CD4+ T cells from the PB. To investigate the importance of SF in maintaining IL-17A expression from CD4+ T cells, a future experiment could involve adding Golgi-Stop directly to SF samples. In doing so, the CD4+ T cells present would still be in the inflammatory environment, whilst the Golgi-Stop is

retaining any IL-17A protein being expressed in the cell. Following an incubation period, cells can then be washed and stained for flow cytometry.

In contrast to IL-17A, few IL-17F<sup>+</sup> CD4<sup>+</sup> T cells were detected. Moreover, different *ex vivo* stimulation conditions did not reveal the presence of IL-17F expressing CD4<sup>+</sup> T cells. Similarly, low frequencies of IL-17AF<sup>+</sup> CD4<sup>+</sup> T cells were observed. In light of these data it can be concluded IL-17F<sup>+</sup> CD4<sup>+</sup> T cells are either undetectable or present at very low frequencies following *ex vivo* stimulation. *In vitro* stimulation of RA and PsA PB and SF was performed to assess whether CD4<sup>+</sup> T cells from the SF demonstrated an enhanced ability to express IL-17F vs. PB CD4<sup>+</sup> T cells. However, no consistent difference was observed in IL-17F or IL-17A expression or secretion between PB and SF CD4<sup>+</sup> T cells.

As discussed in the introduction, literature describes numerous other immune cells as IL-17A and IL-17F producers, including CD8<sup>+</sup> T cells. In line with a previous report from the lab<sup>374</sup>, analysis of CD8<sup>+</sup> T cells revealed the presence of IL-17A<sup>+</sup> CD8<sup>+</sup> T cells present at higher levels in SF vs. PB in PsA patients. In one out of seven PsA patients, IL-17F<sup>+</sup> CD8<sup>+</sup> T cells frequencies were observed. This suggests that IL-17F<sup>+</sup> CD8<sup>+</sup> T cells may be present in the SF of a small percentage of inflammatory arthritis patients. To address this hypothesis a significantly larger cohort should be analysed. It is important to acknowledge that back-gating on *ex vivo* stimulated RA PBMC and SMFC revealed the enrichment of IL-17A<sup>+</sup> CD8<sup>+</sup> T cells in the SF of three out of eight RA patients. Currently, the literature implicates that IL-17A<sup>+</sup> CD8<sup>+</sup> T cells are enriched in the SF of PsA and not RA patients<sup>244,374</sup>. As two out of the three RA patients with SF IL-17A<sup>+</sup> CD8<sup>+</sup> T cells were seronegative, this suggests there may be a link between the presence of IL-17A<sup>+</sup> CD8<sup>+</sup> T cells in the inflamed joint and seronegative RA.

*IL17A* or *IL17F* mRNA was not detectable in PB or SF sorted CD4<sup>+</sup> or CD8<sup>+</sup> T cells from RA patients. Additionally, microarray analysis revealed undetectable *IL17A* or *IL17F* gene expression levels in CD25<sup>-</sup> CD4<sup>+</sup> T cells from RA PB or SF. These *IL17A*

gene expression results are in contrast to our robust flow cytometry data which detected elevated levels of IL-17A<sup>+</sup> CD4<sup>+</sup> T cells in the SF of RA patients. The lack of *IL17A* gene expression may be attributed to the fact that only a small percentage of bulk CD4<sup>+</sup> T cells express IL-17A, therefore *IL17A* gene expression is masked by the majority of IL-17A<sup>-</sup> CD4<sup>+</sup> T cells present in the RA SF. Another possibility for the lack of *IL17A* mRNA expression may be due to the absence of a PMA and ionomycin re-stimulation. Flow cytometry revealed the presence of IL-17A<sup>+</sup> CD4<sup>+</sup> T cells in SF samples following a 3 hour re-stimulation with PMA/ionomycin or  $\alpha$ CD3 and  $\alpha$ CD28. However, IL-17A<sup>+</sup> CD4<sup>+</sup> T cells were undetectable with the addition of Golgi-Stop alone. Future experiments could involve re-stimulating CD4<sup>+</sup> T cells from the RA and/or PsA with either PMA/ionomycin or  $\alpha$ CD3/ $\alpha$ CD28 and then extracting RNA for analysis of *IL17A* and *IL17F* mRNA expression.

Investigating the presence of IL-17 cytokines in RA or PsA synovial tissue may reveal results not observed in the SF. This is because there are cells such as T<sub>RM</sub>S which remain embedded in the tissue and do not re-circulate in the SF. T<sub>RM</sub> cells have been shown to produce IL-17A<sup>420</sup>. To the best of my knowledge, these cells have not yet been shown to produce IL-17F. Studies in the literature have demonstrated the presence of *IL-17A* and *IL-17F* mRNA or IL-17A and IL-17F producing cells in the synovial tissue of RA and PsA patients. A recent study showed that out of 14 PsA synovial tissue samples, *IL17A* mRNA was detectable in 9 samples and *IL17F* mRNA was detectable in 6 samples<sup>121</sup>. However, the cellular source of IL-17A and IL-17F in this study remains unknown. Moreover, immunohistochemical analysis demonstrated the presence of IL-17A and IL-17F producing cells in the RA but not OA synovial tissue<sup>112</sup>. Similar to the Glatt *et al.*, study the IL-17 producing cells were unidentified. In this chapter, flow cytometry results of cells isolated from RA synovial tissue revealed the presence of IL-17A<sup>+</sup> CD4<sup>+</sup> T cells, however with low cell events, further experiments are required to confirm this. The number of events in all IL-17F<sup>+</sup> gates was very low; therefore an accurate conclusion on the presence of IL-17F<sup>+</sup> cells in RA tissue cannot be reached with this data. To progress this work further additional RA and PsA tissues could be analysed. Moreover, it would be of interest to include T<sub>RM</sub> markers such as CD103 in the staining panel, to investigate whether any IL-17 producing cells are T<sub>RM</sub>S.

Limited experiments in this thesis were performed to investigate the presence of IL-17B, IL-17C, IL-17D and IL-17E in inflammatory arthritis. Data in chapter 4 demonstrated a synergistic effect between IL-17E and TNF $\alpha$  in eliciting IL-6 and IL-6 secretion by fibroblasts. Therefore, we were keen to examine the presence of IL-17E in the SF. Luminex results show low levels of IL-17E present in some serum and SF samples from RA and PsA patients. Furthermore, undetectable IL-17E gene expression levels were observed in CD25- CD4+ T cells from RA PB and SF samples. Following on from the discussion regarding protein analysis of human samples, it would be worthwhile to repeat analysis of IL-17E protein with an ELISA and/or luminex including steps to prevent heterophilic interference. Moreover, flow cytometry could be used to analyse the presence of IL-17E+ CD4+ T cells in *ex vivo* stimulated RA and PsA PBMC and SFMC. IL-17D has been shown to be produced by resting CD4+ T cells, however *IL17D* gene expression levels were undetectable in CD25- CD4+ T cells from RA PB and SF. Additionally, *IL17B* and *IL17C* gene expression was undetectable in RA PB and SF CD25- CD4+ T cells. However, this is expected as CD4+ T cells have not been reported to be their cellular source. During this study one of the limitations to studying the remaining human IL-17 family members has been the lack of reagents available, for example ELISA kits and flow cytometry antibodies. However, more reagents are now becoming available. In the final discussion I discuss how future experiments can investigate the presence of the remaining cytokines in inflammatory arthritis.

To summarise, this section demonstrates that while IL-17A and IL-17A+ T cells are robustly and consistently detected in the RA and PsA inflamed joint, IL-17F, IL-17AF and IL-17F+ and IL-17AF+ CD4+ T cells are not and therefore may not play a significant immunopathological role in this disease setting.

## **6 Final discussion**

To conclude this thesis, this final chapter summarises key findings, highlights outstanding questions and discusses potential areas of future research.

### **6.1 Summary of Chapter 3 results**

Data presented in this chapter show that in the presence of IL-1 $\beta$  and IL-23, a higher concentration of anti-CD28 mAb in CD4<sup>+</sup> T cell cultures leads to an increase in IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells, while frequencies of IL-17A<sup>+</sup> IL-17F<sup>-</sup> CD4<sup>+</sup> T cells are decreased. This anti-CD28 mAb was shown to be mediated by IL-2 and in part IFN $\gamma$ . Similarly, frequencies of IL-17F<sup>+</sup> CD4<sup>+</sup> T cells were preferentially induced with a higher concentration of anti-CD3 mAb. These data led us to hypothesise that a strong T cell stimulation drives IL-17F expression. Moreover, we hypothesise IL-17A and IL-17F are differentially regulated. Finally, I show IL-17A<sup>+</sup> and IL-17F<sup>+</sup> CD4<sup>+</sup> T cells display different cytokine profiles. Notably, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells display significantly lower frequencies IL-10 and higher frequencies of IFN $\gamma$  when compared with IL-17A<sup>+</sup> IL-17F<sup>-</sup> CD4<sup>+</sup> T cells.

### **6.2 Summary of Chapter 4 results**

Fibroblast assays demonstrated that the inflammatory potential of IL-17F is enhanced in the presence of TNF $\alpha$  leading to synergistically increased levels of IL-6 and IL-8 secretion by fibroblasts. However, in the presence of IL-17A, IL-17F is redundant. Nevertheless, in line with other reports I show that in an environment whereby IL-17A and IL-17F are present, dual blockade of these cytokines is more effective at reducing inflammation. This chapter described the development of an IL-17A and IL-17F secretion assay. Following further validation this will be a useful tool to further characterise IL-17A and IL-17F producing cells. Finally, data presented in this chapter demonstrate that IL-17E also synergises with TNF $\alpha$  increasing IL-6 and IL-8 secretion by fibroblasts.

### 6.3 Summary of Chapter 5 results

This chapter provides robust evidence that IL-17A protein and IL-17A+ CD4+ T cells are increased in the SF vs. PB of RA and PsA patients. Moreover, elevated frequencies of IL-17A+ CD8+ T cells were observed in PsA SF vs. PB. IL-17A+ CD4+ and CD8+ T cells were also identified in RA synovial tissue. Across all approaches IL-17F protein or IL-17F+ T cells were not consistently or reliably detected. Contradicting luminex and ELISA results meant the presence of IL-17F protein in inflammatory arthritis remained inconclusive. *Ex vivo* flow cytometry analysis showed that IL-17F+ CD4+ T cells were either undetectable or present at very low frequencies. In one out of seven PsA patients, IL-17F+ CD8+ T cells were observed but this was not seen in the other six patients. No IL-17F+ T cells were reliably detected in RA synovial tissue. *In vitro* cultures demonstrated that in a particular environment these RA and PsA PB and SF CD4+ T have the potential to express IL-17F.

### 6.4 Outstanding questions and future research

#### 6.4.1 Are IL-17A and IL-17F differentially regulated in CD4+ T cells?

Studies have shown that high-strength T cell activation suppresses the induction of IL-17A+ CD4+ T cells<sup>221,225</sup>. In some reports this suppressive effect has been attributed to IL-2<sup>221</sup>. In this study, titration of anti-CD28 mAb did not affect the frequency of total IL-17A+ CD4+ T cells. Moreover, blockade of IL-2 in CD4+ T cell cultures led to no consistent differences in total IL-17A+ CD4+ T cells percentages. In contrast, our data clearly demonstrated that higher concentrations of anti-CD28 mAb induced IL-17F expression and this effect was shown to be mediated by IL-2. Collectively, data from chapter 3 led us to hypothesise that high-strength T cell activation promotes IL-17F+ CD4+ T cells and that IL-17A and IL-17F expression in CD4+ T cells are differentially regulated. As previously mentioned, IL-2 has been shown to mediate its effects via STAT5<sup>223</sup>. Reports have also suggested IL-2 down-regulates IL-17A expression in CD4+ T cells via the ability of STAT5 to directly compete with STAT3 to enhancers within the *IL17A* gene locus<sup>222</sup>. In contrast, data in this thesis suggest STAT5 is important for driving IL-17F expression in CD4+ T cells. We hypothesise IL-17A and IL-17F may be differentially regulated by STAT3 and STAT5.



To investigate this hypothesis future research could firstly involve examining whether other cytokines which act via STAT5, including IL-15<sup>421</sup> induce IL-17F+ CD4+ T cell expression similar to IL-2. The IL-17A and IL-17F capture assay described in this thesis can be utilised to sort IL-17A+ IL-17F-, IL-17A+ IL-17F+ and IL-17F+ IL-17A- CD4+ T cells following a 3 day *in vitro* CD4+ T cell culture and examining STAT3 and STAT5 phosphorylation. Phosphorylation of STATs can be examined by lysing the sorted cells and performing a western blot with pSTAT3 and pSTAT5 antibodies. If our hypothesis is correct, we may observe a strong signal for pSTAT3 in IL-17A+ IL-17F- CD4+ T cells and conversely a strong pSTAT5 signal in sorted IL-17F+ IL-17A- CD4+ T cells. For a more sophisticated approach, direct STAT3/STAT5 binding to the *IL17A* or *IL17F* promoter could be assessed in the different IL-17 populations via chromatin immunoprecipitation interrogated with next-generation sequencing (ChIP-seq). ChIP is a method used to study protein-DNA interactions. It involves cross-linking proteins to their bound DNA via formaldehyde treatment. An antibody specific to the protein of interest (in this case STAT3 and STAT5) is then used to selectively co-immunoprecipitate the protein-bound DNA fragments that were covalently cross-linked. Finally, the immunoprecipitated protein-DNA links are reversed and the recovered DNA is analysed to determine the sequences bound by that protein. Although multiple approaches are available to analyse the released DNA fragments such as microarray hybridisation (ChIP-chip), next-generation sequencing technology (ChIP-seq) offers distinct advantages<sup>422</sup>. Therefore, IL-17A+ IL-17F-, IL-17A+ IL-17F+ and IL-17F+ IL-17A- CD4+ T cells could be sorted by the described IL-17A and IL-17F capture assay following a three day *in vitro* culture. Sorted cells could then undergo the ChIP-seq protocol and STAT3 and STAT5 binding on the *IL17A* and *IL17F* locus can be assessed in the different IL-17 expressing subsets.

Another approach to investigate the hypothesis that IL-17A and IL-17F are differentially regulated could include mouse model studies especially as the importance of STAT3 in the generation of IL-17A+ CD4+ T cells was demonstrated in mouse studies<sup>203</sup>. Laurence *et al.* demonstrated that *STAT5* deficient mouse T cells produced significantly increased amounts of IL-17A and elevated levels of IL-17A were detectable in the serum of *STAT5a/b* vs. wild type mice<sup>218</sup>. To date, no studies

have investigated the effect of IL-17F in *STAT5* or *IL-2* deficient mice. It would be of interest to investigate whether, in contrast to IL-17A, IL-17F is significantly decreased in these mouse models. In line with this, a study by Lin and colleagues generated transgenic mice that express hyper-active STAT5 in haematopoietic stem cells and derived lineages in a temporally and spatially controlled manner. They demonstrated that hyper-active STAT5 resulted in abnormally high numbers of circulating granulocytes that led to severe airway destruction. Granulocyte accumulation was reduced and the air-way was restored upon down-regulation of excessive STAT5<sup>423</sup>. The mechanism of the granulocyte accumulation was unknown, however, it is in line with studies reporting the role of IL-17F in inducing neutrophilia, especially in asthmatic patients<sup>108,109</sup>. Therefore, it could be speculated that hyper-active STAT5 led to excessive production of IL-17F and subsequently increased granulocyte accumulation. I believe investigating the hypothesis that IL-17A and IL-17F are differentially regulated is interesting to pursue as currently no studies prove this. It may also help us understand when IL-17F can be present in disease, for example where there is an increase in STAT5-signalling proteins.

#### **6.4.2 Is there a functional difference between IL-17A+ and IL-17F+ CD4+ T cells?**

We have shown that by increasing anti-CD28 mAb concentration in CD4+ T cell cultures, the frequency of IL-17A+ IL-17F- CD4+ T cells are decreased, whilst IL-17A+ IL-17F+ and IL-17F+ IL-17A- CD4+ T cell frequencies are increased. One of the key remaining questions is what is the functional impact of skewing Th17 cells into different IL-17A/ IL-17F expressing populations? As IL-17F is considered a weaker inducer of inflammation, one plausible explanation could be IL-17A+ IL-17F- CD4+ T cells are skewed to IL-17F+ IL-17A- CD4+ T cells to dampen the inflammatory effect of IL-17A. In the event of high T cell stimulation, driving a Th17 population from predominantly IL-17A+ IL-17F- CD4+ T cells to IL-17F+ IL-17A- CD4+ T cells will limit the potent inflammatory effect of IL-17A, whilst carrying out similar roles. However, it is important to note that in this culture system, as IL-17A+ IL-17F+ CD4+ T cell frequencies were also increased with a higher concentration of anti-CD28 mAb, the frequency of total IL-17A expressing CD4+ T cells remained unchanged.

Interestingly, data in this thesis demonstrated that IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells have a lower frequency of IL-10<sup>+</sup> cells and higher frequencies of IFN $\gamma$ <sup>+</sup> cells when compared to IL-17A<sup>+</sup> IL-17F<sup>-</sup> CD4<sup>+</sup> T cells. It would be of interest to examine whether the differences in cytokine profiles lead to different functional properties. Future studies could firstly expand upon the characterisation and comparison of IL-17A<sup>+</sup> and IL-17F<sup>+</sup> CD4<sup>+</sup> T cells. Following a 3-day CD4<sup>+</sup> T cell culture with IL-1 $\beta$ , IL-23, anti-CD28 mAb and anti-CD3 mAb, IL-17A<sup>+</sup> IL-17F<sup>-</sup>, IL-17A<sup>+</sup> IL-17F<sup>+</sup>, IL-17F<sup>+</sup> IL-17A<sup>-</sup> and IL-17A<sup>-</sup> IL-17F<sup>-</sup> CD4<sup>+</sup> T cell populations could be sorted (using the method and reagents described in this thesis). Next, the gene expression profile of the sorted IL-17 populations could be analysed by RNA-seq. Briefly, a typical RNA-seq experiment would involve converting RNA from cells of interest into a library of cDNA fragments. Sequencing adaptors are added to each cDNA fragment and a short sequence is obtained from each cDNA using high-throughput sequencing technology. The resulting sequence reads are aligned with a reference genome or transcriptome <sup>424</sup>. Performing RNA-seq would allow a wide-range of genes encoding cytokines, markers and transcription factors to be analysed and compared between the different IL-17 subsets. Firstly, differences in IL-10 and IFN $\gamma$  expression can be validated at the RNA level. Next, it would be of interest to compare the gene expression of homing markers e.g. *CLA*, *CCR4*,  $\alpha 4\beta 7$ , and *CCR5* to investigate whether the different IL-17 subsets might have different migratory properties. T-cell subsets that bear cutaneous lymphocyte antigen (*CLA*) <sup>425</sup> are specialised for skin homing and *CCR4* is a skin homing marker <sup>426</sup>. The integrin  $\alpha 4\beta 7$  is responsible for homing into gut-associated lymphoid tissues <sup>427</sup> and *CCR5* directs cells to sites of inflammation <sup>428</sup>. The rationale for looking at the expression of homing markers is that studies have reported the presence of IL-17F in psoriasis <sup>108,382</sup> and gut inflammation <sup>429</sup>. Therefore, it may be that IL-17F<sup>+</sup> CD4<sup>+</sup> T cells may play a more predominant role at specific sites of inflammation.

The next important experiment would be to assess whether IL-17A<sup>+</sup> IL-17F<sup>-</sup>, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells elicit different responses from target cells. To investigate this, following a 3-day *in vitro* culture, an IL-17A and IL-17F

secretion assay can be performed, sorting the different IL-17 populations. Sorted IL-17 populations can then be cultured for 24 hours in RPMI media and collected. Firstly, a Luminex assay can be performed on the supernatants to assess any differences in cytokines secreted by IL-17A<sup>+</sup> IL-17F<sup>-</sup>, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells. Next, similar to previous experiments, the supernatants from IL-17A<sup>+</sup> IL-17F<sup>-</sup>, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells can be added to fibroblast cultures and the levels of IL-6 and IL-8 secretion can be assessed. However, as IL-17F<sup>+</sup> CD4<sup>+</sup> T cells displayed significantly lower co-expression levels of IL-10, it would be of interest to assess how the supernatant from the different sorted IL-17 subsets affects other immune cells including monocytes and/or macrophages. IL-10 is a potent anti-inflammatory cytokine, which plays an important role in regulating the immune response. The importance of IL-10 in regulating the immune response is evidenced by the IL-10 knock-out mouse phenotype, which displays dysregulated inflammatory responses<sup>28</sup>. One of the mechanisms by which IL-10 exerts anti-inflammatory effects is by potently inhibiting monocyte/macrophage activation. Addition of IL-10 to J744 mouse macrophage cells in the presence of LPS, inhibited macrophage activated by LPS<sup>29</sup>. IL-10 potently inhibits monocyte and macrophage production of TNF $\alpha$  and CC and CXC chemokines, therefore reducing chemokine-driven recruitment of neutrophils and other immune cells to sites of inflammation<sup>30</sup>. To examine whether the different IL-10 co-expression levels observed in the IL-17A<sup>+</sup> IL-17F<sup>+</sup> CD4<sup>+</sup> T cells and IL-17F<sup>+</sup> IL-17A<sup>-</sup> vs. IL-17A<sup>+</sup> IL-17F<sup>-</sup> CD4<sup>+</sup> T cells have a functional effect, the supernatants of the sorted IL-17 populations can be added to cultures of CD14<sup>+</sup> monocytes in the absence or presence of LPS. It can then be assessed whether addition of IL-17A<sup>+</sup> IL-17F<sup>-</sup> CD4<sup>+</sup> T cell supernatants reduces the secretion of TNF $\alpha$  or chemokines from monocytes, whereas IL-17F<sup>+</sup> CD4<sup>+</sup> T cell supernatant has no effect due to the absence/ low levels of IL-10 present. These results would indicate whether skewing a Th17 population towards predominantly IL-17F expressing CD4<sup>+</sup> T cells could allow for a more inflammatory environment.

### **6.4.3 Is IL-17F present in inflammatory arthritis?**

In this thesis, I phenotypically characterised healthy IL-17F<sup>+</sup> CD4<sup>+</sup> T cells and showed the potential benefit of IL-17A and IL-17F dual blockade in reducing inflammation. Moreover, I describe the development of an IL-17F secretion assay which will allow for further characterisation IL-17F<sup>+</sup> cells. We sought to apply this knowledge into understanding the role of IL-17F in inflammatory arthritis. However, the presence of IL-17F in the serum or SF of RA and PsA patients remained inconclusive. Furthermore, out of eight RA and seven PsA patients, only one PsA patient revealed observable frequencies of IL-17F<sup>+</sup> CD8<sup>+</sup> T cells in the SF. Overall, the data in this thesis suggest that while IL-17A<sup>+</sup> T cells are consistently present in RA and PsA SF, only a small percentage of RA or PsA patients may exhibit low frequencies of IL-17F<sup>+</sup> T cells. In this thesis, the predominant focus is IL-17F<sup>+</sup> T cells, however, other cells such as mast cells and macrophages have been reported to secrete IL-17F. In our RA and PsA *ex vivo* dataset, flow cytometry analysis was performed to identify any non-T cell IL-17F<sup>+</sup> producing cells, however, none were found. To conclude work on the presence of IL-17F in inflammatory arthritis future experiments could involve performing an ICCS on all the cells (no Lymphoprep step) present in RA PB and SF. In the case of PB samples, red blood cells would need to be lysed. Performing this experiment would allow for the presence of IL-17F<sup>+</sup> neutrophils or mast cells present in RA/PsA SF to be examined. To analyse a wide-array of cell surface markers, CyTOF staining and acquisition could be performed.

### **6.4.4 Does IL-17F contribute to the pathology of other diseases?**

Future research could explore the role of IL-17F in other diseases and investigate whether dual blockade of IL-17A and IL-17F may alleviate disease symptoms. Evidence has linked IL-17F to the immunopathology of other disease including psoriasis, asthma, inflammatory bowel disease and periodontitis. Here, I review the presence of IL-17F in other diseases and discuss future experiments in different diseases that may be of interest.

#### 6.4.4.1 Psoriasis

As previously discussed in the introduction, psoriasis is a chronic inflammatory skin disorder. While the pathology of psoriasis is complex, studies have suggested the initiation and perpetuation of psoriasis is driven by interactions between T cells, dendritic cells, neutrophils and cytokines secreted by immune cells<sup>430</sup>. IL-17A+ T cells have been implicated as a key contributor to the pro-inflammatory state of psoriasis<sup>378,379</sup> and as previously mentioned in the introduction, studies have identified the presence of *IL17F* mRNA and protein in psoriasis<sup>108,382</sup>. Targeting IL-17A in psoriasis by secukinumab and ixekizumab has led to dramatic success in the clearance of psoriatic lesions<sup>398,431</sup>. Moreover, similar success has been observed with brodalumab, whereby IL-17RA is blocking thereby inhibiting the function of IL-17A, IL-17F, IL-17C and IL-17E<sup>432</sup>. Glatt *et al.* shown that psoriatic patients receiving bimekizumab demonstrated significant disease improvement<sup>121</sup>. However, head-to-head trials are necessary to demonstrate that a combined blockade of IL-17A and IL-17F is more effective at clearing psoriasis than blockade of IL-17A alone.

#### 6.4.4.2 Asthma

Asthma is characterized by chronic deregulated airway inflammation and bronchial hyper-responsiveness. Patients suffer with recurrent chest wheezing, coughing and shortness of breath. Immunohistochemistry staining for IL-17A and IL-17F in bronchial epithelium samples revealed elevated frequencies of IL-17A+ and IL-17F+ cells in severe asthma patients vs. healthy control. While IL-17A was predominantly expressed in both mononuclear cells, IL-17F+ cells were both mononuclear and epithelial cells. In concordance, *IL17A* and *IL17F* mRNA was detectable in bronchial biopsies from severe asthmatic patients<sup>433</sup>. Moreover, another study detected elevated levels of IL-17F protein in the serum of asthma patients vs. non-asthmatic controls<sup>434</sup>.

Although the pathology of asthma remains to be fully elucidated, eosinophilic and neutrophilic inflammation in the airways are characteristic features. Research has demonstrated that neutrophils are present at a two-fold higher concentration in bronchoalveolar lavage fluid (BALF) when compared to mild/moderate asthmatics or normal controls<sup>435</sup>. Linking IL-17 cytokines to the pathology of asthma, studies have

shown overexpression of IL-17A or IL-17F in mouse airways leads to increased numbers of neutrophils in BALF<sup>436,437</sup>. This is likely due to the ability of IL-17A and IL-17F to induce the secretion of chemokines such as IL-8. Additionally, reports have demonstrated the ability of IL-17F to induce pro-fibrotic cytokines including IL-11 and insulin-like growth factor-1 (IGF-1) in bronchial epithelial cells<sup>438,439</sup>. These cytokines can contribute to airway remodelling in asthma. Airway structural changes include sub-epithelial fibrosis and increased smooth muscle mass which contribute to the thickening of airway walls, which can lead to airway narrowing and bronchial hyper-responsiveness<sup>440</sup>. In the airways of asthma patients, a positive correlation between the number of CD4<sup>+</sup> T cells and neutrophil infiltration has been observed<sup>441</sup>. Moreover, in the asthma rat model, depletion of CD4<sup>+</sup> T cells significantly inhibited neutrophil infiltration<sup>442</sup>. Collectively, these reports suggest that CD4<sup>+</sup> T cells in asthma regulate neutrophilic inflammation. It may be possible that IL-17F<sup>+</sup> and IL-17A<sup>+</sup> T cells are encompassed in this CD4<sup>+</sup> T cell population.

The use of biologics as a treatment for severe asthma is being explored, including inhibition of IL-17. Kirsten *et al.* studied the effect of secukinumab in ozone-induced airway neutrophilia in healthy volunteers. They reported no difference in secukinumab vs. placebo group in the number of neutrophils in induced sputum<sup>443</sup>. In a double-blind placebo-controlled phase II study, 302 patients with inadequately controlled moderate to severe asthma were randomized to brodalumab or placebo. Across all patients, no clinical differences in lung function or asthma symptoms were observed. However, when assessing asthma sub-groups, patients with high bronchodilator reversibility demonstrated a clinical response<sup>444</sup>. Since this clinical trial study, studies investigating the therapeutic potential of IL-17 cytokines in asthma have been limited. As asthma is a heterogeneous disease with many other factors such as Th2 cytokines and IgE levels playing a key role, the benefit of dual IL-17A and IL-17F blockade in alleviating asthma symptoms may be revealed in clinical trials of specific asthma sub-types.

#### **6.4.4.3 Inflammatory bowel disease**

Inflammatory bowel disease encompasses ulcerative colitis and crohn's disease, which are both long-term conditions that involve gut inflammation. Ulcerative colitis affects the colon, whereas crohn's disease can affect any part of the digestive system. A report by Seiderer *et al.* showed that *IL17F* gene expression is up-regulated in active crohn's disease but not ulcerative colitis when compared to uninflamed lesion samples from the same patients <sup>429</sup>. Although, animal models suggested dual IL-17A and IL-17F blockade may reduce intestinal inflammation <sup>363</sup>, a clinical trial which investigated the effect of brodalumab in patients with active crohn's disease yielded negative results <sup>445</sup>. Finally, a double-blind, randomised, placebo-controlled proof-of-concept study in 59 crohn's disease patients demonstrated that treatment with secukinumab is ineffective <sup>446</sup>.

#### **6.4.4.4 Periodontitis**

Studies have implicated IL-17A to the immunopathology of periodontitis <sup>198,447</sup>. Periodontitis is a chronic inflammatory disease characterised by the destruction of the supporting structures of teeth (alveolar bone and periodontal ligament). Although evidence is not strong, IL-17F has been linked to periodontitis. A study by Luo *et al.* suggested that IL-17A+ IL-17F- and IL-17F+ IL-17A- CD4+ T cells are elevated in the PB of chronic periodontitis patients vs. healthy controls <sup>448</sup>. However, care must be taken when interpreting these data as the representative flow cytometric plot is not convincing for an IL-17F positive stain. To the best of my knowledge, blockade of IL-17A or IL-17RA has not been tested as a therapy for periodontitis.

#### **6.4.4.5 The role of IL-17F in non-inflammatory arthritis diseases: Future work**

After reviewing the current literature of IL-17F in other diseases, in my view it would be of interest to expand upon the role of IL-17F in psoriasis. This is because initial studies have detected IL-17F in psoriatic lesions and brodalumab and secukinumab have demonstrated significant success in clearing psoriatic lesions. In contrast, although evidence has implicated IL-17A and IL-17F in asthma and inflammatory bowel disease, clinical trials have shown blockade of IL-17A or IL-17RA is not effective at ameliorating disease. Although psoriasis is associated with PsA, in this



thesis psoriatic lesions were not studied. Future experiments could involve isolating cells from psoriatic lesions and examining the presence of IL-17F and IL-17A expressing cells by flow cytometry. If indeed IL-17A<sup>+</sup> and IL-17F<sup>+</sup> CD4<sup>+</sup> T cells are present, flow cytometry analysis could be performed to investigate whether they display different cytokine profiles as demonstrated in this thesis. Furthermore, sorting IL-17A<sup>+</sup> IL-17F<sup>-</sup>, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells from psoriatic lesions for gene expression profiling would be of interest, although a significant limitation would be the low cell numbers. However, this limitation could be overcome by performing single-cell RNA-seq<sup>449</sup>.

#### **6.4.5 Investigating the presence of IL-17B, IL-17C, IL-17D and IL-17E in inflammatory arthritis**

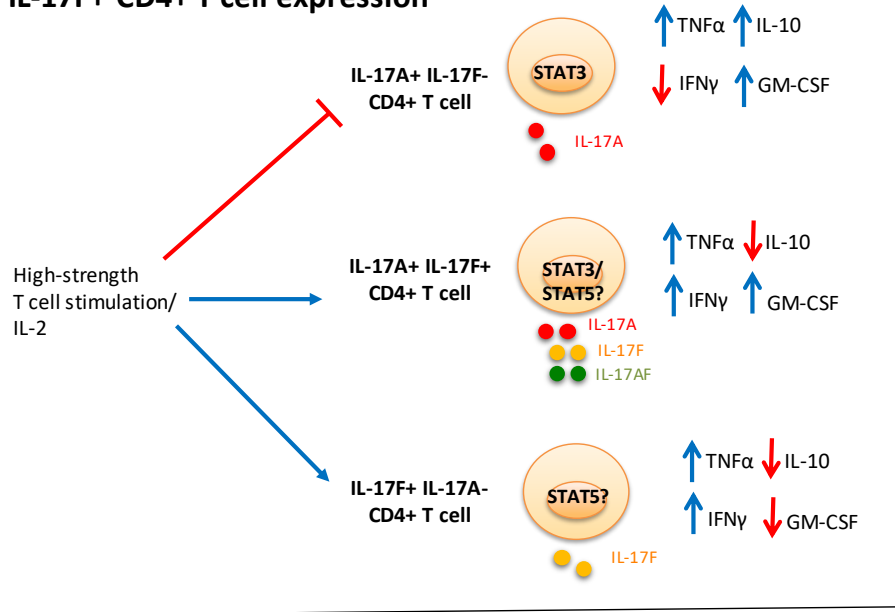
A lot remains unexplored regarding the presence and role of IL-17B, IL-17C, IL-17D and IL-17E in human inflammatory arthritis and this may be interesting for future work to pursue. Future work could involve performing CyTOF to analyse the expression of all IL-17 family members in all immune cell subsets present in inflammatory arthritis PBMC and SFMC. Additionally, new technology (Hyperion Imaging System) has combined CyTOF with imaging capability which allows for visualisation of protein markers in the context of a tissue micro-environment. Using this approach, the presence of all IL-17 cytokines could be analysed in human tissue samples from inflamed arthritic joints.

#### **6.5 Concluding remarks**

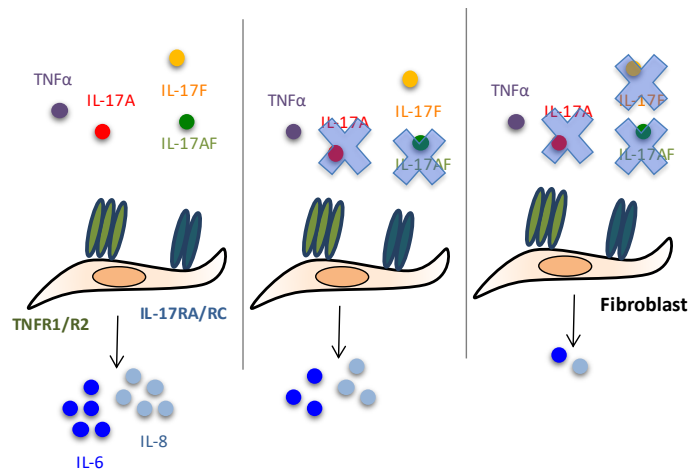
The main findings of this thesis are summarized in Figure 6.1. Data in this thesis suggest that high-strength T cell stimulation drives IL-17F expressing CD4<sup>+</sup> T cells. It is demonstrated that addition of anti-CD28 drives IL-17F<sup>+</sup> CD4<sup>+</sup> T cells via an IL-2 dependent mechanism. We hypothesise that STAT5 plays a role in IL-17F induction. Results also show that IL-17A<sup>+</sup> and IL-17F<sup>+</sup> CD4<sup>+</sup> T cells display different cytokine profiles and may be functionally distinct. These data suggest there is more to IL-17F in addition to being co-expressed with IL-17A. Finally, the pro-inflammatory functions of IL-17F are enhanced in the presence of TNF $\alpha$  and the induced inflammation will persist during blockade of IL-17A alone. We show that in an

environment whereby IL-17A, IL-17F and TNF $\alpha$  are present at sufficient levels, dual IL-17A and IL-17F blockade is more effective at reducing inflammation than blockade of IL-17A alone.

## IL-17F+ CD4+ T cell expression



## Role of IL-17F in inflammation



## Figure 6.1 Insights into the expression and function of IL-17F

Data presented in this thesis demonstrate that in the presence of IL-1 $\beta$  and IL-23, anti-CD28 mAb enhances the frequency of IL-17A+ IL-17F+ and IL-17F+ IL-17A- CD4+ T cells in an IL-2 dependent mechanism. In contrast, the frequency of IL-17A+ IL-17F- CD4+ T cells is reduced. We hypothesise IL-17A and IL-17F are differentially regulated and a strong T cell stimulation drives IL-17F expressing cells, of which STAT5 plays an important role. Results also demonstrate IL-17A+ and IL-17F+ CD4+ T cell populations display different cytokine profiles. Regarding the function of IL-17F, we show the ability of IL-17F to induce the pro-inflammatory mediators IL-6 and IL-8 from target fibroblast cells is enhanced in the presence of TNF $\alpha$ , however, when IL-17A is present the effect of IL-17F is limited. Nevertheless, in an environment where IL-17A, IL-17F, IL-17AF and TNF $\alpha$  are all present combined blockade of IL-17A (which also blocks IL-17AF) is more effective at reducing inflammation than blockade of IL-17A alone.

## **Acknowledgments**

Firstly, I would like to give a big thank you to my supervisor Professor Leonie Taams for her fantastic support, guidance and constructive feedback throughout my PhD. I have thoroughly enjoyed working with Leonie and enjoy her passion for science and engaging scientific discussions. I would also like to thank my second supervisor Bruce Kirkham, my pharmaceutical supervisor Diane Marshall and my PhD thesis committee their continued support. I am grateful to BBSRC programme and UCB for their funding support.

The Taams lab members past and present have been a huge part of my PhD life. Thank you for the warm welcome into the group, lab training, for scientific ideas and contributions and for the continued encouragement. Also thank you for all the fun in Taams lab days out! To Abigail and Ushani, I'm happy I got to share this PhD experience with you both. A big thank you to UCB colleague Ash Maroof for the continued collaboration, support and development of scientific ideas throughout my PhD. Thank you for the supervision during my three month placement at UCB and collaboration in developing a secretion assay protocol. Similarly, thank you to all UCB colleagues who were welcoming and helpful during my placement.

Thank you to all the members of CIBCI for a lovely working environment, assistance and sharing of knowledge. Similarly thank you to members of the Faculty of Life Sciences and Medicine and BRC Flowcore who have assisted me during my PhD. The majority of this work has been dependent of healthy and patient blood and synovial fluid donors – thank you for these donations.

Finally, I am so grateful for the unconditional support of my family and close friends. In particular, my Mum, Steve, Kayleigh, Phil & co. and Anna. Thank you!

## References

- 1 Dinarello, C. A. Historical insights into cytokines. *European journal of immunology* **37**, S34-S45 (2007).
- 2 Ozaki, K. & Leonard, W. J. Cytokine and cytokine receptor pleiotropy and redundancy. *Journal of Biological Chemistry* (2002).
- 3 Bartee, E. & McFadden, G. Cytokine synergy: an underappreciated contributor to innate anti-viral immunity. *Cytokine* **63**, 237-240 (2013).
- 4 Dinarello, C. A. Immunological and inflammatory functions of the interleukin-1 family. *Annual review of immunology* **27**, 519-550 (2009).
- 5 Pulliam, S. R., Uzhachenko, R. V., Adunyah, S. E. & Shanker, A. Common gamma chain cytokines in combinatorial immune strategies against cancer. *Immunology letters* **169**, 61-72 (2016).
- 6 Mahmud, S. A., Manlove, L. S. & Farrar, M. A. Interleukin-2 and STAT5 in regulatory T cell development and function. *Jak-stat* **2**, e23154 (2013).
- 7 Malek, T. R. The main function of IL-2 is to promote the development of T regulatory cells. *Journal of leukocyte biology* **74**, 961-965 (2003).
- 8 Gaffen, S. L. & Liu, K. D. Overview of interleukin-2 function, production and clinical applications. *Cytokine* **28**, 109-123 (2004).
- 9 Mingari, M. *et al.* Human interleukin-2 promotes proliferation of activated B cells via surface receptors similar to those of activated T cells. *Nature* **312**, 641 (1984).
- 10 Siegel, J. P., Sharon, M., Smith, P. L. & Leonard, W. J. The IL-2 receptor beta chain (p70): role in mediating signals for LAK, NK, and proliferative activities. *Science* **238**, 75-78 (1987).
- 11 Refaeli, Y., Van Parijs, L., London, C. A., Tschopp, J. & Abbas, A. K. Biochemical mechanisms of IL-2-regulated Fas-mediated T cell apoptosis. *Immunity* **8**, 615-623 (1998).
- 12 Chen, C.-J. *et al.* Identification of a key pathway required for the sterile inflammatory response triggered by dying cells. *Nature medicine* **13**, 851 (2007).
- 13 Garlanda, C., Dinarello, C. A. & Mantovani, A. The interleukin-1 family: back to the future. *Immunity* **39**, 1003-1018 (2013).
- 14 Piccioli, P. & Rubartelli, A. in *Seminars in immunology*. 425-429 (Elsevier).
- 15 Black, R. A. *et al.* Generation of biologically active interleukin-1 beta by proteolytic cleavage of the inactive precursor. *Journal of Biological Chemistry* **263**, 9437-9442 (1988).
- 16 Hazuda, D. J., Strickler, J., Kueppers, F., Simon, P. & Young, P. Processing of precursor interleukin 1 beta and inflammatory disease. *Journal of Biological Chemistry* **265**, 6318-6322 (1990).
- 17 Chung, Y. *et al.* Critical regulation of early Th17 cell differentiation by interleukin-1 signaling. *Immunity* **30**, 576-587 (2009).
- 18 Kryczek, I. *et al.* Induction of IL-17+ T cell trafficking and development by IFN- $\gamma$ : mechanism and pathological relevance in psoriasis. *The Journal of Immunology* **181**, 4733-4741 (2008).
- 19 Ben-Sasson, S. Z. *et al.* IL-1 acts directly on CD4 T cells to enhance their antigen-driven expansion and differentiation. *Proceedings of the National Academy of Sciences* **106**, 7119-7124 (2009).
- 20 O'Sullivan, B. J. *et al.* IL-1 $\beta$  breaks tolerance through expansion of CD25+ effector T cells. *The Journal of Immunology* **176**, 7278-7287 (2006).
- 21 Cray, C., Zaias, J. & Altman, N. H. Acute phase response in animals: a review. *Comparative medicine* **59**, 517-526 (2009).

- 22 Castell, J. V. *et al.* Interleukin-6 is the major regulator of acute phase protein  
synthesis in adult human hepatocytes. *FEBS letters* **242**, 237-239 (1989).
- 23 Veldhoen, M., Hocking, R. J., Atkins, C. J., Locksley, R. M. & Stockinger, B. TGF $\beta$  in  
the context of an inflammatory cytokine milieu supports de novo differentiation of  
IL-17-producing T cells. *Immunity* **24**, 179-189 (2006).
- 24 Bettelli, E. *et al.* Reciprocal developmental pathways for the generation of  
pathogenic effector T H 17 and regulatory T cells. *Nature* **441**, 235 (2006).
- 25 Hirano, T. *et al.* Purification to homogeneity and characterization of human B-cell  
differentiation factor (BCDF or BSFp-2). *Proceedings of the National Academy of  
Sciences* **82**, 5490-5494 (1985).
- 26 Romano, M. *et al.* Role of IL-6 and its soluble receptor in induction of chemokines  
and leukocyte recruitment. *Immunity* **6**, 315-325 (1997).
- 27 Sabat, R. IL-10 family of cytokines. *Cytokine & growth factor reviews* **21**, 315-324  
(2010).
- 28 Rennick, D., Davidson, N. & Berg, D. Interleukin-10 gene knock-out mice: a model of  
chronic inflammation. *Clinical immunology and immunopathology* **76**, S174-S178  
(1995).
- 29 O'Farrell, A. M., Liu, Y., Moore, K. W. & Mui, A. L. F. IL-10 inhibits macrophage  
activation and proliferation by distinct signaling mechanisms: evidence for Stat3-  
dependent and-independent pathways. *The EMBO journal* **17**, 1006-1018 (1998).
- 30 Moore, K. W., de Waal Malefyt, R., Coffman, R. L. & O'Garra, A. Interleukin-10 and  
the interleukin-10 receptor. *Annual review of immunology* **19**, 683-765 (2001).
- 31 Gaffen, S. L. Structure and signalling in the IL-17 receptor family. *Nature Reviews  
Immunology* **9**, 556 (2009).
- 32 Choi, U. Y., Kang, J.-S., Hwang, Y. S. & Kim, Y.-J. Oligoadenylate synthase-like (OASL)  
proteins: dual functions and associations with diseases. *Experimental & molecular  
medicine* **47**, e144 (2015).
- 33 Ivashkiv, L. B. & Donlin, L. T. Regulation of type I interferon responses. *Nature  
reviews Immunology* **14**, 36 (2014).
- 34 Nguyen, K. B. *et al.* Coordinated and distinct roles for IFN- $\alpha\beta$ , IL-12, and IL-15  
regulation of NK cell responses to viral infection. *The Journal of Immunology* **169**,  
4279-4287 (2002).
- 35 Schroder, K., Hertzog, P. J., Ravasi, T. & Hume, D. A. Interferon- $\gamma$ : an overview of  
signals, mechanisms and functions. *Journal of leukocyte biology* **75**, 163-189 (2004).
- 36 Wack, A., Terczyńska-Dyla, E. & Hartmann, R. Guarding the frontiers: the biology of  
type III interferons. *Nature immunology* **16**, 802 (2015).
- 37 Old, L. J. Tumor necrosis factor (TNF). *Science* **230**, 630-632 (1985).
- 38 Carswell, E. *et al.* An endotoxin-induced serum factor that causes necrosis of  
tumors. *Proceedings of the National Academy of Sciences* **72**, 3666-3670 (1975).
- 39 MacEwan, D. J. TNF ligands and receptors—a matter of life and death. *British journal  
of pharmacology* **135**, 855-875 (2002).
- 40 Trinchieri, G. *et al.* Tumor necrosis factor and lymphotoxin induce differentiation of  
human myeloid cell lines in synergy with immune interferon. *Journal of  
Experimental Medicine* **164**, 1206-1225 (1986).
- 41 Higuchi, M. *et al.* Membrane tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) expressed on HTLV-I-  
infected T cells mediates a costimulatory signal for B cell activation—  
characterization of membrane TNF- $\alpha$ . *Clinical immunology and immunopathology*  
**82**, 133-140 (1997).
- 42 Vilcek, J. *et al.* Fibroblast growth enhancing activity of tumor necrosis factor and its  
relationship to other polypeptide growth factors. *Journal of Experimental Medicine*  
**163**, 632-643 (1986).

- 43 Shalaby, M., Waage, A., Aarden, L. & Espevik, T. Endotoxin, tumor necrosis factor- $\alpha$  and interleukin 1 induce interleukin 6 production in vivo. *Clinical immunology and immunopathology* **53**, 488-498 (1989).
- 44 Philip, R. & Epstein, L. B. Tumour necrosis factor as immunomodulator and mediator of monocyte cytotoxicity induced by itself,  $\gamma$ -interferon and interleukin-1. *Nature* **323**, 86 (1986).
- 45 Brouckaert, P., Spriggs, D. R., Demetri, G., Kufe, D. W. & Fiers, W. Circulating interleukin 6 during a continuous infusion of tumor necrosis factor and interferon gamma. *Journal of Experimental Medicine* **169**, 2257-2262 (1989).
- 46 Xaus, J. *et al.* LPS induces apoptosis in macrophages mostly through the autocrine production of TNF- $\alpha$ . *Blood* **95**, 3823-3831 (2000).
- 47 Zlotnik, A. & Yoshie, O. Chemokines: a new classification system and their role in immunity. *Immunity* **12**, 121-127 (2000).
- 48 Proost, P., Wuyts, A. & Van Damme, J. The role of chemokines in inflammation. *International Journal of Clinical and Laboratory Research* **26**, 211-223 (1996).
- 49 Deshmane, S. L., Kremlev, S., Amini, S. & Sawaya, B. E. Monocyte chemoattractant protein-1 (MCP-1): an overview. *Journal of interferon & cytokine research* **29**, 313-326 (2009).
- 50 Li, A., Dubey, S., Varney, M. L., Dave, B. J. & Singh, R. K. IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis. *The Journal of Immunology* **170**, 3369-3376 (2003).
- 51 Yoshimura, T. *et al.* Purification and amino acid analysis of two human glioma-derived monocyte chemoattractants. *Journal of Experimental Medicine* **169**, 1449-1459 (1989).
- 52 Wang, T., Dai, H., Wan, N., Moore, Y. & Dai, Z. The role for monocyte chemoattractant protein-1 in the generation and function of memory CD8+ T cells. *The Journal of Immunology* **180**, 2886-2893 (2008).
- 53 Clark, S. C. & Kamen, R. The human hematopoietic colony-stimulating factors. *Science* **236**, 1229-1237 (1987).
- 54 Metcalf, D. The colony-stimulating factors and cancer. *Nature Reviews Cancer* **10**, 425 (2010).
- 55 Liongue, C. & Ward, A. C. Evolution of class I cytokine receptors. *BMC evolutionary biology* **7**, 120 (2007).
- 56 Renaud, J.-C. Class II cytokine receptors and their ligands: key antiviral and inflammatory modulators. *Nature Reviews Immunology* **3**, 667 (2003).
- 57 Subramaniam, S., Stansberg, C. & Cunningham, C. The interleukin 1 receptor family. *Developmental & Comparative Immunology* **28**, 415-428 (2004).
- 58 Weber, A., Wasiliew, P. & Kracht, M. Interleukin-1 (IL-1) pathway. *Sci. Signal.* **3**, cm1-cm1 (2010).
- 59 Hsu, H., Shu, H.-B., Pan, M.-G. & Goeddel, D. V. TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. *Cell* **84**, 299-308 (1996).
- 60 Harper, N., Hughes, M., MacFarlane, M. & Cohen, G. M. Fas-associated death domain protein and caspase-8 are not recruited to the tumor necrosis factor receptor 1 signaling complex during tumor necrosis factor-induced apoptosis. *Journal of Biological Chemistry* **278**, 25534-25541 (2003).
- 61 Murdoch, C. & Finn, A. Chemokine receptors and their role in inflammation and infectious diseases. *Blood* **95**, 3032-3043 (2000).
- 62 Jatiani, S. S., Baker, S. J., Silverman, L. R. & Reddy, E. P. Jak/STAT pathways in cytokine signaling and myeloproliferative disorders: approaches for targeted therapies. *Genes & cancer* **1**, 979-993 (2010).

- 63 Rawlings, J. S., Rosler, K. M. & Harrison, D. A. The JAK/STAT signaling pathway. *Journal of cell science* **117**, 1281-1283 (2004).
- 64 Imada, K. & Leonard, W. J. The jak-STAT pathway. *Molecular immunology* **37**, 1-11 (2000).
- 65 Leonard, W. J. Cytokines and immunodeficiency diseases. *Nature Reviews Immunology* **1**, 200 (2001).
- 66 Meraz, M. A. *et al.* Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. *Cell* **84**, 431-442 (1996).
- 67 Durbin, J. E., Hackenmiller, R., Simon, M. C. & Levy, D. E. Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. *Cell* **84**, 443-450 (1996).
- 68 Liu, T., Zhang, L., Joo, D. & Sun, S.-C. NF- $\kappa$ B signaling in inflammation. *Signal transduction and targeted therapy* **2**, 17023 (2017).
- 69 Sun, S.-C. The non-canonical NF- $\kappa$ B pathway in immunity and inflammation. *Nature Reviews Immunology* **17**, 545 (2017).
- 70 Rouvier, E., Luciani, M., Mattei, M., Denizot, F. & Golstein, P. CTLA-8, cloned from an activated T cell, bearing AU-rich messenger RNA instability sequences, and homologous to a herpesvirus saimiri gene. *The Journal of Immunology* **150**, 5445-5456 (1993).
- 71 Yao, Z. *et al.* Human IL-17: a novel cytokine derived from T cells. *The Journal of Immunology* **155**, 5483-5486 (1995).
- 72 Li, H. *et al.* Cloning and characterization of IL-17B and IL-17C, two new members of the IL-17 cytokine family. *Proceedings of the National Academy of Sciences* **97**, 773-778 (2000).
- 73 Lee, J. *et al.* IL-17E, a novel proinflammatory ligand for the IL-17 receptor homolog IL-17Rh1. *Journal of Biological Chemistry* **276**, 1660-1664 (2001).
- 74 Pappu, R., Ramirez-Carrozzi, V., Ota, N., Ouyang, W. & Hu, Y. The IL-17 family cytokines in immunity and disease. *Journal of clinical immunology* **30**, 185-195 (2010).
- 75 Starnes, T. *et al.* Cutting edge: IL-17F, a novel cytokine selectively expressed in activated T cells and monocytes, regulates angiogenesis and endothelial cell cytokine production. *The Journal of Immunology* **167**, 4137-4140 (2001).
- 76 Starnes, T., Broxmeyer, H. E., Robertson, M. J. & Hromas, R. Cutting edge: IL-17D, a novel member of the IL-17 family, stimulates cytokine production and inhibits hemopoiesis. *The Journal of Immunology* **169**, 642-646 (2002).
- 77 Chang, S. H. & Dong, C. A novel heterodimeric cytokine consisting of IL-17 and IL-17F regulates inflammatory responses. *Cell research* **17**, 435-440 (2007).
- 78 Wright, J. F. *et al.* Identification of an interleukin 17F/17A heterodimer in activated human CD4<sup>+</sup> T cells. *Journal of Biological Chemistry* **282**, 13447-13455 (2007).
- 79 Hymowitz, S. G. *et al.* IL-17s adopt a cystine knot fold: structure and activity of a novel cytokine, IL-17F, and implications for receptor binding. *The EMBO journal* **20**, 5332-5341 (2001).
- 80 Gerhardt, S. *et al.* Structure of IL-17A in complex with a potent, fully human neutralizing antibody. *Journal of molecular biology* **394**, 905-921 (2009).
- 81 Ely, L. K., Fischer, S. & Garcia, K. C. Structural basis of receptor sharing by interleukin 17 cytokines. *Nature immunology* **10**, 1245 (2009).
- 82 McDonald, N. Q. *et al.* New protein fold revealed by a 2.3-Å resolution crystal structure of nerve growth factor. (1991).



- 83 Fossiez, F. *et al.* T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. *The Journal of experimental medicine* **183**, 2593-2603 (1996).
- 84 Fallon, P. G. *et al.* Identification of an interleukin (IL)-25–dependent cell population that provides IL-4, IL-5, and IL-13 at the onset of helminth expulsion. *The Journal of experimental medicine* **203**, 1105-1116 (2006).
- 85 Wright, J. F. *et al.* The human IL-17F/IL-17A heterodimeric cytokine signals through the IL-17RA/IL-17RC receptor complex. *The Journal of Immunology* **181**, 2799-2805 (2008).
- 86 Toy, D. *et al.* Cutting edge: interleukin 17 signals through a heteromeric receptor complex. *The Journal of Immunology* **177**, 36-39 (2006).
- 87 Rickel, E. A. *et al.* Identification of functional roles for both IL-17RB and IL-17RA in mediating IL-25-induced activities. *The Journal of Immunology* **181**, 4299-4310 (2008).
- 88 Song, X. *et al.* IL-17RE is the functional receptor for IL-17C and mediates mucosal immunity to infection with intestinal pathogens. *Nature immunology* **12**, 1151 (2011).
- 89 Gaffen, S. L., Jain, R., Garg, A. V. & Cua, D. J. The IL-23-IL-17 immune axis: from mechanisms to therapeutic testing. *Nature Reviews Immunology* **14**, 585-600 (2014).
- 90 Gu, C., Wu, L. & Li, X. IL-17 family: cytokines, receptors and signaling. *Cytokine* **64**, 477-485 (2013).
- 91 Taams, L. S., Steel, K. J., Srenathan, U., Burns, L. A. & Kirkham, B. W. IL-17 in the immunopathogenesis of spondyloarthritis. *Nature Reviews Rheumatology*, 1 (2018).
- 92 May, M. J. IL-17R signaling: new players get in on the Act1. *Nature immunology* **12**, 813 (2011).
- 93 Gaffen, S. L., Jain, R., Garg, A. V. & Cua, D. J. IL-23-IL-17 immune axis: discovery, mechanistic understanding, and clinical testing. *Nature reviews. Immunology* **14**, 585 (2014).
- 94 Kao, C.-Y. *et al.* Up-regulation of CC chemokine ligand 20 expression in human airway epithelium by IL-17 through a JAK-independent but MEK/NF- $\kappa$ B-dependent signaling pathway. *The Journal of Immunology* **175**, 6676-6685 (2005).
- 95 Shahrara, S. *et al.* IL-17–mediated monocyte migration occurs partially through CC chemokine ligand 2/monocyte chemoattractant protein-1 induction. *The Journal of Immunology*, ji\_0901942 (2010).
- 96 Hartupee, J., Liu, C., Novotny, M., Li, X. & Hamilton, T. IL-17 enhances chemokine gene expression through mRNA stabilization. *The Journal of Immunology* **179**, 4135-4141 (2007).
- 97 Schwarzenberger, P. *et al.* IL-17 stimulates granulopoiesis in mice: use of an alternate, novel gene therapy-derived method for in vivo evaluation of cytokines. *The Journal of Immunology* **161**, 6383-6389 (1998).
- 98 Liang, S. C. *et al.* Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *The Journal of experimental medicine* **203**, 2271-2279 (2006).
- 99 Koenders, M. I. *et al.* Blocking of interleukin-17 during reactivation of experimental arthritis prevents joint inflammation and bone erosion by decreasing RANKL and interleukin-1. *The American journal of pathology* **167**, 141-149 (2005).
- 100 Chabaud, M. *et al.* Contribution of interleukin 17 to synovium matrix destruction in rheumatoid arthritis. *Cytokine* **12**, 1092-1099 (2000).

- 101 Udagawa, N., Kotake, S., Kamatani, N., Takahashi, N. & Suda, T. The molecular mechanism of osteoclastogenesis in rheumatoid arthritis. *Arthritis Research & Therapy* **4**, 281 (2002).
- 102 Simonet, W. *et al.* Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* **89**, 309-319 (1997).
- 103 Kotake, S. *et al.* IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis. *The Journal of clinical investigation* **103**, 1345-1352 (1999).
- 104 Adamopoulos, I. E. *et al.* Interleukin-17A upregulates receptor activator of NF- $\kappa$ B on osteoclast precursors. *Arthritis research & therapy* **12**, R29 (2010).
- 105 Lin, D. *et al.* Interleukin-17 regulates the expressions of RANKL and OPG in human periodontal ligament cells via TRAF 6/TBK 1-JNK/NF- $\kappa$ B pathways. *Immunology* **144**, 472-485 (2015).
- 106 Yang, X. O. *et al.* Regulation of inflammatory responses by IL-17F. *The Journal of experimental medicine* **205**, 1063-1075 (2008).
- 107 Kawaguchi, M. *et al.* Induction of granulocyte-macrophage colony-stimulating factor by a new cytokine, ML-1 (IL-17F), via Raf I-MEK-ERK pathway. *Journal of Allergy and Clinical Immunology* **114**, 444-450 (2004).
- 108 Watanabe, H. *et al.* Functional characterization of IL-17F as a selective neutrophil attractant in psoriasis. *Journal of Investigative Dermatology* **129**, 650-656 (2009).
- 109 Sorbello, V. *et al.* Nasal IL-17F is related to bronchial IL-17F/neutrophilia and exacerbations in stable atopic severe asthma. *Allergy* **70**, 236-240 (2015).
- 110 Hou, A. & Tong, L. Expression, Regulation, and Effects of Interleukin-17f in the Human Ocular Surface. *Ocular immunology and inflammation*, 1-9 (2017).
- 111 Zrioual, S. *et al.* IL-17RA and IL-17RC receptors are essential for IL-17A-induced ELR+ CXC chemokine expression in synoviocytes and are overexpressed in rheumatoid blood. *The Journal of Immunology* **180**, 655-663 (2008).
- 112 Zrioual, S. *et al.* Genome-wide comparison between IL-17A-and IL-17F-induced effects in human rheumatoid arthritis synoviocytes. *The Journal of Immunology* **182**, 3112-3120 (2009).
- 113 McAllister, F. *et al.* Role of IL-17A, IL-17F, and the IL-17 receptor in regulating growth-related oncogene- $\alpha$  and granulocyte colony-stimulating factor in bronchial epithelium: implications for airway inflammation in cystic fibrosis. *The Journal of Immunology* **175**, 404-412 (2005).
- 114 Iyoda, M. *et al.* IL-17A and IL-17F stimulate chemokines via MAPK pathways (ERK1/2 and p38 but not JNK) in mouse cultured mesangial cells: synergy with TNF- $\alpha$  and IL-1 $\beta$ . *American Journal of Physiology-Renal Physiology* **298**, F779-F787 (2009).
- 115 Hot, A., Zrioual, S., Lenief, V. & Miossec, P. IL-17 and tumour necrosis factor  $\alpha$  combination induces a HIF-1 $\alpha$ -dependent invasive phenotype in synoviocytes. *Annals of the rheumatic diseases*, annrheumdis-2011-200867 (2012).
- 116 Fischer, J. A. *et al.* Combined Inhibition of Tumor Necrosis Factor  $\alpha$  and Interleukin-17 As a Therapeutic Opportunity in Rheumatoid Arthritis: Development and Characterization of a Novel Bispecific Antibody. *Arthritis & rheumatology* **67**, 51-62 (2015).
- 117 Chabaud, M. & Miossec, P. The combination of tumor necrosis factor  $\alpha$  blockade with interleukin-1 and interleukin-17 blockade is more effective for controlling synovial inflammation and bone resorption in an ex vivo model. *Arthritis & Rheumatism: Official Journal of the American College of Rheumatology* **44**, 1293-1303 (2001).

- 118 Chabaud, M., Fossiez, F., Taupin, J.-L. & Miossec, P. Enhancing effect of IL-17 on IL-1-induced IL-6 and leukemia inhibitory factor production by rheumatoid arthritis synoviocytes and its regulation by Th2 cytokines. *The Journal of Immunology* **161**, 409-414 (1998).
- 119 Kawashiri, S. Y. *et al.* Proinflammatory cytokines synergistically enhance the production of chemokine ligand 20 (CCL20) from rheumatoid fibroblast-like synovial cells in vitro and serum CCL20 is reduced in vivo by biologic disease-modifying antirheumatic drugs. *J Rheumatol* **36**, 2397-2402, doi:10.3899/jrheum.090132 (2009).
- 120 Teunissen, M. B., Bos, J. D., Koomen, C. W., de Waal Malefyt, R. & Wierenga, E. A. Interleukin-17 and interferon- $\gamma$  synergize in the enhancement of proinflammatory cytokine production by human keratinocytes. *Journal of Investigative Dermatology* **111**, 645-649 (1998).
- 121 Glatt, S. *et al.* Dual IL-17A and IL-17F neutralisation by bimekizumab in psoriatic arthritis: evidence from preclinical experiments and a randomised placebo-controlled clinical trial that IL-17F contributes to human chronic tissue inflammation. *Annals of the rheumatic diseases* **77**, 523-532 (2018).
- 122 Hennessy, S. *et al.* IL-17A acts via p38 MAPK to increase stability of TNF- $\alpha$ -induced IL-8 mRNA in human ASM. *American Journal of Physiology-Lung Cellular and Molecular Physiology* **290**, L1283-L1290 (2006).
- 123 Friday, S. C. & Fox, D. A. Phospholipase D enzymes facilitate IL-17- and TNF $\alpha$ -induced expression of proinflammatory genes in rheumatoid arthritis synovial fibroblasts (RASf). *Immunology letters* **174**, 9-18 (2016).
- 124 Fort, M. M. *et al.* IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo. *Immunity* **15**, 985-995 (2001).
- 125 Kleinschek, M. A. *et al.* IL-25 regulates Th17 function in autoimmune inflammation. *The Journal of experimental medicine* **204**, 161-170 (2007).
- 126 Constantinescu, C. S., Farooqi, N., O'Brien, K. & Gran, B. Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS). *British journal of pharmacology* **164**, 1079-1106 (2011).
- 127 Xu, G. *et al.* Opposing roles of IL-17A and IL-25 in the regulation of TSLP production in human nasal epithelial cells. *Allergy* **65**, 581-589 (2010).
- 128 Ramirez-Carrozzi, V. *et al.* IL-17C regulates the innate immune function of epithelial cells in an autocrine manner. *Nature immunology* **12**, 1159 (2011).
- 129 Reynolds, J. M. *et al.* Cutting edge: regulation of intestinal inflammation and barrier function by IL-17C. *The Journal of Immunology*, 1103014 (2012).
- 130 Chang, S. H. *et al.* Interleukin-17C promotes Th17 cell responses and autoimmune disease via interleukin-17 receptor E. *Immunity* **35**, 611-621 (2011).
- 131 Johansen, C. *et al.* Characterization of the interleukin-17 isoforms and receptors in lesional psoriatic skin. *British Journal of Dermatology* **160**, 319-324 (2009).
- 132 Johnston, A. *et al.* Keratinocyte overexpression of IL-17C promotes psoriasiform skin inflammation. *The Journal of Immunology*, 1201505 (2013).
- 133 Bie, Q., Jin, C., Zhang, B. & Dong, H. IL-17B: A new area of study in the IL-17 family. *Molecular immunology* **90**, 50-56 (2017).
- 134 Kouri, V.-P. *et al.* Neutrophils produce interleukin-17B in rheumatoid synovial tissue. *Rheumatology* **53**, 39-47 (2013).
- 135 Ferretti, E., Ponzoni, M., Doglioni, C. & Pistoia, V. IL-17 superfamily cytokines modulate normal germinal center B cell migration. *Journal of leukocyte biology* **100**, 913-918 (2016).

- 136 Yamaguchi, Y. *et al.* IL-17B and IL-17C are associated with TNF- $\alpha$  production and contribute to the exacerbation of inflammatory arthritis. *The Journal of Immunology* **179**, 7128-7136 (2007).
- 137 O'Sullivan, T. *et al.* Interleukin-17D mediates tumor rejection through recruitment of natural killer cells. *Cell reports* **7**, 989-998 (2014).
- 138 Saddawi-Konefka, R., O'Sullivan, T., Gross, E. T., Washington Jr, A. & Bui, J. D. Tumor-expressed IL-17D recruits NK cells to reject tumors. *Oncoimmunology* **3**, e954853 (2014).
- 139 Seelige, R., Washington Jr, A. & Bui, J. D. The ancient cytokine IL-17D is regulated by Nrf2 and mediates tumor and virus surveillance. *Cytokine* **91**, 10-12 (2017).
- 140 Zhang, N. & Bevan, M. J. CD8+ T cells: foot soldiers of the immune system. *Immunity* **35**, 161-168 (2011).
- 141 Zhu, J. & Paul, W. E. CD4 T cells: fates, functions, and faults. *Blood* **112**, 1557-1569 (2008).
- 142 Klein, L., Kyewski, B., Allen, P. M. & Hogquist, K. A. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). *Nature Reviews Immunology* **14**, 377 (2014).
- 143 Fahl, S. P., Coffey, F. & Wiest, D. L. Origins of  $\gamma\delta$  T cell effector subsets: a riddle wrapped in an enigma. *The Journal of Immunology* **193**, 4289-4294 (2014).
- 144 Murphy, K. & Weaver, C. *Janeway's immunobiology*. (Garland Science, 2016).
- 145 Belz, G. T., Carbone, F. R. & Heath, W. R. Cross-presentation of antigens by dendritic cells. *Critical Reviews™ in Immunology* **22** (2002).
- 146 Howland, K. C., Ausubel, L. J., London, C. A. & Abbas, A. K. The roles of CD28 and CD40 ligand in T cell activation and tolerance. *The Journal of Immunology* **164**, 4465-4470 (2000).
- 147 Ranheim, E. A. & Kipps, T. J. Activated T cells induce expression of B7/BB1 on normal or leukemic B cells through a CD40-dependent signal. *Journal of Experimental Medicine* **177**, 925-935 (1993).
- 148 Gimmi, C. D., Freeman, G. J., Gribben, J. G., Gray, G. & Nadler, L. M. Human T-cell clonal anergy is induced by antigen presentation in the absence of B7 costimulation. *Proceedings of the National Academy of Sciences* **90**, 6586-6590 (1993).
- 149 Parry, R. V. *et al.* CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. *Molecular and cellular biology* **25**, 9543-9553 (2005).
- 150 Buchbinder, E. I. & Desai, A. CTLA-4 and PD-1 pathways: similarities, differences, and implications of their inhibition. *American journal of clinical oncology* **39**, 98 (2016).
- 151 Angus, K. L. & Griffiths, G. M. Cell polarisation and the immunological synapse. *Current opinion in cell biology* **25**, 85-91 (2013).
- 152 Monks, C. R., Freiberg, B. A., Kupfer, H., Sciaky, N. & Kupfer, A. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature* **395**, 82 (1998).
- 153 Bromley, S. K. *et al.* The immunological synapse. *Annual review of immunology* **19**, 375-396 (2001).
- 154 Freiberg, B. A. *et al.* Staging and resetting T cell activation in SMACs. *Nature immunology* **3**, 911 (2002).
- 155 Glaichenhaus, N., Shastri, N., Littman, D. R. & Turner, J. M. Requirement for association of p56lck with CD4 in antigen-specific signal transduction in T cells. *Cell* **64**, 511-520 (1991).
- 156 Appleby, M. W. *et al.* Defective T cell receptor signaling in mice lacking the thymic isoform of p59fyn. *Cell* **70**, 751-763 (1992).

- 157 Zhang, W., Sloan-Lancaster, J., Kitchen, J., Tribble, R. P. & Samelson, L. E. LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. *Cell* **92**, 83-92 (1998).
- 158 Wardenburg, J. B. *et al.* Phosphorylation of SLP-76 by the ZAP-70 protein-tyrosine kinase is required for T-cell receptor function. *Journal of Biological Chemistry* **271**, 19641-19644 (1996).
- 159 Liu, S. K., Fang, N., Koretzky, G. A. & McGlade, C. J. The hematopoietic-specific adaptor protein gads functions in T-cell signaling via interactions with the SLP-76 and LAT adaptors. *Current Biology* **9**, 67-75 (1999).
- 160 Smith-Garvin, J. E., Koretzky, G. A. & Jordan, M. S. T cell activation. *Annual review of immunology* **27**, 591-619 (2009).
- 161 Golubovskaya, V. & Wu, L. Different subsets of T cells, memory, effector functions, and CAR-T immunotherapy. *Cancers* **8**, 36 (2016).
- 162 Pennock, N. D. *et al.* T cell responses: naive to memory and everything in between. *Advances in physiology education* **37**, 273-283 (2013).
- 163 Berard, M. & Tough, D. F. Qualitative differences between naive and memory T cells. *Immunology* **106**, 127-138 (2002).
- 164 MacLeod, M. K., Clambey, E. T., Kappler, J. W. & Marrack, P. in *Seminars in immunology*. 53-61 (Elsevier).
- 165 Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A. & Coffman, R. L. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *The Journal of immunology* **136**, 2348-2357 (1986).
- 166 Romagnani, S. T-cell subsets (Th1 versus Th2). *Annals of allergy, asthma & immunology* **85**, 21 (2000).
- 167 Ma, D., Huang, H. & Huang, Z. STAT1 signaling is required for optimal Th1 cell differentiation in mice. *Chinese science bulletin* **55**, 1032-1040 (2010).
- 168 Magram, J. *et al.* IL-12-deficient mice are defective in IFN $\gamma$  production and type 1 cytokine responses. *Immunity* **4**, 471-481 (1996).
- 169 Lighvani, A. A. *et al.* T-bet is rapidly induced by interferon- $\gamma$  in lymphoid and myeloid cells. *Proceedings of the National Academy of Sciences* **98**, 15137-15142 (2001).
- 170 Walker, J. A. & McKenzie, A. N. J. TH2 cell development and function. *Nature Reviews Immunology* **18**, 121, doi:10.1038/nri.2017.118 (2017).
- 171 Zheng, W.-p. & Flavell, R. A. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* **89**, 587-596 (1997).
- 172 Szabo, S. J. *et al.* A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* **100**, 655-669 (2000).
- 173 Vignali, D. A., Collison, L. W. & Workman, C. J. How regulatory T cells work. *Nature Reviews Immunology* **8**, 523 (2008).
- 174 Davidson, T. S., DiPaolo, R. J., Andersson, J. & Shevach, E. M. Cutting edge: IL-2 is essential for TGF- $\beta$ -mediated induction of Foxp3+ T regulatory cells. *The Journal of Immunology* **178**, 4022-4026 (2007).
- 175 Chen, W. *et al.* Conversion of peripheral CD4+ CD25- naive T cells to CD4+ CD25+ regulatory T cells by TGF- $\beta$  induction of transcription factor Foxp3. *Journal of Experimental Medicine* **198**, 1875-1886 (2003).
- 176 Hori, S., Nomura, T. & Sakaguchi, S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* **299**, 1057-1061 (2003).
- 177 Fontenot, J. D., Gavin, M. A. & Rudensky, A. Y. Foxp3 programs the development and function of CD4+ CD25+ regulatory T cells. *Nature immunology* **4**, 330 (2003).

- 178 Chen, Y., Kuchroo, V. K., Inobe, J.-i., Hafler, D. A. & Weiner, H. L. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science* **265**, 1237-1240 (1994).
- 179 Lourenço, E. V. & La Cava, A. Natural regulatory T cells in autoimmunity. *Autoimmunity* **44**, 33-42 (2011).
- 180 Dardalhon, V. *et al.* IL-4 inhibits TGF- $\beta$ -induced Foxp3<sup>+</sup> T cells and, together with TGF- $\beta$ , generates IL-9<sup>+</sup> IL-10<sup>+</sup> Foxp3<sup>-</sup> effector T cells. *Nature immunology* **9**, 1347 (2008).
- 181 Veldhoen, M. *et al.* Transforming growth factor- $\beta$ 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nature immunology* **9**, 1341 (2008).
- 182 Schmitt, E. *et al.* IL-9 production of naive CD4<sup>+</sup> T cells depends on IL-2, is synergistically enhanced by a combination of TGF-beta and IL-4, and is inhibited by IFN-gamma. *The Journal of Immunology* **153**, 3989-3996 (1994).
- 183 Chang, H.-C. *et al.* The transcription factor PU. 1 is required for the development of IL-9-producing T cells and allergic inflammation. *Nature immunology* **11**, 527 (2010).
- 184 Staudt, V. *et al.* Interferon-regulatory factor 4 is essential for the developmental program of T helper 9 cells. *Immunity* **33**, 192-202 (2010).
- 185 Trifari, S., Kaplan, C. D., Tran, E. H., Crellin, N. K. & Spits, H. Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from T H-17, T H 1 and T H 2 cells. *Nature immunology* **10**, 864 (2009).
- 186 Duhren, T., Geiger, R., Jarrossay, D., Lanzavecchia, A. & Sallusto, F. Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells. *Nature immunology* **10**, 857 (2009).
- 187 Ramirez, J. M. *et al.* Activation of the aryl hydrocarbon receptor reveals distinct requirements for IL-22 and IL-17 production by human T helper cells. *European journal of immunology* **40**, 2450-2459 (2010).
- 188 Crotty, S. T follicular helper cell differentiation, function, and roles in disease. *Immunity* **41**, 529-542 (2014).
- 189 Mangan, P. R. *et al.* Transforming growth factor- $\beta$  induces development of the T H 17 lineage. *Nature* **441**, 231 (2006).
- 190 Huang, G., Wang, Y. & Chi, H. Regulation of T H 17 cell differentiation by innate immune signals. *Cellular & molecular immunology* **9**, 287 (2012).
- 191 Korn, T. *et al.* IL-21 initiates an alternative pathway to induce proinflammatory T H 17 cells. *Nature* **448**, 484 (2007).
- 192 Evans, H. G., Suddason, T., Jackson, I., Taams, L. S. & Lord, G. M. Optimal induction of T helper 17 cells in humans requires T cell receptor ligation in the context of Toll-like receptor-activated monocytes. *Proceedings of the National Academy of Sciences* **104**, 17034-17039 (2007).
- 193 Wilson, N. J. *et al.* Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nature immunology* **8**, 950 (2007).
- 194 Chen, Z., Tato, C. M., Muul, L., Laurence, A. & O'shea, J. J. Distinct regulation of interleukin-17 in human T helper lymphocytes. *Arthritis & Rheumatism: Official Journal of the American College of Rheumatology* **56**, 2936-2946 (2007).
- 195 Yang, L. *et al.* IL-21 and TGF- $\beta$  are required for differentiation of human T H 17 cells. *Nature* **454**, 350 (2008).
- 196 Liu, H. & Rohowsky-Kochan, C. Regulation of IL-17 in human CCR6<sup>+</sup> effector memory T cells. *The Journal of Immunology* **180**, 7948-7957 (2008).

- 197 Evans, H. G. *et al.* In vivo activated monocytes from the site of inflammation in  
humans specifically promote Th17 responses. *Proceedings of the National Academy  
of Sciences* **106**, 6232-6237 (2009).
- 198 Cheng, W. C. *et al.* Periodontitis-associated pathogens *P. gingivalis* and *A.*  
*actinomycetemcomitans* activate human CD14<sup>+</sup> monocytes leading to enhanced  
Th17/IL-17 responses. *European journal of immunology* **46**, 2211-2221 (2016).
- 199 van Beelen, A. J. *et al.* Stimulation of the intracellular bacterial sensor NOD2  
programs dendritic cells to promote interleukin-17 production in human memory T  
cells. *Immunity* **27**, 660-669 (2007).
- 200 Ivanov, I. I. *et al.* The orphan nuclear receptor ROR $\gamma$ t directs the differentiation  
program of proinflammatory IL-17<sup>+</sup> T helper cells. *Cell* **126**, 1121-1133 (2006).
- 201 Yang, X. O. *et al.* T helper 17 lineage differentiation is programmed by orphan  
nuclear receptors ROR $\alpha$  and ROR $\gamma$ . *Immunity* **28**, 29-39 (2008).
- 202 Durant, L. *et al.* Diverse targets of the transcription factor STAT3 contribute to T cell  
pathogenicity and homeostasis. *Immunity* **32**, 605-615 (2010).
- 203 Yang, X. O. *et al.* STAT3 regulates cytokine-mediated generation of inflammatory  
helper T cells. *Journal of Biological Chemistry* **282**, 9358-9363 (2007).
- 204 Schraml, B. U. *et al.* The AP-1 transcription factor Batf controls T H 17  
differentiation. *Nature* **460**, 405 (2009).
- 205 Brüstle, A. *et al.* The development of inflammatory T H-17 cells requires interferon-  
regulatory factor 4. *Nature immunology* **8**, 958 (2007).
- 206 Li, P. *et al.* BATF–JUN is critical for IRF4-mediated transcription in T cells. *Nature*  
**490**, 543 (2012).
- 207 Ciofani, M. *et al.* A validated regulatory network for Th17 cell specification. *Cell*  
**151**, 289-303 (2012).
- 208 Dang, E. V. *et al.* Control of T H 17/T reg balance by hypoxia-inducible factor 1. *Cell*  
**146**, 772-784 (2011).
- 209 Harrington, L. E. *et al.* Interleukin 17–producing CD4<sup>+</sup> effector T cells develop via a  
lineage distinct from the T helper type 1 and 2 lineages. *Nature immunology* **6**,  
1123 (2005).
- 210 Park, H. *et al.* A distinct lineage of CD4 T cells regulates tissue inflammation by  
producing interleukin 17. *Nature immunology* **6**, 1133 (2005).
- 211 Yeh, W.-I., McWilliams, I. L. & Harrington, L. E. IFN $\gamma$  inhibits Th17 differentiation  
and function via Tbet-dependent and Tbet-independent mechanisms. *Journal of  
neuroimmunology* **267**, 20-27 (2014).
- 212 Villarino, A. V., Gallo, E. & Abbas, A. K. STAT1-Activating Cytokines Limit Th17  
Responses through Both T-bet–Dependent and Independent Mechanisms. *The  
Journal of Immunology*, 1001343 (2010).
- 213 Bach, E. A., Aguet, M. & Schreiber, R. D. The IFN $\gamma$  receptor: a paradigm for cytokine  
receptor signaling. *Annual review of immunology* **15**, 563-591 (1997).
- 214 Cypowyj, S., Picard, C., Maródi, L., Casanova, J. L. & Puel, A. Immunity to infection  
in IL-17-deficient mice and humans. *European journal of immunology* **42**, 2246-  
2254 (2012).
- 215 Mathur, A. N. *et al.* T-bet is a critical determinant in the instability of the IL-17-  
secreting T-helper phenotype. *Blood* **108**, 1595-1601 (2006).
- 216 Stumhofer, J. S. *et al.* Interleukin 27 negatively regulates the development of  
interleukin 17–producing T helper cells during chronic inflammation of the central  
nervous system. *Nature immunology* **7**, 937 (2006).
- 217 Batten, M. *et al.* Interleukin 27 limits autoimmune encephalomyelitis by  
suppressing the development of interleukin 17–producing T cells. *Nature  
immunology* **7**, 929 (2006).

- 218 Laurence, A. *et al.* Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. *Immunity* **26**, 371-381 (2007).
- 219 Zhou, L. *et al.* TGF- $\beta$ -induced Foxp3 inhibits T H 17 cell differentiation by antagonizing ROR $\gamma$ t function. *Nature* **453**, 236 (2008).
- 220 Elias, K. M. *et al.* Retinoic acid inhibits Th17 polarization and enhances FoxP3 expression through a Stat-3/Stat-5 independent signaling pathway. *Blood* **111**, 1013-1020 (2008).
- 221 Bouguermouh, S., Fortin, G., Baba, N., Rubio, M. & Sarfati, M. CD28 co-stimulation down regulates Th17 development. *PLoS one* **4**, e5087 (2009).
- 222 Yang, X.-P. *et al.* Opposing regulation of the locus encoding IL-17 through direct, reciprocal actions of STAT3 and STAT5. *Nature immunology* **12**, 247 (2011).
- 223 Moriggl, R. *et al.* Stat5 is required for IL-2-induced cell cycle progression of peripheral T cells. *Immunity* **10**, 249-259 (1999).
- 224 Kim, H. S., Sohn, H. & Lee, G. R. (Am Assoc Immunol, 2018).
- 225 Purvis, H. A. *et al.* Low strength T-cell activation promotes Th17 responses. *Blood*, blood-2010-2003-272153 (2010).
- 226 Annunziato, F. *et al.* Phenotypic and functional features of human Th17 cells. *Journal of Experimental Medicine* **204**, 1849-1861 (2007).
- 227 Acosta-Rodriguez, E. V. *et al.* Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nature immunology* **8**, 639 (2007).
- 228 Liao, F. *et al.* CC-chemokine receptor 6 is expressed on diverse memory subsets of T cells and determines responsiveness to macrophage inflammatory protein 3 $\alpha$ . *The Journal of Immunology* **162**, 186-194 (1999).
- 229 Sallusto, F. *et al.* Switch in chemokine receptor expression upon TCR stimulation reveals novel homing potential for recently activated T cells. *European journal of immunology* **29**, 2037-2045 (1999).
- 230 Fergusson, J. R., Fleming, V. M. & Klemerman, P. CD161-expressing human T cells. *Frontiers in immunology* **2**, 36 (2011).
- 231 Cosmi, L. *et al.* Human interleukin 17-producing cells originate from a CD161+ CD4+ T cell precursor. *Journal of Experimental Medicine* **205**, 1903-1916 (2008).
- 232 Poggi, A., Costa, P., Zocchi, M. R. & Moretta, L. Phenotypic and functional analysis of CD4+ NKR1A+ human T lymphocytes. Direct evidence that the NKR1A molecule is involved in transendothelial migration. *European journal of immunology* **27**, 2345-2350 (1997).
- 233 Maggi, L. *et al.* CD161 is a marker of all human IL-17-producing T-cell subsets and is induced by RORC. *European journal of immunology* **40**, 2174-2181 (2010).
- 234 Sallusto, F., Zielinski, C. E. & Lanzavecchia, A. Human T h17 subsets. *European journal of immunology* **42**, 2215-2220 (2012).
- 235 Zielinski, C. E. *et al.* Pathogen-induced human T H 17 cells produce IFN- $\gamma$  or IL-10 and are regulated by IL-1 $\beta$ . *Nature* **484**, 514 (2012).
- 236 Annunziato, F., Cosmi, L., Liotta, F., Maggi, E. & Romagnani, S. Defining the human T helper 17 cell phenotype. *Trends in immunology* **33**, 505-512 (2012).
- 237 McGeachy, M. J. *et al.* TGF- $\beta$  and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T H-17 cell-mediated pathology. *Nature immunology* **8**, 1390 (2007).
- 238 Ghoreschi, K. *et al.* Generation of pathogenic T H 17 cells in the absence of TGF- $\beta$  signalling. *Nature* **467**, 967 (2010).
- 239 Boniface, K. *et al.* Prostaglandin E2 regulates Th17 cell differentiation and function through cyclic AMP and EP2/EP4 receptor signaling. *Journal of Experimental Medicine* **206**, 535-548 (2009).



- 240 zu Horste, G. M. *et al.* RBPJ controls development of pathogenic Th17 cells by  
regulating IL-23 receptor expression. *Cell reports* **16**, 392-404 (2016).
- 241 El-Behi, M. *et al.* The encephalitogenicity of T H 17 cells is dependent on IL-1-and  
IL-23-induced production of the cytokine GM-CSF. *Nature immunology* **12**, 568  
(2011).
- 242 Lee, Y. *et al.* Induction and molecular signature of pathogenic T H 17 cells. *Nature  
immunology* **13**, 991 (2012).
- 243 Gaublomme, J. T. *et al.* Single-cell genomics unveils critical regulators of Th17 cell  
pathogenicity. *Cell* **163**, 1400-1412 (2015).
- 244 Srenathan, U., Steel, K. & Taams, L. S. IL-17+ CD8+ T cells: differentiation,  
phenotype and role in inflammatory disease. *Immunology letters* **178**, 20-26 (2016).
- 245 Kondo, T., Takata, H., Matsuki, F. & Takiguchi, M. Cutting edge: Phenotypic  
characterization and differentiation of human CD8+ T cells producing IL-17. *The  
Journal of Immunology* **182**, 1794-1798 (2009).
- 246 Wanke, F. *et al.* Expression of IL-17F is associated with non-pathogenic Th17 cells.  
*Journal of Molecular Medicine* **96**, 819-829 (2018).
- 247 Le Bourhis, L. *et al.* Mucosal-associated invariant T cells: unconventional  
development and function. *Trends in immunology* **32**, 212-218 (2011).
- 248 Martin, B., Hirota, K., Cua, D. J., Stockinger, B. & Veldhoen, M. Interleukin-17-  
producing  $\gamma\delta$  T cells selectively expand in response to pathogen products and  
environmental signals. *Immunity* **31**, 321-330 (2009).
- 249 Coquet, J. M. *et al.* Diverse cytokine production by NKT cell subsets and  
identification of an IL-17-producing CD4- NK1. 1- NKT cell population. *Proceedings  
of the National Academy of Sciences* **105**, 11287-11292 (2008).
- 250 Noordenbos, T. *et al.* Interleukin-17-positive mast cells contribute to synovial  
inflammation in spondylarthritis. *Arthritis & Rheumatism* **64**, 99-109 (2012).
- 251 Hueber, A. J. *et al.* Cutting edge: mast cells express IL-17A in rheumatoid arthritis  
synovium. *The Journal of Immunology* **184**, 3336-3340 (2010).
- 252 Lin, A. M. *et al.* Mast cells and neutrophils release IL-17 through extracellular trap  
formation in psoriasis. *The Journal of Immunology*, 1100123 (2011).
- 253 Noordenbos, T. *et al.* Human mast cells capture, store, and release bioactive,  
exogenous IL-17A. *Journal of leukocyte biology* **100**, 453-462 (2016).
- 254 Almahmoudi, R. *et al.* Extracellular interleukin-17F has a protective effect in oral  
tongue squamous cell carcinoma. *Head & neck* (2018).
- 255 Hu, S. *et al.* IL-17 Production of neutrophils enhances antibacteria ability but  
promotes arthritis development during Mycobacterium tuberculosis infection.  
*EBioMedicine* **23**, 88-99 (2017).
- 256 Ferretti, S., Bonneau, O., Dubois, G. R., Jones, C. E. & Trifilieff, A. IL-17, produced by  
lymphocytes and neutrophils, is necessary for lipopolysaccharide-induced airway  
neutrophilia: IL-15 as a possible trigger. *The Journal of Immunology* **170**, 2106-2112  
(2003).
- 257 Katayama, M. *et al.* Neutrophils are essential as a source of IL-17 in the effector  
phase of arthritis. *PLoS One* **8**, e62231 (2013).
- 258 Huppler, A. R., Verma, A. H., Conti, H. R. & Gaffen, S. L. Neutrophils do not express  
IL-17A in the context of acute oropharyngeal candidiasis. *Pathogens* **4**, 559-572  
(2015).
- 259 Tamarozzi, F., Wright, H. L., Thomas, H. B., Edwards, S. W. & Taylor, M. J. A lack of  
confirmation with alternative assays questions the validity of IL-17A expression in  
human neutrophils using immunohistochemistry. *Immunology letters* **162**, 194-198  
(2014).

- 260 Ward, N. L. & Umetsu, D. T. A new player on the psoriasis block: IL-17A-and IL-22-  
producing innate lymphoid cells. *Journal of Investigative Dermatology* **134**, 2305-  
2307 (2014).
- 261 Paustian, A. M. S. *et al.* Continuous IL-23 stimulation drives ILC3 depletion in the  
upper GI tract and, in combination with TNF $\alpha$ , induces robust activation and a  
phenotypic switch of ILC3. *PloS one* **12**, e0182841 (2017).
- 262 Withers, D. R. & Hepworth, M. R. Group 3 innate lymphoid cells: communications  
hubs of the intestinal immune system. *Frontiers in immunology* **8**, 1298 (2017).
- 263 Takahashi, N. *et al.* IL-17 produced by Paneth cells drives TNF-induced shock.  
*Journal of Experimental Medicine* **205**, 1755-1761 (2008).
- 264 Wang, Y.-H. *et al.* IL-25 augments type 2 immune responses by enhancing the  
expansion and functions of TSLP-DC-activated Th2 memory cells. *The Journal of  
experimental medicine* **204**, 1837-1847 (2007).
- 265 Ikeda, K. *et al.* Mast cells produce interleukin-25 upon Fc $\epsilon$ RI-mediated activation.  
*Blood* **101**, 3594-3596 (2003).
- 266 Zaph, C. *et al.* Commensal-dependent expression of IL-25 regulates the IL-23-IL-17  
axis in the intestine. *The Journal of experimental medicine* **205**, 2191-2198 (2008).
- 267 Kang, C.-M. *et al.* Interleukin-25 and interleukin-13 production by alveolar  
macrophages in response to particles. *American journal of respiratory cell and  
molecular biology* **33**, 290-296 (2005).
- 268 Moore, E. E. *et al.* Expression of IL-17B in neurons and evaluation of its possible  
role in the chromosome 5q-linked form of Charcot-Marie-Tooth disease.  
*Neuromuscular Disorders* **12**, 141-150 (2002).
- 269 Smolen, J. S. *et al.* Rheumatoid arthritis. *Nature Reviews Disease Primers* **4**, 18001,  
doi:10.1038/nrdp.2018.1 (2018).
- 270 Gladman, D., Shuckett, R., Russell, M., Thorne, J. & Schachter, R. Psoriatic arthritis  
(PSA)-an analysis of 220 patients. *QJM: An International Journal of Medicine* **62**,  
127-141 (1987).
- 271 Pereira, D., Ramos, E. & Branco, J. Osteoarthritis. *Acta Med Port* **28**, 99-106 (2015).
- 272 Sugiyama, D. *et al.* Impact of smoking as a risk factor for developing rheumatoid  
arthritis: a meta-analysis of observational studies. *Annals of the rheumatic diseases*  
(2009).
- 273 MacGregor, A. J. *et al.* Characterizing the quantitative genetic contribution to  
rheumatoid arthritis using data from twins. *Arthritis & Rheumatism: Official Journal  
of the American College of Rheumatology* **43**, 30-37 (2000).
- 274 Consortium, W. T. C. C. Genome-wide association study of 14,000 cases of seven  
common diseases and 3,000 shared controls. *Nature* **447**, 661 (2007).
- 275 van der Woude, D. *et al.* Quantitative heritability of anti-citrullinated protein  
antibody-positive and anti-citrullinated protein antibody-negative rheumatoid  
arthritis. *Arthritis & Rheumatism: Official Journal of the American College of  
Rheumatology* **60**, 916-923 (2009).
- 276 Kurkó, J. *et al.* Genetics of rheumatoid arthritis—a comprehensive review. *Clinical  
reviews in allergy & immunology* **45**, 170-179 (2013).
- 277 Gregersen, P. K., Silver, J. & Winchester, R. J. The shared epitope hypothesis. An  
approach to understanding the molecular genetics of susceptibility to rheumatoid  
arthritis. *Arthritis & Rheumatism: Official Journal of the American College of  
Rheumatology* **30**, 1205-1213 (1987).
- 278 Moreno, I. *et al.* Association of the shared epitope with radiological severity of  
rheumatoid arthritis. *The Journal of rheumatology* **23**, 6-9 (1996).
- 279 Gonzalez-Gay, M. A., Garcia-Porrúa, C. & Hajeer, A. H. in *Seminars in arthritis and  
rheumatism*. 355-360 (Elsevier).

- 280 Firestein, G. S. Evolving concepts of rheumatoid arthritis. *Nature* **423**, 356 (2003).
- 281 Huizinga, T. W. *et al.* Refining the complex rheumatoid arthritis phenotype based  
on specificity of the HLA–DRB1 shared epitope for antibodies to citrullinated  
proteins. *Arthritis & Rheumatism* **52**, 3433-3438 (2005).
- 282 Carrier, N. *et al.* The DERA HLA–DR alleles in patients with early polyarthritis:  
protection against severe disease and lack of association with rheumatoid arthritis  
autoantibodies. *Arthritis & Rheumatism: Official Journal of the American College of  
Rheumatology* **60**, 698-707 (2009).
- 283 Farago, B. *et al.* Protein tyrosine phosphatase gene C1858T allele confers risk for  
rheumatoid arthritis in Hungarian subjects. *Rheumatology international* **29**, 793-  
796 (2009).
- 284 Hollis-Moffatt, J. E. *et al.* Evidence for association of an interleukin-23 receptor  
variant independent of the R381Q variant with rheumatoid arthritis. *Annals of the  
rheumatic diseases* (2008).
- 285 Kochi, Y. *et al.* A regulatory variant in CCR6 is associated with rheumatoid arthritis  
susceptibility. *Nature genetics* **42**, 515 (2010).
- 286 Winchester, R. *et al.* HLA associations reveal genetic heterogeneity in psoriatic  
arthritis and in the psoriasis phenotype. *Arthritis & Rheumatism* **64**, 1134-1144  
(2012).
- 287 Bowes, J. *et al.* Cross-phenotype association mapping of the MHC identifies genetic  
variants that differentiate psoriatic arthritis from psoriasis. *Annals of the rheumatic  
diseases*, annrheumdis-2017-211414 (2017).
- 288 Haroon, M., Winchester, R., Giles, J. T., Heffernan, E. & FitzGerald, O. Certain class I  
HLA alleles and haplotypes implicated in susceptibility play a role in determining  
specific features of the psoriatic arthritis phenotype. *Annals of the rheumatic  
diseases*, annrheumdis-2014-205461 (2014).
- 289 Hermann, E., zum Büschenfelde, K. M., Fleischer, B. & Yu, D. HLA-B27-restricted  
CD8 T cells derived from synovial fluids of patients with reactive arthritis and  
ankylosing spondylitis. *The Lancet* **342**, 646-650 (1993).
- 290 McHugh, K. & Bowness, P. The link between HLA-B27 and SpA—new ideas on an  
old problem. *Rheumatology* **51**, 1529-1539 (2012).
- 291 Allen, R. L., O’Callaghan, C. A., McMichael, A. J. & Bowness, P. Cutting edge: HLA-  
B27 can form a novel  $\beta$ 2-microglobulin-free heavy chain homodimer structure. *The  
Journal of Immunology* **162**, 5045-5048 (1999).
- 292 Kollnberger, S. *et al.* Cell-surface expression and immune receptor recognition of  
HLA–B27 homodimers. *Arthritis & Rheumatism: Official Journal of the American  
College of Rheumatology* **46**, 2972-2982 (2002).
- 293 Bowness, P. *et al.* Th17 cells expressing KIR3DL2+ and responsive to HLA-B27  
homodimers are increased in ankylosing spondylitis. *The Journal of Immunology*,  
1002653 (2011).
- 294 Bowes, J. *et al.* Confirmation of TNIP1 and IL23A as susceptibility loci for psoriatic  
arthritis. *Annals of the rheumatic diseases*, annrheumdis150102 (2011).
- 295 Filer, C. *et al.* Investigation of association of the IL12B and IL23R genes with  
psoriatic arthritis. *Arthritis & Rheumatism: Official Journal of the American College  
of Rheumatology* **58**, 3705-3709 (2008).
- 296 Cargill, M. *et al.* A large-scale genetic association study confirms IL12B and leads to  
the identification of IL23R as psoriasis-risk genes. *The American Journal of Human  
Genetics* **80**, 273-290 (2007).
- 297 Di Meglio, P. *et al.* The IL23R R381Q gene variant protects against immune-  
mediated diseases by impairing IL-23-induced Th17 effector response in humans.  
*PloS one* **6**, e17160 (2011).

- 298 Cénit, M. C. *et al.* Influence of the STAT3 genetic variants in the susceptibility to psoriatic arthritis and Behcet's disease. *Human immunology* **74**, 230-233 (2013).
- 299 Bowes, J. *et al.* Dense genotyping of immune-related susceptibility loci reveals new insights into the genetics of psoriatic arthritis. *Nature communications* **6**, 6046 (2015).
- 300 Ospelt, C. Synovial fibroblasts in 2017. *RMD open* **3**, e000471 (2017).
- 301 Lefèvre, S. *et al.* Synovial fibroblasts spread rheumatoid arthritis to unaffected joints. *Nature medicine* **15**, 1414 (2009).
- 302 Croft, A. P. *et al.* Rheumatoid synovial fibroblasts differentiate into distinct subsets in the presence of cytokines and cartilage. *Arthritis research & therapy* **18**, 270 (2016).
- 303 Mizoguchi, F. *et al.* Functionally distinct disease-associated fibroblast subsets in rheumatoid arthritis. *Nature communications* **9**, 789 (2018).
- 304 Frank-Bertoncelj, M. *et al.* Epigenetically-driven anatomical diversity of synovial fibroblasts guides joint-specific fibroblast functions. *Nature communications* **8**, 14852 (2017).
- 305 Bainbridge, P. Wound healing and the role of fibroblasts. *Journal of wound care* **22** (2013).
- 306 Brentano, F., Schorr, O., Gay, R. E., Gay, S. & Kyburz, D. RNA released from necrotic synovial fluid cells activates rheumatoid arthritis synovial fibroblasts via Toll-like receptor 3. *Arthritis & Rheumatism* **52**, 2656-2665 (2005).
- 307 Yamamura, Y. *et al.* Effector function of resting T cells: activation of synovial fibroblasts. *The Journal of Immunology* **166**, 2270-2275 (2001).
- 308 Salmon, M. *et al.* Inhibition of T cell apoptosis in the rheumatoid synovium. *The Journal of clinical investigation* **99**, 439-446 (1997).
- 309 Carrión, M. *et al.* RNA sensors in human osteoarthritis and rheumatoid arthritis synovial fibroblasts: immune regulation by vasoactive intestinal peptide. *Arthritis & Rheumatism* **63**, 1626-1636 (2011).
- 310 Cho, M. L. *et al.* Effector function of type II collagen-stimulated T cells from rheumatoid arthritis patients: Cross-talk between T cells and synovial fibroblasts. *Arthritis & Rheumatism* **50**, 776-784 (2004).
- 311 Rengel, Y., Ospelt, C. & Gay, S. Proteinases in the joint: clinical relevance of proteinases in joint destruction. *Arthritis research & therapy* **9**, 221 (2007).
- 312 Araki, Y. *et al.* Histone Methylation and STAT-3 Differentially Regulate Interleukin-6-Induced Matrix Metalloproteinase Gene Activation in Rheumatoid Arthritis Synovial Fibroblasts. *Arthritis & rheumatology* **68**, 1111-1123 (2016).
- 313 Korb-Pap, A. *et al.* Early structural changes in cartilage and bone are required for the attachment and invasion of inflamed synovial tissue during destructive inflammatory arthritis. *Annals of the Rheumatic diseases*, annrheumdis-2011-200386 (2012).
- 314 Falgarone, G., Jaen, O. & Boissier, M.-C. Role for innate immunity in rheumatoid arthritis. *Joint Bone Spine* **72**, 17-25 (2005).
- 315 Iguchi, T., Kurosaka, M. & Ziff, M. Electron microscopic study of HLA-DR and monocyte/macrophage staining cells in the rheumatoid synovial membrane. *Arthritis & Rheumatism: Official Journal of the American College of Rheumatology* **29**, 600-613 (1986).
- 316 Koller, M. *et al.* Expression of adhesion molecules on synovial fluid and peripheral blood monocytes in patients with inflammatory joint disease and osteoarthritis. *Annals of the Rheumatic Diseases* **58**, 709 (1999).

- 317 Katschke, K. J. *et al.* Differential expression of chemokine receptors on peripheral blood, synovial fluid, and synovial tissue monocytes/macrophages in rheumatoid arthritis. *Arthritis & Rheumatism* **44**, 1022-1032 (2001).
- 318 Roberts, C. A., Dickinson, A. K. & Taams, L. S. The interplay between monocytes/macrophages and CD4+ T cell subsets in rheumatoid arthritis. *Frontiers in immunology* **6**, 571 (2015).
- 319 Nie, H. *et al.* Phosphorylation of FOXP3 controls regulatory T cell function and is inhibited by TNF- $\alpha$  in rheumatoid arthritis. *Nature medicine* **19**, 322 (2013).
- 320 Van Der Voort, R. *et al.* Elevated CXCL16 expression by synovial macrophages recruits memory T cells into rheumatoid joints. *Arthritis & Rheumatism* **52**, 1381-1391 (2005).
- 321 van Lieshout, A. W. *et al.* Regulation of CXCL16 expression and secretion by myeloid cells is not altered in rheumatoid arthritis. *Annals of the rheumatic diseases* (2008).
- 322 Bosco, M. C. *et al.* Hypoxic synovial environment and expression of macrophage inflammatory protein 3 $\gamma$ /CCL20 in juvenile idiopathic arthritis. *Arthritis & Rheumatism: Official Journal of the American College of Rheumatology* **58**, 1833-1838 (2008).
- 323 Li, Q., Laumonnier, Y., Syrovets, T. & Simmet, T. Recruitment of CCR6-expressing Th17 cells by CCL20 secreted from plasmin-stimulated macrophages. *Acta Biochim Biophys Sin* **45**, 593-600 (2013).
- 324 Samuels, J., Ng, Y.-S., Paget, D. & Meffre, E. Impaired early B-cell tolerance in patients with rheumatoid arthritis. *Arthritis research & therapy* **7**, P78 (2005).
- 325 Zvaifler, N. J. Rheumatoid synovitis. An extravascular immune complex disease. *Arthritis & Rheumatism: Official Journal of the American College of Rheumatology* **17**, 297-305 (1974).
- 326 Jasin, H. E. Autoantibody specificities of immune complexes sequestered in articular cartilage of patients with rheumatoid arthritis and osteoarthritis. *Arthritis & Rheumatism: Official Journal of the American College of Rheumatology* **28**, 241-248 (1985).
- 327 Zvaifler, N. J. in *Advances in immunology* Vol. 16 265-336 (Elsevier, 1973).
- 328 Silverman, G. J. & Carson, D. A. Review Roles of B cells in rheumatoid arthritis. (2003).
- 329 Takemura, S., Klimiuk, P. A., Braun, A., Goronzy, J. J. & Weyand, C. M. T cell activation in rheumatoid synovium is B cell dependent. *The Journal of Immunology* **167**, 4710-4718 (2001).
- 330 Meednu, N. *et al.* Production of RANKL by memory B cells: a link between B cells and bone erosion in rheumatoid arthritis. *Arthritis & rheumatology* **68**, 805-816 (2016).
- 331 van Boxel, J. A. & Paget, S. A. Predominantly T-cell infiltrate in rheumatoid synovial membranes. *New England Journal of Medicine* **293**, 517-520 (1975).
- 332 Duke, O., Panayi, G., Janossy, G. & Poulter, L. An immunohistological analysis of lymphocyte subpopulations and their microenvironment in the synovial membranes of patients with rheumatoid arthritis using monoclonal antibodies. *Clinical and experimental immunology* **49**, 22 (1982).
- 333 Beacock-Sharp, H., Young, J. L. & Gaston, J. H. Analysis of T cell subsets present in the peripheral blood and synovial fluid of reactive arthritis patients. *Annals of the rheumatic diseases* **57**, 100-106 (1998).
- 334 Cohen, I. R., Holoshitz, J., van Eden, W. & Frenkel, A. T lymphocyte clones illuminate pathogenesis and affect therapy of experimental arthritis. *Arthritis &*

- Rheumatism: Official Journal of the American College of Rheumatology* **28**, 841-845 (1985).
- 335 Sakaguchi, N. *et al.* Altered thymic T-cell selection due to a mutation of the ZAP-70 gene causes autoimmune arthritis in mice. *Nature* **426**, 454 (2003).
- 336 Quayle, A. *et al.* Rheumatoid inflammatory T-cell clones express mostly Th1 but also Th2 and mixed (Th0-like) cytokine patterns. *Scandinavian journal of immunology* **38**, 75-82 (1993).
- 337 Miltenburg, A., Van Laar, J., De Kuiper, R., Daha, M. & Breedveld, F. T cells cloned from human rheumatoid synovial membrane functionally represent the Th 1 subset. *Scandinavian journal of immunology* **35**, 603-610 (1992).
- 338 Morita, Y. *et al.* Expression of interleukin-12 in synovial tissue from patients with rheumatoid arthritis. *Arthritis & Rheumatism: Official Journal of the American College of Rheumatology* **41**, 306-314 (1998).
- 339 Brand, D. D., Latham, K. A. & Rosloniec, E. F. Collagen-induced arthritis. *Nature protocols* **2**, 1269 (2007).
- 340 Malfait, A. *et al.* Blockade of IL-12 during the induction of collagen-induced arthritis (CIA) markedly attenuates the severity of the arthritis. *Clinical and experimental immunology* **111**, 377 (1998).
- 341 Leonard, J., Waldburger, K. & Goldman, S. Prevention of experimental autoimmune encephalomyelitis by antibodies against interleukin 12. *Journal of Experimental Medicine* **181**, 381-386 (1995).
- 342 Segal, B. M., Dwyer, B. K. & Shevach, E. M. An interleukin (IL)-10/IL-12 immunoregulatory circuit controls susceptibility to autoimmune disease. *Journal of Experimental Medicine* **187**, 537-546 (1998).
- 343 Vermeire, K. *et al.* Accelerated collagen-induced arthritis in IFN-gamma receptor-deficient mice. *The Journal of Immunology* **158**, 5507-5513 (1997).
- 344 Guedez, Y. B. *et al.* Genetic ablation of interferon- $\gamma$  up-regulates interleukin-1 $\beta$  expression and enables the elicitation of collagen-induced arthritis in a nonsusceptible mouse strain. *Arthritis & Rheumatism* **44**, 2413-2424 (2001).
- 345 Murphy, C. A. *et al.* Divergent pro-and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. *Journal of Experimental Medicine* **198**, 1951-1957 (2003).
- 346 Cua, D. J. *et al.* Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* **421**, 744 (2003).
- 347 Langrish, C. L. *et al.* IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *Journal of Experimental Medicine* **201**, 233-240 (2005).
- 348 Veale, D. *et al.* Reduced synovial membrane macrophage numbers, ELAM-1 expression, and lining layer hyperplasia in psoriatic arthritis as compared with rheumatoid arthritis. *Arthritis & Rheumatism* **36**, 893-900 (1993).
- 349 Kruithof, E. *et al.* Synovial histopathology of psoriatic arthritis, both oligo- and polyarticular, resembles spondyloarthropathy more than it does rheumatoid arthritis. *Arthritis research & therapy* **7**, R569 (2005).
- 350 Mease, P. J. *et al.* Efficacy and safety of abatacept, a T-cell modulator, in a randomised, double-blind, placebo-controlled, phase III study in psoriatic arthritis. *Annals of the rheumatic diseases* **76**, 1550-1558 (2017).
- 351 Olivieri, I. *et al.* in *Seminars in arthritis and rheumatism*. 36-43 (Elsevier).
- 352 Buch, M. H., Vital, E. M. & Emery, P. Abatacept in the treatment of rheumatoid arthritis. *Arthritis research & therapy* **10**, S5 (2008).
- 353 Njobvu, P. & McGILL, P. Psoriatic arthritis and human immunodeficiency virus infection in Zambia. *The Journal of rheumatology* **27**, 1699-1702 (2000).

- 354 Costello, P., Bresnihan, B., O'Farrelly, C. & FitzGerald, O. Predominance of CD8+ T lymphocytes in psoriatic arthritis. *The Journal of rheumatology* **26**, 1117-1124 (1999).
- 355 Ross, E. L., D'Cruz, D. & Morrow, W. Localized monocyte chemotactic protein-1 production correlates with T cell infiltration of synovium in patients with psoriatic arthritis. *The Journal of rheumatology* **27**, 2432-2443 (2000).
- 356 Costello, P. J. *et al.* Psoriatic arthritis joint fluids are characterized by CD8 and CD4 T cell clonal expansions that appear antigen driven. *The Journal of Immunology* **166**, 2878-2886 (2001).
- 357 Gaffen, S. L. The role of interleukin-17 in the pathogenesis of rheumatoid arthritis. *Current rheumatology reports* **11**, 365 (2009).
- 358 Lubberts, E. *et al.* IL-1-independent role of IL-17 in synovial inflammation and joint destruction during collagen-induced arthritis. *The Journal of Immunology* **167**, 1004-1013 (2001).
- 359 Corneth, O. B. *et al.* Absence of interleukin-17 receptor a signaling prevents autoimmune inflammation of the joint and leads to a Th2-like phenotype in collagen-induced arthritis. *Arthritis & Rheumatology* **66**, 340-349 (2014).
- 360 Lubberts, E. *et al.* Treatment with a neutralizing anti-murine interleukin-17 antibody after the onset of collagen-induced arthritis reduces joint inflammation, cartilage destruction, and bone erosion. *Arthritis & Rheumatism* **50**, 650-659 (2004).
- 361 Lubberts, E. *et al.* Requirement of IL-17 receptor signaling in radiation-resistant cells in the joint for full progression of destructive synovitis. *The Journal of Immunology* **175**, 3360-3368 (2005).
- 362 Sarkar, S. *et al.* Interleukin (IL)-17 A, F and AF in inflammation: a study in collagen-induced arthritis and rheumatoid arthritis. *Clinical & Experimental Immunology* **177**, 652-661 (2014).
- 363 Schmidt, E. G. W. *et al.* TH17 cell induction and effects of IL-17A and IL-17F blockade in experimental colitis. *Inflammatory bowel diseases* **19**, 1567-1576 (2013).
- 364 Chabaud, M. *et al.* Human interleukin-17: AT cell-derived proinflammatory cytokine produced by the rheumatoid synovium. *Arthritis & Rheumatism: Official Journal of the American College of Rheumatology* **42**, 963-970 (1999).
- 365 Van Baarsen, L. *et al.* Heterogeneous expression pattern of interleukin 17A (IL-17A), IL-17F and their receptors in synovium of rheumatoid arthritis, psoriatic arthritis and osteoarthritis: possible explanation for nonresponse to anti-IL-17 therapy? *Arthritis research & therapy* **16** (2014).
- 366 Metawi, S. A., Abbas, D., Kamal, M. M. & Ibrahim, M. K. Serum and synovial fluid levels of interleukin-17 in correlation with disease activity in patients with RA. *Clinical rheumatology* **30**, 1201-1207 (2011).
- 367 Gullick, N. J. *et al.* Linking power Doppler ultrasound to the presence of th17 cells in the rheumatoid arthritis joint. *PLoS One* **5**, e12516 (2010).
- 368 Leipe, J. *et al.* Role of Th17 cells in human autoimmune arthritis. *Arthritis & Rheumatism* **62**, 2876-2885 (2010).
- 369 Chen, D.-Y. *et al.* Increasing levels of circulating Th17 cells and interleukin-17 in rheumatoid arthritis patients with an inadequate response to anti-TNF- $\alpha$  therapy. *Arthritis research & therapy* **13**, R126 (2011).
- 370 Jain, M. *et al.* Increased Plasma IL-17F Levels in Rheumatoid Arthritis Patients Are Responsive to Methotrexate, Anti-TNF, and T Cell Costimulatory Modulation. *Inflammation*, 1-7 (2014).

- 371 Schofield, C. *et al.* Characterization of IL-17AA and IL-17FF in rheumatoid arthritis and multiple sclerosis. *Bioanalysis* **8**, 2317-2327 (2016).
- 372 Jandus, C. *et al.* Increased numbers of circulating polyfunctional Th17 memory cells in patients with seronegative spondylarthritides. *Arthritis & Rheumatism* **58**, 2307-2317 (2008).
- 373 Raychaudhuri, S. P., Raychaudhuri, S. K. & Genovese, M. C. IL-17 receptor and its functional significance in psoriatic arthritis. *Molecular and cellular biochemistry* **359**, 419-429 (2012).
- 374 Menon, B. *et al.* Interleukin-17+ CD8+ T Cells Are Enriched in the Joints of Patients With Psoriatic Arthritis and Correlate With Disease Activity and Joint Damage Progression. *Arthritis & Rheumatology* **66**, 1272-1281 (2014).
- 375 Guggino, G. *et al.* Interleukin (IL)-9/IL-9R axis drives  $\gamma\delta$  T cells activation in psoriatic arthritis patients. *Clinical & Experimental Immunology* **186**, 277-283 (2016).
- 376 Soare, A. *et al.* Cutting edge: homeostasis of innate lymphoid cells is imbalanced in psoriatic arthritis. *The Journal of Immunology*, j11700596 (2018).
- 377 Leijten, E. F. *et al.* Brief report: enrichment of activated group 3 innate lymphoid cells in psoriatic arthritis synovial fluid. *Arthritis & Rheumatology* **67**, 2673-2678 (2015).
- 378 Di Cesare, A., Di Meglio, P. & Nestle, F. O. The IL-23/Th17 axis in the immunopathogenesis of psoriasis. *Journal of Investigative Dermatology* **129**, 1339-1350 (2009).
- 379 Lowes, M. A. *et al.* Psoriasis vulgaris lesions contain discrete populations of Th1 and Th17 T cells. *Journal of Investigative Dermatology* **128**, 1207-1211 (2008).
- 380 Li, J., Chen, X. e., Liu, Z., Yue, Q. & Liu, H. Expression of Th17 cytokines in skin lesions of patients with psoriasis. *Journal of Huazhong University of Science and Technology* **27**, 330-332 (2007).
- 381 Harper, E. G. *et al.* Th17 cytokines stimulate CCL20 expression in keratinocytes in vitro and in vivo: implications for psoriasis pathogenesis. *Journal of Investigative Dermatology* **129**, 2175-2183 (2009).
- 382 Kolbinger, F. *et al.*  $\beta$ -Defensin 2 is a responsive biomarker of IL-17A-driven skin pathology in patients with psoriasis. *Journal of Allergy and Clinical Immunology* **139**, 923-932. e928 (2017).
- 383 Di Meglio, P. *et al.* Targeting CD8+ T cells prevents psoriasis development. *Journal of Allergy and Clinical Immunology* **138**, 274-276. e276 (2016).
- 384 Senra, L. *et al.* Keratinocyte-derived IL-17E contributes to inflammation in psoriasis. *Journal of Investigative Dermatology* **136**, 1970-1980 (2016).
- 385 Wilsdon, T. D. & Hill, C. L. Managing the drug treatment of rheumatoid arthritis. *Australian prescriber* **40**, 51 (2017).
- 386 Hazlewood, G. S. *et al.* Methotrexate monotherapy and methotrexate combination therapy with traditional and biologic disease modifying antirheumatic drugs for rheumatoid arthritis: abridged Cochrane systematic review and network meta-analysis. *bmj* **353**, i1777 (2016).
- 387 Maini, R. Infliximab treatment of rheumatoid arthritis. *Rheumatic Disease Clinics* **30**, 329-347 (2004).
- 388 Maini, R. *et al.* Infliximab (chimeric anti-tumour necrosis factor  $\alpha$  monoclonal antibody) versus placebo in rheumatoid arthritis patients receiving concomitant methotrexate: a randomised phase III trial. *The Lancet* **354**, 1932-1939 (1999).
- 389 Antoni, C. *et al.* Infliximab improves signs and symptoms of psoriatic arthritis: results of the IMPACT 2 trial. *Annals of the rheumatic diseases* **64**, 1150-1157 (2005).
- 390 Wijbrandts, C. & Tak, P. in *Mayo Clinic Proceedings*. 1129-1143 (Elsevier).



- 391 Leonardi, C. L. *et al.* Efficacy and safety of ustekinumab, a human interleukin-12/23 monoclonal antibody, in patients with psoriasis: 76-week results from a randomised, double-blind, placebo-controlled trial (PHOENIX 1). *The Lancet* **371**, 1665-1674 (2008).
- 392 Papp, K. A. *et al.* Efficacy and safety of ustekinumab, a human interleukin-12/23 monoclonal antibody, in patients with psoriasis: 52-week results from a randomised, double-blind, placebo-controlled trial (PHOENIX 2). *The Lancet* **371**, 1675-1684 (2008).
- 393 Ritchlin, C. *et al.* Efficacy and safety of the anti-IL-12/23 p40 monoclonal antibody, ustekinumab, in patients with active psoriatic arthritis despite conventional non-biological and biological anti-tumour necrosis factor therapy: 6-month and 1-year results of the phase 3, multicentre, double-blind, placebo-controlled, randomised PSUMMIT 2 trial. *Annals of the rheumatic diseases* **73**, 990-999 (2014).
- 394 Griffiths, C. E. *et al.* Comparison of ustekinumab and etanercept for moderate-to-severe psoriasis. *New England Journal of Medicine* **362**, 118-128 (2010).
- 395 Smolen, J. S. *et al.* A randomised phase II study evaluating the efficacy and safety of subcutaneously administered ustekinumab and guselkumab in patients with active rheumatoid arthritis despite treatment with methotrexate. *Annals of the rheumatic diseases*, annrheumdis-2016-209831 (2017).
- 396 McInnes, I. B. *et al.* Secukinumab, a human anti-interleukin-17A monoclonal antibody, in patients with psoriatic arthritis (FUTURE 2): a randomised, double-blind, placebo-controlled, phase 3 trial. *The Lancet* **386**, 1137-1146 (2015).
- 397 van de Kerkhof, P. C. *et al.* Secukinumab long-term safety experience: a pooled analysis of 10 phase II and III clinical studies in patients with moderate to severe plaque psoriasis. *Journal of the American Academy of Dermatology* **75**, 83-98. e84 (2016).
- 398 Gordon, K. B. *et al.* Phase 3 trials of ixekizumab in moderate-to-severe plaque psoriasis. *New England Journal of Medicine* **375**, 345-356 (2016).
- 399 Nash, P. *et al.* Ixekizumab for the treatment of patients with active psoriatic arthritis and an inadequate response to tumour necrosis factor inhibitors: results from the 24-week randomised, double-blind, placebo-controlled period of the SPIRIT-P2 phase 3 trial. *The Lancet* **389**, 2317-2327 (2017).
- 400 Tlustochowicz, W. *et al.* Efficacy and safety of subcutaneous and intravenous loading dose regimens of secukinumab in patients with active rheumatoid arthritis: results from a randomized phase II study. *The Journal of rheumatology* **43**, 495-503 (2016).
- 401 Genovese, M. C. *et al.* A phase II randomized study of subcutaneous ixekizumab, an anti-interleukin-17 monoclonal antibody, in rheumatoid arthritis patients who were naive to biologic agents or had an inadequate response to tumor necrosis factor inhibitors. *Arthritis & rheumatology* **66**, 1693-1704 (2014).
- 402 Durez, P. Is There a Future for Interleukin 17 Blocking Agents in Rheumatoid Arthritis? *The Journal of Rheumatology* **43**, 465-467 (2016).
- 403 Mease, P. J. *et al.* Brodalumab, an anti-IL17RA monoclonal antibody, in psoriatic arthritis. *New England Journal of Medicine* **370**, 2295-2306 (2014).
- 404 Papp, K. *et al.* A prospective phase III, randomized, double-blind, placebo-controlled study of brodalumab in patients with moderate-to-severe plaque psoriasis. *British Journal of Dermatology* **175**, 273-286 (2016).
- 405 Pavelka, K. *et al.* A study to evaluate the safety, tolerability, and efficacy of brodalumab in subjects with rheumatoid arthritis and an inadequate response to methotrexate. *The Journal of rheumatology*, jrheum. 141271 (2015).

- 406 Spitzer, M. H. & Nolan, G. P. Mass cytometry: single cells, many features. *Cell* **165**,  
780-791 (2016).
- 407 Zhou, L. *et al.* IL-6 programs TH-17 cell differentiation by promoting sequential  
engagement of the IL-21 and IL-23 pathways. *Nature immunology* **8**, 967 (2007).
- 408 Lorré, K., Fransen, L. & Ceuppens, J. Interleukin-2 induces tumor necrosis factor-  
alpha production by activated human T cells via a cyclosporin-sensitive pathway.  
*European cytokine network* **3**, 321-330 (1992).
- 409 Melton, A. C. *et al.* Regulation of IL-17A production is distinct from IL-17F in a  
primary human cell co-culture model of T cell-mediated B cell activation. *PLoS one*  
**8**, e58966 (2013).
- 410 Gomez-Rodriguez, J. *et al.* Differential expression of IL-17A and IL-17F is coupled to  
TCR signaling via Itk-mediated regulation of NFATc1. *Immunity* **31**, 587 (2009).
- 411 Aschenbrenner, D. *et al.* An immunoregulatory and tissue-residency program  
modulated by c-MAF in human TH 17 cells. *Nature immunology* **19**, 1126 (2018).
- 412 Sawyer, L. *et al.* The comparative efficacy of brodalumab in patients with  
moderate-to-severe psoriasis: a systematic literature review and network meta-  
analysis. *Journal of Dermatological Treatment*, 1-12 (2018).
- 413 Carlin, C. S., Feldman, S. R., Krueger, J. G., Menter, A. & Krueger, G. G. A 50%  
reduction in the Psoriasis Area and Severity Index (PASI 50) is a clinically significant  
endpoint in the assessment of psoriasis. *Journal of the American Academy of*  
*Dermatology* **50**, 859-866 (2004).
- 414 Papp, K. A. *et al.* Dual Neutralization of Both Interleukin 17A and Interleukin 17F  
with Bimekizumab in Patients with Psoriasis: Results from BE ABLE 1, 12-Week  
Randomized, Double-Blinded, Placebo-Controlled Phase 2b Trial. *Journal of the*  
*American Academy of Dermatology* **79**, 277 (2018).
- 415 Puel, A. *et al.* Chronic mucocutaneous candidiasis in humans with inborn errors of  
interleukin-17 immunity. *Science* **332**, 65-68 (2011).
- 416 Ziolkowska, M. *et al.* High levels of IL-17 in rheumatoid arthritis patients: IL-15  
triggers in vitro IL-17 production via cyclosporin A-sensitive mechanism. *The*  
*Journal of Immunology* **164**, 2832-2838 (2000).
- 417 DeForge, L. E. *et al.* Evaluation of heterophilic antibody blocking agents in reducing  
false positive interference in immunoassays for IL-17AA, IL-17FF, and IL-17AF.  
*Journal of immunological methods* **362**, 70-81 (2010).
- 418 Soderstrom, C. *et al.* Ultra-Sensitive Measurement of IL-17A and IL-17F in Psoriasis  
Patient Serum and Skin. *The AAPS journal* **19**, 1218-1222 (2017).
- 419 Chen, V., Croft, D., Purkis, P. & Kramer, I. Co-culture of synovial fibroblasts and  
differentiated U937 cells is sufficient for high interleukin-6 but not interleukin-  
1beta or tumour necrosis factor-alpha release. *British journal of rheumatology* **37**,  
148-156 (1998).
- 420 Cheuk, S. *et al.* CD49a expression defines tissue-resident CD8+ T cells poised for  
cytotoxic function in human skin. *Immunity* **46**, 287-300 (2017).
- 421 Pandiyan, P. *et al.* The role of IL-15 in activating STAT5 and fine-tuning IL-17A  
production in CD4 T lymphocytes. *The Journal of Immunology*, 1201476 (2012).
- 422 Mardis, E. R. ChIP-seq: welcome to the new frontier. *Nature methods* **4**, 613 (2007).
- 423 Lin, W.-c., Schmidt, J. W., Creamer, B. A., Triplett, A. A. & Wagner, K.-U. Gain-of-  
function of Stat5 leads to excessive granulopoiesis and lethal extravasation of  
granulocytes to the lung. *PLoS one* **8**, e60902 (2013).
- 424 Wang, Z., Gerstein, M. & Snyder, M. RNA-Seq: a revolutionary tool for  
transcriptomics. *Nature reviews genetics* **10**, 57 (2009).

- 425 Fuhlbrigge, R. C., Kieffer, J. D., Armerding, D. & Kupper, T. S. Cutaneous lymphocyte antigen is a specialized form of PSGL-1 expressed on skin-homing T cells. *Nature* **389**, 978 (1997).
- 426 Campbell, J. J., O'Connell, D. J. & Wurbel, M.-A. Cutting edge: chemokine receptor CCR4 is necessary for antigen-driven cutaneous accumulation of CD4 T cells under physiological conditions. *The Journal of Immunology* **178**, 3358-3362 (2007).
- 427 Gorfu, G., Rivera-Nieves, J. & Ley, K. Role of  $\beta$ 7 integrins in intestinal lymphocyte homing and retention. *Current molecular medicine* **9**, 836-850 (2009).
- 428 Oppermann, M. Chemokine receptor CCR5: insights into structure, function, and regulation. *Cellular signalling* **16**, 1201-1210 (2004).
- 429 Seiderer, J. *et al.* Role of the novel Th17 cytokine IL-17F in inflammatory bowel disease (IBD): upregulated colonic IL-17F expression in active Crohn's disease and analysis of the IL17F p. His161Arg polymorphism in IBD. *Inflammatory bowel diseases* **14**, 437-445 (2007).
- 430 Nickoloff, B. J., Qin, J.-Z. & Nestle, F. O. Immunopathogenesis of psoriasis. *Clinical reviews in allergy & immunology* **33**, 45-56 (2007).
- 431 Langley, R. G. *et al.* Secukinumab in plaque psoriasis—results of two phase 3 trials. *New England Journal of Medicine* **371**, 326-338 (2014).
- 432 Lebwohl, M. *et al.* Phase 3 studies comparing brodalumab with ustekinumab in psoriasis. *New England Journal of Medicine* **373**, 1318-1328 (2015).
- 433 Al-Ramli, W. *et al.* TH17-associated cytokines (IL-17A and IL-17F) in severe asthma. *Journal of Allergy and Clinical Immunology* **123**, 1185-1187 (2009).
- 434 Bazzi, M. *et al.* 9 Interleukin 17A and F and Asthma in Saudi Arabia: Gene Polymorphisms and Protein Levels. *Journal of Investigational Allergology and Clinical Immunology* **21**, 551 (2011).
- 435 Wenzel, S. E. *et al.* Bronchoscopic evaluation of severe asthma: persistent inflammation associated with high dose glucocorticoids. *American journal of respiratory and critical care medicine* **156**, 737-743 (1997).
- 436 Hurst, S. D. *et al.* New IL-17 family members promote Th1 or Th2 responses in the lung: in vivo function of the novel cytokine IL-25. *The Journal of Immunology* **169**, 443-453 (2002).
- 437 Oda, N. *et al.* Interleukin-17F induces pulmonary neutrophilia and amplifies antigen-induced allergic response. *American Journal of Respiratory and Critical Care Medicine* **171**, 12-18 (2005).
- 438 Kawaguchi, M. *et al.* IL-17F-induced IL-11 release in bronchial epithelial cells via MSK1-CREB pathway. *American Journal of Physiology-Lung Cellular and Molecular Physiology* **296**, L804-L810 (2009).
- 439 Kawaguchi, M. *et al.* Induction of insulin-like growth factor-I by interleukin-17F in bronchial epithelial cells. *Clinical & Experimental Allergy* **40**, 1036-1043 (2010).
- 440 Bergeron, C., Tulic, M. K. & Hamid, Q. Airway remodelling in asthma: from benchside to clinical practice. *Canadian respiratory journal* **17**, e85-e93 (2010).
- 441 Gavett, S. H., Chen, X., Finkelman, F. & Wills-Karp, M. Depletion of murine CD4+ T lymphocytes prevents antigen-induced airway hyperreactivity and pulmonary eosinophilia. *American journal of respiratory cell and molecular biology* **10**, 587-593 (1994).
- 442 Renzi, P. M., Yang, J. P., Diamantstein, T. & Martin, J. G. Effects of depletion of cells bearing the interleukin-2 receptor on immunoglobulin production and allergic airway responses in the rat. *American journal of respiratory and critical care medicine* **153**, 1214-1221 (1996).

- 443 Kirsten, A. *et al.* The anti-IL-17A antibody secukinumab does not attenuate ozone-induced airway neutrophilia in healthy volunteers. *European Respiratory Journal* **41**, 239-241 (2013).
- 444 Busse, W. W. *et al.* Randomized, double-blind, placebo-controlled study of brodalumab, a human anti-IL-17 receptor monoclonal antibody, in moderate to severe asthma. *American journal of respiratory and critical care medicine* **188**, 1294-1302 (2013).
- 445 Targan, S. R. *et al.* Mo2083 A randomized, double-blind, placebo-controlled study to evaluate the safety, tolerability, and efficacy of AMG 827 in subjects with moderate to severe Crohn's disease. *Gastroenterology* **143**, e26 (2012).
- 446 Hueber, W. *et al.* Secukinumab, a human anti-IL-17A monoclonal antibody, for moderate to severe Crohn's disease: unexpected results of a randomised, double-blind placebo-controlled trial. *Gut* **61**, 1693-1700 (2012).
- 447 Azman, R. *et al.* Clinical associations between IL-17 family cytokines and periodontitis and potential differential roles for IL-17A and IL-17E in periodontal immunity. *Inflammation Research* **63**, 1001-1012 (2014).
- 448 Luo, Z., Wang, H., Wu, Y., Sun, Z. & Wu, Y. Clinical significance of IL-23 regulating IL-17A and/or IL-17F positive Th17 cells in chronic periodontitis. *Mediators of inflammation* **2014** (2014).
- 449 Tang, F. *et al.* mRNA-Seq whole-transcriptome analysis of a single cell. *Nature methods* **6**, 377 (2009).

## 7 Appendix

### 7.1 Analysis of IL-17A/IL-17F CD4<sup>+</sup> T cells via CyTOF

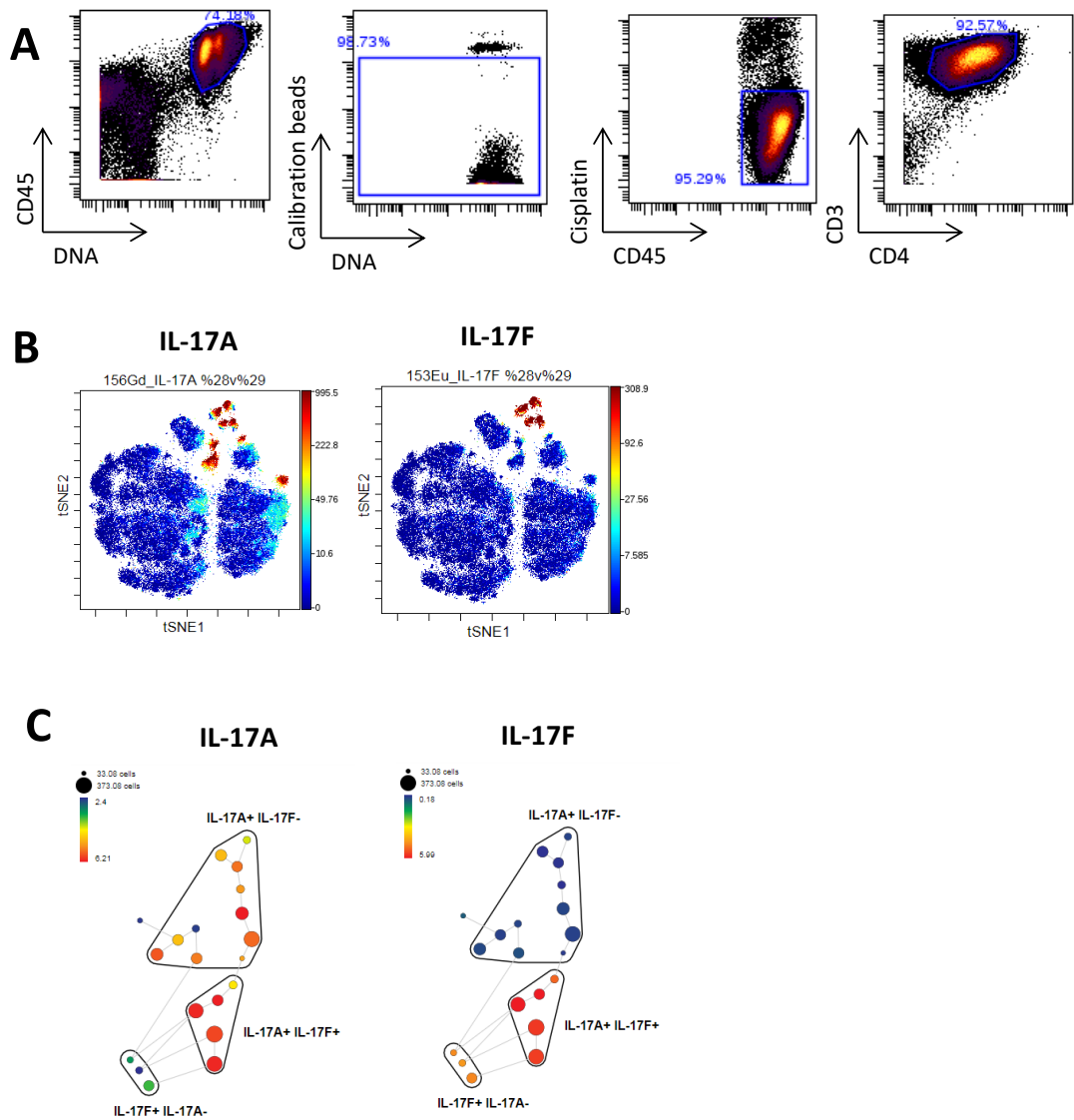
Mass cytometry (CyTOF) analysis was performed to provide a broad overview of the co-expression of cytokines in IL-17A and IL-17F CD4<sup>+</sup> T cell populations. Following a three day culture, cells were re-stimulated with PMA and ionomycin for 3 hours, stained with metal-conjugated antibodies as described in the materials and methods and then acquired on CyTOF Helios. Data were analysed using Cytobank. This experiment was performed with one individual donor.

The gating strategy applied is illustrated in Figure 7.1A. Unlike flow cytometry, cell FSC-A and FSC-W characteristics are unavailable as cells are vaporized during CyTOF acquisition. Therefore, to begin gating CD45 and DNA intercalator expressing cells were selected. Within this population, live cells were selected and CyTOF calibration beads were excluded. Dead cells were excluded based on the presence of cisplatin and CD3<sup>+</sup> CD4<sup>+</sup> T cells were selected. Next, a t-distributed stochastic neighbour embedding (t-SNE) was performed to visually (viSNE) map individual CD4<sup>+</sup> T cells based on their phenotypic relationship. To perform this, all cytokines analysed (IL-17A, IL-17F, IL-17AF, TNF $\alpha$ , IFN $\gamma$ , GM-CSF, IL-21, IL-22, IL-13, IL-4 and IL-10) were selected for viSNE mapping. Cells which have high variability of cytokines will be polarized apart, whereas cells with low cytokine variance will be closer on the viSNE map. The viSNE map generated is illustrated in Figure 7.1B and depicts the CD4<sup>+</sup> T cells which express IL-17A and IL-17F. To compare the cytokine co-expression of IL-17A and IL-17F expressing CD4<sup>+</sup> T cells, IL-17A and IL-17F CD4<sup>+</sup> T cells were selected on the viSNE map and a spanning-tree progression analysis of density-normalized events (SPADE) algorithm was applied. The SPADE analysis applied clusters of cells into populations or 'nodes' based on their cytokine expression. Colouring can then demonstrate the relative cytokine staining intensity in each cluster of cells. Based on IL-17A and IL-17F expression SPADE nodes were grouped into IL-17A<sup>+</sup> IL-17F<sup>-</sup>, IL-17A<sup>+</sup> IL-17F<sup>+</sup> or IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells (Figure 7.1C).

Next, as displayed in Figure 7.2, SPADE maps were used to compare the co-expression of cytokines in IL-17A<sup>+</sup> IL-17F<sup>-</sup>, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells. It is important to note, the SPADE map for each cytokine has an individual scale range which indicates the range of mean staining intensity. This varies from cytokine to cytokine depending on the signal of cytokine present. Broadly analysing the SPADE CyTOF results, it is clear IL-17AF is highly co-expressed by IL-17A<sup>+</sup> IL-17F<sup>+</sup> and not IL-17A<sup>+</sup> IL-17F<sup>-</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cell populations. This is in concordance with previous data demonstrated in Figure 3.8. All IL-17 populations co-expressed TNF $\alpha$ , IFN $\gamma$ , GM-CSF and IL-8. Although co-expressed by the IL-17 populations the total staining intensity of IL-21, IL-22 and IL-6 was low. The Th2 cytokine, IL-13 was undetectable and total levels of IL-4 was low. The anti-inflammatory cytokine, IL-10 appeared to be predominantly co-expressed by IL-17A<sup>+</sup> IL-17F<sup>-</sup> and IL-17A<sup>+</sup> IL-17F<sup>+</sup> CD4<sup>+</sup> T cells.

For validation purposes and to analyse the data in more depth, the dataset acquired by CyTOF was analysed by FlowJo. Here I focus on the co-expression of the cytokines TNF $\alpha$ , IFN $\gamma$ , GM-CSF and IL-10. Using the same gating strategy applied in the Cytobank analysis, the cytokine co-expression of live CD3<sup>+</sup> CD4<sup>+</sup> T cells was analysed. To assist with depicting a positive cytokine expression gate for the sample, flow cytometry plots were compared with a no PMA and ionomycin control sample (Figure 7.3A). Small ‘bleb’ like populations resting on the negative control were observed in the IL-17A and IL-10 sample FACS plots. While it may be a true cytokine population, it is also possible the population could be a result of antibodies sticking together, therefore they were excluded from the positive gate. To compare cytokine expression, TNF $\alpha$ , IFN $\gamma$ , GM-CSF and IL-10 gates were then applied to IL-17A<sup>+</sup> IL-17F<sup>-</sup>, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cell populations. All IL-17 populations highly expressed TNF $\alpha$  (>90% of IL-17 producing cells). Interestingly, higher IFN $\gamma$  co-expression levels were observed in IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells when compared with IL-17A<sup>+</sup> IL-17F<sup>-</sup> CD4<sup>+</sup> T cells. IL-17A<sup>+</sup> IL-17F<sup>+</sup> CD4<sup>+</sup> T cells displayed the highest co-expression levels of GM-CSF and IL-10. No IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells co-expressed IL-10.

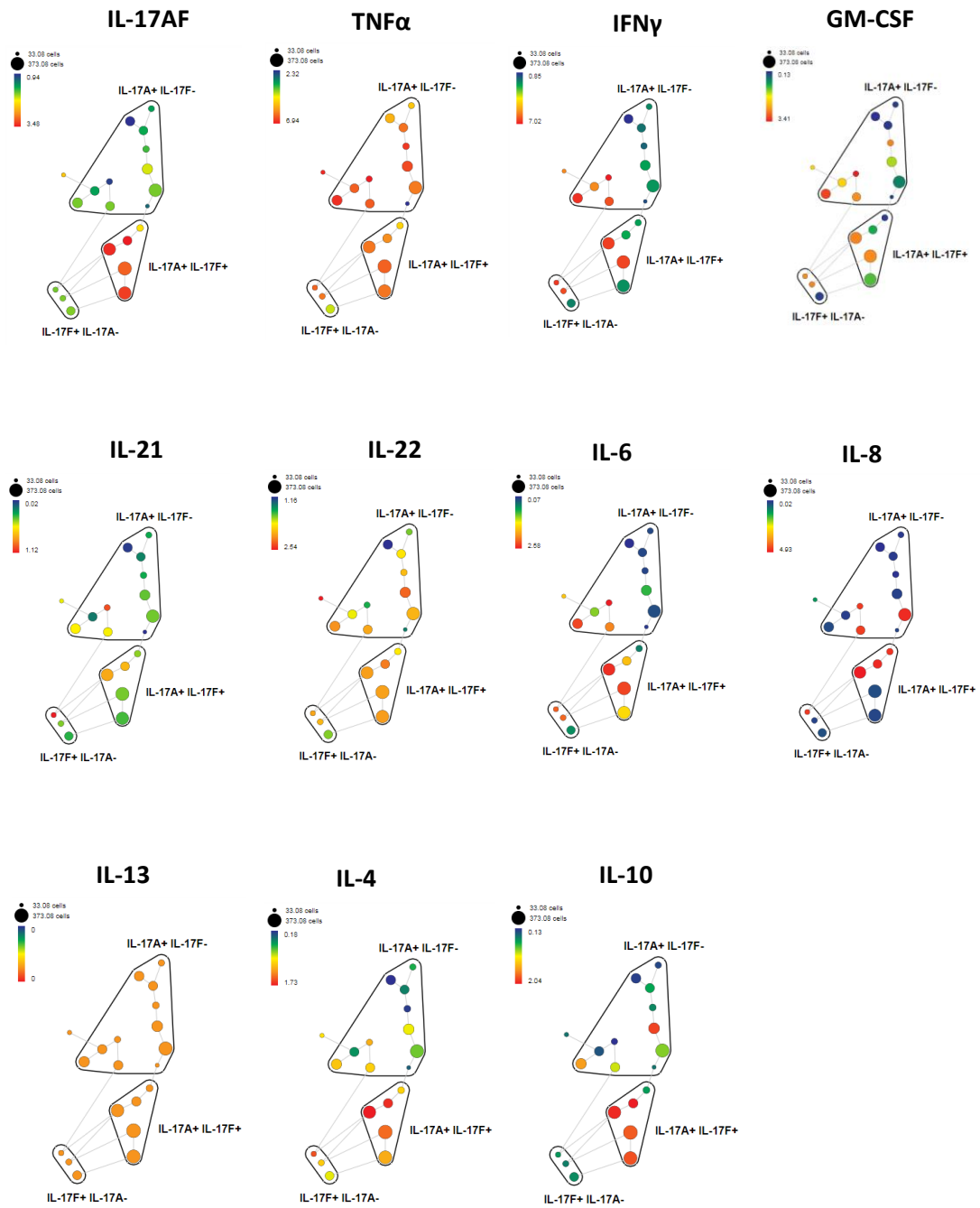
While this CyTOF data provides a good indication IL-17A<sup>+</sup> IL-17F<sup>-</sup>, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells may display a different cytokine profile, it was only an initial analysis showing the results of one individual donor. Moreover, the analysis of the raw data indicates potential ‘stickiness’ of the antibodies which leads to the reliability of the results being questioned. While CyTOF offers the advantage of investigating an extensive panel, in this case it was decided to move forward by analysing the co-expression of TNF $\alpha$ , IFN $\gamma$ , GM-CSF and IL-10 by IL-17A and IL-17F CD4<sup>+</sup> T cell populations by flow cytometry, shown in section 3.2.2.2. Differences in the CyTOF vs. flow cytometry data were observed. This is likely to be attributed to the lack of n numbers for the CyTOF data and possible issues with antibody staining. The flow cytometry data presented in section 3.2.2.2 is robust with multiple n numbers and successful antibody staining.



**Figure 7.1 CyTOF gating strategy.**

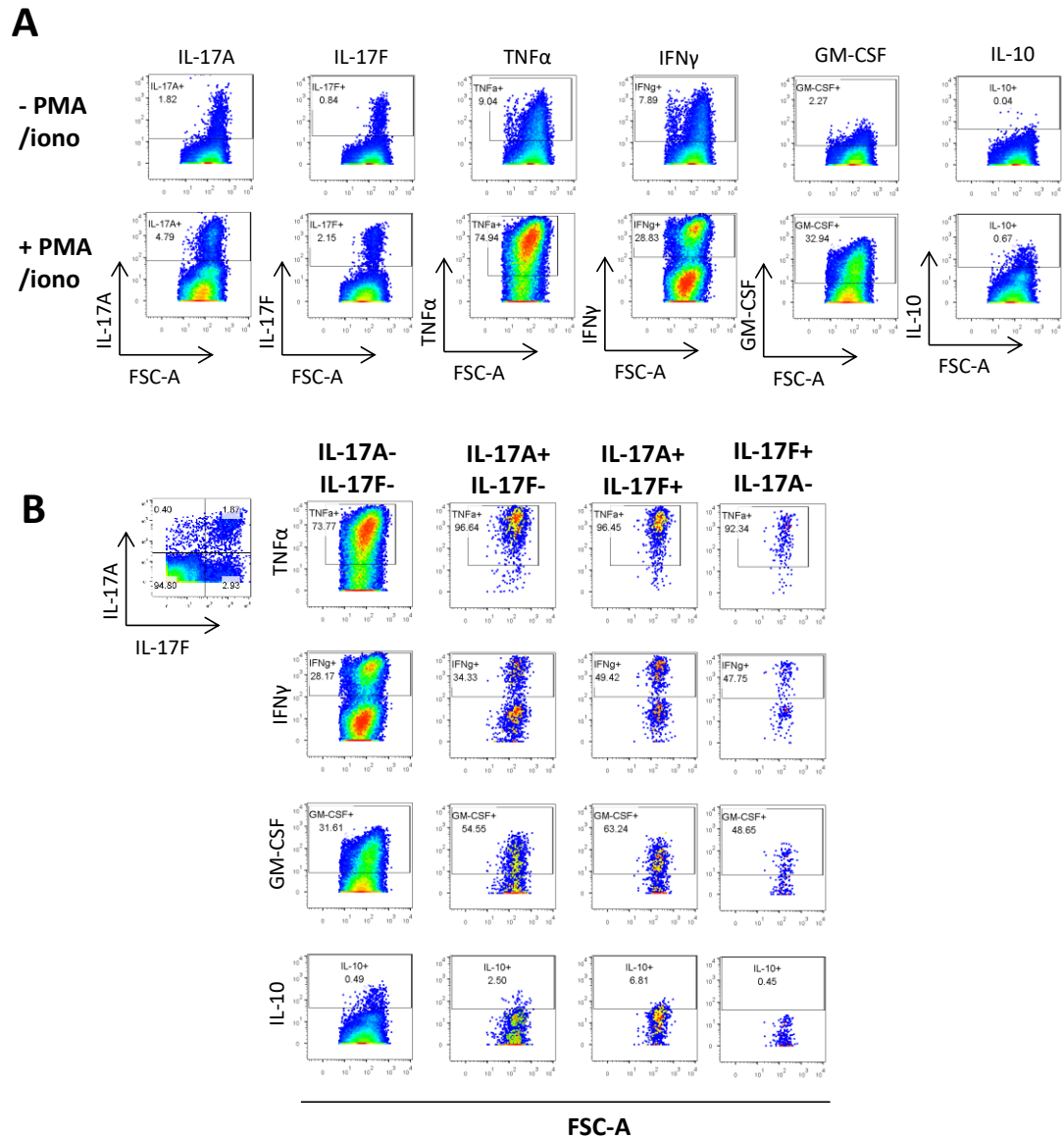
Bulk CD4<sup>+</sup> T cells were cultured with anti-CD3 mAb,  $\alpha$ CD28 mAb, IL-23 and IL-1 $\beta$ . After 3 days, cells were re-stimulated with PMA and ionomycin, stained with metal-conjugated antibodies and acquired on CyTOF Helios. Data was analysed using CytoBank. **(A)** shows the gating strategy applied. CD45<sup>+</sup> and DNA intercolator<sup>+</sup> events were selected. Within this population, live cells were selected and CyTOF calibration beads were excluded. Dead cells were excluded based on the presence of cisplatin and CD3<sup>+</sup> CD4<sup>+</sup> T cells were selected. **(B)** visNE plots of CD4<sup>+</sup> T cells clustering using intracellular cytokines, shown are heatmaps for the expression of IL-17A and IL-17F. **(C)** Gating within IL-17A<sup>+</sup> and IL-17F<sup>+</sup> CD4<sup>+</sup> T cell events on the visNE plot, a spanning-tree progression analysis of density-normalized events (SPADE) algorithm was applied. Nodes are coloured by IL-17A or IL-17F median intensity and size is based on cell number. Grouped together are IL-17A<sup>+</sup> IL-17F<sup>-</sup>, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells. Results are from n=1 donor.





**Figure 7.2 Comparison of IL-17A<sup>+</sup> IL-17F<sup>-</sup>, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells by SPADE analysis.**

Bulk CD4<sup>+</sup> T cells were cultured with anti-CD3 mAb,  $\alpha$ CD28 mAb, IL-23 and IL-1 $\beta$ . After 3 days, cells were re-stimulated with PMA and ionomycin, stained with metal-conjugated antibodies and acquired on CyTOF Helios. Data was analysed using CytoBank. Grouped together are IL-17A<sup>+</sup> IL-17F<sup>-</sup>, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells. Nodes are coloured by the individual cytokine median intensity and size is based on cell number. Results are from n=1 healthy donor.



**Figure 7.3 Flow Jo analysis on CyTOF acquired data.**

Bulk CD4<sup>+</sup> T cells were cultured with anti-CD3 mAb, αCD28 mAb, IL-23 and IL-1β. After 3 days, cells were re-stimulated with PMA and ionomycin, stained with metal-conjugated antibodies and acquired on CyTOF Helios. Data was analysed using Flow Jo. Gated within live CD45<sup>+</sup> CD3<sup>+</sup> CD4<sup>+</sup> T cell events (A) displays the flow cytometric plots of total IL-17A, IL-17F, TNFα, IFNγ, GM-CSF and IL-10 cells present within CD4<sup>+</sup> T cells in conditions unstimulated and stimulated with PMA and ionomycin. (B) Gating within IL-17A<sup>+</sup> IL-17F<sup>-</sup>, IL-17A<sup>+</sup> IL-17F<sup>+</sup> and IL-17F<sup>+</sup> IL-17A<sup>-</sup> CD4<sup>+</sup> T cells the co-expression of TNFα, IFNγ, GM-CSF and IL-10 was assessed. Results are from n=1 healthy donor.