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Transcriptomic analysis of peripheral blood monocytes and synovial macrophages in inflammatory arthritis

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A thesis submitted to the College of Medicine, Veterinary and Life Sciences,
University of Glasgow in fulfilment of the requirements for the degree of
Doctor of Philosophy

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Abstract

Background: Rheumatoid arthritis (RA) and psoriatic arthritis (PsA) are two distinct forms of chronic auto-immunity; understanding the transcriptomic profiles of key leukocyte subsets implicated in these arthritides could improve the diagnosis and treatment of patients. Current microarray analyses of samples derived from RA and PsA patients have examined the genetic profiles of whole blood or diseased tissue which, although informative, can mask the genetic contributions of individual cell types. Monocytes and macrophages are a cellular subset known to play a major role in PsA and RA through the production of pro-inflammatory chemokines, cytokines and destructive proteinases.

Aim: To define the transcriptome in CD14⁺ cells separated from the blood and synovial fluid of PsA and RA patients, and to then compare and contrast that signature in health and disease. Thereafter to define the relevant activities of selected novel moieties described in the foregoing analysis.

Methods & Results: The transcriptomic profiles of healthy, RA and PsA CD14⁺ blood cells were remarkably similar - few genes could distinguish diseased from healthy CD14⁺ cells. Comparison of the genetic signature of the RA and PsA synovial fluid CD14⁺ cells revealed that just over 50% of the differentially expressed genes were shared between the two disease groups. Furthermore, analysing the canonical pathways in the synovial fluid cells compared to the matched peripheral blood of both patient groups surprisingly revealed Liver X receptor (LXR) activation pathway as the most significantly upregulated pathway: this pathway has been previously shown by our group to play a pro-inflammatory role in arthritis.

Examination of specific upregulated mRNAs in the synovial fluid CD14⁺ cells from both disease types revealed two novel genes that had not previously been associated with arthritis, the lysosomal enzyme legumain and the cell surface molecule plexin A1. Legumain was demonstrated to be present in RA and PsA CD14⁺ cells by RNA and protein analysis and was physiologically active. Incubation of CD14⁺ cells with patient synovial fluid under hypoxic conditions also potentiated legumain expression. Plexin A1 was confirmed to be expressed at the mRNA level within RA synovium. siRNA knockdown of plexin A1 suggested

that it may play a pro-inflammatory role within macrophages since subsequent treatment of these macrophages with LPS resulted in decreased TNF α production. However, investigations into the identity of the specific ligands for plexin A1 in arthritis, known as semaphorins, were inconclusive.

I finally generated microarray data to evaluate the transcriptome of macrophages activated via cell contact with activated T cells. Such cells shared only a small percentage of genes with those dysregulated in the RA and PsA synovial fluid derived CD14⁺ cells suggesting that this model at the time points chosen may not be an appropriate *in vitro* representation of articular macrophages. An imaging system of this *in vitro* model was also established to visualise the dynamic nature of the T cell - macrophage interactions and demonstrated that variables such as duration or method of T cell activation could alter the number and duration of interactions between the two cell types.

Conclusions: These studies demonstrate that the CD14⁺ cells isolated from the blood are similar transcriptomically between healthy controls and RA and PsA patients. The synovial fluid CD14⁺ cells from RA and PsA patients exhibit substantial overlap in terms of their genetic profile. Two novel molecules expressed by diseased patients namely plexin A1 and legumain have been identified and their preliminary characteristics in the context of synovitis have been defined.

Table of contents

Abstract	2
Table of contents	4
List of Tables	8
List of Figures	9
List of Accompanying Material	11
Acknowledgements	12
Author's Declaration	14
Abbreviations	15
Chapter 1 Introduction	17
1.1 Inflammatory Arthritis: Rheumatoid Arthritis and Psoriatic Arthritis	18
1.1.1 Definition and Classification	18
1.1.2 Epidemiology	20
1.1.3 Clinical Features	21
1.1.4 Risk Factors	22
1.1.5 Histopathology	28
1.1.6 Immunopathology	30
1.1.7 Mechanisms of inflammation	36
1.1.8 Current therapies in RA and PsA	41
1.2 Monocytes and Macrophages	45
1.2.1 Monocyte and macrophage origin	45
1.2.2 Monocytes	46
1.2.3 Macrophages	51
1.2.4 Monocytes and macrophages in inflammatory arthritis	62
1.2.5 Macrophages in RA and PsA	65
1.3 Gene expression analyses in inflammatory arthritis	80
Chapter 2 Materials and Methods	93
2.1 Patients and controls	94
2.2 Cell Culture	94
2.2.1 Primary Human Cell Culture	94
2.2.2 Purification of mononuclear cells from buffy coats and peripheral blood	94
2.2.3 Purification of mononuclear cells from synovial fluid	94
2.2.4 Isolation of CD3 ⁺ T cells and CD14 ⁺ monocytes	95
2.2.5 CD3 ⁺ T cell and CD14 ⁺ monocyte stimulation	95
2.2.6 T cell / macrophage cell contact	95
2.3 Flow cytometry	96
2.3.1 Assessment of cell purity	96
2.3.2 Semaphorin 6D staining	96
2.4 Enzyme-linked-immunosorbent assay (ELISA)	98
2.5 Microarray	99
2.5.1 Collecting and preparing samples for microarray	99
2.5.2 Isolating cells using the FACS ARIA	99
2.5.3 Manual RNA Isolation from Trizol	100
2.5.4 Single strand cDNA amplification	101
2.5.5 SPIA TM amplification	103
2.5.6 Purification of amplified cDNA	104
2.5.7 Fragmenting and biotin labelling of the amplified cDNA	105
2.5.8 Array hybridization	106
2.5.9 Staining and Scanning	107

2.5.10	Scanning	109
2.6	PCR and SYBR green	109
2.6.1	cDNA preparation	110
2.6.2	PCR.....	110
2.6.3	SYBR green	110
2.7	Immunohistochemical methods	111
2.7.1	Antibodies.....	111
2.7.2	Single immunohistochemical staining.....	112
2.8	Western blotting.....	113
2.8.1	Whole cell lysates.....	113
2.8.2	BCA Assay	113
2.8.3	SDS-PAGE gel electrophoresis.....	113
2.8.4	Western blotting.....	113
2.9	Legumain activity assay.....	114
2.9.1	Collecting samples for activity assay	114
2.9.2	Setting up activity assay	114
2.10	siRNA	115
2.11	Real time cell imaging.....	116
2.11.1	Preparing macrophages for imaging.....	117
2.11.2	Staining macrophages for imaging	117
2.11.3	Staining T cells for imaging	117
2.11.4	Imaging cell contact on microscope.....	117
2.11.5	Analysis of real time images.....	118
2.12	Statistical analysis	118
Chapter 3 Transcriptomic analyses of psoriatic and rheumatoid arthritis blood and synovial fluid CD14⁺ cells		119
3.1	Introduction and Aims	120
3.2	Patients and normal donors.....	122
3.3	Collecting patient samples.....	123
3.3.1	Purity of monocyte samples	125
3.3.2	RNA quality of patient samples.....	125
3.4	Summary of samples analysed on microarray.....	128
3.5	Analysis of microarray results.....	129
3.5.1	Background correction of the Affymetrix chips	130
3.5.2	Signal histogram of normalised data	131
3.5.3	Principle component analysis	132
3.6	Comparing samples.....	135
3.6.1	Comparison 1: Patient blood compared to normal blood.....	135
3.6.2	Comparison 2: Analysis of microarray results comparing psoriatic and rheumatoid patient blood CD14 ⁺ cells to synovial fluid CD14 ⁺ cells	149
3.7	Discussion	181
3.7.1	Comparison 1: Healthy blood versus diseased blood.....	182
3.7.2	Comparison 2: RA and PsA blood versus matched synovial fluid	201
Chapter 4 Investigation into the expression and function of the novel gene plexin A1 in rheumatoid and psoriatic arthritis patients		224
4.1	Introduction.....	225
4.2	Validation of plexin A1 in microarray samples	230
4.3	Expression of plexin A1 in other patient samples	231
4.4	Immunohistochemistry of plexin A1	234
4.5	siRNA knockdown of plexin A1 in M-CSF derived macrophages	236
4.5.1	Expression of plexin A1 in macrophages after 6 days of M-CSF differentiation	236
4.5.2	Transfection efficiency of N-TER reagent.....	237

4.5.3	Testing siRNA 1-4 at 3 concentrations	240
4.5.4	Effect of plexin A1 knockdown in M-CSF derived macrophages stimulated with LPS or Tck	243
4.6	Investigation into the expression of the Plexin A1 ligand semaphorin 6D on T cells	246
4.6.1	Semaphorin 6D FACS analysis	247
4.6.2	Investigation into the level of semaphorin 6D mRNA in T cell samples	251
4.7	Investigation into the expression of other plexin A1 ligands semaphorin 3A, 3F, 6D on RA synovial membrane	254
4.7.1	qRT-PCR on RA synovial membrane	254
4.7.2	SYBR green of RA synovial fibroblasts	255
4.8	Discussion	257
Chapter 5 Investigation into the expression and function of the novel gene legumain in rheumatoid and psoriatic arthritis patients		263
5.1	Introduction	264
5.2	Validation of legumain expression in microarray samples	269
5.3	Expression of legumain in other patient samples	270
5.4	Immunohistochemistry	273
5.5	Western blot for legumain	275
5.5.1	Legumain in M-CSF derived macrophages by RT-PCR	275
5.5.2	Legumain in M-CSF macrophages by western blot	276
5.5.3	Legumain in patient CD14 ⁺ samples by western blot	277
5.6	Legumain activity assay	278
5.6.1	Legumain activity assay of M-CSF derived macrophages	279
5.6.2	Legumain activity assay of patient CD14 ⁺ samples	281
5.7	Analysing legumain expression under normoxic and hypoxic conditions	283
5.8	Discussion	285
Chapter 6 Microarray analysis of cell contact activated macrophages		291
6.1	Introduction	292
6.2	Patients and normal donors	292
6.3	Separating macrophages from the cell contact	293
6.3.1	Live cell contact	293
6.3.2	FACS ARIA sorting of macrophages	295
6.4	Analysing cell contact RNA yield and quality	298
6.4.1	Cell contact activated macrophages have variable RNA quality after 24 hours	298
6.4.2	Cell contact activated macrophage RNA quality over time	300
6.4.3	RNA yield decreases after prolonged cell contact activation	301
6.5	Collecting patient samples	303
6.6	Microarray analysis	304
6.6.1	Signal histogram of normalised data	304
6.6.2	Principle component analysis	305
6.7	Cell contact activated macrophages compared to synovial fluid activated macrophages	307
6.8	Discussion	314
Chapter 7 Validation of an <i>in vitro</i> live cell imaging system		321
7.1	Introduction	322
7.2	Real-time imaging	325
7.2.1	Imaging on the microscope	327
7.2.2	Quantitative measurements of imaging	328
7.3	Comparison of cytokine activated T cells to unstimulated T cells	331

7.3.1	Comparison of Tcks and T cells after 2 hours of co-culture with macrophages	335
7.4	Comparison of fixed and live Tcks after 2 hours co-culture with macrophages.....	340
7.5	Discussion	344
Chapter 8: General Discussion.....		350
References.....		355

List of Tables

Table 1.1 ACR classification criteria for rheumatoid arthritis.....	19
Table 1.2 Classification criteria for psoriatic arthritis (CASPAR).....	20
Table 1.3 Comparison of clinical features of RA and PsA.....	22
Table 1.4 Major cytokines with a role in inflammatory arthritis.....	38
Table 1.5 Chemokines with a role in inflammatory arthritis.....	40
Table 1.6 Characteristics of human monocyte subsets.....	49
Table 1.7 Depending on T cell stimulus, various products are induced in the monocytes/macrophages upon direct cell contact	75
Table 1.8 Genomic studies in RA and PsA	89
Table 2.1 Cytokine analysis by ELISA	99
Table 2.2 Primer sequences used for RT-PCR.....	109
Table 2.3 Antibody source and concentration used for IHC.....	111
Table 3.1 Patient characteristics	122
Table 3.2 Patient and normal donor samples collected	124
Table 3.3 Summary of microarray samples	128
Table 3.4 A list of the 12 upregulated genes in RA PB CD14 ⁺ cells compared to PsA PB CD14 ⁺ cells.....	139
Table 3.5 A list of the 12 upregulated genes in PsA blood CD14 ⁺ cells compared to RA blood CD14 ⁺ cells	142
Table 3.6 The top 5 canonical pathways associated with the RA PB vs RA SF CD14 ⁺ comparison	158
Table 3.7 The top 5 diseases and disorders associated with the RA PB vs RA SF CD14 ⁺ comparison.....	158
Table 3.8 Top 5 molecular and cellular functions associated with the RA PB vs RA SF CD14 ⁺ comparison	158
Table 3.9 Canonical pathways associated with the PsA PB vs PsA SF CD14 ⁺ comparison	162
Table 3.10 Diseases and disorders associated with the PsA PB vs PsA SF CD14 ⁺ comparison	163
Table 3.11 Molecular and cellular functions associated with the PsA PB vs PsA SF CD14 ⁺ comparison.....	163
Table 3.12 Canonical pathways associated with the differentially expressed genes in BOTH RA and PsA synovial fluid	170
Table 3.13 Molecular and cellular functions associated with the differentially expressed genes in BOTH RA and PsA synovial fluid.....	170
Table 3.14 Canonical pathways associated with the differentially expressed genes in PsA only.....	171
Table 3.15 Molecular and cellular functions associated with the differentially expressed genes in PsA only.....	172
Table 3.16 Canonical pathways associated with the differentially expressed genes in RA only	173
Table 3.17 Molecular and cellular functions associated with the differentially expressed genes in RA only	175
Table 3.18 Gene upregulated in BOTH RA and PsA synovial fluid CD14 ⁺ cells	177
Table 6.1 Patient and normal donor samples collected	304

List of Figures

Figure 1.1 Comparison of a normal and arthritic synovium	30
Figure 1.2 Inflammatory cell types within the synovium	36
Figure 1.3 Differentiation of the macrophage/DC progenitor and origin of macrophage and DC subsets	46
Figure 1.4 Innate and acquired immune activation of macrophages.....	61
Figure 1.5 Phenotypic overview of RA and PsA monocytes and macrophages....	73
Figure 3.1 Purity of CD14+ cells isolated from PsA and RA patient blood and synovial fluid samples	126
Figure 3.2 RNA quality of the CD14+ samples from two patients	127
Figure 3.3 Signal histogram of normalised data.....	132
Figure 3.4 Principle component analysis of all microarray samples.....	134
Figure 3.5 Profile plot of entities that passed the quality control filter.	136
Figure 3.6 Up and downregulated groups of genes in RA blood compared to healthy blood.....	147
Figure 3.7 Up and downregulated groups of genes in PsA PB compared to healthy PB	148
Figure 3.8 PCA analysis of PB and SF from RA and PsA patients	151
Figure 3.9 Profile plots of entities that are significantly changed between PB and SF in RA and PsA	152
Figure 3.10 Heatmap of significantly changed genes with a fold change cut off of 4 between the PB and SF of PsA and RA patients	155
Figure 3.11 LXR/RXR activation canonical pathway associated with the RA PB vs SF comparison.....	159
Figure 3.12 Pathogenesis of multiple sclerosis canonical pathway associated with the PsA PB vs PsA SF CD14+ cell comparison.....	162
Figure 3.13 Venn diagram of the differentially expressed genes between RA PB and SF and between PsA PB and SF	165
Figure 3.14 B cell development canonical pathway associated with the genes expressed in PsA synovial fluid only	172
Figure 3.15 ERK5 signalling canonical pathway associated with the genes expressed in RA synovial fluid only.....	174
Figure 4.1 Semaphorin ligands for type A plexin family members	226
Figure 4.2 Plexin A1 expression in microarray samples	231
Figure 4.3 Plexin A1 expression in CD14 ⁺ cells from arthritic patients.....	233
Figure 4.4 Plexin A1 staining in RA synovial membrane.....	235
Figure 4.5 Plexin A1 in M-CSF differentiated macrophages	237
Figure 4.6 siRNA transfection efficiency by imaging fluorescence on the microscope.....	238
Figure 4.7 FACS analysis siRNA transfected macrophages.....	239
Figure 4.8 Testing siRNA PLX 1-4 in M-CSF differentiated macrophages.....	242
Figure 4.9 Testing PLX 1 and PLX 4 siRNA in macrophages	243
Figure 4.10 RT-PCR of plexin A1 knockdown in activated macrophages	245
Figure 4.11 TNF α ELISA of siRNA treated macrophages.....	246
Figure 4.12 Surface and intracellular semaphorin 6D staining of CD3/CD28 stimulated T cells	249
Figure 4.13 Surface and intracellular semaphorin 6D staining of Tcks	250
Figure 4.14 qRT-PCR of semaphorin 6D expression in T cell samples and patient CD3 ⁺ samples	253
Figure 4.15 Semaphorin 3A, 3F, 6D expression in RA synovial membrane	255
Figure 4.16 Semaphorin 3A, 3F and 6D expression in RA synovial fibroblasts ..	256

Figure 5.1 Legumain expression in microarray samples	270
Figure 5.2 Legumain expression in CD14 ⁺ cells from arthritic patients	272
Figure 5.3 Immunohistochemistry staining of legumain in RA synovial membrane	274
Figure 5.4 Legumain expression in M-CSF treated macrophages for 6 days	275
Figure 5.5 Legumain western blot of M-CSF treated macrophages	277
Figure 5.6 Legumain western blot of patient CD14 ⁺ cells	278
Figure 5.7 Legumain activity assay in M-CSF derived macrophages	281
Figure 5.8 Legumain activity assay in CD14 ⁺ patient samples	282
Figure 5.9 Legumain expression in the presence of synovial fluid under hypoxic and normoxic conditions	284
Figure 5.10 Overview of legumain's expression and proposed role in arthritis ...	290
Figure 6.1 Separation of cells from a live cell contact	294
Figure 6.2 Separating the macrophages and Tcks from cell contact using the FACS ARIA	297
Figure 6.3 RNA integrity of macrophages and Tcks after 24 hours of cell contact	299
Figure 6.4 Macrophage RNA quality after different durations of cell contact	301
Figure 6.5 Total RNA yield from macrophages after increasing duration of cell contact activation by Tcks	302
Figure 6.6 Principle component analysis of all microarray samples	306
Figure 6.7 Venn diagram of the significantly differentially expressed genes within the cell contact activated macrophages, RA synovial fluid macrophages and PsA synovial fluid macrophages	309
Figure 6.8 Up- and downregulated biological processes in cell contact, RA SF and PsA SF macrophages	312
Figure 6.9 Up- and downregulated biological processes in cell contact and RA SF macrophages	313
Figure 7.1 Outline of in vitro real time imaging experimental setup	326
Figure 7.2 Example of the images taken by the inverted microscope for the real time imaging	327
Figure 7.3 Volocity software method for determining the macrophage-T cell interactions	330
Figure 7.4 Imaging Tcks or non-activated T cells incubated with macrophages for 24 hours	334
Figure 7.5 Imaging Tcks or non-activated T cells incubated with macrophages for 2 hours	339
Figure 7.6 Imaging fixed or live Tcks incubated with macrophages for 2 hours ..	343

List of Accompanying Material

A DVD disc containing the real time movies referenced in Chapter 7 accompanies this thesis and can be visualised using Apple QuickTime software.

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Author's Declaration

I declare that, except where explicit reference is made to the contribution of others, that this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Signature.....

Printed name.....

Abbreviations

ACPA	Anti-citrullinated protein antibodies
AEP	Asparagine endopeptidase
APC	Allophycocyanin
APC	Antigen presenting cell
APO	Apoplipoprotein
AS	Ankylosing Spondylitis
C.C	Cell contact
CCP	Cyclic citrullinated protein
CD	Cluster of differentiation
CIA	Collagen induced arthritis
CLU	Clusterin
CRP	C-reactive protein
DAVID	Database for annotation, visualisation and integrated Discovery
DC	Dendritic cell
ERK	Extracellular signal-related kinase
ESR	Erythrocyte Sedimentation Rate
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FLS	Fibroblast-like synoviocyte
FOXP1/3	Forkhead box P1/3
FPR1/2	Formyl peptide receptor 1/2
FZ	Frizzled receptor
GM-CSF	Granulocyte macrophage colony-stimulating factor
GSTM1/2	Glutathione S-transferase M1/2
HDL	High density lipoprotein
HLA	Human leukocyte antigen
ICAM	Inter-cellular adhesion molecule
IFN	Interferon
IL	Interleukin
IPA	Ingenuity pathway analysis
ITAM	Immunoreceptor tyrosine-based activation motif
JAK	Janus kinase
KLF	Krupple-like factor
LAMC1	Laminin gamma 1
LPS	Lipopolysaccharide
LXR	Liver X receptor
MAC	membrane attack complex
MAPK	Mitogen-activated protein kinase
MCP-3/4	Monocyte-specific chemokine 3/4
M-CSF	Macrophage colony-stimulating factor
MHC	Major histocompatibility complex
MICA	MHC class I chain-related gene A
MIP-1 α /B	Macrophage inflammatory protein 1 alpha/beta
MM	Mis-match
MMP	Matrix metalloproteinase
Mo	Monocyte
M Φ	Macrophage
MTC	Multiple testing correction

NK	Natural killer
NO	Nitric oxide
OA	Osteoarthritis
PAMP	Pathogen associated molecular pattern
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cells
PCA	Principle component analysis
PDGFA/B	Platelet-derived growth factor alpha/beta polypeptide
PLX	Plexin
PM	Perfect match
PPBP	Pro-platelet basic protein
PsA	Psoriatic arthritis
RA	Rheumatoid arthritis
RANKL	Receptor-activator-of-nuclear-factor- κ B ligand
RANTES	Regulated upon activation, normal T-cell expressed, and secreted
RF	Rheumatoid factor
RNI	Reactive nitrogen intermediates
ROS	Reactive oxygen species
RXR	Retinoid X Receptor
SEMA	Semaphorin
SF	Synovial fluid
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
SOCS	Suppressor of cytokine signalling
SpA	Spondylarthritis
STAT	Signal transducer and activator of transcription
TACE	TNF α -converting enzyme
T1D	Type 1 diabetes
Tck	Cytokine activated T cell
Th1	T helper 1 cell
Th2	T helper 2 cell
TIEG	TGFB early gene-1
TIMP	Tissue inhibitor of MMPs
TNF α	Tumor necrosis factor α
TLR	Toll-like receptor
TRAF	TNF receptor associated factor
Treg	T regulatory cell
TTCF	Tetanus toxin C fragment
UPR	Unfolded protein response
VASH1	Vasohibin 1
VEGF	Vascular endothelial growth factor
ZIA	Zymogen induced arthritis

Chapter 1 Introduction

1.1 Inflammatory Arthritis: Rheumatoid Arthritis and Psoriatic Arthritis

The inflammatory arthritides compose a heterogeneous group of chronic inflammatory rheumatic diseases. The progressive joint inflammation and immune cell infiltration typically seen in these diseases leads to progressive and irreversible destruction of the cartilage and bone. Arthritis affects 1-3% of the general population and is projected to affect 67 million people by 2020 in the United States alone [1]. This disease is not only associated with significant healthcare costs but also severe physical limitations and disability within the individual [2]. Rheumatoid arthritis affects 0.5-1.1% of the population [3]. Psoriatic arthritis is another common inflammatory arthritis affecting 2-3% of the general population and occurring in 7-26% of psoriasis sufferers [4].

1.1.1 Definition and Classification

Rheumatoid arthritis (RA) is a chronic symmetric polyarticular arthritis which primarily affects the small joints of the hands and feet [5]. It is characterised by inflammation of the joint lining and cellular infiltration into the joint, which subsequently causes damage and destruction of bone and cartilage. RA sufferers have a life expectancy decrease of 3 to 10 years compared to the general population [3]. The current accepted criteria for the classification of RA have been defined by the 1987 American College of Rheumatology (ACR) criteria outlined in Table 1.1 [6].

Table 1.1 ACR classification criteria for rheumatoid arthritis

A patient is diagnosed as having RA if they present with at least 4 of the 7 criteria with the first four criteria being present for at least 6 weeks:

1. Morning stiffness
 2. Soft tissue swelling in 3 or more joint areas
 3. Arthritic swelling of the hand joints
 4. Symmetrical presentation of arthritis
 5. Rheumatoid nodules
 6. Serum rheumatoid factor (RF) positive
 7. Radiographic changes including erosions or bony decalcifications.
-

Psoriatic arthritis (PsA) is a (predominantly) seronegative inflammatory disease of joints, entheses and periarticular connective tissue in association with psoriasis [7]. Rheumatoid factor and anti-cyclic citrullinated proteins (anti-CCP) antibodies are seen in 4.7% and 7.6% of cases respectively [8]. PsA constitutes a subset within the spondyloarthropathy (SpA) group due to it sharing common clinical features with the other SpA diseases. Clinical, radiologic and familial evidence has indicated PsA as a distinct disease entity however basic research within this disease has been confounded by the absence of widely agreed-upon, validated disease criteria. Several classification criteria have been proposed by Moll and Wright [9], Vasey and Espinoza [10] and the European Spondyloarthropathy Study Group (ESSG) [11]. A large international study group identified as the classification criteria for psoriatic arthritis or CASPAR study group has since been used to construct new classification criteria [8] (see Table 1.2).

Table 1.2 Classification criteria for psoriatic arthritis (CASPAR)

To meet the CASPAR criteria a patient must have inflammatory articular disease (joint, spine or enthesal) with ≥ 3 points from the following categories (1 point each):

1. Psoriasis (skin, scalp)
2. Psoriasis in patient's history
3. Psoriasis in family history
4. Psoriatic nail involvement (now)
5. Rheumatoid factor negative (determined by ELISA)
6. Dactylitis (inflammation of an entire digit) (now)
7. Dactylitis in patient's history
8. Radiological signs of new bone formation adjacent to the joints

1.1.2 Epidemiology

Numerous population based studies have been performed on the incidence and prevalence of RA and indicate large differences between various communities. Studies performed in North America and Northern Europe have found the prevalence of RA to be 0.5-1.1% with an annual incidence of 29-38 per 100,000 whereas lower prevalence of 0.3-0.7% were demonstrated in Southern Europe which had an annual incidence of 16.5 per 100,000 inhabitants [3]. In the UK the prevalence of RA is estimated to be 0.8% [12] with an incidence of 36 per 100,000 in women and 14 per 100,000 in men [13]. There have been few prevalence studies in developing countries but those that have taken place suggest a significantly lower prevalence of RA than in North America and Northern Europe, of 0.1-0.5% [14].

In comparison, fewer epidemiological studies have been performed on PsA possibly due to a previous lack of a widely accepted classification or diagnostic criteria. Prevalence is estimated at 0.1-1% [15] which increases in patients with psoriasis to 7-26% [4] but has been reported up to 30% in a Swedish study [16]. Few population based incidence studies have been undertaken on PsA however, the various incidence rates have been reported as 6 in 100,000 individuals in Finland [17], 6.59 in 100,000 in the USA [18] and 8 in 100,000 in Sweden [19]. In a study performed in Minnesota the age- and sex-adjusted incidence increased

from 3.6 (1970-1979) to 9.8 (1990-2000), reasons for this increased are unknown but may be due to better classification and diagnosis of the disease [20].

1.1.3 Clinical Features

RA and PsA often present similar overlapping features of disease, as outlined in Table 1.3, such as the presence of rheumatoid factor which occurs in 80% of RA patients but also 4.7% of PsA patients [8]. Secondly, although PsA classically presents with an asymmetric distribution, symmetry has been detected in up to 33% of patients with polyarticular disease [21]. The pattern of joint involvement is not fixed and in a 2 year follow up study of early PsA patients initially classified as polyarticular, 49% were subsequently classified as oligoarticular [22]. In addition psoriasis, which is a defining feature of PsA, develops after the rheumatological manifestations in 20% of patients or does not develop at all [23]. However, there are several differential features between the two diseases that aid in differential diagnosis: PsA shares features in common with the spondyloarthropathies such as HLA-B27 association which are not observed in RA [24-26]; also the involvement of the distal joints, spine involvement and the presence of enthesitis are all typical clinical features specific to PsA [15]. Consequently, several factors must therefore be taken into account when distinguishing PsA from RA.

Table 1.3 Comparison of clinical features of RA and PsA

Finding	RA	PsA
Morning stiffness >1 hour	+++	+
Standard mortality ratio	1.27 [27]	1.62 [28]
Rheumatoid Factor	++ (80%) [6]	+/- (4.7%) [8]
Articular Features		
Symmetrical Arthritis	+++	+/- [21]
Dactylitis	-	+++
New bone formation near joints	-	+++
Affection of distal finger/toe joints	-	+++

Extra-articular features**Rheumatoid arthritis:**

Skin (Rheumatoid nodules, Vasculitis); Cardiac (ischemic heart disease) [29];

Respiratory (interstitial lung disease) [30]; Haematological (anaemia) [31]

Psoriatic Arthritis:

Skin (psoriasis, nail lesions 87% [32]); Ocular (uveitis [33]); Back pain [21].

1.1.4 Risk Factors

The precise cause of RA or PsA is currently unknown, but it is believed to be a combination of numerous risk factors as well as dysregulated inflammatory processes.

1.1.4.1 Genetic Factors

There is overwhelming evidence that the risk of developing RA or PsA is associated with genetic factors.

1.1.4.1.1 First-degree relatives and twin studies

A well established approach to assess and quantify the possible role of genetic factors and disease risk is to measure the concordance of diseases between related individuals. A highly heritable disease will have an increased prevalence in individuals related to the arthritis sufferer compared to the general population. A parameter used to measure this is the λ_R which is the ratio of the

prevalence of a disease among first-degree relatives compared to the general population. The λ_R for RA is 2-17 [34] which is a modest value compared to a highly penetrant diseases such as cystic fibrosis which can have a λ_R value of 500 [35]. A recent study in Sweden demonstrated an increased relative risk (the probability of the disease occurring) of RA in siblings of affected individuals as 4.6 and in children as 3.0 [36]. The λ_R for PsA was determined to be 30.8 in a first-degree relative study of 100 PsA patients in Canada [37] and 47 in a similar study conducted in the UK [38]. A 2009 Icelandic study also demonstrated that first-degree relatives had a 40-fold increased risk of developing PsA whilst second-degree relatives had a 12-fold increased risk [39]. Overall these studies indicate that PsA a more heritable disease in first- and second-degree relatives compared to RA.

Twin studies are a powerful tool to understand the genetics of a disease. An increased incidence of disease amongst monozygotic (identical) twins compared to dizygotic twins offers evidence that inherited factors predispose to disease [35]. In RA two major twin studies have been undertaken using a Finnish [40] cohort and a British cohort [41] and the combined data from these studies suggests that RA is approximately 65% heritable [42]. Unlike RA there are no large twin cohorts available for PsA and the one twin study reported could not demonstrate a genetic effect on PsA as it was underpowered [43] however the results from the first-degree relative studies have demonstrated that PsA is a highly heritable disease.

1.1.4.1.2 HLA associations

The largest genetic contribution to RA susceptibility is a combination of a group of alleles within the HLA-DRB1 gene found within the major histocompatibility complex (MHC) region in chromosome 6p21. These alleles are collectively known as the shared epitope since they encompass a conserved sequence of amino acids in the third hypervariable region of the class II DR β 1 chain (⁷⁰Q/R-K/R-R-A-A⁷⁴) [44]. Many studies have demonstrated that HLA-DRB1 alleles are consistently linked and associated with RA in every population [45, 46] and have been shown to interact with environmental factors such as smoking [47]. Despite being the largest genetic contribution to RA a large UK based study found no PsA susceptibility association with the HLA-DRB1 alleles [48].

There have been far fewer genetic mapping studies performed on PsA compared to psoriasis which was first found to be associated with HLA class 1 antigens in the 1970s [49], however subsequent studies have since identified the same psoriasis risk alleles to be associated with PsA. For example, the HLA-Cw*0602 which comprises the largest genetic contribution to psoriasis is also associated with PsA patients who have psoriasis [48]. HLA-B27 is another molecule containing PsA associated variants [24-26], it is considered indicative of the association of PsA to the spondyloarthropathies and could also be a predictive marker for clinical features such as joint deformity [50]. PsA has also been associated with polymorphisms in the MHC class 1 chain-related gene A (MICA), which was not associated with psoriasis alone [51]. MICA molecules are considered markers of stress and are expressed by epithelial cells [52], they are recognised by the receptor NKG2D found on CD8 $\alpha\beta$ T cells, $\gamma\delta$ T cells and natural killer (NK) cells and therefore polymorphisms within this molecule may subsequently affect the innate immune response.

1.1.4.1.3 Other genetic associations

Despite the MHC locus contributing the single strongest genetic risk to RA, it is estimated to explain only 30% of total genetic component of susceptibility indicating that other genes may also be involved in disease predisposition. Genome wide linkage scans have subsequently identified a number of non-HLA genes associated with disease risk.

PTPN22

A variant within the PTPN22 gene is the second largest genetic risk to the development of RA after the shared epitope. This variant has been associated with RA in numerous studies [53, 54] but has been found to be very rare in Asian populations and is not associated with RA in these populations [55]. PTPN22 codes for a lymphoid-specific phosphatase (Lyp), which is an intracellular protein tyrosine phosphatase (PTP) involved in T cell receptor (TCR) signalling. Following engagement of the TCR complex, protein tyrosine kinases become activated and then phosphorylate immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic tail of the TCR-CD3 chains. Phosphatases dampen this response thereby providing a means of turning the signalling on and off. The genetic variant within the PTPN22 gene associated with RA is a nucleotide change causing an amino acid substitution. This results in a gain of

function for Lyp by increasing its enzymatic activity and causes an overall defect in T cell response to antigen stimulation. It has been proposed that this variant could predispose to RA by suppressing T cell receptor signalling during thymic development resulting in the survival of autoreactive T cells [56].

Despite the PTPN22 variant predisposing to RA and many other autoimmune diseases such as systemic lupus erythematosus (SLE) [54] and systemic sclerosis (SSc) [57] there has been no consistent evidence for an association with PsA [58, 59]. However, different variants within the same gene locus have been associated with psoriasis [60, 61].

STAT4

Signal transducer and activator of transcription 4 (STAT4) is an important transcription factor in the regulation of the immune response. STAT4 is involved in the signalling pathways of IL-12, IL-23 and type 1 interferons in T cells and monocytes leading to a T helper type 1 (Th1) and Th17 differentiation and monocyte activation [62]. A fine-mapping study first identified an RA susceptibility locus within the STAT4 gene in a US population [63] and this association has since been confirmed in other populations of European and Asian descent [64, 65]. Analysis of STAT4 deficient mice have underlined the importance of this transcription factor in RA since these mice lack Th1 responses and do not develop experimental arthritis [66]. However, further studies are required to determine the role of the susceptibility variants in RA. There has been no reported PsA susceptibility locus in STAT4 however, as has been found with PTPN22, a gene polymorphism in STAT4 is associated with psoriasis [67].

IL12B and IL23R

IL12B and IL23R are both proteins which are known to be important in T cell development. Genetic variants within these genes have been identified as psoriasis genetic susceptibility loci and subsequent studies have also identified association with PsA but not RA [68].

1.1.4.2 Gender

Despite PsA showing no gender bias, RA is far more common in women than men with the female to male ratio being 3:1 [3]. Gender differences may also play a role in the disease activity as women with RA have been found to have worse

disease activity compared to men with RA [69]. The higher incidence of RA in women had led many studies to analyse whether or not there is a hormonal influence on disease risk. Studies on hormone levels are often difficult as treatment for the disease or often the disease itself can affect hormone levels and reports on pre-disease hormone levels are not often available. One study found that serum testosterone levels were lower in males with RA compared to healthy controls which is supportive of the hypothesis that male sex hormones could protect against RA [70]. RA frequently goes into remission in women during pregnancy [71] but will flare up again during breastfeeding [72] which is thought to be due to the major hormonal fluctuations taking place. Further studies into the use of the oral contraceptive pill (OCP) in women has found that current or ever-use of OCP has a protective effect against RA development although it has not been determined whether OCP postpones rather than prevents the onset of RA [73].

1.1.4.3 Environmental and lifestyle factors

Both environmental and lifestyle factors are influences that have long been considered as potential triggers for the pathogenesis of RA and PsA.

1.1.4.3.1 *Infectious agents*

A potential involvement of infectious agents in the occurrence of RA and PsA has been speculated as it is thought that such agents could trigger the development of disease in a genetically susceptible host. The identification of bacterial DNA in the RA synovium has fuelled this hypothesis as investigations have demonstrated the presence of *Mycobacterium* [74] and mycoplasma [75] DNA. However, the bacterial species identified frequently have an equal occurrence in control populations therefore the pathogenic role for these bacteria remain to be identified.

Bacterial infections have also been suggested to have a pathogenic role in PsA as there have been reports of increased immunoreactivity in the sera of PsA patients to some streptococcal antigens which is not seen in RA patients or psoriasis patients without arthritis [76]. However, it is difficult to prove a solely pathogenic role for such antigens since secondary infections of psoriatic plaques are common. Anti-enterobacterial antibodies have also been detected in the

sera of PsA patients at a significantly higher level compared to psoriatic patients or healthy controls [77] although the significance of this is yet to be established.

Interest into the role of viral infection in the initiation and propagation of inflammation in inflammatory arthritis has been stimulated through several observations such as acute viral infections are occasionally followed by the development of an acute polyarthritis similar to early RA. Proposed viral triggers associated with RA include parovirus B19, retroviruses, and herpes viruses including Epstein-Barr virus (EBV) [78]. EBV was initially proposed as a potential factor involved in arthritis by the presence of anti-EBV antibodies in the sera of affected patients [78]. Subsequent research has since aimed to address the possible role of EBV in RA, Takeda et al found evidence of EBV infection in 47% of RA synovial samples but none of the 30 OA controls [79]. However, two following studies by Blaschke et al [80] and Niedobitek et al [81] did not find such a high proportion of EBV within the RA synovium (8% and 18% respectively). Consequently, since its expression is highly variable in RA synovium it is unlikely that EBV plays a primary role in this disease. Hepatitis C infection has been put forward as a trigger for PsA since levels of the virus are found at significantly higher levels in the sera of PsA patients compared to that of healthy controls [82] but once again the role of this virus within disease pathogenesis is unknown.

Consequently, there has been no conclusive evidence to date which has clearly defined the role of any infectious agent in RA or PsA. The persistent expression in joint tissue of any one particular pathogen has not been demonstrated but interestingly a study analysing the synovial tissue of a variety of inflammatory arthritis patients demonstrated that several samples were positive for two or more viral species at the same time [83]. Therefore, multiple different infections may be needed in order to drive arthritis pathogenesis.

1.1.4.3.2 Smoking

Cigarette smoking has been demonstrated to significantly increase the risk, severity and outcome of RA in men and women [84, 85]. Smoking is associated with a thirteen fold increase in the incidence of the disease and can induce long term susceptibility as individuals who have ceased smoking for greater than ten years remain twice as likely to develop RA as the general population [86].

Cigarette smoking supports the process of citrullination which is the post-translational modification of the amino acid arginine within a protein to the amino acid citrulline. This process is catalysed by the enzyme peptidylarginine deaminase 4 (PADI4). Citrullinated proteins such as fibronectin, fibrin, α -enolase, fibronectin and type-II collagen have been detected in the RA synovium and have been demonstrated to be targets of the autoantibody response [87, 88]. These anti-citrullinated protein antibodies (ACPAs) have been identified in over 90% of RA patients and correlate with disease activity [89]. Citrullinated proteins have been suggested to play a pathogenic role by modifying self peptide which may then drive an immune response [90]. There is a strong association between smoking and the generation of ACPAs in patients with HLA-DRB1 alleles demonstrating how genetic and environmental factors may interact to drive RA pathology [47].

1.1.5 Histopathology

The normal synovium covers the inner surface of the joints and is relatively acellular consisting of 0.5-5 mm thickness. It comprises of a thin intimal layer of predominantly fibroblasts and macrophages and a thicker sublining layer connective tissue layer. Within the joint, a protective smooth layer of cartilage acts as a shock absorber and provides a low friction surface to allow the bones to gently move over each other. This is lubricated by synovial fluid which within a non-diseased joint is relatively acellular with mean counts of 35 cells/ μ l [91]. The synovial fluid also provides nutrients to the chondrocyte cells within the joint cartilage as the cartilage lacks blood vessels.

During the progression of chronic arthritis, the synovium evolves from a protective structure to an invasive tissue that is characterised by inflammatory cell infiltration into the synovial membrane (synovitis) which drives the typical symptoms of joint pain and stiffness. In RA, the intimal lining layer thickens to over 8 cells deep and in PsA this thickening is 2-3 cells deep [92]. In addition, the synovial fluid expands abnormally acquiring between 10-1000 cell/ μ l [93] consisting of polymorphonuclear cells as well as monocytes, T cells, DCs, B cells and NK cells.

Radiographic examinations reveal a loss of joint space and the destruction of underlying cartilage and bone which is caused, in part, by the release of proteolytic enzymes from macrophages and fibroblasts. MMP-1, MMP-3 and TIMP-1 mRNA have been detected at similar levels in the synovium of both RA and PsA [94]. Moreover, the activation of bone-resorbing osteoclasts by M-CSF and RANK-L, both of which are up-regulated by IL-1 and TNF α , is a critical event for progression of bone damage in RA and PsA and levels of both osteoclast activating factors are found in both the synovium and peripheral blood of RA and PsA patients [95, 96]. In PsA, bony erosions are less common and often appear at a slower rate than in RA and consistent with these findings the levels of aggrecans, which are components of the cartilage structure and an indicator of damage, are lower in PsA synovial fluid compared to RA synovial fluid [97].

This articular destruction is accompanied by pannus formation (intrusion of the bone and cartilage by the proliferating synovial membrane) and the infiltration of inflammatory cells. The influx of these cells can promote synovial inflammation and damage through the production of chemokines, cytokines and proteases which can in turn promote a state of hypoxia and hence pronounced angiogenesis. Angiogenesis is a prominent feature of synovitis and is fundamental to disease pathology by transmitting oxygen and nutrients to the synovium in addition to being a means through which inflammatory cells are recruited to different areas of the diseased tissue. In both RA and PsA pro-angiogenic factors, such as angiopoietin-1 and -2 and VEGF, are detectable at early stages of disease [98, 99] however levels have been found to be higher in PsA than RA which may be explained by the significantly increased number of blood vessels found in PsA compared to RA [92]. See Figure 1.1 for a comparison of an arthritic and normal synovium.

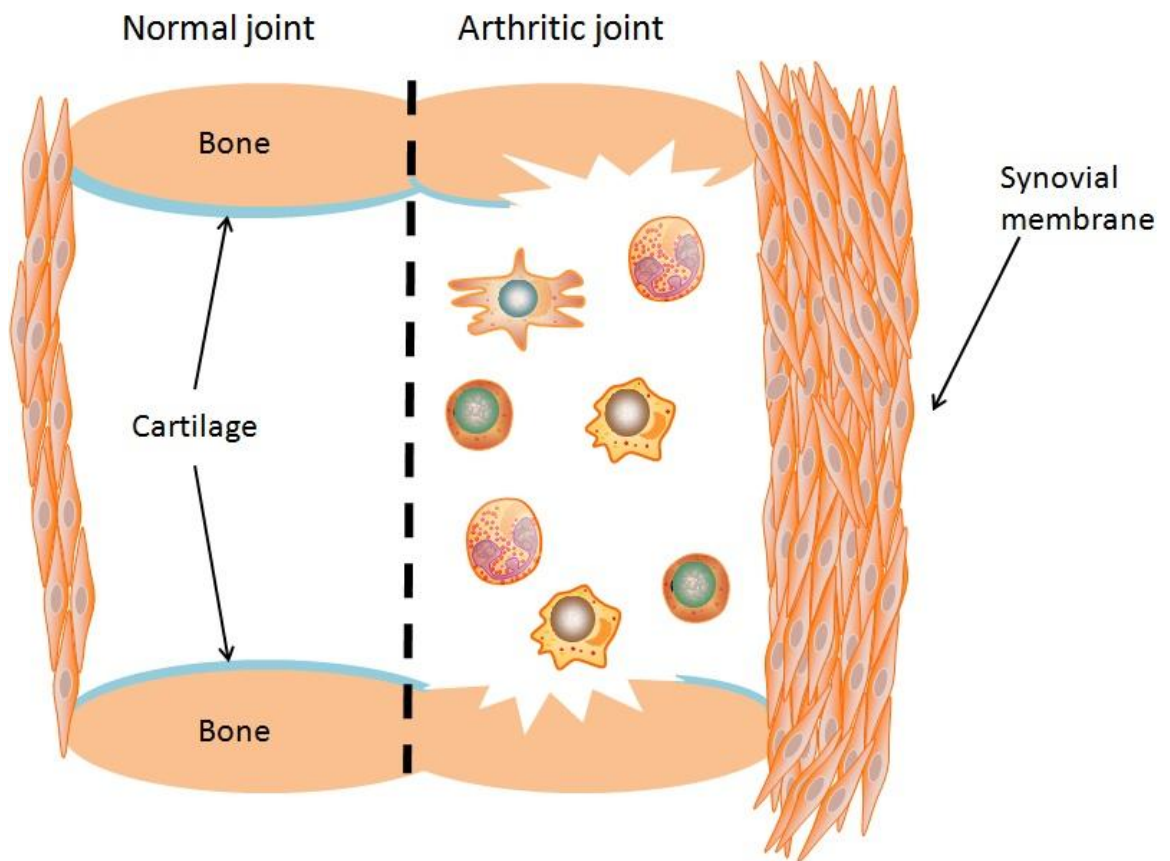


Figure 1.1 Comparison of a normal and arthritic synovium

This illustration demonstrates the presence of inflammatory cell infiltrates characterised by macrophages, B cells, T cells and DCs; synovial membrane hyperplasia and cartilage and bone destruction.

1.1.6 Immunopathology

RA and PsA are both characterised by an inflammatory cell influx into the diseased joints which drives tissue destruction and inflammation. Consequently, in order to elucidate the mechanisms which drive synovitis it is important to identify which cells are present within the synovium and how they may be contributing to the perpetual inflammatory response.

See Figure 1.2 for a summary of the interactions between the inflammatory cells found within the arthritic joint.

1.1.6.1 The monocyte/macrophage lineage

Monocytes and macrophages are found at high levels within the synovium of both RA and PsA. Circulating monocytes of the peripheral blood also demonstrate an increased level of activation compared to healthy controls. The variety of cytokines, chemokines, growth factors and enzymes that are secreted by this cell type ensure that they play a central role in the pathogenesis of arthritis. Due to this cell type being the focus of this thesis; a full and detailed discussion of monocytes and macrophages will take place in section 1.2 of this introduction.

1.1.6.2 Dendritic cells

Dendritic cells (DCs) arise from myeloid progenitor cells and are a highly phagocytic cell type. DCs are professional antigen presenting cells (APCs) that are critical to the cross talk between the innate and adaptive immune response. DCs are found in tissues in an inactive form and upon immune activation by TLR ligation or antigen uptake they downregulate their phagocytic activity and upregulate their ability to process and present antigen and are subsequently directed to lymph nodes via a CCR7 dependent pathway in order to induce T cell activation [100]. There have been two major subsets of DCs identified, myeloid DCs (mDC) and plasmacytoid DCs (pDC). mDCs have a primary function of antigen capture and presentation and are capable of producing a wide variety of cytokines such as IL-23, IL-18, IL-12p70, IL-15 and TNF α and therefore are able to polarise T helper cells depending on the cytokines they secrete. pDCs tend to be present in the circulation at a lower frequency than mDCs and upon viral infection are a major source of type I interferon synthesis. In RA and PsA the levels of circulating pDCs are significantly reduced compared to healthy controls and the levels of mDCs are significantly reduced in RA patients however both mDCs and pDCs are found to be present in the SF of both RA and PsA patients [101]. Depletion of pDCs in a murine model of arthritis has been shown to enhance the severity of articular pathology suggesting that they may mediate an anti-inflammatory function [102] whereas mDC numbers have been demonstrated in RA patients to inversely correlate with disease activity suggesting that they may contribute to RA activity [103].

1.1.6.3 T cells

T cells can be divided into two subgroups, CD4⁺ and CD8⁺. CD4⁺ effector T cells can be divided into distinct subgroups depending on their cytokine secretion profile. Th1 cells are classified according to the secretion of the cytokines IL-2 and IFN γ whilst Th2 cells are known to secrete the cytokines IL-4, IL-5 and IL-13. In addition another subset of T cells designated Th17 has recently been described due to their secretion of IL-17A in addition to IL-17F and IL-22. There is strong evidence which implicates a role for T cells within the pathogenesis of RA and PsA, first CD4⁺ T cells contribute a large proportion of infiltrating cells into the synovium and synovial fluid of RA and PsA. The number of T cells within the synovial fluid are similar in both groups of patients [92]. Secondly, the strong association of HLA genes to aggressive pathology in RA and PsA may reveal the presentation of arthritogenic peptide to autoreactive T cell clones especially since T cells can be detected co-localised to MHC II expressing APCs in RA and PsA synovium. In addition, T cells have been demonstrated to play a primary role in several murine models of arthritis including CIA [104].

RA and PsA were thought to be Th1 mediated diseases due to the predominance of Th1 cytokines such as IFN γ and IL-12 within the synovium and the detection of Th1 CD4⁺ IFN γ ⁺ cells at an elevated ratio compared to Th2 cells [105]. However, more recently, studies in animal models have favoured a mechanism which implicates Th17 cells. IL-6 is known to induce the differentiation of Th17 cells and studies have demonstrated that *IL6*^{-/-} mice are resistant to the development of CIA [106]. In addition, the inhibition or overexpression of IL-17 within the murine joints suppresses or increases joint inflammation respectively [104, 105]. At present it is not known how well these murine models represent human disease as although IL-17 is detected within RA synovial fluid [106] Th17 cells are present in low numbers within the RA synovium and may only be induced to produce IL-17 following cell contact with activated macrophages [107]. Other studies have also demonstrated that the frequency of Th17 within the synovium is low compared to the frequency in patient blood and Th1 cells are the predominant T cell subtype within the diseased joint [108]. Additionally, IL-17 is now known to be secreted by non-CD4⁺ T cells such as $\gamma\delta$ T cells, NK cells, CD8 cells, macrophages, neutrophils and most recently mast cells [109-112] therefore it is unlikely that the observed phenotype upon IL-17 deletion is solely

attributable to Th17 cells but may be a combination of different cellular sources. IL-17 can induce the secretion of numerous effector molecules, including TNF α , IL-23, IL-6, MCP-1 and RANKL from macrophages, fibroblasts and chondrocytes indicating that blockade of IL-17 may be a beneficial therapeutic target for RA and PsA [113].

CD4⁺,CD25⁺ regulatory T cells (Tregs) have become a focus of arthritis research due to their participation in controlling effector CD4⁺ cell function and their ability to regulate the autoimmune response through the production of IL-10 and TGF β [114] and via direct cell contact. Treg cells have been identified within the peripheral blood and synovial tissue of RA patients but they have been found to have a decreased ability to suppress TNF α and IFN γ from effector CD4⁺ cells or monocytes [115]. Interestingly, this ability to suppress cytokine secretion from effector CD4⁺ cells was demonstrated to be mediated through TNF α , since treatment with the anti-TNF α antibody infliximab was able to restore the Treg suppressive capacity.

In contrast to CD4⁺ T cells, CD8⁺ T cells or cytotoxic T cells mediate effector function by MHC I directed antigen specific killing of target cells and through cytokine production, in particular IFN γ and TNF α . Populations of CD8⁺ T cells have been identified in both RA and PsA synovium [116, 117]. Of interest, studies have reported a greater CD8⁺ T cell enrichment in PsA synovial fluid compared to RA synovial fluid which may be reflecting a more important role for CD8⁺ T cells in the former compared to the latter [118] however the role of CD8⁺ T cells in either disease are at present unclear.

1.1.6.4 B cells

B cells mediate adaptive immunity through the secretion of antigen-specific antibodies which promote pathogen elimination, but are also able to present antigen. Upon activation by antigen presentation, B cells will migrate to the lymph nodes or spleen where they will differentiate into antibody secreting plasma cells. The production of auto-antibodies can subsequently induce inflammatory cell activation through the binding of the antibodies to the Fc receptors expressed on leukocytes.

There is a wealth of evidence which points to a key role of B cells within arthritis pathology including the presence of germinal centres within the synovium of both RA and PsA [119, 120]. These germinal centres are in an environment surrounded by pro-inflammatory cytokines and self antigens and therefore provide an ideal location for antigen presentation, plasma cell differentiation and auto-antibody production. In addition, the use of the anti-CD20 monoclonal antibody therapy rituximab has shown clinical benefits in both RA and PsA [121, 122]. Of particular importance, it is recognised that B cells may be critical in the initiation of RA since autoantibodies against self citrullinated proteins, type II collagen and rheumatoid factor are present in the serum of patients up to 10 years before the clinical manifestation of disease [123]. A similar finding has yet to be shown in PsA.

1.1.6.5 Other inflammatory cell types

Neutrophils and Mast cells

Neutrophils are found at a high concentration within the synovial fluid and synovium of RA and PsA with an increased number being detected in the latter disease [124]. At present their primary role within disease pathogenesis is not known. However, neutrophils are activated by complement components and cytokines to release a variety of proinflammatory cytokines such as TNF α , BAFF, IL-6 and IL-18 and therefore may generally contribute to the pro-inflammatory milieu found within the synovium. Mast cells are also detected in the synovial fluid of both RA and PsA [125, 126]. Animal models of arthritis have been contradictory in determining the role that mast cells play within the disease [127, 128]. However, as mentioned above, a recent study has demonstrated that mast cells within the synovium of RA patients express the proinflammatory cytokine IL-17A [109] and therefore may be playing a pathogenic role within the disease by secreting cytokines into the synovium which will activate other inflammatory cells resulting in a positive feedback loop of inflammation.

Fibroblasts

Synovial fibroblasts together with synovial macrophages are the two leading cell types within the hyperplastic synovial tissue that invades and degrades adjacent

cartilage and bone. Fibroblasts are well established effectors of inflammation within arthritis as they are known to produce a wide variety of inflammatory mediators such as IL-1, IL-6, TNF α , MCP-1, IL-8 and M-CSF which in turn can potentiate the influx, survival and activation of several leukocyte subtypes [129]. Synovial fibroblasts are also an important source of MMPs and cathepsins which are enzymes that drive the destruction of articular cartilage and bone. In addition, these cells produce high levels of RANKL in both RA and PsA and therefore can drive articular destruction indirectly through the induction of osteoclasts and hence osteoclastogenesis [130, 131].

Osteoclasts and Chondrocytes

The articular structure in healthy individuals is maintained in stable equilibrium through maintaining a balance of both bone resorption and deposition and collagen deposition. Within the diseased synovium this balance is skewed in favour of bone resorption and collagen removal which leads to articular degradation, furthermore in PsA patients aberrant bone formation may also occur. Osteoclasts are multinucleated cells which mediate bone resorption through the secretion of acid and MMPs, and are differentiated from monocyte precursors in the presence of RANKL and M-CSF [132]. Other cytokines within the inflammatory milieu can drive osteoclast formation such as TNF α , IL-1 β and IL-17 which induce RANKL expression.

Chondrocytes are cells which, under normal conditions, maintain a stable equilibrium between the synthesis and degradation of matrix components such as collagen. However, in arthritis the chondrocyte cell participates in the destruction of its own matrix by releasing a variety of MMPs in response to the proinflammatory cytokines present within the synovium such as IL-1 and TNF α . The chondrocytes themselves can also release pro-inflammatory mediators in response to cytokine activation which can further perpetuate the chronic cartilage destruction in a paracrine and autocrine fashion [133]. Consequently, it is evident that the synovial environment within RA and PsA can promote articular destruction through several mechanisms.

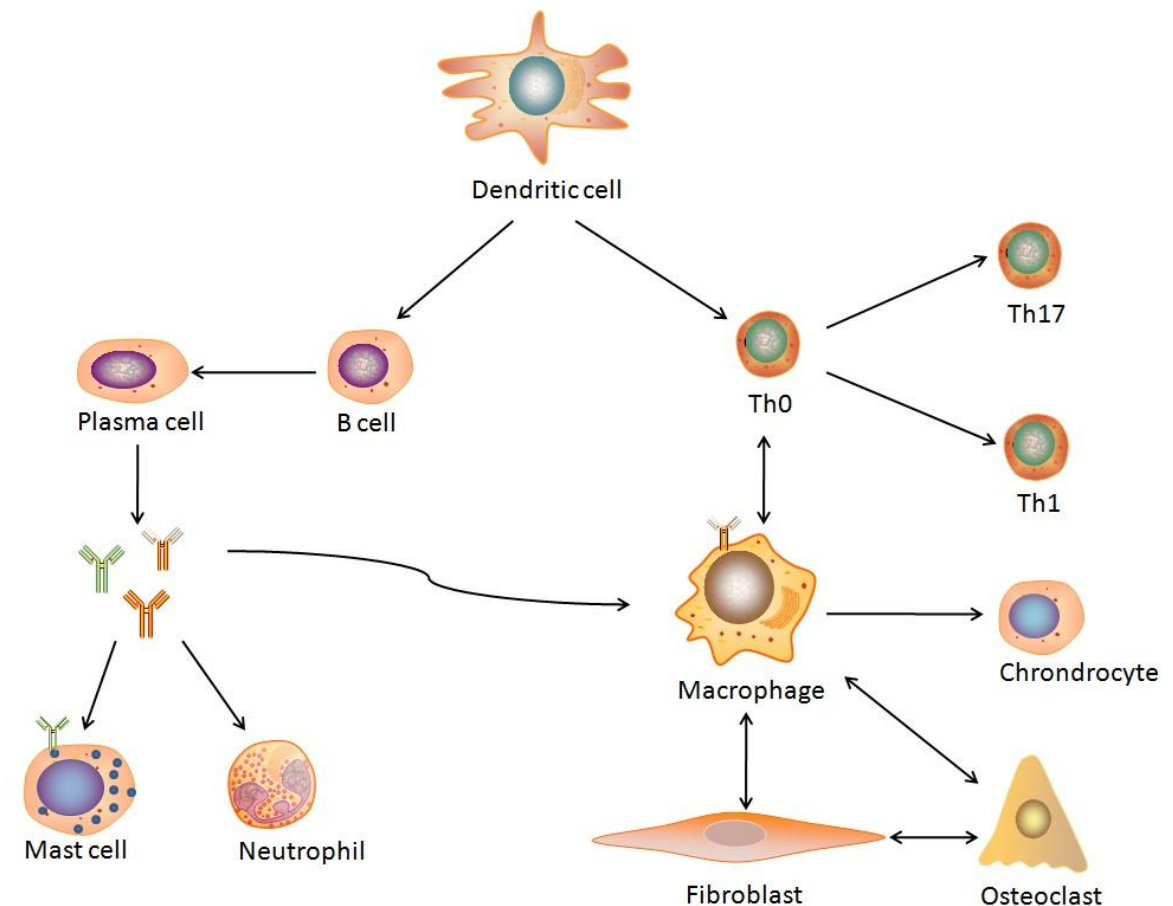


Figure 1.2 Inflammatory cell types within the synovium

An overview of the cellular communication between immune cells within the RA and PsA inflamed joint. Adapted from [132].

1.1.7 Mechanisms of inflammation

The numerous inflammatory cells infiltrating the synovial compartment release soluble factors including cytokines, chemokines and MMPs that are directly responsible for the disease pathology by activating and promoting recruitment of further inflammatory cells. A full explanation of the cytokines and chemokines expressed by monocytes and macrophages in RA and PsA are discussed fully in section 1.2.4 and 1.2.5.

1.1.7.1 Cytokines

Cytokines are small (5 to 50 kDa) proteins or glycoproteins that serve as chemical messengers between cells and thus are of significant importance in almost all aspects of innate and adaptive immunity. Cytokine levels are significantly upregulated in RA and PsA where they regulate the chronic

inflammatory response. Within the synovium cytokines can work in an autocrine fashion and due to the close proximity of cells they can also work in a paracrine (neighbouring cell activation) and juxtacrine (cell-to-cell contact) manner.

In both RA and PsA numerous pro-inflammatory cytokines including TNF α IL-6, IL-1 α / β , IL-17, IL-18, BAFF and IL-23 are elevated in the serum, synovial fluid and synovium [132] (see Table 1.4). The critical role of cytokines within the pathogenesis of both RA and PsA is exemplified by the success of specific cytokine targeted therapies such as anti-TNF α and anti-IL-6R [134, 135]. Of note, anti-inflammatory cytokines such as IL-10, IL-1Ra and soluble TNF-receptor (sTNFR) are also detectable in the inflamed synovial compartment. However, the levels of these are thought to be too low to be biologically active thus providing a disequilibrium of pro- and anti-inflammatory cytokines.

Table 1.4 Major cytokines with a role in inflammatory arthritis

Cytokine	Principle Source	Primary Biological Activity
TNF α	Monocytes, macrophages Fibroblasts, T cells, DC, Neutrophils	activates macrophages and endothelial cells; induces neutrophil apoptosis; increases ICAM-1, VCAM-1 and E selectin expression
IL-1 α /B	Monocytes, macrophages DC, B cell, fibroblasts	induces cytokine secretion from monocytes and fibroblasts; increase endothelial adhesion molecule expression; osteoclast activation
IL-1Ra	Monocytes	antagonize effects of IL-1 α and IL-1B
IL-18	Monocytes, DC, neutrophils	T cell differentiation; NK cell activation; activation of monocytes and macrophages
IL-6	Fibroblasts, monocytes, macrophages, T cells, B cells	B cell proliferation and antibody production; T cell proliferation; acute phase response; thrombopoiesis
IL-10	Monocytes, T cells, B cells, DC, epithelial cells	decrease DC activation and cytokine release; decrease synovial fibroblast cytokine and MMP release
Type I IFN	Widespread	antiviral response; macrophage activation; lymphocyte activation and survival
IL-17	T cells, macrophages, DC and $\gamma\delta$ T cells	induces proinflammatory cytokine release from macrophages; fibroblasts and endothelial cells
IL-15	T cells, monocytes, macrophages, DC	increased T cell and NK cell recruitment, B cell differentiation, synovial fibroblast and macrophage activation
IL-23	monocytes, DC, macrophages	Th17 cell proliferation
RANKL	stromal cells, osteoblasts, T cells	stimulates osteoclast mediated bone resorption

Table 1.4 continued

APRIL	monocytes, macrophages, lymphocytes	promotes T and B cell survival and proliferation
BAFF	T cells, monocytes, macrophages, DC	Promotes B cell survival and maturation
GM-CSF	Monocytes, fibroblasts, T cells, osteoclasts	Stimulates the differentiation and survival of macrophages and neutrophils
M-CSF	Lymphocytes, fibroblasts, monocytes and osteoclasts	regulator of monocyte differentiation and proliferation and of macrophage survival

1.1.7.2 Chemokines

The influx of inflammatory cells into the synovium is mediated by chemokines which are small molecular weight (8-12 kDa) chemoattractant proteins that primarily promote leukocyte recruitment and activation. Over 50 distinct chemokines have been identified and can be classified into four sub-families: 1) CXC which can promote angiogenesis, 2) CC which act primarily on monocytes and T cells, 3) C and 4) CX₃C which consists of the chemokine fractalkine (CX3CL1) [136]. A number of pro-inflammatory chemokines have been detected at elevated levels in both RA and PsA (see Table 1.5).

Table 1.5 Chemokines with a role in inflammatory arthritis

Chemokine ligand	Receptor (s)	Primary source	Physiological Feature
CC Family			
CCL2 (MCP-1)	CCR2	Fibroblasts, macrophages, Osteoclasts	Inflammation, osteoclast differentiation
CCL3 (MIP-1 α)	CCR1, CCR5	Macrophages, fibroblasts	Inflammation
CCL4 (MIP-1B)	CCR5	Monocytes, DCs	Inflammation
CCL5 (RANTES)	CCR3, CCR5, CCR1	T cells, macrophages, Fibroblasts	Inflammation
CCL19 (ECL)	CCR7	Macrophages, neutrophils	Homeostatic, Inflammatory
CCL20 (MIP-3- α)	CCR6	macrophages, Fibroblasts	Inflammation, bone resorption
CCL21 (SLC)	CCR7	endothelial cells	Inflammation, Lymphoid neogenesis
C Family			
XCL1 (lymphotactin)	XCR1	mast cells, activated CD8 ⁺ , NK cells	Inflammation, stimulates T cell Accumulation
CXC Family			
CXCL1 (gro- α)	CXC2R, CXCR1	Fibroblasts	Inflammation, synovial angiogenesis
CXCL5 (ENA-78)	CXCR2	Fibroblasts	Inflammation
CXCL8 (IL-8)	CXCR1, CXCR2	Macrophages, fibroblasts, epithelial cells	Inflammation, synovial angiogenesis
CXCL9 (Mig)	CXCR3	macrophages, Neutrophils	Inflammation, angiostatic
CXCL10 (IP-10)	CXCR3	Fibroblasts, monocytes, DCs, epithelial cells, Endothelial cells	Inflammation, agiostatic
CX₃C Family			
CXC3CL1 (fractalkine)	CXC3CR1	Monocytes, fibroblasts, Macrophages, DC Endothelial cells	Inflammation

MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; RANTES, regulated on activation normal T cell expressed and secreted; ECL, Epstein-Barr virus-induced receptor ligand; SLC, secondary lymphoid tissue chemokine; ENA, epithelial-derived neutrophil-activating peptide; MIG, monokine induced by gamma interferon; IP-10, interferon gamma-induced protein 10kDa.

1.1.8 Current therapies in RA and PsA

Advances in the knowledge of the mechanisms driving RA and PsA pathology have enabled the discovery of new therapeutics. In recent years the purpose of new therapeutics has altered from that of simply inhibiting disease progression to the ability to prevent the initial onset of disease.

1.1.8.1 DMARDS

Disease modifying anti-rheumatic drugs (DMARDs) particularly methotrexate have been widely used for the treatment of RA and PsA for decades. Despite reports of methotrexate treatment reducing the synovial sublining layer within PsA patients [137] it has been increasingly recognised that methotrexate only partially inhibits the progression of structural damage in RA [138]. In addition, a 2-year retrospective study of matched PsA patients either on or off methotrexate therapy did not show any difference in radiologic progression scores between the two groups [139]. Furthermore, up to 60% of RA patients have reported adverse reactions to methotrexate including nausea, diarrhoea, headaches, hepatitis and leukopenia which have only resolved with discontinuation of therapy [140]. As a result methotrexate does not exert therapeutic benefits in a large proportion of patients and may only delay the progression of disease. Other DMARDs in use are sulfasalazine, cyclosporine, leflunomide and hydroxychloroquine which all demonstrate some clinical benefit in a percentage of patients but as with methotrexate are often associated with toxicity and restricted duration of effectiveness.

1.1.8.2 Anti-TNF α

TNF α is a potent pro-inflammatory cytokine that is known to play a critical role in chronic inflammatory diseases such as RA and PsA [141]. Soluble TNF α is produced as a precursor form called transmembrane TNF α (tmTNF α) which is a cell surface protein found on activated macrophages and lymphocytes [142]. This tmTNF α is cleaved and processed by TNF α -converting enzyme (TACE) to release a soluble 17 kDa molecule. The combination of three of these 17 kDa molecules as a homotrimer forms soluble TNF α , which mediates its pro-inflammatory properties through type 1 and 2 TNF receptors (TNFR1 and TNFR2) [143]. Numerous studies have indicated that both soluble TNF α and its precursor form

tmTNF α are involved in the inflammatory response through binding to TNFR1 or TNFR2. Soluble TNF α can act at sites distant from the TNF α -producing cells whereas tmTNF α can exert its function on other cells through direct cell contact [144].

At present there are three anti-TNF α agents, infliximab, etanercept and adalimumab, which are approved worldwide for the treatment of various inflammatory conditions including RA, PsA, psoriasis, Crohn's disease and AS [135]. In addition, other anti-TNF α agents, certolizumab pegol and golimumab, have just been approved for clinical use. Infliximab, adalimumab and golimumab are monoclonal antibodies against human TNF α and etanercept is engineered from human TNF receptors. Adalimumab, infliximab and entanercept all bind to soluble or tmTNF α with a similar affinity [145].

Adalimumab has been demonstrated to have a high level of tissue penetration in RA patients as radioscintigraphy demonstrated that labelled adalimumab rapidly (within minutes) localised to the inflamed joints after intravenous administration [146] and tissue concentrations of the antibody in the synovial fluid range from 31% to 96% of those in the serum [147]. Greater than two thirds of RA patients respond favourably to TNF α inhibitors and administration of adalimumab to RA patients has been shown to significantly slow the radiological progression after one year [148]. PsA patients treated with adalimumab over 48 weeks show reduced radiographic progression, reduced disability and improved skin and joint manifestations [149] therefore this anti-TNF α treatment have proved to be a highly effective treatment for the management of inflammatory arthritis. The antibody's mechanisms of action within the diseased patients include binding and hence neutralising soluble TNF α .

Infliximab is a similar therapy to adalimumab as it is a monoclonal antibody against human TNF α . A phase III study of infliximab in 200 PsA patients showed significant benefit since at week 14, 58% of patients achieved an ACR20 response. The presence of dactylitis and enthesitis decreased significantly and quality of life measures also significantly improved [150]. Within RA patients infliximab has proven to be highly efficacious with ACR20 and ACR50 responses of up to 80% and 60% respectively [151]. Etanercept is a soluble receptor for TNF α and has been demonstrated to be successful in the treatment of PsA and

RA. In a phase III trial of 205 PsA patients an ACR20 response was achieved by 59% of etanercept treated patients and psoriatic skin involvement significantly decreased after 24 weeks of treatment [152]. Furthermore, the successful response of patients to treatment from any of these three anti-TNF α therapies are significantly increased when administered in combination with methotrexate with which it has synergistic benefits [151, 153, 154].

Since TNF α can act independently, additively or synergistically within the arthritic joint its neutralisation can reduce the production of other pro-inflammatory molecules such as IL-6, IL-1 β and MCP-1 from immune cells as well as reducing the expression of ICAM-1 and VCAM-1 adhesion molecules which can prevent cell trafficking into the joint and is the reason for the reduced cellularity seen in the inflamed tissue after anti-TNF α therapy [135].

1.1.8.3 Rituximab and B cell targeted therapies

Rituximab is a chimeric human/mouse monoclonal antibody directed at the CD20 antigen expressed by mature B cells and pre-B cells but not on other cells including plasma cells. It was first used as a treatment for non-Hodgkin's lymphoma but has since been approved for treatment of RA. Although several studies have suggested that CD20 may function as a calcium channel or a cell surface signalling receptor, its function and the mechanisms of action in RA remains unclear [155]. In the phase III REFLEX trial rituximab was administered to 309 RA patients whilst 209 patients received placebo, and it was found that rituximab efficacy versus placebo was evident from 8 weeks onwards and the retardation of joint damage was significantly different from placebo after 56 weeks [122]. Rituximab therapy has also been successful in the treatment of PsA [121].

Several mechanisms for the observed improvements have been suggested such as reduced antibody production; reduced secretion of B cell derived pro-inflammatory cytokines or reduced antigen presentation. Due to the success of rituximab considerable effort has been expended into developing biologics against BLyS (B-lymphocyte stimulator) also known as BAFF (B-cell-activating factor of the TNF family) and APRIL (a proliferation induced ligand) which are TNF superfamily members and promote B cell survival, differentiation and

activation. Belimumab is a fully human monoclonal antibody that specifically targets BlyS, the reports of a phase II study of this antibody in RA demonstrated a reduction in B cells and rheumatoid factor however the ACR responses were low in comparison to those seen with rituximab as only 29% of patients achieved an ACR20 response [156].

1.1.8.4 Anti IL-6 receptor

Since anti-TNF α therapy is ineffective in a proportion of patients, other therapies are required to treat such non-responder patients. Tocilizumab, a human monoclonal antibody against the IL-6 receptor, has provided encouraging results in patients who have previously failed anti-TNF α therapy [134]. In addition, as has been observed with anti-TNF α therapy, the optimal clinical responses are seen when tocilizumab is administered in combination with methotrexate [157]. At present there have been no trials investigating the efficacy of tocilizumab in psoriatic arthritis.

1.1.8.5 Abatacept

Abatacept is a recombinant human fusion protein of the extracellular domain of the cytotoxic T lymphocyte-associated antigen 4 (CTLA4) linked to the Fc portion of human IgG1. Abatacept functions to block T cell activation by binding to the CD80/86 receptor on an antigen presenting cell thus blocking the second signal activation of the CD28 receptor on the T cell. During clinical trials of RA patients abatacept treatment resulted in a high ACR response and it is now approved in Europe for treatment of RA [158]. A recent trial has also found that abatacept is an efficacious therapy for PsA resulting in significant improvements in patients compared to placebo controls [159].

1.1.8.6 Other therapies

Ustekinumab is an IL12/23 inhibitor and its administration has demonstrated significant efficacy in psoriasis since IL-12 and IL-23 are over expressed in psoriasis plaques. This inhibitor has also shown efficacy in preliminary PsA studies by reducing the signs and symptoms of arthritis [160].

1.2 Monocytes and Macrophages

1.2.1 *Monocyte and macrophage origin*

Leukocytes are a diverse group of cells types which mediate the body's immune response. They have a shared origin of hematopoietic stem cells (HSC) and develop along divergent differentiation pathways in response to internal and external cues. The mononuclear phagocyte system represents a population of leukocytes originating as bone marrow derived myeloid cells [161] and comprises of bone marrow monoblasts and promonocytes, peripheral blood monocytes, tissue macrophages and dendritic cells (DCs).

Current models propose that blood monocytes originate *in vivo* from HSC derived progenitors with myeloid restricted differentiation potential. Successive commitment steps include common myeloid progenitors (CMPs) and macrophage/DC progenitors (MDPs). Each of these differentiation steps involves cell fate decisions which subsequently restrict cell development potential [162]. MDPs are a subset of proliferating cells in the bone marrow that differentiate into both monocytes, which give rise to the macrophage subsets, and common DC precursors (CDPs), which give rise to the DC subsets [161]. See Figure 1.3 for an overview of monocyte and macrophage differentiation.

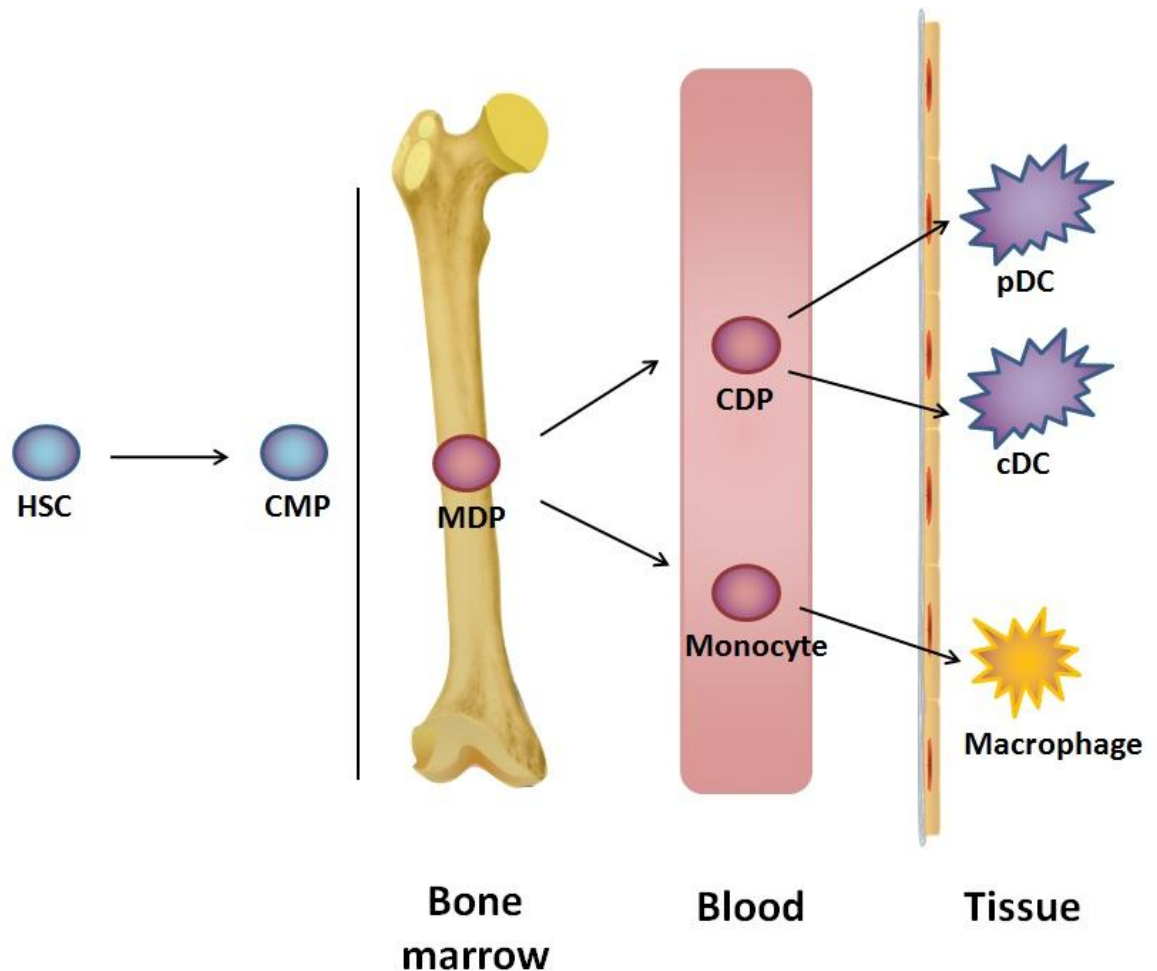


Figure 1.3 Differentiation of the macrophage/DC progenitor and origin of macrophage and DC subsets

Differentiation of monocytes and macrophages in humans. Hematopoietic stem cells (HSC) give rise to common myeloid progenitors (CMPs) which can differentiate into macrophage/DC progenitors (MDPs) within the bone marrow. The MDPs will then give rise to monocytes which will then differentiate into tissue macrophages, alternatively the MDPs will differentiate into common DC precursors (CDPs) within the blood. The CDPs will then differentiate into classical DCs (cDC) or plasmacytoid DCs (pDC).

1.2.2 Monocytes

Circulating blood monocytes supply peripheral tissues with macrophage and DC precursors and within the setting of infection contribute to immune defense against microbial pathogens. Newly formed monocytes are thought to remain in the bone marrow for less than 24 hours before entering into the peripheral blood. In humans and mice, monocytes contribute 4-10% of all circulating leukocytes [163] and share some typical morphological features such as irregular cell shape, cytoplasmic vesicles and high cytoplasm to nucleus ratio.

1.2.2.1 Monocyte subsets

In humans, circulating monocytes are not a homeogenous population and can be divided into two distinct subsets on the basis of the expression of CD14, a component of the LPS receptor complex, and CD16 the Fc γ RIII immunoglobulin receptor [164]. These subsets are designated CD14⁺CD16⁻, otherwise known as CD14⁺ monocytes, and CD14^{low}CD16⁺, otherwise called CD16⁺ cells [165]. These two monocyte subsets express distinct chemokine, immunoglobulin, adhesion and scavenger receptors and have varying levels of cytokine production, antigen presentation and transendothelial migration [166]. See Table 1.6 for the characteristics of these two monocyte subtypes.

CD14⁺ monocytes are large ~18 μ m in diameter and represent 80-90% of circulating monocytes, in contrast CD16⁺ monocytes are smaller ~14 μ m in diameter and constitute ~10% of circulating monocytes [166]. CD14⁺ cells express high levels of the chemokine CCR2 and low levels of CX3CR1; consequently, their phenotype resembles that of mouse Ly6c⁺ (Gr1⁺) monocytes [161, 165] whereas CD16⁺ cells resemble the phenotype of mouse Ly6c^{lo}(Gr1^{lo}) monocytes. TLR4 stimulation of both CD14⁺ and CD16⁺ monocytes with LPS induced similar levels of TNF α mRNA within the two cell types whilst the IL-10 mRNA was low to absent in CD16⁺ monocytes [167]. In addition, intracellular cytokine staining of CD14⁺ and CD16⁺ monocytes demonstrated a higher level of TNF protein within the CD16⁺ in response to TLR4 and TLR2 stimulation [168]. This combination of low IL-10 levels and high TNF levels meant that CD16⁺ monocytes became known as pro-inflammatory monocytes.

Despite monocytes being far less efficient than DCs at MHC class II antigen presentation to CD4⁺ T cells [169], both CD14⁺ and CD16⁺ monocytes demonstrated the ability to present tetanus toxin antigen to T cells [170]. However, CD16⁺ monocytes have demonstrated a higher level of HLA-DR expression compared to CD14⁺ monocytes [164] and have been shown to preferentially develop into DCs with high antigen presentation capacity in response to TLR stimulation [171] suggesting that this monocyte subset and their DC progeny may be superior antigen presenting cells.

A third human monocyte subset has been recently reported that is defined by the expression of CD14⁺ CD16⁺ and CD64⁺ (FcγR-I) [172]. These cells combine characteristics of macrophages and DCs as they have high expression of CD68 and HLA-DR and high T cell stimulatory activity. In comparison to CD14⁺ monocytes, CD14⁺CD16⁺CD64⁺ monocytes have a similarly high phagocytic activity and produce large amounts of cytokines such as TNFα and IL-6. But they also share characteristics with CD16⁺ monocytes such as a greater stimulatory activity in mixed leukocyte reactions (MLR) compared to CD14⁺ cells [173]. At present the origins of this subset are not known but they are speculated to be an intermediate phenotype between monocytes and DCs [172].

Table 1.6 Characteristics of human monocyte subsets

	CD14 ⁺ (CD14 ⁺ CD16 ⁻)	CD16 ⁺ (CD14 ^{low} CD16 ⁺)
Monocyte markers		
CD14	++	+/-
CD16	—	+
CD11b	+	+
Chemokine receptors		
CCR1	+	—
CCR2	+	—
CCR4	+/-	—
CX3CR1	+/-	++
CXCR4	+/-	+
Adhesion molecule		
CD62L	+	—

1.2.2.2 Monocyte diapedesis

A central component of monocyte trafficking is the continuous transition from the blood circulation into the tissue and vice versa, as this process enables the monocyte cells to differentiate and develop into functional macrophages. The endothelium within the vasculature represents the interface between these two compartments and acts as a barrier to leukocyte trafficking whilst also instructing leukocyte adhesion and transmigration. Egress of the monocytes from the bone marrow into the circulation or from the circulation into the tissue requires migration across the monolayer of endothelial cells which line the vascular circulatory system, known as diapedesis.

Extravasation of monocytes can be divided into several steps and the process begins when monocytes accumulate on the luminal side of the endothelium and undergo transient rolling interactions mediated by selectins [163], which facilitates sensing of chemokines presented on the endothelial surface and enables the monocytes to respond to them. This triggers the interaction of monocyte integrin receptors with their endothelial ligands which results in cell arrest, these molecules include junctional adhesion molecule-like (JAML) which has been demonstrated to contribute to monocyte adhesion and transmigration *in vitro* [174]. Once the cells have arrested on the endothelium they undergo actin polarisation and integrin-dependent lateral migration. This process is to allow the monocytes to search for sites permissive for endothelial barrier

penetration [175] and once a site has been found the monocytes breach and transmigrate across the endothelium. The movement across the endothelium may either be paracellular where the monocytes move in between two endothelial cells or transcellular where the monocytes pass directly through an individual endothelial cell through the formation of a transcellular pore [176].

As mentioned previously, CD14⁺ cells preferentially express the chemokine CCR2 which allows monocyte intravasation into the circulation from the bone marrow through interaction with its ligands CCL2 (otherwise known as MCP-1) and CCL7 [177]. This finding is further exemplified in CCR2^{-/-} mice which express a diminished number of Ly6C⁺ mouse monocytes in the circulation under homeostatic conditions [178] demonstrating that the exit of CD14⁺ or Ly6C⁺ monocytes from the bone marrow is mediated in part by CCR2. Inflammation is associated with an increase in CCL2 within the circulation therefore in the presence of a local inflammatory stimulus, circulating CD14⁺ monocytes extravasate into the affected non-lymphoid areas in a CCR2-CCL2 dependent manner where they differentiate into macrophage or DC subsets [179]. These and many other studies has resulted in the human CD14⁺ cells and the equivalent mouse Ly6C⁺ cells becoming known as the “inflammatory” subset.

In contrast, CD16⁺ monocytes lack CCR2 expression and undergo transendothelial migration through the expression of CX3CR1, which is the fractalkine receptor, and CCR5 which is the receptor for CCL3 (otherwise known as MIP1 α) [163]. Studies performed by Auffray et al have demonstrated that the Ly6C⁻ subset of murine monocytes, which are equivalent to the CD16⁺ human monocytes, continuously migrate over the luminal surface of dermal and mesenteric microvascular endothelium where they are thought to be patrolling for signs of infection or tissue damage [180]. This patrolling behaviour was dependent on the integrin ICAM-1 and the chemokine receptor CX3CR1 and is responsible for this cell subset becoming known as “resident” monocytes. Upon tissue damage such as exposure to irritants, aseptic wounding or peritoneal infection these patrolling resident monocytes extravasated rapidly within 1 hour and invaded the surrounding tissue where they were the main producers of the pro-inflammatory cytokines TNF α and IL-1 and were shown to differentiate into macrophages. The Ly6C⁺ cells were shown to enter the immune site at a later time and differentiated into inflammatory DCs. These results elegantly

demonstrated the functions of the two monocyte subsets within an immune response [180]. Since human CD16⁺ monocytes share a phenotype similar to the resident Ly6C⁻ mouse monocytes these cells could have a similar patrolling function within human immune system surveillance.

1.2.2.3 Monocyte function in immune defense

Monocytes were previously considered to be merely a developmental intermediate between bone marrow precursors and tissue macrophages. However, it is now clear that monocytes can carry out specific effector functions during inflammation. Monocytes have been demonstrated to be crucial for host defense against a wide variety of pathogens such as *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Toxoplasma gondii* and various fungi. CCR2^{-/-} mice are susceptible to infections caused by all of the above bacteria/fungi indicating that CCR2 mediated recruitment of Ly6C⁺ monocytes is crucial for host immune defense [166]. This result is verified by experiments performed in MCP-1^{-/-} mice which are also more susceptible to bacterial infections such as *L. monocytogenes* infection, MCP-1 is a ligand for CCR2 and is released during infection [181] and therefore plays a role in monocyte trafficking. Once at the site of infection it has been shown that monocytes produce reactive nitrogen intermediates (RNIs) and reactive oxygen intermediates (ROIs) and increase the production of phagolysosomal enzymes in order to destroy the bacteria and bring the infection to an end [182].

1.2.3 Macrophages

The term “macrophage” was first used over 100 years ago by Elie Metchnikoff to describe the large mononuclear phagocytic cells he observed in tissues [183]. Since this first observation an enormous amount of research to establish the function and importance of this cell type has taken place. It is now known that macrophages, along with DCs, form networks of phagocytic cells throughout most tissues where they play major roles in host development, inflammation, scavenging and antipathogen defences. Most macrophages in the tissue of an adult are derived from circulating monocytes which replenish these populations and there is substantial debate about whether specific monocyte populations develop into specific tissue macrophage subsets [184]. In addition, some macrophages such as those found within the lung and brain, undergo self-

renewal and proliferation and do not need circulating precursor monocytes to maintain their population [185].

1.2.3.1 Macrophage heterogeneity

Macrophages are distributed constitutively throughout the body and known to have a very high degree of heterogeneity which reflects the specialized functions adopted by these cells in different tissue localisations. Apart from the inflammatory monocyte derived macrophages which will be discussed in detail in section 1.2.3.3 there are several other macrophage types that have been identified. Examples of these cell types include: Langerhan cells which are a self-renewing subset found within the epidermis [186]; the bone re-modelling osteoclasts which require M-CSF and RANKL for development [187, 188]; microglia and choroid-plexus macrophages which reside within the CNS; and Kupffer cells which are the resident macrophages of the liver and are involved in the clearance of particulate and soluble substances.

1.2.3.2 Macrophage function

The heterogeneous nature of the macrophage, its diverse distribution and numerous cell subsets indicate that it has several physiological functions. However, there are two main function shared by most macrophage subsets; phagocytosis and antigen presentation.

1.2.3.2.1 *Phagocytosis*

Macrophages represent a major defense against invasion of the host by a wide variety of microorganisms including bacteria, viruses, fungi and protozoa. Macrophages also play a role in the clearance of apoptotic cells and the process by which they carry out these two important functions is phagocytosis. Apoptosis is a feature that is conserved through all eukaryotic cells and constitutes a series of cellular events through which the cells orchestrate their own demise. A critical feature of apoptosis is the ability of the apoptotic cell to be recognised by the innate immune system and phagocytes. One of the most studied apoptotic signals is the change in the plasma membrane phospholipid distribution on the apoptotic cell, especially the exposure of the phospholipid phosphatidylserine (PS). PS is normally found in the inner leaflet of the plasma membrane of viable cells. However during apoptosis PS is exposed on the outer membrane of the cell

and is a marking of an apoptotic cell. It has been proposed that PS exposure is a sufficient signal for macrophage-mediated phagocytosis [189]. Macrophages appear to use several receptors in the recognition and phagocytosis of apoptotic cells such as PS-R, CD36 and CD14 [190] which is thought to maintain the efficient clearance of apoptotic bodies as different receptors may engage with different phases of apoptosis. CD14-dependent clearance of apoptotic cells by macrophages has been demonstrated in both murine and human systems [190, 191] and it has been shown to interact specifically with ICAM-3, a glycosylated leukocyte protein, during the clearance of apoptotic leukocytes [192].

Macrophages also have specialised surface receptors that detect the presence of microorganisms before phagocytosis takes place. These receptors can directly interact with the microorganism through the recognition of pathogen associated molecules such as surface carbohydrates, peptidoglycans or lipoproteins. The macrophage can also recognise the pathogen through opsonins. Opsonins are host factors such as IgG and components of the complement cascade that attach to the pathogen surface. These opsonins are then recognised by receptors on the macrophage such as Fc γ receptors (Fc γ R) and complement receptor 3 (CR3) [193].

Once the macrophage has recognised the apoptotic cell or the microorganism it will engulf the cell or pathogen by advancing pseudopodia over the regions of the cell/microorganism that are linked to the cell surface receptors. This internalisation requires reorganisation of the macrophage cytoskeleton as well as delivery of membrane to accompany the increased surface area of the phagocytic cup which engulfs and surrounds the apoptotic cell/microorganism. A process called phagosome maturation then takes place inside the phagocytic macrophage by which the phagosome undergoes sequential fusion with early endosomes, late endosomes and lysosomes which are increasingly acidified membrane bound structures [194]. The fusion of these structures to the phagosome eventually leads to degradation of the phagocytosed pathogens or cells. Although most pathogens are phagocytosed and destroyed with ease by the macrophages, certain pathogens have developed mechanisms through which they avoid phagocytosis such as *M. Tuberculosis* which inhibits phagosomal maturation and *L.pneumophila* which can redirect the phagosomal maturation pathway in order to allow bacterial replication [193].

1.2.3.2.2 *Antigen processing and presentation*

Macrophages are one of several antigen presenting cells (APCs) which present antigen to T cells or to B cells within follicles of the lymph node [195]. Indeed one of the important functions of the macrophage is to assist in initiating and facilitating cell-mediated immune responses against pathogens through the processes of secreting cytokines and inflammatory mediators as well as antigen presentation. As professional phagocytes, macrophages express a variety of receptors that participate in the phagocytic uptake of antigens including those which recognise complement activation products or Ig Fc fragments. Macrophages express both MHC class I and class II molecules and therefore are able to present antigen derived from cytosolic or extracellular proteins for recognition by the appropriate CD8⁺ (MHC class II expressing) or CD4⁺ (MHC class I expressing) restricted antigen specific T cells. In addition macrophages can deliver the second co-stimulation signal required by the T cell to enhance cellular adhesion and signalling and promote maximal cross-talk between the macrophage and the T cell. These signals include CD80/86 and CD40 which interact with CD28 and CD154 respectively on the T cells [196]. The process of antigen presentation amplifies the inflammatory signal through subsequent T cell cytokine production and clonal expansion.

1.2.3.3 Macrophage activation

Macrophages are an extremely heterogenous cell type and as a result have demonstrated remarkable plasticity which allows them to respond to environmental cues and change their phenotype. These stimuli can be exogenous or endogenous and can be produced by innate immune cells, antigen-specific cells or the macrophages themselves. This extensive network of potential stimuli can give rise to diverse populations of macrophages with distinct physiology and much work has been devoted to phenotyping and understanding these macrophage populations.

One of the first populations of activated macrophages to be described were the classically activated macrophages which designates the effector macrophage population that are produced during the process of cell-mediated immune responses [197]. The first description of a classically activated macrophage was the result of research performed in the 1960s by Mackaness et al which

demonstrated that the infection of mice with *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) or *Listeria monocytogenes* enhanced the antimicrobial ability of macrophages in a stimulus dependent but antigen independent manner [198]. Since this first observation, several studies have shown that classical macrophage activation depends on the pro-inflammatory cytokines produced by T helper 1 (Th1) cells and NK cells, in particular TNF α and IFN γ , and microbial products [199]. The combination of these two signals resulted in a macrophage population which had enhanced microbicidal activity and secreted high levels of pro-inflammatory cytokines [200].

IFN γ can be produced by both innate and adaptive immune cells. NK cells are an early source of this cytokine as they produce IFN γ in response to stress and infection. This cytokine can prime macrophages to secrete pro-inflammatory cytokines and produce oxygen and nitrogen radicals to increase their killing ability [201]. However this production of IFN γ by NK cells is transient and cannot maintain a population of macrophages. Therefore an adaptive immune response is normally needed to sustain the activated macrophages and sustain host defense against intracellular pathogens. In this situation IFN γ can also be provided by Th1 cells which are antigen specific however the microbicidal macrophages that the IFN γ induces can kill and phagocytose randomly.

Other mechanisms of classical macrophage activation include TLR ligation by bacterial pathogens which, acting in a MyD88-dependent manner, induces the transcription of TNF α which will then assist IFN γ and activate the macrophage population in an autocrine manner. In addition to this mechanism of activation, TLR ligands can also activate TIR-domain-containing adaptor protein inducing IFN β (TRIF)-dependent pathways, which signal through IRN-regulatory factor 3 (IRF3) and result in IFN β production [202], this IFN β can induce classical macrophage activation in place of IFN γ indicating that TLR agonists can overcome the IFN γ dependent classical macrophage activation.

The role of classically activated macrophages in the host defense to intracellular pathogens has been well established. Studies have shown that mice lacking in IFN γ expression are more susceptible to various bacterial or viral infections and treating macrophages with IFN γ and TNF α before infection with *Leishmania* parasites results in a population of macrophages which can kill the parasite.

However the complex combination of genes and cytokines that are expressed and produced as a result of classical activation require both the IFN γ receptor ligation and either TNF α receptor ligation or TLR ligation. Since stimulation of macrophages with IFN γ alone resulted in macrophages that could not clear the *Leishmania* parasite effectively but exogenous treatment with TNF α or a TLR ligand restored the macrophage ability to clear the parasite completely [197].

In conclusion, classically activated macrophages can be produced by innate stimuli following a pathogenic infection and cell-mediated immune response. This activation usually requires two stimuli consisting of IFN γ and TNF or TLR ligation but this can be overcome by certain TLR agonists that can induce TNF and IFN β production from the macrophages which acts in an autocrine manner. The resultant macrophages have increased microbicidal activity and can produce high levels of pro-inflammatory cytokines. The pro-inflammatory cytokines produced by classically activated macrophages, such as IL-1, IL-6 and IL-23, are known to induce the development and expansion of Th17 cells [197, 203] which can contribute to numerous inflammatory autoimmune pathologies. Therefore although classically activated macrophages are a fundamental constituent of host defense, their regulation must be tightly controlled to avoid extensive host damage.

Since the identification of classically activated macrophages numerous investigations have demonstrated that macrophages can also be activated by other stimuli which results in the upregulation of cell surface markers and phenotypic changes that are distinct from those induced by IFN γ . These studies analysing different subset of activated macrophages came after reports of the macrophage mannose receptor, which is expressed on resident macrophages, being specifically downregulated by IFN γ but upregulated by the cytokine IL-4 [204, 205]. It was proposed that IL-4 induced an “alternative” form of macrophage activation compared to the classical activation and subsequently other workers introduced the term M1 and M2 for classically and alternatively activated macrophages respectively to emulate the T helper cell polarisation literature [206]. Further work has demonstrated that IL-13 can also induce alternative macrophage activation [207] by upregulating the mannose receptor which is dependent on IL4R α 1 which is the common IL-4 and IL-13 receptor alpha chain [208].

Alternatively activated macrophages are similar to classically activated macrophages as they can develop in response to innate or adaptive signals. IL-4 is thought to be one of the first cytokines released during tissue injury [209] where one of the main sources of this cytokine are basophils and mast cells. However, as has been demonstrated with classically activated macrophages, the transient secretion of activating cytokines by innate cells is not sufficient to maintain the sustained macrophage activation therefore the adaptive immune response is necessary to develop and maintain an alternatively activated macrophage population. In contrast to the classically activated macrophages which require a Th1 immune response to produce the cytokines needed for activation the alternatively activated macrophages require a Th2 immune response. Th2 immune responses are primarily induced in response to disturbance at mucosal surfaces such as the intestine or the lung but can also be induced at non-mucosal surfaces such as in response to helminth infections [210]. The main cytokines which are produced by Th2 cells are IL-4 and IL-13 which directly induce the alternative activation of macrophages, however other cytokines have also been demonstrated to indirectly induce alternative macrophage activation through Th2 cells. IL-33 is one such cytokine and binds to its receptor ST2 on Th2 cells which therefore amplifies Th2 cytokine production and hence alternative macrophage activation [211].

Alternatively activated macrophages act upon inflammatory and immune processes and their main role is to counteract pro-inflammatory and cellular immune mechanisms through regulatory and inhibitory functions. Treating macrophages *in vitro* with IL-4 and IL-13 induces macrophages that fail to present antigen to T cells, produce minimal levels of pro-inflammatory cytokines and are much less efficient at producing oxygen and nitrogen radicals and killing intracellular pathogens compared to classically activated macrophages [212]. IL-4 can also stimulate arginase activity in these macrophages which allows them to convert arginine to ornithine, since ornithine is a precursor of collagen these cells can therefore contribute to the production of extracellular matrix. Consequently, alternatively activated macrophages are often referred to as wound healing macrophages [197].

Alternatively activated macrophages also have a role in parasitic infections where they contribute to the clearance of helminths by aiding in the expulsion of

the worms in an arginase-dependent manner [213]. Recent reports have suggested that this protective effect mediated by alternatively activated macrophages may be due to these macrophages controlling the underlying Th-1 responses during the helminth infections which if left unregulated would contribute to pathogenesis of the infection [210]. Alternatively activated macrophages can also secrete the chemokine CCL22 which induces the recruitment of Th2 cells into affected tissue to aid in the removal of parasitic infection [214]. This macrophage subgroup has also been identified in the lungs of mice with experimental asthma and also in those of humans with asthma where they are thought to play a role in the recruitment of inflammatory cells into the lung through their secretion of the chemokines CCL24 and CCL17. These chemokines recruit several cell types into the lung including basophils, eosinophils, mast cells and Th2 cells which may lead to excessive type 2 inflammation and airway hyperreactivity and therefore contribute to the clinical disease pathogenesis [211, 215].

Previously, during the investigations into the phenotypes and subsets of macrophages the term “alternative activation” was used to describe all forms of non-classical activation of macrophages. This loose definition obscured the phenotypic differences observed in macrophages which were activated by cytokines or factors other than IFN γ in a non-classical manner. Consequently, three subsets of the M2 alternative activation were proposed: M2a are macrophages activated by IL-4 and IL-13; M2b are macrophages that are induced by agonists of TLRs or IL-1 receptors; and M2c macrophages are induced by IL-10 and glucocorticoid hormones [216]. However, a recent review by David Mosser and Justin Edwards proposed a different grouping of macrophage subsets and propositioned a new population of macrophages called regulatory macrophages [197]. These regulatory macrophages would appear to be most like the M2c subset as they are induced by glucocorticoids, IL-10, apoptotic cells and immune complexes and have anti-inflammatory activity. Glucocorticoids are naturally produced hormones which are released by the adrenal gland in response to stress and can inhibit macrophage mediated host defense by preventing the transcription of genes coding for pro-inflammatory cytokines such as IL-1 β and TNF α [217]. Glucocorticoids can exert both direct and indirect effects on immune responses as glucocorticoid treated APCs either fail to present antigen

to T cells or induce the development of regulatory T cells which can inhibit immune responses [218] therefore the macrophages induced by glucocorticoids are primarily anti-inflammatory.

The first report of regulatory macrophages was by Gerber and Mosser in 2001 who demonstrated that following *in vitro* stimulation with immune complexes and the TLR4 agonist LPS, the subsequent macrophage population produced high levels of the anti-inflammatory cytokine IL-10 [219]. Following studies demonstrated that the same IL-10 producing regulatory macrophages could be obtained by activating macrophages with various stimuli including IL-10 and apoptotic cells [197, 220]. Similar to the induction of classically activated macrophages, regulatory macrophages require two signals to induce their anti-inflammatory activity. The first signal of glucocorticoids, immune complexes or apoptotic cells for example will generally have little stimulatory function on the macrophage however when combined with a second signal, such as a TLR ligand, these two signals will induce the macrophages to produce IL-10 which is the most reliable characteristic of regulatory macrophages. In addition, regulatory macrophages also downregulate IL-12 production [219] therefore the ratio of IL-10 to IL-12 can be used to define regulatory macrophage populations. Due to the ability of IL-10 to downregulate the production of pro-inflammatory, regulatory macrophages are effective inhibitors of inflammation although they do maintain their ability to produce pro-inflammatory cytokines [197]. Regulatory macrophages are unable to contribute to the extracellular matrix which is seen in alternatively activated macrophages however they do express high levels of the co-stimulatory molecules CD80 and CD86 and as a result can still present antigens to T cells [212].

In summary, the regulatory macrophages represent a newly defined subset of activated macrophages that have a predominantly anti-inflammatory function through the production of IL-10. However several studies have demonstrated that incorrect regulation of these macrophage can have deleterious effects on the host and regulatory macrophages have been found to play a role in the neoplastic growth of tumours [197]. Tumour associated macrophages have been found to have a regulatory phenotype and produce high levels of IL-10 and can also contribute to angiogenesis and tumour growth [221]. However, the function

and exact role that these regulatory macrophages play in tumour progression is still to be elucidated.

To conclude, the plasticity of macrophages has made classification of activated populations very difficult and there is controversy regarding the phenotype of these populations as research groups denote different identifications of the macrophage subsets. As demonstrated above the classification of macrophages is more complex than an M1 or M2 macrophage because unlike T cells, which undergo major epigenetic modifications during differentiation, macrophages will maintain their plasticity and continue to respond to environmental cues [222]. Therefore rather than being strict phenotypic characteristics the three subsets of macrophages discussed here represent a spectrum of activation and these macrophages will have the ability to change from one subset to another and may even display attributes of two subsets depending on the host environment. See Figure 1.4 for a cartoon representation of the three macrophage populations which arise as a result of different mechanisms of activation.

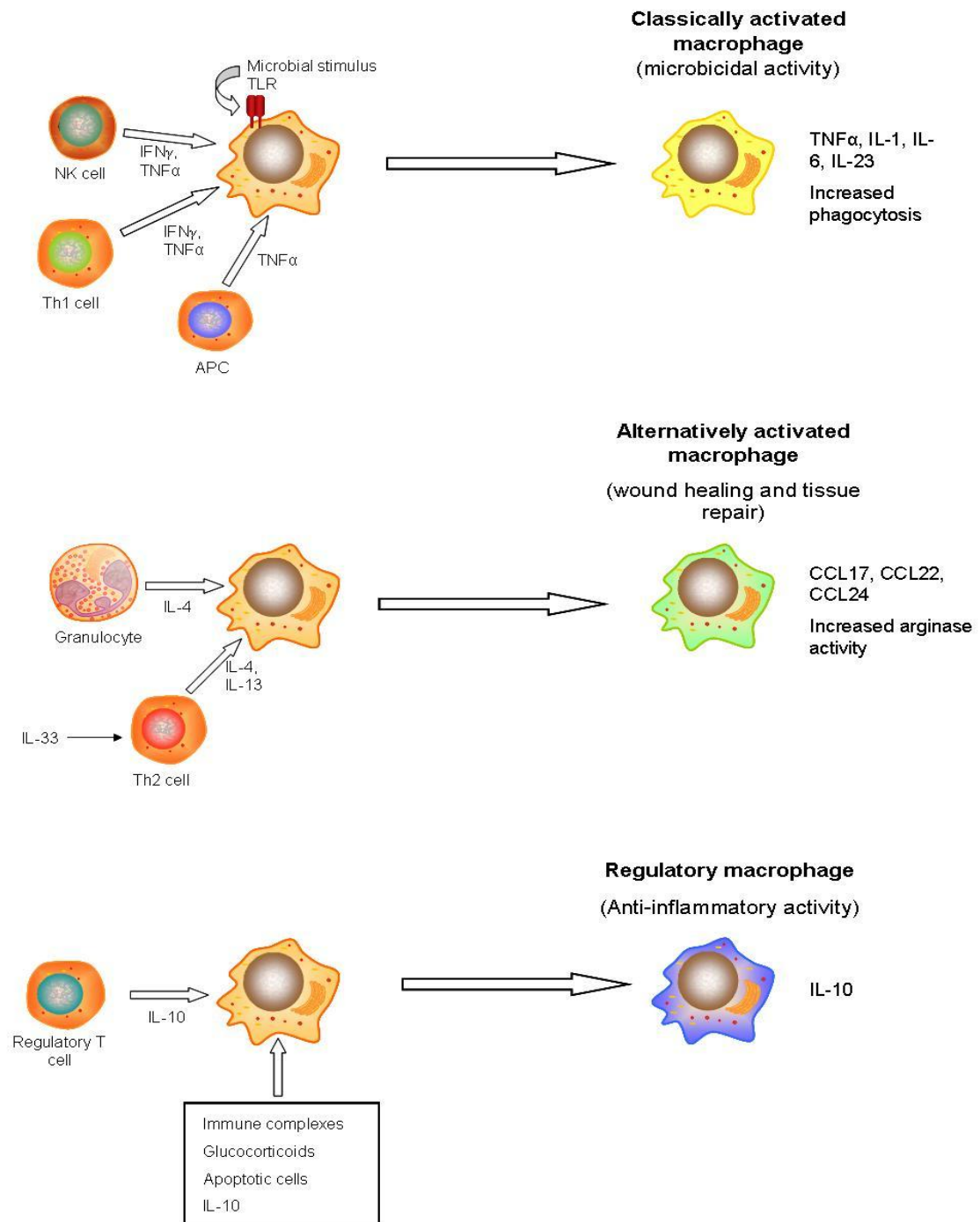


Figure 1.4 Innate and acquired immune activation of macrophages

Classically activated macrophages develop in response to the cytokines IFN γ , which can be produced during an adaptive immune response by Th1 cells or during an innate immune response by NK cells, and TNF α which can be produced by NK cells, Th1 cells or APCs. Alternatively, classical macrophage activation can develop as a result of TLR ligation by microbial products. Alternatively activated macrophages arise in response to IL-4 and IL-13 which are produced during a Th2 adaptive response or during a granulocyte innate response. IL-33 can activate Th2 cells and amplify their cytokine production and therefore alternative macrophage activation. Regulatory macrophages develop in response to various stimuli including IL-10, apoptotic cells, immune complexes and glucocorticoids. Each of these macrophage populations has a different phenotype and role within host defense, classically activated macrophages have microbicidal activity whereas alternatively activated macrophages have a role in tissue repair and parasite expulsion and regulatory macrophages produce high levels of IL-10 to dampen the immune response. The development of the macrophage populations shown here are not permanent changes to the cells and due to remarkable plasticity of macrophage cells they retain the ability to change from one subset to another depending on the environmental stimuli. Adapted from [197].

1.2.4 Monocytes and macrophages in inflammatory arthritis

Autoimmune disorders such as PsA and RA are characterised by the obliteration of self-tolerance and the appearance of self-reactive lymphocytes. Monocytes and macrophages are one of the cell types implicated in the perpetuation of arthritic disease and are found in dense populations within the diseased synovial membrane.

1.2.4.1 Blood monocytes in RA and PsA

Within healthy individuals the circulating blood monocyte population will consist of approximately 90% CD14⁺CD16⁻ cells and 10% CD14^{low}CD16⁺ cells [166]. In comparison to the CD14⁺CD16⁻ monocytes, CD14⁺CD16⁻ (CD16⁺) monocytes have shown distinct patterns of additional surface molecules such as MHC class II, ICAM-1 and LFA-1 [164, 223] and are thought to bear resemblance to tissue macrophages. CD16⁺ monocytes are thought to be of pathological significance since they are expanded in inflammatory conditions such as asthma [224] and sepsis [225] and have been discovered to be at a higher ratio in the blood of RA patients. The percentage of CD16⁺ monocytes is significantly increased in RA blood (11-15%) compared to healthy blood (9-9.5%) [226-229] and the RA diseased blood monocytes also had increased levels of surface TLR2 compared to the healthy controls [227]. CD16⁺ cells produce larger amounts of TNF α in response to TLR2-specific ligands compared to CD16⁻ cells [168] suggesting that the RA blood monocytes may be more proinflammatory and have higher levels of activation compared to those of the healthy controls.

The expression of CD16 on blood monocytes in RA patients has also been shown to be correlated to the disease activity of the patients which also substantiates the hypothesis that CD14⁺CD16⁺ monocytes in RA contribute to the disease pathogenesis [226, 228, 229]. In addition, the plasma concentrations of cytokines which are known to induce CD16⁺ expression such as M-CSF, TGF β 1 and IL-10 are increased in RA patients. In addition, to being at an increased level in the blood of RA patients, CD16⁺ monocytes are also overrepresented in the synovium of patients where they again correlate with disease activity and the degree of joint destruction [229]. Moreover, circulating CD16⁺ monocytes as well as the lining layer in the synovial tissue of RA patients show increased expression of CX3CR1 which is the receptor for fractalkine (CX3CL1). CX3CR1 is capable of

mediating leukocyte migration and firm adhesion, and high levels of fractalkine have been detected in the serum and synovial fluid of RA patients indicating that this receptor - ligand complex may be mediating the migration and recruitment of CD16⁺ monocytes from the blood into the synovium [230].

In addition to the work performed on CD16⁺ overexpression within the blood of RA patients other studies investigating the function of monocytes in RA have further demonstrated their pathogenic role within the disease. For example, RA CD14⁺ monocytes have been shown to inhibit synovial T cell glucocorticoid - mediated apoptosis through the release of soluble factors rather than via direct cell contact. Therefore RA monocytes could provide a mechanism for the increased survival of synovial T cells within the disease and may be a mechanism for the differences in response to glucocorticoid treatment for RA [231].

In comparison to the RA literature there has been little research into the presence of an elevated CD16⁺ monocyte population in PsA patients. However, a very recent study by Chiu et al noted that there was an increased frequency of circulating CD14⁺CD16⁺ monocytes in PsA patients compared to healthy controls (0.91% versus 0.46% respectively) [232]. It had been previously demonstrated that circulating monocytes in PsA were able to mature into osteoclasts, which are cells that directly absorb bone, without any exogenous stimuli [233]. The results of the current study demonstrate that the osteoclast cells were mainly derived from the CD14⁺CD16⁺ cells in PsA and increased CD16 expression was associated with increased bone erosion in PsA [232]. However, this result is in direct contrast to those of Komano et al who isolated monocytes from the blood of RA patients and illustrated that the CD16⁻ monocyte subset but not the CD16⁺ population were able to differentiate into osteoclasts by stimulation of the cells with RANKL in combination with M-CSF. In contrast, the CD16⁺ monocytes expressed larger amounts of TNF α and IL-6 which were enhanced by RANKL stimulation therefore this would suggest that the RA blood monocytes consist of two functionally distinct subsets which respond in alternative ways to RANKL stimulation [234]. The difference demonstrated between the ability of CD16⁺ cells to form osteoclasts in PsA but not in RA is at present inexplicable but may be due to differences in the monocyte subsets between the two diseases.

1.2.4.1.1 Chemokine receptor expression on diseased monocytes

The majority of cells which form the infiltrate in RA and PsA synovial tissue are derived from the circulation and the synovial inflammation seen in both diseases is dependent on the infiltration and retention of these inflammatory cells within the joint. Chemokines and their receptors play a crucial role within these processes as they allow the immune cells to chemotax from the blood to the synovium and the upregulated expression of chemokine receptors is a characteristic of diseased monocytes. The chemokine network represents a large group of chemotactic proteins which are structurally related. To date there are around 50 chemokines signalling through 20 different receptors which can be constitutively expressed on cells or be up- or down-regulated dependent on cell activation.

Arthritic blood monocytes have previously been demonstrated to express chemokine receptors at a significantly increased level compared to normal blood monocytes. As mentioned previously fractalkine (CX3CL1) is expressed at a higher level on RA CD16⁺ monocytes which is thought to mediate the migration of these cells into the diseased joint [230]. An additional study has also found that RA PB monocytes express both CCR1, which will bind a variety of ligands including CCL3 and CCL5, and CCR2, which binds with CCL2, while RA SF macrophages have upregulated levels of CCR5 and CCR3. This differential chemokine receptor expression would suggest that CCR1 and CCR2 may be involved in monocyte recruitment from the blood and CCR3 and CCR5 may be involved in retention of the monocytes within the synovium [235]. In addition, a recent paper had revealed the expression of the chemokine receptor CCR9 on RA PB monocytes as well as RA synovia, the expression of CCR9's only known ligand CCL25 was also detected in RA synovia co-localised with CD14⁺ monocytes and CD68⁺ macrophages. CCL25 induced strong chemotaxis of PB monocytes and also the differentiation of monocytes into macrophages and therefore this receptor-ligand complex may play a dual role in the pathogenesis of monocytes in RA by inducing their movement into the synovium and promoting their differentiation into a destructive macrophage phenotype [236].

The RA and PsA synovium also contains high levels of monocyte chemotactic chemokines including the ligands for the chemokines receptors mentioned above which are expressed on PB monocytes such as CCL5 and CCL3 [237, 238] which

will enable the migration of blood monocytes into the synovium in order to differentiate into pathogenic macrophages and maintain their continual pathogenic secretion of pro-inflammatory cytokines and chemokines.

A recent interesting imaging study which investigated the migration of RA monocytes, isolated CD14⁺ monocytes from patients and labelled them with a radioactive tracer before re-infusing these monocytes back into the patients. The patients were imaged and the results determined that a small number of the monocytes had migrated into the diseased joints after 1 hour and had a continuous movement into the joint [239]. These results demonstrate the short migration time of monocytes from the blood into the synovial compartment and reveal the strong migratory potential of these monocytes possibly led in part by the high levels of chemotactic proteins expressed within both the patient synovium and serum and the upregulation of adhesion molecules on the surface of the cells [230, 240].

The proinflammatory milieu within the serum of arthritis patients can also induce blood monocytes to produce chemokines and pro-inflammatory cytokines therefore the blood monocytes are not only the precursors for the inflammatory macrophages seen in the synovium but can also contribute to the inflammatory environment themselves. For example, a recent study demonstrated that the pro-inflammatory molecule osteopontin which is found at increased levels in the serum of RA patients induced the expression of the chemokines CCL2 and CCL4 in CD14⁺ monocytes [241]. Monocytes have also been shown to secrete TNF α in response to immune complexes found in the serum of arthritis patients [242] indicating that these cells play a role in the elevated serum chemokine and cytokine levels observed in RA and PsA.

1.2.5 Macrophages in RA and PsA

Once the peripheral blood monocytes have entered the arthritic synovium they will mature and develop into macrophages which express broad pro-inflammatory, remodelling and destructive potential and contribute significantly to the inflammation and joint destruction observed in RA and PsA. Similar numbers of CD68⁺ macrophage populations are found within the lining and sublining of the synovium in RA and PsA [243] and upregulated levels of

monocytes and macrophages have been found in the synovial fluid of RA and PsA patients [244]. The importance of macrophages for the pathogenesis of inflammatory arthritis has been demonstrated by several studies such as those performed by Tak et al who illustrated that the number of synovial macrophages correlate with the scores for knee pain and also the levels of TNF α and IL-6 expression [245]. In addition, the radiographic progression of joint destruction in RA has been found to correlate with the degree of synovial macrophage infiltration [246] which underscores the important role the macrophage plays in synovial inflammation. Furthermore, an *ex vivo* cellular model of rheumatoid arthritis which was set up using cultures of synovial tissue from RA patients revealed that the spontaneous reconstruction of inflammatory tissue which was visualised after 4 weeks of culture could be inhibited by depleting CD14⁺ cells from the synovial tissue. They suggested that these results imply a critical role of CD14⁺ monocytes and macrophages in the development of the destructive pannus tissue observed in RA [247]. Studies in animal models have also revealed the importance of macrophages in arthritis as op/op mice which are deficient in the macrophage differentiation cytokine M-CSF fail to develop collagen induced arthritis [248].

1.2.5.1 Stimulation of macrophage activation in RA and PsA

The differentiation and activation of infiltrating monocytes into pathogenic macrophages underlies the systemic inflammation observed in both RA and PsA and is a complex interaction of paracrine mechanisms through cell-cell interaction as well as the result of soluble stimuli and autocrine mechanisms.

1.2.5.1.1 *Cell-cell interaction*

A considerable part of macrophage effector responses is mediated by cell contact dependent signalling with different inflammatory or mesenchymal cells. Due to the large numbers of fibroblasts and macrophages within synovial tissue the interaction of these cell types is essential for the resulting tissue damage and inflammation. The direct cell contact of these two cell types elicits the production of IL-6, GM-CSF and IL-8 which can be enhanced by the addition of other pro-inflammatory cytokines or inhibited by the addition of regulatory cytokines or by neutralising CD14 [249].

The interaction between monocytes and endothelial cells in arthritis is necessary for the sustained influx of cells into the synovium through the interaction of selectin/intergrin pairs on the surface of the two cell types. The synovial cytokine environment upregulates the expression of these ligand pairs and leads to the further influx and activation of peripheral blood monocytes [250]. In addition, cell contact between macrophages and monokine-activated NK cells induces TNF α production from the macrophages demonstrating a further mechanism for macrophage activation [251].

A substantial amount of work has been dedicated to analysing the cell contact dependent activation of macrophages by T cells which will be discussed in detail in section 1.2.5.3. This cell contact activation occurs in the absence of antigen but there have also been reports of macrophages acting as APCs within the joint and presenting autoantigens such as collagen to T cells [252].

1.2.5.1.2 Soluble stimuli

There are numerous soluble stimuli present in the arthritic joint which are able to activate macrophages of these the most notable stimuli are cytokines. Many of the cytokines present have a pro-inflammatory effect on the macrophages and several of them, such as TNF α , IL-1 and IL-15, are produced by macrophages themselves and therefore will activate the macrophages in an autocrine fashion and result in a subset of perpetually activated macrophages. T cells within the joint can also produce inflammatory cytokines that will affect the macrophage population such as IL-17 which has pro-inflammatory and amplifying effects on macrophages in RA [113]. In addition to their inflammatory effects, there are also cytokine stimuli within the joint which have a regulatory effect on the synovial macrophages such as IL-4 and IL-13 which are produced by T cells and are involved in the alternative activation of macrophages as discussed previously and also IL-10 which is produced by macrophages themselves and may be a mechanism of autocrine regulation in order to try and reduce the chronic inflammation taking place.

An interesting study undertaken by Vandooren et al demonstrated that the synovial fluid from SpA patients which included a subset of PsA patients was able to induce the preferential expression of the M2 (alternative activation) markers CD163 and CD200R on peripheral blood monocytes in comparison to synovial fluid

from RA patients [253]. Previous studies had also shown an increased number of CD163 expressing macrophages in the synovium of SpA patients [254, 255], these studies demonstrate that the local inflammatory synovial environment is different in SpA compared to RA and could be preferentially inducing a more wound healing alternative macrophage phenotype which may be contributing towards maintaining tissue integrity although at present no differences in the subsequent destructive processes in SpA and RA have been identified.

1.2.5.2 Macrophage effector molecules in RA and PsA

Once the blood monocytes have been differentiated and fully activated into synovial macrophages they start to produce a number of pro-inflammatory effector molecules that contribute to the continual inflammation and joint destruction seen within the arthritic joint. Subsets of these molecules are outlined below.

1.2.5.2.1 Chemokines

As discussed previously chemokine receptor expression play a significant role in the infiltration of blood monocytes into the synovium. Once in the arthritic joint the differentiated macrophage will upregulate a different profile of chemokine receptors such as CCR3 and CCR5 which may be involved in their retention within the synovium [235] and in addition will also secrete chemokines in response to the pro-inflammatory environment. Synovial macrophages have been shown to produce a range of CXC and CC chemokines and will often produce the ligands for the chemokine receptors expressed on the diseased blood monocytes in order to increase the number of infiltrating monocytes entering the synovium. One such chemokine is CXCL16 which is the sole ligand for CXCR6 and is found in the RA synovial tissue lining and interstitial macrophage cells. CXCL16 is involved in monocyte recruitment into the RA synovial tissue [256].

In addition to secreting monocyte chemotactic chemokines synovial macrophages also produce chemokines such as CXCL8 (IL-8), CXCL1 (Gro α), CXCL7 (CTAP-III) and CCL18 (PARC) which are chemotactic for other inflammatory cells such as neutrophils and T cells [257, 258]. CCL22 is also secreted by macrophages in RA and PsA synovium and is thought to be involved in the migration of CCR4 expressing memory T cells to the joint [259]. These secreted chemokines can

become part of autocrine loops of macrophage activation as their cognate receptors are often found on the surface of the macrophages themselves. Furthermore, some of the macrophage-expressed chemokines such as IL-8 are powerful promoters of angiogenesis and thus provide a link between macrophage activation and the prominent neo-vascularisation of the RA and PsA synovium [136].

1.2.5.2.2 Cytokines, proteinases and growth factors

TNF α

In RA and PsA, TNF α is mostly produced by macrophages at the cartilage pannus junction and in the synovial membrane [250], this cytokine can induce the production of adhesion molecules, collagenase and collagen from synovial cells and can also act on macrophages in an autocrine manner to produce numerous products including IL-6, IL-8, MCP-1 and MIP-1 α [258]. Due to its significant pro-inflammatory capabilities TNF α is of critical importance in the pathogenesis of RA and PsA and neutralisation of the cytokine through the use of anti-TNF α antibodies is now used as a therapy for both diseases [135, 148, 149].

IL-1

IL-1 is another pro-inflammatory cytokine found within the synovial joint that is predominantly secreted by CD14⁺ macrophages [260], and synovial fluid levels of this cytokine have been found to correlate with joint inflammation [261]. IL-1 influences proteoglycan synthesis and degradation and hence articular damage underscoring the pathogenic effect macrophages and their products can have on the chronic nature of arthritis. In addition, the IL-1 type 1 receptor (IL-1R1), which mediates IL-1-induced cell activation, is found on numerous cells within the synovium of RA patients [262] indicating the pro-inflammatory capacity of IL-1 within the synovium. The type II receptor (IL-1R2) which does not induce cellular activation and is therefore anti-inflammatory is found at low levels in the arthritic synovium [263]. However, IL-1 receptor antagonist (IL-1RA) which blocks the action of IL-1 by binding to IL-1R1 is constitutively expressed by differentiated macrophages and is produced at increased levels by the mononuclear cells of RA patients compared to healthy controls [264]. In addition, IL-1RA levels in the sera of PsA patients correlate with the number of swollen joints [265]. These findings may help to explain why therapeutic application of IL-1RA (anakinra) appears to be only modestly effective in RA

[266] and PsA [267] despite being an effective therapeutic for several hereditary autoimmune diseases.

IL-15

IL-15 is an IL-2 family member cytokine that has chemoattractant properties for memory T cells, it is produced by lining layer cells including macrophages and is increased in the synovial fluid of RA patients [268]. Peripheral or synovial T cells stimulated with this cytokine can induce macrophages to produce IL-1 β , TNF α , MCP-1 and IL-8 but not the anti-inflammatory IL-10 [269]. Since IL-15 is produced by the macrophages themselves, this may re-stimulate the T cells and result in a self-perpetuating pro-inflammatory loop [268].

IL-6

IL-6 has also been shown to be produced by synovial macrophages in addition to synovial fibroblasts, it is elevated in the synovial fluid of RA and inflammatory arthritis during the acute phase of disease and has been shown to have phase dependent effects as it protects the cartilage in acute disease but promotes excessive bone formation during chronic disease [250]. However, despite these opposing effects of IL-6 within the disease setting clinical trials of an anti-IL-6 receptor antibody suggest it may be used as an effective treatment for RA [134, 157] although at present no trials have investigated the use of such antibodies in PsA.

IL-18

IL-18 is a member of the IL-1 family of cytokines and its expression is up-regulated within the synovial membrane as well as the serum and synovial fluids of RA and PsA patients [270, 271]. It is expressed by CD68⁺ macrophages contained within lymphoid aggregates as well as by fibroblast-like synoviocytes and RA synovial fluid CD14⁺ macrophages also express the IL-18 receptor (IL-18R) [272]. Several lines of investigation have demonstrated the pro-inflammatory role of IL-18 in arthritis: (a) IL-18 deficient mice exhibit reduced incidence and severity of disease in the CIA and ZIA mouse models of arthritis [273-275] (b) anti-IL-18 antibodies can effectively reduce development and established arthritis in both streptococcal cell wall induced arthritis (a strongly macrophage-dependent model) and CIA [276, 277] (c) treatment with low doses of IL-18 prior to induction of CIA was able to increase the severity and incidence of disease [278] and (d) IL-18 is selectively overexpressed in the bone marrow of patients

with juvenile idiopathic arthritis and macrophage activation syndrome [279]. IL-18 has also been demonstrated to correlate with CRP levels in arthritis patients and their levels decrease in parallel in synovial tissue and serum following effective treatment with DMARDs [271]. A recent study performed by Ruth et al engrafted human synovial tissue into SCID mice and investigated whether the injection of IL-18 into these grafts could induce the migration of human PB monocytes which were administered to the mice. They found that IL-18 could indeed induce monocyte recruitment to the synovial tissue and in addition IL-18 gene knockout mice showed pronounced reductions in joint inflammation during ZIA model of arthritis once again indicating the dominant role of IL-18 within arthritis and its ability to act in an autocrine manner and induce monocyte migration to sites of inflammation [275].

MIF

Macrophage migration inhibitory factor (MIF) is an early-response cytokine abundantly released by macrophages. This cytokine can stimulate macrophage functions in an autocrine manner such as the release of TNF- α and phagocytosis. MIF can also confer resistance to apoptosis in both macrophages and synovial fibroblasts within the synovial joint [250]. In RA, MIF is overexpressed in the serum and synovial fluid of patients and correlates with disease activity and arthritis severity is significantly reduced in MIF^{-/-} mice in comparison to wild type mice and is associated with reduced T cell activation [280].

Proteinases

Matrix metalloproteinases (MMPs) are enzymes involved in extracellular matrix degradation that is a central part of the joint destruction seen in arthritis. There are around 30 known MMPs and of these mainly MMP-1 (collagenase), MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are released by RA synovial macrophages [258, 281]. MMP-1 and MMP-3 (stromelysin) mRNA has also been detected in the synovium of PsA patients [94]. Synovial macrophages also produce tissue inhibitors of MMPs such as TIMP-1 and TIMP-2 that antagonise the effects of MMPs.

Growth factors

Growth factors are involved in the synovial angiogenesis, inflammation and fibrosis occurring in the arthritic joint. Synovial macrophages are known to secrete several such factors including platelet-derived growth factor (PDGF) and

vascular endothelial growth factor (VEGF) [258]. PDGF-C and PDGF-D are the two isoforms expressed by synovial tissue macrophages and the latter isoform has been previously shown to increase the production of the enzyme MMP-1 within the synovium [282]. VEGF levels are significantly increased in the sera of PsA and RA patients and correlate with disease activity [98, 283]. The VEGF receptor (VEGFR-1/Flt-1) has previously been implicated in macrophage activation and angiogenesis in RA [284]. VEGFR-1 deficient mice were shown to obtain diminished clinical symptoms of induced arthritis compared to wild type mice and this VEGFR-1 deficiency was associated with reduced macrophage differentiation and function including a decrease in the secretion of pro-inflammatory mediators [284].

1.2.5.2.3 *Anti-inflammatory cytokines*

In addition to producing a large variety of pro-inflammatory mediators in the synovial joint macrophages also produce anti-inflammatory cytokines, namely IL-1RA as mentioned previously and IL-10. IL-10 is a macrophage and Th2 derived cytokine which reduces antigen presentation by macrophages and prevents the production of pro-inflammatory cytokines by synovial macrophages [250]. The *ex vivo* production of IL-10 by RA PBMCs is negatively correlated with radiographic joint damage suggesting that IL-10 is protective however several studies have found a deficiency of IL-10 in the synovial compartments [285] which proposes that the synovial macrophages may be reducing their regulatory cytokine output in favour of a highly pro-inflammatory profile.

An outline of the characteristics and pro-inflammatory potential of inflammatory arthritis monocytes and macrophages are shown in Figure 1.5.

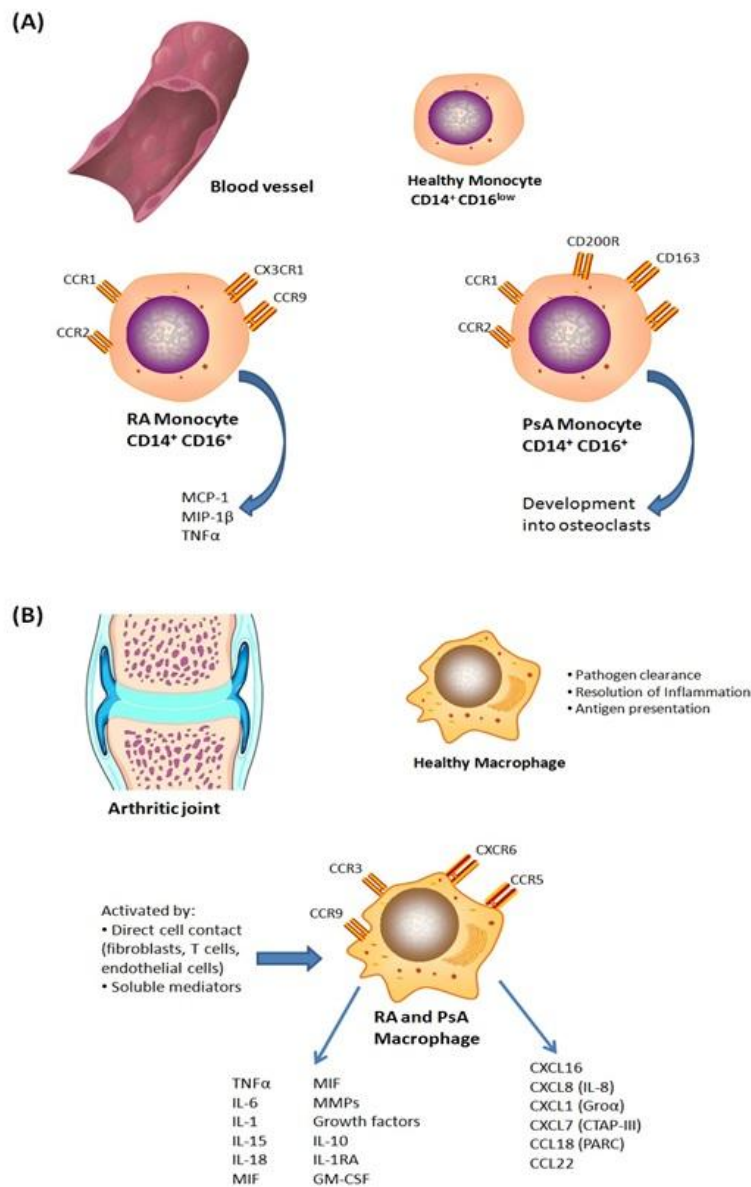


Figure 1.5 Phenotypic overview of RA and PsA monocytes and macrophages

An overview of the functional differences between healthy monocytes and RA or PsA diseased monocytes in the blood (A) and the arthritic joint (B). This figure demonstrates the inflammatory phenotype of these cellular subtypes and the mechanisms of their activation in RA and PsA.

1.2.5.3 In vitro model of inflammatory arthritis

The use of *in vitro* model systems within the investigation of chronic arthritis is valuable for the detailed analysis of patient cells to fully understand how they activate and interact with other cells. One such *in vitro* model has been established to investigate the contact-dependent, antigen independent interactions occurring in the synovial joint between macrophages and stimulated T cells. Since histologically, T cells are often found within close contact with macrophages within RA synovial tissue [286]. In addition, T cell cytokines such as IL-4, IL-10, TGF- β and IL-13 have predominantly anti-inflammatory effects and

IFN- γ alone displays weak activation capacity in terms of IL-1 β and TNF- α induction which suggests that the soluble factors produced by T cells are not pathological mediators. Therefore it was hypothesised that within the synovium, one method through which T cells could exert a pathological effect would be through direct cellular contact with macrophages to induce cytokine and chemokine secretion from the latter cell type.

The history of contact mediated activation of macrophages by stimulated T cells began in the mid-eighties when it was observed that the expression of membrane-associated IL-1 (IL-1 α) in mouse macrophages was mediated by both soluble mediators and direct cell contact with T cells [287]. The same group subsequently demonstrated that this T cell contact mediated IL-1 induction in monocytes took place with both Th1 and Th2 cells in the absence of lymphokine release [288]. Following this result a similar experiment was setup with human cells and it was demonstrated that the production of IL-1 β by human monocytes also required direct cell contact with anti-CD3 stimulated cells [289]. Accordingly, other investigators have shown that the induction of macrophage effector functions mediated through T lymphocytes in living cell co-cultures involved signals from cell-cell contact in addition to IFN γ [290]. Furthermore, by using isolated plasma membranes from T cells stimulated with PHA/PMA Vey et al demonstrated that this contact activation was sufficient for the activation of the human monocytic cell line THP-1 and their subsequent secretion of IL-6, TNF α and IL-1 β in the absence of T cell derived cytokines. This cytokine release did not occur when the cells were physically separated by a permeable membrane [291].

Antigen independent, direct contact mediated activation of monocytes and macrophages by stimulated T cells was found to be as potent as LPS in inducing cytokine production in monocytes and monocytic cell lines [291-293]. However, besides PHA/PMA various stimuli for the T cells were found to activate monocytes by direct cellular contact such as cross-linking CD3 by anti-CD3 mAb and the addition of cytokines to the T cells. In addition, depending on the T cell type and the stimulus, direct cell-cell contact between stimulated T cells and monocytes/macrophages induced different patterns of products secreted from the latter cell type (see Table 1.7).

Table 1.7 Depending on T cell stimulus, various products are induced in the monocytes/macrophages upon direct cell contact

Stimulus	T cell	Monocyte	Products
PHA/PMA	PB T cells, Synovial, Jurkat, Th1 and Th2, HUT-78	PB mo, THP-1	TNF α , IL-1 β , IL-6, IL-8, IL-1 α , MMP-1, MMP-9, TIMP-1 [291, 293-295]
PdBu/ionomycine	PB T cells	PB mo	IL-10, TNF α [296]
Anti-CD3	PB and synovial T cells	PB mo, THP-1	IL-10, IL-1 β , TNF α , MMP-1 [296-299]
Anti-CD3	PB T cells	PMA/IFN γ treated U937	IL-10, IL-12, TNF α , IL-4 [300]
IL-15 or IL-2	PB T cells, Synovial T cells, Th1 and Th2	U937, PB mo, THP-1, synovial mo	TNF α , IL-1 β , IL-10 [301, 302]
IL-2, IL-6, TNF α	PB T cells, Synovial T cells	PB mo	TNF α , MCP-1, MCP-1, MIP-1 α , MIP-1 β , RANTES, IL-8, GRO α , IP-10, IL-1 β [269, 298]

The observations outlined in Table 1.7 suggested that multiple ligands and counter-ligands are involved in the contact mediated activation. It was hypothesised that membrane associated cytokines or soluble cytokines may play a role however the Dayer group consistently demonstrated that neither soluble TNF α receptors or IL-1Ra blocked T cell mediated signalling of the monocyte. Additionally neutralisation of TNF α , IL-1, IL-2, GM-CSF and IFN γ all failed to affect monocyte activation by membranes from stimulated T cells [291, 293, 297]. In addition to membrane-associated cytokines other surface molecules were investigated for their ability to activate macrophages upon contact with stimulated T cells. The CD40/CD40L interaction was shown to be involved in the contact activation of both human and mouse macrophages by T cells stimulated for 6 hours [303] but T cells isolated from a CD40L knockout mouse were also able to trigger macrophage activation although this activation was less than that seen from wild type murine T cells [304]. Furthermore, blocking antibodies to CD40L or soluble CD40 failed to inhibit contact-induced activation of

macrophages by 48 hour activated T cells. HUT-78 cells, which are able to induce cytokine production from macrophages, do not express CD40L mRNA in the resting or activated state [305]. Finally, Th1 but not Th2 clones stimulated with IL-15 were able to induce IL-1 β production from monocytes, in the former system blockade of the CD40/CD40L interaction resulted in inhibition of IL-1 β production despite similar levels of CD40L expression in the Th1 and Th2 cells indicating the selective requirement of CD40 engagement upon monocyte activation by Th1 clones [301]. Other groups have also demonstrated the presence of surface molecules such as CD69, LFA-1 and ICAM-1 on simulated T cells play a role in macrophage activation through the use of blocking antibodies which effectively inhibited macrophage activation [291, 302, 306].

Histological studies of the RA synovium have indicated that this tissue is very cellular and that several different cell types including macrophages and T cells are in close proximity [286]. This could therefore suggest that direct contact between macrophages and T cells could be important *in vivo* in modulating synovial macrophage function. It was first reported in 1994 that a cocktail of cytokines, namely IL-2, IL-6 and TNF α , could activate T cells in the absence of antigen which in turn stimulated B cells [307]. Since the RA synovial joint is a highly pro-inflammatory environment containing a milieu of pro-inflammatory cytokines it was hypothesised that these cytokines could activate the T cells contained within the joint which would subsequently activate the synovial macrophages by direct cell contact and be a mechanism for the increased production of pro-inflammatory mediators.

In 1997, McInnes et al demonstrated that IL-15 alone, which is a cytokine found within RA synovium [268], could activate T cells isolated from the PB or SF of RA patients; these fixed T cells were then able to activate U937 cells or matched RA PB or SF monocytes to produce TNF α . This monocyte activation was cell contact dependent since the TNF α production was abrogated in the presence of a semi permeable membrane. Furthermore, it was demonstrated that the T cell surface molecules CD69, LFA-1 and ICAM-1 were involved in this activation since antibodies against these molecules prevented the secretion of TNF α from the monocyte [302]. In the same year Brennan and Feldmann used the cytokine cocktail IL-2, IL-6 and TNF α to stimulate PB T cells and demonstrated that the manner in which the T cells were activated influenced the profile of cytokines

secreted by the monocytes. Thus, if blood T cells were activated with cross-linked anti-CD3 this induced the production of TNF α and IL-10 from monocytes [296]. However, if the T cells were stimulated with the cocktail of cytokines for 8 days, the monocytes produced TNF α production but not IL-10 [269]. Such observations suggested that these cytokine stimulated T cells, designated Tcks, could be similar to the T cells found in the synovial tissue of RA patients since they induce an unbalanced pro-inflammatory cytokine response from monocytes.

Consequently, further work performed by the Brennan and Feldmann group set out to investigate fully these Tck cells and their relevance to RA. This group demonstrated that the TNF α production in RA synovium was T cell dependent as the removal of CD3-positive T cells from RA synovial mononuclear cells resulted in the significant reduction of macrophage TNF α , in addition this TNF α output was abrogated if physical contact between the two cell types was blocked [298]. It was also shown that Tck cells but not T cell receptor dependent stimulated T cells induced TNF α production in monocytes in an NF- κ B dependent manner. Conversely, phosphatidylinositol 3-kinase (PI3K) inhibitors were found to block T cell receptor stimulated T cell activation of monocytes but did not affect Tck mediated monocyte activation indicating the different signalling mechanisms employed by the two differentially activated T cells [298]. Interestingly, RA synovial T cells were also found to employ the same signalling mechanisms as Tcks since the induction of TNF α in resting PB monocytes by synovial T cells was NF- κ B dependent and were superinduced if PI3K was blocked [298]. These results provided evidence that the IL-2, IL-6, TNF α activated Tcks were similar to the synovial T cells found in RA and provided a basis for an *in vitro* model of inflammatory arthritis.

Subsequent investigations have also substantiated this finding and provided evidence for the Tck cell contact activated macrophages as a model of arthritis. For example, the chemokines produced by M-CSF differentiated macrophages after contact with fixed Tck cells have been analysed and it was revealed that they produce several CC and CXC chemokines such as MCP-1, MIP-1 α , MIP-1 β , RANTES, IL-8, GRO α and IP-10. Interestingly, blockade of NF- κ B signalling through I κ B α overexpression inhibits Tck and synovial T cell induced macrophage secretion of CXC but not CC chemokines, suggesting that RA synovial T cells share similarities in their effector function to Tck cells [308]. Subsequent

phenotyping of this Tck cell subset has revealed that they are a CD4⁺CD45RO⁺CCR7⁻CD49d^{high} population of T cells. After IL-2, IL-6 and TNF α stimulation in culture these Tcks acquire an activated phenotype that resembles the phenotype of RA synovial T cells including the selective upregulation of adhesion molecules and integrins such as very late antigen 4(VLA-4) [309]. In summary, these investigations established the functional and phenotypic similarities between *in vitro* cytokine activated T cells (Tck) and RA synovial T cells and demonstrated the importance of the cell contact mediated activation of monocytes/macrophages as a surrogate model of RA.

The modulation of T cell induced signalling in monocytes may be an important concept as it would maintain a low level of monocyte activation within the blood stream. Apolipoprotein (apo) A-I has been identified as a specific inhibitor of contact-mediated activation of monocytes [310]. Apo A-I is known as a negative acute phase protein and is one of the main proteins of high density lipoprotein (HDL). There have been several reports of variations of apo A-I concentration in inflammatory diseases [311] and in RA the levels of circulating apo A-I and HDL-cholesterol in untreated patients were lower than in healthy controls [312]. Conversely, apo A-I levels were enhanced in the synovial fluid of RA patients [313].

This possible regulatory mechanism within the synovium may be overcome by a positive acute-phase protein which can displace apo A-I from HDL, known as serum amyloid A (SAA) [314]. SAA is produced in the liver but has also been detected in the RA synovium, HDL-associated SAA is a pro-inflammatory molecule and the expression of SAA can be increased by cytokines such as TNF α and IL-1 β . This could therefore lead to a positive feedback loop of inflammation within the synovium as the cell contact dependent activation of synovial macrophages will induce the production of pro-inflammatory cytokines which will therefore increase the levels of SAA and consequently decrease the levels of Apo A-I. The decreased level of Apo A-I, which is an inhibitor of contact mediated monocyte activation, will thereby result in higher levels of monocyte activation and increased levels of pro-inflammatory molecules thus increasing the chronic synovial inflammation [314]. To try to further understand the role of HDL within T cell contact activation of monocytes Gruaz et al performed a microarray analysis of monocytes activated by the plasma membranes of fixed

PHA/PMA stimulated T cells in the presence or absence of HDL. The results of this study demonstrated that T cell activation of monocytes induced the upregulation of 437 probe sets in the monocytes including genes associated with the inflammatory response and inflammatory diseases. It was also shown that the addition of HDL to the monocytes at the time of T cell membrane activation was able to inhibit the expression of 164 (38%) of these probe sets which included probe sets specific for TNF α , IL-6, IL-1 β , CCL4 and CCL3. Consequently this study substantiated the previous reports of the pro-inflammatory nature of T cell contact activation of human monocytes and the subsequent anti-inflammatory capacity of HDL upon this cell contact model [315].

In summary, these studies have demonstrated that stimulated T cells have the ability to activate monocytes and macrophages in an antigen independent but cell contact dependent manner. The method through which the T cells are stimulated can vary (see Table 1.7) and this variation can affect the products secreted by the monocytic cell. However it has been demonstrated that a particular stimulus consisting of a cocktail of cytokines can induce a T cell phenotype and activation pattern similar to that of RA synovial T cells. Due to the close proximity of T cells and macrophages within the RA synovium and the predominantly anti-inflammatory effect of T cell secreted cytokines the T cell - macrophage cell contact activation has been postulated to be a mechanism of increasing the pro-inflammatory cytokine and chemokine levels within the synovium. Therefore the cytokine cocktail method of T cell stimulation within the cell contact model has become an established *in vitro* method of investigating the role T cell - macrophage activation within arthritis.

1.3 Gene expression analyses in inflammatory arthritis

As described in 1.1.1, the rheumatic diseases are a heterogenous group of disorders with a wide clinical spectrum and considerable variability in secondary organ involvement. Every aspect of this heterogenous disease phenotype should be represented in the pattern of genes and proteins which are expressed by the patient. This molecular signature will represent the contribution of the interactions between specific molecules and the individual cell types that are associated with disease characteristics and subtypes and thus will determine the samples' unique molecular biology. A powerful way to gain insight into the molecular signature contained within the cells and tissues of rheumatic diseases has arisen with the advent of DNA microarray technology, which can comprehensively identify the subset of genes which are differentially expressed among patients with clinically defined disease. There were initially several problems in the use of such technology, which requires highly standardised conditions, such as the lack of consistent methods for blood and tissue handling or preparation or the use of different platforms and standardisation procedures. However, after a decade of technical improvement the technology and standardisation procedures have been shown to be robust and allow comparison between properly designed and controlled experiments [316] thereby helping to unravel information that may aid in allowing clinicians to optimise treatment for and understanding further the complexity of rheumatic diseases.

The resultant findings of microarray analyses performed in RA and PsA are summarised in Table 1.8.

1.3.1.1 Rheumatoid arthritis

1.3.1.1.1 *Genetic analysis of affected tissues*

One of the first gene expression profiling studies for RA was conducted on biopsy tissue using high density cDNA arrays [317]. This report identified an increased expression of inflammation associated genes, such as HLA-DR, in the RA synovium compared to normal synovium. However, due to the investigators using pooled samples from three patients with RA and three healthy controls it was impossible to study the individual genetic profiles of the patients or the heterogeneity between samples. In contrast, a study set up by van der Pouw

Kraan analysing the genetic expression of the synovial tissue from RA (n=21) and OA (n=9) patients found the RA patients to be highly heterogeneous in their genetic signatures [318, 319]. Interestingly, they identified two molecularly distinct forms of RA; the synovial tissue from one group of patients had an abundant expression of clusters of genes indicative of ongoing inflammation and involvement in the adaptive immune response. This subgroup was referred to as the RA high inflammation group. The second group, known as the low inflammation subgroup, contained expression profiles of tissue remodelling genes such as increased expression of MMP-11 and MMP-13 and low levels of inflammatory genes which was similar to the patterns seen in the OA tissue [318]. Analysis of the genes in the RA high inflammation subgroup indicated a prominent role of genes involved in activated IFN and the signal transducer and activator of transcription (STAT)-1 pathway [318]. It was determined by histological analysis that the difference obtained in the two subgroups of RA patients were related to differences in cell distribution, as tissues which contained germinal centre-like structures were those found in the high inflammation subgroup.

A further study performed by Devauchelle et al analysed the differences in gene expression between the synovial tissue of RA (n=5) and OA (n=10) patients [320]. A set of 63 genes were identified based on their over- or under-expression in RA tissue compared to the OA tissue, 15 of these genes had unknown function but the expression of the remaining 48 genes allowed the correct classification of additional RA or OA patients therefore the authors proposed that such information could aid in the diagnosis of RA. Of interest, 16 genes from this study overlapped between the microarray performed by van der Pouw Kraan and seven of these genes including the type I IFN regulated GBP1 and CTSL had comparable gene expression profiles indicating the ability to reproduce genetic results between different patient samples and different groups of investigators.

Additional studies have also demonstrated that RA patients have significant tissue heterogeneity both between and within patient samples. A study performed by Tsubaki and colleagues [321] showed that early RA patients were a heterogeneous group in comparison to patients with longstanding RA as the early RA patients could be subdivided into at least two different subgroups depending on their gene expression profiles. A similar study was performed in 2009 [322],

the synovium from 4 early RA patients, 4 treated longstanding RA patients and 7 controls was analysed by microarray and it was found that distinct molecular signatures were expressed during early or longstanding RA suggesting that increased understanding regarding the main biological processes and pathways involved at each stage of the disease may help to decide the most appropriate therapy for the patients. An interesting study by Lindberg et al [323] investigated the variability in synovial gene expression at the biopsy site, between different sites and between patients. Multiple biopsy samples were taken from 13 patients; 7 by orthopaedic surgery and 6 by rheumatic arthroscopy and it was found that the number of differentially expressed genes between pairs of biopsies from the same knee ranged from 6 to 2,133 indicating the considerable level of heterogeneity between biopsy samples. The methods by which these samples were taken had a significant effect on the results since the number of differentially expressed genes between biopsies from the same patient was three times higher when the biopsies were taken by the orthopaedic method compared to the arthroscopic method which was thought to be due to sampling of non-inflamed tissue. Upon removal of biopsies which contained non-inflamed tissue the remaining gene expression signatures were found to be unique to each patient but also contained groups of genes which belonged to gene ontology categories such as cell communication and immune response.

Since the arrival of microarray studies which have analysed the diseased synovium of RA patients there has also been an increase in the number of studies which analyse the gene expression changes of the synovium in response to patient treatment. Lindberg and colleagues recently investigated the transcriptional profile of the synovium of RA patients treated with the anti-TNF α therapy infliximab in order to determine biomarkers which could be predictive of response to treatment [324]. This study found that within the 62 patients analysed no significant differences in genes expression could be detected between responders and non-responders to the infliximab treatment which therefore does not support the idea that microarray analysis of whole synovium biopsy specimens can be used to identify non-responders before the initiation of treatment.

1.3.1.1.2 Genetic analysis of blood derived cells

Although the gene expression analysis of tissue samples of affected target organs offer insight into the genes principally involved in disease progression and activity, it may not be viable to use this approach to study all patients especially large cohorts. Consequently, several groups began to analyse the gene expression profiles of whole blood or peripheral blood mononuclear cells (PBMCs) to obtain disease related gene expression profiles since the systemic nature of RA allows the communication between systemic and organ specific compartments within the disease. In addition, understanding the gene expression of blood cells may allow the use of such profiles for biomarker studies and could also improve diagnosis and personalised therapy.

One of the first microarrays analysing RA PBMCs took place in 2004 by Bovin and colleagues who examined the PBMCs from eight RF-positive RA patients, six RF-negative patients and seven healthy controls [325]. No significant differences between RF-positive and RF-negative patients were detected. However, comparison of the gene expression pattern from all fourteen patients and the healthy controls identified a subset of 25 discriminative genes which included genes associated with the inflammatory response such as defensin alpha and CD14 antigen as well as the calcium binding proteins S100A8 and S100A12. Batliwalla and colleagues investigated the gene expression differences between PBMCs from RA patients (n=29) and those from healthy donors (n=21) [326]. Using cluster analysis they identified a significant alteration in the expression of 81 genes in the PBMCs of RA patients compared to controls. A large number of these genes correlated with differences in the monocyte counts between the two study populations as many of the genes were known to be expressed at high levels in monocytes such as CD14 antigen. A logistic regression analysis revealed that of the remaining genes IL-1RA, S100A12, glutaminyl cyclase and Grb2-associated binding protein (GAB2) were the top discriminator genes in categorising RA patients from controls.

A further study performed by Edwards and colleagues analysing the RNA from the PBMCs of nine RA patients and thirteen healthy controls found 330 genes differentially expressed in RA [327]. These differentially expressed genes belonged to diverse functional classes and included genes involved in several biological groups such as transcription, signal transduction, extracellular matrix,

cytokines, enzymes and proteases. Interestingly, ten genes which had an increased expression in RA PBMCs compared to healthy controls mapped to an RA susceptibility locus, 6p21.3 which the investigators hypothesised could be a mechanism contributing to the etiology of the disease through the coordinated transcription of specific regions of a chromosome in response to stress or inflammation. A study performed by Junta et al evaluated the differential gene expression profile of PBMCs of RA patients and their association with the HLA shared epitope (HLA-SE), anti-CCP antibody levels, DAS-28 score and disease treatment [328]. This research demonstrated that thirteen genes were exclusively associated with the presence of HLA-SE alleles whose major functions were related signal transduction, apoptosis and signal transduction. One hundred and one genes were associated with the presence of anti-CCP antibodies, 91 genes were associated with disease activity including genes involved in DNA damage, signal transduction and response to stress and finally 28 genes were associated with TNF blockade treatment which included genes involved in protein transport and intracellular signalling.

In 2007, van der Pouw Krann and colleagues published a paper investigating the gene expression profiles of whole blood from RA patients (n=35) and healthy controls (n=15) [329]. The microarray data confirmed previous observations of increased expression of genes such as the S100A8 and S100A12 calcium-binding proteins. Pathway analysis software identified the increased expression within RA patients of immune defense genes including the type I IFN response genes indicating that this pathway is activated systemically in RA. A separate study comparing RA PBMCs to those of controls identified a spectrum of genes involved in immunity and defense to be upregulated in the RA patients once again demonstrating that such immune pathways are upregulated systemically in RA [330].

In addition to microarray analyses being performed on synovial biopsies from patients before and after therapeutic intervention there have also been a number of recent studies evaluating the effect of different treatments on the gene profile of PBMCs. Since a significant percentage of patients fail to show a clinical response to treatment, designated non-responders, the main focus of such microarray studies has been to determine molecular discriminators of response in patients. One study which obtained blood from patients before

starting the TNF α blockade therapy infliximab identified an eight-gene expression profile that was able to predict the patient response to the treatment as either a responder or non-responder [331]. This profile, which included the genes HLA-DRB3 and TLR5, was able to predict response of an independent validation set of RA patients with 85.7% accuracy. Another study looking at molecular discriminators of response to the anti-TNF α therapy etanercept found certain genes which when expressed in groups of two or three could have 89-95% prediction accuracy [332]. Finally, a microarray analysis of PBMCs taken from patients before the commencement of the IL-1Ra therapy anakinra identified 52 genes which were expressed as a response to treatment [333]. Seven of these transcripts, as assessed by quantitative RT-PCR, were able to predict 15 of 18 independent responder and non-responder patients with an accuracy of 87.5%. In conclusion, the emergence of such modelling systems could aid the clinician in the selection of the optimal treatment strategy for RA patients and has strong application potential within the clinical setting.

1.3.1.1.3 Gene analysis within cell subsets

The analysis of synovial biopsies and PBMCs from RA patients has been extremely useful in identifying patterns of gene expression within the disease and the response of patient tissues to treatment. However, it has been suggested that some of the genetic signatures identified in such studies may be attributable to differences in the relative abundance of individual cell populations within the sample. In addition cell-specific changes may be missed when analysing a mixed population of cells. Consequently, several groups set out to investigate the genetic signatures of individual cell populations within RA patients.

Fibroblast-like synoviocytes (FLS) are considered to play a major role in joint destruction in RA and also contribute to leukocyte migration into the joint. One of the first gene expression profiles of RA FLS revealed the over-expression of genes responsible for tumour like growth of rheumatoid synovium [334]. This study used a cDNA array containing 588 cDNA-fragments of known cancer related genes to compare the gene expression of FLS from five RA patients and five traumatic control patients and found the increased expression of such cancer-related genes in the RA FLS. An additional study analysing the genetic expression of 19 RA FLS samples correlated the genetic data to that of paired synovial tissue samples and found that the heterogeneity at the synovial tissue level was

associated with a specific FLS phenotype [335]. The high inflammation tissues were associated with an FLS subtype that exhibits similarity with a fibroblast cell called myofibroblasts. The myofibroblast cells are specialised cells that have a key role in connective tissue remodelling and cell infiltration. Addition studies in oncology have demonstrated that these cells play a crucial role in angiogenesis through the production of growth factors and extracellular matrix proteins. Therefore these myofibroblast-like FLS in RA synovium are thought to contribute to the tissue destruction and angiogenesis which is often seen in this disease.

Szodoray and colleagues used a genome scale microarray to identify differentially regulated genes in peripheral blood B cells from eight RA patients with early disease compared to eight healthy controls [336]. They discovered that 305 genes were overexpressed in RA B cells and 231 genes were underexpressed compared to controls. Clusters of functionally associated networks were analysed and five biological classes were defined: autoimmunity; cytokines; cell activation and apoptosis; neuro-immune regulation and angiogenesis. A microarray analysis has also been performed on isolated CD4⁺ T cells, the study by Li et al analysed the microRNA (miRNA) profile of CD4⁺ T cells isolated from the synovial fluid of RA patients compared to CD4⁺ T cells isolated from healthy controls [337]. Analysis of the results demonstrated that miR-146a was upregulated in SF CD4⁺ T cells whilst miR-363 and miR-498 were downregulated. Subsequent studies indicated that miR-146a overexpression suppressed T cell apoptosis since one of the genes it regulated was Fas associated factor 1 (FAF1) and therefore the upregulation of this miR could promote the long-term survival of the pathogenic T cells.

There is also one microarray gene expression study that has been performed specifically on monocytes from RA patients, Stuhlmüller et al compared monocytes from the first leukapheresis pool (activated cells) and third leukapheresis pool (nonactivated cells) of 26 RA patients undergoing leukapheresis treatment to identify known and novel genes in the activated monocytes [338]. This analysis identified 482 differentially expressed genes between the first and third pools, the activated monocytes were found to express inflammatory genes such as IL-1B, IL-6 and TNF α whilst the nonactivated monocytes expressed differentiation and transcription genes such as PU.1.

Despite the enormous advances in the field of transcriptomics and microarray analyses there appears to be very few studies investigating the genetic signature of individual cell types within arthritis which could be masked in a full scale microarray of whole blood or synovium. Since individual cell types are known to play diverse roles in the pathogenesis of arthritis understanding the genetic signal of the different activated cell types may aid in unravelling the causes and different treatment mechanisms of this disease.

1.3.1.2 Psoriatic arthritis

In comparison to the vast amount of microarray analysis that has been performed on RA patients there has been a paucity of research into the genetic signature of PsA. In addition, there has been a great deal of progress into identifying the genetic phenotype of psoriasis but very few studies analysing the arthritis that will often accompany or follow this condition have been performed. One of the few microarray studies analysing PsA took place in 2002, Gu and colleagues investigated the genetic profiles of PBMCs from RA patients (n=6), spondyloarthropathy (SpA) patients (n=7) and PsA patients (n=6) compared to healthy controls (n=7). They established an upregulation of several genes in the three types of arthritis in comparison to healthy controls including those coding for chemokine receptors (CCR1 and CXCR4), cytokines (IL-1 β) and the signalling molecules JAK3 and MAP kinase p38 [339]. They also found an upregulation of the chemokine receptor CXCR4 in the three arthritis groups, the ligand for CXCR4 is SDF-1 and levels of this chemokine were detected in the SF of the SpA and RA patients. Since SDF-1 is a chemokine for lymphocytes and monocytes it was suggested to be potentially important pro-inflammatory mediator within the pathogenesis of RA, SpA and PsA.

Despite the interesting observations of the above study, due to the method by which they grouped the microarray results together for all three different arthritis subsets it proved difficult to discriminate the specific gene profile of the PsA patients. However, two subsequent studies used microarray technology to compare the PBMCs of PsA patients to those of healthy controls. The first study performed by Batliwalla and colleagues analysed the gene expression signature of peripheral blood cells from 19 PsA patients compared to that of age and sex matched healthy donors [340]. They found that 257 genes were

expressed at a lower level in PsA patients and 56 genes were expressed at a higher level in comparison to controls. The genes that were downregulated in the PsA patients were found to be involved in the suppression of innate and acquired immune mechanisms suggesting that these cells favoured a pro-inflammatory response. Pro-inflammatory genes such as S100A8 and S100A12 demonstrated increased expression in the PsA samples. In addition, using a dataset of RA samples for comparison it was found that the combination of two genes MAP3K3 and CACNA1S could correctly classify the RA patients and PsA patients. Therefore this gene expression profiling was able to differentiate PsA from control subjects and PsA from RA patients indicating that PsA has a distinctive blood cell gene expression pattern. Stoeckman et al also performed a similar microarray analysis comparing the PBMCs of PsA patients to those of age and sex matched healthy controls and discovered that 310 genes were differentially expressed with the majority being upregulated in PsA [341]. It was also found that this differential gene expression profile did not overlap with those of RA or SLE patients and that individual genes, such as ZNF395, had high discriminatory potential for disease diagnosis indicating that PsA patients had a unique gene expression profile which could reveal novel disease markers.

At present these three microarray analyses are the only studies to be performed investigating the gene expression pattern of PsA. There have been no studies into the gene expression of synovial tissue or individual cell subsets and the effect of different drug therapies on the genetic signatures of diseased cells, which has been very informative in the field of RA, have not been performed. As a result very little is known about the transcriptomics of PsA and therefore more investigation is required in this field in order to gain more information into the pathogenesis of this disease and to aid clinicians in deciding the optimal therapy for the patients.

See Table 1.8 for a summary of the gene expression studies performed in RA and PsA.

Table 1.8 Genomic studies in RA and PsA

Disease	Tissue	Number of samples	Approx number of genes on array	Comparison	Results	Ref.
RA	Synovium	21 RA and 9 OA	11,500 and 18,000	Within RA and versus OA	Evidence for the existence of multiple pathways for tissue destruction	[318, 319]
RA	Synovium	5 RA and 10 OA	5,760	RA versus OA	Differentially expressed genes between RA and OA	[320]
RA	Synovium	12 early and 4 late	23,040	Early versus longstanding RA	Early RA fell into two groups dependent on the expression of genes critical for proliferate inflammation	[321]
RA	Synovium	4 early untreated, 4 treated longstanding, 7 controls	10,000	Early RA versus LS RA, early RA versus healthy, LS RA versus healthy	Early and LS RA have separate molecular signatures with distinct biological processes participating during the course of disease	[322]
RA	Synovium	13 RA. 7 biopsies obtained by orthopaedic sampling, 6 by arthroscopy	46,000	Biopsies from the same patient compared, orthopaedic versus arthroscopy	There is a substantial level of heterogeneity between pairs of patient samples. Orthopaedic biopsies are unsuitable for gene expression analysis	[323]
RA	Synovium	62 patients before infliximab treatment	17,927	Treatment responders versus non-responders	No difference between responders and non-responders was detected therefore synovial biopsy samples may not be appropriate to identify non-responders to infliximab treatment	[324]

Table 1.8 continued

RA	PBMCs	8 RF ⁺ , 6 RF ⁻ , 7 healthy controls	10,000	RF ⁺ versus RF ⁻ and versus healthy control	No differentially expressed genes between RF ⁺ and RF ⁻ patients. Increased expression of immunoinflammatory response genes in RA	[325]
RA	PBMCs	29 RA and 21 healthy controls	12,626	RA versus HC	Monocyte associated gene signature increased in RA	[326]
RA	PBMCs	9 RA and 13 healthy controls	6937	RA versus HC	Diverse functional subsets of genes were differentially expressed between RA and HC samples. Ten genes with increased expression in RA PBMCs mapped to RA susceptibility locus 6p21.3	[327]
RA	PBMCs	23 RA	4500	Comparison between patients according to anti-CCP level, DAS-28, HLA-shared epitope presence and treatment type	Different numbers of genes were exclusively associated with each disease variable	[328]
RA	Whole blood	35 RA and 12 healthy controls	18,000	within RA and versus HC	Assignment of a type I IFN signature in a subpopulation of patients	[329]
RA	PBMCs	18 RA and 15 healthy controls	48,701	RA versus HC	Elevated expression of genes associated with immunity and defense in RA patients	[330]

Table 1.8 continued

RA	Whole blood	44 RA before infliximab treatment	47,000	treatment responders versus non-responders	An 8-gene expression profile was able to predict treatment response [331]
RA	PBMCs	19 RA	18,500	Before versus 72 hours after etanercept	Gene pairs and triplets predictive for response to treatment at an early stage of treatment [332]
RA	PBMCs	32 RA before anakinra treatment	12,000	treatment responders versus non-responders	A 52-gene expression profile was demonstrated to predict treatment response [333]
RA	FLS	5 RA and 5 HC	588	RA versus HC	Increased expression of genes responsible for tumour-like growth in RA FLS [334]
RA	FLS	19 RA	18,000	Within RA	The heterogeneity between synovial tissue is reflected in the FLS [335]
RA	B cells	8 RA and 8 HC	21,329	RA versus HC	B cell biology in RA is dysregulated due to the differential expression of multiple biological pathways [336]
RA	CD4 T cells	2 RA and 1 HC	47,000	RA SF versus HC PB CD4 ⁺ cells	RA CD4 ⁺ cells overexpress micro-RNA's which help promote the long term survival of the T cells [337]
RA	Monocytes	29 RA	482	Monocytes from first leukapheresis compared to monocytes from third leukapheresis pool	Activated monocytes from first leukapheresis pool had an increased expression of pro-inflammatory genes [338]

Table 1.8 continued

PsA	PBMCs	6 RA, 7 SpA, 6 PsA, 7 HC	588	Three forms of arthritis versus HC	The three forms of arthritis similarly [339] upregulate pro-inflammatory molecules in comparsion to HC
PsA	PBCs	19 PsA, 19 HC	22,215	PsA versus healthy	Downregulated genes in PsA [340] compared to HC consisted of genes associated with suppression of immune mechanisms
PsA	PBMCs	16 PsA, 15 HC	54,000	PsA versus healthy	PsA genetic profiles showed no [341] overlap with RA or SLE profiles and were distinct from control profiles

Chapter 2 Materials and Methods

2.1 Patients and controls

RA and PsA patients who fulfilled the American College of Rheumatology criteria for RA [6] or met diagnostic criteria for PsA [8] were identified from the Rheumatology Clinics in Glasgow Royal Infirmary (GRI) and Glasgow Gartnavel Hospital (GGH) and were invited to take part in the study. All patients and healthy donors gave informed consent and the study protocol was approved by the Ethical Committee, Glasgow Royal Infirmary, Scotland.

2.2 Cell Culture

2.2.1 Primary Human Cell Culture

All tissue culture work was performed in laminar flow hoods and all cells were cultured at 37°C, 5% CO₂ in RPMI-1640 medium (Invitrogen) containing L-glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 µg/ml) and 10% foetal calf serum (complete medium).

2.2.2 Purification of mononuclear cells from buffy coats and peripheral blood

Peripheral blood mononuclear cells (PBMC) were purified by standard density gradient centrifugation. Briefly, blood was obtained from either single donor plateletpheresis residues donated by Gartnavel blood bank or from healthy donors. The blood was then diluted 1:1 in phosphate buffered saline (PBS) (Invitrogen) and 10 mls were overlaid on 4 mls Histopaque®-1077 (Sigma) in 15 ml centrifuge tubes. Tubes were centrifuged at 350 g for 20 minutes at room temperature. The mononuclear cells were then harvested from each tube and washed three times at 200 g for 5 minutes in ice cold PBS containing 2 mM ethylene diamine tetra-acetic acid (EDTA) and 0.5% human serum albumin, (PEA) before cell purification.

2.2.3 Purification of mononuclear cells from synovial fluid

Before the mononuclear cells were isolated from patient synovial fluid (SF) the fluid was centrifuged for at 350 g for 20 minutes in order to pellet the cells. The cell free synovial fluid was collected and stored at -70°C. The pellet of cells from the synovial fluid was resuspended in PEA before being overlaid on 4 mls Histopaque®-1077 and centrifuged at 350 g for 20 minutes. The mononuclear

cells were then harvested and washed three times in PEA before cell purification.

2.2.4 Isolation of CD3⁺ T cells and CD14⁺ monocytes

CD3⁺ T cells and CD14⁺ monocytes were purified by positive selection from mononuclear cell suspension by autoMACS, according to the manufacturer's instructions. Briefly, mononuclear cells were incubated in 90 $\mu\text{l}/10^7$ cells PBS/2 mM EDTA/0.5% human serum albumin (PEA) containing 10 $\mu\text{l}/10^7$ cells anti-CD3-conjugated magnetic microbeads (Miltenyi Biotec), or 10 $\mu\text{l}/10^7$ cells anti-CD14-conjugated magnetic microbeads (Miltenyi Biotec) for 15 minutes at 4°C. After washing in ice cold PEA by centrifugation at 200 g for 5 minutes CD3⁺ T cells or CD14⁺ monocytes were isolated by positive selection using the possel programme on the autoMACS machine. The positive cell fraction was put through the machine twice on possel programme to ensure a higher purity. Purity was assessed by flow cytometry and routinely >95%.

2.2.5 CD3⁺ T cell and CD14⁺ monocyte stimulation

CD3⁺ T cells were purified by positive selection from peripheral blood and activated for 6 days in complete medium containing IL-2 (25 ng/ml), IL-6 (100 ng/ml) and TNF- α (25 ng/ml) (all from Biosource), in order to produce an activated phenotype similar to that displayed in the synovium [309]. CD14⁺ cells were purified by positive selection from the same peripheral blood and cultured in 96, 24 or 12 well plates depending on the experiment at 5×10^5 cells/ml in complete medium supplemented with 50 ng/ml M-CSF (Biosource). These cells were then incubated for 6 days at 37°C.

2.2.6 T cell / macrophage cell contact

After 6 days in culture the cytokine activated T cells (Tcks) were washed three times in PEA and either fixed in PBS/2% paraformaldehyde on ice for 2 hours, or not (as indicated), and added back to the macrophage culture at a ratio of 4:1. This culture was incubated at 37°C for between 1 to 24 hours depending on the experiment.

2.3 Flow cytometry

2.3.1 Assessment of cell purity

All samples which were isolated on the autoMACS were analysed for purity by Flow cytometry. After positive selection cells were washed in PEA and resuspended at a concentration of 1×10^6 cells/ml. 100 μ l of cells was added to two clean FACS tubes, one for the isotype control and one for the antibody staining. For purity checks the following antibodies were used (all from BD biosciences):

Tube 1. Isotype IgG FITC, IgG APC

Tube 2. CD14 FITC, CD3 APC

5 μ l of each antibody was added to the tube and the cells were incubated at room temperature for 15 minutes in the dark. The cells were then washed with PEA and the supernatant discarded. If the cells were to be analysed immediately they were resuspended in 200 μ l PEA or if they were to be analysed the following day they were resuspended in 200 μ l FACS FIX (2% paraformaldehyde in PBS). The cells were analysed on a FACS Calibur (BD Biosciences) using Cell Quest software™. When assessing the purity of the samples, an analysis gate was set to ensure that within the isotype control sample <2.5% of the cells were positive for FITC or APC staining.

2.3.2 Semaphorin 6D staining

Surface and intracellular semaphorin 6D staining was assessed on Tck cells and CD3/CD28 activated cells after 0, 3 and 6 days of activation.

T cells were obtained from donor blood packs as described in 2.2.4 and half of the T cells were stimulated with IL-2, IL-6 and TNF- α as described in 2.2.5 for 3 and 6 days to produce Tcks. The remainder of the T cells were stimulated with T cell activation expansion kit cytoestim beads (Miltenyi Biotec) according to the protocol. Briefly, the MACSibead particles were loaded by pipetting 100 μ l each of CD2-biotin, CD3-biotin and CD28-Biotin into a 1.5 ml eppendorf and mixing. 500 μ l of Anti-biotin MACSibead particles (1×10^8 Anti-biotin MACSibead Particles) were added to the antibody mix with 200 μ l of PEA and the mixture

was incubated for 2 hours at 4°C under constant, gentle rotation. Once the Anti-Biotin MACSibead particles were loaded they were stored at 4°C until required. To activate the T cells with the Anti-Biotin MACSibead particles 25 µl of beads per 5×10^6 T cells were resuspended in 100 µl complete culture medium and centrifuged at 300 g for 5 minutes after which time the beads were resuspended in 100 µl complete medium. The T cells were resuspended at a density of 5×10^6 cells per 900 µl of complete medium and the 100 µl of beads were added. The cells were cultured in 24 well plates at a concentration of 2.5×10^6 cells per well for 3 and 6 days. In order to remove the beads before the FACS staining commenced the T cells were extracted from the plate and washed before being resuspended in PEA and vortexed thoroughly. The cells were then placed in an easystep magnet (Stemcell) for 2 minutes and the supernatant containing the activated T cells was removed and the process was repeated again. Once all the beads had been removed from the cells they were washed twice in PEA buffer and stained for semaphorin 6D.

The following protocol for intracellular and surface staining of semaphorin 6D was used to stain unactivated T cells at day 0 and the Tcks and CD3/CD28 Anti-biotin MACSibead after 3 and 6 days of activation.

2.3.2.1 Surface staining Semaphorin 6D

T cells were washed and approximately 1×10^6 cells were put into each FACS tube. 10 µl of Fc block (Miltenyi Biotec) was added to each tube and the cells incubated for 5 minutes at 4°C. The cells were washed in PEA at 200 g for 5 minutes and 5 µg of semaphorin 6D antibody (MAB2095, R&D) or 5 µg of irrelevant antibody (CD68 mouse anti human DAKO) was added to the tubes before being incubated for 20 minutes at 4°C. The cells were washed in PEA and 6 µl of goat F(ab')₂ anti-mouse IgG APC secondary antibody (F0101B, R&D) was added to the cells for 10 minutes at 4°C before being washed again. CD4 FITC or isotype FITC (BD biosciences) was added to the relevant FACS tubes for 15 minutes at 4°C and the cells were washed a final time before being resuspended in 300 µl PEA and analysed on the FACS Calibur.

2.3.2.2 Intracellular staining of semaphorin 6D

T cells were washed and approximately 1×10^6 cells were put into each FACS tube. 10 µl of Fc block (Miltenyi Biotec) was added to each tube and the cells

incubated for 5 minutes at 4°C. The cells were washed in PEA at 200 g and 100 µl of cytoperm (BD biosciences) was added for 20 minutes at 4°C, after this time 50 µl of permwash (BD Biosciences) was added to the cells which were then spun at 200 g for 5 minutes. The cells were then resuspended in 100 µl of 2% goat serum/perm wash and incubated at 4°C for 20 minutes; 5 µg of semaphorin 6D, CD68 or mouse IgG₁ was added directly to the cells in 2% goat serum/perm wash and incubated at 4°C for 20 minutes. After this time 500 µl of permwash was added to the cells which were then spun at 200 g for 5 minutes. The cells were resuspended in 100 µl of permwash and 5 µl of goat anti-mouse APC (Invitrogen) was added before the cells were incubated at 4°C for 10 minutes. For the final step the cells were washed in permwash and resuspended in 200 µl permwash before being analysed on the FACS Calibur.

2.4 Enzyme-linked-immunosorbent assay (ELISA)

ELISA was used to measure concentration of human IL-6 and TNF α in cell culture supernatants. The concentrations of antibodies and buffers used for each ELISA are shown in Table 2.1.

96 well plates (Thermo Labsystems) were coated with detection antibody in PBS and incubated overnight at 4°C. The plates were then washed with 0.05% PBS/Tween followed by the addition of blocking buffer (0.5% BSA/PBS) to block non-specific binding and incubated at 37°C for 1 hour. An eight point standard curve was made using recombinant human cytokine dissolved in complete media at a top standard of 2000 pg/ml and serially diluted 1:2. 100 µl of each standard was added in duplicate to the plate along with two wells containing only complete media. Samples were then diluted 1:2-4 with complete media (donor dependant) and 100 µl transferred to each well. The plates were covered and incubated for 2 hours at room temperature. The plates were washed several times in 0.05% PBS/Tween followed by addition of secondary antibody and incubated at 37°C for 1 hour. After incubation the plates were washed and 100 µl TMB chromagen (Biosource) was added to each well. The reaction was stopped by addition of 100 µl stop solution (Biosource) and the intensity of the signal was read at 450 nm on a microplate reader (Dynex Technology).

Cytokine	Concentration of primary antibody	Concentration of secondary antibody	Streptavidin HRP dilution	Kit supplier
TNF α	2 μ g/ml (100 μ l/well)	1.5 μ g/ml (100 μ l/well)	1:1250	Invitrogen
IL-6	2 μ g/ml (100 μ l/well)	1.5 μ g/ml (100 μ l/well)	1:1250	Invitrogen

Table 2.1 Cytokine analysis by ELISA

2.5 Microarray

2.5.1 Collecting and preparing samples for microarray

All CD14⁺ blood and synovial fluid samples were collected by density gradient centrifugation and magnetic bead positive selection as described in 2.2.2 and 2.2.3. An aliquot of each sample was taken to check for cell purity and the sample was lysed in trizol (Invitrogen) (2x10⁶ cells/ml trizol) and stored at -70°C.

2.5.2 Isolating cells using the FACS ARIA

For the cell contact activated macrophages the following protocol was used. Cytokine activated T cells (Tcks) were washed three times in PEA, CFSE (Invitrogen) dye was made up in Hank's buffered salt solution (HBSS) media (Invitrogen) at a concentration of 10 μ M and 1 ml was added to the Tcks before incubating for 10 minutes at 37°C. Tcks were washed three times in PEA and then fixed in PBS/2% paraformaldehyde on ice for two hours. After washing three times in PEA the fixed CFSE stained Tcks were added to macrophages in a 24 well plate at a 4:1 ratio and left in cell contact for 2 hours. Following the 2 hour cell contact the supernatant was collected and retained for cytokine analysis by ELISA and 0.5 ml cell dissociation solution (Sigma) was added to each well. The plate was incubated for 15 minutes at 37°C to remove the adherent macrophage cells. Any remaining cells that were seen to be still adhering to the plate on inspection under a brightfield microscope were incubated with additional cell dissociation solution. All of the cell suspensions were washed in

PEA and pooled together for isolating on the FACS ARIA. The two cell fractions of macrophages and fixed CFSE stained Tcks were separated on the basis of FITC positive and FITC negative therefore all the FITC negative cells were believed to be macrophages. Cells were counted as they were being separated on the FACS ARIA and cell fractions were lysed in Trizol (2×10^6 cells/ml Trizol) and stored at -70°C .

A small aliquot of each cell fraction was taken for cytopins to ensure we had collected the correct cell type in each fraction.

2.5.2.1 Cytopins

An aliquot of cells isolated from the FACS ARIA were resuspended at a concentration of 0.5×10^6 cell/ml in PEA. Glass slides were labelled and placed into the metal holders with a piece of filter paper and a cuvette, 200 μl of the cell suspension was loaded into the cuvette and the cells were spun at 350 rpm for 6 minutes. The slides were then removed and the cuvette was carefully detached to avoid damaging the smear of cells. The cells were immediately stained with RAPI-DIFF reagents (Ready reagents). The slides were placed in a Coplin jar containing solution A for 15 seconds and then placed in a Coplin jar containing solution B for 15 seconds finally the slides were washed twice in Phosphate buffer pH 6.8 for 10 seconds and then left to dry before being imaged on a light microscope.

2.5.3 Manual RNA Isolation from Trizol

The cells isolated from the cell contact experiments and patient samples were stored in Trizol and transferred to GlaxoSmithKline (GSK) in Stevenage for mRNA extraction. QIAgen mini columns were used for the RNA clean-up with an incorporated on column DNase step. Briefly, 200 μl of chloroform was added to each epindorff of cells containing 1 ml of Trizol and centrifuged at 12,000 g for 3 minutes. The aqueous supernatant was removed to a clean epindorff and an equal volume of 70% ethanol added. This solution was applied to a QIAgen RNeasy mini-column and the protocol supplied with the QIAgen kit was followed to extract the RNA. Briefly, the RNeasy column was centrifuged at 13,000 g for 30 seconds at room temperature and the flow through discarded. The sample was then washed by adding 700 μl of solution RWT and the column was spun at

13,000 g for 1 minute at room temperature and the flow through was discarded. A DNase-1 stock solution (Qiagen) was prepared in 550 µl RNase-free water and 10 µl of this solution was added to 70 µl buffer RDD. 80 µl of DNase-1/RDD buffer was pipetted onto each column and incubated at room temperature for 15 minutes. Following this step 500 µl of buffer RW1 was added to each column which was subsequently spun at 12,000 g for 1 minute. The sample was then washed twice by adding 500 µl of solution RPE and spinning the column at 13,000 g for 1 minute. The column was then transferred to an RNase-free microfuge tube and 35 µl of RNase-free water was added to the column making sure the liquid was centred on the membrane. The sample was allowed to stand for 1 minute to allow the RNA to dissolve into the water and the microfuge tube was spun for 1 minute at 13,000 g. The RNA sample was then re-pipetted onto the column membrane and allowed to stand for 1 minute in order to increase RNA yield before being centrifuged at 13,000 g for 1 minute. All RNA samples were subsequently stored at -70°C.

The concentration of each RNA sample was measured on a nanodrop spectrophotometer and the quality of the RNA was analysed on an Agilent 2100 bioanalyser using the agilent RNA 6000 pico kit (Agilent Technologies). When using the Agilent bioanalyser the sample RNA was diluted to 5 ng/ml in deionised water before being put on the pico chip for analysis.

RNA was then diluted to 10 ng/µl using water on a Beckman Coulter Biomek FX machine. 5 µl of this RNA was transferred to a 96 well cone plate (Greiner) for amplification.

2.5.4 Single strand cDNA amplification

Single strand amplification was performed using NuGEN RNA amplification system V2.

2.5.4.1 First strand cDNA synthesis

For the primer annealing stage 2.5 µl of A1 primer was added to each 5 µl RNA sample and the 96 well plate was placed in a thermal cycler for 5 minutes at 65 °C. After this time the samples were removed and snapcooled by placing them on ice.

The first strand master mix was prepared as follows (volumes listed are for a single sample)

First strand buffer mix	12 μ l
First strand enzyme mix	1 μ l
<u>Total Volume</u>	<u>13 μl</u>

13 μ l of the first strand master mix was added to each sample and the samples placed in a thermal cycler programmed to run as follows:

Incubate at 48°C for 60 minutes

Heat at 70°C for 15 minutes

Cool to 4°C

Once the temperature had reached 4°C the plate was removed from the thermal cycler and spun in a centrifuge to collect condensation before being placed on ice.

2.5.4.2 Second strand cDNA synthesis

The second strand mastermix was prepared as follows (volumes listed are for a single sample)

Second strand buffer mix (B1)	18 μ l
Second strand enzyme mix (B2)	2 μ l
<u>Total Volume</u>	<u>20 μl</u>

A multichannel pipette was used to add 20 μ l of second strand master mix to each well containing the first strand reaction mixture in the 96 well plate. The plate was briefly centrifuged for 2 seconds to remove air bubbles before being placed in a pre-warmed thermal cycler set to the following programme:

Incubate at 37°C for 30 minutes

Continue heating at 75°C for 15 minutes

Cool to 4°C

Once the programme had completed the plate was removed from the thermal cycler and spun briefly before being placed on ice.

2.5.5 SPIA™ amplification

The SPIA mastermix was prepared as follows (volumes are listed for a single sample):

SPIA buffer mix (C2)	72 µl
SPIA primer mix (C1)	2 µl
Water	4 µl
SPIA enzyme mix (C3)	40 µl
<u>Total Volume</u>	<u>118 µl</u>

A multichannel pipette was used to add 118 µl of the SPIA mastermix to each well of the 96 well plate containing the second strand reaction mixture. The plate was briefly centrifuged before being placed in a thermal cycler programmed to incubate the samples at 48°C for 30 minutes. After this time a further 6 µl of SPIA primer mix (C1) was added to each well of the 96 well plate containing the SPIA reaction mixture. The 96 well plate was centrifuged for 2 seconds and placed back into the thermal cycler to run on the following programme:

Incubate 48°C for 30 minutes

Continue heating at 95°C for 5 minutes

Cool to 4°C

The plate was removed from the thermal cycler and centrifuged for 2 seconds before being placed on ice.

2.5.6 Purification of amplified cDNA

Purification of the amplified cDNA is required if it is intended for fragmentation, labelling and GeneChip array analysis. All amplified cDNA was purified according to the Agencourt RNAClean magnetic beads protocol.

The magnetic beads were resuspended by gently agitating the RNAClean bottle before being dispensed into a clean reagent reservoir. 288 µl of beads were added to one matrix tube per sample. The amplified cDNA sample (160 µl) was then pipetted into a matrix tube and incubated at room temperature for 10 minutes to allow the cDNA to bind to the magnetic beads.

A round bottomed 96 well plate was placed onto the plate magnet and for each sample half of the bead/cDNA mixture (220 µl) was added into each well and incubated for 10 minutes at room temperature. After this time the supernatant was carefully removed and discarded using a multichannel pipette.

The remaining 220 µl of bead/cDNA mixture was added to the same wells and again the plate was incubated for 10 minutes at room temperature before removing the supernatant from the wells.

Each sample was washed by dispensing 200 µl of 80% ethanol to each well for 30 seconds after which time the ethanol was removed and discarded. This step was repeated ensuring that all ethanol was removed before leaving the samples to air dry for 2 minutes.

34 µl of water was added to each sample on the plate and the plate was left undisturbed for 5 minutes, after this time the plate was tapped gently to mix and left for another 5 minutes. The plate was then placed onto the magnet and incubated for 10 minutes.

The cDNA concentration of each sample was measured on a nanodrop spectrophotometer and the quality of the cDNA was analysed on an Agilent 2100 bioanalyser using the agilent RNA 6000 pico kit. When using the Agilent bioanalyser the sample cDNA was diluted to 5 ng/ml in deionised water before being put on the pico chip for analysis.

2.5.7 Fragmenting and biotin labelling of the amplified cDNA

For fragmenting and labelling, 5 µg of cDNA in a total volume of 25 µl was used. Fragmenting and labelling was performed using the FL-Ovation™ cDNA Biotin Module V2 kit from Nugen.

2.5.7.1 Fragmentation

The fragmentation master mix was prepared as follows (volumes listed are for a single sample)

FL1	5 µl
FL2	2 µl
<u>Total Volume</u>	<u>7 µl</u>

7 µl of master mix was added to each cDNA sample in a 96 well plate and mixed by pipetting the sample several times. The plate was centrifuged briefly to remove any air bubbles and then was placed into a thermal cycler set to the following programme:

Incubate 37⁰C for 30 minutes

Incubate 95⁰C for 2 minutes

Cool to 4⁰C.

The plate was centrifuged briefly and placed on ice before proceeding with the labelling step.

2.5.7.2 Labelling

The labelling buffer mix (FL3), labelling reagent (FL4) and labelling enzyme (FL5) were obtained from the product box stored at -20⁰C 15 minutes prior to completion of the fragmentation reaction to allow the products to thaw to room temperature.

The labelling master mix was prepared as follows (volumes listed are for a single sample):

FL3	15 µl
FL4	1.5 µl
FL5	1.5 µl
<u>Total Volume</u>	<u>18 µl</u>

18 µl of master mix was added to each cDNA sample in a 96 well plate and mixed by pipetting the sample several times. The plate was centrifuged briefly to remove any air bubbles and was then placed into a thermal cycler set to the following programme:

Incubate 37⁰C for 60 minutes

Incubate 70⁰C for 10 minutes

Cool to 4⁰C

After completion of the programme the plate was removed from the thermal cycler and centrifuged briefly before continuing with the hybridization step.

2.5.8 Array hybridization

Array hybridization was performed using the GeneChip Hybridization Control Kit (Affymetrix). The biotin-labelled cDNA was hybridized onto Human genome U133 Plus 2.0 array cartridges (Affymetrix).

The Affymetrix cartridges were removed from the refrigerator one hour before use to allow them to equilibrate to room temperature.

The hybridization cocktail was prepared as follows (volumes listed are for a single sample):

2x hybridization buffer	110 µl
Affymetrix B2	3.7 µl
Affymetrix 20X controls	11 µl
Invitrogen BSA	2.2 µl
Invitrogen herring sperm DNA	2.2 µl
DMSO	22 µl
Water	19 µl
<u>Total volume</u>	<u>170.1 µl</u>

170 µl of hybridization cocktail was added to a 96 well plate (one well per sample) into which 50 µl of fragmented and labelled cDNA was added. After pipetting several times to mix the sample the 96 well plate was placed in a thermal cycler set to the following programme:

Incubate 95⁰C for 3 minutes

Incubate 45⁰C for 5 minutes

Cool to 4⁰C

The plate was removed from the thermal cycler and centrifuged at 350 g for 5 minutes.

For each sample 200 µl of sample/hybridization cocktail was removed from the 96 well plate and dispensed into an Affymetrix chip. The chips were secured in a 45⁰C hybridization oven and spun at 60 rpm for 16 hours.

2.5.9 Staining and Scanning

Genechip fluidic station 450 (Affymetrix) was used to wash and stain the probe chips.

Wash buffers were made up the day prior to staining and scanning the chips. Wash buffer A was prepared as follows:

20% SSPE	300 ml
10% Tween	1 ml
Water	699 ml
<u>Total volume</u>	<u>1000 ml</u>

150 ml of wash A was aliquoted into wash station bottles (one bottle per one Affymetrix chip).

Wash buffer B was prepared as follows:

MES buffer	83.2 ml
NaCl	5.3 ml

10% Tween	1 ml
Water	910.5 ml
<u>Total Volume</u>	<u>1000 ml</u>

150 ml of wash buffer B was aliquoted into wash station bottles (one bottle per Affymetrix chip).

The fluidics stations were primed and for each fluidic station three bottles containing wash A buffer, wash B buffer and water were placed in the fluidic stations.

The staining solutions to stain the chips were prepared as follows.

Streptavidin Phycoerythrin (SAPE) solution (volumes listed are for a single sample, 600 μ l each for the first and third stain):

2x stain buffer	600 μ l
BSA	48 μ l
SAPE	12 μ l
Water	540 μ l
<u>Total Volume</u>	<u>1200 μl</u>

Antibody solution (volumes listed are for a single sample):

2x stain buffer	300 μ l
BSA	24 μ l
Goat IgG	6 μ l
Antibody	3.6 μ l
Water	266.4 μ l
<u>Total Volume</u>	<u>600 μl</u>

The empty vials on the fluidics station were replaced with 600 μ l each of SAPE (first and third stain) and antibody (second stain).

The Affymetrix chips were removed from the hybridization oven and the hybridization mix was extracted from the chip. Each chip was inserted into a fluidics station where they were washed and stained, after this time the chips were removed and checked for air bubbles. If air bubbles were found the chips

were put back on the fluidic station to be re-filled with wash buffer, if no air bubbles were found the chips were then scanned.

2.5.10 Scanning

Tough-spot (Cole-Parmer) labels were used to cover the two septa on the affymetrix chip to prevent leakage of wash buffer. Smudges and dust were removed from the glass front of the chip and the chips were loaded into the chip carousel. The chips were scanned using GeneChip® Scanner 3000 and analysed using Affymetrix GeneChip Command Console software.

2.6 PCR and SYBR green

The primer sequences used for SYBR green are shown in Table 2.2.

Table 2.2 Primer sequences used for RT-PCR

Primer name	Forward sequence	Reverse sequence
GAPDH	TCG ACA GTC AGC CGC ATC TTC TTT	ACC AAA TCC GTT GAC TCC GAC CTT
B-actin	AAT GTG GCC GAG GAC TTT GAT TGC	AGG ATG GCA AGG GAC TTC CTG TAA
Legumain	CGC ACA CCA ACA CCA GCC AC	AGG GGG ACG GGA GAA CTG GC
Plexin A1	AGG GAG AAC GGC TGC CTG GT	AGC AGG GAG CGC ACG TTG TC
Sema 3A	TGG GGT TGC CCA GCT CCC TT	TGT GCG TCT CTT TGC AGT GGG
Sema 3F	GGA CAC AGC ATC GGC CCT CA	GAC GGG TCG TTC AGC CAC CG
Sema 6D	GCC GTT CCA CCC ATT GCC GA	ACC ATG CCA GCT TCA GAG CCA

RNA isolation was performed on samples as described in 2.5.3.

2.6.1 cDNA preparation

cDNA was prepared from RNA samples according to AffinityScript™ multiple temperature cDNA synthesis kit. Briefly 100-500 ng of RNA, depending on experiment, was mixed with 3 µl random primers and RNase-free water to a total volume of 15.7 µl. The reaction was incubated at 65°C for 5 minutes before being cooled to room temperature for 10 minutes to allow the primers to anneal to the RNA. The following components were then added to each sample for a total volume of 20 µl.

2.0 µl of 10x AffinityScript RT buffer

0.8 µl of dNTP mix

0.5 µl of RNase Block Ribonuclease Inhibitor (40 U/µl)

1 µl of AffinityScript Multiple Temperature Reverse Transcriptase (RT)

A minus RT (-RT) control was also set up with each set of samples along with a water control. In the case of the -RT control 1 µl of RNase free water was added instead of the AffinityScript Multiple Temperature RT.

The reaction was then incubated for 10 minutes at 25°C to extend the primers prior to increasing the reaction temperature to 42°C for 60 minutes for cDNA synthesis. The reaction was then terminated by incubating it at 70°C for 15 minutes.

2.6.2 PCR

2.6.3 SYBR green

SYBR green was performed using SYBR green mastermix (Applied Biosystems). Prior to setting up the SYBR green the cDNA was diluted 1 in 5 using RNase-free water. The reaction mix was prepared as follows (volumes listed are for a single sample):

Mastermix	10 µl
Primer mix	0.8 µl
Water	8 µl

Total volume 18.8 µl

Each sample was measured in triplicate therefore 3 lots of reaction mix were prepared for each cDNA sample. For the triplicate samples 54.5 µl reaction mix was mixed with 6 µl cDNA and 19.8 µl of this total mixture was added to each of the three wells of a MicroAmp fast optical 96 well reaction plate (Applied Biosystems). A non template control was added to the plate where 6 µl of water was mixed with the 56.4 µl reaction mix and a minus RT was also included in the plate using the -RT control cDNA.

Once the plate had been loaded with the cDNA samples it was covered using a MicroAmp optical adhesive cover (Applied Biosystems) and spun at 200 g for 5 minutes. The plate was then transferred to an ABI 7900 H7 Fast-real-time PCR machine (Applied Biosystems) where it was read on the following cycle:

Incubate 48°C for 30 minutes

Incubate 95°C for 10 minutes

40 cycles of 95°C for 15 seconds followed by 60°C for 1 minute

A dissociation curve was subsequently measures on the following cycle:

Incubate 95°C for 15 seconds

Incubate 60°C for 15 seconds

Incubate 95°C for 15 seconds

Using the results of this analysis, the expression of target genes relative to the housekeeping gene was then quantified.

2.7 Immunohistochemical methods

2.7.1 Antibodies

Table 2.3 Antibody source and concentration used for IHC

Antibody	Manufacturer	Source	Working concentration
Legumain	Prestige Antibodies	Rabbit	0.5 µg/ml
Plexin A1	Cell Signalling	Rabbit	1.02 µg/ml

Table 2.3 shows the antibodies that were used for single immunohistochemical staining for light microscopy and the concentration of the antibody used.

2.7.2 Single immunohistochemical staining

Sections containing sections of RA synovial membrane were initially heated to 65°C for 35 minutes after which time they were de-waxed in xylene and rehydrated through an ethanol gradient to water/TBS Tween (TBST). To reduce non-specific background staining endogenous peroxidase activity was blocked using hydrogen peroxide (H₂O₂) and methanol. Fixation of tissue specimens forms cross-links that mask the antigenic sites, thereby giving weak or false negative staining for immunohistochemical (IHC) detection of proteins. Antigen retrieval using citrate buffer is designed to break the cross-links thereby unmasking the antigens and epitopes in the tissue. Antigen retrieval was performed in 0.01 M citrate buffer pH 6.0, the buffer was heated in a microwave for 5 minutes then the sections were added to the buffer and were heated for 8 minutes before being left to cool for 15 minutes. The sections were then blocked for 1 hour at room temperature in 2.5% serum of the species in which the secondary antibody was raised. The relevant primary antibody or isotype antibody was applied to the sections which were left overnight at 4°C.

The following day the species specific reagent from the ImmPRESS kit (Vector) was added to the sections for 30 minutes before being washed in TBST and developed with ImmPACT DAB (Vector) for up to 2 minutes until a brown reaction product appeared. Sections were then washed in water and counterstained using hematoxylin. Finally sections were dehydrated in ethanol, cleared in xylene to remove fats and oils and then mounted in DPX (Cellpath Ltd) before being imaged on a light microscope.

2.8 Western blotting

2.8.1 Whole cell lysates

CD14⁺ monocytes from the peripheral blood or synovial fluid of patients and CD14⁺ monocytes and M-CSF matured macrophages from normal donors were washed in PBS. Subsequently, 100 µl of M-PER (Thermo Scientific) with the addition of 1 µl Halt Protease Inhibitor Cocktail (Thermo Scientific) was added to the cells. The cells were solubilised for 30 minutes on ice before centrifugation of the lysates at 16,000 g for 15 minutes. The resulting whole cell lysate supernatants were removed from the cell debris pellet and were stored at -20°C before being used for Western blot analysis.

2.8.2 BCA Assay

To determine the protein concentration of the cell lysates a BCA assay (Thermo Scientific) was performed. For each sample 2.5 µl was added to 72.5 µl of BCA reagent in triplicate on a 96 well plate and left at room temperature for 30 minutes. A standard curve was also set up on the plate using known concentrations (2000 µg/ml - 25 µg/ml) of the protein bovine serum albumin (BSA). The plate was read on a plate reader at 502 nm. The amount of total protein in each sample was determined by comparing the OD value of each sample to a standard curve of values.

2.8.3 SDS-PAGE gel electrophoresis

5µg of protein from the whole cell lysates were incubated with the appropriate volume of 4x NuPAGE LDS sample buffer (Invitrogen) and 10 x NuPAGE reducing agent (Invitrogen) at 70°C for 10 minutes. Samples and seeblue plus2 protein standard (Invitrogen) were run on NuPAGE Bis-Tris gels (4-12%) (Invitrogen) with NuPAGE MES running buffer (Invitrogen) at 100 V for 1 hour. The gel was then transferred onto a nitrocellulose membrane using the i-blot transfer system (Invitrogen).

2.8.4 Western blotting

Following transfer, nitrocellulose membranes were washed in TBS/Tween (0.5 M NaCl and 20 mM Tris pH 7.0 with 0.1% (v/v) Tween-20) and blocked for 1 hour in TBS/Tween containing 5% non-fat milk protein. Membranes were then incubated

with 0.2 µg/ml legumain primary antibody (R&D, AF2199) diluted in TBS/Tween containing 5% non-fat milk protein overnight at 4°C. Following the incubation with the primary antibody the nitrocellulose membranes were washed (5 x 5mins) with TBS/Tween and incubated in the an anti-goat HRP-conjugated antibody (abcam, ab6741) containing 5% non-fat milk containing protein for 1 hour at room temperature. The membranes were then washed in TBS/Tween (5 x 5 mins) and protein bands were visualised using the ECL detection system (GE Life Sciences). Nitrocellulose membranes were incubated in a mixture of equal volumes of ECL solution A (2.5 mM luminol, 0.4 mM p-coumaric acid and 100 mM Tris pH 8.5) and ECL solution B (0.002% hydrogen peroxide and 100 mM Tris pH 8.5) for 1 minute before exposing the membranes to Kodak X-Ray film. Nitrocellulose membranes were then stripped and re-probed with a β-actin primary antibody (1/1000 dilution ab8229 Abcam). Membranes were stripped at room temperature for 1 hour with stripping buffer (100 mM 2-mercaptoethanol, 2% SDS and 62.5 mM Tris pH 7). Nitrocellulose membranes were washed thoroughly in PBS/Tween and checked for residual signal before re-starting the western blotting protocol.

2.9 Legumain activity assay

To measure the activity of the enzyme legumain in our samples an activity assay was performed. The activity of legumain was measured on its ability to cleave a substrate Z-Ala-Ala-Asn-AMC (AnaSpec). Cleavage of this substrate releases the AMC (7-amino-4-methylcoumarin) flurophore which can be detected at 441 nm upon excitation at 342 nm.

2.9.1 Collecting samples for activity assay

M-CSF differentiated samples were washed in PEA prior to lysis. Patient synovial and blood CD14+ cells were isolated by magnetic bead separation and washed in PEA before lysis. All samples were lysed in lysis buffer containing 50 mM citrate, 0.1% CHAPS (pH 5.5), 5 mM of DTT and 0.5% Triton-X 100 [342]. Cell lysates were collected and frozen at -70°C.

2.9.2 Setting up activity assay

Human recombinant legumain (rhLGM) (R&D, 2199-CY-010) was used as a positive control and is supplied as the pro form of the enzyme. To activate the

rhLGM it was diluted to 100 µg/ml in activation buffer containing 50 mM sodium acetate and 100 mM NaCl, pH 4.0 and incubated for 2 hours at 37°C. After this time the rhLGM was diluted to 1 ng/µl in assay buffer containing 20 mM citric acid, 60 mM Na₂HPO₄, 0.1% CHAPS (pH 5.5), 1 mM EDTA, and 1 mM DTT. 50 µl of the rhLGM was loaded into a well of a black welled 96 well plate.

Cell lysate samples were freeze thawed three times before being centrifuged at 10,000 g for 5 minutes, 50 µl of each sample was loaded into a black welled 96 well plate.

The substrate Z-Ala-Ala-Asn-AMC was diluted to 200 µM in assay buffer and the reaction was started by adding 50 µl of substrate to all wells containing sample and rhLGM. A blank well containing 50 µl assay buffer and 50 µl substrate was also put on the plate.

The plate was read on a fluorescent plate reader programmed to take readings at times 0, 5, 10, 15, 20, 25, 30, 60, 90, 120, 150, 180, 210 minutes, the plate was incubated at 37°C for the duration of the readings.

2.10 siRNA

N-TER Nanoparticle siRNA transfection system (Sigma) was used to knockdown the plexin A1 gene in M-CSF derived macrophages using 4 Flexitube plexin A1 siRNA's (Qiagen). To check the transfection system was working in the macrophage cells an Allstars negative control with an Alexa Fluro 555 modification (Qiagen) was used to visualise the cells after transfection. If the nanoparticle transfection system had worked and the siRNA negative control had entered the cells then the cells were seen to be fluorescing orange by light microscope or by FACS analysis. The Allstars negative control siRNA is a nonsilencing siRNA which has no known homology to any known mammalian gene; therefore it is used to check whether the transfection of the cells has any effect on the expression of the gene being investigated.

To set up the siRNA transfection the target (plexin A1) and negative control (Allstars negative control) siRNA were prepared as follows:

5 µM target/control siRNA	13 µl
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siRNA dilution buffer	37 μ l
<u>Total volume</u>	<u>50 μl</u>

The N-TER peptide was then prepared as follows (volumes listed are for each siRNA being tested)

N-TER peptide	8 μ l
Water	42 μ l
<u>Total Volume</u>	<u>50 μl</u>

The 50 μ l of N-TER peptide was added to each 50 μ l siRNA preparation and incubated at room temperature for 20 minutes; the concentration of siRNA at this point was 650 nM.

To transfect the M-CSF differentiated macrophages the siRNA was diluted to a concentration of 5 - 30 nM in complete medium depending on the experiment. The culture medium was then removed from the macrophages and 500 μ l of diluted siRNA was added to macrophages. The cells were incubated for 24 hours at 37°C and after this duration the supernatants were collected for cytokine analysis and the cells were put in 0.5 ml trizol. Alternatively after siRNA treatment, the macrophages were stimulated with Tcks at 4:1 ratio or were stimulated with 10 ng/ml LPS for 24 hours at 37°C. After this period of stimulation the supernatants were collected for cytokine analysis and the cells were put into 0.5 ml trizol.

All 4 siRNA's were tested individually on the macrophages for knockdown of the plexin A1 gene, however in some experiments a mixture of 2 siRNA's were used to see their combined effect. In this case when preparing the siRNA 7.5 μ l of each siRNA was added to the siRNA dilution buffer.

2.11 Real time cell imaging

Macrophages and T cells used for cell imaging were autoMACS purified as described in section 2.2.4.

2.11.1 *Preparing macrophages for imaging*

AutoMACS purified CD14⁺ monocytes were cultured in 24 well plates in complete medium supplemented with 50 ng/ml M-CSF (Biosource) for 5 days. To remove the macrophages 0.5 ml of cell dissociation solution (Sigma) was added to each well and the plate was placed into a 37°C incubator for 10 minutes. The wells were then washed with PEA and scraped to remove any remaining macrophages still adhering to the wells. Macrophages were washed, counted and resuspended in complete medium at concentrations between 2x10⁵ and 6x10⁵ depending on the experiment and plated out on either a 2-well or 4-well chamberslide (VWR). The macrophages were left to adhere overnight in a 37°C incubator.

2.11.2 *Staining macrophages for imaging*

A red fluorochrome called CMTPIX (Invitrogen) was used to stain the macrophages, the dye was diluted to a concentration of 2.5 µM in HBSS media (Invitrogen) and 1 ml was added to each chamberslide well of macrophages. The chamberslides were placed in a 37°C incubator for 20 minutes. After this time the media was removed from each well and 1 ml of complete medium added, the chamberslides were then incubated for a further 30 minutes in a 37°C incubator to allow any excess CMTPIX dye to be secreted from the cells.

2.11.3 *Staining T cells for imaging*

Depending on the experiment, either T cell purified from the blood of a healthy donor on the day of the imaging or Tcks that had been activated in a cytokine cocktail for 6 days were used. If Tcks were to be used they were washed thoroughly three times in PEA before being stained. The T cells/Tcks were dyed green with 1 ml of 10 µM CFSE (Invitrogen) which had been diluted in HBSS medium. The cells were incubated for 10 minutes at 37°C, after this period the cells were washed three times in PEA to remove any excess CFSE. Tcks were either fixed in PBS/2% paraformaldehyde on ice for two hours, or not (as indicated) and the cells were added to the macrophages at a ratio of 1:1 or 4:1.

2.11.4 *Imaging cell contact on microscope*

The T cell - macrophage cell contact was left for varying amounts of time depending on the experiment but was usually imaged after 2 hours on a Carl

Zeiss Axiovert S100 inverted microscope. An automated sequence of events using Openlab software was setup in order to record time lapse images of the cell contact. The sequence of events consisted of an image taken in green, followed by a brightfield image followed by a red image. This series of pictures was taken every 12 seconds for up to 1 hour and recorded the movement of the T cells/Tcks along with their interactions with the macrophages.

2.11.5 *Analysis of real time images*

Analysis of the movies produced from the time lapse images was done on Volocity software (PerkinElmer). Two different types of analysis were carried out measuring the number and duration of interactions and the co-localisation coefficient of interactions.

2.12 Statistical analysis

All microarray results were background corrected by the GC-RMA algorithm and analysed by paired T test or 1-way ANOVA unequal variance (Welsh ANOVA) using Genespring GX 11 software. In addition for specific comparisons both DAVID and IPA software, which use a modified version of Fisher's exact test, were employed. The remaining results are displayed as mean \pm standard deviation and statistical analysis was done by students T test or 1-way ANOVA test, as indicated in figure legends, using the GraphPad Prism 4 software. A p value of < 0.05 was considered statistically significant.

Chapter 3 Transcriptomic analyses of psoriatic and rheumatoid arthritis blood and synovial fluid CD14⁺ cells

3.1 Introduction and Aims

Rheumatoid arthritis (RA) and Psoriatic arthritis (PsA) are both chronic, autoimmune arthritides. The cause of neither disease is, at present, known with both genetic and environmental factors thought to play a role in disease susceptibility and progression thereafter. The synovial tissue in both RA and PsA is characterised by marked cellular infiltration, pronounced angiogenesis and increased cytokine and protease expression [132, 343]. Therapies used clinically in both RA and PsA induce significant improvement in disease activity status but many patients do not respond to existing standard of care [132], consistent with the heterogeneous nature of the diseases: this in turn underlines the need for further research and better therapies specifically targeted at patients who are non-responders to current treatments.

In order to address this gap in the knowledge and understanding of RA and PsA many genome-wide gene expression studies have been undertaken in both diseases comparing the genetic profiles of patients to those of healthy controls in order to acquire a genetic signature unique to each disease [325-327, 329, 330, 340, 341]. Gene expression studies have also been performed on patients undergoing various forms of therapy to identify discriminatory genetic markers which could predict the patient response to therapy [332]. Many of these genetic studies have been performed on peripheral blood mononuclear cells (PBMCs) or whole synovial tissue, which provides interesting information on the genetic expression status of the diseased state as a whole but cellular heterogeneity contained therein may be masking the contribution of individual cell types. Recently a small number of RA gene expression studies have specifically examined B cells [336], CD4⁺ T cells [337] and fibroblasts [334] from diseased patients in order to obtain an understanding of how these individual cell types are contributing to disease.

There is also one microarray gene expression study that has been performed specifically on monocytes from RA patients, Stuhlmüller et al compared monocytes from the first leukapheresis pool (activated cells) and third leukapheresis pool (nonactivated cells) of 26 RA patients undergoing leukapheresis treatment to identify known and novel genes in the activated monocytes [338]. However, at present there are no gene expression studies

looking at CD14⁺ cells in PsA and as yet there have been no studies performed on both blood and synovial CD14⁺ monocyte/macrophage cells from RA or PsA patients. These cells are known to play an important role in disease progression as they are a major source of proinflammatory cytokines in the joint [344] and the number of synovial macrophages has been found to correlate with the degree of disease activity in RA [345]. The inflamed synovia in both RA and PsA have been found to contain similar numbers of macrophages upon comparison [243] therefore elucidating the molecular signature of this cell type in both PsA and RA may lead to a better understanding of its critical role in disease activity.

Due to the lack of transcriptome data from CD14⁺ cells from PsA and RA I therefore sought to determine the gene expression pattern of CD14⁺ cells from the matched peripheral blood and synovial fluid of RA and PsA as well as CD14⁺ cells isolated from healthy volunteer derived blood. A priori comparisons were set up between disease and non-diseased state (healthy versus diseased blood), the site of cell isolation (peripheral blood versus synovial fluid) and the two disease types (RA versus PsA).

3.2 Patients and normal donors

Inflammatory arthritis patients attending Gartnavel General Hospital (GGH) or Glasgow Royal Infirmary (GRI) fulfilled the American college of Rheumatology criteria for RA [6], or met diagnostic criteria for PsA [346]. Information regarding patient characteristics is shown in Table 3.1.

Age-matched normal donors were recruited from the Glasgow Biomedical Research Centre (GBRC) building and all blood samples were taken with informed consent.

Table 3.1 Patient characteristics

Characteristic	RA patients	PsA patients
Mean age (years)	68	51
Female:Male	5:3	4:4
Mean Disease duration (years)	12.2	1.8
ESR mm/hr (mean)	86	37
CRP (mean)	107	115
RF positive (%)	87.5	0
% of patients using DMARDS	62.5	37.5
Anti-TNF therapy (number of patients)	1*	1*

*Anti-TNF therapy was stopped due to patients becoming anti-TNF resistant

3.3 Collecting patient samples

For the microarray study, matched synovial fluid and blood samples were collected from 8 RA patients and 8 PsA patients. CD14⁺ cells were positively selected from the samples using Miltenyi beads and an aliquot of cells were tested by FACS analysis for purity before being put into Trizol. CD14⁺ Miltenyi beads were used since the CD14 surface antigen is present on both monocytes and macrophages [190, 347] therefore these beads should isolate blood monocytes as well as the synovial fluid macrophages. Normal donors who were age and sex matched to patients (Table 3.2) were recruited and from these donors CD14⁺ cells were positively selected from the blood only since by definition no synovial compartment derived population could be ethically derived. An aliquot of unactivated CD14⁺ cells were put into Trizol and the remainder of the monocytes were stimulated with M-CSF for 6 days and then cell contact activated (see chapter 6). Once all the samples had been collected the RNA was extracted from all samples at GSK Stevenage and analysed for quality.

Table 3.2 Patient and normal donor samples collected

Patient	Gender	Age	Age and sex matched normal donor
PsA 1	Female	53	Yes
PsA 2	Female	40	Yes
PsA 3	Male	34	Yes
PsA 4	Male	34	No
PsA 5	Male	80	No
PsA 6	Female	58	Yes
PsA 7	Male	54	Yes
PsA 8	Female	58	No
RA 1	Female	71	No
RA 2	Male	56	Yes
RA 3	Female	62	No
RA 4	Male	69	No
RA 5	Female	78	No
RA 6	Male	69	No
RA 7	Female	69	No
RA 8	Female	77	No

3.3.1 Purity of monocyte samples

The CD14⁺ cells isolated from each patient blood and synovial fluid sample were analysed on a FACS Calibur to check that there were no other contaminating cell types that would affect the transcriptomic profile obtained. Figure 3.1 shows a purity check from one representative PsA patient (A) and one RA patient (B).

3.3.2 RNA quality of patient samples

The RNA quality of the samples was analysed on an Agilent 2100 Bioanalyser which is a microfluidics based platform that analyses RNA qualitatively; RNA samples and a standard (ladder) are loaded into wells on a specifically designed chip containing a fluorescent dye. The dye molecules become incorporated into the RNA and a voltage gradient is applied to the chip allowing the RNA to become separated by size. By comparing the separation of the RNA to that of the ladder, which contains components of known size, a standard curve of migration time versus fluorescence units is plotted called an electropherogram. Viewing the electropherogram can give an indication of the integrity of the RNA sample.

Figure 3.2 (A) is an electropherogram showing an example of good quality RNA. It is characterised by three clear peaks, a peak for the RNA marker (1) and a peak for the 18 S (2) and 28 S (3) ribosomal RNA subunits. There should be a relatively flat baseline between the two rRNA peaks and minimal low molecular weight noise adjacent to the RNA marker peak. Figure 3.2 also shows the RNA quality from the blood and synovial fluid CD14⁺ cells of a representative PsA patient (B) and RA patient (C). All four of the samples show good quality RNA evident by the two clear peaks and no low molecular weight noise. After checking the RNA from each sample was of good quality, cDNA was generated from each RNA sample before being purified, fragmented and labelled. The labelled cDNA was then hybridised onto an Affymetrix GeneChip.

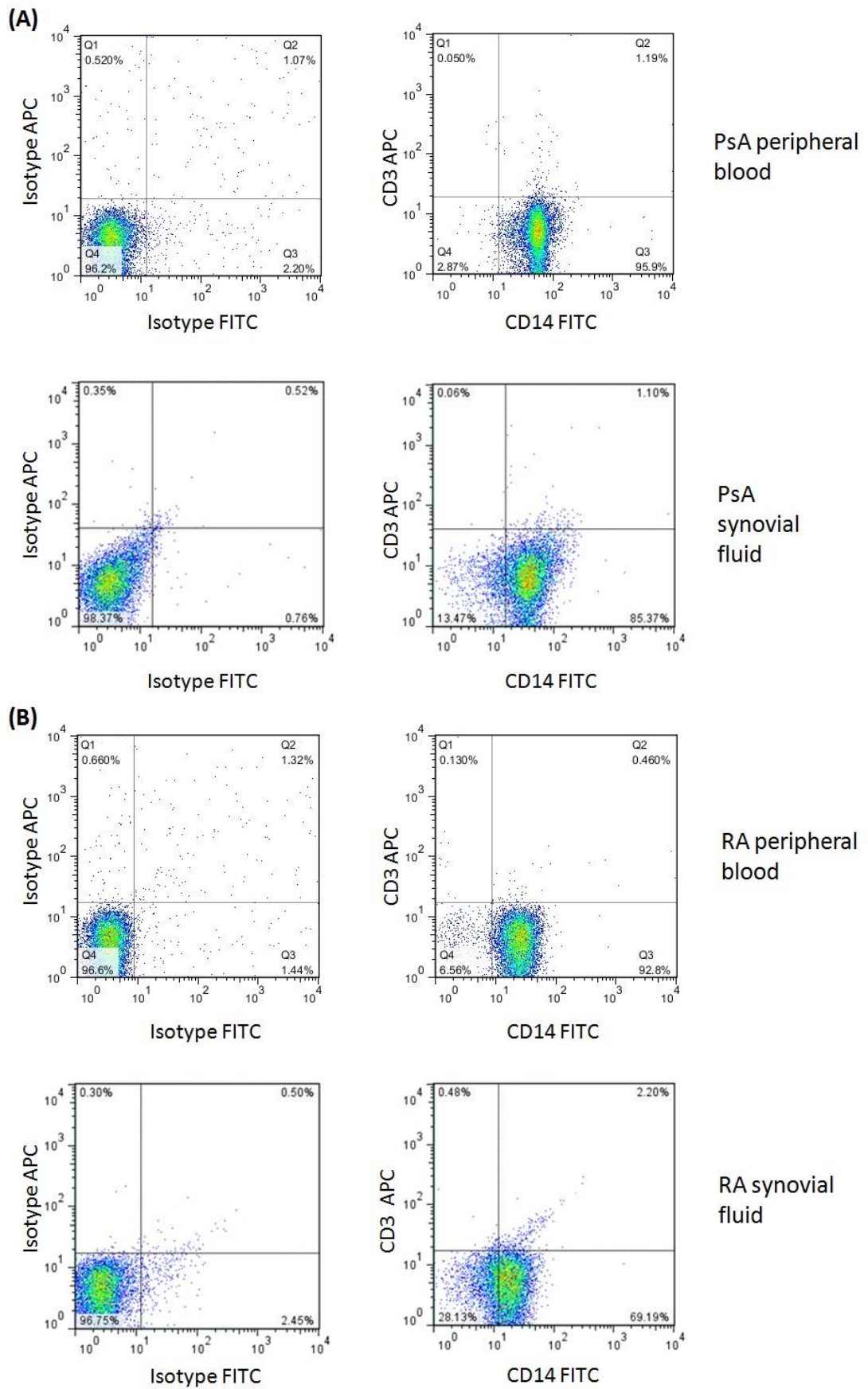


Figure 3.1 Purity of CD14⁺ cells isolated from PsA and RA patient blood and synovial fluid samples

These graphs are a representative of the CD14⁺ cells isolated from the 8 PsA and the 8 RA patients that were analysed on the microarray

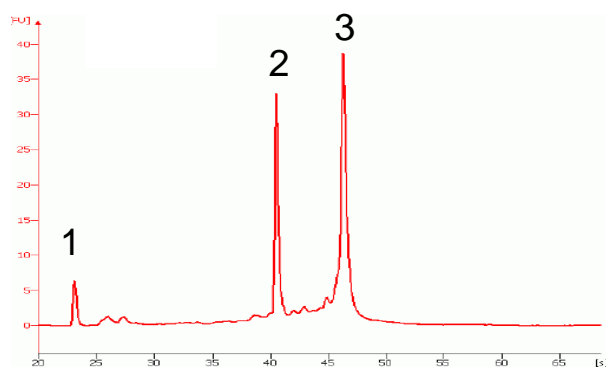
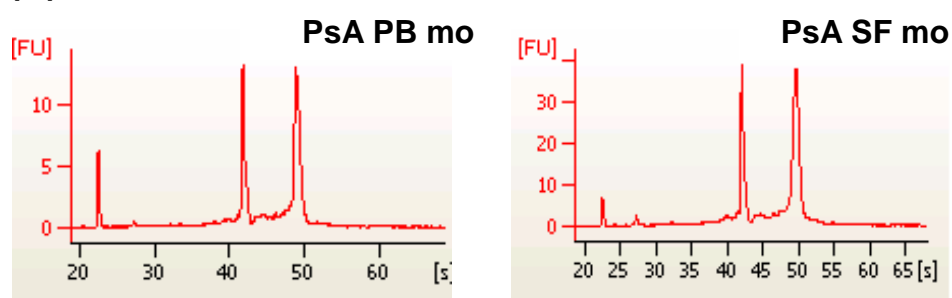
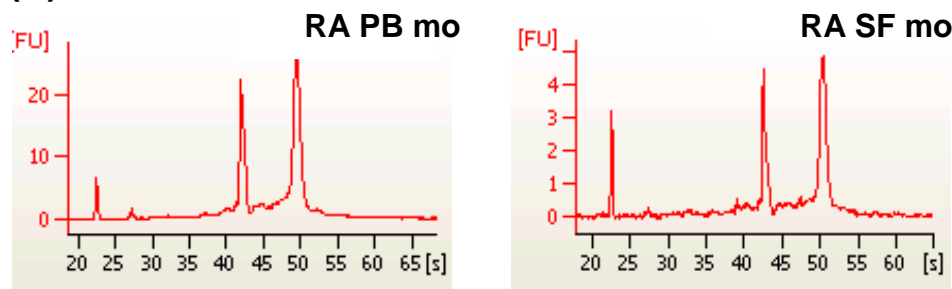
(A)**(B)****(C)**

Figure 3.2 RNA quality of the CD14+ samples from two patients

RNA was extracted and analysed for quality using an Agilent 2100 Bioanalyser. Figure (A) represents an example of good quality RNA. It is characterised by three clear peaks: a peak for the RNA marker (1) and a peak for the 18 S (2) and 28 S (3) ribosomal RNA subunits. The quality of representative PsA patient samples (B) and RA patient samples (C) are shown here and both demonstrate good quality RNA.

3.4 Summary of samples analysed on microarray

The samples that were examined using the microarray GeneChips are summarised in Table 3.3. Eight matched PsA and RA blood and synovial fluid samples were obtained along with 6 age and sex matched healthy controls, unactivated blood CD14⁺ cells were not collected from one control but macrophage cells before and after Tck cell contact activation were collected for all 6 healthy controls. These latter samples will be discussed and analysed in chapter 6.

Table 3.3 Summary of microarray samples

Monocyte/macrophage sample analysed	Number of samples
PsA PB CD14 ⁺	8
PsA SF CD14 ⁺	8
RA PB CD14 ⁺	8
RA SF CD14 ⁺	8
Healthy control PB CD14 ⁺	5
Healthy control M-CSF activated Mø	6
Tck cell contact activated Mø	6

3.5 Analysis of microarray results

Affymetrix GeneChip microarrays are one of the most popular high-density oligonucleotide (oligo) gene expression arrays currently available and have been widely validated for the purposes of the present study - this was therefore our selected methodology of choice. Each gene on an Affymetrix GeneChip is represented by a probe set consisting of 11 pairs of 25-bp oligos covering the transcribed region of the gene. Each probe set pair consists of a perfect match (PM) and a mismatch (MM) oligonucleotide. The PM probe matches exactly the sequence of the gene while the MM probe differs in a single substitution in the central 13th base, the MM probe is designed to distinguish noise caused by non-specific hybridisation from the actual specific hybridisation signal [348]. Affymetrix GeneChips have many sources of variation, for example non-biological variation can arise due to: differences in sample preparation, unequal quantities of starting RNA, differences between chips or differences in hybridisation between chips [349], therefore pre-processing must take place to adjust for these sources of non-biological variation.

Affymetrix microarrays are pre-processed in three steps:

Background correction - this is to adjust for hybridisation effects that are not related to the interaction between probes and target DNA

Normalisation - this is to remove systematic errors and bias, it is necessary as it allows multiple chips to be compared to each other and analysed together. It is performed under the assumption that all chips have approximately the same distribution of PM values.

Summarisation - This is performed using the 11 individual probe intensities from a probe set, combined to yield a single value for each gene that best represents the expression level of the sample RNA transcript.

There are many different algorithm tools that can be applied to the background correction step of Affymetrix data pre-processing: an appropriate choice is dependent upon the experimental design and dataset. Each phase will now be described.

3.5.1 Background correction of the Affymetrix chips

At present there are several different algorithm tools that are used to background correct Affymetrix chips such as MAS5 [350], RMA [351], GC-RMA [352] and PLIER. Upon careful consideration it was decided to use GC-RMA, which is a modified version of RMA, as the background correction for my experiment design and dataset. As mentioned above each gene is represented on the GeneChip by 11 probe pairs made up of a MM and a PM probe, the MM probe is designed to distinguish noise and non-specific background signal from the specific hybridisation signal. However a problem arises when using MM data because in approximately 1/3 of probes the MM intensity levels are higher than the PM intensity levels. This suggests that the MM probes are detecting real signal. In these cases subtracting the MM signal from the PM signal results in a negative signal value and the loss of interesting signal value for those probes. Due to this problem several pre-processing algorithms were designed which only took into account the PM values, an example of which is RMA. RMA excludes MM data completely which reduces noise but also loses information therefore GC-RMA was designed which takes into account non-specific hybridisation using the signal intensity of representative MM probes possessing the same GC nucleotide content as the PM probe of interest.

The background correction in GC-RMA consists of three sequential steps:

1. Optical background correction, this is an important step as the scanner measuring the hybridisation strength of each GeneChip will introduce optical noise
2. Probe intensity adjustment through non-specific binding (NSB) using the optical noise adjusted representative MM probe intensities
3. Probe intensity adjustment through gene-specific binding where the NSB adjusted PM intensities are corrected for the effect of PM probe affinities.

GC-RMA is currently considered to be one of the best methods for estimating expression values of genes. A recent study comparing several pre-processing algorithms found GC-RMA to be the algorithm of choice for detecting differential expression of genes and also the algorithm which produced gene expression data

that was highly correlated to qRT-PCR validation data [353]. Hence, GC-RMA was chosen for pre-processing the Affymetrix GeneChips data produced in the current study.

3.5.2 *Signal histogram of normalised data*

Once the data normalisation had been performed and prior to formal data analysis, all the GeneChips must be analysed to check for outliers that may affect or skew the results interpretation. One method is to examine the signal histogram of the normalised data which plots a line for each of the GeneChips with the intensity of the probes contained on the chip on the x-axis and the frequency of the probe intensity on the y-axis. This then allows the visualisation of the distribution of the signal intensity to identify any outliers. Figure 3.3 illustrates the signal histogram of all the samples that were tested, each line represents one GeneChip and they are coloured by condition with blue representing the cell contact activated macrophage samples, red the M-CSF treated macrophages, purple the healthy donor blood CD14⁺, green the patient blood CD14⁺ and gold represents the synovial fluid CD14⁺ cells. It can clearly be seen on the histogram that all the samples have a similar signal intensity expression apart from one sample which is a GeneChip containing a cell contact activated macrophage sample. The reasons for this outlying sample are unknown but since it is only one sample and the signal intensities of the other samples are relatively similar to each other it is unlikely to represent a problem with the hybridisation, washing or staining of the chips. As a result it is most likely to be due to a problem with the individual sample or the labelling of that particular sample. Since this sample is an outlier and may potentially skew the data interpretation it was removed from further data analysis.

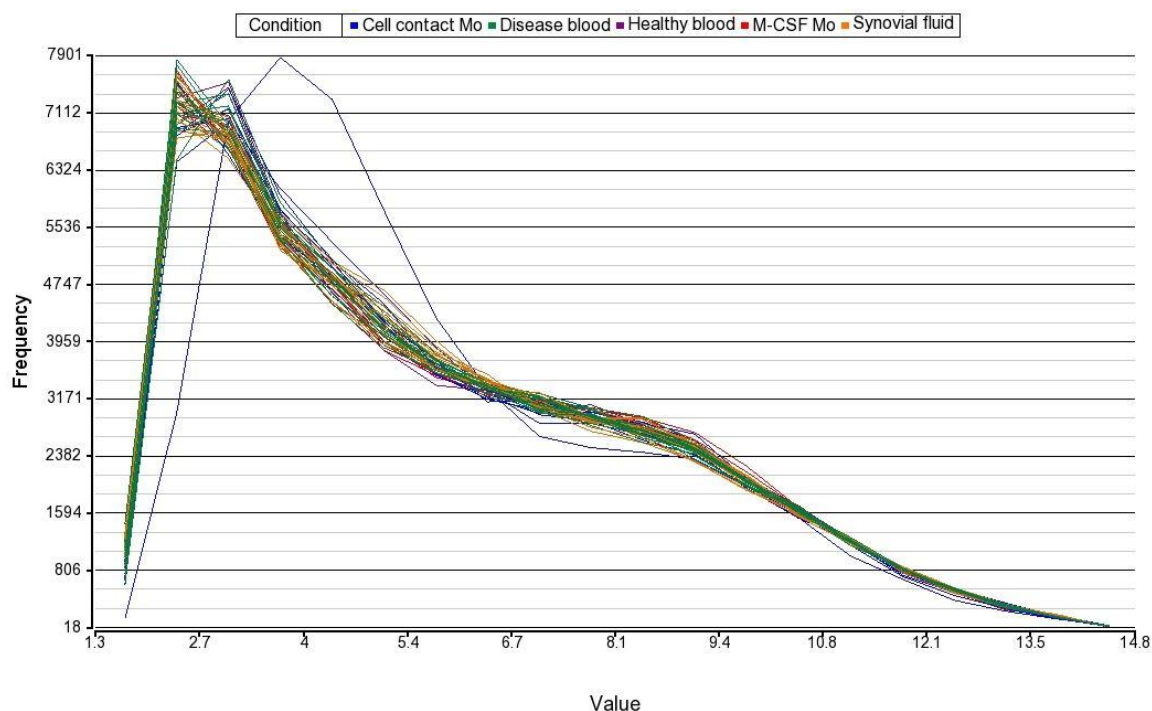


Figure 3.3 Signal histogram of normalised data

This graph represents the signal intensity of all the GeneChips that were analysed in this experiment. It is used to identify outliers in the samples. Each line represents one GeneChip and the lines are coloured according to the monocyte sample that was loaded onto each GeneChip. A blue line represents a cell contact activated macrophage, green is CD14⁺ cells from the patients, purple is healthy donor blood CD14⁺, red is M-CSF activated macrophages and gold is synovial fluid CD14⁺ cells.

3.5.3 Principle component analysis

Phenotypes of diseases or experimental conditions can be classified using Principle Components Analysis (PCA) [354]. This is an analytical method that identifies distinct patterns in the observed transcriptomic differences of samples. It transforms multidimensional data into a lower dimensional space using as few variables as possible. The resultant PCA graph can be used to look for trends in the data and also identify samples which may be outliers as samples clustered within the same area of the PCA graph are considered to be genetically similar.

All the samples from the microarray were analysed by PCA based on conditions applied (Figure 3.4). Each circle on the graph represents a sample and each colour represents a condition. Figure (A) shows the PCA plot of all samples including the outlier sample that was identified in the signal histogram to illustrate how different the genetic profile of that sample was compared to all the other samples. This is visualised on the PCA plot by the one blue circle

representing the outlier sample being isolated on the left hand side of the graph while all the remaining samples are clustered on the right hand side of the graph. This graph provides further support for the removal of this one sample from the analysis as its genetic profile and signal intensity vary widely from the other samples.

Figure 3.4 (B) demonstrates the PCA plot of the samples once the outlier had been removed. It can clearly be seen that SF CD14⁺ cells (gold circles) from both disease types cluster tightly together indicating that CD14⁺ cells from RA and PsA SF share a similar expression transcriptomic profile. This same pattern can also be seen for the patient blood CD14⁺ cells (green circles); both diseases have similar genetic profiles as indicated by the clustering of the green circles. This cluster of patient blood samples is distinct from the synovial fluid cluster of samples and more 'transcriptomically similar' to the healthy donor CD14⁺ blood cells (purple circles) due to the healthy donor CD14⁺ samples being located in the same area of the PCA graph as the diseased blood CD14⁺ cells. The red circles and blue circles represent M-CSF activated macrophages and cell contact activated macrophages respectively and will be fully discussed in chapter 6.

This PCA analysis helped to visualise the trends in the data and these trends were used to determine further comparisons to be performed. These were:

- comparing patient blood to normal blood
- comparing diseased blood CD14⁺ cells to synovial fluid CD14⁺ cells of both RA and PsA to see if there are genes that are similar in both diseases and conversely if each disease also has a unique distinguishing genetic subset.

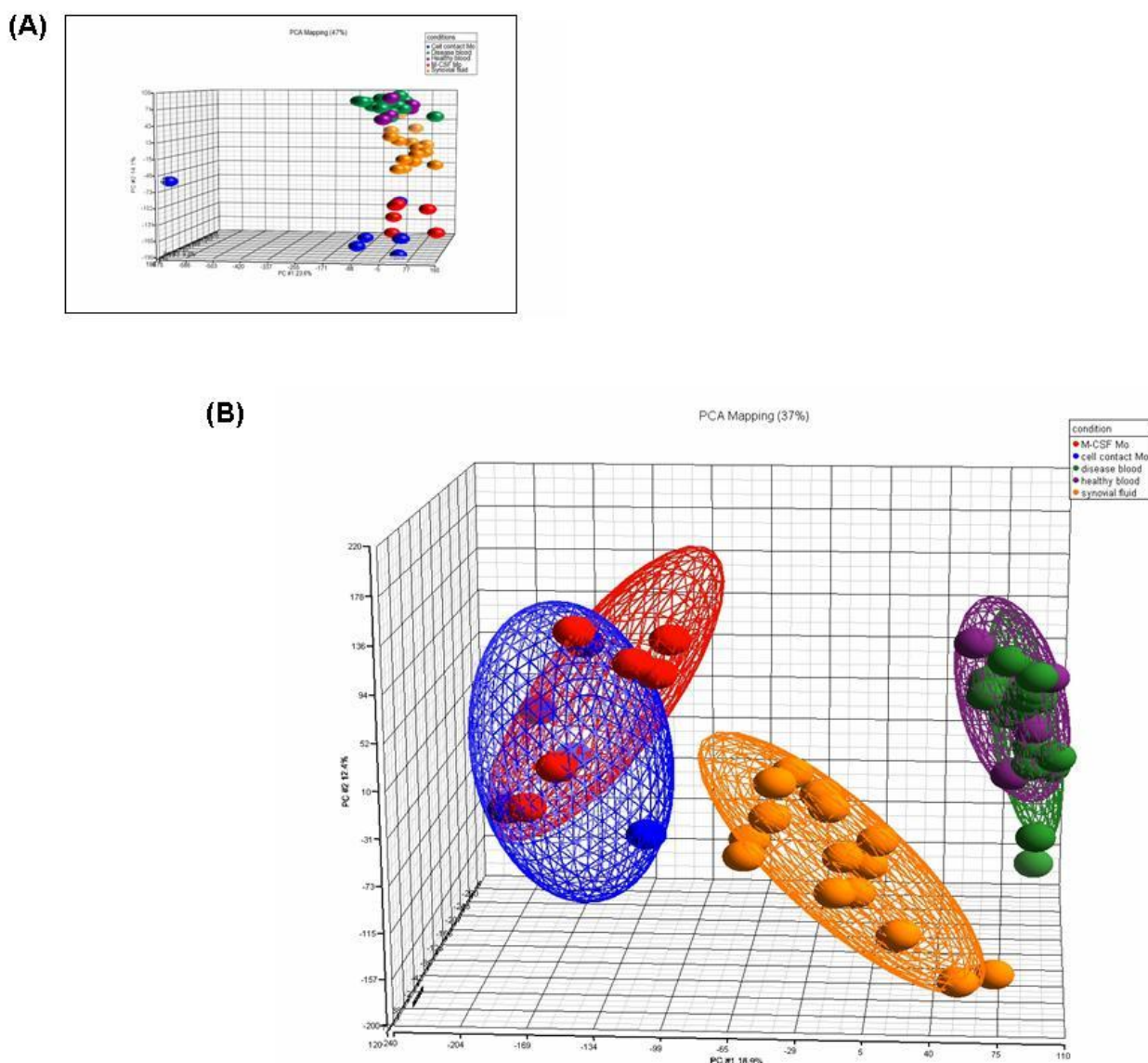


Figure 3.4 Principle component analysis of all microarray samples

PCA was performed on the microarray samples to try and identify trends in the data. Graph (A) indicates the normalised samples before the outlier was removed. The blue circle on the left hand side of the graph represents the outlying sample and it can be seen that all the other samples are clustered away from that area of the graph indicating that general genetic profile of the outlying sample does not match those of the remaining samples. Figure (B) shows PCA graph once the outlying cell contact sample had been removed. Each circle represents a sample and samples clustered in the same area of the graph are considered to be genetically similar. Each colour represents a sample condition; red is M-CSF treated macrophages, blue is cell contact activated macrophages, gold is synovial fluid derived CD14+ cells, green is patient blood CD14+ cells and purple is healthy blood CD14+ cells.

3.6 Comparing samples

3.6.1 Comparison 1: Patient blood compared to normal blood

Analysing the PCA graph indicated that the CD14⁺ cells from both the 8 RA and 8 PsA patients have a similar gene expression to the 5 healthy control CD14⁺ cells. It was decided to analyse the genetic profiles of these samples further to see how similar they were and to determine if there were any disease-specific genes not detected in the healthy controls as has been found by many previous studies [325-327, 329, 330, 340, 341].

All samples were pre-processed by the GC-RMA algorithm and quality control on the entities was performed. To pass the quality control analysis each entity had to have a signal value between 20 and 100% in order to remove any background signal and each entity had to be expressed in all 21 samples (8 RA PB, 8 PsA PB and 5 healthy PB). This is due to the considerable heterogeneity in the patient samples with regards to treatment and disease duration; we were only interested in genes that had changed in all disease samples rather than genes that changed for example, only in response to a certain treatment type.

41424 entities passed the quality control analysis that can be visualised as shown in Figure 3.5. The y value is the normalised signal intensity of the entity where the signal is normalised to the median value of that entity across all the samples. It is a visualisation tool that allows entities with very high or very low signal values to be visualised on the same graph. The graph indicates that whilst there are many entities that do not change their signal expression levels between non-diseased and diseased states there are some outliers that have varying levels of intensity in each of the three conditions.

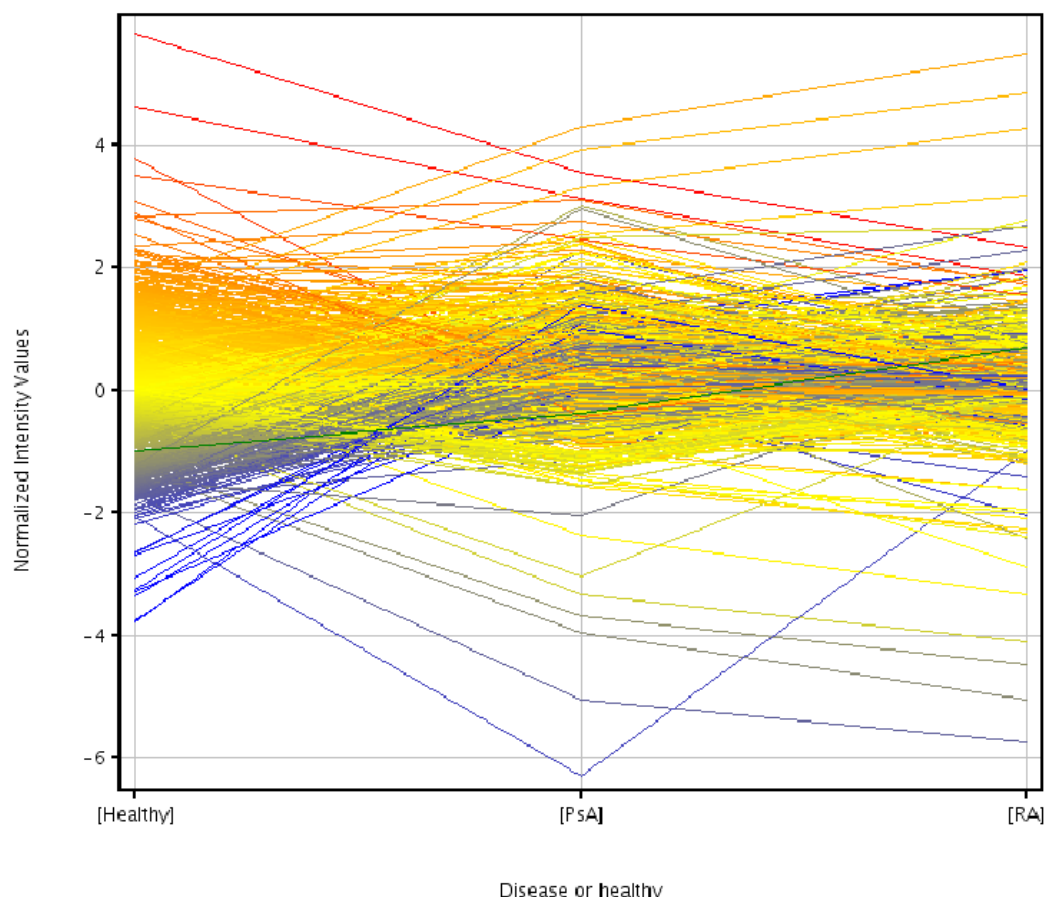


Figure 3.5 Profile plot of entities that passed the quality control filter.

Each line represents an entity and this graph shows the change in the signal intensity value of each entity in all healthy samples (left hand side of the graph), PsA PB samples (middle of the graph) and RA PB samples (right hand side of the graph). The colour of the line denotes the signal intensity of the entity in healthy peripheral blood; blue represents a signal intensity of -6 to -1, yellow -1 to 1 and red 1 to 6.

3.6.1.1 Statistical analysis of healthy and diseased blood CD14⁺ cells

During microarray data analysis, multiple testing corrections (MTC) should be applied where possible to decrease the number of false positives. Any statistical tests (e.g. T test, ANOVA) that are performed on microarray data perform one test per gene therefore the number of false positives increases proportionally to the number of tests performed. For example analysing 10000 genes with a cut-off p value of 0.05 will mean performing 10000 individual statistical tests and as a result 500 genes (0.05×10000) are likely to appear significant by chance alone. The Benjamini and Hochberg (false discovery rate) MTC gives a corrected p value for each gene depending on the number of genes being tested [355].

A one-way ANOVA unequal variance (Welch ANOVA) with a Student-Newman-Keuls (SNK) post Hoc test was used to analyse the 3 microarray conditions of

healthy blood, RA blood and PsA blood. A MTC was unable to be used in this instance as when the Benjamini and Hochberg MTC was used there were no significantly changed genes between the three groups. Since a p value cut off of 0.05 was used this means that 5% of the differentially expressed genes may be false positives and appearing by chance alone, a way to overcome this is to look at the p values of each gene as the higher the p value of the gene the less chance of it being a false positive.

The one-way ANOVA with a p value cut off of 0.05 gave the following results:

494 genes significantly changed between RA and PsA PB cells

1457 genes significantly changed between healthy blood and RA blood

1384 genes significantly changed between healthy blood and PsA blood

These results indicate that although the PCA graph showed all of the blood CD14⁺ samples to be genetically similar, there are groups of genes that are significantly changed between samples indicating that there are likely to be disease-specific genes being expressed.

3.6.1.2 Fold change cut off analysis of healthy and diseased blood derived CD14⁺ cells

Since there were many genes that had significantly changed between groups a fold change analysis was performed to look at differential expression. A cut off value of 2 was chosen, consequently if a gene had a fold change difference in expression between two conditions that was ≥ 2 then it would be considered differentially expressed. The fold change cut off gave the following results:

43 genes changed between RA and PsA blood CD14⁺ cells (22 genes up-regulated in RA compared to PsA PB and 21 genes up-regulated in PsA compared to RA PB)

239 genes changed between healthy blood and RA blood (158 genes up-regulated in RA compared to healthy PB and 81 genes down-regulated in RA compared to healthy PB)

281 genes changed between healthy blood and PsA blood (153 genes up-regulated in PsA blood compared to healthy and 128 genes downregulated in PsA blood compared to healthy)

These results demonstrate that of the 54,000 probe sets on the Affymetrix GeneChips there are very few of these (43 probes/genes) that are differentially expressed between RA blood and PsA blood. This result reinforces the PCA results which showed the RA and PsA blood samples to be grouped together therefore implying these samples were transcriptionally similar. Hence from our data it appears that the blood monocytes from RA and PsA have similar transcriptomic profiles and there are very few genes that are expressed by these monocytes that could distinguish them as belonging to either disease.

Nevertheless there are 43 genes that were distinguished by this analysis as being differentially expressed between RA and PsA PB CD14⁺ cells with a fold change difference of ≥ 2 . Of these 43 gene hits there were only 24 individual genes that had changed. This is because on the affymetrix GeneChips each gene can be represented by more than one probe set and one gene can appear several times in the differentially changed gene list, also the gene lists often contain transcribed loci that have no known function or gene name and therefore cannot be analysed further. Of the 24 genes that were differentially expressed between RA and PsA blood CD14⁺ cells 12 were upregulated in RA blood compared to PsA and 12 were upregulated in PsA blood compared to RA.

Table 3.4 outlines the functions of the 12 genes upregulated in RA PB CD14⁺ cells compared to PsA PB CD14⁺ cells.

Table 3.5 outlines the functions of the 12 genes upregulated in PsA PB CD14⁺ cells compared to RA PB CD14⁺ cells.

Table 3.4 A list of the 12 upregulated genes in RA PB CD14⁺ cells compared to PsA PB CD14⁺ cells

Symbol	Gene Name	Fold change upregulation in RA PB compared to PsA PB (p value)	Description of Gene
NRP1	Neuropilin 1	5.1 (5.72 X 10 ⁻⁵)	A single pass transmembrane protein which has various ligands including semaphorins and VEGF family members. They have principle functions in cell motility, including neural growth cone collapse mediated by semaphorins, and vascular and lymphatic sprouting triggered by VEGFs [356]. NRP-1 is the major VEGF 165 receptor in fibroblast like synoviocytes in RA. It is also expressed in the lining layer, endothelial cells and infiltrating leukocytes in RA [357].
NCAM1	Neural cell adhesion molecule 1	3.8 (0.017)	A cell surface glycoprotein which mediates homotypic and heterotypic cell-cell adhesion through a homophilic binding mechanism. It is present in the nervous system where it participates in neural outgrowth and cell migration [358]. NCAM may also play a role in blood vessel stability as NCAM deficiency results in unstable endothelial cell - pericyte interaction and hence vessel stability [359].
CYB5R2	Cytochrome b5 reductase 2	3.5 (0.021)	Cytochrome b5 is an electron transfer component in oxidative reactions such as anabolic metabolism of fats and steroids [360]. The CYB5R2 isoform is a membrane bound form of cytochrome b5 reductase which partakes in fatty acid elongation and desaturation [361] and cholesterol biosynthesis. It may also play a role in mitochondrial respiration [362].
GPR34	G protein-coupled receptor 34	2.8 (0.048)	G protein-coupled receptors are the largest family of receptor proteins in mammals and are known to bind to a variety of extracellular ligands such as chemokines, enzymes and hormones [363]. GPR34 is highly abundant in mast cells where it acts as the functional receptor for lysophosphatidylserine which enhances mast cell degranulation [364].
HPGD	hydroxyprostaglandin dehydrogenase 15-(NAD)	2.4 (0.0062)	An enzyme responsible for the inactivation of prostaglandins and lipoxins by catalysing the oxidation of 15(S)-Hydroxyl group resulting

in metabolites with reduced biological activity [365]. It is downregulated in human epithelial tumours and shows tumour suppressor activity [366].

PKP2 Plakophilin 2 2.3 (0.046)

A protein found co-localised to desmosomes, which are adhesive junctions linking intermediate filament networks to sites of strong intercellular adhesion. These junctions provide strength to tissue undergoing mechanical stress such as cardiac and epidermal tissue [367]. Plakophilin 2 has been detected in the adherens junctions of cardiomyocytes [368] and can co-localise with β -catenin at adherens junctions in epithelial cells [369].

CA5B carbonic anhydrase VB, mitochondrial 2.2 (0.045)

A metalloenzyme expressed in mitochondria which is involved in several biosynthetic processes and plays a key role in fatty acid biosynthesis

[370].

IL7 Interleukin 7 2.1 (0.011)

A member of the IL-2 family expressed by epithelial cells in lymphopoietic tissues, epithelial cells, keratinocytes, DCs and monocytes [371]. IL-7 plays an important role in T cell homeostasis such as naïve T cell survival but has also been found to induce bone loss by inducing osteoclast formation from human monocytes in a T cell dependent manner [372]. Serum levels of IL-7 are higher in RA patients compared to healthy controls and correlate with markers of inflammation [371]. IL-7⁺ cells correlate with the number of CD68⁺ cells in RA synovial lining and sub-lining layers and double staining demonstrates that CD68⁺ cells are a major producer of IL-7 [373].

LAMC1 Laminin, gamma 1 2.1 (0.016)

Laminins are extracellular matrix components and are a major constituent of basement membranes. Laminin and collagen provide the basic scaffold to which other basement membrane proteins, such as proteoglycan, integrate [374]. Laminin is also expressed in the cerebral cortex and laminin γ 1 has been shown to play a critical role in neuronal morphogenesis and migration [375]. Laminin γ 1 is also required for the deposition of matrix molecules in cardiomyocytes [376].

LYCAT	Lysocardiolipin acyltransferase	2.1 (0.011)	In embryonic stem cells (ESC) differentiation lysocardiolipin acyltransferase plays an essential role in hematopoietic and endothelial lineage development [377]. It is also involved in the remodelling of the phospholipid cardiolipin. Cardiolipin is sensitive to oxidative damage by reactive oxygen species and lysocardiolipin acyltransferase plays a regulatory role in remodelling cardiolipin in response to stress [378].
LRP11	Low density lipoprotein receptor-related protein 11	2.1 (0.0077)	The LDL receptor related protein (LRP) is a heterodimeric endocytic cell surface receptor which transports a variety of macromolecules into cells through a process called receptor mediated endocytosis. LRP11 is expressed mainly in the brain and it has been shown to play a role in cell-cell adhesion in neurons however it has also been shown to be present in vascular smooth muscle cells and can induce enhanced migration and invasion activities [379]. LRP11 may play a role in Alzheimer disease (AD) as its expression is significantly reduced in the brains of AD patients, in this setting LRP11 is thought to be a sorting protein that protects β -amyloid precursor protein (APP) processing into amyloid- β ($A\beta$) which is the peptide found accumulated within the brains of AD patients [380].
RNF34	Ring finger protein 34	2.0 (0.049)	Otherwise known as human ring finger homologous to inhibitor of apoptosis (hRFI). hRFI is an apoptosis regulator protein. Transfection of hRFI into HeLa cells resulted in an increase in cell survival after TNF α treatment [381] and transfection of hRFI into human colorectal cancer cells significantly inhibited death receptor mediated apoptosis with decreased caspase activity [382]. Thus indicating that hRFI has anti-apoptotic activity.

Table 3.5 A list of the 12 upregulated genes in PsA blood CD14⁺ cells compared to RA blood CD14⁺ cells

Symbol	Gene Name	Fold change upregulation in PsA PB compared to RA PB (p value)	Description of Gene
GSTM2	glutathione S-transferase M2	3.0 (0.040)	Glutathione S-transferases (GSTs) are a broadly expressed family of enzymes which protect against endogenous oxidative stress as well as exogenous toxins. They detoxify a variety of electrophilic compounds including oxidised lipids and DNA products generated by reactive oxygen species damage to intracellular molecules [383]. Glutathione S-transferase M2 is a muscle specific enzyme.
GSTM1	Glutathione S-transferase M1	2.9 (0.035)	(See above for a description of GSTs). Allelic variant within the GSTM1 gene are associated with differences in detoxification efficiency, the GSTM1 gene has a non-functioning allele termed GSTM1-null. RA patients who carry this GSTM1-null polymorphism and are smokers are associated with worse disease severity [384]. A further study in a Korean population indicated that the GSTM1-null polymorphism is associated with increased susceptibility to RA [385].
PPP1R16B	protein phosphatase 1, regulatory (inhibitor) subunit 16B	2.9 (0.036)	Protein phosphatase 1 (PP1) is a eukaryotic serine/threonine phosphatase which regulates a large variety of cellular functions through the interaction of its catalytic subunit with over 50 different regulatory subunits [386]. The PPP1R16B gene encodes a protein called TGF- β -inhibited membrane-associated protein (TIMAP). TIMAP is expressed at high levels in endothelial cells, phosphorylation of TIMAP controls its association with PP1 which in turn regulates extension of filapodia within the endothelial cells [387].
VASH1	Vasohibin 1	2.9 (0.0088)	An angiogenesis inhibitor upregulated in endothelial cells [388].

			Vasohibin-1 has also been detected in RA synovium in synovial lining cells, synovial fibroblast and endothelial cells. <i>In vitro</i> stimulation of RA synovial fibroblasts with inflammatory cytokines induced vasohibin-1 mRNA expression in hypoxic conditions [388].
CHST13	Carbohydrate (chondroitin 4) sulfotransferase 13	2.8 (0.013)	Chondroitin sulfotransferases synthesize the diverse sulphated structures found in the glycosaminoglycans (GAGs) chondroitin sulphate [389]. As present there is no defining information on the specific role of carbohydrate sulfotransferase 13.
FOXP1	forkhead box P1	2.7 (0.017)	A member of the FOX family of transcription factors. FOXP1 has a role in B cell development [390], thymocyte development [391] and monocyte differentiation as downregulation of FOXP1 is necessary for monocyte differentiation and hence macrophage function [392].
KLF9	Kruppel-like factor 9	2.3 (0.011)	The kruppel-like factor family are zinc finger transcription factors. KLF9 regulates estrogen and progesterone action by modulating the activity of the progesterone receptor (PGR). It directly interacts with PGR to mediate progesterone responsive genes [393] and acts as a transcriptional repressor of estrogen receptor signalling [394].
KIF1B	Kinesin family member 1B	2.3 (0.031)	The kinesin superfamily of proteins play a role in organelle transport in cells such as neurons and epithelial cells [395]. KIF1B is a plus end directed motor that transports synaptic vesicle precursors in the axon from the cell body to the synapse [396], it has also been shown to transport mitochondria in nerve cells [397].
BIN1	Bridging integrator 1	2.3 (0.035)	BIN1 contains a BAR domain that allows it to interact with cellular membranes and induce membrane curvature [398]. BIN1 is a muscle specific isoform of BAR domain containing proteins. It is highly expressed during muscle development and helps

generate the membrane curvature needed to form T-tubules [399].

MED26	Mediator complex subunit 26	2.1 (8.6 X 10 ⁻⁴)	Once of several subunits that comprise the Mediator complex. The Mediator complex forms a crucial part of the RNA polymerase II transcriptional machinery that is essential for the transcription of almost all protein coding genes [400].
ACSL3	Acyl-CoA synthetase long-chain family member 3	2.1 (0.039)	An enzyme isoform involved in lipid synthesis, fatty acid catabolism and membrane remodelling [401].
EIF2AK3	Eukaryotic translation initiation factor 2-alpha kinase 3	2.1 (0.022)	An ER transmembrane protein kinase which functions during cellular stress as an unfolded protein response (UPR) sensor. It is activated by accumulated misfolded proteins in the ER and reduces cellular translation to allow the cell to correct for the misfolded proteins causing the ER stress before synthesising additional proteins [402].

3.6.1.3 Database for annotation, visualisation and integrated discovery (DAVID) analysis of genes changed between healthy and patient CD14⁺ cells

Following on from the statistical and fold change analysis of the blood CD14⁺ samples an analysis of the groups of genes which were changed between healthy and patient samples took place. This analysis will identify the function of the expressed genes and subsequently group them according to their function. This may be more informative rather than studying each individual gene and could indicate the phenotype of the diseased CD14⁺ cells.

The database for annotation, visualisation and integrated discovery (DAVID) analysis was used to investigate the gene lists to see if there was a significant enrichment of group of genes required for a specific biological function. DAVID is a publicly available software that can systematically map a large number of genes to their associated biological annotation and then statistically highlight the most overrepresented enriched biological processes [403].

DAVID analyses gene lists in regard to the reference background, which in this analysis is all of the genes expressed on the Affymetrix GeneChip. To decide the degree of biological enrichment within the input gene list DAVID compares it to the reference background for example, if 10% of the gene list are kinases versus 1% of the genes on the Affymetrix GeneChip then kinases would be classed as overrepresented within the gene list. The DAVID software uses a modified Fisher's exact test to measure the gene-enrichment in the biological process groups and groups with a p value <0.05 were considered to be specifically associated with the gene list. Each gene is represented by more than one probe on the Affymetrix GeneChip and therefore there may be several entities in the gene list which correspond to the same gene, DAVID analysis takes this into account and will only analyse each gene once irrespective of how many times it appears in the gene list.

The gene lists which were analysed using DAVID were the up- and down-regulated genes in RA blood CD14⁺ compared to healthy blood CD14⁺ and the up- and down-regulated genes in PsA blood CD14⁺ compared to healthy blood. Related groups of genes with a p value <0.05 were considered to be significantly enriched within the gene list, if there were several gene groups associated with

the gene list only the top 10 most significantly associated groups were analysed. Figure 3.6 shows the biological process gene groups which were expressed at a higher level in RA blood compared to healthy blood CD14⁺ (A) and those that were expressed at a lower level in RA blood (B). The upregulated groups of genes include genes associated with chemotaxis, which could be in preparation of the monocytes extravasating from the blood into the diseased joints, and genes associated with response to external stimulus. This latter gene group may be due to the proinflammatory stimuli found within the serum of arthritis patients but not healthy controls to which the RA blood monocytes are exposed [404, 405]. The downregulated groups of genes in RA PB monocytes include those that regulate intracellular protein complex localisation and negative regulation of cell migration which reinforces the results shown in graph (A) suggesting that RA blood monocytes are upregulating groups of genes which will enable the cell to migrate and respond to chemotaxis.

Figure 3.7 indicates the biological process groups which were upregulated in PsA PB CD14⁺ compared to healthy controls (A) and downregulated in PsA PB compared to healthy control blood (B). The results of this analysis indicate that in comparison to healthy control monocytes the PsA blood monocytes have an increased expression of genes involved in DNA packaging, chromatin assembly and nucleosome assembly suggesting an overall reduction of DNA transcription and translation within these cells. The PsA blood monocytes are also upregulating genes involved in chemotaxis, which was also shown in the RA blood monocytes in the previous figure, and may be due to an increase in chemokines within the serum of PsA patients in comparison to healthy controls [265, 406]. The downregulated genes in the PsA blood samples are mainly involved in cell projection, morphogenesis, differentiation and metabolic processes which all involve a large amount of energy expenditure therefore the cells could be trying to conserve energy and resources.

In summary, the purpose of this healthy blood and diseased blood comparison was to determine if there were any disease-specific genes not detected in the healthy controls as the PCA analysis had demonstrated that the blood CD14⁺ samples in healthy and diseased patients had similar genetic profiles. Despite the possibility that 5% of the genes within the gene lists generated are false positives due to not being able to apply a multiple testing correction (MTC) to

the data there were a considerable number of differentially expressed genes between the healthy blood samples and diseased samples. This indicates that there are disease specific genes being expressed in the monocytes which in the case of both RA and PsA included groups of genes required for chemotaxis and cell movement. This analysis also generated the surprising result that there are only 24 individual genes that are differentially expressed between RA and PsA blood monocytes, this would suggest that the blood monocytes are remarkably similar in the two forms of arthritis and may suggest that they play similar roles within the diseases.

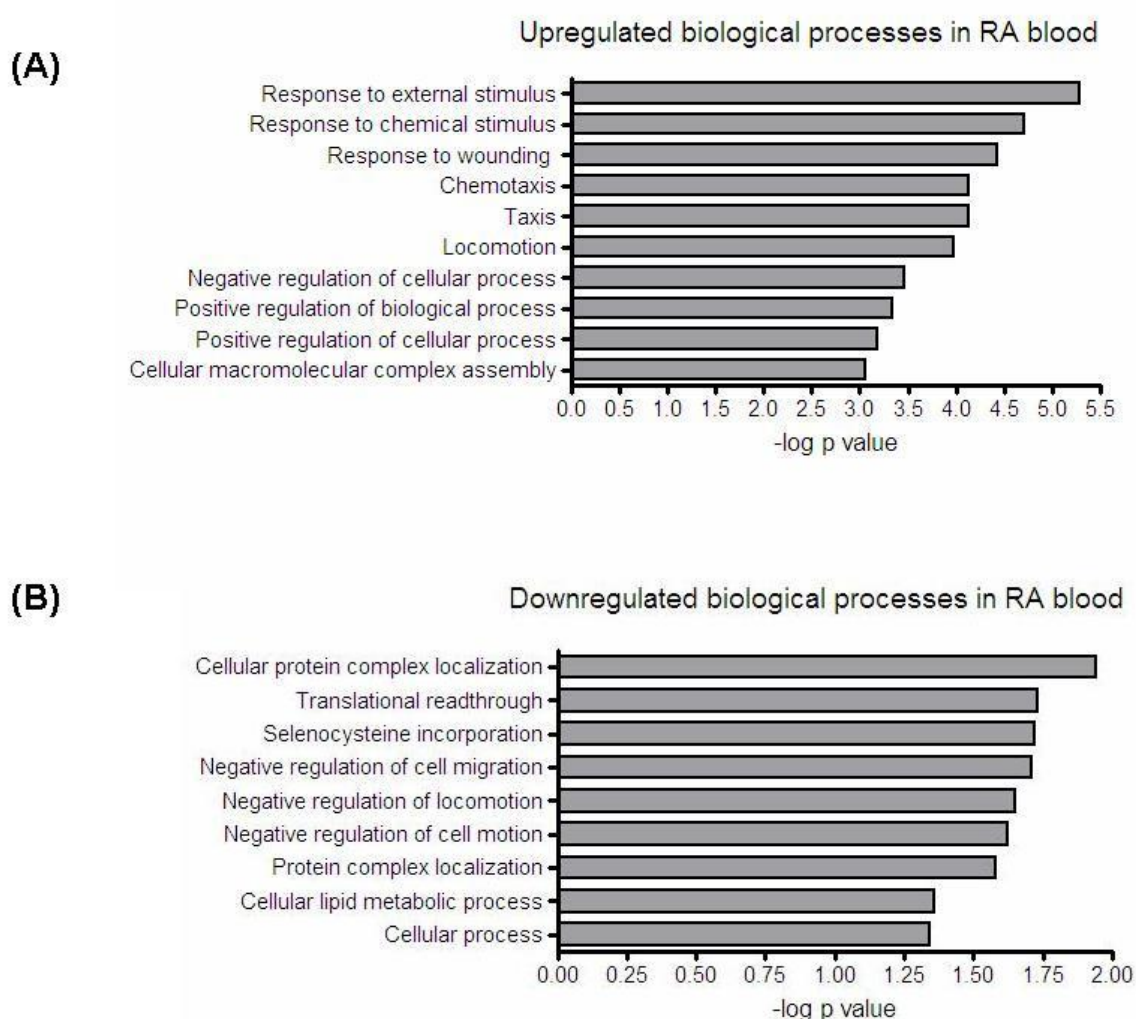


Figure 3.6 Up and downregulated groups of genes in RA blood compared to healthy blood
DAVID analysis was used to discover the significantly up- and downregulated biological process gene groups as identified by gene ontology (GO). The 10 most significant biological process groups are shown which all have a p value <0.05 which is equivalent to a $-\log p$ value >1.30.

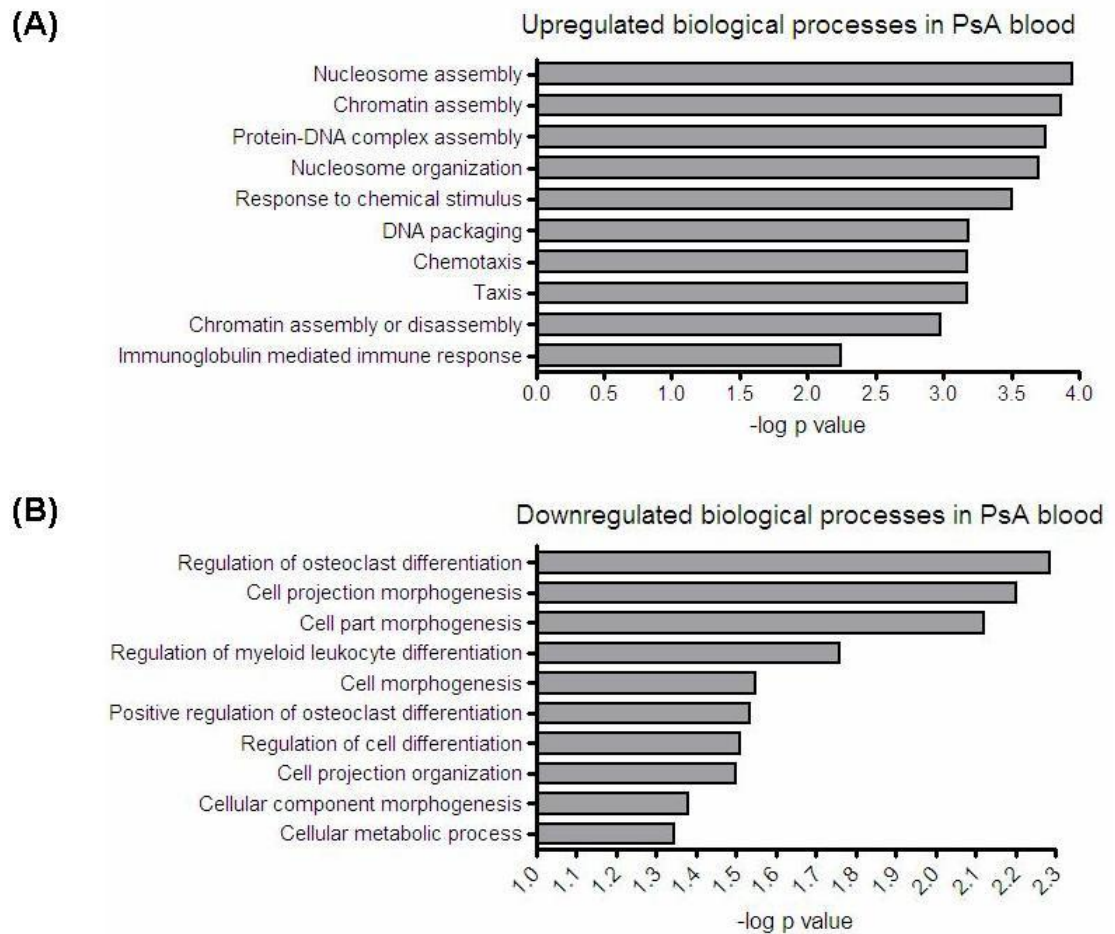


Figure 3.7 Up and downregulated groups of genes in PsA PB compared to healthy PB

DAVID analysis was used to discover the significantly up- and downregulated biological process gene groups as identified by gene ontology (GO). The 10 most significant biological process groups are shown which all have a p value <0.05 which is equivalent to a $-\log p$ value >1.30.

3.6.2 Comparison 2: Analysis of microarray results comparing psoriatic and rheumatoid patient blood CD14⁺ cells to synovial fluid CD14⁺ cells

My major questions concerned changes occurring in the genetic profile as the CD14⁺ cells of PsA and RA patients move from the blood into the synovial compartment. The principle component analysis (PCA) plot of the RA and PsA PB and SF CD14⁺ (Figure 3.8) illustrates that there are numerous genetic differences between the PB and SF of both diseases indicated by the PB samples (blue and red circles) occupying a separate area of the PCA graph as the SF samples (green and purple circles). However this graph also shows that the PB CD14⁺ cells of both diseases and the SF CD14⁺ of the two diseases are transcriptionally similar to each other. This observation indicates that there are large genetic differences between the site from which the CD14⁺ were isolated (i.e. blood and synovial fluid) however the genetic differences between diseases at these sites is small (i.e. RA and PsA SF CD14⁺ cells are transcriptomically similar).

3.6.2.1 Statistical analysis of peripheral blood CD14⁺ compared to synovial fluid CD14⁺

All samples were pre-processed with the GC-RMA algorithm and quality control was performed on the 16 matched RA samples (8 PB and 8 SF) and then performed on the 16 matched PsA samples (8 PB and 8 SF). In order to pass the quality control filter each gene had to have a signal value between 20 and 100% and be expressed in all 16 samples. Again, this was selected due to the high heterogeneity of the 8 RA and 8 PsA patients with regard to disease duration and patient treatment. Ensuring that each gene was present in all of the 16 RA or PsA samples being tested meant that the analysis was only looking at genes changed in the disease condition as a whole and was not affected by an individual treatment type.

Once the quality control had been performed a paired T test was undertaken for each disease comparing the matched PB and SF samples of each patient. The p value cut off was 0.05 and the Benjamini and Hochberg multiple testing correction was applied to the data which gives the genes a corrected p value.

Following this analysis there were:

10763 significantly changed genes between RA PB CD14⁺ cells and RA SF CD14⁺ cells

9533 significantly changed genes between PsA PB CD14⁺ cells and PsA SF CD14⁺ cells

Figure 3.9 shows the profile plots of the significantly changed genes between RA PB and SF (A) and between PsA PB and SF (B). The profile plot is a visualisation tool similar to a heatmap to allow trends in genetic expression data to be identified, however its benefit over a heatmap is that it allows thousands of genes to be analysed simultaneously. On these graphs each line represents one gene and the colour of the line denotes the median GeneChip intensity value of that gene from the 8 blood samples; with blue signifying a low intensity value, yellow denotes an intermediate value and red denotes a high level of intensity. The profile plots indicate that there are a large number of genes in both diseases which are upregulated in the SF CD14⁺ cells compared to PB CD14⁺ cells; there are also a smaller number of genes which are downregulated in the SF cells compared to the PB CD14⁺ cells.

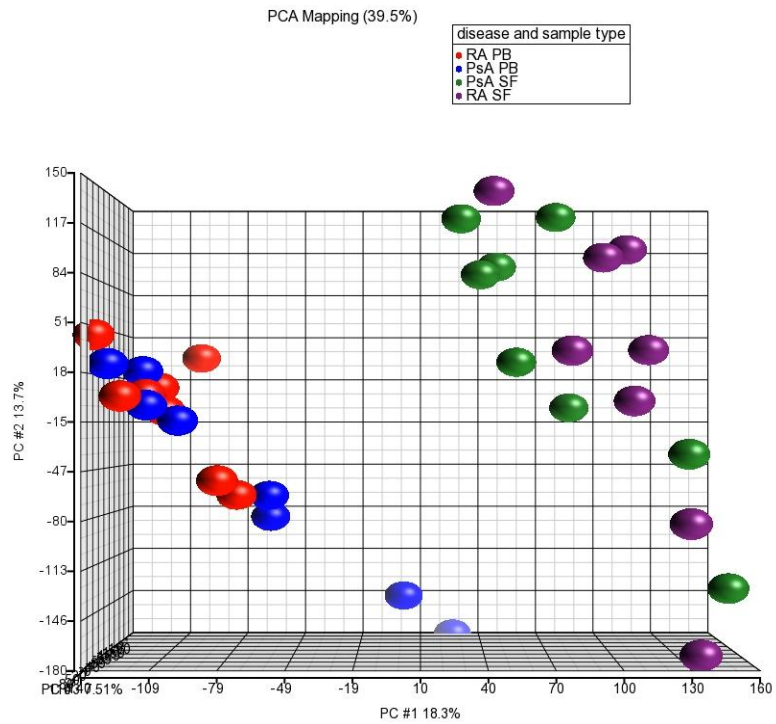


Figure 3.8 PCA analysis of PB and SF from RA and PsA patients

PCA analysis was performed on the 32 samples to try and discover trends in the data as samples in the same area of the graph are considered to be genetically similar. Each circle represents an individual sample and all samples are colour coded per condition, red corresponds to RA PB, blue to PsA PB, purple to RA SF and green to PsA SF.

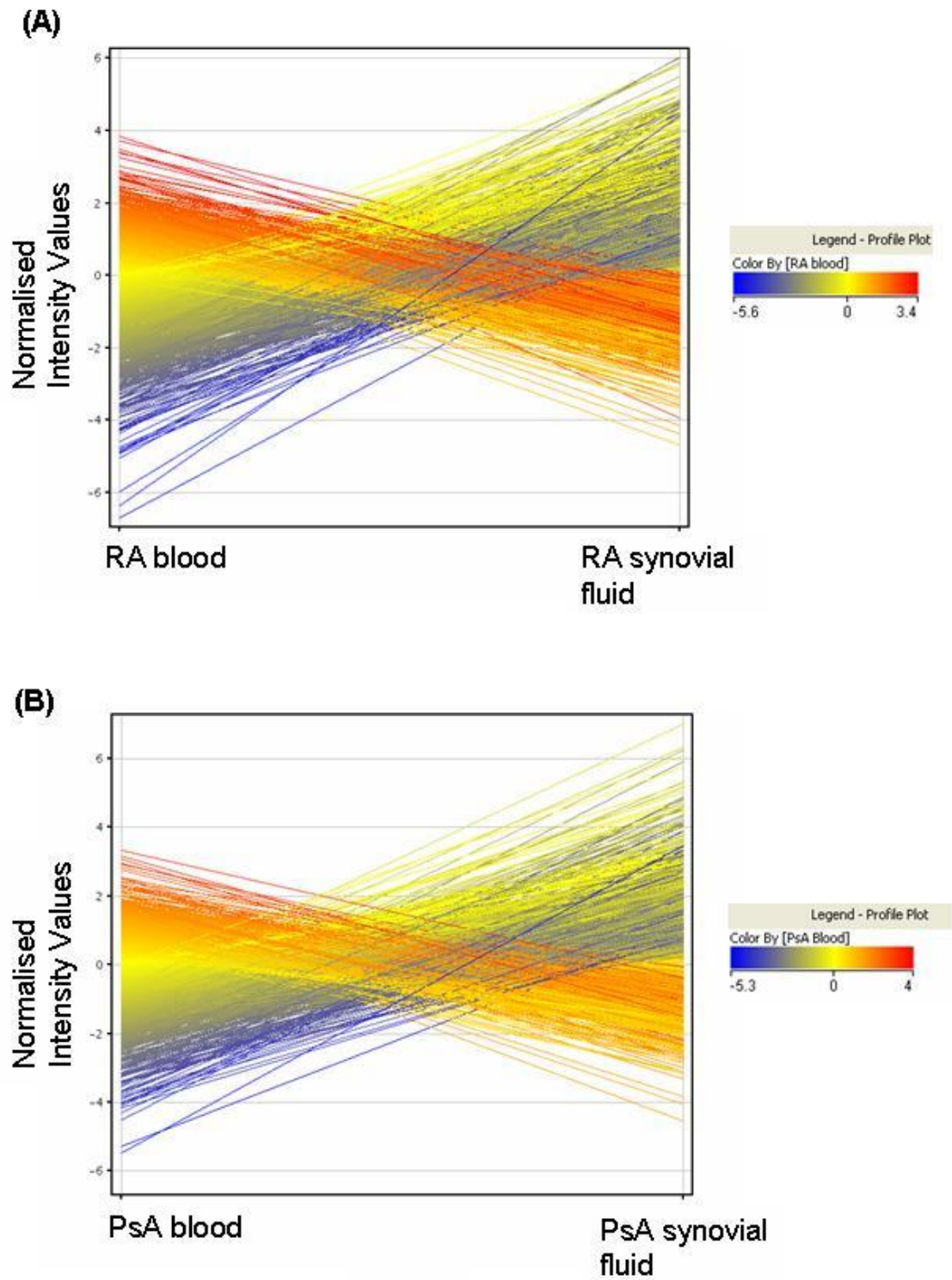


Figure 3.9 Profile plots of entities that are significantly changed between PB and SF in RA and PsA

Each line on the profile plot represents one gene and the graphs indicate how the normalised signal intensities of the gene change between the PB samples and the SF samples. The colour of the line represents the median signal intensity of each gene in the PB samples. A blue line indicates the gene was expressed at a low level in the PB, a yellow line indicates an intermediate level and a red line a high level of signal intensity.

3.6.2.2 Fold change analysis and Ingenuity pathway analysis (IPA) of RA PB vs RA SF and PsA PB vs PsA SF

The two gene lists which had been generated from the paired T test analysis were subjected to fold change analysis. Due to the substantial number of significantly expressed genes in both diseases a fold change cut off of ≥ 4 was applied to the data. In microarray data analysis the fold change cut off value is arbitrary and we were interested in genes with a high fold change difference between PB and SF.

The fold change analysis gave the following results:

- 1686 genes significantly changed between RA PB and RA SF with a fold change ≥ 4
- 1149 genes significantly changed between PsA PB and PsA SF with a fold change ≥ 4

The varying expression levels of these genes is depicted in Figure 3.10 which is a heatmap of all the significantly changed genes between the PB and SF samples of RA and PsA with a fold change cut off of 4. The heatmap displays the normalised signal intensity of each gene within the blood and synovial fluid samples of each patient. The range in colour represents the relative expression levels of the genes in each sample with green representing a low level of expression and red representing a high level of relative expression. Heatmaps are used as a visualisation tool and this figure is demonstrating there appear to be many genes which are upregulated in the synovial fluid of the patients in comparison to the peripheral blood as demonstrated by the large number of genes which are green (low level of expression) in the peripheral blood cell but are red (high level of expression) in the synovial fluid cells.

The two gene lists were then subjected to ingenuity pathway analysis (IPA) which may be used to analyse complicated gene sets and identify genes that are part of a common regulatory network. This software has advantages over DAVID analysis in addition to identifying the overrepresented molecular and cellular functions within the gene list IPA can also identify the canonical pathways as well as the diseases and disorders associated with the genes. IPA is similar to

DAVID analysis as it uses Fisher's exact test to determine whether an association of a biological process or a pathway with the dataset is due to random chance. The smaller the p value of the process or pathway the less likely that the association is random and the more significant the association, in this analysis p values less than 0.05 indicate a statistically significant non-random association. IPA determines the association of processes and pathways within the gene list by comparing it to a reference gene set, which in this case are all the genes expressed on the Affymetrix GeneChips, and calculating whether the pathways or processes are overrepresented in the gene list and whether this association is statistically significant using Fisher's exact test.

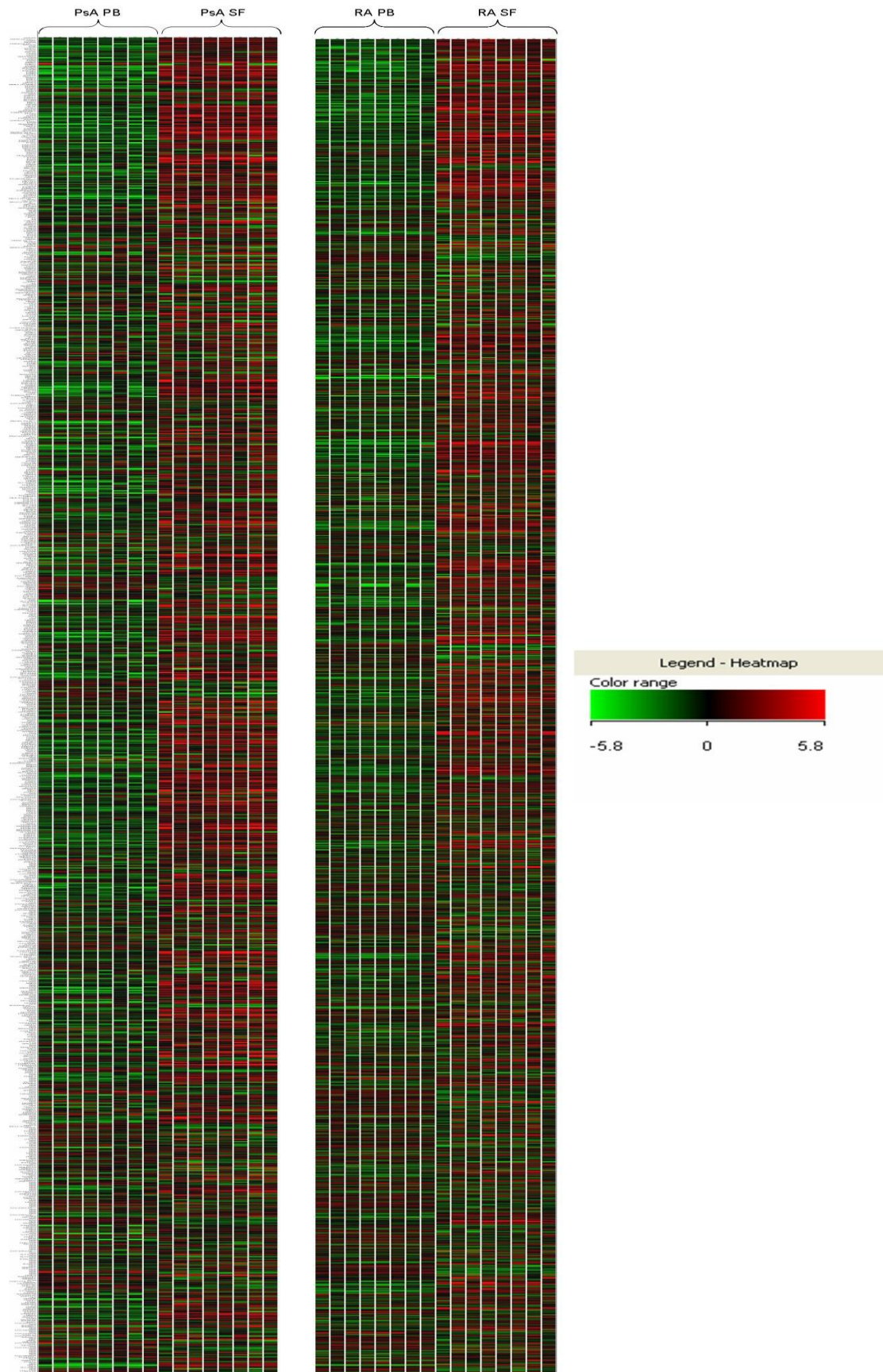


Figure 3.10 Heatmap of significantly changed genes with a fold change cut off of 4 between the PB and SF of PsA and RA patients

The normalised signal intensity of each differentially expressed gene with a fold change cut off of 4 or above is shown. The degree of colour represent the relative level of expression for each gene in each sample ranging from green - low relative expression, to red - high relative expression (see legend). It can be visualised that there are genes which are expressed at a low level in the blood and therefore a high level in the synovial fluid and vice versa.

3.6.2.3 IPA of RA blood vs RA synovial fluid CD14⁺

The 1686 differentially expressed genes between the RA PB and RA SF CD14⁺ samples with a fold change cut off of 4 were analysed using IPA. This analysis generated three sets of results associated with the gene lists; the top 5 canonical pathways, the top 5 diseases and disorders and the top 5 molecular and cellular functions.

Table 3.6 indicates the top 5 canonical pathways associated with the up- and downregulated genes observed in the RA synovial fluid samples. This analysis includes pathways which have previously been associated with RA such as the coagulation system and the role of macrophages in RA but also includes other pathways such as LXR/RXR activation.

Figure 3.11 outlines the canonical pathway of LXR/RXR activation which was the most significant pathway associated with the RA PB vs SF gene list. All of the genes highlighted in the pathway are the 17 genes from the gene list associated with the RA comparison; all of these genes are highlighted in red which indicates that these genes were upregulated in the synovial fluid CD14⁺ cells compared to the PB CD14⁺ cells. These genes range from pro-inflammatory chemokines such as MCP-1 to the cholesterol efflux genes ApoE and ApoC1. The relevance of these genes to RA pathogenesis will be analysed in detail in the discussion section of this chapter.

Table 3.7 outlines the diseases and disorders associated with the up- and down-regulated genes expressed in RA SF CD14⁺ compared to RA PB CD14⁺. As would be expected all the diseases and disorders are related to or involve RA. The range of p values shown determines the significance of the gene list to different terms encompassed by the diseases and disorders. For example the term inflammatory disease includes more specific terms such as inflammatory disorder of the joint, arthritis or multiple sclerosis so depending on the association of the gene list with each term tested an individual p value is given. Due to the large number of terms that can be significant within one category a range of p values is outlined in the table.

Table 3.8 outlines the top 5 cellular functions associated with the gene list. The gene list comprises genes that are differentially expressed within the RA synovial fluid CD14⁺ cells and the cellular functions significantly overrepresented in this gene list are those which we would expect to be upregulated within the RA SF cells in comparison to the PB cells. Two of the cellular functions are cellular movement and immune cell trafficking which could be upregulated as a result of the CD14⁺ transendothelial migration from the blood vessels into the synovium [258, 407]. Since macrophages within the synovial compartment are known to participate in cellular interactions with several cell types such as fibroblasts [249], T cells [269, 314, 408], endothelial cells [407] and natural killer (NK) cells [251] it is no surprise that one of the top cellular functions associated with the RA SF CD14⁺ cells is cell-to-cell signalling and interaction. The final two cellular functions associated with RA SF CD14⁺ compared to PB cells are cellular growth and proliferation and cellular development which is logical due to the maturation that the CD14⁺ cells undergo into fully activated macrophages once they are within the synovium and exposed to the pro-inflammatory and hypoxic environment [409, 410].

Table 3.6 The top 5 canonical pathways associated with the RA PB vs RA SF CD14⁺ comparison

Canonical pathway	p value (number of associated genes/total number of genes in pathway)
LXR/RXR activation	3.96×10^{-7} (17/86)
Hepatic fibrosis/ hepatic stellate cell activation	2.27×10^{-5} (21/131)
Role of mφ, fibroblasts and endothelial cells in RA	7.20×10^{-5} (36/366)
LPS/IL-1 mediated inhibition of RXR function	1.07×10^{-4} (25/205)
Coagulation system	1.98×10^{-4} (9/37)

Table 3.7 The top 5 diseases and disorders associated with the RA PB vs RA SF CD14⁺ comparison

Diseases and Disorders	p value
Connective Tissue Disorders	4.68×10^{-15} - 4.77×10^{-4}
Inflammatory Diseases	4.68×10^{-15} - 5.90×10^{-4}
Skeletal and Muscular Disorders	4.68×10^{-15} - 4.77×10^{-4}
Immunological Diseases	9.70×10^{-14} - 4.43×10^{-5}
Inflammatory Response	2.79×10^{-13} - 7.03×10^{-4}

Table 3.8 Top 5 molecular and cellular functions associated with the RA PB vs RA SF CD14⁺ comparison

Molecular and Cellular Functions	p value
Cellular movement	1.00×10^{-23} - 5.90×10^{-4}
Cell-to-cell signalling and interaction	1.40×10^{-17} - 7.03×10^{-4}
Cellular growth and proliferation	9.08×10^{-17} - 7.05×10^{-4}
Immune cell trafficking	1.72×10^{-15} - 1.35×10^{-4}
Cellular development	7.08×10^{-13} - 6.89×10^{-4}

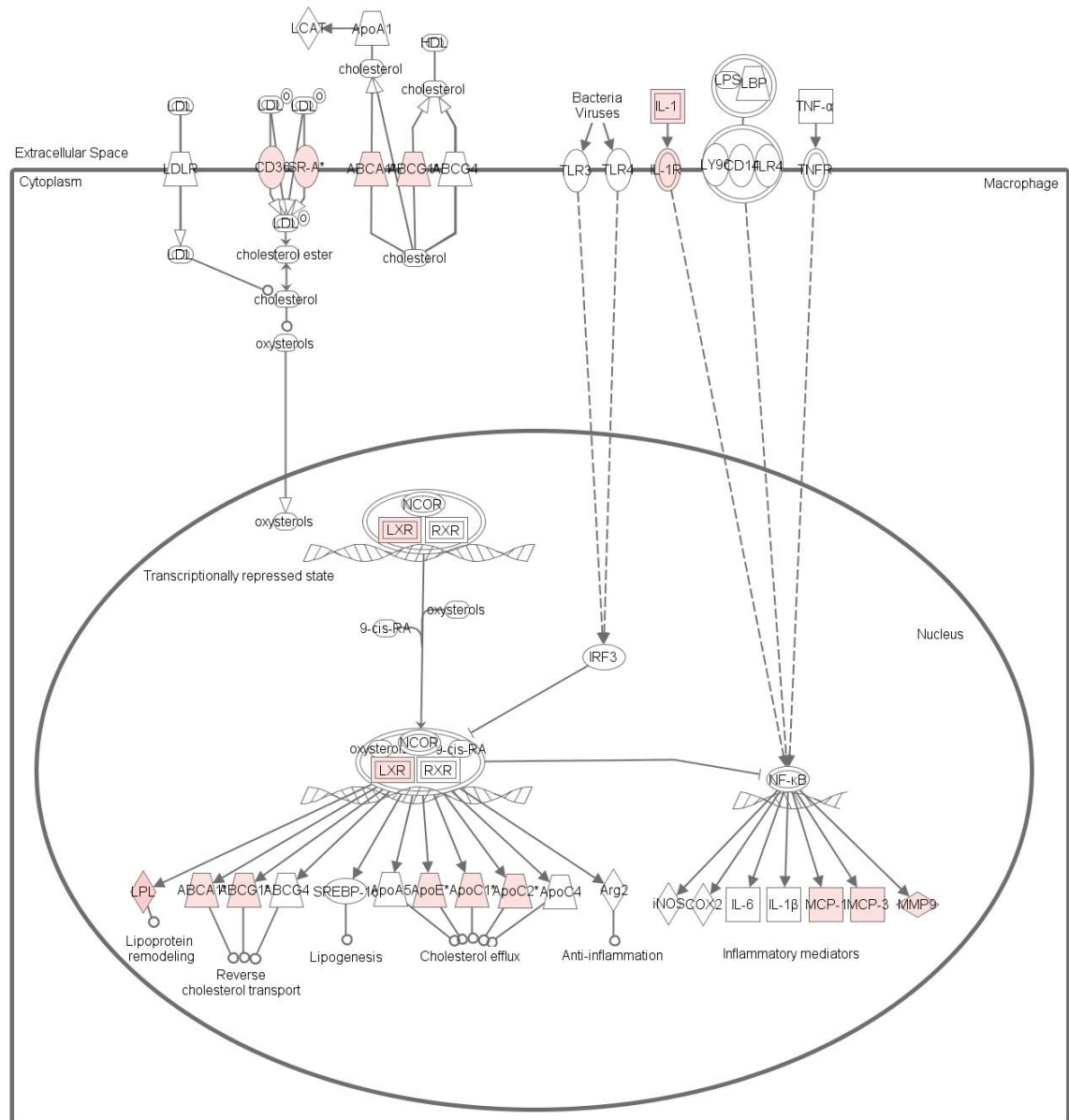


Figure 3.11 LXR/RXR activation canonical pathway associated with the RA PB vs SF comparison

This diagram outlines the genes involved in LXR and RXR activation which were differentially expressed in the RA PB vs RA SF comparison. The genes which are coloured red are those which were expressed at a higher level in the RA SF cells and therefore are genes which are upregulated once the blood CD14⁺ cells enter the synovial environment.

3.6.2.4 IPA analysis of PsA blood vs PsA synovial fluid CD14⁺

The 1149 genes differentially expressed with a fold change ≥ 4 in the PsA synovial fluid compared to the PsA blood CD14⁺ were analysed using IPA. As with the RA blood vs synovial fluid investigation this analysis yielded three sets of results associated with the gene list: the top 5 canonical pathways, the top 5 diseases and disorders and the top 5 molecular and cellular functions.

Table 3.9 demonstrates the top 5 canonical pathways associated with the differentially expressed genes in the PsA SF CD14⁺ samples compared to the PsA CD14⁺ blood samples. Three of the pathways overlap with the results of RA SF associated pathways once again emphasizing the similarities between the synovial fluid macrophages of both RA and PsA. The PsA SF specific canonical pathways included the complement system and intriguingly the pathogenesis of multiple sclerosis. The elements of this canonical pathway along with the PsA genes associated with it can be visualised in Figure 3.12. This diagram demonstrates that the PsA SF differentially expressed genes associated with the pathogenesis of multiple sclerosis pathway are all chemokines and emphasises the overlap in inflammatory gene expression between immune mediated diseases.

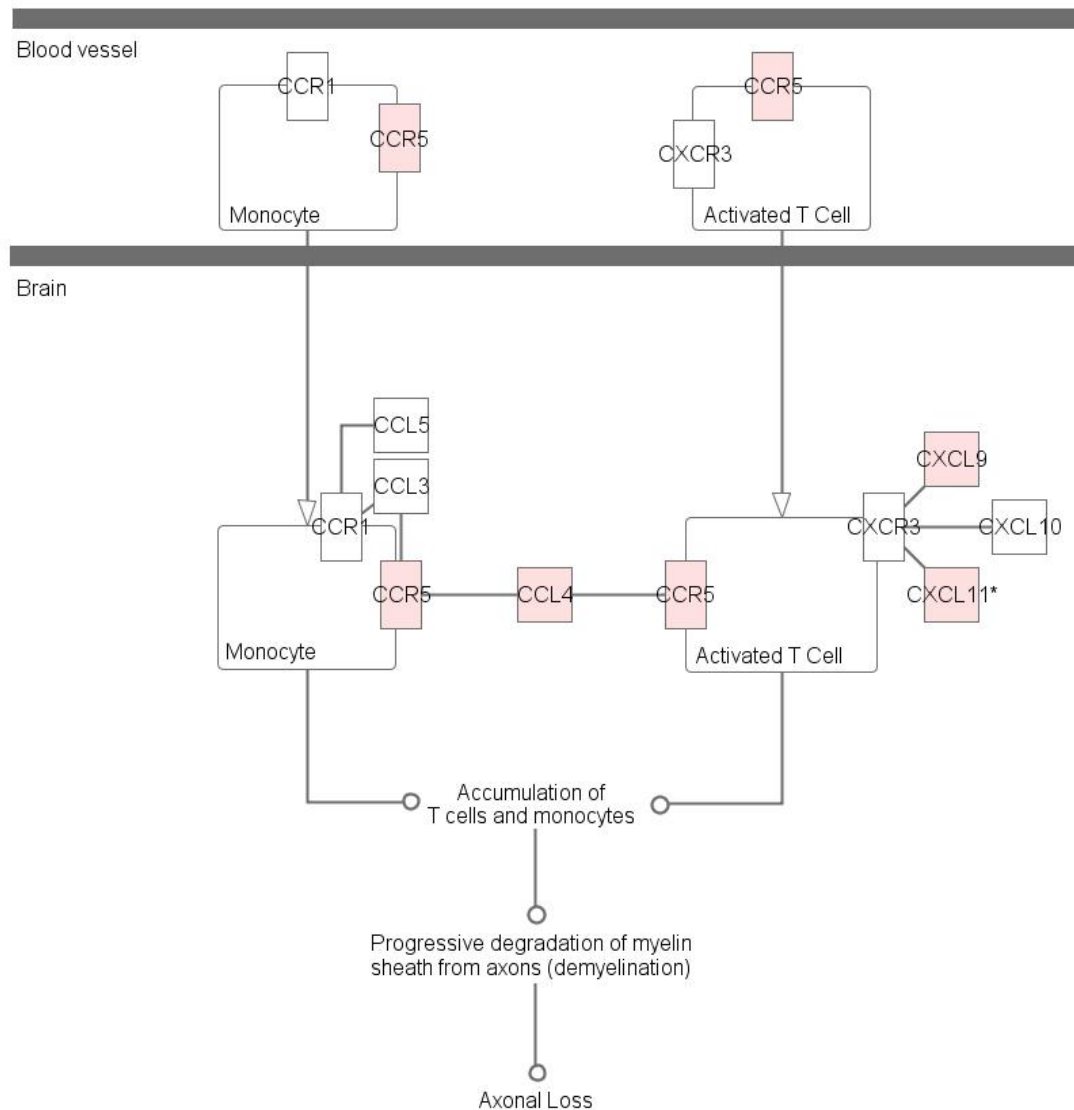
Table 3.10 outlines the diseases and disorders associated with the differentially expressed genes in PsA SF CD14⁺ cells compared to PsA PB CD14⁺ cells. As was observed with the RA SF gene set in Table 3.7 all of the diseases and disorders associated with immunological disease and the inflammatory response.

Table 3.11 outlines the cellular functions associated with PsA SF CD14⁺ cell gene expression. There is considerable overlap between the cellular functions associated with PsA SF and RA SF CD14⁺ cells as four of the top five functions associated with RA are also associated with PsA. All of the five cellular functions would be expected to be differentially expressed in PsA SF CD14⁺ cells compared to the matched peripheral blood cells. In PsA, monocytes are known to migrate from the blood into the inflamed joint [254, 411] (demonstrated by the functions cellular movement and immune cell trafficking) where they interact with other cell types and illicit an immune response [412] (demonstrated by the functions cell-to-cell signalling and interactions and cell mediated immune response), the pro-inflammatory environment also enables the differentiation of these cells into activated macrophages (demonstrated by the function cellular growth and proliferation). Hence, the cellular functions being undertaken by the PsA SF CD14⁺ cells as they move from the blood into the synovium are the functions needed to transform them into an activated, pro-inflammatory immune cell capable of sustaining the chronic inflammation seen in psoriatic arthritis.

Table 3.9 Canonical pathways associated with the PsA PB vs PsA SF CD14⁺ comparison

Canonical pathway	p value (number of associated genes/total genes in pathway)
LXR/RXR activation	8.52×10^{-6} (13/86)
Complement system	7.12×10^{-5} (8/35)
LPS/IL-1 mediated inhibition of RXR function	2.09×10^{-4} (20/205)
Pathogenesis of multiple sclerosis	4.50×10^{-4} (4/9)
Hepatic fibrosis/hepatic stellate cell activation	1.67×10^{-3} (14/131)

Pathogenesis of Multiple Sclerosis



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Figure 3.12 Pathogenesis of multiple sclerosis canonical pathway associated with the PsA PB vs PsA SF CD14⁺ cell comparison.

Component of the pathway coloured red are genes that are upregulated in the PsA SF CD14⁺ cells. Four of the nine pathway components are upregulated in PsA SF macrophages.

Table 3.10 Diseases and disorders associated with the PsA PB vs PsA SF CD14⁺ comparison

Diseases and Disorders	p value
Inflammatory Disease	$2.58 \times 10^{-15} - 2.21 \times 10^{-3}$
Connective Tissue Disorder	$2.76 \times 10^{-15} - 2.08 \times 10^{-3}$
Skeletal and Muscular Disorders	$2.76 \times 10^{-15} - 1.70 \times 10^{-3}$
Immunological Disease	$6.64 \times 10^{-14} - 2.08 \times 10^{-3}$
Inflammatory Response	$4.83 \times 10^{-10} - 2.21 \times 10^{-3}$

Table 3.11 Molecular and cellular functions associated with the PsA PB vs PsA SF CD14⁺ comparison

Molecular and Cellular Function	p value
Cellular Movement	$2.98 \times 10^{-20} - 2.31 \times 10^{-3}$
Cell-to-cell signalling and interaction	$1.51 \times 10^{-16} - 2.15 \times 10^{-3}$
Cellular Growth and Proliferation	$3.41 \times 10^{-13} - 2.21 \times 10^{-3}$
Immune Cell Trafficking	$1.18 \times 10^{-13} - 1.14 \times 10^{-3}$
Cell Mediated Immune Response	$7.86 \times 10^{-9} - 2.15 \times 10^{-3}$

3.6.2.5 Depicting RA and PsA differentially expressed genes

Analysis of the foregoing results identified the interesting observation that the synovial fluid CD14⁺ cells of both the RA and PsA patients shared substantial similarity in gene expression. This observation was first identified by examining the PCA plot (Figure 3.8) depicting the SF samples of both diseases were co-localised indicating a similarity in the overall transcriptomes of the two diseases. The subsequent IPA analysis then identified that many of the same canonical pathways and cellular functions appeared in both diseases therefore signifying a similarity in gene expression. Consequently, it was decided to further analyse the genes that had changed in the RA and PsA PB and SF samples as we hypothesized that there would be a subset of genes that changed in both diseases as well as subsets of genes that had only changed in RA or PsA. To answer this hypothesis a Venn diagram was constructed (Figure 3.13) using the following gene lists:

All entities (shown in blue), which is the list of genes that had a positive signal on the GeneChip in any of the samples. This is the starting list of genes before any quality control analysis has been done.

PsA PB vs PsA SF (shown in green), these are the differentially expressed genes between PsA PB and PsA SF. According to a paired T test and Benjamini and Hochberg MTC each gene has a corrected p value ≤ 0.05 .

RA PB vs RA SF (shown in red), this list represents the differentially expressed genes between RA PB and RA SF. The p values were calculated in the same manner as those for the PsA comparison.

Figure 3.13 indicates the Venn diagram, analysing the results proved our hypothesis to be correct as the diagram produced three gene lists:

1. Genes that are specifically changed in only the RA PB vs RA SF analysis (5158 genes)
2. Genes that are specifically changed only in the PsA PB vs PsA SF analysis (3928 genes)

3. Genes that are changed in both diseases (5605 genes)

These gene lists also demonstrate that 58% (5605 of 9533 total genes) of the PsA SF CD14⁺ differentially expressed genes and 52% (5605 of 10763 total genes) of the RA SF CD14⁺ differentially expressed genes are present in both of the diseases. This indicates that there is a significant and substantial amount of overlap between the transcriptomes of the synovial fluid CD14⁺ cells of the two diseases.

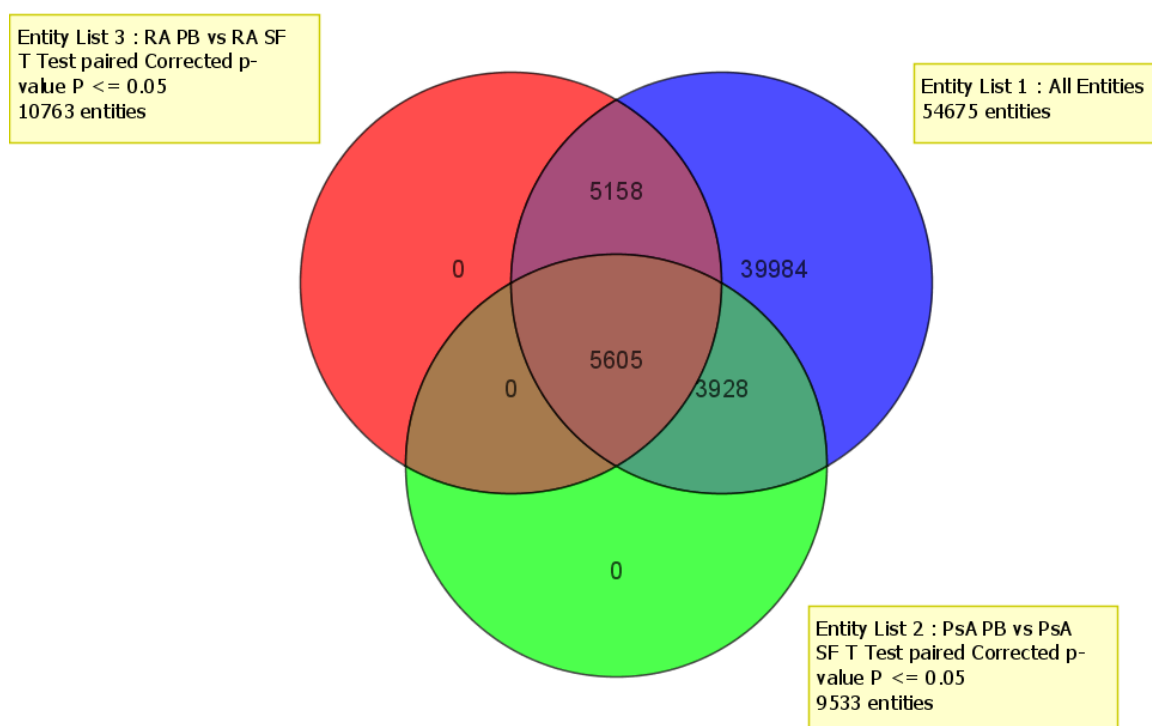


Figure 3.13 Venn diagram of the differentially expressed genes between RA PB and SF and between PsA PB and SF

Gene lists were applied to the Venn diagram in order to distinguish genes that were changed in both RA and PsA and genes that were changed in only one of the diseases. The blue circle represents all of the genes which were expressed on the Affymetrix GeneChips. The red circle represents differentially expressed genes between RA PB and RA SF which had a p value ≤ 0.05 . The green circle represents the differentially expressed genes between the PsA PB and PsA SF which had a p value ≤ 0.05 . The regions where these three circles overlap represent genes which are expressed in both RA and PsA (5605 genes), in RA only (5158 genes) and in PsA only (3928 genes).

Prior to any further analysis a fold change cut off of 4 was applied to the data. Since the fold change cut off in microarray data analysis is arbitrary we decided a high fold change cut off to specifically focus on the genes that were differentially expressed to a large extent. The fold change cut off gave the following three gene lists:

1. Up- and downregulated genes that are changed in both diseases (873 genes)
2. Up- and downregulated genes that are changed in the PsA PB vs PsA SF analysis only (154 genes)
3. Up- and downregulated genes that are changed in the RA PB vs RA SF analysis only (348 genes)

IPA analysis was subsequently performed on these three gene lists to determine the canonical pathways associated with both diseases and each disease individually. Specifically the genes that were up- and down-regulated in the synovial fluid compared to the peripheral blood CD14⁺ cells of the diseases were analysed to try to determine what pathways were activated within the synovial fluid macrophages once they were exposed to the proinflammatory environment of the joint. The diseases and disorders analyses in the previous IPA comparisons were not very informative as they highlighted that the diseases associated with our gene lists were related to arthritic and inflammatory disorders. Therefore in this IPA analysis only the canonical pathways and molecular functions associated with the genes lists were investigated.

Table 3.12 shows the canonical pathways associated with the differentially expressed genes that were similarly expressed in both RA and PsA synovial fluid. These pathways are the same as those that were identified in the IPA analysis of the RA PB vs SF and PsA PB vs SF comparison (Table 3.6 and Table 3.9 respectively). Table 3.13 demonstrates the molecular and cellular functions associated with the gene list which was differentially expressed in both RA and PsA synovial macrophages. These functions have previously been identified in the RA PB vs SF and the PsA PB vs SF comparisons and demonstrate the shared macrophage functions between the two diseases.

Table 3.14 identified the canonical pathways that are significantly associated with the subset of genes which are differentially expressed specifically in the synovial fluid CD14⁺ cells of the PsA patients but not the RA patients. It outlines pathways that would not usually be associated with macrophages such as B cell development and altered B and T cell signalling in RA. Investigating the differentially expressed genes from the gene list which are associated with these pathways identifies genes that are expressed in both macrophages and B cells/T cells such as CD40, MHC II and TLR3. This result is highlighted in Figure 3.14 which demonstrates that the two genes within B cell development canonical pathway associated with the PsA only gene list, are MHC II and CD40 which are two genes that are known to be expressed in both B cell and macrophages.

Table 3.15 outlines the molecular and cellular functions associated with the differentially expressed genes found specifically in the PsA SF CD14⁺ cells compared to the PsA PB CD14⁺ cells. Two of these functions, cellular movement and cell-to-cell signalling and interaction, were previously associated with the broad PsA PB vs SF CD14⁺ comparison, however the other three functions which consist of DNA replication, cell cycle and cell death are unique to the PsA SF CD14⁺ expressed genes and demonstrate the disease specific cellular functions.

Table 3.16 outlines the canonical pathways significantly associated with the subset of genes differentially expressed specifically within the RA synovial fluid CD14⁺ cells. The top two pathways were previously associated with the differentially expressed genes in RA PB vs SF (see Table 3.6) however this analysis of the unique genes expressed in RA SF cells identifies three other pathways involving intracellular signalling within the macrophages including atherosclerotic, p38 and ERK5 signalling. Figure 3.15 demonstrates the ERK5 canonical pathway and outlines the five genes from the RA gene list which were associated with the pathway. In this figure, genes highlighted in green were expressed at a higher level in the RA PB CD14⁺ cells compared to the SF cells whereas genes highlighted in red denote the genes which were expressed at a higher level in the RA SF CD14⁺ cells compared to RA PB CD14⁺ cells. Table 3.17 outlines the molecular and cellular functions associated with the gene list expressed by RA SF CD14⁺ cells only. All of these functions were previously associated with the broad analysis of RA PB vs SF CD14⁺ apart from the function

of cell morphology. Hence cell morphology is a disease specific function and is only associated with RA SF CD14⁺ cells.

Table 3.12 Canonical pathways associated with the differentially expressed genes in BOTH RA and PsA synovial fluid

Canonical Pathways expressed in BOTH RA and PsA synovial fluid CD14⁺	P value (number of associated genes/total number of genes in pathway)
LXR/RXR activation	3.28x10 ⁻⁶ (12/76)
LPS/IL-1 mediated inhibition of RXR function	7.07x10 ⁻⁵ (18/197)
Complement system	1.00x10 ⁻⁴ (7/34)
Hepatic fibrosis/Hepatic stellate cell activation	1.52x10 ⁻³ (12/128)
Pathogenesis of Multiple Sclerosis	3.11x10 ⁻³ (3/9)

Table 3.13 Molecular and cellular functions associated with the differentially expressed genes in BOTH RA and PsA synovial fluid

Molecular and cellular functions associated with BOTH RA and PsA synovial fluid CD14⁺	P value
Cellular movement	9.80x10 ⁻¹⁷ - 1.15x10 ⁻²
Cellular growth and proliferation	3.51x10 ⁻¹² - 1.15x10 ⁻²
Cell-to-cell signalling and interaction	1.09x10 ⁻¹¹ - 1.15x10 ⁻²
Cellular development	3.96x10 ⁻⁷ - 1.15x10 ⁻²
Cellular function and maintenance	5.32x10 ⁻⁷ - 1.15x10 ⁻²

Table 3.14 Canonical pathways associated with the differentially expressed genes in PsA only

Canonical pathways expressed in PsA synovial fluid CD14 ⁺ only	P Value (number of associated genes/total number of genes in pathway)
B cell development	8.08x10 ⁻⁴ (3/30)
Allograft rejection signalling	1.53x10 ⁻³ (3/51)
Pathogenesis of Multiple Sclerosis	1.75x10 ⁻³ (2/9)
Autoimmune Thyroid disease signalling	1.79x10 ⁻³ (3/53)
Altered T and B cell signalling in Rheumatoid Arthritis	2x10 ⁻³ (4/85)

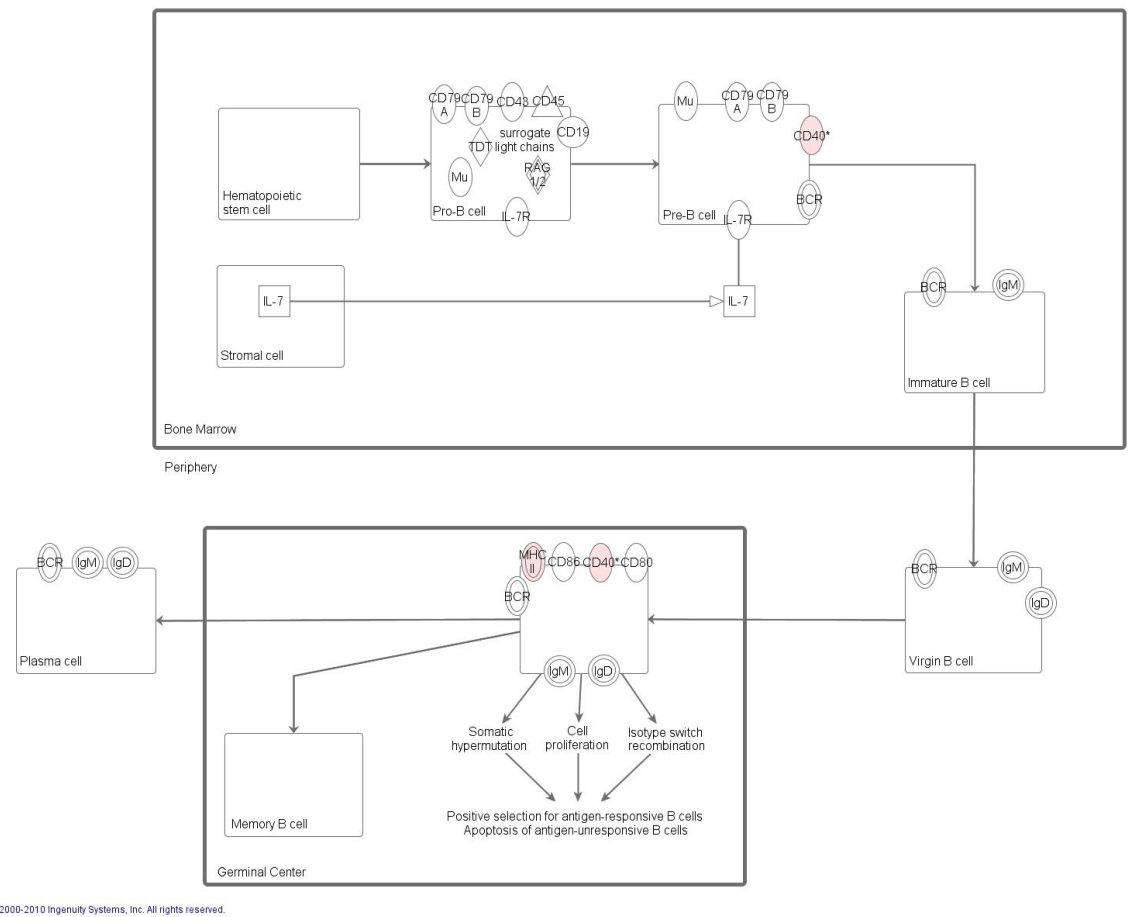


Figure 3.14 B cell development canonical pathway associated with the genes expressed in PsA synovial fluid only

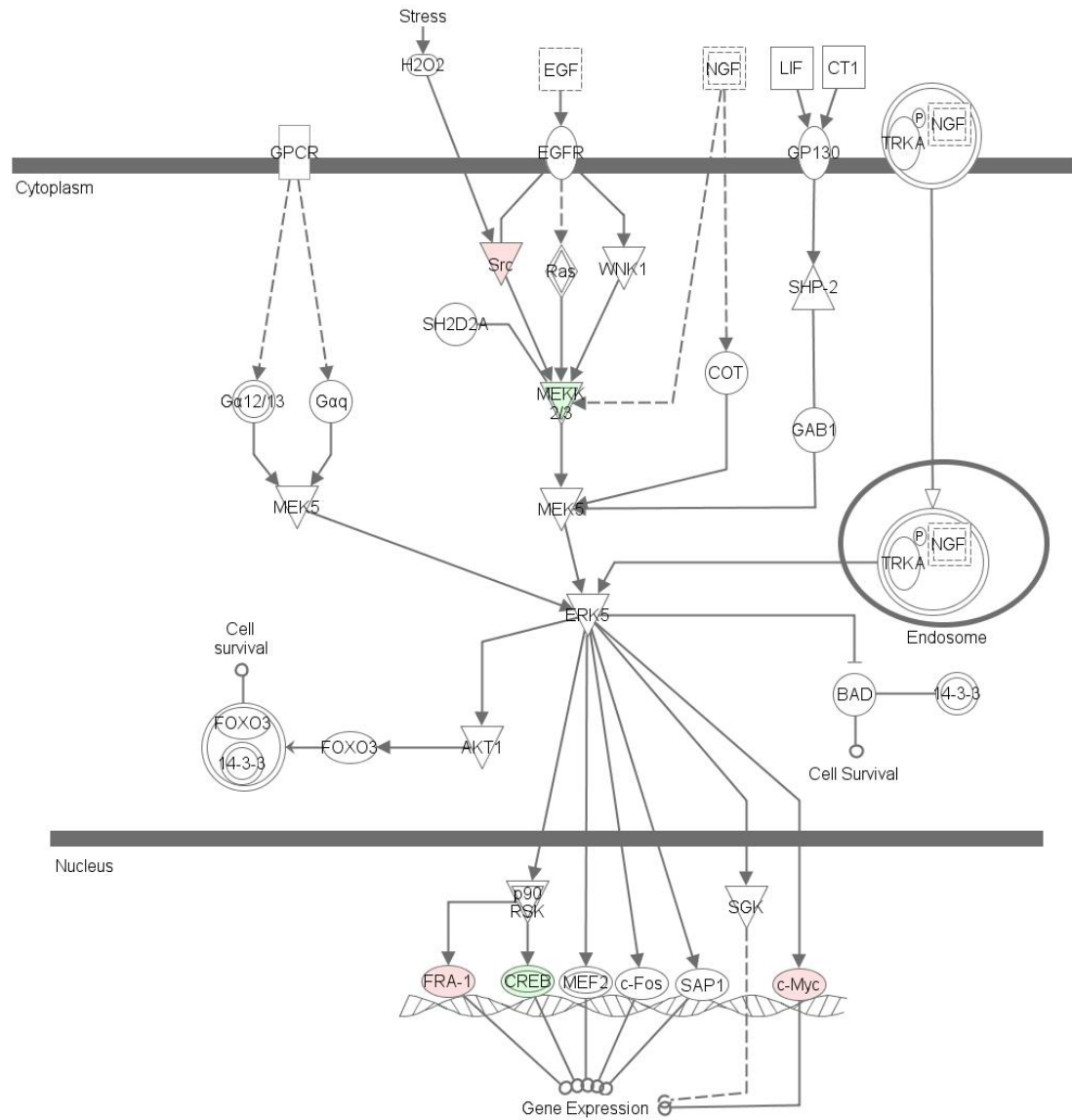
The two genes highlighted within this pathway (MHC II, CD40) are expressed in the PsA only gene list and are associated with this pathway. These genes are highlighted in red which indicates that they were upregulated in the PsA SF compared to PsA PB.

Table 3.15 Molecular and cellular functions associated with the differentially expressed genes in PsA only

Molecular and cellular functions associated with PsA synovial fluid CD14 ⁺ only	P value
Cellular movement	3.34x10 ⁻⁷ - 2.16x10 ⁻²
DNA replication, recombination and repair	1.08x10 ⁻⁵ - 1.91x10 ⁻²
Cell-to-cell signalling and interaction	1.74x10 ⁻⁵ - 2.16x10 ⁻²
Cell cycle	2.46x10 ⁻⁵ - 2.16x10 ⁻²
Cell death	3.47x10 ⁻⁵ - 2.16x10 ⁻²

Table 3.16 Canonical pathways associated with the differentially expressed genes in RA only

Canonical pathways expressed in RA synovial fluid CD14 ⁺ only	p value (number of associated genes/total number of genes in pathway)
Heptatic fibrosis/hepatic stellate cell activation	5.27×10^{-5} (9/128)
Role of mØ/fibroblasts and endothelial cells in RA	2.19×10^{-4} (13/329)
Atherosclerosis signalling	2.95×10^{-4} (7/107)
p38 MAPK signalling	1.52×10^{-3} (6/96)
ERK5 signalling	1.54×10^{-3} (5/69)



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Figure 3.15 ERK5 signalling canonical pathway associated with the genes expressed in RA synovial fluid only

The highlighted genes are those that were differentially expressed only in the RA PB vs RA SF comparison. Genes highlighted in green indicate genes which were downregulated in RA SF CD14⁺ cells and therefore were expressed at a higher level in RA PB CD14⁺ cells. Genes which are highlighted in red demonstrate genes which were expressed at a higher level in RA SF CD14⁺ cells compared to the RA PB CD14⁺ cells.

Table 3.17 Molecular and cellular functions associated with the differentially expressed genes in RA only

Molecular and cellular functions associated with RA synovial fluid CD14 ⁺ only	P value
Cellular movement	1.09×10^{-14} - 4.09×10^{-3}
Cellular growth and proliferation	3.19×10^{-11} - 3.75×10^{-3}
Cellular development	1.38×10^{-9} - 4.12×10^{-3}
Cell-to-cell signalling and interaction	4.87×10^{-9} - 3.57×10^{-3}
Cell morphology	9.64×10^{-8} - 4.90×10^{-3}

3.6.2.6 Table of genes highly upregulated in both RA and PsA SF samples

The IPA analysis was very informative in defining the groups of genes and the pathways involved in the RA and PsA synovial CD14⁺ cells however we also wanted to study individual genes and their expression within each disease to try and identify novel pathways of arthritis pathogenesis. The aim of the subsequent analysis therefore was to locate novel genes that were increased in the CD14⁺ SF samples of both diseases. The list of genes upregulated in both RA and PsA SF cells compared to their matched blood cells was obtained from the Venn diagram analysis and analysed in order of descending fold change. Starting from the most highly expressed genes in both RA and PsA synovial fluid literature searches were performed on genes to determine their function and expression. Unsurprisingly, many genes had been previously associated with or found to be highly expressed in either RA or PsA; however some novel genes were also found. Table 3.18 lists several of the genes that were analysed.

Table 3.18 Gene upregulated in BOTH RA and PsA synovial fluid CD14+ cells

Symbol	Gene Name	Fold change upregulation in RA SF (p value)	Fold change upregulation in PsA SF (p value)	Description of Gene
SPP1	Secreted phosphoprotein 1 (osteopontin)	1762 (3.8×10^{-4})	484 (3.2×10^{-4})	An extracellular matrix glycoprotein expressed by many inflammatory cells. It is up-regulated in response to injury and inflammation [413]. Thrombin cleaved OPN is found in RA SF at a 30 times higher concentration compared to OA SF [414].
CCL18	Chemokine ligand 18	326 (6.4×10^{-5})	375 (2.7×10^{-4})	A T cell attracting chemokine expressed by monocytes/macrophages and DCs [415]. Levels of CCL18 in RA were higher than in OA in serum, synovial fluid, cartilage and synovial tissue. These levels were positively correlated with disease activity and rheumatoid factor levels [416, 417].
APOC1	Apolipoprotein C-I	482 (3.3×10^{-4})	344 (5.4×10^{-4})	Apo C-I along with Apo C-II are constituents of high-density lipoprotein (HDL) that slows the clearance of triglyceride-rich lipoproteins [418].
PLTP	Phospholipid transfer protein	98 (8.9×10^{-4})	246 (2.7×10^{-4})	PLTP facilitates the transfer of phospholipids from triglyceride-rich lipoproteins to HDL via an enzyme lipoprotein lipase (LPL) [419]. PLTP may play a role in atherosclerosis depending on its expression. Systemic over-expression of PLTP is associated with increased atherosclerosis susceptibility [420] whereas in an LDLr ^{-/-} mouse model, which is susceptible to atherosclerosis, macrophage derived PLTP was found to be atheroprotective [421].

AXL	AXL receptor tyrosine kinase	72 (0.002)	203 (2.4×10^{-4})	AXL subfamily is involved in cell adhesion, motility, angiogenesis and signal transduction. It may play a role in the invasiveness of cancers [422].
FN1	Fibronectin 1	263 (8.1×10^{-4})	172 (5.6×10^{-4})	A glycoprotein that has roles in adhesion of multiple cell types to the extracellular matrix (ECM), migration, proliferation, matrix remodelling and tissue repair [423]. Activated T cells and macrophages secrete fibronectin [424] and RA SF contains 2 to 3 times more fibronectin than RA plasma [425]. Fibronectin is present in the SF, synovial membrane and plasma of PsA patients [426-428].
LGM	Legumain	167 (1.7×10^{-4})	164 (2.7×10^{-4})	A lysosomal cysteine proteinase that is specific for the hydrolysis of asparaginyl bonds [429]. It can cleave and activate MMP2 [429]; α -thymosin [430]; fibronectin [431] and cathepsin B, H and L [432]. It is over-expressed in tumour associated macrophages in breast cancer tumours [433]; unstable atherosclerotic plaque macrophages [434]; and lesional atopic dermatitis and psoriasis [435].
AQP3	Aquaporin 3	62 (5.5×10^{-4})	30 (4.4×10^{-4})	A small hydrophobic membrane protein involved in the transport of water and glycerol across the cell membrane [436]. AQP3 is expressed in the basal layer of keratinocytes mammalian skin [437, 438]. AQP3 has been found in OA cartilage [439] and in PBMCs from RA patients where it was associated with DAS28-CRP levels [440].
NRP1	Neuropilin 1	11 (0.002)	40 (7.0×10^{-4})	Single pass transmembrane glycoprotein involved in cell motility and whose ligands include semaphorins and VEGF family members [356]. Neuropilin 1 is the VEGF 165 receptor in fibroblast like

synoviocytes in RA and is also expressed on infiltrating leukocytes [357]. Neuropilin 1 mRNA has also been detected in OA but not healthy cartilage samples [441].				
PLXNA1	Plexin A1	26 (4.9×10^{-4})	27 (9.9×10^{-4})	Single membrane spanning proteins which, along with neuropilins, are receptors for semaphorins [442]. Plexin A1 expression is increased during M-CSF differentiation [443] and it is thought to be important for T cell – DC interactions as plexin A1 deficient DCs Induced 50% less T cell proliferation than control DCs [444].
PDPN	Podoplanin	6 (0.02)	22 (0.001)	A mucin-type transmembrane glycoprotein that is involved in lymphatic vessel formation. It is a specific marker for lymphatic endothelium and is not expressed in blood vessels [445]. TNF α blockade therapy promotes the proliferation of lymphatic vessels, the inflamed synovium of RA and SpA patients receiving TNF blockade therapy stained for podoplanin [446].

Table 3.18 demonstrates the subset of genes that were explored *in silico* in depth from the list of genes upregulated in SF CD14⁺ cells of both diseases. They were analysed based on their large fold change increased in the SF; this analysis was set up to try and discover novel genes that as yet had not been analysed in the context of RA and PsA arthritis. Several novel genes were found but after performing comprehensive literature searches two of these genes, an enzyme called legumain and a surface receptor called plexin A1, appeared to be candidate genes which may have particular relevance to the pathogenesis of the synovial macrophage in both PsA and RA. It was decided to concentrate further research on these two genes to try and discover why they were highly upregulated in the SF CD14⁺ cells of both RA and PsA. The following two chapters demonstrate the experimental work performed to try and understand the role of these genes within the synovial environment.

3.7 Discussion

Microarrays have become a standard method to investigate differential disease states on the basis of genome-wide expression. Previously, there have been numerous microarray studies analysing the two diseases RA and PsA. However, these studies mainly focused on the gene expression of PBMCs or whole synovial tissue and as yet very few studies have measured the gene expression within individual immune cell types. Macrophages are an essential component of the inflammatory cell influx into the joint, being one of the major cell types producing the pro-inflammatory cytokines, chemokines and extracellular matrix components which all contribute to the destruction and degradation of the arthritic joint [258]. Since very little is known about the transcriptomic profile of this cell type within RA or PsA we decided to microarray chip analyse several different samples to try and elucidate which genes were being expressed in (1) diseased blood monocytes compared to healthy controls, and (2) synovial fluid macrophages compared to blood monocytes.

Once all the patient and normal samples had been collected, prepared and individually hybridized onto Affymetrix GeneChips the results were subjected to quality control to detect any outliers. Quality control must take place during microarray analysis to ensure that: i) the data is suitable to answer the experimenter's question and ii) the data is suitable for subsequent analysis [447]. For the current analysis quality control consisted of examining the signal intensity of the GeneChips on a signal histogram which resulted in the detection of a GeneChip which had an abnormal signal intensity. This outlier was further confirmed by analysing the principle component analysis (PCA) plot which graphs the overall transcriptomic signature of each sample. Quality control problems can come from several sources; for example the hybridisation step can cause uneven fluorescence on the chip and differences in RNA quality can produce variable signal intensity and reports have shown that removing outlier GeneChips can improve the detection of differentially expressed genes [447], therefore the outlier sample identified in this study was removed from further analysis.

Once the outlier was removed a PCA plot of the samples was setup to identify trends in the data since samples clustered within the same area of the PCA graph are considered to be genetically similar [354]. This analysis identified that

the blood CD14⁺ cells from both RA and PsA were genetically similar and also the synovial fluid CD14⁺ cells from both diseases had similar genetic profiles, which was a surprising result as previous studies had identified differential subsets of macrophages in RA compared to PsA [253, 255]. On the basis of the PCA plot and the original hypotheses we decided to perform two main comparisons: i) the differentially expressed genes in the patient blood CD14⁺ cells compared to those of the healthy blood CD14⁺ cells, and ii) the differentially expressed genes in the patient synovial fluid CD14⁺ compared to the matched blood CD14⁺ cells.

3.7.1 Comparison 1: Healthy blood versus diseased blood

The first data comparison to take place was analysing CD14⁺ cells isolated from the blood of diseased patients and healthy controls. Previously there have been several microarray analyses comparing peripheral blood mononuclear cells (PBMCs) or peripheral blood cells (PBCs) of healthy donors to those of patients with rheumatic diseases such as RA, PsA and JIA [325-327, 329, 330, 340, 341]. These studies have been very informative in distinguishing disease specific expression profiles such as the microarray analysis work by van der Pouw Krann et al which discovered a type I IFN subpopulation of RA patients who had a higher expression of type I IFN specific genes and a distinct biomolecular phenotype compared to healthy controls [329]. Batliwalla et al performed a similar microarray analysis comparing the PBCs of PsA patients to those of healthy controls and identified specific genes that could discriminate healthy controls from PsA patients, which they suggested could act as diagnostic biomarkers for PsA [340]. Consequently, microarray analysis comparing healthy and diseased peripheral blood cells have provided a wealth of information for rheumatic disease and identified many disease specific genes and biological processes.

However, this type of analysis is not without problems and several analyses have reported skewing of the overall data and gene expression due to differing numbers of cell subtypes within the whole blood. Batliwalla et al came across this problem when examining the gene expression profiles of PBCs from RA patients to those isolated from healthy controls. They found 40% of the genes differentially upregulated in the RA cells were classed as “monocyte enriched” which they later discovered was due to significantly higher numbers of

monocytes in the RA patient PBCs compared to those of healthy controls [326]. Thus whilst microarray analysis on whole blood cells is informative, difficulties can arise when trying to account for differences in the number of cell subtypes which can cause complications when trying to compare and verify work between groups.

Whole blood microarray profiling may also be masking the individual contribution of cell subtypes to the disease therefore microarray analysis of individual cell types from diseased patients may be a useful strategy in understanding the genetic makeup of these cells. Recently, a small number of studies have been performed looking at the gene expression profiles of specific cell types in RA such as B cells [336], CD4⁺ T cells [337] and fibroblasts [334]. One study performed by Stuhlmuller et al has also analysed the gene expression of RA blood monocytes isolated during the course of leukapheresis treatment [338]. However, at present there have been no studies analysing the blood monocytes of RA and PsA compared to healthy donor blood monocytes. Given the importance of the monocyte/macrophage in the pathogenesis of RA and PsA we hypothesised that analysing the gene expression profile of this cell subset in comparison to healthy controls may elucidate disease specific genes and also discriminate differences in gene expression between the two diseases.

Peripheral blood isolated CD14⁺ cells were analysed from 8 RA patients, 8 PsA patients and 5 healthy controls. When these samples were analysed on the PCA plot (Figure 3.4) we discovered the intriguing result that all of the peripheral blood samples were clustered together indicating a similar gene expression profile expressed by the healthy and diseased sample conditions. Since most of the previous microarray experiments of healthy vs diseased PBMCs have demonstrated a significant difference in the genetic profiles of the two conditions this result may suggest that the large genetic differences seen in these experiments are not the result of differences in the monocytes but may be due to large genetic differences between other cell subtypes.

Of the 12 genes which are upregulated in RA blood monocytes compared to PsA monocytes three (NCAM1, PKP2 and LAMC1) are associated with cell adhesion and interaction. Neural cell adhesion molecule 1 (NCAM1) otherwise known as CD56 is usually expressed on cytotoxic cells such as natural killer (NK) cells and

NK-T cells. However, a small population of CD56⁺ monocytes have been identified in healthy individuals which can present antigen and produce a variety of monocyte cytokines in response to LPS stimulation [448]. CD56⁺ NK cells are enriched at the inflammatory sites in RA where they have been found to interact with monocytes and induce TNF α expression in a cell contact dependent manner [251, 449]. Conversely, CD56⁺ CD8⁺ CD28⁻ T cells displayed strong anti-inflammatory properties in a SCID mouse chimera model of human RA by interacting with APCs and causing the subsequent down-regulation of CD80 and CD86 [450]. NCAM1 is known to mediate cell-cell adhesion and the data above suggests that it plays a role in cell adhesion and interaction therefore we can hypothesise that NCAM1 expression in RA blood monocytes may be playing a role in cell-cell interaction.

PKP2 and LAMC1 are two novel genes not previously associated with RA. Plakophilin 2 (PKP2) is normally found co-localised with adhesive junctions in cardiomyocytes and epithelial cells [368, 369] therefore its possible role in RA blood monocytes is unclear at present. LAMC1 (laminin gamma 1) is a subunit of all laminin molecules which are major constituents of basement membranes and, along with collagen, provide the basic scaffold to which other basement membrane proteins, such as proteoglycan, integrate [374]. Laminins have structural, adhesive and cell signalling functions and are able to modulate cell signalling behaviour and migration by interacting with cell surface integrin receptors [451]. Laminin has also previously been found to affect monocyte phagocytosis, and play a role in monocyte migration by inhibiting their adhesion to basement membranes thereby assisting their migration across the membrane and also by inducing spontaneous migration within the cells [452-455]. LAMC1 has previously been detected in healthy blood monocytes, which was used to produce the molecule laminin-8, monocyte adhesion to laminin-8 also produced spontaneous and chemokines-induced migration [455]. Hence, the upregulation of LAMC1 in RA blood monocytes could help play a role in the migration of these cells across the basement membrane of the blood vessels thereby assisting their infiltration into the joint.

Two genes which were upregulated in RA PB compared to PsA PB have previously been shown to be associated with RA. These genes are NRP1 (neuropilin-1) and IL7 (Interleukin 7). Neuropilin-1 is a receptor for vascular endothelial growth

factor (VEGF) and is expressed in the synovium of RA patients where it was specifically associated with the lining layer, infiltrating leukocytes and endothelial cells [357]. Recent work has shown that the interaction of neuropilin-1 with VEGF prevented apoptosis and was therefore crucial to rheumatoid synovial cell survival [357]. VEGF is detected in the serum of RA patients [456] and has been found to correlate with the development of radiographic damage and CRP level [457]. Since neuropilin-1 interaction with VEGF has been shown to prevent apoptosis in synoviocytes we hypothesise that neuropilin-1 is playing the same role in the blood monocytes and the upregulation of NRP1 in the RA monocytes could be a mechanism to prolong their survival.

IL-7 has also previously been described in RA, serum levels of the cytokine are higher in RA patients compared to healthy controls and correlate with markers of inflammation [371]. IL-7⁺ cells correlate with the numbers of CD68⁺ cells in RA synovial lining and double staining has demonstrated that CD68⁺ cells are the major producer of IL-7 [371]. In RA blood and synovial fluid IL-7R is primarily expressed on T cells, with the highest expression on CD4⁺ cells. Numerous studies have focused on the role of IL-7 in RA, synovial fluid isolated CD4⁺ T cells are hyperresponsive to IL-7 and IL-7 enhanced cell-contact dependent activation of T cells and macrophages resulting in TNF α production [373]. IL-7 also enhances IFN γ and TNF α production from T cells [458]. Hence, the RA blood monocytes positive for the IL7 mRNA in this present study may be a source of the increased RA serum IL-7 levels and due to the cytokine's pro-inflammatory properties could be contributing to increased inflammation seen in these patients.

Analysing the group of 12 genes that are upregulated in PsA PB monocytes compared to RA PB monocytes two groups of genes emerge, genes that are associated with response to stress and toxins (GSTM1, GSTM2 and EIF2AK3) and genes involved in transcription and its initiation (FOXP1, KLF9 and MED26). Glutathione S-transferase M1 and M2 (GSTM1 and GSTM2 respectively) are part of a broadly expressed family of enzymes which catalyse the conjugation of glutathione and are involved in the detoxification of cytotoxic carcinogens and metabolites. Glutathione is an intracellular antioxidant and is a prominent defence against reactive oxygen species, it acts as a radical quencher in cells by

removing superoxide anions and hydrogen peroxide and works to maintain the equilibrium between free radical production and antioxidant defence thereby reducing oxidative stress damage [383]. Oxidative stress has previously been shown to be implicated in the pathogenesis of RA and PsA where an excess of free radicals causes oxidation and subsequent damage to cartilage, extracellular matrix and DNA [459] and markers of oxidation have been found in the serum and synovial fluid of patients [460, 461]. Hassan et al measured the level of the antioxidant glutathione in RA serum compared to healthy controls and found it to be decreased whereas the levels of the enzyme glutathione S-transferase (GST) were increased, which the authors suggested could be a compensatory mechanism in the absence of its substrate glutathione [462]. Since our data has shown an increase in the levels of two GST proteins, GSTM1 and GSTM2, in PsA blood monocytes compared to RA blood monocytes these PsA patients may also be increasing their levels of these enzymes to due to the increased oxidative stress associated with this disease and the decrease in antioxidant glutathione. Of interest, polymorphisms in GST genes have been associated with RA susceptibility, these genes include GST Theta (GSTT1), GST Pi (GSTP1) and in particular one of our genes of interest GST Mu (GSTM1) [385]. Some of the polymorphisms also show an additive effect with smoking and patients carrying the polymorphisms who also smoke were found to be at an increased risk of disease with higher disease severity [463]. Despite no susceptibility being reported for PsA these findings highlight the potential importance of GST enzymes within the pathogenesis of psoriatic arthritis.

Eukaryotic translation initiation factor 2-alpha kinase 3 (EIF2AK3) otherwise known as PERK is an endoplasmic reticulum (ER) transmembrane protein kinase. The function of the ER is to correctly fold and process proteins which are intended for the secretory pathway before they are transported to the Golgi apparatus. If the secretory demand exceeds the folding capacity of the ER, the accumulated mis- or unfolded proteins, often called ER stress, activates the unfolded protein response (UPR). The UPR aims to enhance the processing, assembly and transport of secretory proteins. PERK is a UPR sensor, its role is to reduce global translation thereby allowing the cell to correct for the impaired protein folding resulting from the ER stress before allowing additional secretory proteins to be synthesised [402]. PERK does this by phosphorylating eIF2 which

prevents the release of the translation factor from the ribosomal machinery and thus reduces translation [464]. At present there have been no studies performed on the role of the UPR in psoriatic arthritis, however another spondyloarthropathy ankylosing spondylitis (AS) has shown associations between the molecule HLA-B27, which is present in up to 96% of patients [465], and the UPR. Research into AS in HLA-B27 transgenic rats has shown that HLA-B27 has the propensity to mis-fold resulting in ER stress and the activation of the UPR [466]. Consequently, the upregulation of the UPR sensor PERK in PsA compared to RA blood monocytes may be an indication that the PsA monocytes are more exposed to mediators of ER stress such as metabolic stress or mutant/over expression of secreted proteins as is seen in the spondyloarthropathy AS which shares some clinical features with PsA.

As mentioned above there are also a group of genes upregulated in PsA blood monocytes which are associated with transcription initiation namely FOXP1, KLF9 and MED26. Forkhead box P1 (FOXP1) is a member of the FOX family of transcription factors and is involved in B cell development [390], thymocyte development [391] and monocyte differentiation *in vitro* and *in vivo* [392, 467]. FOXP1 must be downregulated by the monocyte in order for it to fully differentiate into a macrophage, because mice which are transgenic for FOXP1 have reduced M-CSF receptor expression, impaired migratory capacity and diminished macrophage function such as cytokine production [392]. Since PsA monocytes expressed FOXP1 as 2.7 fold change increase compared to RA monocytes this could suggest that at the time of the samples being taken the RA monocytes were more differentiated towards a macrophage phenotype compared to the PsA monocytes.

Kruppel-like factor 9 (KLF9) is a member of the Kruppel-like family (KLF) of zinc finger transcription factors. There are 25 KLFs proteins known at present, which bind to GC/GT boxes found in promoter regions and enhancer/silencer regions of multiple chromosomal genes [468]. KLF9 has been identified as having a stimulatory role in neuronal cell process formation and differentiation [469] and endometrial cell proliferation [470]. A recent study has also shown that mice lacking KLF9 have dysregulated intestinal crypt cell proliferation and villus cell migration [471]. The exact role of KLF9 within PsA blood monocytes is unclear at present but it could play a role in monocyte migration into the diseased joint.

Mediator complex subunit 26 (MED26) is one of at least 30 subunits that make up the mediator complex. The mediator complex is a required part of transcriptional activation and serves as an adaptor between the transcription factors bound at the regulatory elements on the DNA and both RNA polymerase II and the general transcription factors [472]. At present the exact role of MED26 within the mediator complex is unknown therefore it is difficult to speculate on the role of MED26 on PsA blood monocytes however it could indicate an increase in the amount of general transcription taking place within the monocytes.

Database for annotation, visualisation and integrated discovery (DAVID) software was used in this analysis to determine if there is a significant enrichment of different biological processes within the two remaining gene lists: (1) differentially expressed genes between RA PB monocytes and healthy blood monocytes (2) differentially expressed genes between PsA PB monocytes and healthy blood monocytes.

The DAVID analysis of the genes which were expressed at a higher level in RA blood compared to healthy blood monocytes (Figure 3.6) demonstrated two distinct groups of genes, response to stimulus (indicated by the three biological processes response to external stimulus, response to chemical stimulus and response to wounding) and cellular movement (indicated by the three biological processes chemotaxis, taxis and locomotion). The other biological processes associated with this RA gene list were negative regulation of cellular process and positive regulation of cellular process, which both contain many of the same genes. Since these biological processes conflict highly with each other they were not analysed further.

The RA blood monocytes may be up-regulating genes associated with response to stimulus function due to the pro-inflammatory stimuli present in the serum of RA patients which are not found in healthy controls. The three biological process groups: response to stimulus, response to chemical stimulus and response to wounding contain many of the same genes including; TSP1 (thrombospondin-1), SOCS3 (suppressor of cytokine signalling 3) and CLU (clusterin). Thrombospondin-1 has previously been associated with RA [473] and a recent study indicated that serum thrombospondin-1 levels were increased in RA patients compared to healthy controls and correlated with increased serum levels of pro-inflammatory

cytokines [474]. This study validates the data found in this experiment which indicates thrombospondin-1 is expressed at a higher level in RA PB monocytes compared to healthy controls and is significantly associated with biological processes involving response to environmental stimuli.

Another gene associated with all three response to stimuli related biological processes is suppressor of cytokine signalling 3 (SOCS3). The SOCS family consists of SOCS1-SOCS7 and cytokine-inducible SH2 (CIS) and their role is to tightly regulate the activity of the JAK-STAT pathway [475]. They are not constitutively expressed in cells but their expression is induced by a range of cytokines via activated STATs [476]. Once expressed they inhibit the JAK-STAT pathway by several mechanisms such as prevention of STAT recruitment to activated cytokine receptors, induction of substrate degradation and suppression of JAK catalytic activity [477]. SOCS3 levels are significantly higher in RA PBMCs compared to healthy controls, which was found to be due to the upregulation of SOCS3 specifically in the RA blood monocytes [478]. SOCS3 overexpression is effective in preventing the development of murine collagen induced arthritis (CIA) [479] and SOCS3 knockout mice, which have a constitutive lack of SOCS3 in hematopoietic and endothelial cells, developed a more severe form of acute IL-1 dependent inflammatory arthritis [480]. Taken together these results suggest that SOCS3 is a critical negative regulator in inflammatory arthritis. Our data indicate that SOCS3 is upregulated in RA PB compared to healthy control monocytes which was also found by Isomaki et al [478]. This upregulation is no doubt caused by the increased pro-inflammatory environment to which the RA monocytes are exposed to within the blood and may be a regulatory mechanism of the cells to try and reduce the overactive inflammatory response. However, SOCS expression is known to alter cellular responsiveness to cytokines therefore up-regulation of SOCS3 may be involved in disease progression by causing unresponsiveness to anti-inflammatory genes.

Clusterin (CLU) was another gene associated with the response to stimulus processes outlined in the DAVID analysis. It was the most differentially expressed genes in the RA PB vs healthy blood monocyte comparison with a fold change increase in RA PB of 40 it also had the second highest p value of 1.80×10^{-4} . Since a multiple testing correction was unable to be used in the analysis a high p value indicates that this change is more likely to be a true result and not a false

positive. CLU is a secreted heterodimeric protein which was initially described as a complement inhibitor protein, where its role is to protect cells from the membrane attack complex (MAC) by binding to the MAC and rendering it physiologically inactive [481]. It has since been found to interact with a variety of molecules including lipids, amyloid proteins, and Igs. However, unravelling the function of CLU is complicated by its ability to interact with several molecules and its differential expression in many diseased states such as neurodegenerative disease and cancer [482, 483]. Recently it became apparent that different functions of CLU might depend on its final maturation and localisation, and whilst its predominant form is a secreted 80 kDa heterodimeric protein (sCLU) a nuclear form of the protein (nCLU) has also been reported which is thought to be responsible for its functions involving NF- κ B and apoptosis [484]. This suggests that CLU can have different functions depending on whether it is expressed inside or outside of the cell.

Santilli et al reported that the intracellular level of nCLU could be essential for regulation of NF- κ B activity through its ability to stabilise inhibitors of NF- κ B (I κ B) [485]. Consequently, Devauchelle et al further demonstrated that nCLU interacts with phosphorylated I κ B and prevented it from being degraded by the E3 ubiquitin ligase β TrCP and therefore blocking NF- κ B activation [486]. This latter study was based on the result of a previous microarray experiment which demonstrated significantly lower amounts of CLU in the synovial membrane of RA patients compared to OA [320]. Subsequently it was concluded that the lack of CLU within the RA synovium would result in enhanced I κ B degradation and therefore a greater activation status of NF- κ B enabling pro-inflammatory gene expression. This finding was also supported by their observation that siRNA induced knockdown of CLU in cultured fibroblast-like synoviocytes (FLS) resulted in increased production of IL-8 and IL-6 cytokines which are regulated by NF- κ B [486].

Numerous studies have analysed the role of CLU in the apoptosis of cancer cells but once again the function of CLU may depend on its form and localisation. sCLU inhibits apoptosis in human cancer cells by interfering with Bax activation in mitochondria [487] however, nCLU has been found to be pro-apoptotic as overexpression of nCLU resulted in cell death in both the presence or absence of ionizing radiation [488]. It is now accepted that a shift from the cell producing

sCLU to nCLU would result in cell death as nCLU has been found to induce cell death in many experimental conditions [489].

The finding that RA blood monocytes highly upregulate CLU compared to healthy blood monocytes is a significant one and has not been demonstrated previously. Of note the same pattern of upregulated CLU expression is also seen in PsA blood monocytes compared to healthy controls. Many reports conclude that CLU is an anti-inflammatory molecule in arthritis as CLU has been found at a reduced level in RA synovium compared to OA synovium [320] and CLU knockout mice induced with the K/BN model of arthritis developed arthritis faster which also lasted for longer compared to the control mice [490]. Hence the reasons for the large upregulation of CLU in RA PB (40 fold change increase) and PsA PB (20 fold change increase) compared to healthy controls are unknown. Since most reports demonstrate that CLU is anti-inflammatory the monocytes may be upregulating CLU as a counteractive mechanism as a result of the pro-inflammatory environment. Two of the most studied functions of CLU are inhibition of the NF- κ B pathway and induction of apoptosis. However, since several of the NF- κ B target genes, such as IL-6, are consistently upregulated in the serum of RA patients and there have been no reports of increased apoptosis levels in PsA or RA PB monocytes, to the contrary the antiapoptotic molecule Mcl-1 has been found to be increased in CD14⁺ cells isolated from RA SF [491], this may suggest that CLU may have a different function within arthritic blood monocytes. CLU is a heterogeneous molecule with many differing, often conflicting, functions and further work into the expression of this molecule within the cells and serum of RA and PsA patients is necessary.

The second group of biological processes upregulated in RA PB monocytes compared to healthy controls were associated with cellular movement, indicated by the three biological processes chemotaxis, taxis and locomotion. This group of biological processes is significant for the pathogenesis of RA as one of the earliest events in RA is the ingress of leukocytes into the inflamed synovial tissue. In order for the cells to enter the synovium from the blood they must adhere to the endothelial cells and then migrate into the interstitial matrix which is aided by the upregulation of chemokines and chemokines receptor on the leukocytes and the endothelial cells [136]. Once again due to the heterogeneous nature of genes and their various functions many of the same

genes were significantly associated with all three chemotaxis associated biological processes which indicate the importance of cellular movement to the RA PB monocytes. Some of the genes associated with these biological processes are LAMC1, FPR1, FPR2 and PPBP.

Laminin gamma 1 (LAMC1) has been described earlier on in this discussion as it was significantly upregulated in RA PB compared to PsA PB. It is a basement membrane molecule and may be helping the RA monocytes migration across the basement membrane of the blood vessels thereby allowing their subsequent infiltration into the joint. The two genes formyl peptide receptor 1 (FPR1) and formyl peptide receptor 2 (FPR2) were also both included in the chemotaxis, taxis and locomotion biological process groups. The protein product of FPR1 is known as formyl peptide receptor (FPR) and the protein product of FPR2 is denoted as formyl peptide receptor-like 1 (FPRL-1) which both belong to the seven transmembrane domain Gi-protein-coupled receptor (GPCR) family. Two of the main sources of ligands for this family of receptors are bacterial [492] and mitochondrial proteins [493] therefore the main function of these receptors is thought to be to mediate trafficking of phagocytes to sites of bacterial invasion, tissue damage or cell necrosis, although anti-inflammatory functions of some ligands has also been proposed [494]. FPR and FPRL-1 are detected on phagocytic leukocytes especially monocytes and neutrophils and activation of these receptors by their agonists results in Ca^{2+} mobilisation and cellular migration [495].

There are numerous ligands for FPR and FPRL-1 and several of them have been previously found in the serum and synovial fluid of RA, one such ligand is soluble form of the urokinase-type plasminogen activator receptor (suPAR). suPAR is a ligand for FPRL-1 and has been demonstrated to be released by neutrophils isolated from the PB and SF of RA patients. Cell lines expressing FPRL-1 migrated towards the supernatants harvested from RA neutrophils thereby suggesting that neutrophils may be involved in the recruitment of FPRL-1 expressing cells into the diseased synovium [496]. Consequently, the upregulation of FPR and FPRL-1 in RA blood monocytes in comparison to healthy control blood may be due to pro-inflammatory environment that the RA blood monocytes are exposed to which has been shown to contain the ligands for these receptors. Activation of FPR and FPRL-1 has been demonstrated to induce monocyte migration therefore

the upregulation of these genes may be a mechanism to induce monocyte recruitment into the synovial joint.

Pro-platelet basic protein (PPBP) otherwise known as CXCL7 is the third gene associated with the cell migration group of biological processes upregulated in RA monocytes. It is translated as a 14 kDa propeptide called PPBP or leukocyte derived growth factor (LDGF) which can then be cleaved into several smaller forms called platelet basic protein (PBP), β -thromboglobulin (β -TG) connective tissue activating protein-3 (CTAP-III) and neutrophil activating peptide-2 (NAP-2) [497]. Peptidases such as cathepsin G, are known to play a role in the post-transcriptional modifications of PPBP [498]. The derivatives of PPBP have amazing functional diversity: NAP-2 is a potent neutrophil chemoattractant [497]; PBP and CTAP-III have antimicrobial activity and LDGF itself is a fibroblast mitogen [497]. PBP was originally thought to be expressed only by cells of the megakaryocyte lineage but it has since been shown to be expressed by monocytes in response to various stimuli such as exposure to microbial components LPS or zymosan [499]. CTAP-III has previously been found in the blood circulation of RA patients, deposition of CTAP-III was found in the synovium and is associated with a partial processing to NAP-2 isoforms [500]. It is difficult to speculate on the importance of the upregulation of PPBP in RA blood monocytes due to its ability to be cleaved into several smaller forms which all have differing functions especially since cathepsin G, which can cleave PPBP, is present in RA serum [501]. Its main function may be to act as a neutrophil chemoattractant however further investigations are needed.

The biological processes downregulated in RA PB compared to healthy control PB monocytes display three main functions: protein complex localisation (demonstrated by the two biological processes cellular protein complex localisation and protein complex localisation), selenocysteine incorporation (translational readthrough and selenocysteine incorporation) and negative regulation of cell movement (demonstrated by the three biological processes negative regulation of cell migration, locomotion and cell motion). A downregulation in protein complex localisation was indicated by the decrease in expression of SMAD7 (SMAD family member 7) and TMEM48 (transmembrane protein 48). SMAD7 is an inhibitor of TGF- β signalling, TGF- β is a multifunctional cytokine which regulates cell growth, adhesion and differentiation in a variety of

cell types and it is also a potent immunosuppressor. Therefore the RA PB monocytes downregulating SMAD7, the inhibitor of TGF- β , may be an autoregulatory mechanism to compensate for the increased inflammatory processes taking place. However SMAD7 had also been demonstrated to negatively regulate signalling intermediaries in pro-inflammatory signalling pathways such as NF- κ B, in this pathway SMAD7 plays a role in increasing I κ B expression [502]. Consequently the downregulation of SMAD7 by the RA PB monocytes may conversely enable NF- κ B signalling and increase the production of pro-inflammatory cytokines therefore contributing to the increased pro-inflammatory cytokines detected within RA serum. The other gene associated with protein complex localisation is TMEM48 that codes for the protein nucleoporin NDC1. This protein is required for nuclear pore complex assembly which are large multiprotein complexes that span the nuclear envelope forming a selective channel between the cytoplasm and the nucleus. There is very little information regarding nucleoporin NDC1 therefore at present the relevance of the downregulation of this gene in RA blood monocytes is unclear.

Selenocysteine incorporation was also process downregulated by the RA PB monocytes indicated by the downregulation of the genes SEPSECS (Sep (O-phosphoserine) tRNA:Sec (selenocysteine) tRNA synthase) and TRSPAP1 (tRNA selenocysteine associated protein 1). Selenocysteine incorporation it is an essential process required for the production of at least 25 selenoproteins. Selenocysteine is comprised of the trace element selenium which is an antioxidant, deficiency of this element results in increase reactive oxygen species (ROS) [503]. Selenium levels are reduced in the serum and synovial fluid of RA patients compared to controls and this reduced selenium is associated with increased risk of the disease [504]. Hence, a reduction in the selenium levels in the serum of the RA patients could be the reason for the downregulation of genes associated with selenocysteine incorporation in RA PB monocytes. Of note, selenium deficiency in RAW 264.7 macrophages had significantly increased inducible nitric oxide synthase activity (iNOS) and subsequent nitric oxide (NO) production after stimulation with LPS [505] therefore a reduction in selenium and selenocysteine incorporation in RA PB monocytes may be one mechanism causing the increased iNOS and NO seen in RA [506].

The third group of biological processes downregulated in RA PB monocytes was negative regulation of cell movement which again emphasises the importance of the process of chemotaxis and extravasation for the diseased monocytes. The downregulated RA genes associated with this biological process are SMAD7, which has been previously discussed, and VASH1 (vasohibin 1). Vasohibin 1 is a negative feedback regulator of angiogenesis which was originally identified in a microarray analysis designed to analyse the upregulated genes induced by vascular endothelial growth factor (VEGF) stimulation in endothelial cells [388]. Subsequent immunohistochemistry analysis has identified vasohibin 1 in the synovium of RA patients associated with synovial lining cells, endothelial cells and synovial fibroblasts [507]. At present there has been no vasohibin 1 identified specifically in monocytes therefore our observation may represent a novel one. Inflammatory cytokines were shown to induce vasohibin 1 expression in RA synovial fibroblasts [507] and its expression is thought to help co-ordinate the formation of blood vessels and angiogenesis in the synovium. Consequently, the downregulation of vasohibin 1 in RA blood monocytes could be due to it not being required by the blood cells and may be upregulated again once the monocytes enter the inflamed synovium. Analysing the RA SF macrophage samples (which will be discussed thoroughly in the next comparison of this chapter) this hypothesis is correct. The RA PB CD14⁺ express a low level of vasohibin 1 compared to healthy controls and this expression is increased by a fold change of 13 in the patient matched RA SF macrophages. This result indicates that the monocytic cells downregulate their vasohibin 1 expression until they migrate into the synovial fluid of RA patients where its anti-angiogenic properties are needed to regulate and co-ordinate the formation of blood vessels.

DAVID analysis was also performed on the differentially expressed genes between PsA PB monocytes and healthy blood monocytes. After removal of gene repeats in the list and genes with no designated function the list of 154 genes upregulated in PsA blood monocytes was reduced to 92 and the list of 129 genes downregulated in PsA blood monocytes was reduced to 66 genes. The two biological processes associated with the 92 upregulated genes in PsA were DNA packaging (demonstrated by the groups nucleosome assembly, chromatin assembly, protein-DNA complex assembly, DNA packaging and chromatin

assembly and disassembly) and chemotaxis (demonstrated by the groups chemotaxis and taxis). Again because genes can be part of several biological process groups each of the six biological process groups which had the shared general function of DNA packaging all had the same six genes from the PsA gene list associated with them. These genes were HIST1H2BK (histone cluster 1, H2bk), TSPYL5 (TSPY-like 5), HIST1H2AC (histone cluster 1, H2ac), HIST3H2A (histone cluster 3, H2a), NAP1L5 (nucleosome assembly protein 1-like 5) and a probe on the Affymetrix GeneChip which targets six histone cluster 1 genes. Surprisingly there is very little to no information on the function or role of any of these genes particularly the histone genes. The function of NAP1L5 is also unknown but it shows homology to nucleosome assembly protein 1 which is involved in the translocation of histones from the cytoplasm into the nucleus [508]. Given that the role of histones is to provide a core around which DNA is packaged to form a nucleosome, the basic unit of chromatin, it could be suggested that the PsA blood monocytes upregulating genes responsible for DNA packaging would therefore prevent any further gene transcription. However, the importance of this observation cannot be fully understood until the roles of the upregulated genes are discovered.

The second group of biological processes associated with the genes upregulated in PsA PB monocytes had the general function of chemotaxis. This result is the same as that seen in the upregulated genes in RA PB monocytes, and the same genes such as FRP1, FRP2 and PPBP are upregulated in both cell subsets. Once again this highlights importance of chemotaxis to the arthritic blood monocyte as it is via chemotaxis that the cell will move into the diseased joint. The increased levels of chemokines and pro-inflammatory mediators detected in the serum of PsA patients compared to healthy controls may be one of the triggers causing the upregulation of chemotaxis related genes in the PsA monocytes [406, 509].

The two main biological processes associated with the downregulated genes in the PsA blood monocytes have the main functions of cell morphogenesis (demonstrated by the biological processes cell projection morphogenesis, cell part morphogenesis, cell morphogenesis, cell projection organisation and cellular component organisation) and osteoclast differentiation (demonstrated by the biological processes regulation of osteoclast differentiation, regulation of

myeloid leukocyte differentiation and positive regulation of osteoclast differentiation). Downregulated PsA genes associated with the cell morphogenesis biological processes include DYNC1I2 (dynein, cytoplasmic 1, intermediate chain 2) and DST (dystonin). Dynein is an energy dependent motor protein found to be associated with microtubules which moves cellular cargo, such as organelles, vesicles and RNA, towards the minus end of the microtubules. DYNC1I2 is one of two intermediate chains which are involved in intracellular dynein cargo transport by binding to specific cargo although the mechanisms of this are not fully understood [510]. Dystonin is a cytoskeletal interacting protein which is known to play important roles in maintaining cytoarchitecture integrity in skin and in the neuromuscular system, it is thought to act as a “linker” protein linking elements of the cytoskeleton to each other or to subcellular components such as adhesion structures [511]. Since there are no reports of dysregulated cytoskeletal or microtubule transport in PsA the importance of these downregulated genes in PsA is yet to be understood.

Downregulated genes associated with the osteoclast differentiation include GNAS (GNAS complex locus) and KLF10 (Kruppel-like factor 10). GNAS codes for the protein G_s α -subunit ($G_s\alpha$) which is a subunit of G proteins. G proteins are membrane associated complexes that are primarily involved in signalling at the plasma membrane. $G_s\alpha$ plays a central role in receptor mediated signal transduction, coupling receptor activation with the production of cAMP [512]. It is well known that G-protein coupled receptors play a substantial role in arthritis as the signal transducing receptors for many molecules such as MMPs and chemokines [513, 514]. However, the GNAS complex locus has recently been identified as an incredibly complex gene which encodes multiple gene products through the use of alternative promoters and first exons [515] such as extra-large isoform $G_s\alpha$, which is thought to be localised to the Golgi apparatus, and NESP55 amongst others [512]. Consequently, this multiple gene product complexity renders it impossible to try and understand the significance of the down-regulation of this gene in PsA blood monocytes since we do not know which end product of the gene the PsA and healthy monocytes are translating and producing.

KLF10 otherwise known as TGF β early gene-1 (TIEG) is a zinc finger transcription factor which plays an important role in mediating the TGF β Smad signalling

pathway. TIEG overexpression enhances Smad 4 activity and the induction of TGF β target genes [516]. Overexpression of TIEG in an epithelial cell line induced apoptosis [517] and reduced cell proliferation in a human osteosarcoma cell line which was also seen in control cells stimulated with TGF β [518]. Hence the PsA monocytes may be downregulating the TIEG gene in order to avoid TGF β mediated apoptosis and suppression of cellular activation and proliferation. Of note, this mechanism is seen in cancer cells which are known to downregulate their TIEG expression at the protein and mRNA level in order to avoid cell death and reduced proliferation [519].

In conclusion the diseased and healthy blood comparison has been very informative in defining the biological functions of the groups of genes up- or downregulated by each monocyte subset as well and allowing the investigation of individual genes. The PCA analysis performed on the entire set of microarray samples indicated the surprising result that the peripheral blood monocytes from healthy donors, RA patients and PsA patients all had a very similar genetic profile. In addition, performing statistical and fold change analyses on the data did result in the identification of just 24 genes which were differentially expressed between the RA and PsA blood monocytes. This low result demonstrates that within the blood the monocyte may not a cell type responsible for the differing clinical features seen in both diseases since this cell subset is showing a similar genetic profile in both diseases.

The 12 genes which were upregulated in RA blood compared to PsA blood monocytes contained a group of genes which had roles in cell adhesion and interaction, such as the laminin subunit LAMC1, as well as genes that had been previously associated with RA including the IL-7 gene. This latter result is encouraging as the presence of differential expression in genes previously associated with RA validates this dataset. The 12 genes which were upregulated in PsA blood compared to RA blood monocytes contained two groups of genes, one group had functions involved in the response to stress and toxins such as EIF2AK3/PERK and the other group of genes were involved in transcription and its initiation. It would be interesting to perform validation experiments on some of these genes especially those which have not been previously associated with monocytes in inflammatory disease. Laminin and its receptor expression has previously been associated with synovial fibroblasts in RA [520] but we believe

this is the first time the expression of a laminin subunit (LAMC1) has been found in RA blood monocytes and further experiments are needed in order to elucidate the function of LAMC1 in particular whether the monocyte is producing and releasing laminin molecules. The upregulated expression of EIF2AK3/PERK in PsA monocytes is another novel and interesting finding since this molecule is involved in the ER stress induced unfolded protein response (UPR). The UPR has not previously been implicated in the pathogenesis of PsA but has been well researched in another spondyloarthropathy ankylosing spondylitis (AS) [466, 521] which shares some clinical features with PsA. Unfortunately time restrictions meant that no further experimental or validation assays were able to be performed on these genes.

The RA PB vs healthy PB monocyte comparison was also very revealing in illustrating the overall functions of the differentially expressed genes. In comparison to healthy blood monocytes the RA PB cells showed an upregulation of genes involved in response to stimulus such as CLU and genes involved in cellular movement such as CXCL7. These two groups of genes are not surprising given that it is well known that the RA serum contains many pro-inflammatory mediators compared to healthy control serum which will be partly responsible for the upregulation of the response to stimulus genes. It is also known that the monocyte cells in RA need to undergo chemotaxis and migration in order to transmigrate from the blood vessels into the synovial joint where they accumulate and play a role in the damage and destruction of the joint.

The downregulated genes in RA PB had functions in complex localisation, selenocysteine incorporation and negative regulation of cell movement. The down-regulation of two genes involved in selenocysteine incorporation was interesting as selenium, the main component of selenocysteine, is decreased in the serum and synovial fluid of RA patients but also selenium deficiency has been shown to induce increased iNOS and subsequent nitric oxide (NO) production in RAW 264.7 macrophages. Therefore a lack of serum selenium may directly be contributing to the increased NO and NO-induced damage seen in RA. Down-regulation of genes associated with the negative regulation of cell movement also reinforces the importance of the ability of the RA monocytes to undergo chemotaxis and cell migration.

The upregulated genes in PsA blood monocytes compared to healthy blood monocytes had roles in DNA packaging and chemotaxis. An upregulation of genes associated with DNA packaging could suggest that the PsA monocytes are reducing their overall transcription levels by packing their DNA into nucleosomes and chromatin. However, since there is little to no information on any of the six upregulated genes associated with the DNA packaging function it is difficult to speculate further on the reasons for their increased expression compared to healthy controls. The upregulated genes associated with chemotaxis are the same genes upregulated in RA PB monocytes such as CXCL7, FPR1 and FPR2. The increased expression of these genes in PsA blood compared to healthy control demonstrates the importance of the arthritis specific requirement for the monocyte to migrate out of the blood and into the diseased joint. The downregulated genes in PsA monocytes identified an interesting transcription factor TIEG which is known to induce apoptosis; therefore the PsA monocytes may be down-regulating molecules like TIEG in order to maintain and prolong cell survival as has been seen in cancer cells.

Due to time restrictions only 5 healthy control blood monocyte samples were obtained which mainly were age- and sex-matched to the PsA patients. It is recognised that obtaining additional healthy donor samples which were age-matched to the RA patients would have been advantageous and allowed us to investigate the age specific genes that may have changed in the monocytes. Nevertheless, the analysis performed with the 5 healthy donors has been very informative in identifying the differentially regulated genes in the monocytes including novel genes not previously associated with inflammatory arthritis. This analysis has also identified that the blood monocytes in RA and PsA have very similar genetic profiles which was also previously unknown.

3.7.2 Comparison 2: RA and PsA blood versus matched synovial fluid

Given the important role of synovial macrophages in the pathogenesis of both RA and PsA one important question to be answered by this microarray analysis was what changes occur in the arthritic monocyte as it exits the blood and enters the synovium? This is an important question as it aims to identify not only the monocyte differentiation markers as it changes from a monocyte into an inflammatory macrophage but it will also identify the inflammatory genes that are upregulated once the monocyte is exposed to the pro-inflammatory synovial environment.

In order to answer this question paired T tests were performed between the matched RA blood and synovial fluid samples and a separate set of paired T tests were performed between the matched PsA blood and synovial fluid samples in order to determine the up- and down-regulated genes in the synovial macrophages compared to the blood monocytes. Due to the substantial amount of differentially expressed genes between the PB and SF of each disease (10763 in RA and 9533 in PsA) we applied a fold change cut off of 4 to the data since fold change values in microarray analysis are arbitrary and we were interested in genes with a high fold change difference between the blood and the SF. Ingenuity pathway analysis (IPA) was then used to determine the canonical pathways and molecular functions significantly associated with the differentially expressed genes in the RA SF compared RA PB CD14⁺ cells and those significantly associated with the differentially expressed gene in the PsA SF compared to the PB CD14⁺.

3.7.2.1 RA PB CD14⁺ versus RA SF CD14⁺

Table 3.6 shows the top 5 canonical pathways significantly associated with the differentially expressed genes in the RA SF macrophages compared to the RA PB monocytes. The most significantly associated pathway is LXR/RXR activation pathway. LXR is a nuclear receptor ligand-activated transcription factor, it forms heterodimers with RXR before binding to the DNA sequences of target genes [522]. Analysing the list of RA SF differentially expressed genes associated with this pathway demonstrates the novel finding that one of the LXR isoforms LXR α which has been previously been shown to be expressed in

monocytes/macrophages [523] is upregulated in RA SF macrophages compared to RA PB. Other RA SF upregulated genes associated with this pathway include a range of LXR target genes such as ABCA1, ABCG1, lipoprotein lipase (LPL), ApoE, ApoC1 and ApoC2 which are all involved in reverse cholesterol transport and cholesterol efflux [524, 525]. This process ensures the efflux of cholesterol from peripheral cells and its transport to the liver for metabolism and biliary excretion hence the LXR activation pathway is considered to be central in maintaining cholesterol homeostasis and is considered protective against atherosclerosis.

However, LXR agonism has also been considered pro-inflammatory since LPS-stimulated human macrophages treated with two synthetic LXR agonists increased TNF α expression though the ability of LXRs to increase Toll-like receptor 4 (TLR4) expression; this agonism also increased reactive oxygen species generation from the macrophages [526]. LXR agonism has also been demonstrated by our group to exacerbate articular damage in a murine model of arthritis suggesting LXRs have a pro-inflammatory role in arthritis [527]. In this study, LXR agonism induced a dose-dependent enhancement of the prevalence, incidence and severity of the collagen induced arthritis (CIA) mouse model. Ex vivo culture of RA PB derived monocytes which were LPS stimulated and treated with two LXR agonists showed an increase in IL-6 and TNF α production. Macrophages stimulated in the *in vitro* inflammatory model of arthritis by direct cell contact with Tcks also demonstrated an increase in pro-inflammatory cytokine and chemokine production in response to LXR agonism. Low density lipoprotein (LDL) is a natural agonists for LXR activation and has been found in the synovial fluid of RA patients [528] therefore the up-regulation of the LXR pathway in RA SF macrophages compared to PB monocytes along with the presence of its agonist in the synovial fluid indicates that this pathway may be promoting articular destruction through inducing the increased production of pro-inflammatory cytokines and chemokines from the synovial macrophages. However more work on the role of LXR activation in RA SF macrophages is required to test this hypothesis.

The hepatic stellate cell activation canonical pathway is the second most significant pathway differentially expressed in RA SF CD14⁺ cells. This is due to the overlap of several MMPs, chemokines, cytokines and growth factors that are

implicated in both hepatic stellate activation and synovial macrophage activation. For example platelet-derived growth factor alpha polypeptide (PDGFA) and PDGFB were both upregulated in the RA SF macrophages and these genes have also been implicated in hepatic stellate cell induced hepatic fibrosis [529, 530]. PDGF is a potent mitogen, chemoattractant and angiogenesis promoter [531] and the level of this growth factor is increased in RA synovial tissue and fluid [532, 533]. PDGF has been implicated in RA through its function as a growth factor for fibroblast-like synoviocytes (FLS) [532, 533]. It has recently been shown that in combination with TGF- β , PDGF augmented TNF α or IL-1 β induced MMP3, IL-6, IL-8 and macrophage inflammatory protein 1 alpha (MIP1 α) secretion by FLS suggesting PDGF induces a more pro-inflammatory, aggressive phenotype in the FLS in response to TNF α [534]. Consequently, the increased expression and possible secretion of PDGFA and PDGFB by RA SF macrophages may be contributing to articular damage by activating synovial FLS.

The remaining genes differentially expressed in RA SF macrophages which were associated with the hepatic stellate cell activation canonical pathway consist of many inflammatory cytokines, chemokines and growth factors which have previously been connected with RA. These genes include FN1 (fibronectin) which is detected in the synovial fluid of RA and can induce MMP expression from fibroblasts [425]; the gelatinases MMP2 and MMP9 (matrix metalloproteinase 2 and 9) which are both upregulated in the synovium of RA patients [535] and their levels are associated with increased joint erosions [536]; CCL2 (monocyte chemoattractant protein 1, MCP1) is also increased in the synovium and is a potent chemoattractant for immune cells [537]; and CSF1 (macrophage colony stimulation factor, M-CSF). M-CSF is a growth factor which controls the proliferation, survival and differentiation of mononuclear phagocytes, it is normally expressed by the tissue environment however macrophages can also produce and secrete M-CSF if the tissue cells are unable to supply large amounts of the growth factor [538]. In conclusion, the association of the hepatic stellate cell activation pathway with the genes differentially expressed in the RA SF macrophages compared to RA PB monocytes has highlighted the upregulation of several important inflammatory genes discussed above. However not only do the SF macrophages up-regulate genes which will contribute to the pro-inflammatory environment but they also demonstrate increased expression of the macrophage

differentiation and survival factor M-CSF which could help differentiate monocytes infiltrating into the joint into pro-inflammatory macrophages.

The third canonical pathway is defined as the role of macrophages, fibroblasts and endothelial cells in RA. The association of this canonical pathway validates the genes that are differentially expressed by the RA SF macrophages as many of these genes are known to be expressed in the RA synovium. This pathway contains many of the upregulated genes which were associated with the hepatic stellate cell activation pathway such as fibronectin, MMP2, MMP9 and the two PDGFs as well as other genes such as TRAF5 (TNF receptor-associated factor 5), WNT5A (wingless type MMTV integration site family member 5A) and FZD7 (frizzled homolog 7).

TRAFs are cytoplasmic adaptor proteins for the TNF/IL-1/TLR receptor superfamily, they are responsible for transducing extracellular signals from cell surface receptors and activating intracellular signalling cascades such as the NF- κ B and the JNK signalling pathways [539]. The RA SF macrophages demonstrated a 5 fold increase in TRAF5 expression compared to matched blood monocytes and interestingly a SNP upstream of TRAF5 has been identified as an RA susceptibility gene [540]. TRAF5 interacts with lymphotoxin β receptor (LT β R) and CD40 and has shown functional redundancy with TRAF2 [541]. Both TRAF2 and TRAF5 can mediate TNF-induced NF- κ B activation and protection from cell death [539]. Hence, an upregulation of TRAF5 in the RA SF macrophage may imply that it plays a proinflammatory role such as increased cellular activation and transcription of other proinflammatory cytokines. However, a recent study into the role of macrophage TRAF5 in atherosclerosis found it to be anti-inflammatory as murine monocytes deficient for TRAF5 demonstrated an upregulation of cell adhesion molecules implicated in cell adhesion and rolling and also enhanced migration and chemokine production [542]. Since macrophage TRAF5 has not been examined in RA at present further studies are required to distinguish whether TRAF5 plays a pro- or anti-inflammatory role in this disease.

WNT5A was also upregulated in the RA SF macrophages compared to the blood monocytes, Wnt-5a is one of 19 Wnt proteins which are a family of secreted lipid-modified glycoproteins that display a highly regulated pattern of expression and have distinct roles during development and tissue homeostasis. The

importance of Wnt-5a in various developmental pathways is demonstrated in Wnt-5a knockout mice which die at birth and display many defective features such as facial abnormalities, truncated bodies and shortened limbs [543]. Wnt signalling pathways are activated by the binding of secreted Wnt to the cystolic domain of a family of transmembrane receptors called the Frizzled receptor proteins (Fz) [544]. The binding of Wnts to their receptors determines the type of downstream signalling pathway either canonical, which involves the transcriptional activation of β -catenin, or non-canonical which does not involve β -catenin. Wnt-5a is classed as a non-canonical Wnt which are generally considered to control morphogenic movement. In RA Wnt-5a has been shown to be upregulated in synovial fibroblasts where it binds to frizzled 5 (Fz5), and the downregulation of Wnt-5A or Fz5 both resulted in inhibition of synovial fibroblast activation [545].

Wnt-5a has also been demonstrated to be upregulated in human macrophages stimulated with bacterial pathogens *in vitro* as it was demonstrated that activation of TLR4 signalling cascade induced Wnt-5a expression. These macrophages also expressed Fz5 and it was demonstrated that Wnt-5a and Fz5 regulated the microbially induced IL-12 release from these cells [546]. Wnt-5a has been demonstrated to be expressed in macrophages of murine and human atherosclerotic plaques associated with TLR4 [547] and has also been demonstrated to be critical for invasion and MMP7 and TNF α release from tumour associated macrophages which were found to be positive for Wnt-5a [548]. Consequently the upregulation of Wnt-5a in the RA SF macrophages may be as a result of increased TLR4 signalling since TLR4 ligands are present in the RA synovium [549]. Both synovial fibroblasts and macrophages have been demonstrated to express a Wnt-5a receptor Fz5 therefore the secretion of Wnt-5a by synovial macrophages could be acting in an autocrine or paracrine manner by stimulating surrounding cells. This activation by Wnt-5a could cause inflammatory cytokine and proteinase secretion by synovial fibroblasts or macrophages thereby contributing to the synovial joint damage.

FZD7 (frizzled homolog 7) was another gene upregulated by the RA SF macrophages which was associated with role of macrophages and fibroblasts in RA canonical pathway. As mentioned above frizzled molecules are the cell surface receptors for Wnt proteins and the subsequent binding of these two

molecules activates the Wnt signalling pathways. Frizzled 7 (Fz7) has previously been detected in RA synovial tissue [550] and a recent paper has demonstrated the Fz7 can associate with Ror2 to form a receptor complex to mediate Wnt-5a signalling and activation of the JNK signalling pathway [551]. Put together this novel data demonstrates the upregulation of a Wnt protein (Wnt-5a) and part of its receptor complex (Fz7) specifically in RA synovial macrophages, further work is required to elucidate the importance of these molecules in RA pathogenesis however they could play a role in the invasive potential, inflammatory mediator secretion and sustained activation of these cells.

The differentially associated genes with the role of macrophages and fibroblasts in RA canonical pathway was also associated with genes that were downregulated in RA SF macrophages compared to their matched PB monocytes. Two of these downregulated RA SF macrophage genes were TLR5 and TLR7. The activation of TLRs by pathogen-associated molecular patterns (PAMPs) alters the host to presence of infection and triggers a variety of defense mechanisms, such as the production of pro-inflammatory cytokines and chemokines, depending on the receptor and cell type. Several TLRs have been detected in RA synovium as well as candidate endogenous TLR ligands [552] therefore the perpetual activation of TLR signalling has been identified as a potential mechanism for the maintenance of the chronic inflammation seen in RA. TLR5 has not previously been identified in the synovium of RA however stimulation of FLS from RA and JIA patients with the TLR5 ligand flagellin did induce IL-6, MMP1 and MMP3 production suggesting that these FLS expressed TLR5 [553].

TLR7 has been demonstrated to be expressed within the RA synovium [554]. However, when DCs isolated from RA patients were stimulated with TLR7 ligands the level of cytokine production was similar between DCs from RA and those from healthy patients. This was in marked contrast to TLR2 or TLR4 stimulation which induces a much higher production of inflammatory mediators for RA DCs compared to healthy controls [554]. This result suggests that TLR7 may not as essential to the perpetuation of arthritis as TLR2 and TLR4, which is further verified by the observation that TLR2 and TLR4 deficient mice do not develop serum transfer or streptococcal cell wall-induced arthritis [555, 556]. Consequently, the downregulation of TLR5 and TLR7 by the RA SF macrophages

may be due to these receptors not being as vital in the pathogenesis of RA as some of the other Toll-like receptor members such as TLR2/TLR4.

The LPS/IL-1 mediated inhibition of RXR was the fourth most significant canonical pathway associated with the RA SF differentially expressed genes. The associated genes include many of the same genes which were included in the LXR activation pathway such as APOC1, APOC2 and APOE. All three of these apolipoprotein molecules are constituents of the anti-inflammatory and athero-protective high density lipoprotein (HDL) [418]. ApoE and another apolipoprotein ApoA1 have been detected at an increased level in the synovial fluid of RA patients compared to serum levels and total cholesterol was also at an increased level in the synovial fluid [557]. Accumulation of HDL particles in the inflamed joint has anti-inflammatory effects since HDL can block the pro-inflammatory cytokine production from monocytes induced by T cell contact through binding to the T cell surface activating factors [558]. However, it has also been proposed that the accumulation of HDL within the synovium of RA patients and thereby reducing the amount of circulating HDL may contribute to the increased cardiovascular risk seen in these patients [559]. Consequently, the upregulation of these genes in the RA SF macrophages as well as the lipid transporter ABCA1 needed to assemble the HDL particles may be as a result of the activation of the LXR/RXR pathway, as indicated by the canonical pathway analysis, or it may be as a result of the macrophage trying to dampen down the immune response by producing anti-inflammatory HDL particles which will inhibit the local production of inflammatory cytokines.

The presence of the LPS/IL-1 mediated inhibition of RXR canonical pathway also indicates that the LPS pathway may play a role in the RA SF CD14⁺. LPS is one of the ligands for TLR4 which is a protein expressed on the surface of immune cells such as monocytes and its activation triggers signalling pathways which in turn lead to the induction of inflammatory cytokines such as TNF- α [560]. TLR4 has been detected in the RA synovium [549] and a TLR4 agonist has shown therapeutic effect in two murine models of arthritis [561] indicating that it may play a role in pathogenesis. Therefore the association of this canonical pathway with RA SF CD14⁺ is unsurprising since many TLR4 agonists such as hyaluronan, fibrinogen and heat shock proteins have been detected in the RA synovium.

The coagulation system is the fifth canonical pathway that is significantly associated with the differentially expressed genes in the RA SF CD14⁺ cells due to the upregulation of genes such as coagulation factors and plasminogen activators such as UPA (plasminogen activator, urokinase). UPA can convert plasminogen into the less specific plasmin which has various substrates including fibrin, cytokines and proMMPs, and also has a role in matrix degradation [562]. It has been demonstrated that systemic inflammation in arthritis can activate the coagulation cascade [563]. Levels of UPA are increased in RA [564] and induction of CIA in UPA^{-/-} mice resulted in a mild disease indicating a pro-inflammatory role for UPA in RA [565]. Therefore these results indicate that RA synovial fluid macrophages are expressing genes which are contributing to the increased coagulation cascade seen in RA as well as the increased matrix degradation and inflammation.

The diseases and disorders associated with the differentially expressed genes in RA SF macrophages compared to their matched PB monocytes are all related to rheumatoid arthritis such as inflammatory disease and skeletal and muscular disorders. The presence of these diseases significantly associated with the differentially expressed gene list validates this dataset as they are all related to our disease of interest.

The top 5 cellular functions significantly overrepresented in the RA SF gene list comprise functions which we would expect to be upregulated in the monocytes once they move from the blood into the synovium. This is exemplified by two of the cellular functions being cellular movement and immune cell trafficking which would be expected to be upregulated as a result of the CD14⁺ transendothelial migration from the blood vessels into the synovium [258, 407]. Transendothelial migration is the mechanism via which leukocytes infiltrate into the RA joint and several adhesion molecules which assist in this process have been identified in RA [179]. Another cellular function significantly associated with the up- and down-regulated genes in the RA SF gene list was cell-to-cell signalling and interaction which is unsurprising since it has been well documented that macrophages within the synovial compartment are known to participate in cellular interactions with several cell types such as fibroblasts [249], T cells [269, 314, 408], endothelial cells [407] and natural killer (NK) cells [251]. The final two cellular functions associated with RA SF CD14⁺ compared to

PB cells are cellular growth and proliferation and cellular development which is logical due to the maturation that the CD14⁺ cells undergo into fully activated macrophages once they are within the synovium and exposed to the pro-inflammatory and hypoxic environment [409, 410]. An upregulation of genes such as CSF1 (M-CSF) which was previously discussed will be contributing to the maturation of the blood monocytes into synovial macrophages.

In conclusion the RA PB versus RA SF analysis to determine the effect of the migration of the monocytes from the blood into the synovial fluid has been very informative. The IPA analysis demonstrated the association of the differentially expressed genes with several pathways such as the LXR/RXR activation pathway. This pathway could be contributing towards an anti-inflammatory effect, though the production of HDL, or a pro-inflammatory effect since research by our group has demonstrated LXR activation is pro-inflammatory in a murine arthritis model [527]. Further analysis of the IPA canonical pathways also demonstrated the upregulation of novel receptor ligand complexes in the RA SF macrophages such as Fz7 and Wnt-5a which warrant further investigation. The cellular and molecular functions associated with the RA SF gene list consisted of cellular migration, cell-to-cell interaction and cellular development. This result demonstrates that once the RA PB monocytes migrate into the synovial compartment they are expressing transcripts of genes involved in cellular interactions, which are known to promote the production of pro-inflammatory mediators, and also genes associated with monocyte maturation into a fully functional synovial macrophage.

3.7.2.2 PsA PB CD14⁺ versus PsA SF CD14⁺

The IPA analysis of the differentially expressed genes in the PsA SF CD14⁺ cells compared to the PB CD14⁺ cells resulted in remarkably similar results to those obtained from the IPA analysis of the RA SF gene list. Three of the five significantly associated canonical pathways were the same pathways found associated with RA SF macrophages: LXR/RXR activation, LPS/IL-1 mediated inhibition of RXR function and hepatic stellate cell activation. Many of the individual genes associated with these pathways were the same in the PsA and RA gene lists indicating similarities between the two types of arthritic synovial macrophages. LXR activation has not previously been demonstrated to be associated with PsA therefore this finding is a novel one. The LXR activation pathway could be playing one of two roles; it could be an anti-inflammatory pathway in PsA by producing components of HDL which prevents cytokine secretion from monocytes [558] or it is more likely to be playing a pro-inflammatory role in PsA. As discussed in the previous section LXR agonism in LPS-stimulated monocytes resulted in increased TNF α production and LXR agonism also exacerbates a murine model of arthritis possibly through the increased cytokine and chemokine production [526, 527]. A natural agonist of the LXR activation pathway is LDL, which has been detected in the plasma of PsA patients therefore indicating a mechanism for the LXR activation [566]. The association of the LPS/IL-1 mediated inhibition of RXR pathway with the differentially expressed PsA SF macrophage genes is due to the upregulation of the same apolipoproteins and lipid transporter genes which were upregulated in the RA SF macrophages including APOE, APOC1, APOC2, ABCA1 and ABCG1. As discussed for the RA SF CD14⁺ cells the upregulation of these genes would result in an increase in HDL which prevents monocyte cytokine secretion and therefore mediate an anti-inflammatory effect from the PsA SF macrophage.

The association of the hepatic stellate cell activation canonical pathway with the PsA gene list is due the upregulation of growth factors, MMPs and chemokines which are also involved in hepatic stellate cell activation. It includes genes which have been previously associated with PsA such as MMP2, MMP9 [567], fibronectin [426] and CCL2 [509] but also novel genes such as SMAD7 and TGFB2 (transforming growth factor beta 2). TGF β exists in three isoforms TGF β 1, TGF β 2 and TGF β 3, it is a multifunctional cytokine which regulates cell growth,

adhesion and differentiation in a variety of cell types, it is a potent immunosuppressor but also helps to drive the differentiation of Th17 which can promote inflammation and augment autoimmune conditions [568]. Of note there are increased levels of circulating Th17 cells in the blood of PsA patients [569]. TGF β has not previously been associated with PsA but one of its isoforms TGF β 1 is present in the epidermis and serum of psoriasis patients and correlates with the psoriasis area and severity index (PASI), although the same study found no correlation between PASI and our TGF isoform of interest TGF β 2 [570]. Overexpression studies of TGF β 1 in keratinocytes of mice induces a psoriasis-like skin inflammation suggesting TGF β is pro-inflammatory [571] however the mechanisms for this are not understood. In addition to an upregulation of TGF β 2, the PsA SF macrophages also upregulated SMAD7 which paradoxically is a negative regulator of TGF β 2 [572]. SMAD7 had also been demonstrated to negatively regulate signalling intermediaries in pro-inflammatory signalling pathways such as NF- κ B, in this pathway SMAD7 plays a role in increasing I κ B expression [502]. Therefore the upregulation of SMAD7 in the PsA macrophages may be as an auto-regulatory anti-inflammatory mechanism in order to decrease the amount of NF- κ B pro-inflammatory gene transcription. TGF β 2 and SMAD7 expression have not been analysed in PsA previously therefore our observation is an interesting one. It is difficult to determine the overall net effect of the upregulation of the potential pro- and anti-inflammatory molecule TGF β 2 along with its intracellular inhibitor SMAD7 therefore further expressional and functional studies would be required.

The two canonical pathways which are specifically associated with the PsA SF macrophage differentially expressed gene list are pathogenesis of multiple sclerosis and the complement system. Numerous studies have implicated the complement system within rheumatoid arthritis; elevated levels of complement activation products have been detected within RA synovial fluid [573] and several animals models have provided evidence for the involvement of complement in arthritis such as C5^{-/-} and C3^{-/-} mice being resistant to CIA [574, 575]. In comparison there are very few studies implicating the complement system in PsA, one study has found the presence of terminal complement complex in PsA serum [576] and another study indicated that erythrocytes from psoriatic patients had reduced expression of surface CD59 compared to healthy

controls and it was hypothesised that this may be an indicator of low tissue CD59 expression [577]. CD59 (protectin) is an inhibitor of the membrane attack complex (MAC) therefore a reduction in its expression could result in the perpetual activation of the complement system and increased lysis of erythrocytes and synovial cells through the MAC. Contrary to this observation the data generated by our microarray indicates that the PsA SF actually have an increased level of CD59 expression compared to PB monocytes which may be due to the macrophage trying to regulate the amount of complement activation taking place in order to prevent perpetual activation. Brought together these data suggest that the complement system may be involved in PsA as well as RA and the synovial macrophage could be a key cell type producing complement system components.

The pathogenesis of multiple sclerosis canonical pathway is significantly associated with the differentially expressed genes in PsA SF CD14⁺ due to four chemokines which play a role in this small nine component pathway being present in the gene list (see Figure 3.12). The four chemokines involved in this pathway are CCL4, CCR5, CXCL9 and CXCL11. CCL4 otherwise known as macrophage-inflammatory protein-1 beta (MIP-1 β) is produced by activated macrophages [578]. CCL4 has been identified as one of the chemokines significantly upregulated in the serum of PsA patients compared to healthy controls and it is significantly associated with cellular synovial tissue markers in PsA [237]. Studies performed in RA have demonstrated that mediators found within RA synovial fluid, such as osteopontin, can induce CCL4 production from monocytes [241] and there is also evidence that the interaction between CCL4 and its receptor CCR5 plays a crucial role for trafficking Th1 and other immune cells into the RA joint [235, 579]. Apart from the identification of CCL4 within PsA serum and synovial fluid there is little known about the role of CCL4 within PsA; however it could be hypothesised that this chemokine plays a similar role in inflammatory cell recruitment that has been described for RA.

CCR5 otherwise known as CC-CKR5 is a receptor found on macrophages and T cells which binds RANTES, MIP-1 α and MIP-1 β (CCL4) with high affinity [580]. As has been illustrated above it has been implicated in Th1 cell trafficking into the RA joint and its expression has been determined within the RA synovial tissue and on synovial fluid monocytes and T cells [581]. At present there is no

evidence for the presence of CCR5 within the PsA synovial compartment apart from the data presented here, however since its high affinity ligand CCL4 has been demonstrated to be present in the synovial fluid we can hypothesise that CCR5 is also present also and contributing to the inflammatory cell influx into the diseased joint.

The third gene upregulated in PsA SF CD14⁺ cells and associated with pathogenesis of MS pathway was CXCL9 otherwise known as monokine induced by IFN- γ (Mig). CXCL9 is produced by activated monocytes [582] and mRNA levels of this chemokines have previously been detected in the synovium of PsA [238] where it is thought to help regulate the influx of inflammatory cells. CXCL11 otherwise known as IFN-inducible T-cell alpha chemoattractant (I-TAC) was the fourth chemokine upregulated in PsA SF CD14⁺ cells, this chemokines is upregulated by LPS stimulated macrophages [583] and had been detected in PsA synovial fluid [584]. Consequently, from this result it is not assumed that the pathogenesis of multiple sclerosis (MS) plays a role in PsA only that several of the chemokines which are involved in the pathogenesis of MS are also upregulated in PsA synovial fluid macrophages.

The diseases and disorders correlated with the PsA SF macrophage differentially expressed genes are the same as those found associated with the RA SF macrophage genes and are all related to arthritic disease such as inflammatory diseases and inflammatory response. Again the significant association of these disorders with the gene list validates our PsA dataset.

The molecular and cellular functions associated with the up- and down-regulated PsA SF macrophages are highly similar to those correlated with the RA SF macrophage genes as 4 of the 5 associated functions are the same in RA and PsA. This data further substantiates the finding that the RA and PsA macrophages have very similar genetic profiles and upregulate analogous genes once the monocytes migrate out of the blood and into the synovial fluid. The molecular and cellular functions which were significantly associated with the PsA genes included cell movement and immune cell trafficking which will be due to the presence of up- and downregulated genes involved in transendothelial migration and movement of the cells into the synovial joint. The synovium of PsA patients is characterised by an inflammatory cell influx [7] and monocytes isolated from

PsA patients have been demonstrated to interact with cultured endothelial cells whilst down-regulating adhesion molecules which is thought to aid in their migration [411]. PBMCs from PsA patients are also have a significant increase in their adhesion to endothelial cells compared to healthy PBMCs [585] and the level of chemokines is significantly increased in the serum and synovium of PsA patients [259, 406, 509]. These data emphasize the importance of transendothelial migration in the pathogenesis of PsA and also emphasises why the PsA SF have a significant change in their migratory associated genes compared to PB monocytes.

Another molecular and cellular function associated with the PsA SF macrophage expressed genes was cell-to-cell signalling and interaction. In comparison to RA there have been only two studies into the role of macrophage direct cell contact in PsA. These studies demonstrated that direct cell contact between PsA monocytes and NK cells was able to differentiate the monocytes into DCs [586] and direct interaction of PsA monocytes with endothelial cells aided in their transendothelial migration [411]. Despite the lack of information regarding the role of direct macrophage cell contact in PsA, due to the close proximity of macrophages to other immune cells in the synovium [124] we can hypothesise that direct cell interaction is one method through which macrophages exert their pro-inflammatory potential as has been extensively demonstrated in RA. The two other molecular and cellular functions associated with the PsA SF macrophage gene list are cellular growth and proliferation and cell mediated immune response. A reason for genes associated with both of these functions being differentially expressed in the synovial macrophages could be due the pro-inflammatory environment into which the cells migrate. Previous studies have demonstrated the PsA synovium to be a hypoxic environment [587] containing many cytokines, chemokines and inflammatory mediators [237, 259] which would in turn cause the monocytes to differentiate into macrophages and illicit an immune response.

In summary the PsA PB versus PsA SF microarray comparison to establish the effect of the PB monocyte migration into the synovium has identified the activation of novel pathways in the macrophages such as LXR/RXR activation as well as the identification of novel genes which have not previously been associated with PsA such as TGF β 2 and its inhibitor SMAD7. Nevertheless, the

most interesting observation to come out of this analysis is the similarity between the transcriptomes of the RA and PsA synovial macrophages which is investigated further in the following comparison.

3.7.2.3 Identification of genes expressed in: both RA and PsA synovial fluid macrophages, only in RA macrophages or only in PsA macrophages

The similarity between the transcriptomes of the RA and PsA samples first became apparent during the PCA comparison which indicated that the synovial fluid samples of both PsA and RA were clustered in the same area of the graph indicating that these samples had similar genetic expression. Investigating the two diseases individually during IPA analysis then demonstrated that the synovial macrophages in both RA and PsA shared many of the same canonical pathways and molecular functions. These results let us to hypothesise that the synovial fluid macrophages of PsA and RA shared many of the same genes. To investigate this hypothesis the differentially expressed genes from both disease PB versus SF comparison were subjected to a Venn diagram comparison to determine genes which were expressed in both diseases and also genes which were specific to the synovial fluid macrophages of each disease.

Interestingly the Venn diagram demonstrated that 58% of the PsA differentially expressed genes and 52% of the RA differentially expressed genes were present in both diseases indicating that just under half of the genes expressed in the RA or PsA synovial fluid cell were disease specific. This result is in contrast to those of Dominique Baeten and his group who have previously described a differential macrophage phenotype in SpA (including PsA) compared to RA patients through revealing an increased number of CD163⁺ cells in the synovium of SpA patients [255] and demonstrating that the synovial fluid of SpA but not RA was able to polarise blood monocytes to express CD163 [253]. CD163 is a cell surface glycoprotein which is classed as a marker of alternatively activated macrophages (M2), M2 macrophages have been implicated in tissue remodelling and immune regulation and are in stark contrast to classically activated macrophages (M1) which are thought to be the main source of pro-inflammatory cytokines which are associated with tissue damage [185]. Consequently, Baeten et al suggest that the macrophage phenotype in RA and SpA are significantly different due to the presence of different markers of macrophage polarisation and the ability of the

diseased synovial fluid to induce these macrophage phenotypes. Our venn diagram data illustrates that in contrast to results of Baeten et al the genetic profile and therefore the phenotype of both RA and PsA macrophages are very similar and over 50% of the genes expressed by each diseased macrophage are shared between the diseases. This observation is further exemplified by analysing for the presence of genes associated with M1 and M2 phenotypes in the macrophage gene lists from both diseases. As although CD163 is not present in either the PsA or RA SF macrophages, possibly due to the strict inclusion criteria for the microarray analysis, other markers of M2 phenotype such as the mannose receptor (MRC1 and MRC2) and macrophage scavenger receptor (CD204) are upregulated by a similar fold change in both RA and PsA. MRC1 has a fold change (FC) increase of 73 in PsA and 61 in RA, MRC2 (FC 32 PsA, 32 RA) and CD204 (FC 10 PsA, 8 RA). However, analysis of markers of M1 polarisation demonstrates that these markers are also upregulated to similar levels of magnitude in both RA and PsA SF macrophages such as CD86 (FC 11 PsA, 15 RA) and MHC class II molecules HLA-DOA (FC 7 PsA, 4 RA). Our results would suggest that rather than the RA and PsA SF macrophages being two separate phenotypes, as implicated by work of Baeten et al, they are both similar cell types and at the RNA transcript level appear to have no preferential expression of an M1 or M2 phenotype. This could be an indication of the high level of plasticity of macrophage cells and their ability to constantly change their phenotype in response to their environment. Despite there being very little differences in the expression of several M1 and M2 macrophage markers the results of the venn diagram did also demonstrate that both the RA and PsA have a subset of genes that were disease specific which could account for some of the differences in seen in the pathogenesis and cytokine profiles of the two diseases.

The results of the Venn diagram were saved as three individual datasets; i) genes which were differentially expressed in both diseases, ii) genes which were expressed only in PsA and iii) genes which were expressed only in RA. These gene lists were then analysed by IPA to identify the canonical pathways and the molecular and cellular functions which were significantly associated with them. The canonical pathways associated with the gene list expressed by both PsA and RA consisted of pathways which had already been discovered from the previous IPA analyses of RA PB vs RA SF and PsA PB vs PsA SF. These five pathways were:

LXR activation which has been previously been described as being a potential pathogenic pathway in RA [527] but as yet no association has been demonstrated for PsA; LPS/IL-1 inhibition of RXR function is also associated with the gene list due to an upregulation of HDL constituents in both RA and PsA macrophages, the significance of this pathway is not fully understood but since HDL had been demonstrated to prevent monocytes from releasing pro-inflammatory mediators [315, 558] this pathway may be an anti-inflammatory mechanism through which the macrophages counter-balance their disproportionate pro-inflammatory response. The complement system was the third pathway associated with the genes expressed in both diseases, complement components have been detected in the serum and synovial fluid of RA and PsA patients [573, 576] and several animals models have already provided evidence for the involvement of complement in arthritis such as C5^{-/-} and C3^{-/-} mice being resistant to CIA [574, 575]. Therefore the combination of this data with our microarray data indicate that synovial macrophages may be a specific cell type in both RA and PsA to be involved in the activation of the complement pathway.

The hepatic stellate cell activation was the fourth associated canonical pathway, as described previously the presence of this pathway is due to the occurrence of pro-inflammatory chemokines, cytokines and growth factors that are expressed in both hepatic stellate cells and synovial macrophages during cellular activation. The final canonical pathway was the pathogenesis of multiple sclerosis, the association of this pathway to the gene list is due to the presence of three chemokines which play a role in both multiple sclerosis as well as RA and PsA pathogenesis. The three chemokines were CCL4 (MIP-1 β) and its receptor CCR5 which are both associated with macrophages and play a role in trafficking immune cells into the arthritic joint [235, 579], and CXCL11 which has been previously detected in RA synovium [588] and PsA synovial fluid [584].

The molecular and cellular functions associated with the gene list expressed in both RA and PsA SF CD14⁺ cells had all previously been associated with the SF macrophages such as cell-to-cell signalling and interaction, cellular development and cellular movement. The one function which had not previously been correlated to the SF macrophages was cellular function and maintenance which may be due to genes associated with cellular homeostasis being expressed by the RA and PsA macrophages.

The canonical pathways associated with the genes specifically expressed in PsA SF macrophages were: B cell development, pathogenesis of multiple sclerosis, autoimmune thyroid disease signalling, altered T and B cell signalling in rheumatoid arthritis, and allograft rejection signalling. The pathogenesis of multiple sclerosis was previously associated with the PsA PB vs SF comparison but the four other canonical pathways are unique to the PsA SF macrophage gene list. All four of these novel canonical pathways appear to be specifically T and B cell related, however they are associated with the PsA SF specific gene list due to the presence of three genes within the gene list. These genes are CD40, HLA-DQA1 and HLA-DQA2 which were all upregulated in PsA SF macrophages compared to PB monocytes and these genes are found in all four canonical pathways.

CD40 is a cell surface receptor found on macrophages/monocytes, B cells and myeloid DCs and it is activated by ligation with CD154 found on CD4⁺ T cells, platelets, mast cells and basophils [589]. Ligation of CD40 on monocytes and macrophages results in the induction of pro-inflammatory cytokine and chemokine synthesis such as IL-1 α , TNF α , IL-6, IL-8, CCL2 and CCL4 [589]. CD40 ligation on monocytes or macrophages also upregulates the expression of MHC class II and co-stimulatory molecules CD80 and CD86, as well as CD40 itself [590]. CD40 has previously been demonstrated to play a role in arthritis as blockade of the CD40:CD154 interaction inhibited the onset of collagen induced arthritis (CIA) [591]. Once again, in comparison to RA there have been few studies analysing the role of CD40 in PsA. However, soluble CD40 ligand (sCD40L) is present in the serum of PsA patients [592] and interestingly Stoeckman et al discovered that CD40 and its signalling intermediates were downregulated in PsA PBMCs compared to healthy controls which they suggested could be due to the migration of CD40-expressing cells out of the blood and into the synovium. Our data agrees with this hypothesis as we demonstrate that in comparison to PsA PB CD14⁺ cells there is an upregulation of CD40 gene expression in PsA SF CD14⁺ cells. The presence of CD40 on PsA synovial macrophages will be contributing to the inflammatory milieu within the synovium through its ligation with CD154 present on the synovial T cells and sCD40L present within PsA patients.

The remaining two genes associated with the four canonical pathways specific to the PsA macrophage differentially expressed genes are HLA-DQA1 and HLA-DQA2.

Human leukocyte antigen (HLA) is coded for by the major histocompatibility complex (MHC) gene region and HLA molecules are critical for distinguishing self from non-self. Cell-surface HLA molecules function to present processed antigenic peptide, which is held within a groove in the HLA molecule, to T cell receptor molecules expressed on the surface of T cells. HLA class II DR, DQ and DP molecules are found on B cells and professional antigen presenting cells and present processed peptides derived from exogenous antigens to CD4⁺ T cells [593]. An increased expression of HLA genes within the PsA SF macrophage could indicate an increase in the amount of antigen presentation taking place within the synovium and is also a marker of increased cellular activation. HLA-DQA1 alleles have been associated with susceptibility to a wide variety of inflammatory conditions such as multiple sclerosis [594] and coeliac disease [595]. The expression of this molecule has also been upregulated in oligoarticular JIA [596] and different alleles have been demonstrated to be a risk factor for susceptibility to RA [597] and susceptibility to psoriasis [598]. These results indicate that HLA-DQA1 has a role in immune disease and arthritis pathogenesis and despite there being no direct link to PsA increased levels of HLA-DQA1 in PsA macrophages may be playing a role in disease susceptibility and antigen presentation. The other gene associated with the PsA specific canonical pathways is HLA-DOA which is another HLA molecule involved in antigen presentation and is upregulated at the RNA and protein level in monocytes treated with GM-CSF [599]. A single nucleotide polymorphism (SNP) in HLA-DOA which is significantly associated with RA susceptibility has recently been identified [600] again emphasising the importance of antigen presentation in the pathogenesis of arthritic disease.

The cellular and molecular functions specifically associated with PsA SF macrophages included two functions, cellular movement and cell-to-cell signalling and interaction, which have been previously discussed. However, the other three pathways: DNA replication, recombination and repair; cell cycle and cell death represent functions which are specific to PsA SF macrophages. Despite there being no direct link of DNA replication, recombination and repair to PsA pathogenesis this molecular function is associated with the PsA macrophages due to an increase in the expression of genes such as TOP2A (topoisomerase (DNA) II alpha 170kDa). TOP2A is an enzyme which transiently breaks and rejoins double

stranded DNA in order to resolve topological problems which arise as a result of supercoiling or knotting which can occur naturally in the DNA [601].

The molecular function of cell cycle is associated with the PsA gene list due to the upregulation of genes such as TOP2A (described above) and CDK1 (cyclin-dependent kinase 1) which regulates cell division and controls cell-cycle transitions by associating with different cell-cycle stage specific cyclins [602]. The association of the cell death function with the PsA gene list is due to the differential expression of apoptosis related genes including the down-regulation of clusterin (CLU) in SF macrophages. Clusterin was discussed previously in this chapter as a protein which was highly upregulated in RA PB monocytes compared to healthy controls. Clusterin's cellular functions are context dependent and nuclear associated clusterin (nCLU) is a pro-apoptotic factor [489]. Hence the down-regulation of this gene within the SF macrophages may be a mechanism through which these cells evade apoptosis. This data demonstrates that as well as the proinflammatory pathways which are being upregulated in the synovial macrophages, as demonstrated by the pathways correlated with the genes expressed in both RA and PsA SF macrophages, the PsA macrophages are also up-regulating cellular functions associated with the maintenance of homeostasis such as cell cycle and DNA replication as well as pro-survival factors.

The canonical pathways associated with the gene list expressed specifically in RA SF macrophages compared to the PB monocytes consisted of two pathways, hepatic stellate cell activation and the role of macrophages, endothelial cells and fibroblasts in RA, which were previously associated with the primary RA PB vs RA SF comparison. This is due to the differential expression of genes such as CSF1 (M-CSF) and VEGFA which play a role in these pathways and the pathogenesis of RA. However, the IPA analysis of the genes expressed only in RA SF macrophages also revealed three RA specific pathways: atherosclerosis signalling, ERK5 signalling and p38 MAPK signalling. The presence of the atherosclerosis signalling pathway within the IPA analysis of the RA SF specific genes is interesting as inflammatory pathways have been implicated in both atherosclerosis and RA and this result emphasises the overlap in immune dysregulation which exists in both diseases. RA patients also have a 30-60% higher risk of cardiovascular events compared to osteoarthritis patients or patients without arthritis [603]. This phenomenon is thought to be due to RA patients

being exposed to chronic inflammation which is known to be a driver of atherosclerosis.

One of the RA SF macrophage genes correlated to this atherosclerosis signalling pathway was TNFRSF12A (tumour necrosis factor receptor superfamily, member 12A) which codes for the TWEAK (TNF-like weak inducer of apoptosis) receptor otherwise known as fibroblast growth factor inducible 14 (Fn14). TWEAK has been demonstrated to induce proliferation as well as cell adhesion molecule and cytokine expression in human umbilical vein endothelial cells (HUVECs) [604] and has also been implicated in angiogenesis *in vivo*. Recently, studies using an anti-human Fn14 monoclonal antibody have demonstrated that TWEAK-induced proliferation and migration is solely mediated by its Fn14 receptor [604]. The TWEAK/Fn14 interaction has been implicated in the pathogenesis of arthritis as serum levels of TWEAK were elevated in murine CIA and neutralising TWEAK in this model significantly reduced the clinical severity, paw swelling and infiltration of inflammatory cells [605, 606]. Recently, Fn14 has been detected in the RA synovium and on synovial fibroblasts where its ligation with TWEAK induced the production of pro-inflammatory cytokines and the expression of intracellular adhesion molecule (ICAM-1) [607]. This pro-inflammatory cytokine release has since been demonstrated to be mediated through the activation of the NF- κ B pathway [608]. Fn14 is detected within the synovium of both RA and PsA where it has been demonstrated to be specifically expressed on macrophages and fibroblasts [609] and its ligand TWEAK is present in the serum of RA patients and correlates with disease activity [610]. Consequently, the upregulation of the Fn14 gene TNFRSF12A by the RA SF macrophages in our study demonstrates a mechanism through which these macrophages can contribute to the inflammatory environment in RA synovium. The TWEAK/Fn14 has been shown to play a role in the pathogenesis of atherosclerosis and cardiac dysfunction due to its ability to induce angiogenesis, exacerbate vascular damage and mediate lipid uptake by macrophages [611-613] and therefore may be a pathway causing the increased risk of cardiovascular events seen in RA.

The remaining two canonical pathways associated with the RA specific gene list were ERK5 signalling pathway and p38 MAPK pathway, both of these pathways are members of the MAPK signalling family and their association with RA SF macrophages is due to the up- and down-regulation of genes coding for signalling

intermediates and transcription factors involved in these pathways. ERK5 signalling pathway has not previously been associated with RA pathogenesis but some of its signalling components which were differentially expressed in the RA SF macrophages have been demonstrated to play a role. For example, FOSL1 is upregulated in the RA SF macrophages and this gene codes for the Fra-1 protein which dimerises with c-Jun to form the AP-1 transcription factor. AP-1 has been demonstrated to be constitutively active in rheumatoid synovium [614] and treatment of rheumatoid synoviocytes with TNF α and IL-1 β induced the nuclear translocation of Fra-1 and induction of AP-1 transcription [615]. Consequently, despite no evidence for the ERK5 pathway being implicated in RA the components of this pathway have been demonstrated to play a role in the pathogenic phenotype of synovial cells.

The p38 MAPK pathway is also correlated to the RA SF macrophages which is due to the upregulation of genes coding for IL-1 receptor type I, IL-1 receptor type II, IL-1 receptor-associated kinase 2 (IRAK2) and the anti-inflammatory molecule IL-1 receptor antagonist. The ligation of IL-1 α or IL-1 β to the IL-1 receptor type I on the cell surface activates IRAK and commences the activation of the p38 MAPK signalling pathway and the subsequent translation of genes coding for pro-inflammatory mediators such as IL-6, IL-8, CCL2 (MCP-1), IL-1 α and IL-1 β [616]. Interestingly the RA SF macrophages have also upregulated levels of IL-1 receptor antagonist (IL-1RA) and the decoy receptor IL-1 receptor II (IL-1R2) which are inducible negative regulators of IL-1 signalling and can terminate or limit IL-1 effects. Hence the RA SF macrophages are not only up-regulating activator components of the p38 MAPK pathway but also negative regulators of this pathway, the net effect of this gene expression is not known since IL-1RA binds IL-1R1 with a similar specificity and affinity as IL-1 α and IL-1 β [616]. Of interest, a recombinant version of human IL-1RA designated anakinra is used as a therapy for RA demonstrating the significant anti-inflammatory properties of IL-1RA [266].

The molecular and cellular functions of the RA SF specific gene list highlight the same group of functions which were associated with the previous RA PB vs RA SF comparison such as cellular movement, cell-to-cell signalling and cellular growth. As discussed previously the differential expression of genes associated with these functions demonstrates the increased cellular migration undertaken

by the macrophages as well as the physiological growth and development which the PB monocytes undergo to mature into the synovial macrophages.

In summary, the analysis of the genes similarly expressed in both RA and PsA SF macrophages as well as the genes expressed specifically in PsA SF macrophages or RA SF macrophages has been crucial in defining groups of genes specifically expressed in each disease. The Venn diagram analysis also revealed the significant similarity in gene expression in both RA and PsA macrophages compared to their matched disease blood monocytes with over 50% of the genes being shared in each disease.

Apart from the identification of the shared expression of the LXR activation pathway which was an interesting finding this analysis unfortunately did not reveal any novel genes which change in expression as the RA monocytes move from the blood into the synovial fluid. For that reason it was decided to analyse the differentially expressed genes that changed in both RA and PsA SF macrophages to try and detect novel genes that had not previously been associated with arthritis. This was in the hope of finding novel pathways regulating the pathogenesis of the arthritic synovial macrophage. The genes which had been upregulated in the synovial macrophages compared to the blood monocytes were analysed starting with those that had the largest fold change difference. Analysing the genes using this method unsurprisingly resulted in the identification of genes coding for molecules which have previously been associated with arthritis such as FN1 (fibronectin), SPP1 (osteopontin) and CCL18. However, through this analysis we also discovered two novel genes; LGM which codes for the enzyme legumain and PLXA1 which codes for the cell surface receptor plexin A1. These two candidate genes were subsequently validated and further analysis was performed to determine their function within the arthritic synovium. These results are discussed in full detail in the following chapters.

Chapter 4 Investigation into the expression and function of the novel gene plexin A1 in rheumatoid and psoriatic arthritis patients

4.1 Introduction

Plexin A1 is one of the genes found to be upregulated in RA and PsA SF CD14⁺ cells in the preceding microarray experiment; it has not been previously associated with arthritis and is one of the two genes that I selected for intensive investigation in the context of rheumatoid and psoriatic arthritis pathogenesis. Plexins are a homogenous family of proteins, which when first identified were shown to be neuronal cell surface molecules with a function in cell adhesion [442]. They are grouped into four categories (A-D) based on their overall homology, there are 4 type-A, three type-B, one type-C and one type D found in vertebrates and two plexins found in invertebrates (A and B) [617].

Plexins are the receptors for semaphorins, which are both secreted and membrane associated proteins named for their properties which are analogous to the system of flags and lights used in rail and maritime communication for the direction of movement. Semaphorins were originally characterised in the developing nervous system as chemorepellent molecules which acted to initiate axonal collapse in areas where they were found in high concentration, thus resulting in axonal guidance [618, 619]. However, more recently they have been shown to have diverse functions in several physiological processes such as angiogenesis [620], tumor metastasis [621, 622], osteoclastogenesis [623] and immune regulation [624]. More than 20 types of semaphorins have been identified and are grouped into eight classes on the basis of their structural elements and amino acid sequence similarity [619]. The semaphorins found in invertebrates are grouped into classes 1 and 2, classes 3-7 are vertebrate semaphorins and the final group, group V, are viral semaphorins. Proteins in the semaphorin classes 1, 4, 5, 6 and 7 are membrane associated whereas those in classes 2, 3 and the viral semaphorins are secreted [617]. All semaphorins contain a conserved ~500 amino acid extracellular domain termed a sema (semaphorin) domain and a class specific C terminus.

Plexins are single membrane spanning proteins that have an extracellular domain consisting of a ~500 amino acid sema domain, that is conserved among all semaphorin family members, followed by a cysteine-rich motif and several glycine-proline rich motifs [442]. Most plexin-semaphorin interactions are mediated through the sema domains of both proteins except for class 3

semaphorins, which, with the exception of sema 3E, bind to neuropilin-1 or neuropilin-2 along with plexin in a co-receptor complex [625-627]. Neuropilins have a very short cytoplasmic domain that cannot transduce intracellular signals, therefore the plexin receptor must act as the signal transducing components of the semaphorin-neuropilin-plexin complex [628]. Plexin A family members are the main receptors for the secreted class 3 semaphorins as shown in Figure 4.1, semaphorin 3A will only bind neuropilin-1 (NRP-1) in the plexin-neuropilin receptor complex whereas semaphorin 3C and 3F bind both NRP-1 and NRP-2 [443].

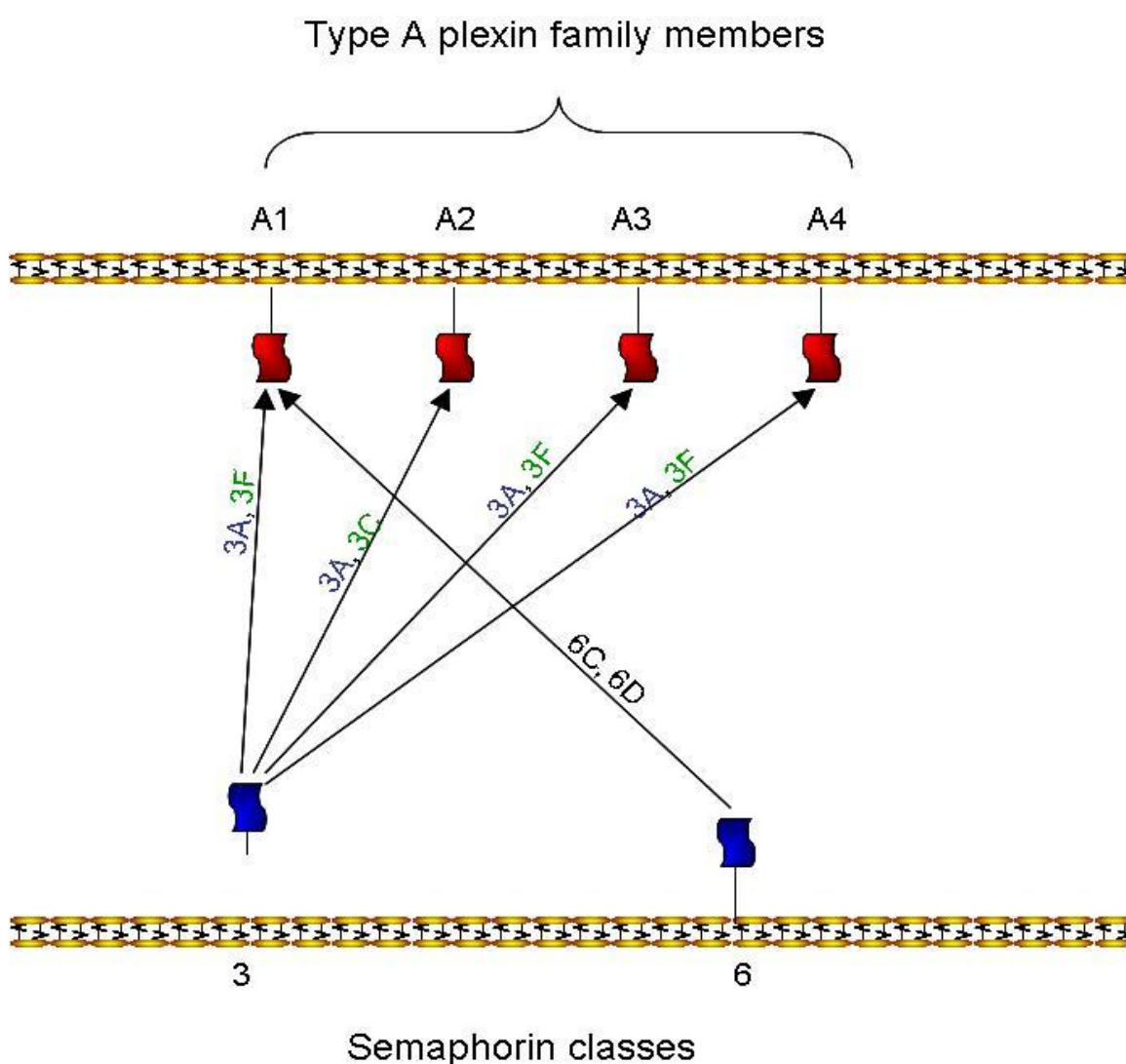


Figure 4.1 Semaphorin ligands for type A plexin family members

There are four members of the plexin A family which interact primarily with two classes of semaphorins, class 3 and class 6. Both plexins and semaphorins are characterised by a sema domain shown in red for the plexins and blue for the semaphorins. Arrows indicate binding interactions detected between semaphorins and plexins. Labels on the arrows indicate which specific semaphorins have been shown to interact with which plexin [443, 444, 617, 629-632]. Blue labels of semaphorin names indicate the necessity for neuropilin-1 (dark blue) or neuropilin-1/neuropilin-2 (green).

Plexin A1 is the only receptor in the type A family of plexins that interacts with two different classes of semaphorins, class 3 and class 6 (Figure 4.1). It is our gene of interest based on the results of the microarray and it is one of the semaphorin receptors whose function has been widely investigated. It is known to be expressed in the heart, liver, brain, kidney, spleen and lung of 4 week old mice [623] and is specifically expressed on plasmacytoid DCs [633], mature dendritic cells [444] and at low levels on T and B cells [623]. Recently plexin A1 has also been shown to increase in expression during M-CSF differentiation of human monocytes into macrophages [443], the same group also showed LPS stimulation suppressed plexin A1 expression in macrophages. Toyofuku et al first identified semaphorin 6D (sema 6D) as one of plexin A1's ligands during chick cardiac development. Plexin A1 was found to form site specific receptor complex with vascular endothelial receptor type 2 (VEGFR2) in the conotruncal segment or with Off-track in the ventricle segment. These specific complexes bound to sema 6D and were responsible for its specific effects in these regions [631]. It is thought that semaphorin binding relieves plexin autoinhibition, as plexin A1 lacking its sema domain is constitutively active [634].

Plexin A1 has been previously shown to play a role in axonal guidance in the developing nervous system by acting as a co-receptor with neuropilin-1 to bind sema 3A resulting in a chemorepulsive signal [635]. It also has a role in chemotaxis of carcinoma cells as expression of constitutively active plexin A1 in breast carcinoma cells impaired their ability to chemotax [636]. However, the first report of plexin A1 in immune cell function came as a result of a microarray that identified plexin A1 as a gene down regulated in CIITA-deficient DCs to 5% of that found in wild type cells [444]. CIITA (class II transactivator) is a transcription factor that controls MHC class II expression [637], it functions as a transcriptional scaffold to coordinate NF- κ B and RFX5 transcription factors in binding the conserved promoter regions found in MHC class II promoters. The same group found plexin A1 to be highly expressed in mature DCs from wild type mice and it was established that depletion of plexin A1 in mature DCs by RNA interference led to a 50% reduction in T cell stimulation *in vitro* and *in vivo* indicating that plexin A1 expression is important for T cell-DC interactions. This reduced proliferation could not be attributed to decreased antigen processing, MHC expression or peptide loading; however a suggested function for plexin A1

on the DCs was to serve as a co-stimulatory marker that binds its partner ligand on the T cells therefore enhancing the T cell-DC interaction.

A further study, [623], into plexin A1's role in immune responses found that plexin A1^{-/-} DCs had an impaired ability to stimulate antigen-specific T cells as they had reduced proliferative responses and cytokine production, consistent with the previous work performed by Wong et al. The plexin A1^{-/-} mice had reduced cellularity in their long bones compared to wild type litter mates and it was revealed that plexin A1 deficiency resulted in increased bone mass and the development of osteopetrosis. It was also shown that plexin A1^{-/-} mice had decreased osteoclast numbers, lower osteoclast surface to bone ratio and reduced osteoclast bone turnover therefore implicating a role for plexin A1 in bone homeostasis. This group had previously identified sema 6D as the ligand for plexin A1 in chick cardiac development [631] and found sema 6D to be highly expressed in T cells, therefore they incubated DCs with soluble recombinant sema 6D and showed that it induced IL-12 production and upregulation of MHC class II suggesting that sema 6D is the ligand for plexin A1 in T cell- DC cellular interactions. Sema 6D was also found to be expressed on osteoclasts and soluble recombinant sema 6D promoted osteoclast differentiation again suggesting that sema 6D is the ligand for plexin A1 in bone homeostasis. To try and further understand the plexin receptor complex, the group screened molecules associated with plexin A1 in DCs and osteoclasts and identified TREM-2. Previous studies have indicated that TREM-2 forms a receptor complex with DAP12, an ITAM-bearing activating adaptor protein, through a positively charged amino acid in its transmembrane domain [638]. Several experiments using RNA interference and DAP12^{-/-} cells suggested that plexin A1, DAP-12 and TREM-2 are the sema 6D functional receptor components on DCs and osteoclasts [623]. Interestingly, genetic mutations in TREM-2 or DAP12 result in a bone fracture syndrome called Nasu-Hakola disease further underscoring the importance of the association of plexin A1 with DAP12 and TREM-2 in bone homology [639].

Further studies into the localisation of plexin A1 in DCs found it to be associated with the DC-T cell immune synapse in approximately 70% of T cell- DC cellular conjugates where it was found to regulate actin polarisation in the DCs via activation of the small GTPase Rho [640]. Plexin A1 has also been found to associate with DAP12 and another TREM family member found specifically on

pDCs designated PDC-TREM. Sema 6D binding to the plexin A1-DAP12-PDC-TREM complex on pDCs induced the production of type I IFN [633].

Plexin A1 has recently been shown to play a crucial role in allowing murine DCs to enter the lymphatic system and track to the lymph node. This migration is dependent on the presence of semaphorin 3A (sema 3A), one of plexin A1's ligands, in the lymphatic system. Sema 3A is thought to promote DC transmigration by inducing actomyosin contraction, which allows the cells to pass through constricted areas, by regulating myosin II activity and phosphorylation. This was demonstrated by treating the DCs with the myosin II inhibitor blebbistatin or the Rho kinase ROCK inhibitor Y-27632 which abolished sema 3A induced transmigration across lymphatic endothelial cell monolayers [632].

Due to the identification of a novel gene, plexin A1, expressed in RA and PsA SF CD14⁺ cells, and its plausible biologic profile, I therefore sought to validate this expression by RT-PCR in the microarray samples and samples from other patients. I then sought to identify its potential function in macrophage cells by siRNA and to investigate the expression of its semaphorin ligands on stimulated T cells and synovial membrane.

4.2 Validation of plexin A1 in microarray samples

Plexin A1 was one of the genes identified when the group of genes upregulated in both RA and PsA SF were analysed. It had a fold change increase from the PB to the SF of 26 in RA patients and 27 in PsA patients. Figure 4.2 (A) demonstrates the raw fluorescent data signals of plexin A1 obtained from the Affymetrix GeneChips. The PB samples from both disease groups have very low levels of signal intensity indicating that these samples had low levels of plexin A1 mRNA. In contrast the patient synovial fluid samples have much higher levels of signal intensity and therefore contained higher levels of plexin A1 mRNA. The reason for the variable levels of the mRNA expression may be due to different disease duration in the patients or differences in disease therapy. To verify and validate this expression qRT-PCR was used to analyse the expression of plexin A1 in the 8 RA and 8 PsA matched PB and SF CD14⁺ samples. All of the samples were able to be analysed apart from one RA PB sample which consistently produced unusual results which could not be included in the analysis. This may have been due to a problem with the RNA sample therefore that PB sample and its matched SF were not analysed. Figure 4.2 (B) shows the results of qRT-PCR, the fold change values were calculated by comparing the level of plexin A1 expression in the synovial fluid compared to the PB. Each circle represents one patient and the line is the mean fold change, the RA samples had a mean fold change increase of 8 from the PB to the SF and the PsA samples had a mean fold change increase of 13. The results demonstrate that there was an increase in plexin A1 expression in all of the synovial fluid samples compared to their matched peripheral blood samples which agree with the results obtained from the microarray.

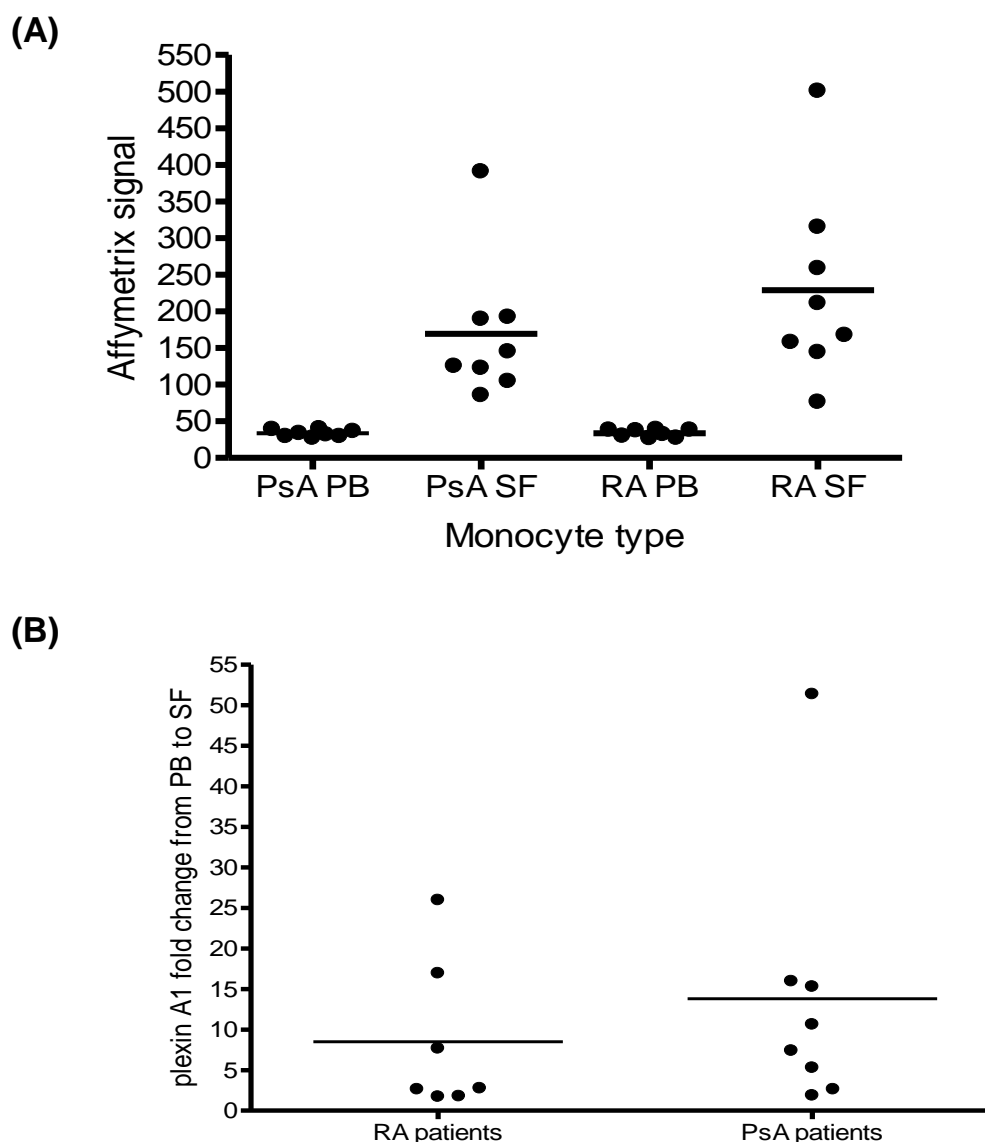


Figure 4.2 Plexin A1 expression in microarray samples

(A) Demonstrates the raw signal intensities of the plexin A1 gene locus on the Affymetrix GeneChips. Each circle represents one patient sample which was hybridised to one genechip and the line represents the mean signal intensity. Plexin A1 mRNA levels were then measured in the RA and PsA paired PB and SF microarray samples by qRT-PCR. (B) The fold change of plexin expression in the SF samples was normalised to the matched patient PB sample, each circle represents one patient and the line the mean fold change

4.3 Expression of plexin A1 in other patient samples

Once the expression of plexin A1 had been validated in the microarray patient samples we then analysed the expression of plexin A1 in CD14⁺ of other arthritic patients to check that the increase in plexin A1 expression was generalizable. Figure 4.3 shows the results of this analysis, graph (A) indicated all the CD14⁺ cells that were obtained and tested for relative expression plexin A1 which was normalised to the housekeeping gene GAPDH. PsA SF cells have varying

expressions of plexin A1 depending on the individual patient but the mean relative expression is higher than the mean relative expression of the PsA PB samples. The RA SF samples also have variable amount of plexin A1 but the mean expression is similar to that seen in PsA SF. An inflammatory polyarthritis and a juvenile idiopathic arthritis (JIA) SF sample were also obtained for this analysis and both samples contain plexin A1 RNA, unfortunately no matched peripheral blood CD14⁺ cells were acquired for either patient so we cannot determine whether the plexin A1 expression increases in the SF cells compared to the PB cells as is seen with RA and PsA patients. However, matched PB and SF CD14⁺ cells from a patient with osteoarthritis (OA) were also analysed and shows that, as with the RA and PsA from the microarray, plexin A1 expression is higher in the SF compared to the PB. This indicates that expression of plexin A1 on CD14⁺ cells is not confined to PsA and RA SF and could also be expressed in other arthritic diseases. These analyses should however be considered anecdotal and are included here for completeness at this stage. Five of the PsA patients analysed had matched SF and PB CD14⁺ cells therefore the fold change increase of plexin A1 from the PB samples to the SF samples was determined (graph B). In summary, these data indicate that in the majority, but not all, PsA patients plexin A1 RNA levels are higher in the SF compared to the PB CD14⁺ cells as was seen in the microarray experiment.

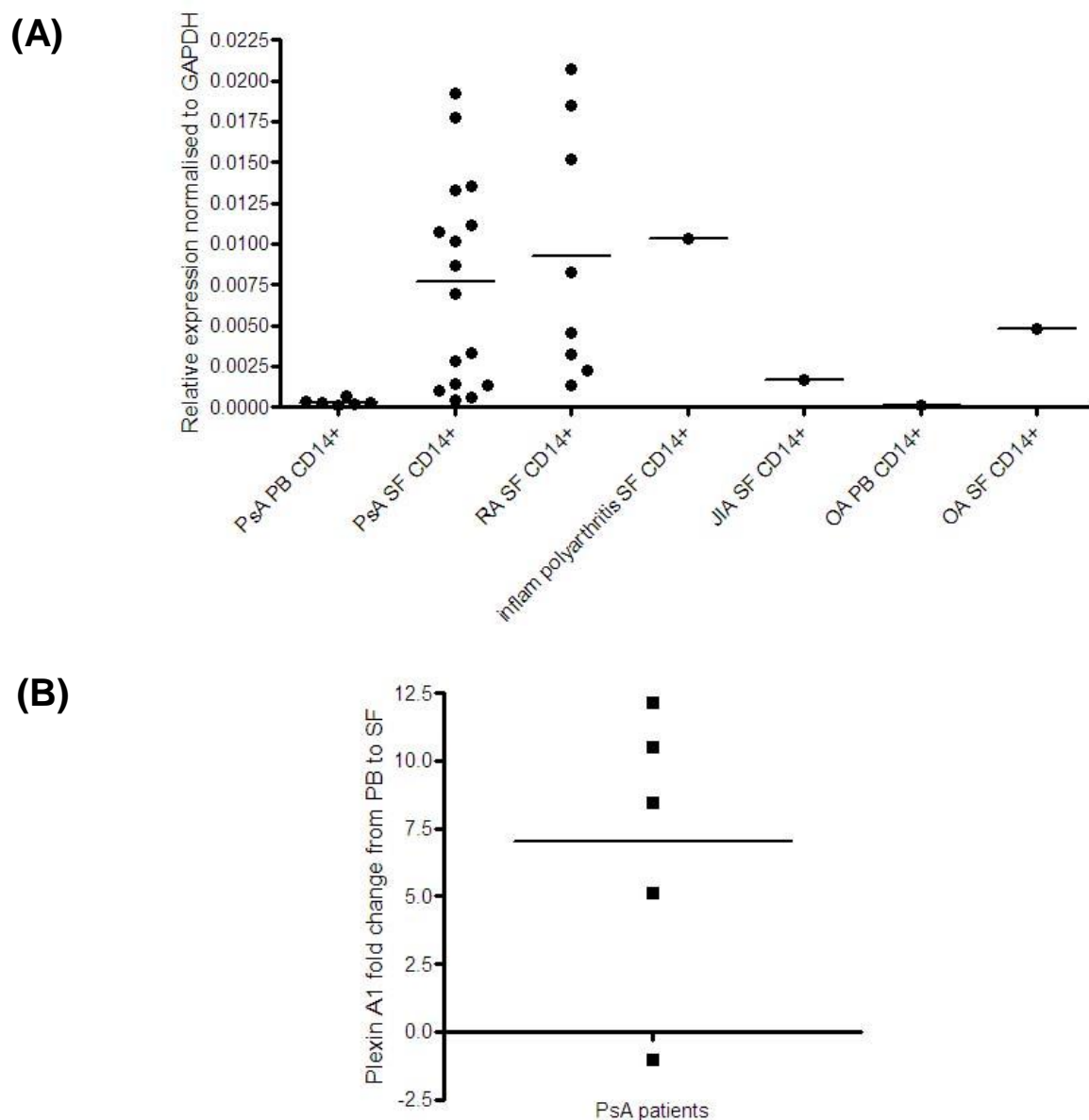


Figure 4.3 Plexin A1 expression in CD14⁺ cells from arthritic patients

To verify the microarray results plexin A1 RNA levels were measured in CD14⁺ cells from several other arthritic patients (A). Five of the PsA patients analysed in (A) had matched PB and SF samples therefore the fold change increase of plexin A1 expression from the PB sample to the SF sample was measured for each of these patients and is shown in (B) each square represents one patient. Lines on each graph indicate the mean.

4.4 Immunohistochemistry of plexin A1

Once plexin A1 expression had been validated as being present in the microarray samples and other patient samples by RT-PCR, immunohistochemistry (IHC) was used to investigate the presence of plexin A1 protein in RA synovial membrane tissue. Five RA membranes stained positively for plexin A1; Figure 4.4 shows the pattern of staining seen in a representative membrane. Panel (B) shows an area of the membrane at x10 magnification on the left and x40 magnification on the right. The synovial membrane can be seen to be positively stained (indicated by brown staining) around vascular structures and within cells in the area surrounding the vasculature possibly indicative of infiltrating cells (indicated by black arrows). Panel (C) indicates two more images taken from the same membrane and again demonstrates that the areas staining positively for plexin A1 are those surrounding blood vessels and in cells around vasculature area but there are also cells within the synovial membrane structure that have stained for plexin A1. The staining appears to be present in both the cytoplasm and the nucleus of the cells. The positive staining surrounding the vasculature could be indicative of endothelial cells but the identity of the positive cells within the membrane require double staining of plexin A1 and other cellular markers. Such experiments would be the focus of future analysis in order to confirm the extent of plexin A1 expression.

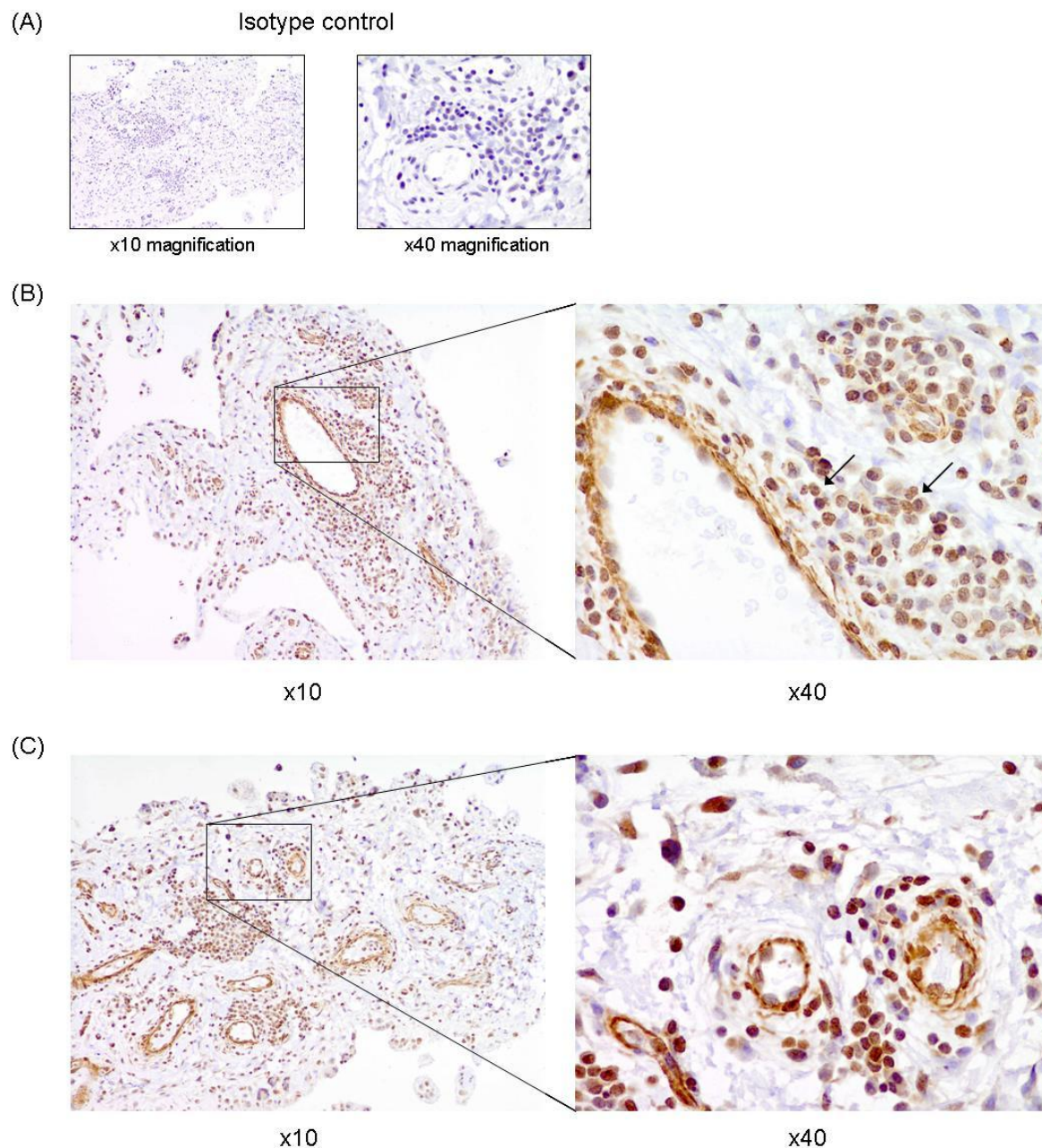


Figure 4.4 Plexin A1 staining in RA synovial membrane

Five RA synovial membrane tissues were stained for the presence of plexin A1. This figure indicates the staining pattern seen in one of these membranes. Panel (A) indicates the isotype control at x 10 and x 40 magnification. Panel (B) and (C) show the positive plexin A1 staining (shown in brown) of two areas within the membrane (arrows identify infiltrating cells), the staining is shown at x 10 magnification on the left and x 40 magnification on the right.

4.5 siRNA knockdown of plexin A1 in M-CSF derived macrophages

I next decided to establish the role of plexin A1 in macrophage biology using RNA interference (RNAi). RNAi is a pathway in eukaryotic cells by which sequence specific small interfering RNA (siRNA) is able to find and cleave complementary mRNA [641]. An enzyme known as Dicer can cleave double stranded RNA into siRNA fragments [642]. Once siRNA is present in the cell it is incorporated into a protein complex called the RNA induced silencing complex (RISC) [643] where argonaute-2 unwinds the siRNA. The activated RISC complex containing the antisense strand of siRNA will then find and degrade mRNA that is complementary to this strand resulting in specific gene silencing. Synthetic siRNA specific to a gene can be directly introduced into a cell allowing gene silencing of almost any mammalian gene thereby allowing analysis of specific gene function.

So far most experimental work on plexin A1 has looked at plexin A1 expression and function in DCs, since our results showed that plexin A1 is upregulated in monocytes/macrophages in the arthritic synovial fluid we wanted to determine the role of plexin A1 in this cell type with the use of siRNA. M-CSF differentiated macrophages were used to work up the siRNA experiments as these are a relatively accessible cell source compared to the limited number of synovial fluid patient samples we obtain in the lab.

4.5.1 Expression of plexin A1 in macrophages after 6 days of M-CSF differentiation

A preliminary experiment before starting the siRNA knockdown of plexin A1 was to first determine that macrophages expressed plexin A1 mRNA. A previous study had demonstrated that plexin A1 was upregulated in macrophages differentiated in M-CSF for 2 days [443]. In our experimental setup monocytes were differentiated with 50 ng/ml M-CSF for 6 days and analysed at day 0 and 6 for plexin A1 expression. Figure 4.5 shows the fold change in plexin A1 expression in day 6 differentiated macrophages compared to day 0. Once the presence of plexin A1 had been determined in these cells we could then set up the siRNA experiments.

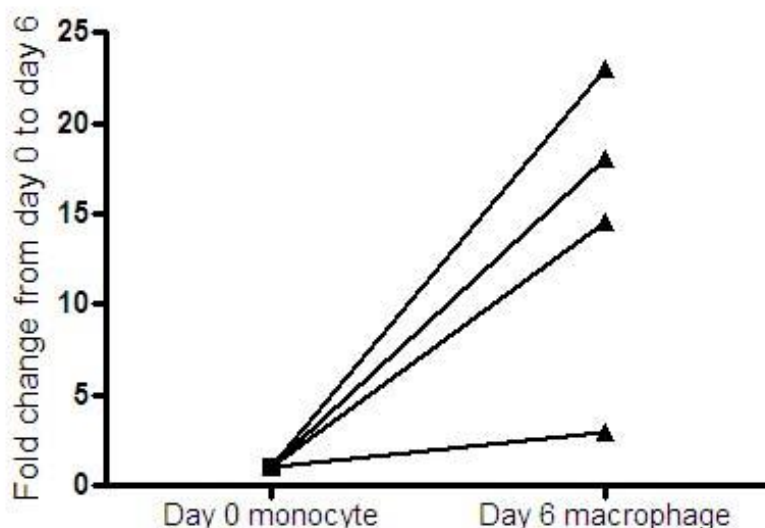


Figure 4.5 Plexin A1 in M-CSF differentiated macrophages

Monocytes were differentiated with 50 ng/ml M-CSF for 6 days. Fold change in plexin A1 mRNA expression was measured by qPCR.

4.5.2 Transfection efficiency of N-TER reagent

An N-TER nanoparticle transfection system was used to knockdown the plexin A1 gene. In this transfection system the N-TER peptide binds siRNA non-covalently forming a nanoparticle. The nanoparticle then interacts with the lipids on the surface of the plasma membrane thus allowing the nanoparticle to diffuse across the cell membrane and delivering the siRNA to the cytoplasm.

To test the efficiency of this transfection system a negative control siRNA with an Alexa Fluro 555 modification was used. The negative control siRNA will not knockdown any genes in the cell since it is a non-silencing siRNA with no known homology to any known mammalian gene. Since it is modified with a fluorescent tag it can be used to measure the transfection efficiency of the N-TER peptide as any cell that has been successfully transfected will fluoresce. To determine the concentration at which the N-TER peptide worked most effectively, cells were transfected with the negative control siRNA and analysed on a fluorescent microscope and by FACS.

4.5.2.1 Inverted microscope images

Day 6 M-CSF differentiated macrophages were transfected with varying concentrations of negative control siRNA for 24 hours in the presence or absence of fetal calf serum (FCS) as FCS can sometimes affect siRNA transfection. The

cells were then imaged on a fluorescent inverted microscope as seen in Figure 4.6. In the cells transfected in medium plus FCS all concentrations of siRNA have resulted in positively transfected cells as shown by the orange fluorescence. However, the cells did not transfect as well at the 5 nM concentration of siRNA indicated by the less intense fluorescent staining of those macrophages. A similar staining pattern can be seen in the cells which were transfected without serum in the medium as all the concentrations of siRNA show positive transfection and fluorescent cells but some cells in the 10 nM and 5 nM concentrations are not stained as brightly as the cells in the 30 nM and 20 nM concentrations. The cells that were incubated without serum contained lots of cellular debris as can be seen in the 30 nM image indicating that this could be detrimental to the cells. Therefore, siRNA transfection was performed in the presence of FCS.

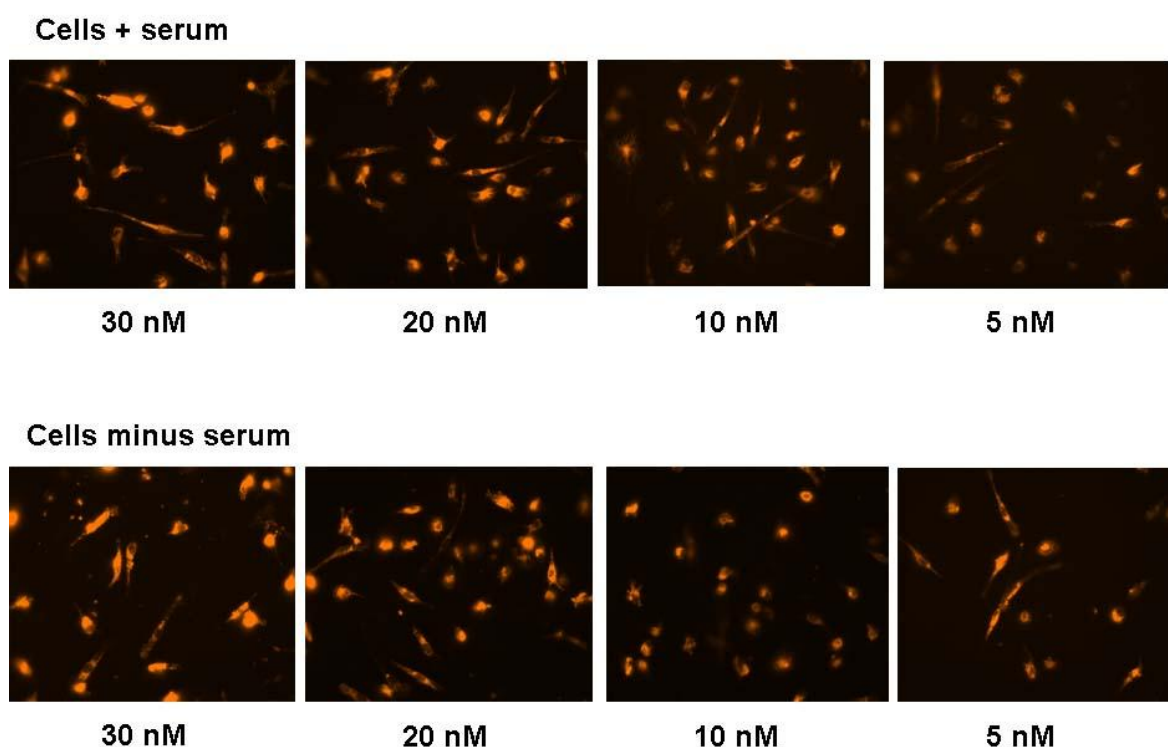


Figure 4.6 siRNA transfection efficiency by imaging fluorescence on the microscope

Macrophages were treated with M-CSF for 6 days and transfected with varying concentrations (30 - 5 nM) of negative control siRNA for 24 hours in the presence or absence of fetal calf serum (FCS). The negative control siRNA has a fluorescent tag therefore cells that been successfully transfected cells will fluoresce.

4.5.2.2 FACS plots

To quantify the amount of positive transfection with the N-TER transfection system macrophages were treated with the negative control siRNA for 24 hours in serum-containing media, the cells were then collected and analysed on the FACS for Alexa Fluro-555 fluorescence compared to untransfected cells. Figure 4.7 shows the results of this FACS analysis and demonstrates that at least 93% of macrophages were positive for negative control siRNA in all of the concentrations tested. This indicates that the N-TER transfection system allows siRNA to successfully enter the macrophage cells even at 5 nM concentration.

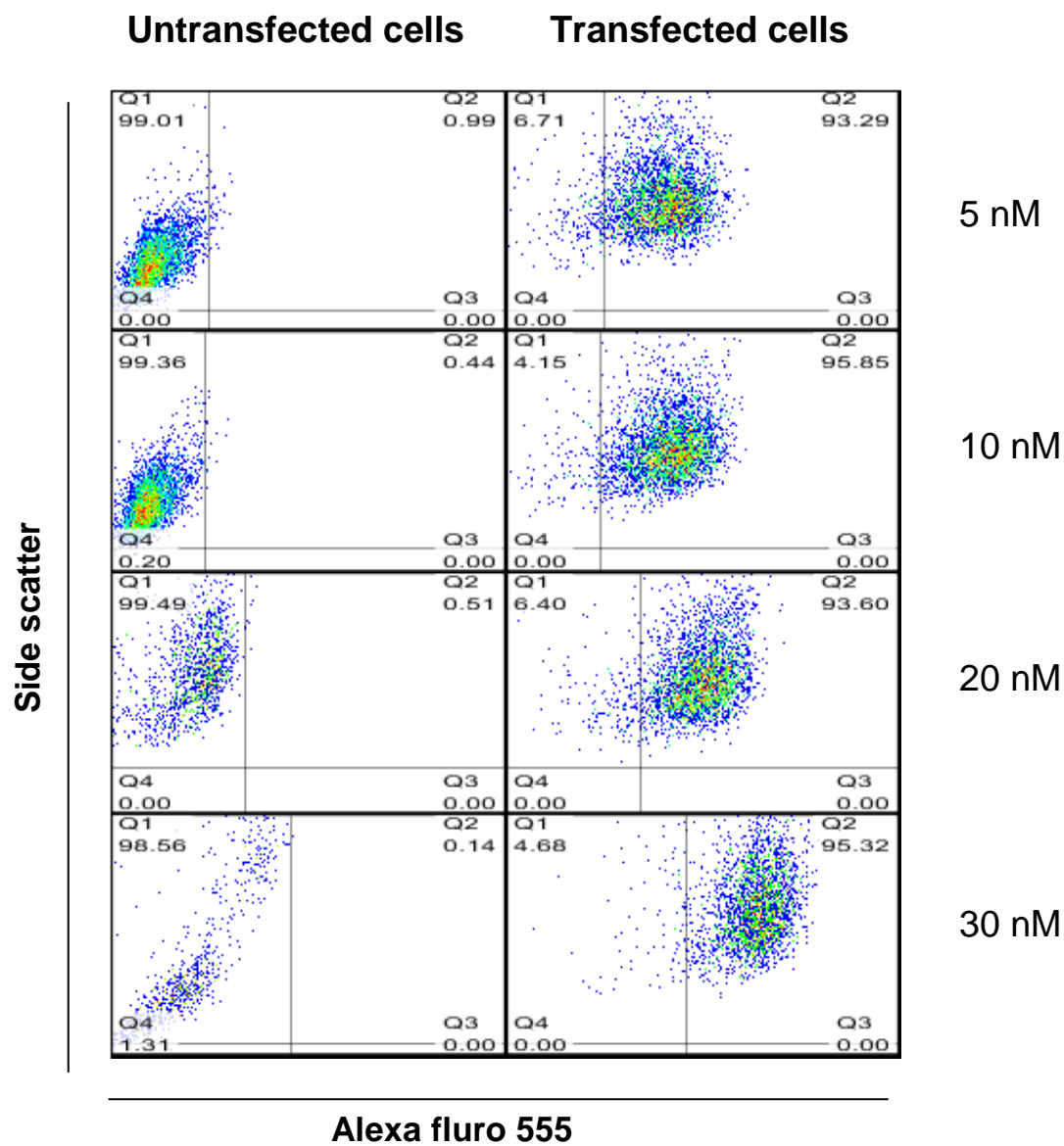


Figure 4.7 FACS analysis siRNA transfected macrophages
 Monocytes were differentiated with M-CSF for 6 days and transfected with varying concentrations of a fluorescent tagged negative control siRNA for 24 hours in complete medium. Cells were then

collected and analysed by FACS for fluorescence which was indicative of positive siRNA transfection.

4.5.3 Testing siRNA 1-4 at 3 concentrations

Once the transfection system had been validated in macrophage cells the plexin A1 siRNA was tested in the macrophage cells to see if it changed the expression of plexin A1. The plexin A1 siRNA received from Qiagen consisted of four individual siRNA's designated 1-4. Each individual siRNA was tested on the macrophages at three concentrations 10, 20 and 30 nM. It was decided not to test the siRNA at 5 nM as from the fluorescent microscope experiment the macrophages did not appear to be as intensely stained at the 5 nM concentration as they did at the other concentrations.

4.5.3.1 PLX1- 4 at 30, 20, 10 nM

Macrophages were M-CSF treated for 6 days and then treated with negative control siRNA (designated control) and the four plexin siRNA (designated PLX 1-4) at 10, 20 and 30 nM concentrations for 24 hours. After this time the cells were collected and put into trizol and the qRT-PCR was performed on the samples to determine whether the siRNA had decreased the amount of plexin A1 siRNA being expressed by the cells. Since the negative control siRNA is a non-silencing siRNA it should not affect the amount of plexin A1 expression and therefore the plexin A1 mRNA levels in the samples treated with siRNA PLX 1-4 were compared back to the negative control siRNA to look for a relative increase or decrease in expression. Figure 4.8 shows the results of this experiment; graph (A) demonstrates the expression of plexin A1 as a result of siRNA treatment. The values of the negative control siRNA are 1 and the line drawn on the graph is set at 1. It can be seen that at 30 nM concentration PLX 1, -2 and -3 siRNAs are causing an increase in Plexin A1 expression rather than knocking it down and at the 10 nM concentration PLX 2 and 3 siRNA are also causing an increase in plexin A1 expression in the cells compared to the control.

Figure 4.8 (B) shows the percentage inhibition of plexin A1 as a result of adding the 4 different siRNAs. The percentage inhibition was calculated relative to the negative control and a negative value indicates an increase in plexin A1 expression whereas a positive value indicates a decrease in plexin A1 expression. At the 30 nM concentration PLX 1, PLX 2 and PLX 3 siRNA induced an increase in

plexin A1 expression of 89, 83 and 84% respectively. Also at the 10 nM concentration PLX 2 and PLX 3 siRNA increased the amount of plexin A1 mRNA by 100 and 67% respectively. The reason for these siRNAs increasing the amount of expression is unknown. A recent study has shown when an siRNA sequence is complementary to the promoter region of a gene it can increase transcription of that gene [644]. However, the siRNAs used in this study are unlikely to target the promoter region of plexin A1 as the phenomenon of the PLX siRNA inducing expression of plexin A1 does not occur at all of the concentrations tested and only occurs at 30 nM and 10 nM concentrations.

Due to the PLX 2 and PLX 3 siRNA producing an increase in plexin A1 levels at 30 and 10 nM concentrations they were omitted from further experiments. PLX 1 siRNA induced an increase of plexin A1 at the 30 nM concentration but otherwise induced a high amount (75%) of inhibition at the 20 nM concentration and also decreased plexin A1 levels at the 10 nM concentration. PLX 4 siRNA was the only siRNA to consistently decrease the level of plexin A1 mRNA. Consequently only PLX 1 and 4 were used in additional experiments.

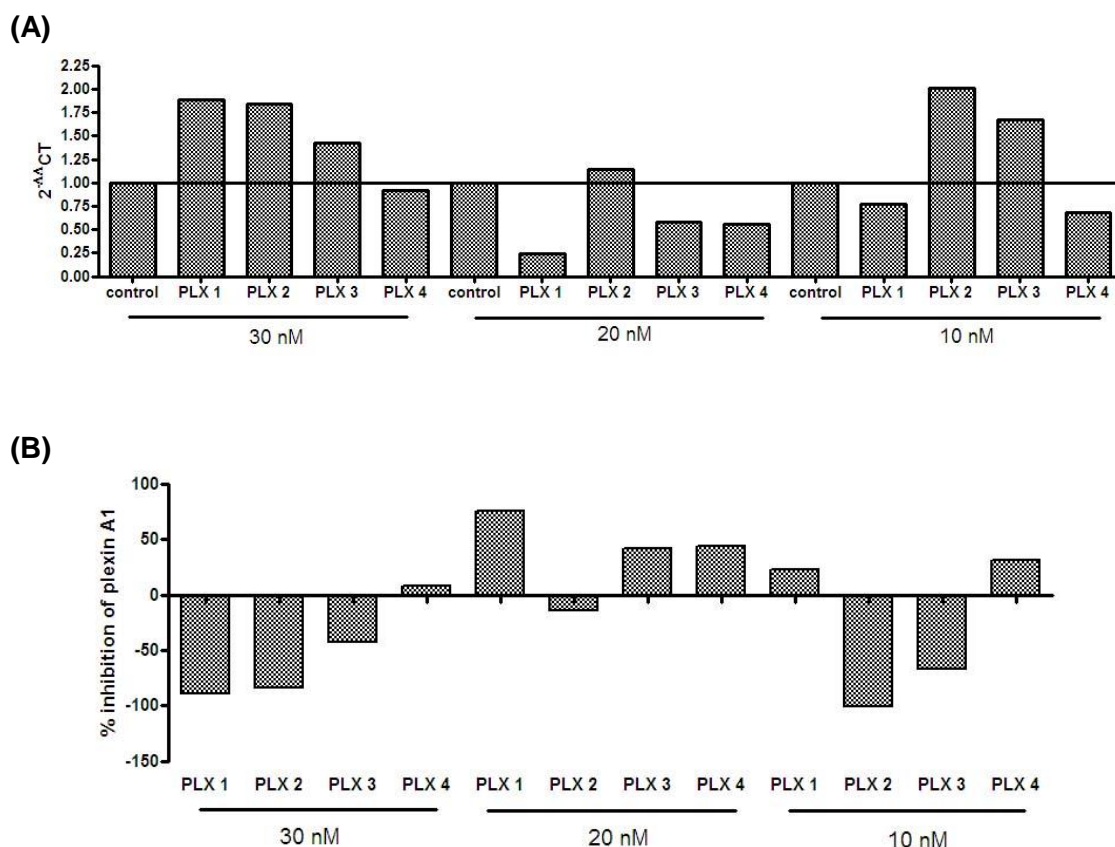


Figure 4.8 Testing siRNA PLX 1-4 in M-CSF differentiated macrophages

M-CSF differentiated macrophages were treated with 4 siRNAs PLX 1-4 and a negative control siRNA that should not affect the expression of plexin A1 at 3 different concentrations 10, 20 and 30 nM for 24 hours. Using qRT-PCR the amount of expression of plexin A1 in the cells relative to the negative control, which is set at 1, was determined in graph (A). The line on the graph is set at 1 therefore any bars above the line are showing an increase in plexin A1 expression and any lines below are a decrease in plexin A1 expression. The percentage inhibition of plexin A1 was also determined for this experiment as can be seen in graph (B). Positive values for the percentage inhibition indicate that there was a decrease in plexin A1 expression compared to the negative control.

4.5.3.2 PLX 1 and 4 at 30, 20, 10 nM

To check the level of plexin A1 inhibition by the two siRNAs PLX 1 and PLX 4, M-CSF derived macrophages were treated with 10, 20 and 30 nM siRNA for 24 hours. Figure 4.9 (A) indicates that both PLX 1 and PLX 4 decreased the amount of plexin A1 mRNA expression in the macrophages compared to the negative control in all of the three conditions. The percentage inhibition of plexin A1, graph (B), ranged from 14% to 54%. The concentration at which the percentage inhibition was greatest was 20 nM, at this concentration PLX 1 and PLX 4 induced 49 and 54% inhibition respectively compared to the negative control. Hence the 20 nM concentration of PLX 1 and PLX 4 was used in the further experiments, a mixture of both siRNAs was also used to determine if combining the two siRNAs results in a greater amount of gene silencing.

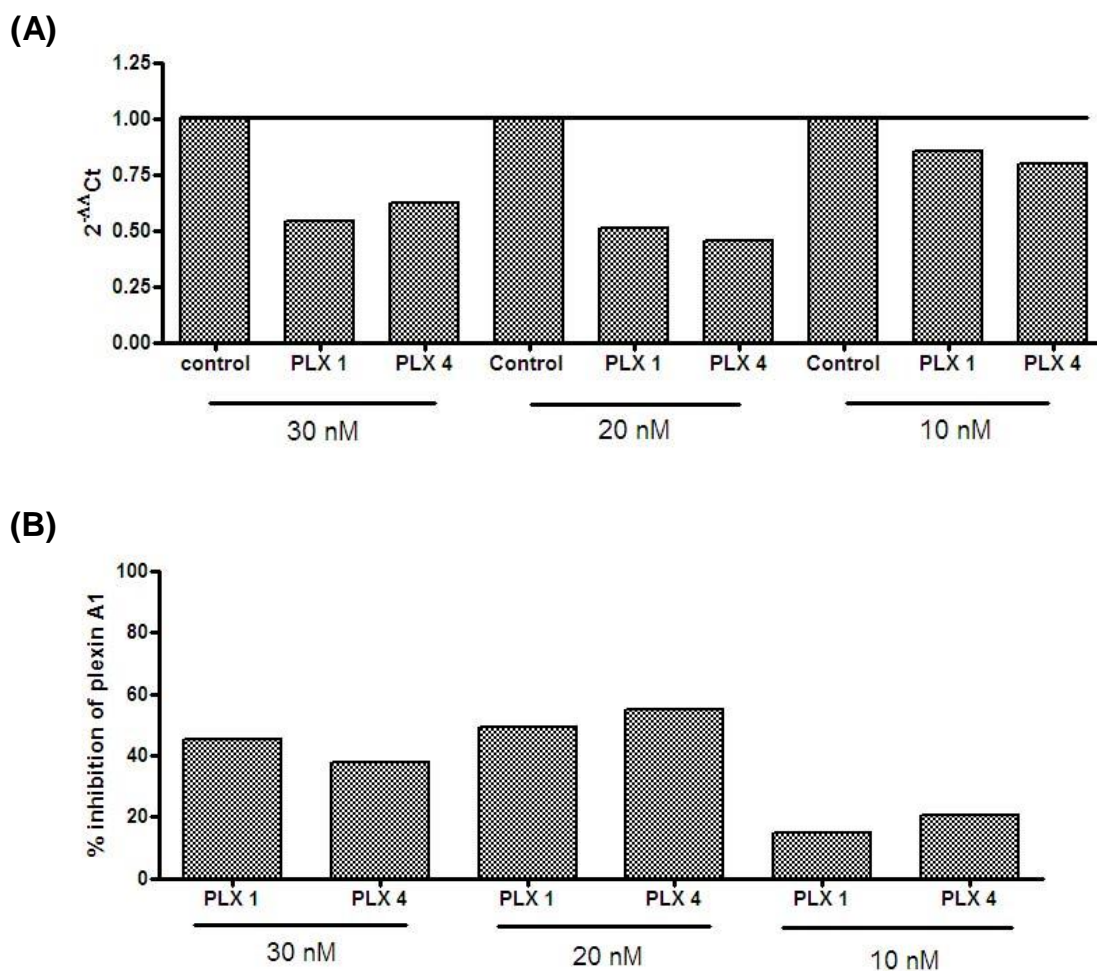


Figure 4.9 Testing PLX 1 and PLX 4 siRNA in macrophages

PLX 1 and PLX 4 siRNA were tested again to ensure that they were decreasing the amount of plexin A1 expression in M-CSF derived macrophages. The relative expression compared to the negative control is shown (A) as well as the percentage inhibition of plexin A1 expression (B).

4.5.4 Effect of plexin A1 knockdown in M-CSF derived macrophages stimulated with LPS or Tck

I next examined the effect of plexin A1 knockdown on macrophage activation. M-CSF derived macrophages were treated with 20 nM PLX 1, PLX 4 and PLX 1 + PLX 4 for 24 hours, the transfection media was then removed and the cells were either left unstimulated or stimulated with 10 ng/ml LPS or Tcks at a ratio of 4:1 for 24 hours. The supernatants were then removed for cytokine analysis and the cells were analysed by qRT-PCR. This experiment was repeated three times and the following results are a representative example of the results achieved.

4.5.4.1 qRT-PCR data

Prior to analysing the TNF ELISA data it was necessary to check that the plexin A1 expression had decreased as a result of treating the cells with siRNA. Figure 4.10 shows the relative expression of plexin A1 in the macrophage samples relative to the negative control. In the unstimulated macrophages which were incubated with media after the siRNA treatment there is a decrease in the amount of plexin A1 being expressed by the cells which were treated with PLX 1 siRNA. The amount of plexin A1 expression in macrophages treated with PLX 4 siRNA is also decreased compared to control but the siRNA treatment producing the highest amount of gene silencing is the combined treatment of PLX 1+4. This same pattern is seen with the macrophages which were stimulated with LPS, where the greatest level of gene silencing is seen when the combination of PLX1+4 were used. The percentage inhibition of plexin A1 in the macrophages treated with PLX 1+4 siRNA was 43% and 55% for the macrophage samples which were subsequently incubated with media or LPS respectively. Despite this being a large percentage inhibition in the context of the experiment it indicates that there is still over 50% or just under 50% of the total amount of plexin A1 being expressed in the macrophages, which will therefore bias the results when trying to determine effect of silencing the plexin A1 gene in macrophages. It also indicates that there is a discrepancy of over 10% in the amount of gene knockdown obtained each time the macrophages are treated with PLX siRNA; this variability will also bias the results of the siRNA experiment.

In Figure 4.10 the results from the macrophages which were subsequently stimulated with Tcks in a cell contact (c.c) indicate that there was very little gene silencing of plexin A1 in these cells since the level of plexin A1 relative expression in the cells treated with siRNA is similar to the level of plexin A1 in the cells treated with the negative control siRNA. This may be due to contaminating Tcks in the sample with the macrophages as although every effort was made to remove the Tcks before putting the cells in trizol some may have remained. T cells have been shown to express plexin A1 [623] and the presence of contaminating Tcks in the macrophage sample could have diluted out the effect of the siRNA gene silencing therefore producing the result of little or no plexin A1 gene knockdown in these samples.

Once the result of 43% and 55% gene knockdown in the macrophage samples had been determined, the pro-inflammatory output from these stimulated macrophages could be analysed.

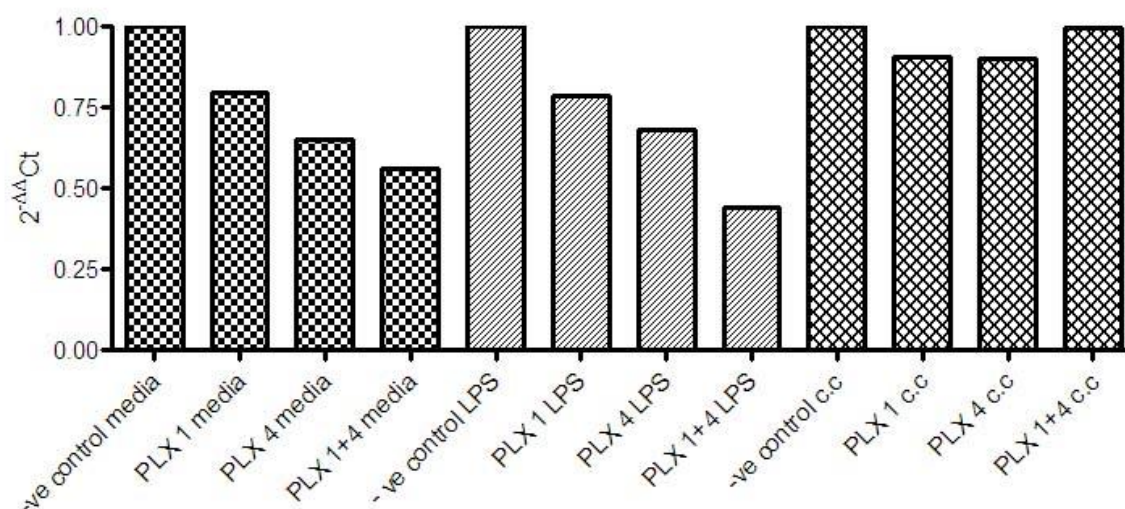


Figure 4.10 RT-PCR of plexin A1 knockdown in activated macrophages

PLX 1, 4 or 1+4 siRNA were used to silence the plexin A1 gene in macrophages that were subsequently stimulated with LPS or Tcks.

4.5.4.2 TNF α ELISA data

To establish whether the plexin A1 knockdown had any effect on the pro-inflammatory output of the macrophages stimulated with LPS or Tcks a TNF α ELISA was performed on the cell supernatants. Figure 4.11 shows the results of the ELISA, while there were small amounts of TNF α produced by the macrophages incubated for 24 hours in media alone the stimulated macrophages both produced elevated quantities of TNF α . The LPS stimulated macrophages produced less TNF α in the samples which had been treated with PLX siRNA compared to the samples which had been treated with the negative control siRNA. The lowest amount of TNF α produced is from the macrophage sample that was treated with PLX 4 which from the previous figure induced 31% inhibition of plexin A1 therefore inhibiting a relatively small percentage of plexin A1 in macrophages may be enough to change its pro-inflammatory profile. This decrease in TNF α using PLX 4 siRNA compared to the negative control sample is statistically significant using a 1 way ANOVA and a Turkey's multiple comparison test.

The Tck cell contact activated macrophages do not appear to have changed their proinflammatory output as a result of siRNA silencing of plexin A1 since the amount of TNF produced by the cells treated with PLX siRNA is not significantly different to the TNF production from the negative control treated macrophages. This could indicate that plexin A1 may not be involved in the cellular cross talk between macrophages and T cells however plexin A1 was not fully silenced with the PLX siRNA and it is possible that in order to elucidate the role plexin A1 plays in the cellular contact between macrophages and T cells the plexin A1 gene must be fully knocked down.

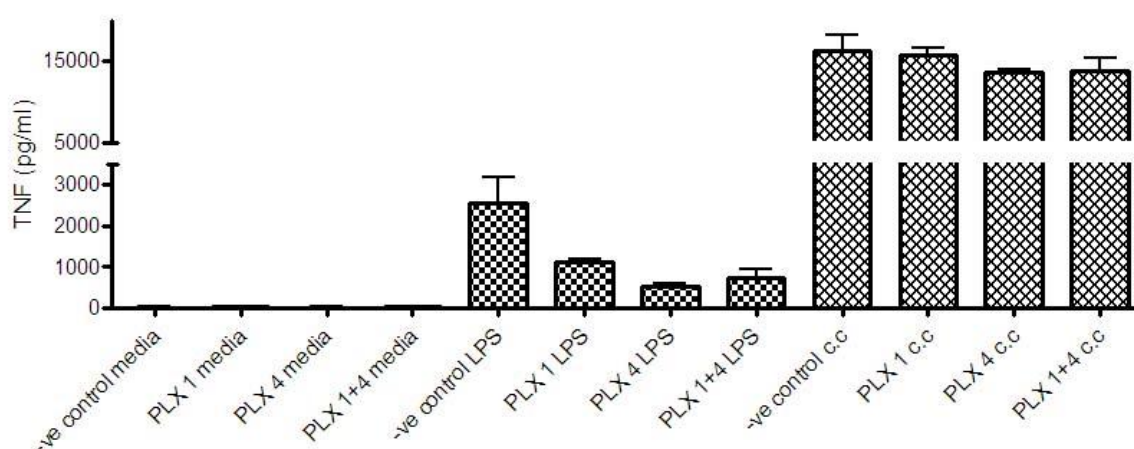


Figure 4.11 TNF α ELISA of siRNA treated macrophages

A TNF α ELISA was performed on macrophages that had been treated with various siRNAs before being incubated with media alone, 10 ng/ml LPS or Tcks (c.c). Error bars are mean \pm SEM. This is a representative of three individual experiments. Using a 1 way ANOVA with Tukey's multiple comparison test on the LPS treated group there is a statistically significant difference between the -ve control LPS group and the PLX 4 LPS group in addition to a statistically significant difference between -ve control LPS and PLX1+4 LPS group ($p < 0.05$).

4.6 Investigation into the expression of the Plexin A1 ligand semaphorin 6D on T cells

Plexin A1 is known to have several semaphorin ligands including semaphorin 6D. In the context of the immune system semaphorin 6D is the most well studied plexin A1 ligand to date and several studies have indicated that plexin A1 expressed on DCs may interact with T cells expressing semaphorin 6D [623]. A recent study investigated the regulation of semaphorin 6D expression on T cells and found that *in vitro* activation of murine CD4⁺ T cells using plate-bound anti-CD3 and anti-CD28 antibody increased mRNA levels of sema 6D by 24 hours and

after 5 days induced 71% surface expression of sema 6D compared to control during the late phase of activation [645].

Since plexin A1 is upregulated in RA and PsA synovial fluid CD14⁺ cells and macrophages are known to interact with T cells in the arthritic joint [408] we hypothesised that semaphorin 6D, one of plexin A1's specific ligands, is expressed on arthritic T cells and is one of the receptor-ligand interactions taking place within the synovium of diseased patients. It was decided to first test for the presence of sema 6D on cytokine activated T cells (Tcks) since these are an *in vitro* model of activated synovial T cells [298, 309]. As a positive control human T cells were also activated with anti-CD3 and anti-CD28 antibodies as a previous study had shown 71% of murine cells stimulated in this manner were found to express semaphorin 6D after 5 days [645].

4.6.1 Semaphorin 6D FACS analysis

T cells were isolated from normal donors and stimulated for 6 days with a cytokine cocktail to produce Tcks or CD3/CD28 microbeads to produce activated T cells. T cells were analysed for semaphorin 6D expression at day 0 (unactivated T cells), day 3 and day 6. Since the semaphorin 6D antibody was unconjugated a secondary APC antibody was incubated with the cells to detect whether sema 6D had bound to the cells. As a control, an irrelevant antibody (CD68) that should not have bound to the T cells was incubated with the cells before adding the secondary antibody in order to detect any non-specific staining of the secondary antibody.

4.6.1.1 Semaphorin 6D expression on CD3/CD28 stimulated T cells

Figure 4.12 demonstrates representative results of the FACS staining for the T cells stimulated for 0, 3 and 6 day with anti-CD3, anti-CD28 microbeads. Isotype control is shown as the grey histogram, semaphorin 6D surface and intracellular staining is shown in blue on the left hand histograms. Irrelevant antibody staining is shown in red on the right hand panel of histograms.

Unstimulated T cells on day 0 appear to have approximately 3.5% semaphorin 6D surface staining however upon comparison to the irrelevant antibody, which has

3.16% positive staining, it appears that the majority of the staining seen at day 0 is non-specific secondary antibody staining. The same pattern is seen at day 3 where there is 3.5% positive surface staining from the irrelevant antibody which indicates that the 4.39% positive semaphorin 6D staining is unlikely to be a valid observation. The same observation is seen within the day 6 activated T cells.. The intracellular staining at day 6 is also negative for both semaphorin 6D and irrelevant antibody. However, the intracellular staining in the T cells at day 3 appears to show a small increase in semaphorin 6D expression compared to isotype control as 9% of cells are positive for semaphorin 6D. The irrelevant antibody staining at this timepoint is 1.9% indicating that the semaphorin 6D expression could be valid observation.

Consequently, day 3 intracellular staining is the only timepoint which shows expression of semaphorin 6D, the intracellular levels are negative at day 6 and there is no surface expression of semaphorin 6D at any of the three timepoints examined. These observations conflict with the results shown by O'Connor et al [645] who demonstrated that over 70% of murine CD4⁺ T cells express semaphorin 6D on the surface after 5 days of stimulation with anti-CD3 and anti-CD28 antibodies.

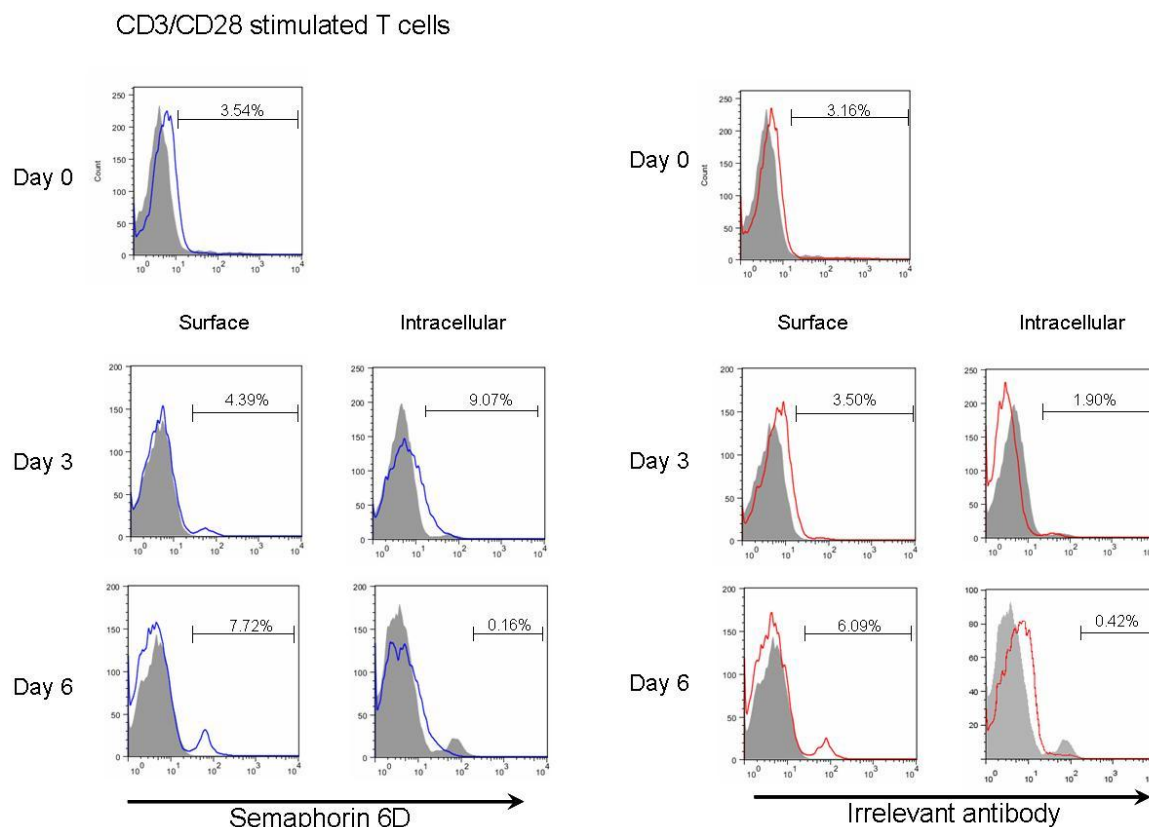


Figure 4.12 Surface and intracellular semaphorin 6D staining of CD3/CD28 stimulated T cells
T cells were analysed at day 0, day 3 and day 6 after stimulation for surface and intracellular staining of semaphorin 6D (blue lines). The semaphorin 6D was not a directly conjugated FACS antibody therefore a secondary APC conjugated antibody was incubated with the cells after semaphorin 6D staining to detect the presence of the surface ligand. As a control an irrelevant antibody (CD68) was incubated with the cells before adding the secondary antibody in order to check for non-specific staining of the secondary antibody (red lines). Isotype control is shown as the grey histograms. The surface staining is a representative of n=6 experiments and the intracellular staining is a representative of n=2 experiments.

4.6.1.2 Semaphorin 6D expression in cytokine stimulated T cell (Tcks)

Tcks were also analysed for semaphorin 6D expression after 0, 3 and 6 days of activation (Figure 4.13). Semaphorin 6D staining is shown by the blue lines on the left hand panel of histograms and irrelevant antibody staining is shown by the red lines on the right hand panel of histograms. Since the same unactivated T cells were used as a control for both CD3/CD28 and Tck activated T cells before the cells were activated with either stimulus, day 0 T cells show the same results as in the previous figure. There is no surface semaphorin 6D expression due to a similar percentage of positive staining for the irrelevant antibody, indicating that the secondary antibody is binding non-specifically. There was no induction of surface semaphorin 6D expression after 3 and 6 days of cytokine activation (2.1% and 2.5% positive respectively) considering that the

secondary antibody was also positive (0.68% and 1.7% respectively) indicating non-specific staining. There was also no intracellular semaphorin 6D expression at day 6 since the percentage of positive cells for semaphorin 6D and irrelevant antibody were almost identical (3.93% and 3.75%). However, as was seen with the CD3/CD28 activated T cells the intracellular expression of semaphorin 6D in Tcks was increased at day 3, 17.1% of Tcks were positive for semaphorin 6D. The irrelevant antibody had a considerably lower expression at this timepoint (3.97%) suggesting that the semaphorin 6D staining was specific.

As was seen with the CD3/CD28 activated T cells semaphorin 6D is only expressed in Tcks intracellularly after 3 days of activation but is not expressed on the surface of the cells during the 6 days of activation.

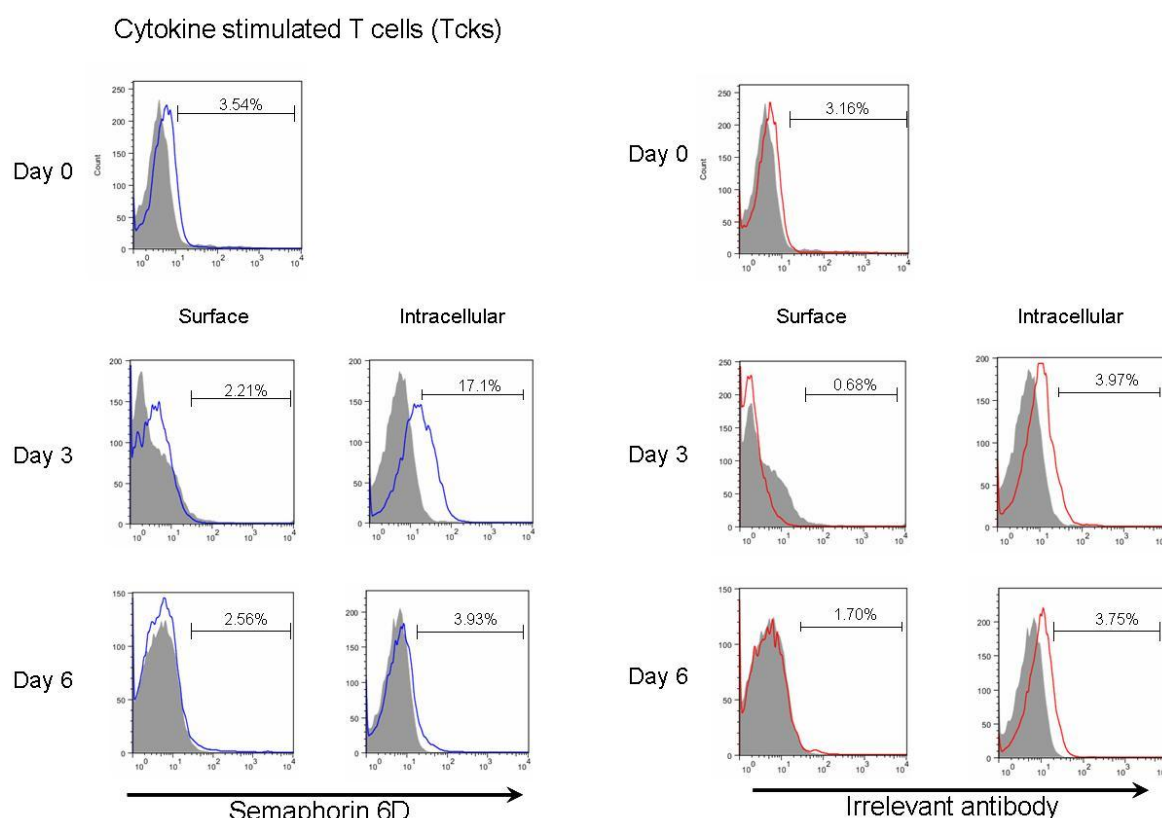


Figure 4.13 Surface and intracellular semaphorin 6D staining of Tcks

T cells were analysed at day 0, 3 and 6 after cytokine stimulation for surface and intracellular expression of semaphorin 6D (blue lines). Since the semaphorin 6D was not a conjugated FACS antibody a secondary APC conjugated antibody was incubated with the cells after semaphorin 6D staining to detect the presence of the ligand. As a control an irrelevant antibody (CD68) was incubated with the cells before adding the secondary antibody in order to check for non-specific staining of the secondary antibody (red lines). Isotype control is shown as the grey histograms. The surface staining is a representative of n=6 experiments and the intracellular staining is a representative of n=2 experiments.

4.6.2 Investigation into the level of semaphorin 6D mRNA in T cell samples

Due to problems with repeated non-specific staining of the secondary antibody during the FACS analysis we therefore went on to analyse the levels of semaphorin 6D mRNA in cytokine- and CD3/CD28-activated T cells. A PCR was set up with these samples and no detectable bands could be observed (data not shown) therefore to verify this result the samples were analysed by qRT-PCR.

4.6.2.1 qRT-PCR of T cell samples

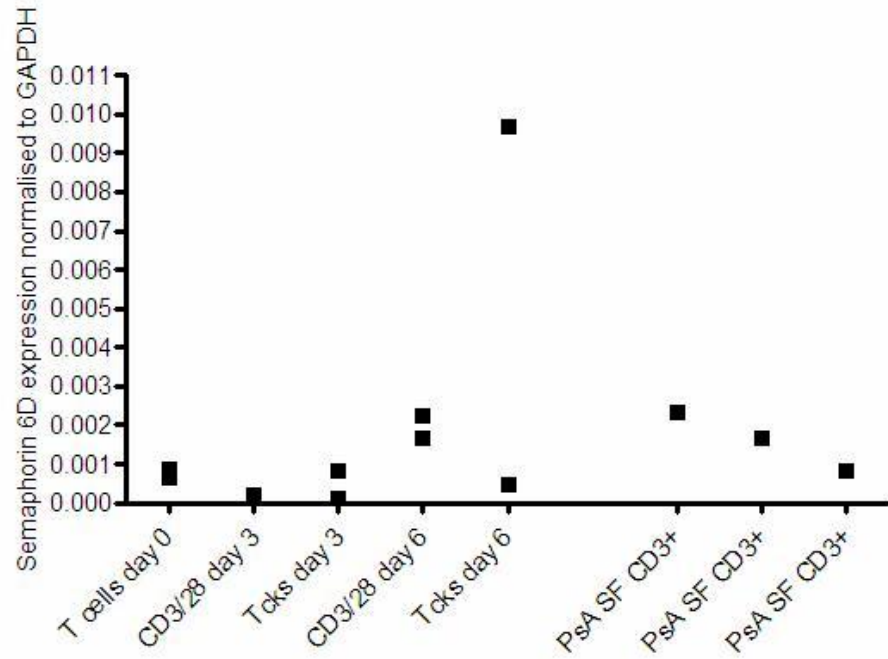
T cells were isolated from 2 donors and activated with cytokines or CD3/CD28 for 6 days. CD3⁺ cells were also isolated from 3 PsA synovial fluids. The relative expression of semaphorin 6D compared to the GAPDH of each sample is shown in graph (A) of Figure 4.14. All Tck and CD3/CD28 samples have very low relative expression values indicating that semaphorin 6D is almost certainly not being expressed. The Tck day 6 sample of one of the donors has a much higher relative expression compared to all of the other samples which could suggest semaphorin 6D mRNA is being expressed. The three PsA synovial fluid CD3⁺ samples have varying levels of semaphorin 6D relative expression but the levels are still relatively low, again indicating that semaphorin 6D is unlikely to be expressed in these cells.

The fold change of semaphorin 6D expression in the Tck and CD3/CD28 activated cells can be determined by comparing the semaphorin 6D expression of the activated samples to that of the corresponding inactivated day 0 T cell sample. Graph (B) of Figure 4.14 illustrates the fold change in semaphorin 6D in the activated T cell samples compared to inactivated T cells. The Tcks and CD3/CD28 T cells activated after 3 days in both donors demonstrated a decrease in the amount of semaphorin 6D compared to inactivated T cells. The day 6 Tcks and CD3/CD28 T cells had a decrease in the amount of semaphorin 6D (fold change of -141 and -28 respectively) in donor 1 and a slight increase in donor 2 (fold change 11 and 1.9 respectively).

The combined FACS and qRT-PCR data suggest that in the activated T cells there are donor specific variances in the mRNA level of semaphorin 6D, however these expression levels are very low and on the whole demonstrate a decrease in the

amount of semaphorin 6D mRNA compared to inactivated T cells. The levels of semaphorin 6D expression within CD3⁺ cells from PsA synovial fluid are also very low. Semaphorin 6D protein appears to be transiently expressed within the T cell during activation. Plexin A1 is a receptor for semaphorin 6D and can only form an interaction if its ligand is present on the cell surface. During the FACS analysis surface semaphorin 6D could not be detected in the Tck samples that have been previously demonstrated to be an *in vitro* model of synovial T cells [298, 309]. This data combined with the very low levels of semaphorin 6D mRNA in the CD3⁺ synovial fluid samples suggests that T cells within the arthritic joint may not be the source of plexin A1's ligand semaphorin 6D.

(A)



(B)

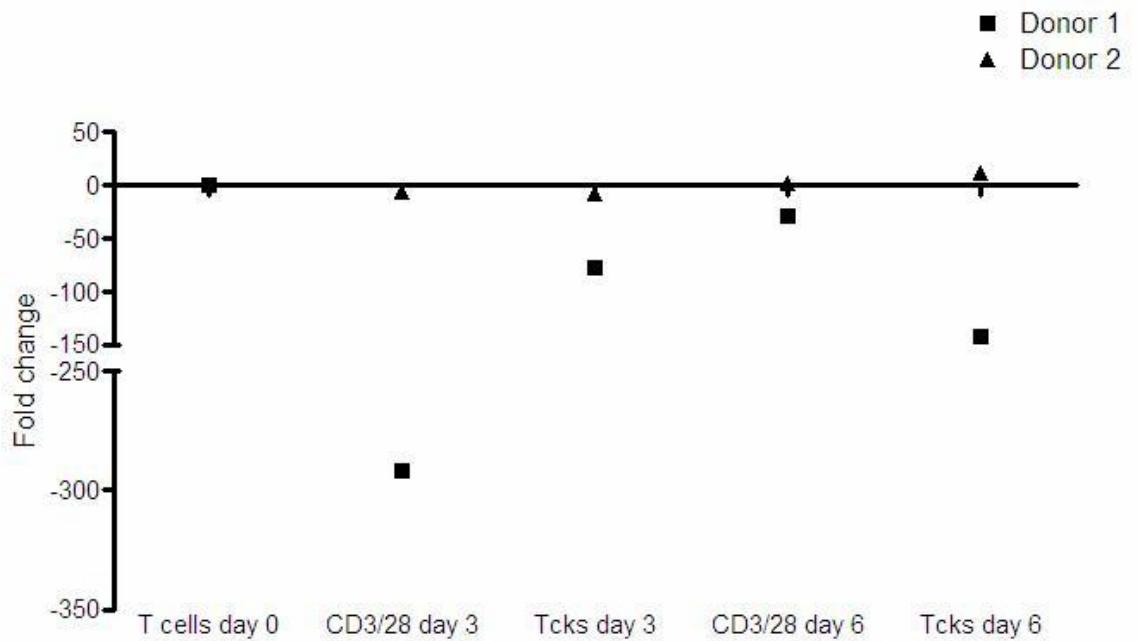


Figure 4.14 qRT-PCR of semaphorin 6D expression in T cell samples and patient CD3⁺ samples

T cells from two donors were stimulated for 3 and 6 days with cytokines (Tcks) or CD3/CD28 microbeads. These activated T cells and inactivated T cell were then analysed for semaphorin 6D expression by qRT-PCR. T cells were also isolated from 3 PsA synovial fluids and analysed for semaphorin 6D. Graph (A) shows the relative expression of semaphorin 6D compared to the GAPDH levels in each sample. Graph (B) shows the fold change in expression in the Tck or CD3/CD28 activated T cells compared to the inactivated T cells (T cells d=0).

4.7 Investigation into the expression of other plexin A1 ligands semaphorin 3A, 3F, 6D on RA synovial membrane

The previous results suggested that semaphorin 6D is unlikely to be expressed on the T cells in the arthritic joint therefore it was decided to analyse the synovial membrane of RA patients for semaphorin 6D. If the membrane was positive for semaphorin 6D expression, this would imply that plexin A1's ligand is being expressed by other cell types rather than T cells.

Plexin A1 is also known to interact with class 3 semaphorins namely semaphorin 3A and semaphorin 3F [617]. As mentioned in the introduction class 3 semaphorins will only bind plexin A1 if neuropilin is present as a co-receptor, semaphorin 3A will specifically bind neuropilin-1 (NRP-1) and semaphorin 3F will bind NRP-1 and NRP-2 [443]. Analysing the microarray data (presented in chapter 4) for genes that are upregulated in addition to plexin A1 in the RA and PsA SF CD14⁺ cells reveals that these cells have upregulated NRP-1 with a fold change increase of 40 in PsA SF and 11 in RA SF and NRP-2 with a fold change increase of 24 in PsA SF and 31 in RA SF. Since these cells had upregulated plexin A1 and the two co-receptors needed for semaphorin 3A and 3F signalling it was possible that plexin A1 could be binding to either of these ligands hence the presence of semaphorin 3A and 3F were also analysed in the RA synovial membrane.

4.7.1 qRT-PCR on RA synovial membrane

SYBR green analysis was performed on 10 RA synovial membranes to investigate the presence of three plexin A1 ligands. The results are shown in Figure 4.15 and indicate that all three semaphorins are expressed in the RA membranes however semaphorin 3A has the greatest relative expression in the samples compared to its housekeeping gene. Semaphorin 6D is also expressed in the RA membranes indicating that although we could not detect it on T cells it may be present on other cell types within the membrane. Consequently the level of these semaphorins on another cell type known to be present in the RA synovium was investigated.

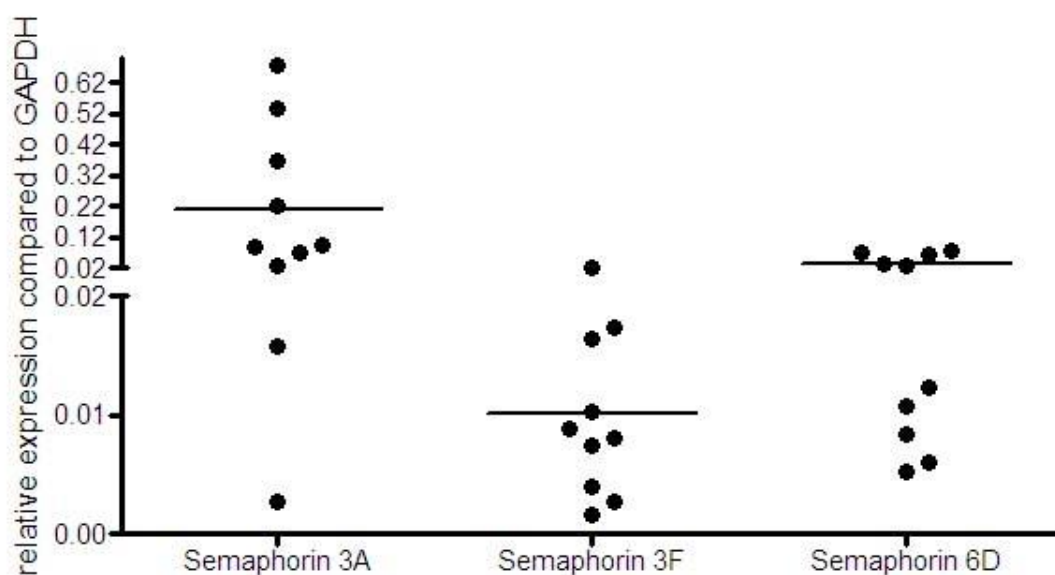


Figure 4.15 Semaphorin 3A, 3F, 6D expression in RA synovial membrane

qRT-PCR was used to determine the relative expression of 3 of plexin A1's ligands in 10 RA synovial membranes. Each dot represents one patient sample and the line indicates the mean expression.

4.7.2 SYBR green of RA synovial fibroblasts

Given that it had been determined that three of the plexin A1 ligands were expressed in the RA synovial membrane it was hypothesised that fibroblasts may be a cell type expressing these semaphorins since macrophages and fibroblasts are known to interact in the inflamed synovial joint [646]. Consequently, the relative expression of semaphorin 3A, 3F and 6D compared to GAPDH was determined for RA synovial fibroblasts from 5 patients. Figure 4.16 outlines the results and reveals that semaphorin 3F was the most highly expressed semaphorin and all five samples expressed it at a similar level. Semaphorin 3A was also expressed but the levels varied between samples with 3 samples having a high level of expression comparable to semaphorin 3F samples whereas the remaining 2 samples having a low level of expression. The five RA fibroblast samples expressed semaphorin 6D but at a lower level compared to the other semaphorin's examined. The overall expression of the three semaphorin molecules is very low with all of the relative expression values being lower than 0.02. Further analysis with more samples would need to be performed to determine whether synovial fibroblasts could be expressing the plexin A1 ligands.

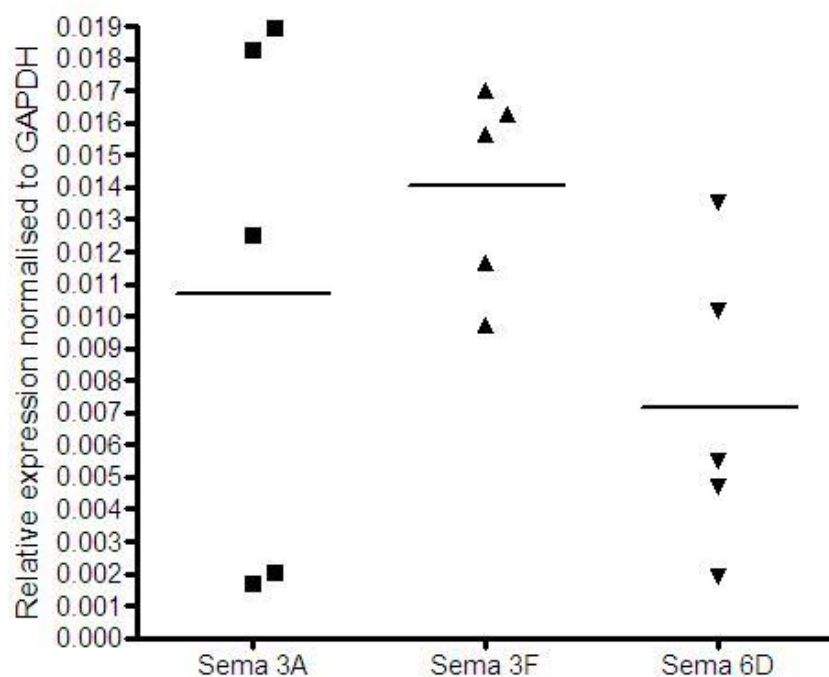


Figure 4.16 Semaphorin 3A, 3F and 6D expression in RA synovial fibroblasts

Synovial fibroblasts from 5 RA patients were analysed for semaphorin (sema) 3A, 3F and 6D. qRT-PCR was used to determine the relative expression of the semaphorins which were then normalised to GAPDH levels of each sample. The line represents the mean value.

4.8 Discussion

Plexin A1 is a semaphorin receptor known to participate in axonal guidance [635], chick cardiac development [631], bone homeostasis [623], T cell-DC interactions [444, 623] and DC trafficking to the lymph node [632]. Plexin A1 had been previously shown to be expressed on human M-CSF differentiated macrophages [443] but has not been shown to be present or play a role in PsA or RA. After discovering plexin A1 to be a highly upregulated gene in RA and PsA CD14⁺ cells, showing a fold change increase of 26 and 27 respectively, we were interested in its previously identified role in T cell-DC interactions. In this setting plexin A1 is expressed on the DCs and is thought to act as a co-stimulatory marker for a T cell ligand because plexin A1^{-/-} DCs have a reduced ability to stimulate T cells [623] and plexin A1 has been shown to be localised at the T cell-DC interface [640]. Since macrophages have been demonstrated to interact with many cellular subtypes within the synovium of arthritis patients [249, 408, 646] we hypothesized that the increased levels of plexin A1 on synovial fluid CD14⁺ cells may play a role in enhancing cellular interactions within the diseased joint. In order to investigate this hypothesis we needed to demonstrate that plexin A1 was present within the synovial fluid cells and the synovium of arthritis patients, determine which plexin A1 ligands were present within the joint and investigate the effects of silencing plexin A1 on the pro-inflammatory cytokine level obtained from activated macrophages.

Plexin A1 was expressed at the mRNA level in all of the CD14⁺ cells isolated from the synovial fluid of RA and PsA examined which included the samples that had been analysed on the microarray and other patient samples. Plexin A1 mRNA was also detected in the CD14⁺ synovial fluid cells of a JIA, an inflammatory polyarthritis and an OA patient indicating that this cell surface molecule may also be playing a role in other arthritic diseases, although more patients would have to be analysed to verify this. In the patients where a matched synovial fluid and blood sample were available the mRNA level of plexin A1 was consistently increased in the synovial fluid CD14⁺ cells compared to the blood CD14⁺ cells verifying the microarray results and suggesting the pro-inflammatory environment within the diseased joint causes the CD14⁺ cells to upregulate plexin A1 once they exit the blood and enter the synovium.

We observed the presence of plexin A1 in five RA synovial membranes where it was mainly located in cells outlining vascular structures, possibly indicative of endothelial cells, and within cells surrounding the vasculature structures which could represent cells infiltrating into the membrane via the blood vessels. However, due to a lack of PsA synovial membrane this disease was unable to be analysed for plexin A1 staining.

Plexin A1 is a receptor molecule for semaphorin ligands and previous work has indicated that the specific ligands which interact with plexin A1 are semaphorin 3A, 3F and 6D [624]. The main focus of research involving plexin A1's role in the immune response has been its interaction with semaphorin 6D on T cells [623, 640]. O'Connor et al demonstrated that CD3/CD28 activation of murine CD4⁺ cells induced the surface expression of semaphorin 6D [645] therefore we investigated whether an *in vitro* system of cytokine activation previously shown to induce a T cell phenotype similar to that of synovial T cells [298, 309] could induce the expression of semaphorin 6D. The interaction of macrophages and T cells in the arthritic joint has been well documented [314, 408] therefore a plexin A1 - semaphorin 6D receptor ligand complex could be one of the interactions taking place. We observed no surface semaphorin 6D expression on the cytokine activated or CD3/CD28 activated T cells and no mRNA expression of semaphorin 6D in these cells or synovial fluid CD3⁺ T cells. The lack of surface semaphorin 6D on CD3/CD28 activated T cells conflicted with the results of the O'Connor paper which demonstrated CD3/CD28 antibody stimulation induced semaphorin 6D expression in 70% of T cells after 5 day [645]. However, this discrepancy may be explained by a species specific response as the O'Connor paper analysed murine T cells whereas we investigated semaphorin 6D in human T cells.

Despite semaphorin 6D being undetected in cytokine activated and synovial T cells there was a possibility that semaphorin 6D was being expressed in the synovium on other cell types. There was also the prospect of the macrophage plexin A1 interacting with one of its other ligands in the synovium such as semaphorin 3A or 3F. However, both of these semaphorins only interact with plexin A1 in the presence of neuropilin-1 or -2 acting as a co-receptor [647]. Analysis of the microarray study data indicated that compared to blood CD14⁺ cells RA and PsA SF CD14⁺ had increased levels of neuropilin-1 and -2 as well as

plexin A1 indicating that binding of semaphorin 3A or 3F would be possible in these synovial macrophages. We observed that 10 RA synovial membranes expressed semaphorin 3A mRNA at a higher level than that of semaphorin 3F or 6D and so it appeared that semaphorin 3A could be the main receptor for plexin A1 in the synovial compartment. To try and elucidate which cell type expressed the plexin A1 ligands we investigated five RA synovial fibroblasts samples since synovial macrophages have been demonstrated to interact with synovial fibroblasts [249]. In these cells semaphorin 3F mRNA was expressed at a higher level compared to semaphorin 3A and 6D. However, the relative expression of these semaphorins were very low and further studies to analyse protein levels of expression should be conducted before a definitive conclusion can be made about the presence of semaphorins on synovial cell types.

Despite being unable to define which cells were expressing the plexin A1 ligands we had shown that these ligands were present in the synovium of RA patients and therefore could hypothesize that the upregulation of plexin A1 in synovial macrophages was significant and may be playing a role in the progression of disease in RA and PsA. To try and elucidate the function of plexin A1, small interfering (siRNA) was used to silence the plexin A1 gene in M-CSF derived macrophages which had been shown to upregulate plexin A1 mRNA during their differentiation. The transfection efficiency was over 90% indicating that the siRNA was effectively entering the cells using the nanoparticle transfection system. Unexpectedly, two of the four siRNA induced an increase in plexin A1 expression rather than knocking it down. siRNA's are known to have off-target effects as a result of a lack of specificity and can therefore silence other genes within the cell if the siRNA imperfectly pairs with sequence motifs residing in the 3' UTR of cellular mRNA [648]. There has been one report of siRNA treatment resulting in the upregulation of mRNA within cells. This was thought to be due to competition between the transfected siRNA and the cell's own endogenous miRNA. Hence the mRNAs targeted by the endogenous miRNA are not degraded and the cell demonstrates an upregulation of these mRNA's [649]. However, despite this report of upregulation of mRNA levels occurring as a result of siRNA treatment there has been no report of upregulation of the siRNA target gene which is what occurred during the plexin A1 knockdown experiments. The reason for the observed upregulation of plexin A1 mRNA is unknown but could

have been due to badly designed siRNA which did not target the plexin A1 mRNA for degradation. As a result the two PLX siRNA which demonstrated increased plexin A1 expression were not used in further experiments.

The TNF α cytokine profile of siRNA treated macrophages subsequently stimulated with 10 ng/ml of LPS or cell contact activated with Tcks for 24 hours were analysed. It was thought that these two treatments may mimic the pro-inflammatory environment to which synovial macrophages may be exposed as TLR signalling may play a role in arthritis [650] and cellular contact with activated T cells is known to take place within the joint.

In the experiment shown, treating macrophages with 20 nM PLX 1+PLX 4 resulted in a 43% inhibition of plexin A1 in the cells which were subsequently cultured with media alone and 55% inhibition in the macrophages which were subsequently stimulated with LPS. This discrepancy of over 10% in the amount gene silencing demonstrated the large variability of the siRNA treatment which was seen throughout all three siRNA experiments. The small amount of gene silencing also indicated that potentially there could be as much as 50% of the total amount of plexin A1 mRNA still present in the macrophage cells before activation which could therefore bias the results of the cytokine analysis. In the siRNA treated macrophages which were activated with Tcks there appeared to be little to no gene silencing with the maximal amount of inhibition being 10% with PLX 4 treatment. This observation could be the result of contaminating Tcks remaining in the culture wells as T cells have been shown to express plexin A1 [623]. Consequently, contaminating T cells in the macrophage sample may have diluted out any effect of the siRNA treatment which resulted in the appearance of no plexin A1 knockdown. Due to this discrepancy we must assume that the level of gene silencing in the Tck-stimulated was at a similar level to the gene silencing seen in the other macrophage samples i.e. around 43 - 55% inhibition.

The subsequent TNF α analysis of the macrophage samples demonstrated that the cytokine level did not change in the macrophages incubated in media alone, indicating that the siRNA treatment did not adversely activate the macrophages. The LPS stimulated macrophages produced less TNF α in the samples which had been treated with siRNA compared to the negative control. The sample producing the lowest amount of TNF α was the sample which had a 31%

knockdown of the plexin A1 gene suggesting that even a small decrease in the amount of plexin A1 may be enough to change the pro-inflammatory cytokine profile of the macrophage. This result would imply that plexin A1 is playing a pro-inflammatory role within the macrophage as a decrease in plexin A1 expression causes less TNF α to be released by the cell as a result of TLR4 stimulation. This observation is in contrast to that of Ji et al [443] who previously demonstrated that LPS stimulation of human monocytes alongside M-CSF stimulation for 24 hours resulted in the inhibition of plexin A1 mRNA expression. The inconsistency could be due to the stage of monocyte activation as the Ji et al paper investigated day 0 monocytes which were M-CSF and LPS stimulated at the same time for 24 hours while our investigation analysed the effect of plexin A1 silencing in macrophages that had been treated with M-CSF for 6 days and therefore would have a more activated phenotype. It must also be noted that the plexin A1 inhibition in the macrophages of this experiment was 55% therefore there is still 45% of the total plexin A1 mRNA level being expressed within these cells. Subsequently, it is difficult to draw definitive conclusions about the role of plexin A1 with regards to TLR-4 stimulation until complete knockdown of the plexin A1 gene is established.

The TNF α levels measured in the siRNA treated macrophage activated with Tcks did not appear to have changed significantly in comparison to siRNA negative control macrophages. This result could indicate plexin A1 on the macrophages is not involved in the cellular cross talk which occurs between Tcks and macrophages since its knockdown did not affect the pro-inflammatory output of the macrophages. This conclusion is supported by our finding that semaphorin 6D, one of plexin A1's most studied ligands, is not present on Tcks or synovial CD3⁺ cells at the surface protein or mRNA level. However, as with the siRNA-treated LPS stimulated macrophages it is difficult to draw conclusions about the role of plexin A1 within the macrophage - Tck cell contact when the plexin A1 gene is incompletely silenced. The true function of plexin A1 may only become apparent once macrophage cells with 100% knockdown of plexin A1 are studied.

In conclusion, the plexin A1 investigation has demonstrated that plexin A1 mRNA is highly upregulated in synovial fluid macrophage cells of PsA and RA and is also upregulated in synovial CD14⁺ cells of other arthritic conditions such as OA, JIA and inflammatory polyarthritis. It is present within the synovial membrane of all

5 RA patients examined although double staining is necessary to determine if the plexin A1 positive cells are predominantly macrophages. One of plexin A1's ligands semaphorin 6D could not be measured on cytokine activated T cells (Tcks) or CD3/CD28 activated T cells as had been previously described [645] and its mRNA levels could not be determined in the above cell types or synovial fluid CD3⁺ cells suggesting that macrophage plexin A1 was not interacting with T cells in the synovium. However, another of plexin A1's ligands, semaphorin 3A, was found to be highly expressed at the mRNA level in 10 RA synovial membranes indicating that this could be plexin A1's main ligand within the arthritic joint. At present the cell type expressing this semaphorin has not been discovered and analysis of RA synovial fibroblasts indicated that they expressed low levels of semaphorin. Investigation into the function of plexin A1 using siRNA has hinted at a possible pro-inflammatory role in macrophages as LPS induced TNF α secretion decreased in siRNA treated cells. However further investigations are needed to fully confirm this observation.

Chapter 5 Investigation into the expression and function of the novel gene legumain in rheumatoid and psoriatic arthritis patients

5.1 Introduction

The second ‘novel articular’ gene to be discovered during the analysis of the upregulated genes in RA and PsA SF CD14⁺ cells was legumain. Legumain, otherwise known as asparagine endopeptidase (AEP), is a lysosomal cysteine protease that specifically cleaves protein substrates on the C-terminal side of asparagine [651]. The mammalian form was cloned and sequenced in 1997 [651] after being described in leguminous plants [652] and the blood fluke *Schistosoma mansoni* [653]. There are four families of cysteine endopeptidases found in mammals, the most numerous of those belong to the papain family (C1), which are predominantly lysosomal enzymes and include cathepsin S, L, F, B, K and C [654]. These enzymes are responsible for proteolysis in the lysosomal/endosomal system and can also be secreted to act extracellularly. The other families of cysteine endopeptidases consist of calpain (family C2), caspase and legumain (family C13) [655].

Legumain is produced as an inactive zymogen that requires proteolytic cleavage to gain activity. Legumain activates itself autocatalytically which requires the sequential cleavage of a C-terminal 110-residue propeptide and then a shorter N-terminal 8-residue propeptide, this activation is pH dependent occurring optimally between pH 4.0-4.5 [656]. Initial studies of legumain found it to have the highest activity in the kidney of rat, rabbit and pig [651]. However, further studies of mouse legumain found it be expressed in all samples examined including placenta, spleen, kidney, heart, lung, liver, testis and thymus but the active legumain was particularly abundant in the kidney and placenta [657]. Legumain deficient mice have enlarged proximal renal tubule cells suggesting that material to be degraded is accumulating in the lysosomes; previous hypotheses proposed that legumain has a role in the biosynthesis of lysosomal enzymes [432]. Legumain has since been shown to be ubiquitously expressed in a large panel of human tissue at mRNA level [435] and is constitutively expressed in most cells types including macrophages and DCs [658].

Legumain is known to cleave and activate several substrates such as MMP-2 [429]; fibronectin [431]; α -thymosin [430]; cathepsin B, H and L [432]; TLR9 in DCs [658] and it also processes microbial tetanus toxin antigen [659]. Due to this wide variety of substrates, the functional role of legumain is cell and tissue

context dependent. Manoury et al [659] first described a role for legumain as the enzyme which processes microbial tetanus toxin antigen (TTCF) for antigen presentation; legumain was isolated from disrupted lysosomes of the human B cell line EDR and exposed to TTCF *in vitro*. Peptide inhibitors to legumain slowed TTCF presentation to T cells while pre-processing of the peptide with legumain accelerated its presentation. The same group later showed that T cell responses to TTCF were still mounted in legumain-deficient mice but the *in vivo* kinetics of TTCF presentation was slower. Boosting legumain levels in the primary APCs accelerated TTCF processing and presentation *in vitro* indicating that legumain is the most efficient protease for TTCF processing but in its absence other enzymes can substitute to enable a slower immune response [660]. Recently, Sepulveda et al [658] showed that legumain plays a role in the processing of TLR9 in DCs as legumain deficiency resulted in decreased secretion of proinflammatory cytokines in response to umCpG DNA. They also showed that transfection of TLR9 deficient DCs with a mutant TLR9 lacking a legumain cleavage site did not restore TLR9 signalling, suggesting that TLR9 is a direct substrate for legumain.

As well as its roles in antigen processing and TLR9 cleavage, legumain has also been shown to directly degrade fibronectin *in vitro* and in cultured mouse renal proximal tubular cells [431]. The processing of fibronectin was abrogated by chloroquine, an inhibitor of lysosomal degradation, and was enhanced by the overexpression of legumain revealing that fibronectin degradation occurs in the presence of legumain in the lysosomes of renal proximal tubular cells. Similarly, legumain-deficient mice exhibit renal injury induced protein accumulation of fibronectin and enhanced renal interstitial fibrosis which led to the hypothesis that renal proximal tubular cells take up and degrade fibronectin intracellularly thereby linking legumain to extracellular matrix remodelling. Legumain may also play an indirect role in matrix remodelling as it can cleave pro-MMP-2 into active MMP-2 [429]. MMP-2 is known to degrade the extracellular matrix due its wide variety of substrates including fibronectin, several MMPs and collagen types [661].

Despite playing an important role in extracellular matrix turnover and antigen processing legumain is also linked to various pathological conditions such as cancer, atherosclerosis and skin barrier function. Legumain was first

demonstrated to be involved in cancer when Liu et al showed that it was overexpressed in a panel of human solid tumours including breast, colon, lung, prostate and ovarian carcinoma [662]. The same study used fluorescent microscopy in tumour cells to show legumain expression associated with intracellular membranous vesicles, consistent with its function as a lysosomal protease, as well as on the cell surface and in membranous vesicles concentrated at the invadopodia of the tumour cell. HEK 293 cells stably expressing legumain were found to have increased migration and invasion *in vitro* and legumain expression was correlated with tumour invasion and metastasis *in vivo* [662]. Legumain was also found to be expressed specifically in tissue sections of a mouse tumour line CT26 where it was detected in vascular endothelial cells, macrophages and in the extracellular matrix associated with collagen I [663]. Cystatin E/M is a cysteine protease inhibitor and its relationship with legumain was investigated in human melanoma, legumain was expressed and active in most of the cell lines but was absent or at low levels in cell lines secreting cystatin E/M. When melanoma lines lacking cystatin E/M secretion were transfected with a plasmid containing full length cystatin E/M their intracellular legumain activity was inhibited, suggesting that it regulates legumain activity and hence its invasive potential [664].

Legumain was first associated with atherosclerosis when a microarray study comparing stable and unstable atherosclerotic plaques found legumain to be one of the genes upregulated in the unstable areas of plaques [434]. The same group demonstrated legumain mRNA to be co-localised with CD68 macrophages in the shoulder region of the plaque by *in situ* hybridisation and IHC. Another group followed on from this initial observation by using a microarray to study the aorta of aging Apolipoprotein E deficient mice with developed atherosclerosis, they found that legumain mRNA expression increased with the development of atherosclerosis while its expression remained at low unchanged levels in aged matched C57BL/6 control mice [665]. In both Apolipoprotein E deficient mouse arteries and human advanced atherosclerotic plaques legumain was predominantly expressed in macrophages. It was also shown to induce chemotaxis of primary human monocytes and human umbilical vein endothelial cells (HUVECs). The macrophage-specific expression of legumain *in vivo* as well as the ability of legumain to induce chemotaxis of cells *in vitro* suggests

legumain could play a functional role in atherogenesis. It has also subsequently been shown that human unstable plaques express twice the amount of legumain as stable plaques [666].

Cystatin C and cystatin E/M both bind to legumain with high affinity *in vitro* thereby inhibiting its activation [667]. Since cystatin E/M plays a regulatory role in epidermal differentiation, misregulation of this pathway leads to abnormal stratum corneum and hair follicle formation as well as severe disturbances of skin barrier formation [668]. Legumain is found in the epidermis and hair follicles of wild type and Cystatin E/M null mice (*ichq* mouse) but active legumain is only found in the epidermis of the *ichq* mouse suggesting that a role for cystatin E/M *in vivo* is to regulate legumain activity [669]. Regulation of legumain activity is important as it cleaves and activates cathepsin L which in turn activates transglutaminase 3 (TGM3). Active TGM3 was detected in skin extracts of cystatin E/M null mice at a time where skin lesions developed and caused abnormal cornification [669], therefore unrestricted legumain activation could play a role in irregular skin barrier formation. *Ichq* mice are a model of the human disorder type 2 harlequin ichthyosis which is a severe type of skin disease and as yet the presence of legumain in this disorder has not been determined. However, legumain mRNA has been detected in atopic dermatitis and psoriasis suggesting it may be contributing to the abnormal skin barrier seen in these conditions [435].

Since legumain was expressed at high levels in the SF of RA and PsA in my microarray samples and it has been demonstrated to cleave and activate several substrates found in the arthritic joint, such as fibronectin [425-428], MMP-2 [670] and cathepsin B and L [671-673], it was decided to analyse legumain and its expression and activity in RA and PsA samples.

Due to identification of a novel gene legumain which was highly upregulated in RA and PsA CD14⁺ SF cells I therefore sought to validate this expression in the microarray samples and samples from other patients. I also sought to determine the level of protein expression of legumain in the synovial membrane of diseased patients and in the CD14⁺ cells of diseased patients by IHC and western blot. Once the presence of legumain had been verified I sought to determine

whether the legumain that was present was active and still able to cleave substrates.

5.2 Validation of legumain expression in microarray samples

Legumain had a fold change increase from PB CD14⁺ samples to the matched SF CD14⁺ samples of 167 in RA and 164 in PsA. However, since microarray results require validation and verification by an alternative and complementary gene expression profiling method qRT-PCR was used to determine the expression of legumain in the 8 RA and 8 PsA pairs of samples that had been used for the microarray study. Figure 5.1 (A) shows the raw data signals of legumain on the affymetrix GeneChips for each patient sample. The signal values of legumain in the synovial fluid of both sets are considerably higher than those of the blood samples however the signal intensity varies between the synovial fluid samples of each patient indicating that the levels of legumain in the synovial fluid CD14⁺ cells are not consistent possibly due to differences in disease duration and treatment type of the patients.

To validate these results qRT-PCR was used to determine the fold change in legumain expression in the SF samples compared to the PB samples. One RA patient was unable to be analysed due to a problem with the peripheral blood sample, as repeated measurements gave unusual results which could not be analysed. This could have been due to a problem with the RNA for that sample therefore the matched PB and SF samples from that particular patient were not analysed. Figure 5.1 (B) shows the results of the qRT-PCR and clearly demonstrates that legumain expression increases from the PB to the SF of each patient which reflects the results obtained from the microarray.

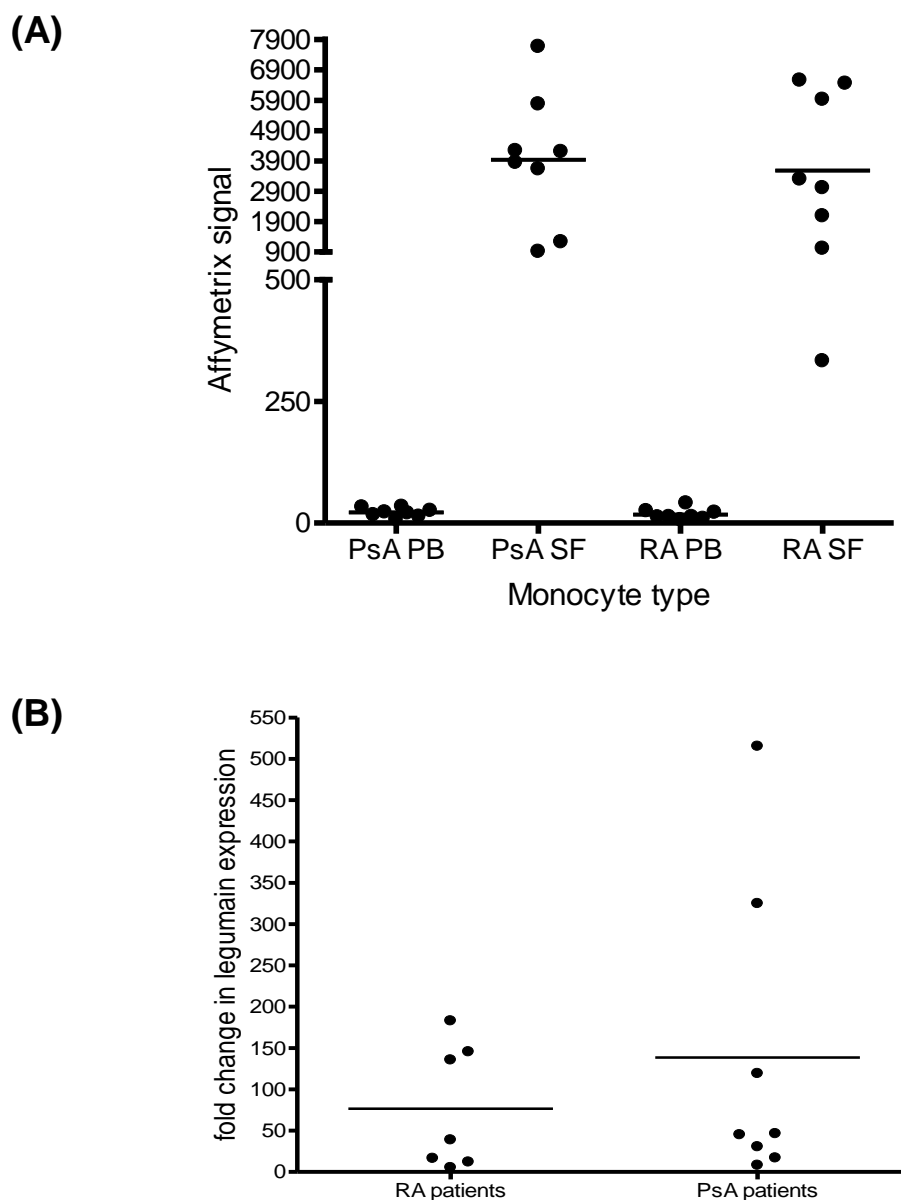


Figure 5.1 Legumain expression in microarray samples

(A) Demonstrates the raw signal intensities of the legumain gene locus on the Affymetrix GeneChips. Each circle represents one patient sample and the line represents the mean signal intensity. Legumain mRNA levels were then measured in the RA and PsA paired PB and SF microarray samples by qRT-PCR. (B) The fold change of legumain expression in the SF samples was normalised to the matched patient PB sample, each circle represents one patient and the line the mean fold change.

5.3 Expression of legumain in other patient samples

Once it had been verified that legumain was expressed in the samples used in the microarray we then analysed legumain expression in more patient samples to check that increased legumain expression in the SF of RA and PsA occurred in other patients and not just the 16 who were examined on the microarray. Figure 5.2 shows the legumain expression in all of the CD14⁺ samples tested. In graph A

it can be seen that there are varying levels of legumain in the PsA CD14⁺ SF samples but the mean level of expression is higher than that seen in PsA PB CD14⁺. Five of these PsA patients tested had matched PB and SF CD14⁺ cells and therefore the fold change increase in legumain expression from the PB to the SF was able to be measured as seen in Figure 5.2 (B). As was observed in the microarray samples some of the patients had a substantial fold change differences but the mean fold change difference in legumain expression was 133 which is similar to the mean fold difference of 138 seen from the microarray samples. We were unable to obtain matched PB samples for the RA patients analysed however, in part (A) of Figure 5.2 the RA SF CD14⁺ and PsA SF CD14⁺ samples both have a similar mean relative expression of legumain which concurs with the results observed in the microarray samples.

Graph (A) of Figure 5.2 also demonstrates the other arthritic patient samples from which we obtained CD14⁺ cells to analyse for legumain expression. The inflammatory arthritis and JIA CD14⁺ samples both appear to have lower levels of legumain expression compared to the RA and PsA SF samples however legumain is present in the samples. Unfortunately we were not able to obtain PB CD14⁺ samples from each patient to distinguish whether the legumain expression level increased from PB to SF CD14⁺ as has been seen with the RA and PsA patients. Matched OA PB and SF CD14⁺ cells from the same patient were measured for legumain expression and as with the RA and PsA microarray samples the level of mRNA expression increased from the PB to the SF. These data demonstrate that legumain is not specific to RA and PsA but is also expressed in other arthritic diseases.

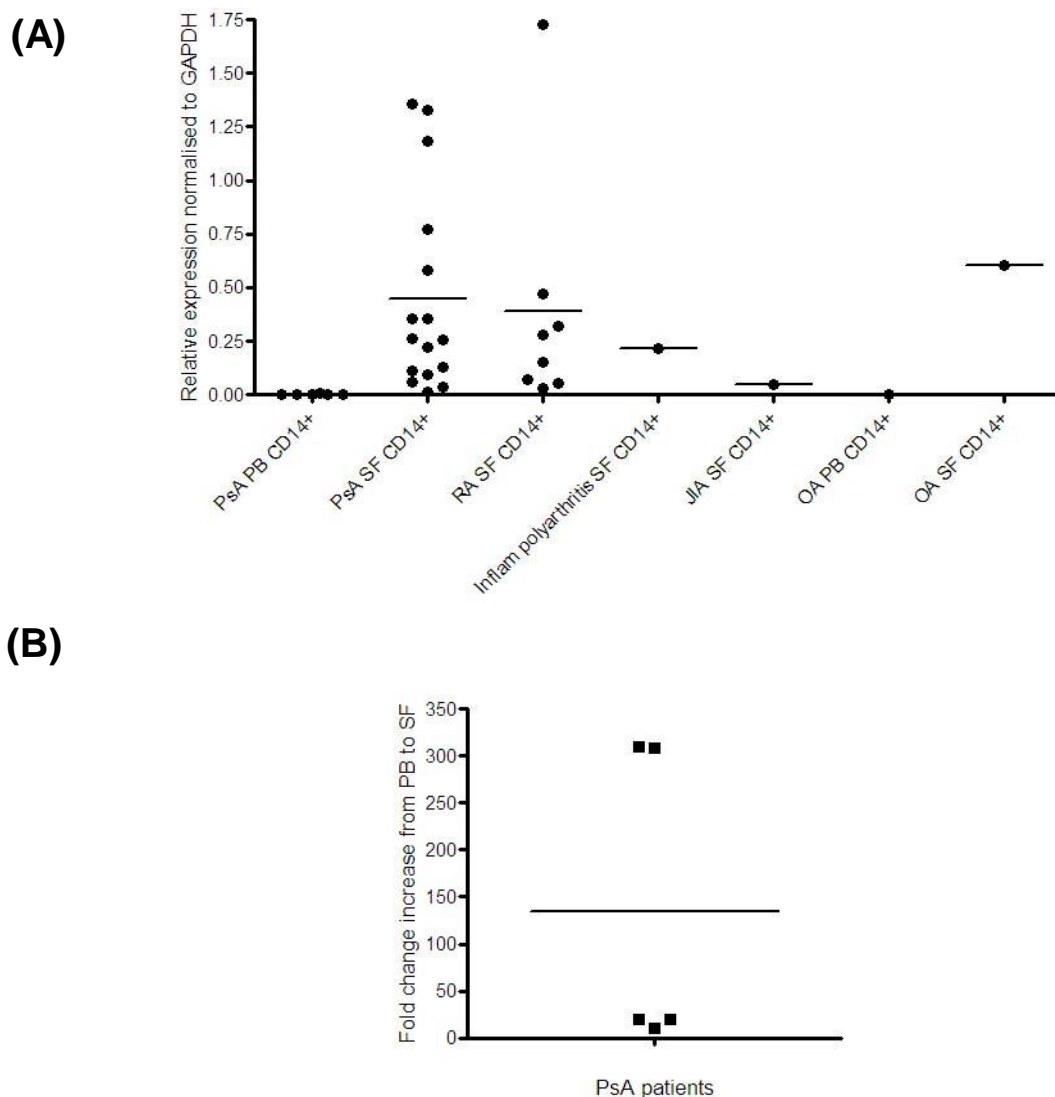


Figure 5.2 Legumain expression in CD14⁺ cells from arthritic patients

Legumain RNA expression was measured in the CD14⁺ cells from the PB or SF of arthritic patients. (A) Expression was measured relative to GAPDH. Five of the PsA patients had matched PB and SF samples, in these patients the fold change increase of legumain expression was measured and plotted in graph (B).

5.4 Immunohistochemistry

Next immunohistochemistry (IHC) was performed to detect the enzyme in RA synovial membrane. The antibody staining was first optimised on tonsil sections before the RA synovial membranes were stained for legumain (data not shown). Five individual RA membranes were stained and they all displayed specific positive staining of legumain. Figure 5.3 shows representative staining from one of the RA synovial membranes. The x10 and x40 magnification isotype controls are shown in panel (A). Positive legumain staining at x10 and x40 magnification from two different areas of the same RA synovial membrane are shown in panel (B) and (C). The isotype controls show no non-specific staining indicating the antibody is specific for legumain. In panel (B) legumain staining is seen to be constitutively expressed throughout the membrane and analysing the x40 magnification the staining is cytoplasmic with no nuclear staining and within some cells the staining is granular indicative of lysosomal staining.

Panel (C) shows staining from the same tissue and demonstrates that positive legumain staining is seen on the edge of the membrane where it is contained within the cytoplasm of cells. Positive staining can also be seen in cells within follicular regions of the tissue (indicated by arrows). The staining appears to be present in macrophage-like cells but this cannot be verified until the membrane is double stained for legumain and CD68.

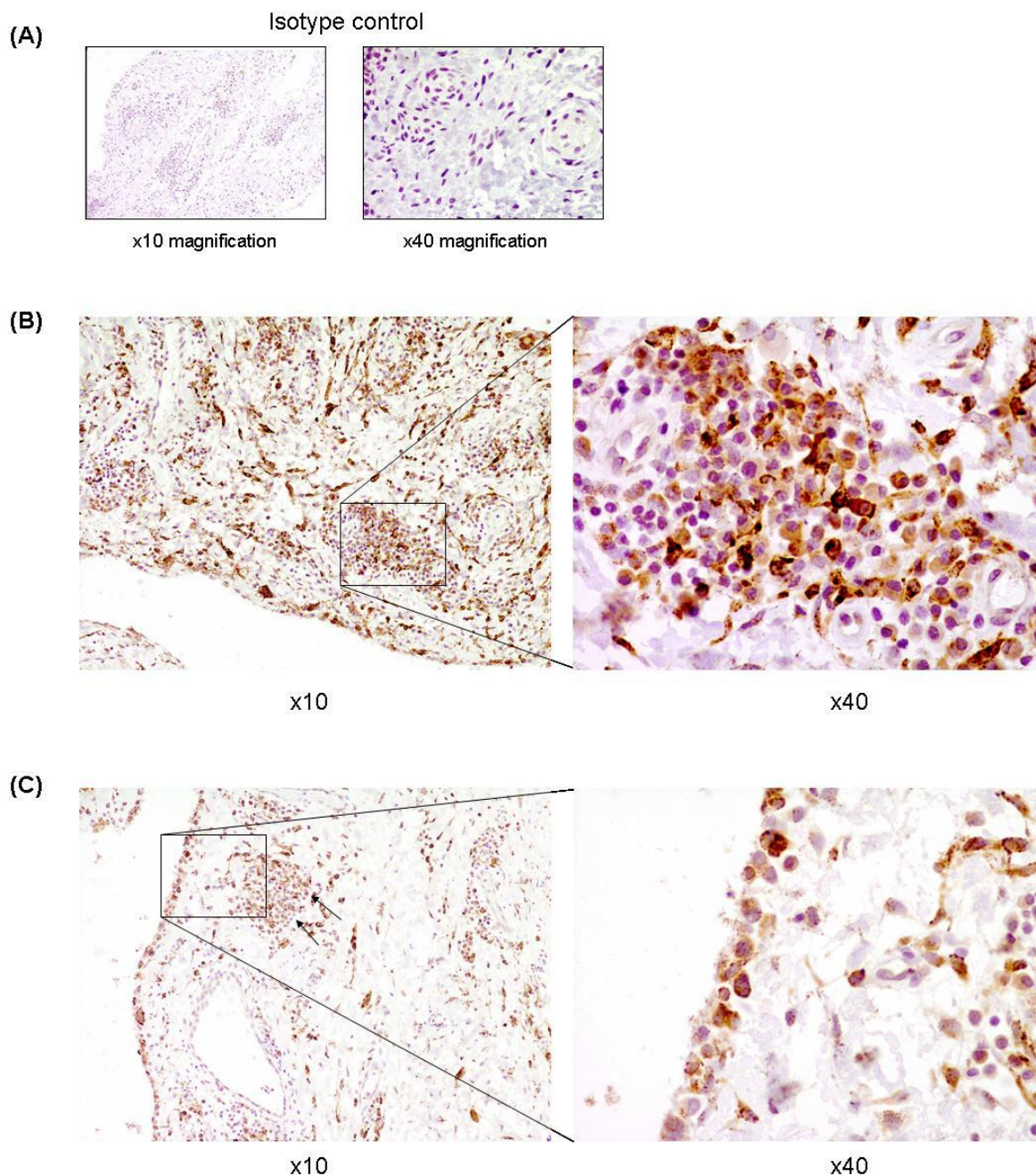


Figure 5.3 Immunohistochemistry staining of legumain in RA synovial membrane

Five individual membranes were stained for legumain; this figure shows the results from one membrane that represents the staining pattern of legumain seen in the membranes. The isotype controls are shown in (A) at the x10 and x40 magnification. Images (B) and (C) define the positive legumain staining (shown in brown) seen in the RA membrane. Arrows in panel C denote positive follicular staining. The x10 magnification is shown on the left and the x40 magnification of an area of the x10 image defined by the box is shown on the right.

5.5 Western blot for legumain

Since we had determined that legumain RNA was expressed in synovial CD14⁺ cells and IHC had indicated that legumain was present in RA synovial membrane we wanted to determine if arthritic synovial CD14⁺ cells were expressing the legumain enzyme at the protein level. Legumain has already been demonstrated to be upregulated in M-CSF macrophages [665, 674] therefore M-CSF treated macrophages were also analysed as a positive control.

5.5.1 Legumain in M-CSF derived macrophages by RT-PCR

To determine whether the presence of legumain increased as a result of M-CSF treatment, day 6 M-CSF treated macrophages from 5 individual donors were analysed by RT-PCR. As can be seen from Figure 5.4, M-CSF treatment induced legumain mRNA production in all 5 donors. The fold change difference between untreated monocytes and treated macrophages is donor dependent and varies between the lowest fold change of 26 and the highest fold change of 3567.

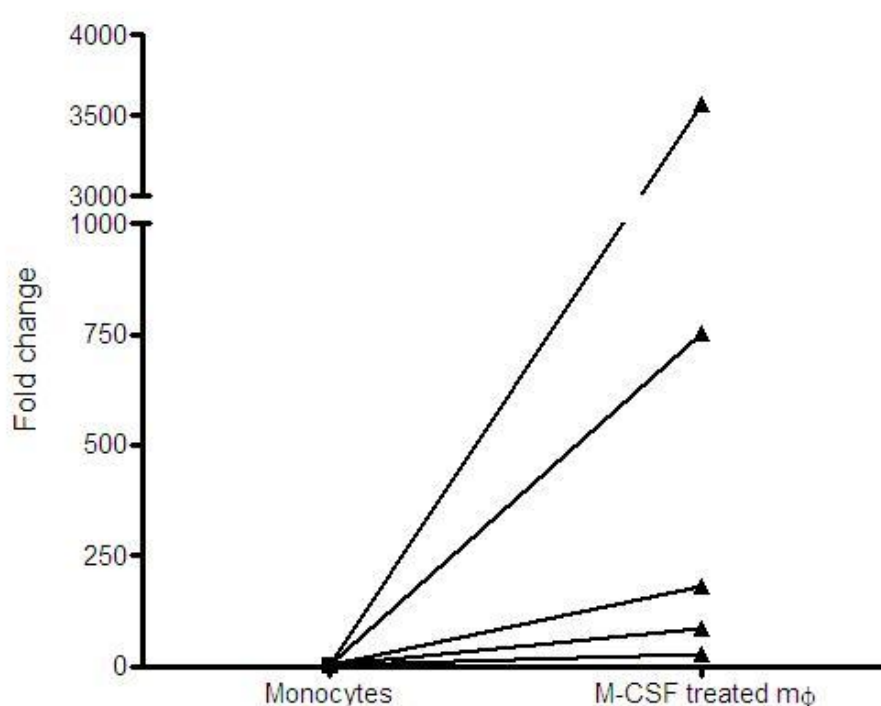


Figure 5.4 Legumain expression in M-CSF treated macrophages for 6 days

Monocytes were obtained from 5 healthy donors and treated with M-CSF for 6 days. Monocyte samples were taken at day 0 and compared to the M-CSF treated macrophages for legumain expression by RT-PCR. The graph shows the relative fold change in legumain expression from day 0 monocytes to day 6 treated macrophages.

5.5.2 Legumain in M-CSF macrophages by western blot

Once we had shown that legumain is present in M-CSF derived macrophages at the mRNA level we next wanted to verify expression at the protein level. Monocytes were isolated from three individual donors and analysed at day 0, 3 and 6 days after M-CSF treatment for legumain and also for β -actin as a loading control (Figure 5.5). As described in the introduction legumain can exist in two forms, a pro form and an active form, to convert to the active form legumain must autocatalytically cleave itself in a pH dependent process [656]. In Figure 5.5 the upper panel indicates the presence or absence of legumain in each sample. There appears to be no legumain in any of the day 0 - monocyte samples however day 3 M-CSF treated monocytes exhibit two bands representing pro-legumain at 52 kDa and active legumain at 36 kDa. At day 6 in all three donors the amount of pro-legumain is reduced as well as the amount of active legumain. The lower panel of Figure 5.5 demonstrates the β -actin loading control of each macrophage sample. Despite the samples being analysed by BCA for protein concentration and 5 μ g of protein being loaded to each well, the β -actin blot indicates that the amount of protein loaded into each well varies due to the different intensity of the β -actin band in each sample. Also, there appears to be very little sample in day 0 monocytes of donor 1 due to a lack of a band for the β -actin control. However, regardless of the loading control we have demonstrated that macrophages express the pro- and active form of legumain when induced via M-CSF differentiation of monocytes.

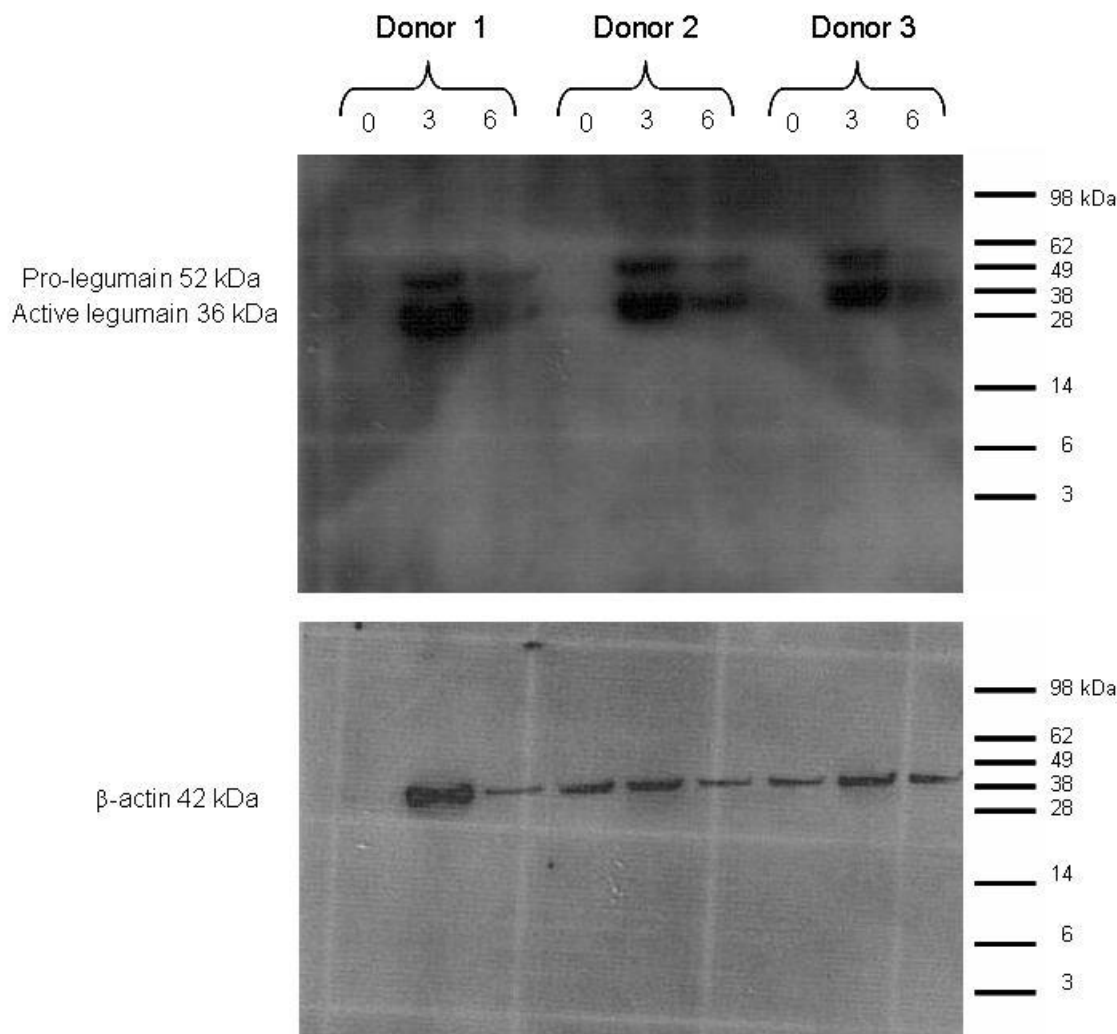


Figure 5.5 Legumain western blot of M-CSF treated macrophages

Three donors were analysed after day 0, 3 and 6 of M-CSF treatment for the presence of legumain (top blot) or β -actin as a loading control (bottom blot). Both pro- and active legumain were present at day 3 in all three donors whereas by day 6 the pro-legumain levels had decreased.

5.5.3 Legumain in patient CD14⁺ samples by western blot

We next wished to detect legumain by western blotting in RA synovial CD14 cells. The western blot assay had already been optimised for the detection of legumain using M-CSF derived macrophages as a control. CD14⁺ cells were isolated from five individual RA bloods, one PsA blood, two PsA synovial fluids and one RA synovial fluid and run on a western blot to detect legumain and the loading control β -actin.

The upper panel in Figure 5.6 illustrates the presence of active legumain in 4 of the 5 RA bloods and in the PsA blood CD14⁺ cells which was not seen in the CD14⁺ cells at day 0 of the normal donors in Figure 5.5. Large amounts of pro- and active legumain were present in both of the PsA synovial fluid macrophage

samples however, there does not appear to be any legumain in the RA synovial fluid CD14⁺ cells. The lower panel of Figure 5.6 demonstrates the β -actin loading control of the samples as observed in the M-CSF macrophage western blot despite 5 μ g of protein being added to each well there appears to be varying levels of protein in each well as indicated by the difference in intensity of the β -actin bands. Also the two samples which appeared to contain no legumain in the upper panel, the RA PB sample and the RA SF sample, do not have a β -actin band suggesting that not enough of sample was loaded into these wells. These samples would have to be repeated to determine if they do contain the enzyme legumain. We have therefore demonstrated that active legumain is present in the peripheral blood CD14⁺ cells of arthritic patients which was not seen in normal donors day 0 CD14⁺ cells (Figure 5.5). We have also demonstrated that pro- and active legumain is expressed in the PsA synovial fluid CD14⁺ cells.

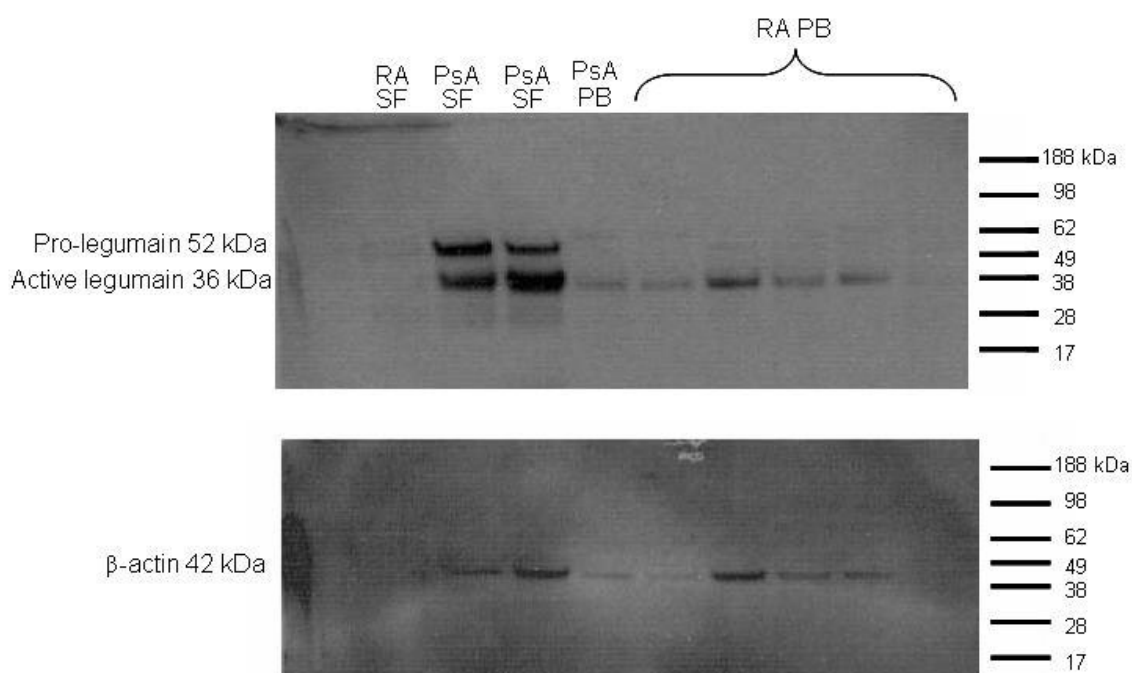


Figure 5.6 Legumain western blot of patient CD14⁺ cells

CD14⁺ cells from five RA bloods, one PsA blood, two PsA synovial fluids and one RA synovial fluid were analysed by western blot for the presence of legumain (upper panel) and β -actin control (lower panel).

5.6 Legumain activity assay

Legumain is a lysosomal enzyme that in its active form specifically cleaves protein substrates after an asparagine residue [651], despite showing that M-CSF derived macrophages and synovial fluid derived CD14⁺ cell express the active

form of legumain it was necessary to establish that this enzyme was still physiologically active and could still cleave its substrates. A legumain activity assay was therefore employed which uses a substrate Z-Ala-Ala-Asn-AMC. Incubating active legumain with this substrate results in its cleavage after the asparagine residue (Asn) thereby releasing the AMC (7-amino-4-methylcoumarin) fluorophore. The amount of fluorophore released can be detected on a luminescence reader.

To determine the presence of active legumain in M-CSF derived macrophages and patient derived CD14⁺ samples, cells were resuspended in lysis buffer and then freeze thawed three times to disrupt the lysosomes where legumain is thought to reside [432].

5.6.1 Legumain activity assay of M-CSF derived macrophages

Given that large amounts of the active form of legumain were visualised in the M-CSF derived macrophages by western blot it was decided to analyse these samples to determine if the legumain in these samples was physiologically active and still able to cleave its substrate. Clerin et al [665] previously demonstrated that legumain is more active and can cleave its substrate in day 3 M-CSF differentiated macrophages compared to monocytes however a timecourse of day 0, 3 and 6 activated macrophages has not been reported to my knowledge.

Day 0, 3 and 6 M-CSF treated macrophages from two donors were analysed using the activity assay; the same number of cells (1×10^6) were examined per condition. Recombinant human (rh) legumain was also analysed on the assay as a positive control. Figure 5.7 illustrates the results of the activity assay; the rh legumain (black line) shows an increase in fluorescence units (FU) over time indicating that the legumain is active. The two donors tested are shown by the red line for donor 1 and blue line for donor 2 with the triangle symbol indicating day 0 monocytes, the square symbol indicating macrophages treated with M-CSF for 3 days and the diamond symbol indicates day 6 M-CSF treated macrophages. In both donors a similar pattern in legumain activity can be observed. The day 0 monocytes have little or no legumain activity for the duration of the assay as the FU detected were less than the background fluorescence of the assay. This corroborates the legumain western blot of M-CSF macrophages (Figure 5.5) as

there was no legumain detected in the day 0 monocytes. The fluorescence units of day 3 M-CSF activated macrophages in both donors is high and increases over time to a maximum of 670 FU and 592 FU in donors 1 and 2 respectively indicating a high level of legumain activity. This discrepancy in the amount of legumain activity in these samples despite having the same number of macrophages may be due to donor specific variability or it is possible that the macrophages from donor 1 contain more legumain.

In both day 6 activated macrophage samples the amount of fluorescence and therefore legumain activity is lower compared to the day 3 activated macrophages. The FU from donor one decreases by almost two thirds from 670 to 177 FU at the 210 minute timepoint and the FU from donor two decreases from 592 to 346. This indicates that there could be less legumain present in these cells and therefore legumain may undergo a cyclic form of expression with no active legumain present in day 0 monocytes, a high level of activity and expression in 3 day differentiated macrophages which then decreases to a lower level at day 6. This observation agrees with the western blot of the M-CSF differentiated macrophages as there was a greater amount of active legumain expressed in the day 3 macrophages compared to the day 6 macrophages. Overall it has been demonstrated that legumain is expressed at varying levels in M-CSF activated macrophages depending on the duration of activation, this legumain is the active form of the enzyme and as a result is able to fulfil its physiological role and cleave its endogenous substrate.

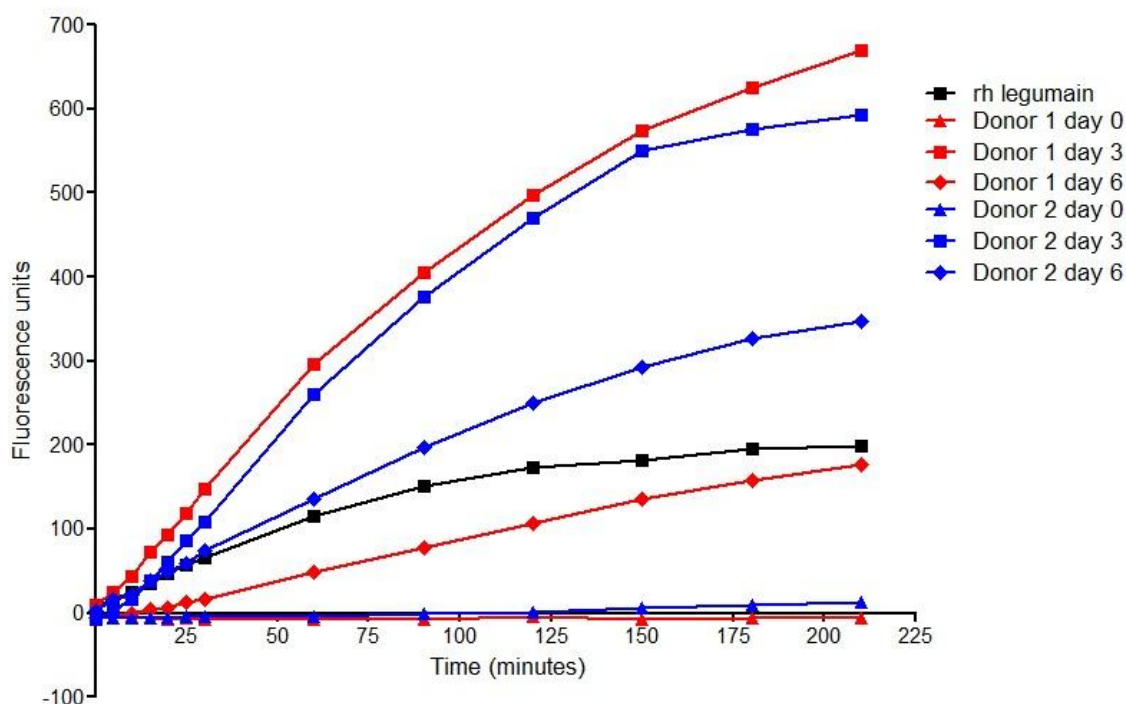


Figure 5.7 Legumain activity assay in M-CSF derived macrophages

M-CSF activated macrophages for 0, 3 and 6 days from two separate donors were analysed using a legumain activity assay to determine whether the legumain detected in these samples was active could cleave a substrate. Once the substrate is cleaved it releases a fluorophore which can be detected on a luminescence plate reader. The assay is incubated at 37°C for 3.5 hours and the amount of fluorescence (measured in fluorescent units) is measured at set time points. Human recombinant legumain was used as a positive control and is indicated by the black line. Cells from donor 1 are shown by the red line and cells from donor 2 are shown by the blue line with the triangle symbol indicating day 0 monocytes, square symbol indicating M-CSF treated macrophages for 3 days and diamond symbol indicating M-CSF macrophages treated for 6 days. Each donor sample contained 1×10^6 cells.

5.6.2 Legumain activity assay of patient CD14⁺ samples

Once we had determined that legumain was present and active in the M-CSF macrophage samples we then went on to establish whether the large amounts of legumain present in the CD14⁺ patient samples as seen on the western blot was active and able to cleave its substrate. Two RA synovial fluid samples, one with a matched blood, were obtained and CD14⁺ cells were isolated from each. Due to differences in the amount of synovial fluid obtained and donor variation there were slight differences in the total number of cells, analysed, RA patient 1 had 7.2×10^5 SF CD14⁺ cells whereas RA patient 2 had 8.8×10^5 SF CD14⁺ cells.

The results for the patient legumain activity assay are shown in Figure 5.8. As in the previous experiment rh legumain was used as a positive control. Despite having the lowest total cell number RA patient 1 SF (red line) exhibited the largest amount of legumain activity shown by the steady increase over time of

FU up to a maximum of 830 FU. However, the PB sample (red line) from the same patient contained a much lower amount of legumain; the fluorescence level of this sample was very low and did not increase above the background level of fluorescence indicated by the negative FU values. Nevertheless, it appears that this sample did contain a small amount of legumain as the FU can be seen to slowly increase towards the baseline which would suggest that legumain is present but there is not enough enzyme to create a strong fluorescent signal. This same pattern of activity can be visualised in the patient 2 RA SF sample (blue line) where the fluorescence unit increase from -30 to -1 FU over the duration of the assay indicating that active legumain is present in this sample but in small amounts.

Synovial fluid from several patients including six RA, six PsA, one OA, one JIA and one inflammatory polyarthritis were analysed using the activity assay to investigate whether these samples contained active legumain that may have been excreted from the synovial cells. However, no activity was determined for any of the samples (data not shown).

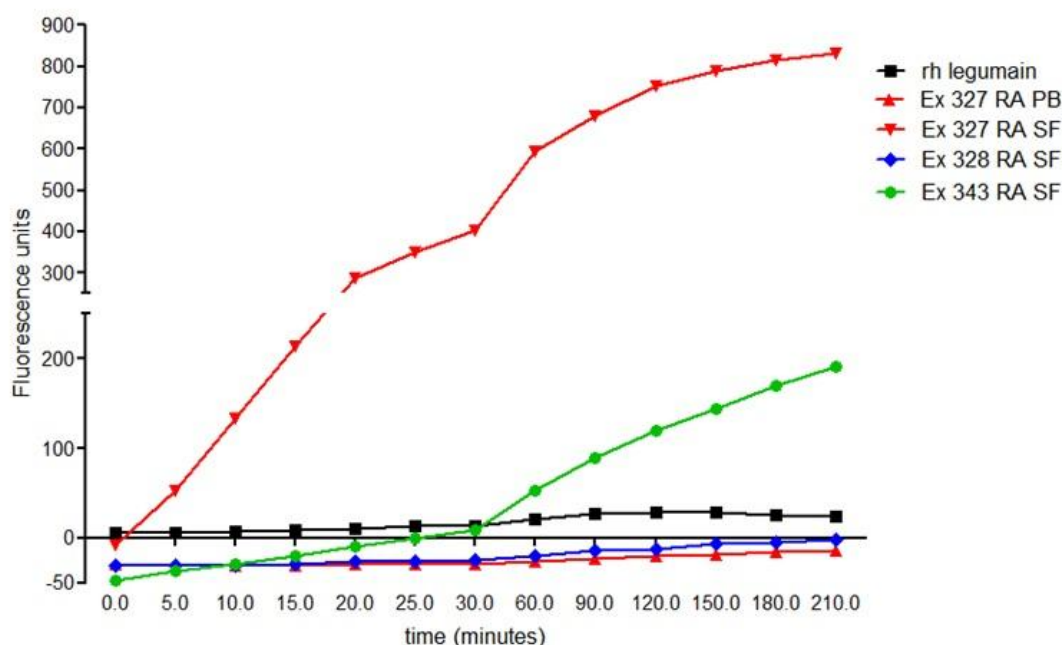


Figure 5.8 Legumain activity assay in CD14⁺ patient samples

CD14⁺ cells isolated from patient blood or synovial fluid samples were analysed using a legumain activity assay to determine whether the legumain that was present in these cells was active and able to cleave a fluorescent substrate. The assay is incubated at 37°C and the amount of fluorescence measured in fluorescence units (FU) is quantified at set time points. Human recombinant legumain (black line) was also assayed as a positive control. RA patient 1 is shown in red with the upwards facing triangle indicating blood CD14⁺ cells and the downwards facing triangle indicating synovial fluid CD14⁺ cells. RA patient 2 is shown by the blue line indicating synovial fluid CD14⁺ cells.

5.7 Analysing legumain expression under normoxic and hypoxic conditions

Once we had established that legumain was present in RA synovial fluid macrophages and was physiologically active we decided to try and determine what potential factors could be inducing legumain expression. To do this we cultured healthy donor M-CSF differentiated macrophages in complete media or in complete media supplemented with 10% RA or PsA synovial fluid for 24 hours under normoxic (24% oxygen) or hypoxic (1% oxygen) conditions. This was in order to represent the conditions that the arthritic macrophages may be exposed to once they exit blood and enter the synovial compartment. The synovium has been shown to be a hypoxic environment [409] and the synovial fluid of arthritic patients contains many cytokines, chemokines, antibodies and extracellular matrix proteins [410, 537] which could interact with and induce differential gene expression in the macrophages. Therefore, we hypothesized that exposing macrophages to patient synovial fluid under hypoxic conditions may induce the expression of legumain within these cells. Three RA and three PsA synovial fluids were tested on the macrophages and the experiment was repeated three times.

Figure 5.9 shows the fold change in legumain mRNA expression relative to the macrophage in complete media alone control. Graph (A) demonstrates those cells which were incubated with the six synovial fluids for 24 hours under normoxic conditions. The fold change in legumain expression increases with 2 of the RA synovial fluids but the addition of RA SF 2 seems to decrease the amount of legumain expression resulting in a negative fold change. In contrast, only one PsA SF appeared to increase the amount of legumain expression under normoxic conditions while PsA SF 1 and PsA SF 3 decreased the amount of legumain expression.

On the other hand, culturing macrophages with synovial fluid under hypoxic condition increased legumain expression in all synovial fluid conditions relative to the macrophages incubated with media alone. The hypoxic conditions are shown in graph (B) of Figure 5.9; the three RA synovial fluids and three PsA synovial fluids all induced an increase in legumain expression compared to control indicated by the positive fold change values.

These results suggest that the combination of both synovial fluid and hypoxia are needed in order to induce legumain expression in macrophages. As the addition of synovial fluid under normoxic conditions induces both up and down regulation of legumain expression compared to control whilst the macrophages cultured under hypoxic conditions show an increase in legumain expression with all of the six synovial fluids examined.

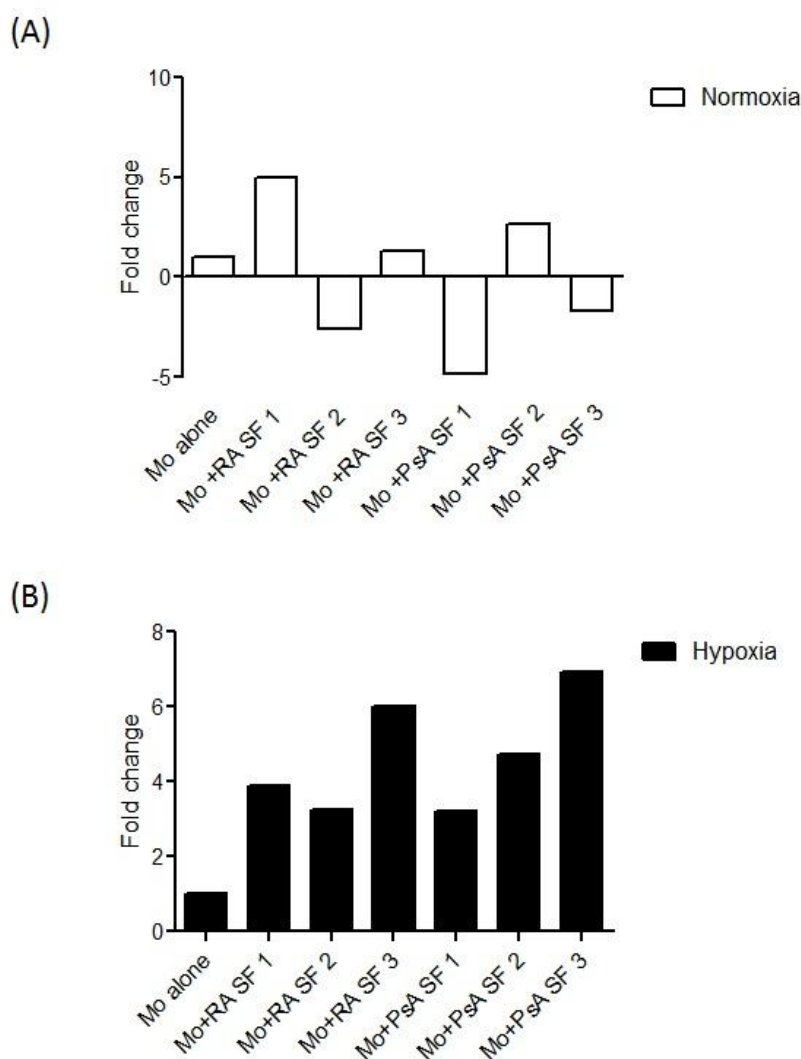


Figure 5.9 Legumain expression in the presence of synovial fluid under hypoxic and normoxic conditions

M-CSF differentiated macrophages were cultured individually with three different RA synovial fluids or three PsA synovial fluids for 24 hours under normoxic (24% oxygen) (A) or hypoxic (1% oxygen) (B) conditions. The fold change in legumain expression is shown relative to the mo alone control for either the hypoxic or normoxic condition. This is a representative of three independent experiments.

5.8 Discussion

The second gene identified from the microarray experiment as being highly expressed in both RA and PsA synovial macrophages was the enzyme legumain. It is an enzyme that specifically cleaves its substrates after an asparagine residue and has a role in the cleavage and activation of several substrates as well as in the processing of microbial antigen for MHC II presentation [659]. Legumain is known to be dysregulated in several inflammatory conditions and diseased states such as breast cancer where it is specifically overexpressed on tumour associated macrophages [433]; atherosclerosis where it is upregulated in macrophages in unstable areas of plaques [434] and in inflamed skin where it is highly upregulated in lesional atopic dermatitis and psoriasis compared to normal skin [435].

Legumain has not been previously associated with inflammatory arthritis however several of its substrates that it can cleave and activate are present within the synovium of arthritis patients. These substrates include fibronectin [431] a glycoprotein that has been found in the synovia of RA and PsA patients [426] whose cleaved fragments acquire catalytic activities which can stimulate cartilage chondrolysis [675]; legumain can activate gelatinase A (MMP2) [429] which has been found at higher levels in RA patient blood compared to controls [670] and is also upregulated in the RA and PsA SF CD14⁺ cells in my microarray experiment (see chapter 3). Legumain has also been demonstrated to cleave several cathepsins including cathepsin B and L which are upregulated in RA synovial fluid [671]. Since active fibronectin, gelatinase A, cathepsin B and L are all known to degrade collagen and other components of the extracellular matrix we hypothesised that legumain is upregulated by synovial fluid macrophages as a result of the pro-inflammatory environment to function as an extracellular matrix degradation enzyme and therefore may be contributing to the cartilage and bone degradation that is often seen in these two diseases.

These are the first data to investigate legumain expression in RA and PsA and using qRT-PCR we observed that all of the RA and PsA synovial fluid samples analysed were expressing legumain at varying levels and the amount of synovial fluid CD14⁺ legumain was always elevated in comparison to the CD14⁺ blood cells. We also demonstrated that legumain was expressed in the synovial fluid

CD14⁺ cells of an OA patient, a JIA patient and an inflammatory polyarthritis patient indicating that this enzyme was not exclusively expressed in RA and PsA and appeared to be a marker of other arthritic synovial macrophages. Research into the control of mRNA expression has uncovered small non-coding RNAs termed microRNAs (miRNA) that target and degrade mRNA thereby suppressing proteins synthesis [676] therefore observing mRNA expression of a gene such as legumain does not imply that it will be expressed at the protein level. Consequently, further studies took place to investigate the expression of legumain. Immunohistochemistry revealed a novel observation that legumain was constitutively expressed in five RA synovial membranes. At a higher magnification legumain was observed specifically in the cytoplasm of the cells in a granular pattern which is indicative of its role as a lysosomal enzyme [432] and cell surface staining was also observed. Legumain has previously been shown to be expressed on the cell surface and along the invadopodia of invading tumour cells where it correlated with increased tumour invasion and metastasis [662]. Further double staining of legumain and the plasma membrane would need to be carried out to verify legumain cell surface expression on synovial arthritic cells but a role for this surface expression could be to help invading synovial cells to further degrade the cartilage and extracellular matrix. Positive staining was also observed within follicular regions of the membrane and within cells on the outside edge of the synovium. We propose that the legumain positive cells on the outside of the membrane are well positioned to uptake substrates from the synovial fluid, such as fibronectin, in order to cleave them intracellularly. To establish whether this synovial legumain was present mainly in macrophages double staining would need to be performed but time restrictions meant this could not be performed.

It had been observed that synovial fluid CD14⁺ macrophages expressed legumain at the mRNA level and arthritic synovial membrane expressed legumain at the protein level therefore we wanted to determine whether synovial fluid macrophages expressed legumain at the protein level by western blot. Legumain has been previously shown to be upregulated in M-CSF differentiated macrophages [665, 674] therefore these cells were used as a positive control for the western blot. We confirmed that M-CSF differentiated monocytes had increased legumain mRNA expression, the fold change increase in legumain

ranged from 26 to 3573 indicative of donor specific variability. A western blot was performed on 3 donors consisting of monocyte samples which were treated for 0, 3 and 6 days with M-CSF. We observed no legumain in the day 0 CD14⁺ monocytes but pro- and active legumain were visible at day 3 and 6 of all three donors analysed. There appeared to be more active legumain in all of the day 3 samples but it is difficult to remark on levels of the enzyme since the β -actin loading control was not consistent among all samples despite the same amount of protein (5 μ g) being loaded onto the assay. Repetition of these experiments would be helpful to formally define this issue - time precluded this analysis.

Once we had validated the western blot assay, we analysed CD14⁺ cells from the blood and synovial fluid of RA and PsA patients. There has been no previous work illustrating legumain's presence in PB monocytes, however it has been demonstrated that other cysteine proteases are upregulated in monocytes in response to pro-inflammatory cytokines [677, 678]. The serum of PsA and RA patients is known to contain pro-inflammatory cytokines, growth factors, MMPs and adhesion molecules [679-681] which could be inducing active legumain in diseased blood monocytes, a phenomenon that is not seen in healthy donor blood monocytes. The two PsA synovial fluid CD14⁺ samples tested on the western blot were positive for high levels of pro- and active legumain but the RA SF CD14⁺ tested did not develop any protein bands. This result indicates that the legumain positive cells detected by IHC in the synovium are likely to be macrophages however the possibility that other cells express legumain cannot be discounted. The two RA PB and SF samples which did not develop a legumain band were also negative for a β -actin loading control band indicating either a problem with the samples or that perhaps not enough sample was loaded onto the western blot. Further analysis of the presence of legumain within RA SF CD14⁺ samples would be useful to verify that these cells express legumain at the protein level but time restrictions meant this was not possible.

An activity assay was set-up to determine if the legumain protein detected in the samples was active. Clerin et al [665] had previously demonstrated that monocytes treated with M-CSF for 3 days contained physiologically active legumain however a timecourse of M-CSF treated monocytes had not been examined. The results of the western blot were replicated in the activity assay as day 0 monocytes showed no activity on the assay and no protein on the

western blot. Day 3 activated monocytes had high levels of active legumain which decreased in the day 6 activated macrophages. These observations demonstrate that legumain may undergo a cyclic form of expression with no active legumain present in day 0 monocytes, a high level of activity and expression in 3 day differentiated macrophages which then decreases to a lower level at day 6. At present there have been no other reports of legumain undergoing cyclic expression dependent on macrophage maturation as all other studies have analysed legumain levels at one timepoint. Further studies are required to understand the significance of this observation.

Patient derived CD14⁺ cells were analysed on the activity assay to ascertain whether these cells contained physiologically active legumain. We examined matched RA PB and SF CD14⁺ samples and a RA SF CD14⁺ sample due to the variation in isolated cell numbers in patient samples the number of cells analysed in each sample was slightly different. Nevertheless, the SF patient sample containing the least number of cells had the highest amount of active legumain illustrating that individual macrophage cells may contain varying levels of legumain. The matched PB sample from this patient showed a low level of active legumain as did the other RA SF sample analysed again indicating that the level of legumain varies between patients. It would be interesting to determine if treatment type or disease duration was a factor determining how much active legumain was present within the synovial macrophages of arthritic patients. Unfortunately we did not receive any PsA patient samples to assay and time restrictions meant we could not continue collecting samples.

The inflammatory synovium is known to be a hypoxic environment [409] and the synovial fluid of RA patients contains many cytokines, chemokines, antibodies and extracellular matrix proteins [410, 537, 682-685] which could interact with and induce differential gene expression in the cells entering the synovium. Establishing what factors could be causing the upregulation of active legumain in macrophages once they have transmigrated from the blood system into the diseased joint is vital in understanding legumain's role in disease pathology. Proinflammatory cytokines have been previously shown to induce expression of other cysteine proteases in monocytes [677, 678] therefore we hypothesised that the proinflammatory mediators and environmental stresses within the synovial environment may be inducing the large upregulation of legumain once the

macrophages entered the synovial compartment. To address this question we differentiated healthy monocytes with M-CSF for 6 days and cultured them in the presence of three RA synovial fluids and three PsA synovial fluids for 24 hours in hypoxic or normoxic conditions. We observed that under normoxic conditions macrophages had increased legumain mRNA expression when incubated with two of the three RA synovial fluids and one of the three PsA synovial fluids compared to macrophages incubated alone. However, under hypoxic conditions legumain mRNA expression increased in all of the macrophages incubated with the six synovial fluids indicating that a combination of the hypoxic environment with the proinflammatory mediators contained within the synovial fluid is necessary to induce upregulation of legumain.

In summary, legumain is an enzyme which plays a physiological role in the biosynthesis of lysosomal enzymes in the proximal tubule cells of the kidney [431] and is expressed in macrophages and DCs where it is thought to play a role in cleaving peptides for antigen presentation [658, 659]. However it has now become apparent that legumain is involved in many inflammatory diseases and conditions including arthritis. We propose that legumain is a marker for cell activation demonstrated by its upregulation in macrophages but it is also an inflammatory enzyme induced and deregulated during inflammation. The perpetual inflammation that occurs during arthritis causes the upregulation of this enzyme. Legumain is induced in the arthritic blood monocytes by the pro-inflammatory mediators contained within the serum and is also induced within the synovial fluid macrophages by the hypoxic inflammatory milieu found within the joint. Once upregulated within the synovial macrophages legumain is expressed on the cell surface and may be assisting the cells to invade and degrade the surrounding joint cartilage and extracellular matrix. Pro- legumain has recently been found to be excreted from macrophages and has been shown to induce chemotaxis of primary monocytes [665], therefore if the legumain positive macrophages visualised within the synovium were also excreting legumain this could be playing a role in increasing the number of infiltrating monocytes into the arthritic joint.

Legumain is also expressed within the lysosomal compartment of the cells where it may cleave and active glycoproteins such as fibronectin either being produced by the cell or which have been taken up by the cell. One of legumain's primary

roles during homeostasis is to cleave microbial antigen for MHC II presentation and to activate several cathepsins which are also involved in antigen presentation [432, 686]. T cells specific to autoantigens such as collagen type II have been found within the arthritic joint [687] therefore an increase of legumain within synovial macrophages may play a role in the presentation of autoantigens to these T cells thereby aiding the perpetuation inflammation and destruction seen in RA and PsA. Figure 5.10 summaries these findings.

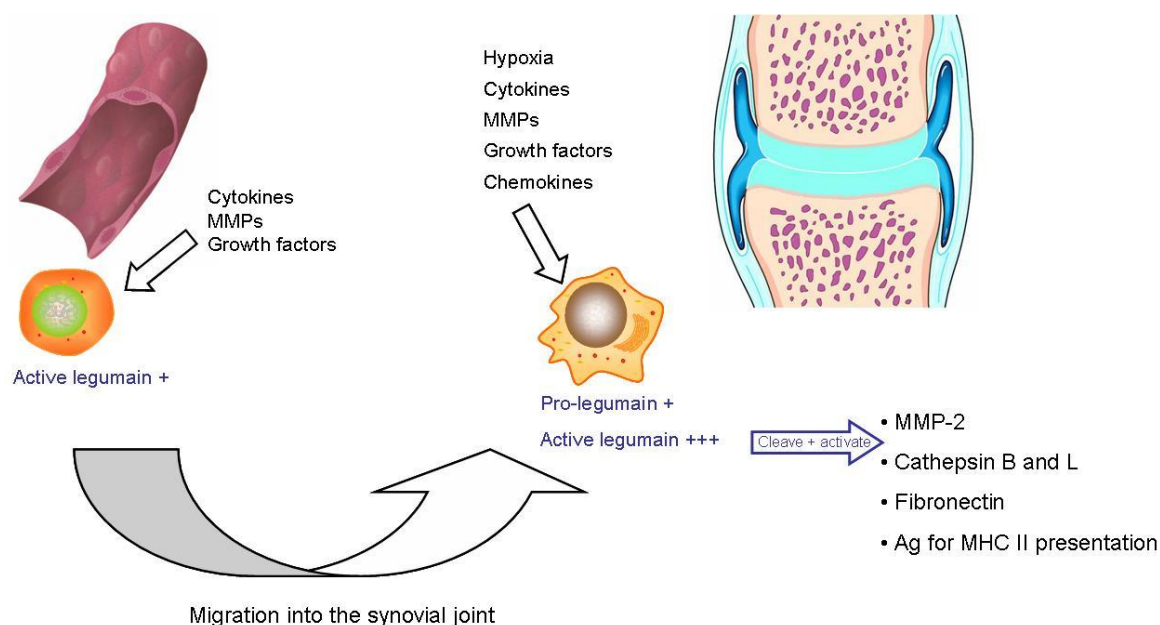


Figure 5.10 Overview of legumain's expression and proposed role in arthritis

A summary of legumain's expression in patient blood monocytes and synovial fluid macrophages, the factors that could be inducing its expression and its proposed role in the synovium.

Chapter 6 Microarray analysis of cell contact activated macrophages

6.1 Introduction

One critical mechanism whereby macrophages are thought to exert their proinflammatory potential in the synovium is by direct cell-cell interaction with T cells. We and other groups have investigated this interaction in an *in vitro* model system of inflammatory arthritis. In order to set up this model monocytes and T cells are isolated from normal donors before activating T cells with a cytokine cocktail (IL-2, IL-6, TNF α) and maturing the monocytes into macrophages with M-CSF for 6 days each. After this time the two cell types are put into direct contact for a period of 24 hours after which the supernatants are collected and analysed for cytokine release. Several groups have investigated this *in vitro* system and found it to be a robust model of inflammatory arthritis since both cytokine activated T cells (Tcks) and RA synovial T cells can induce production of TNF α and several CC and CXC chemokines in resting M-CSF treated macrophages in a cell contact dependent manner [269, 298, 308]. Also Brennan et al found that after 8 days of stimulation Tcks acquire an activated phenotype resembling RA synovial T cells, such as upregulation of adhesion molecules and activation markers. Neutralising these markers in both T cell types abolished the cell contact dependent TNF α production from resting macrophages [309]. However, apart from cytokine and chemokine production little is known about the effects of Tck direct cellular contact activation on the macrophages.

Due to the lack of transcriptomic information of the cell contact activated macrophages it was decided to collect normal donor samples, which were age and sex matched to the RA and PsA patient samples collected for the microarray, and activate these using the cell contact model of inflammatory arthritis. These macrophages were then analysed on the microarray and the results were compared to the synovial CD14⁺ cell samples to determine how similar these Tck activated macrophages were to the synovial macrophages. The results should inform us how comparable the macrophages from the *in vitro* model of inflammatory arthritis are to synovial macrophages from RA and PsA patients.

6.2 Patients and normal donors

As described previously, inflammatory arthritis patients attending Gartnavel General Hospital (GGH) or Glasgow Royal Infirmary (GRI) fulfilled the American

college of Rheumatology criteria for RA [6], or met diagnostic criteria for PsA [346].

Age-matched normal donors were recruited from the Glasgow Biomedical Research Centre (GBRC) building and all blood samples were taken with informed consent.

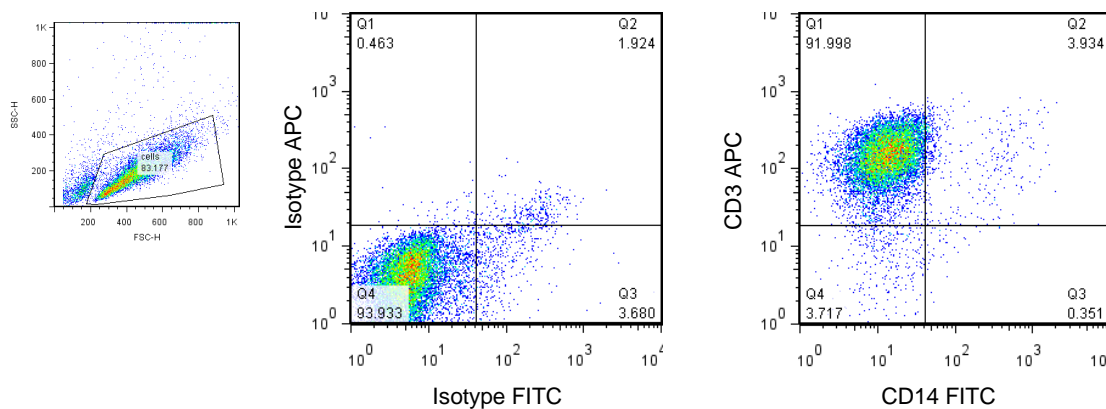
6.3 Separating macrophages from the cell contact

In order to analyse the transcriptomic profile of the activated macrophages after they had been in contact with the Tcks we had to first gain a pure population of macrophages that did not have any contaminating Tcks which would affect the results of the microarray.

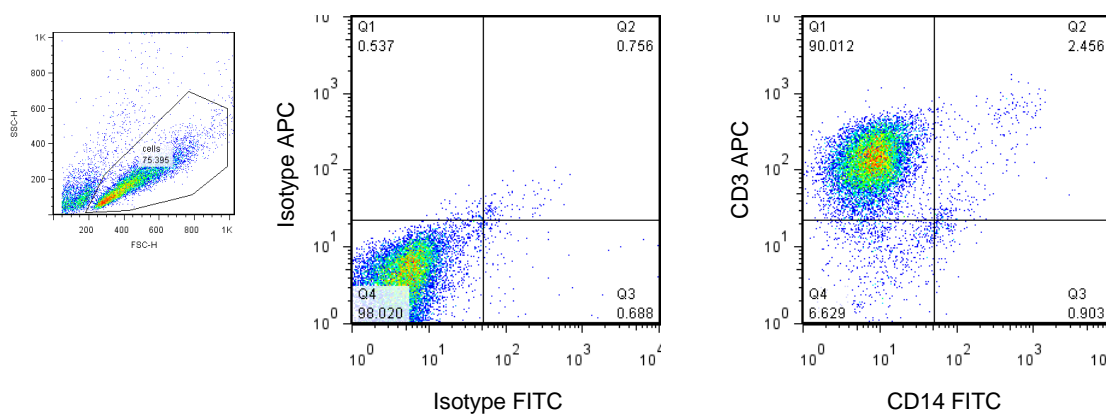
6.3.1 Live cell contact

The *in vitro* cell contact model of inflammatory arthritis can be set up in a number of ways including using T cell membranes [294], fixed Tcks or live Tcks to activate the macrophages. For the microarray experiment we wanted to compare cell contact activated macrophages with patient derived SF CD14⁺ cells and therefore decided to set up the cell contact experiment with live Tcks. This would allow us to see the effects of direct cellular contact along with the soluble mediators that the Tcks released upon the activation of macrophages. The cell contact was set up at a ratio of 4 Tcks to 1 macrophage for 24 hours, the cells were separated by washing off the non-adherent Tcks (Tck fraction) and scraping the adherent macrophages from the wells (macrophage fraction). However, when the two cell fractions were analysed on the FACS for cell purity (Figure 6.1) the macrophage fraction (A) was 91% positive for CD3 cells and had no CD14 positive cells but a very small percentage (3.7%) of CD3 negative cells. This experiment was repeated n=5 times with the same or similar results occurring each time. The same experiment was set up with fixed Tcks as it was thought that fixed cells may be less likely to stick to the macrophages, however we obtained similar Tck contamination in the macrophage fraction in these experiments (data not shown).

(A) Macrophage Fraction



(B) Tck fraction

**Figure 6.1 Separation of cells from a live cell contact**

Cells were separated by washing the non-adherent Tcks from the wells designated Tck fraction (B) and scraping the adherent macrophages from the wells designated macrophage fraction (A). This is a representative FACS plot from n=5 experiments

6.3.2 FACS ARIA sorting of macrophages

Due to the problem of being unable to fully separate the macrophages from the Tcks using the method of washing and scraping the wells, it was decided to try and separate the two cell fractions from the cell contact using the FACS ARIA. The Tcks were stained with carboxyfluorescein succinimidyl ester (CFSE) and fixed before being put into cell contact with the macrophages. After incubating the cell contact all the cells were collected and put through the FACS ARIA machine, separating the cells based on FITC-positive cells (Tcks) and FITC-negative cells (presumably macrophages). Figure 6.2 shows the plots from one FACS ARIA experiment; figure A shows all the cells that were isolated from the cell contact experiment and the histogram shows the counts of cells that were FITC positive and FITC negative. On the histogram the gates p1 and p2 indicate the gates that were used to differentiate and collect FITC-negative (macrophage cells) and FITC-positive (Tck cells) respectively. The two gates did not overlap and a substantial gap was left between them, cells included in this area were not collected as it was important for the purpose of the microarray to be able to collect a pure population of macrophages with no contaminating Tcks. Figure B and C show the plots and histograms of the two separated cell fractions, Tcks and macrophages respectively. The cells collected in the Tck fraction (B) were all FITC-positive, as indicated by the histogram, and the forward and side scatter plot indicates the cells collected in this fraction are of the correct size and granularity for activated T lymphocytes. As expected all cells collected in the macrophage fraction (C) were FITC-negative and on the forward and side scatter plots show the cellular size and granularity of macrophage like cells. However, it must be noted from panel (C) that a large proportion of the events on the forward side scatter graph are in the bottom left hand corner which is indicative of cellular debris. This debris may be as a result of the removal of the macrophages from the culture wells after cell contact activation and the presence of this debris may ultimately affect the purity of the macrophages used for the microarray.

Subsequently an aliquot of the FACS ARIA separated cells was used for cytopins which were stained with RAPI-DIFF reagents to check for cell purity and morphology. As can be seen in figure (D) the cells from the macrophage fraction had the correct shape and morphology of macrophage cells with no

visible contaminating Tck cells whereas the cells from the Tck fraction (E) were the morphology and size of T lymphocytes.

It was decided therefore that using the FACS ARIA to gain a pure population of cell contact activated macrophages was the optimal method of separation for the normal donor samples that would be age and sex matched to the patient samples.

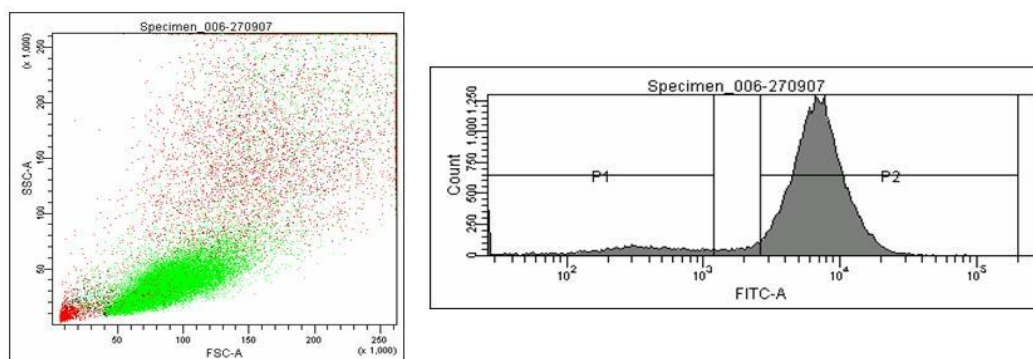
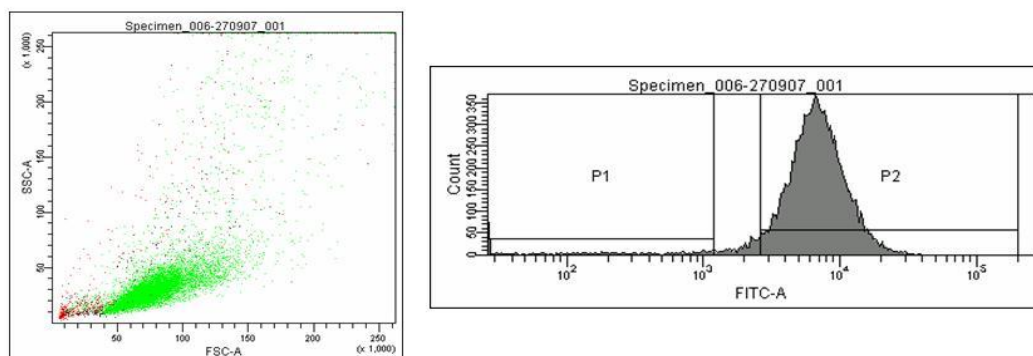
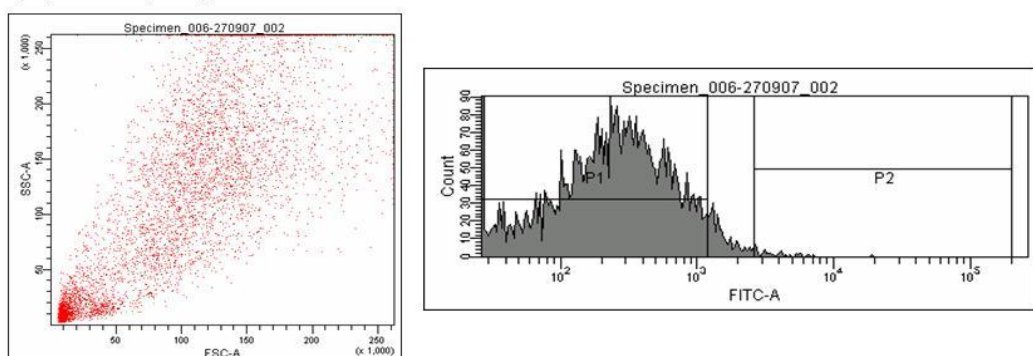
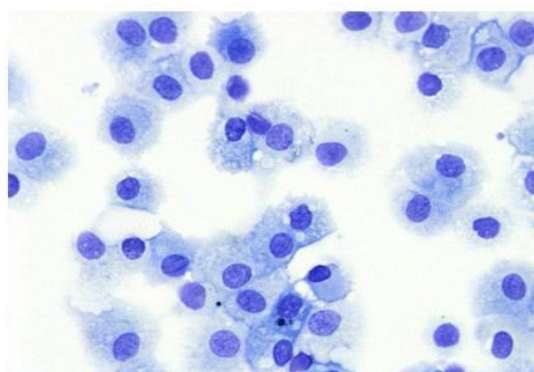
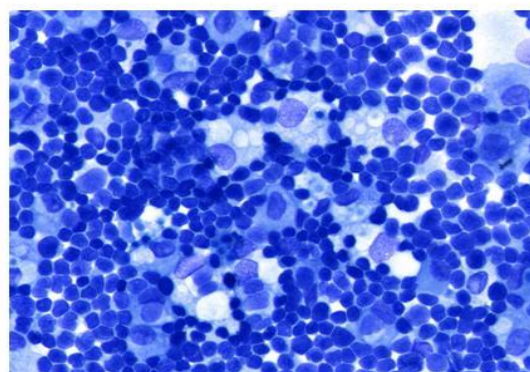
(A) All cells before separation**(B) Tck fraction****(C) Macrophage fraction****(D) Macrophage fraction****(E) Tck fraction**

Figure 6.2 Separating the macrophages and Tcks from cell contact using the FACS ARIA
 The FACS ARIA was used to separate fixed CFSE stained Tcks from the macrophages after cell contact. (A) All cells from the cell contact experiment showing the forward and side scatter plot of the cells and the histogram plot with the gates used to separate the cells, gate p1 (FITC negative cells) and gate p2 (FITC positive cells). (B) Tck cell fraction after separation and (C) macrophage cell fraction. An aliquot of the two cell fractions from the macrophage fraction (D) and the Tck fraction (E) was stained with RAPI-DIFF to check for cell morphology.

6.4 Analysing cell contact RNA yield and quality

Microarray experiments demand a high quality of RNA in order for correct interpretation of results and the amount of RNA obtained from the cells should be consistent between samples. Therefore before the cell contact activated cells are analysed on a microarray the RNA quality and yield from the macrophage cells must be examined (described in detail in chapter 3).

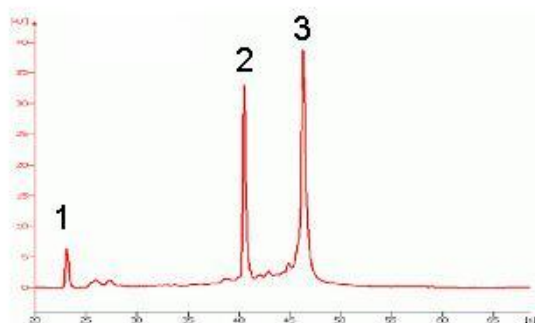
6.4.1 *Cell contact activated macrophages have variable RNA quality after 24 hours*

Fixing the Tcks before putting them into cell contact was found to help recover a more pure population of macrophages after FACS ARIA separation thus it was decided that fixed Tcks should be used in all cell contact experiments. Three independent macrophage - Tck cell contact experiments were set up at a 4:1 ratio of fixed Tcks to macrophages, after 24 hours the Tcks and macrophages were separated and both cell types were put into Trizol reagent. The RNA was isolated from the cells and the quality was analysed on an Agilent 2100 Bioanalyser. The principles of the agilent 2100 bioanalyser are described in section 3.3.2 of chapter 3, but briefly this equipment is used to ascertain the quality of the RNA and allows the user to obtain a plot indicating the RNA integrity. Figure 6.3 (A) shows an example of good quality RNA. It is characterised by three clear peaks, a peak for the RNA marker (1) and a peak for the 18 S (2) and 28 S (3) ribosomal RNA subunits. There should be a relatively flat baseline between the two rRNA peaks and minimal low molecular weight noise adjacent to the RNA marker peak.

Figure 6.3 (B) shows the electropherogram of the three fixed Tck and macrophage samples after 24 hours of cell contact. The Tcks in all three experiments had degraded RNA indicated by the low molecular weight noise and the absence of 18 S and 28 S peaks. The degraded RNA for the Tcks was thought to be a consequence of fixing the Tcks with paraformaldehyde. However, the RNA from the macrophages was of poor quality in two of the three experiments. The macrophage RNA from experiment 2 shows good quality RNA with two sharp rRNA peaks and no low molecular weight noise but the RNA from experiment 1 and 3 is of poor integrity with the complete absence of rRNA peaks. Due to the

large variability in quality of the macrophages RNA it was decided that a timepoint cell contact should be set up to try and discover why the macrophage RNA is degraded after 24 hours of cell contact.

(A)



(B)

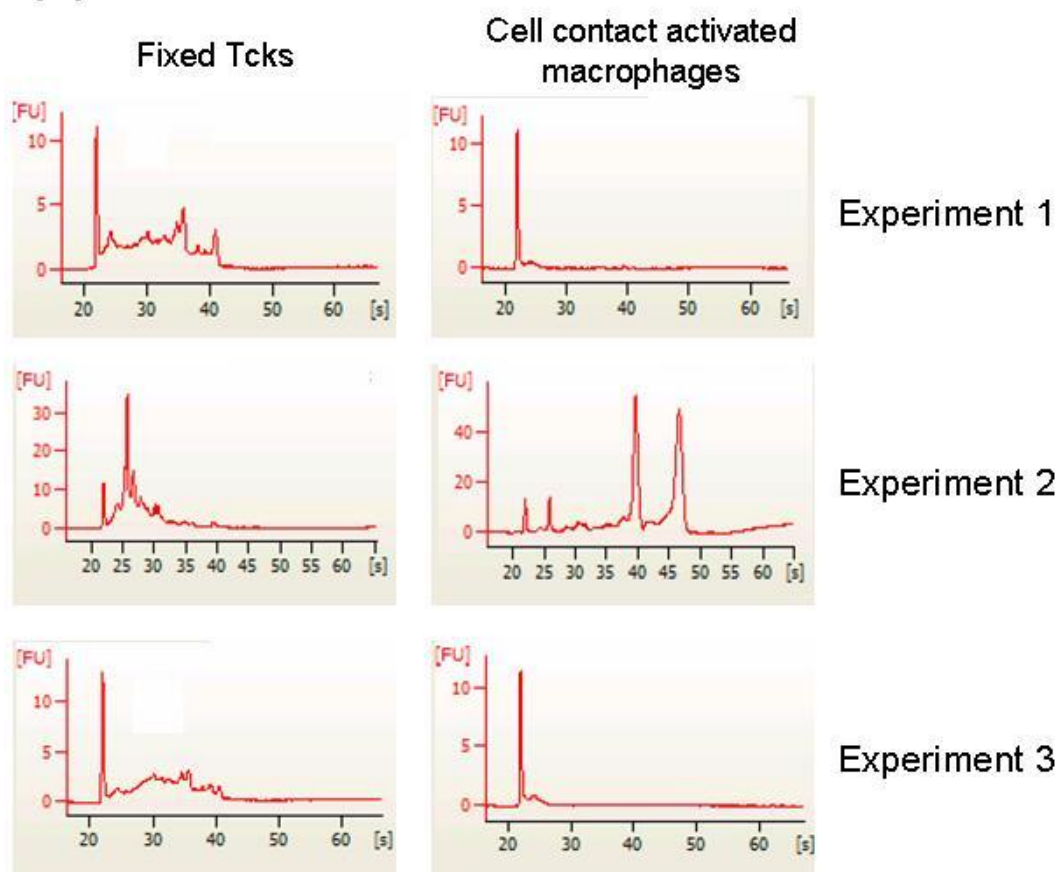


Figure 6.3 RNA integrity of macrophages and Tcks after 24 hours of cell contact

An Agilent 2100 Bioanalyser was used to analyse the quality of the RNA from 3 macrophage and 3 Tck samples after 24 hours of cell contact. (A) an example of good quality RNA (B) the agilent profile electropherograms of the three cell contact experiments.

6.4.2 Cell contact activated macrophage RNA quality over time

A timecourse cell contact was set up three times and the macrophages were activated with Tcks for between 1 and 48 hours. Figure 6.4 shows the results of one timecourse experiment and consist of RNA electropherograms for the fixed Tcks, unactivated macrophages (MØ) and macrophages that have been cell contact activated for varying amounts of time. The fixed Tck RNA is once again badly degraded due to the paraformaldehyde fixation process. The RNA from the macrophages that had not been cell contact activated (MØ) was of good integrity as indicated by the two sharp rRNA peaks and the absence of low molecular weight noise. The RNA from the macrophages that had been in cell contact for 1 hour up until 8 hours is also of good quality showing similar electropherogram plots to the one for the inactivated macrophages. With the exception of 28 hr cell contact activated macrophages (t=28hrs) the plots for RNA isolated from macrophages after 8 hours of cell contact all showed blank electropherogram plots, apart from the RNA marker peak, indicating that the RNA was either degraded or in such low concentration that the Agilent machine could not detect it. t=28 hrs RNA was the only exception to this observation as that sample was of high quality with no degradation.

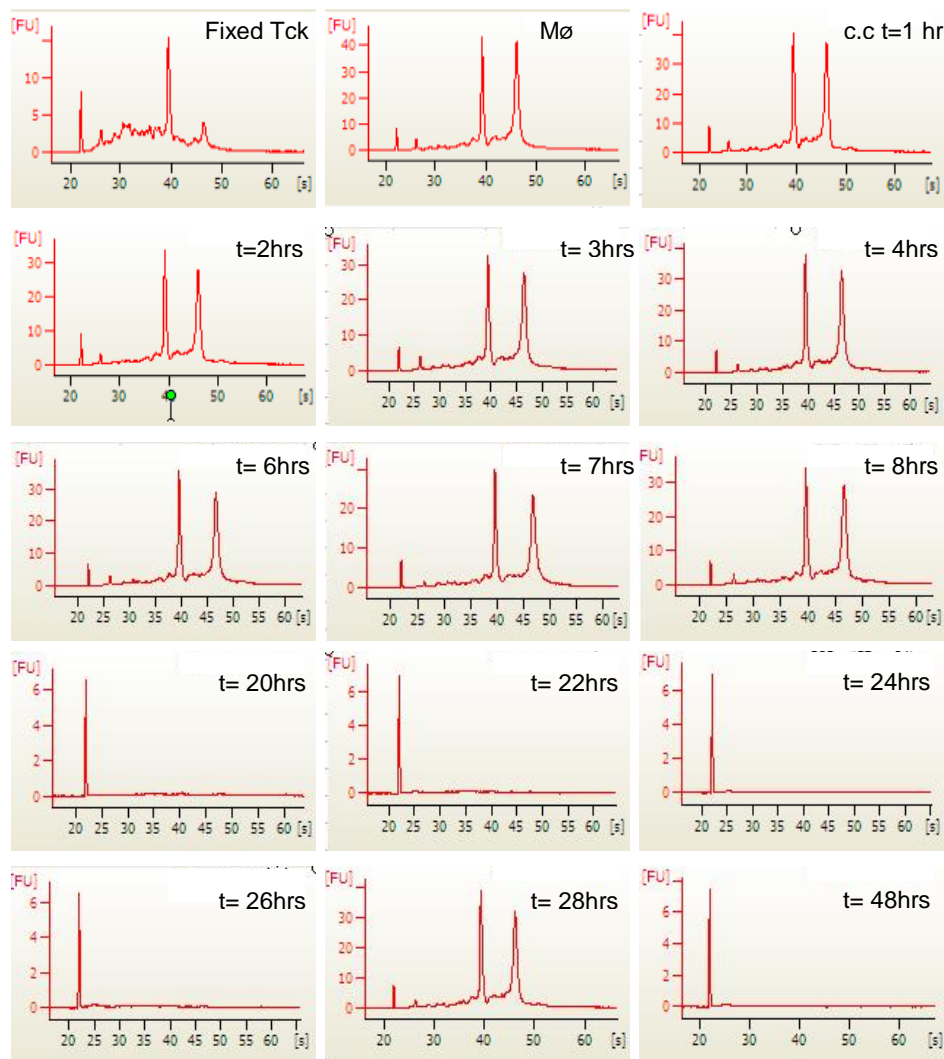


Figure 6.4 Macrophage RNA quality after different durations of cell contact

Macrophages were exposed to direct cellular contact with fixed Tcks for between 1 and 48 hours. The Tcks were removed from the macrophages and the quality of the Tck and macrophage RNA were analysed on an Agilent 2100 Bioanalyser

6.4.3 RNA yield decreases after prolonged cell contact activation

The total amount of RNA that was being recovered from the macrophages during the timecourse cell contact experiments was also investigated and measured on a nanodrop. Figure 6.5 shows a representative experiment from the three experiments that were set up, for comparison the amount of RNA from the fixed Tcks and macrophages alone (t=0) was also measured. It can be seen that compared to the unstimulated macrophages, designated t=0, the total amount of RNA decreases with increasing duration of cell contact activation. This trend continues until 26 hours of cell contact where the total amount of RNA increases

from 202 ng at cell contact $t=26$ hrs to 608 ng at cell contact $t=28$ hrs, however after this one timepoint cell contact the amount of RNA recovered from the sample decreases to 252 ng at $t=48$ hrs. This trend of decreasing total yield of RNA from cell contact activated macrophages compared to the unstimulated macrophages was seen in the two other timecourse experiments set up (data not shown). Based upon the preceding results it was decided to analyse macrophages which had been cell contact activated for 2 hours on the microarray as well as unactivated macrophages from the same donor. The reasons for this were due to the large variability in the yield and quality of macrophage RNA after 8 hours and also because we were interesting in the early response genes that changed in a macrophage after Tck contact.

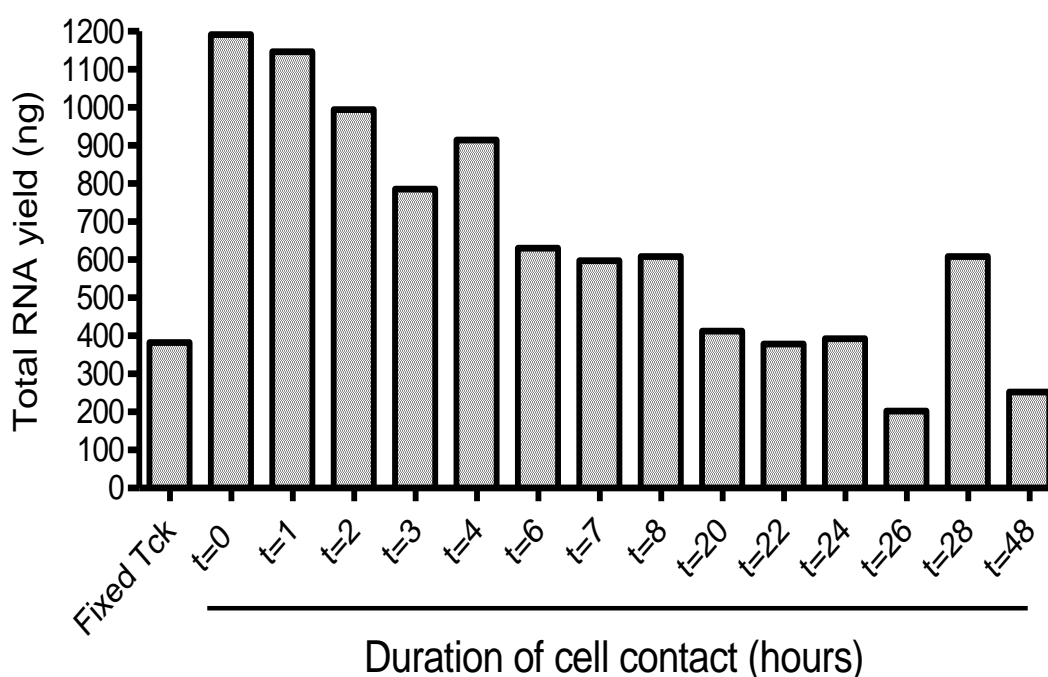


Figure 6.5 Total RNA yield from macrophages after increasing duration of cell contact activation by Tcks

A fixed 4:1 cell contact between Tcks and macrophages was set up for varying amounts of time between 1 and 48 hours. Each cell contact was set up in an individual well of a culture plate. After the specified time the macrophages and Tcks were separated and the macrophages placed into Trizol reagent. RNA was extracted from all samples and the total yield measured. RNA was also extracted from Tcks (fixed Tcks) and macrophages alone ($t=0$). This graph is a representative of $n=3$ experiments.

6.5 Collecting patient samples

Normal donors who were age and sex matched to patients (Table 3.2) were recruited and from these donors CD14⁺ cells were positively selected from the blood. An aliquot of unactivated CD14⁺ cells were put into trizol and the rest of the cells were differentiated with M-CSF for 6 days. After this duration the macrophages were cell contact activated for 2 hours with fixed Tcks that had been obtained from the same donor. The cell contact activated macrophages were isolated using the FACS ARIA and put into trizol as well as unactivated M-CSF derived macrophages from the same donor. Once all the samples had been collected the RNA was extracted from all samples at GSK Stevenage and analysed for quality. The samples which were analysed by the microarray are summarised in table 3.3 of chapter 3. Six age and sex matched healthy controls were recruited and macrophage cells before and after Tck cell contact activated were collected from all of these controls.

Patient	Gender	Age	Age and sex matched normal donor
PsA 1	Female	53	Yes
PsA 2	Female	40	Yes
PsA 3	Male	34	Yes
PsA 4	Male	34	Yes
PsA 5	Male	80	No
PsA 6	Female	58	Yes
PsA 7	Male	54	No
PsA 8	Female	58	No
RA 1	Female	71	No
RA 2	Male	56	Yes
RA 3	Female	62	No
RA 4	Male	69	No
RA 5	Female	78	No
RA 6	Male	69	No
RA 7	Female	69	No
RA 8	Female	77	No

Table 6.1 Patient and normal donor samples collected

6.6 Microarray analysis

6.6.1 Signal histogram of normalised data

The signal histogram of all the samples which were analysed by the microarray is shown in figure 3.3 of chapter 3. Upon inspection of the histogram it was discovered that one of the cell contact activated samples was an outlier as it clearly had a distinct signal intensity compared to the other samples which were analysed. The reasons for this sample being an outlier are unknown but since the other samples have similar signal intensities it is most likely to be due to a problem with the individual sample or the labelling of that particular sample. Due to this sample being an outlier it may potentially skew the data interpretation and therefore was removed from further data analysis.

6.6.2 Principle component analysis

The PCA plot shown in Figure 6.6 has previously been shown in chapter 3 but it is represented in this chapter to demonstrate the outlier cell contact activated macrophage sample indicated by the blue circle on the left hand side of the graph in (A). Once this sample had been removed the PCA analysis graph was analysed to look for trends in the data since samples represented in the same area of the graph are considered to be genetically similar. Each circle on the graph represents a sample and each colour represents a condition.

Figure 6.6 (B) demonstrates the PCA plot of the samples once the cell contact outlier sample had been removed. This plot demonstrates that the cell contact activated macrophages (blue circles) are genetically distinct from the M-CSF activated macrophages (red circles) indicating that transcriptional changes take place once the macrophages are put into cell contact with Tcks for 2 hours. However, it can also be seen that the cell contact activated samples (blue circles) appear to be genetically distinct from the synovial fluid activated macrophage samples (yellow circles) which could argue against the cell contact macrophages not having a similar genetic profile to the synovial fluid. This will be explored further in the following sections.

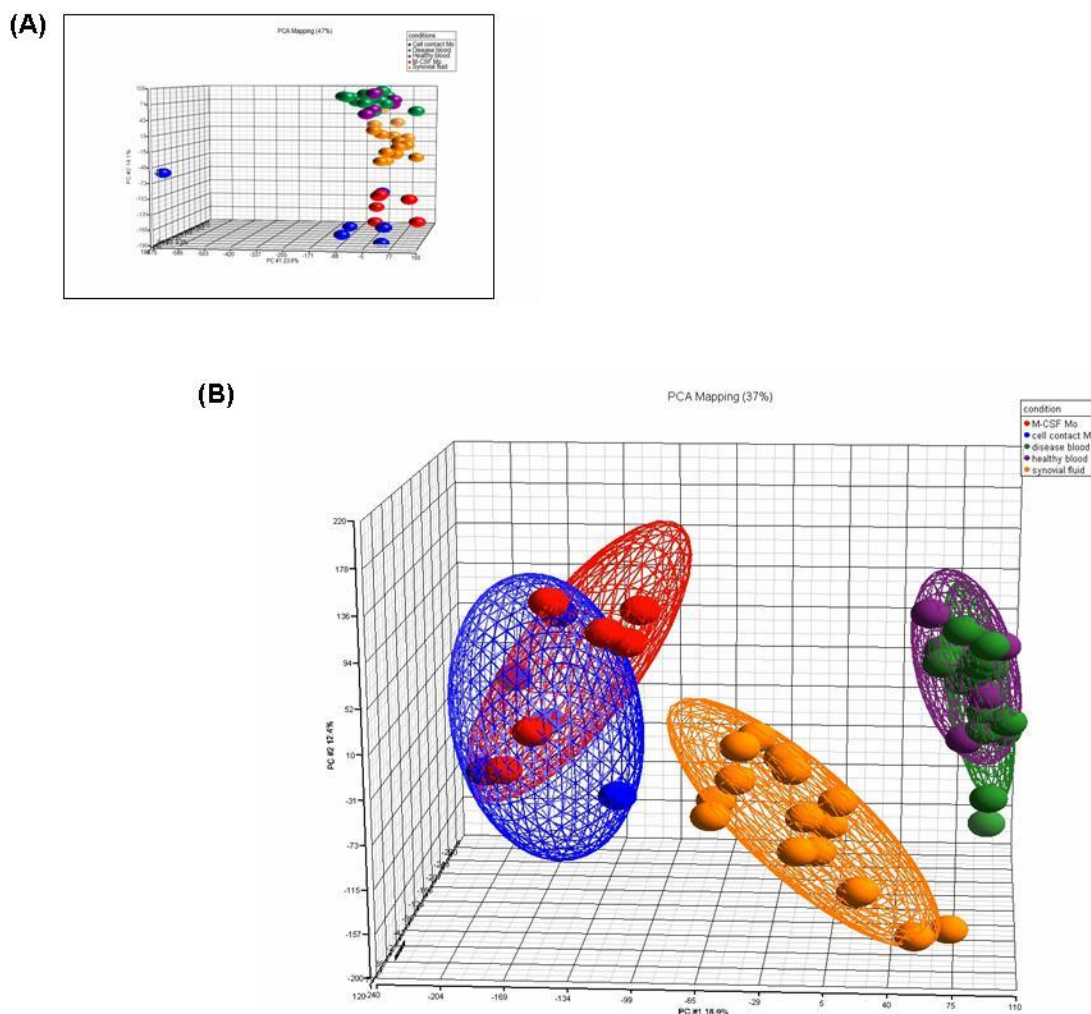


Figure 6.6 Principle component analysis of all microarray samples

PCA was performed on the microarray samples to try and identify trends in the data. Graph (A) indicates the normalised samples before the outlier was removed. The blue circle on the left hand side of the graph represents the outlying sample and it can be seen that it is distinct from all the other samples indicating that its genetic profile is dissimilar to that of the remaining microarray samples. Figure (B) shows PCA graph once the outlying cell contact sample had been removed. Each circle represents a sample and samples clustered in the same area of the graph are considered to be genetically similar. Each colour represents a sample condition; red is M-CSF treated macrophages, blue is cell contact activated macrophages, gold is synovial fluid derived CD14+ cells, green is patient blood CD14+ cells and purple is healthy blood CD14+ cells. This graph was previously shown in chapter 3.

6.7 Cell contact activated macrophages compared to synovial fluid activated macrophages

Cell contact dependent activation of macrophages by synovial T cells has previously been shown to play a role in arthritis and as a result an *in vitro* model of inflammatory arthritis has been developed by our group and others to mimic the cellular interactions taking place within the diseased joint. Investigations into the proinflammatory cytokines and chemokines released by the macrophages as a result of T cell interactions have taken place [269, 298, 308] as well as analysing the activated T cells [309]. One study by Gruaz et al [315] has also analysed the genetic profile of the macrophages after being stimulated by the isolated membranes of the human T cell line HUT-78. However there has been no comparative analysis of the *in vitro* cell contact stimulated macrophages and macrophages isolated from the arthritic joint.

The cell contact activated macrophages were stimulated with fixed cytokine activated T cells (Tcks) for 2 hours as we were interested in the early response genes that were differentially expressed in the macrophages. Previous analyses indicated that one of the cell contact activated macrophage samples was an outlier due to its abnormal signal intensity. Therefore this sample and its matched unactivated macrophage sample was removed from further analysis.

6.7.1.1 Statistical analysis of genes and elucidating similarly expressed genes between conditions using a Venn diagram

All samples were pre-processed using the GC-RMA algorithm and quality control was performed on the three macrophage conditions i.e. cell contact, RA or PsA. In order to pass the quality control filter each gene had to have a signal value between 20 and 100% and be expressed in all of the samples of each condition. To establish the presence of differentially expressed genes in the three activated macrophage conditions paired T Tests were first performed with a p value cut off of 0.05 followed by the Benjamini and Hochberg multiple testing correction (MTC). However an MTC was unable to be used in the cell contact comparison as there were no significantly changed genes between the unactivated macrophages and cell contact activated macrophages. Since a p value cut off of 0.05 was used this means that 5% of the differentially expressed

genes may be false positives and appearing by chance alone, a way to overcome this is to look at the p values of each gene as the higher the p value of the gene the less chance of it being a false positive. This statistical analysis gave the following results:

- Unactivated M-CSF macrophages vs cell contact activated macrophages (n=5 matched samples) 3902 significantly expressed gene entities
- RA PB vs RA SF (n=8 matched samples) 10879 significantly expressed gene entities
- PsA PB vs PsA SF (n=8 matched samples) 7401 significantly expressed gene entities

The up- and downregulated genes with a p value <0.05 from each of the three comparisons were then inputted into a Venn diagram to distinguish if there were similar genes being expressed by the activated macrophages conditions.

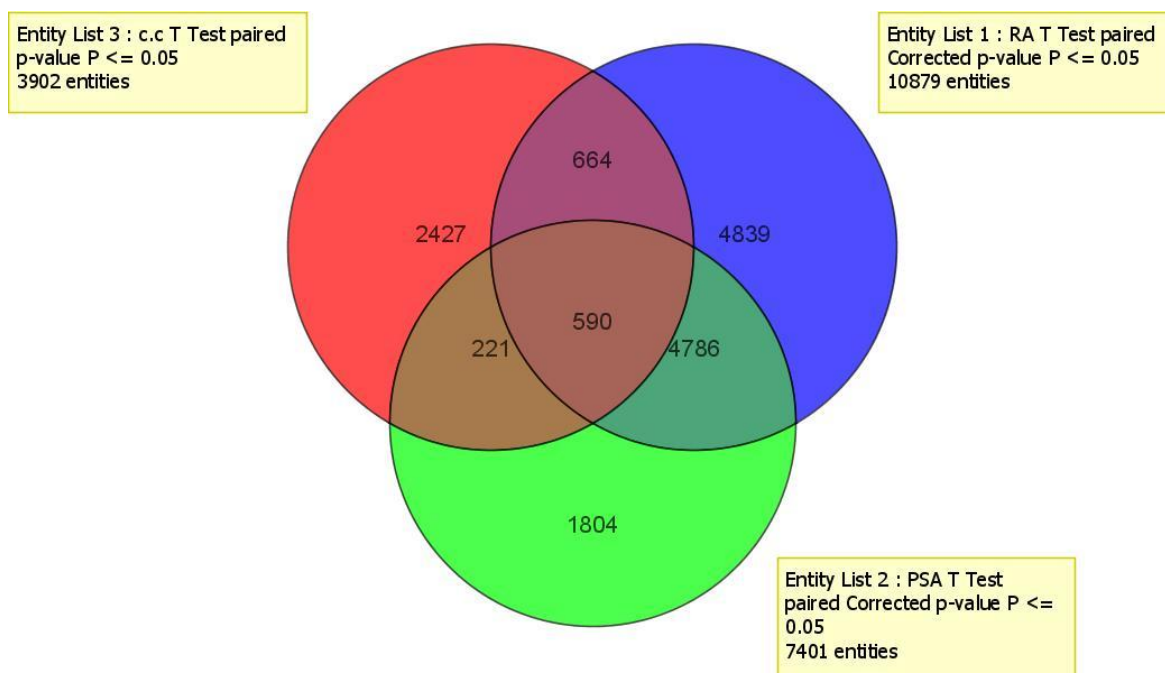


Figure 6.7 Venn diagram of the significantly differentially expressed genes within the cell contact activated macrophages, RA synovial fluid macrophages and PsA synovial fluid macrophages

Paired T Tests were performed between the unactivated and cell contact activated macrophages, RA blood and RA synovial fluid macrophages and between PsA blood and PsA synovial fluid macrophages. The up and downregulated genes in the activated macrophage samples were then put into a Venn diagram, blue represents the differentially expressed genes in the RA condition, green represents the PsA condition and red represents the cell contact activated macrophages. The genes that were expressed by cell contact macrophage samples and PsA or RA or by all three conditions were analysed further.

Figure 6.7 shows differentially expressed genes in all three conditions, blue represents the significantly expressed genes between RA PB and RA SF CD14⁺ samples, green represents the significantly changed genes between PsA PB and PsA SF while red represents the significantly expressed genes between unactivated M-CSF macrophages and cell contact activated macrophages. Analysing the Venn diagram it can be seen that there are three groups of genes being expressed in cell contact activated and synovial fluid macrophages:

- Genes expressed in all conditions (cell contact, RA SF and PsA SF macrophages). 590 genes
- Genes expressed in cell contact macrophages and PsA SF macrophages. 221 genes
- Genes expressed in cell contact macrophages and RA SF macrophages. 664 genes

The Venn diagram also indicates that only a small proportion of the genes expressed by the RA or the PsA synovial fluid are expressed within the cell contact activated macrophages. 10% (811 of 7401 genes) of PsA genes and 11% (1254 of 10879) of RA genes are expressed in the cell contact activated macrophages.

The three gene groups listed above were then subjected to fold change analysis where the cut off threshold was set at a fold change ≥ 2 . This analysis gave the following results:

- 149 genes expressed in all three conditions (c.c, RA SF, PsA SF)
- 40 genes expressed in both cell contact macrophages and PsA SF
- 146 genes expressed in both cell contact macrophages and RA SF

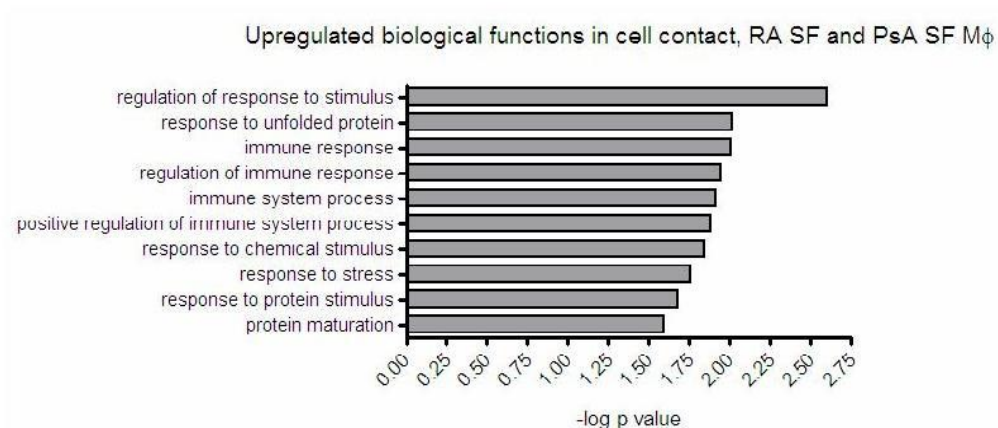
Since the basis of this investigation was to determine how similar the cell contact activated macrophages were to the synovial fluid macrophages, the gene lists generated from the fold change analysis were scrutinised further to only include genes that were up or downregulated in both the cell contact samples and the synovial fluid samples. This is due to the discovery that many genes were being expressed in different directions, for example a gene that was upregulated in the cell contact activated samples was downregulated in the synovial fluid samples. As a result the number of similar genes from cell contact and synovial fluid macrophages decreased further:

- 110 genes expressed in all three conditions (72 downregulated genes and 38 upregulated genes)
- 18 genes expressed in cell contact macrophages and PsA SF (6 downregulated genes and 12 upregulated genes)
- 128 genes expressed in cell contact macrophages and RA SF (88 downregulated genes and 40 upregulated genes)

6.7.1.2 DAVID analysis of the differentially expressed genes in cell contact activated macrophages and synovial fluid macrophages

DAVID software was used to analyse the up- and downregulated genes that were changed in the cell contact macrophages and the synovial fluid macrophages to determine the overall biological functions of these genes. DAVID analysis did not find any significantly overrepresented processes within the gene list expressed in both the cell contact and PsA SF macrophage samples, which was likely to be due to the small number of genes within the list. Figure 6.8 demonstrates the top ten overrepresented biological processes associated with the upregulated genes found in all 3 macrophage conditions (A) and the downregulated genes found in all 3 macrophage conditions (B). The results of this analysis indicate that the macrophage samples are upregulating genes associated with immune response and response to stimulus and stress. The same macrophage samples are also downregulating genes associated with intracellular signalling cascade and regulation of myeloid cell differentiation. Figure 6.9 shows the biological processes associated with the upregulated genes found in both the cell contact and RA SF macrophages conditions (A) and the biological processes associated with the downregulated genes in both the cell contact and RA SF macrophage conditions (B). The upregulated genes are involved in processes such as intracellular signal transduction and phosphorylation. The downregulated genes are involved in processes such as cellular metabolism and T cell differentiation.

(A)



(B)

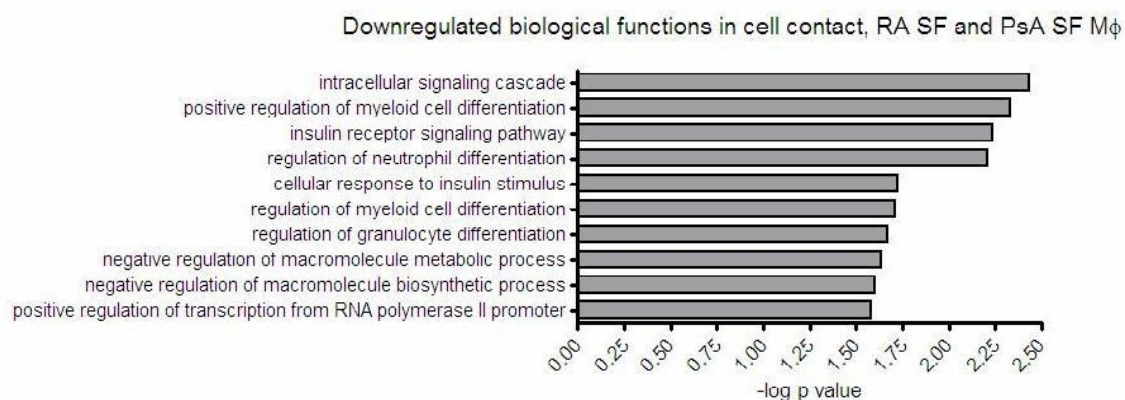
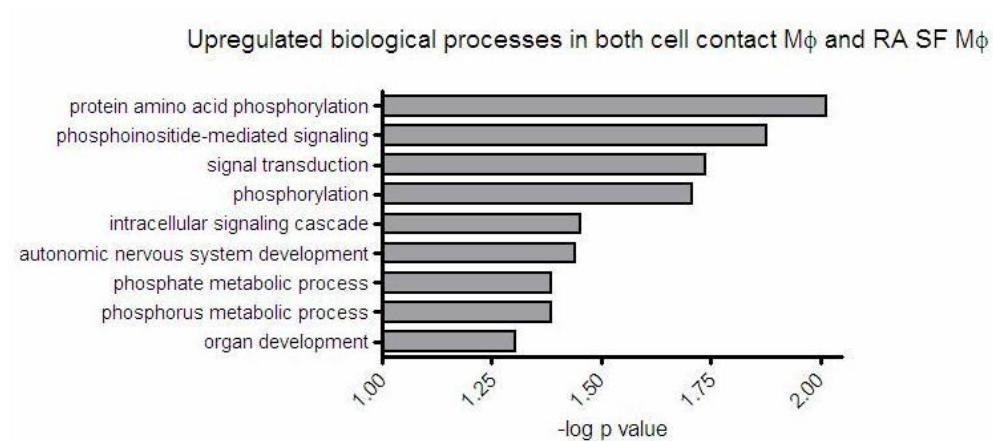


Figure 6.8 Up- and downregulated biological processes in cell contact, RA SF and PsA SF macrophages

DAVID software was used to analyse the genes which were differentially expressed in all three macrophage conditions to determine overrepresented biological processes associated with the genes. The top ten significantly overrepresented processes are shown here with a p value <0.05 which is equivalent to a $-\log p$ value > 1.30

(A)



(B)

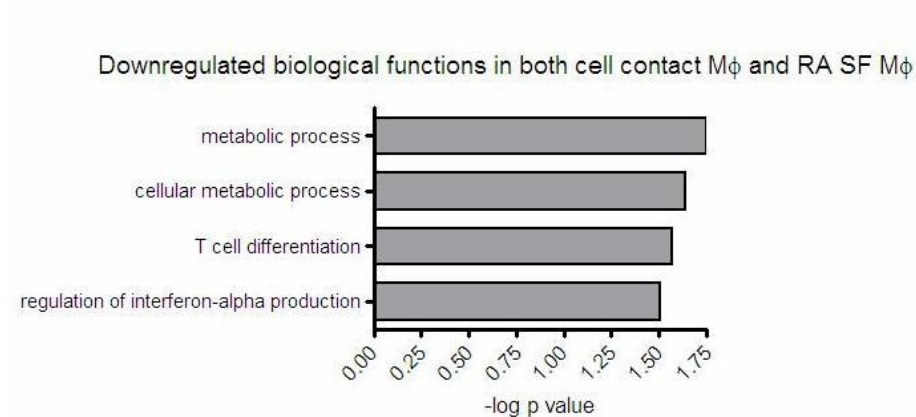


Figure 6.9 Up- and downregulated biological processes in cell contact and RA SF macrophages

DAVID software was used to analyse the genes which were differentially expressed in cell contact and RA SF macrophage conditions to determine overrepresented biological processes associated with the genes. The top significantly overrepresented processes are shown here with a p value <0.05 which is equivalent to a $-\log p$ value > 1.30

6.8 Discussion

The *in vitro* model of inflammatory arthritis consists of direct cell contact activating M-CSF differentiated macrophages with cytokine activated T cells. Microarray analysis of one cell type requires a pure cell population as contaminating cells would have an effect on the genetic profiles obtained. Therefore we devised a method of isolating the activated macrophages from the T cells by CFSE staining the T cells and using the FACS ARIA to separate the cells based on their fluorescence. This method ensured that no antibody or CFSE staining of the macrophages took place as any manipulation of these cells may have altered their genetic profile. However, the FACS ARIA plots indicated that a large amount of cellular debris had been collected in the macrophage cell fraction which may have ultimately affected the macrophage purity and subsequently the transcriptomic profile of the cell contact activated macrophages.

A 2 hour timepoint was demonstrated to be the appropriate duration of cell contact activation for the macrophages due to variability in the quality and yield of RNA after this time. It is difficult to fully explain these variable findings. However, the low quality RNA in the cell contact activated macrophages, as visualised by the empty electropherogram plots seen after $t = 8$ hours in Figure 6.4, may be due to very low amounts of RNA being run through the Agilent Bioanalyser. The sequential decrease in the yield of macrophage RNA over the duration of the cell contact activation was also an unusual finding but may be due to increased apoptosis and cell loss which would therefore decrease the yield of RNA from the cells. As a result of these findings, we decided to analyse the macrophages after they had been cell contact activated for 2 hours, it was also decided that this timepoint was more appropriate for analysing the early response genes that would be differentially expressed within the macrophages. Crucially however this may not represent the gene profile present in a chronically activated macrophage derived from within the synovial compartment and in this sense the outcome was perhaps predictable.

After completion of the microarray experiment and removal of the outlier cell contact macrophage sample from further analysis a PCA plot of the samples was setup to identify trends in the data since samples clustered within the same area

of the PCA graph are considered to be genetically similar [354]. This analysis identified that the M-CSF differentiated macrophages were genetically distinct from the Tck activated macrophages which suggests that being in cell contact with the Tcks for 2 hours induced a transcriptional change in the macrophages. However, the PCA analysis also demonstrated that the cell contact macrophages were genetically distinct from the synovial fluid macrophages.

Previous work has demonstrated that the cytokine activated T cells (Tcks) used in this *in vitro* system are similar to RA synovial T cells, as they up-regulate the same cell surface activation markers and adhesion molecules and also induce the production of various cytokines and chemokines from macrophages in a cell contact dependent manner [298, 308, 309]. Recently, a microarray analysis of monocytes activated for 24 hours by PMA and PHA stimulated T cell membrane fragments was performed by Gruaz et al who analysed the anti-inflammatory effects of high-density lipoproteins (HDL) on the transcriptome of the activated monocytes. They discovered that 666 probe sets were altered in the activated monocytes compared to unactivated monocytes indicating that T cell contact activation of monocytes does have an effect on the transcriptome of the cells [315]. Hence, for our study it was hypothesised that cell contact activation of macrophages by fixed Tcks for 2 hours should indicate the early response genes upregulated by the macrophages. Given that the Tcks have been demonstrated to be similar to synovial T cells, the genes upregulated in these macrophages should be similar to those expressed in the synovial fluid CD14⁺ cells.

The three macrophage conditions of cell contact, PsA synovial fluid and RA synovial fluid were compared to their matched unactivated or baseline samples, which in this comparison were unactivated M-CSF macrophages, RA PB CD14⁺ cells and PsA CD14⁺ cells. After determining the differentially up- and down-regulated genes in these activated conditions a Venn diagram was used to discover if any genes overlapped with those of the other conditions. The initial results of the Venn diagram were disappointing as it demonstrated that only 10% of the total significantly expressed genes in the PsA SF condition and 11% of the total expressed genes in the RA SF condition were expressed in the cell contact macrophages indicating that these conditions were not significantly similar. A 2-fold change analysis was subsequently performed on the gene lists which were then examined further to ensure that they were all differentially expressed in

the same direction. For example, if a gene was differentially expressed in both the cell contact activated and PsA synovial fluid conditions it had to be either up-regulated or down-regulated in both conditions but could not be up-regulated in one condition and down-regulated in the other since we were investigating the similarities between the conditions. This resulted in further decreasing the size of the gene lists and of the 54,675 probe sets on the Affymetrix GeneChips less than 130 were expressed in each of the three gene lists of i) expressed in cell contact, RA SF and PsA SF conditions, ii) expressed in cell contact and RA SF, iii) expressed in cell contact and PsA SF.

DAVID analysis was performed on the up- and down-regulated genes in each of the three comparisons to determine the biological function of the similarly expressed genes. DAVID analysis of the genes present in all conditions indicated that all three macrophage types were up-regulating genes involved in response to stimuli. In the cell contact macrophages this would be as a result of Tck contact and in the synovial fluid macrophages this would be as a result of direct cell contact as well as the pro-inflammatory environment. All three cell conditions are also up-regulating genes associated with the immune response and its regulation, this would be expected in the synovial fluid macrophages since it has been well documented that the cellular influx into the joint is exposed to many pro-inflammatory mediators [537, 682-685] which would result in the up-regulation of these genes. However, it is reassuring to observe that the cell contact activated macrophages also up-regulate genes involved in immune system regulation since these cells are used as an *in vitro* model of arthritis. This also reflects the observations seen in the Gruaz et al microarray analysis of monocytes activated with PMA and PHA stimulated T cells which demonstrated an up-regulation of cytokines and mediators involved in the immune system [315]. The biological processes of the down-regulated genes in all three conditions include intracellular signalling cascade, RNA polymerase II transcription, and regulation of myeloid cell differentiation suggesting that the macrophages could be trying to regulate the increased transcription and signalling observed in cell contact activated and synovial cells as a result of extracellular stimuli.

DAVID analysis of the genes up- and down-regulated in the cell contact macrophages and the RA synovial fluid macrophages demonstrated that the up-

regulated genes were involved in processes such as signal transduction and intracellular signalling cascade. Signal transduction pathways provide an intracellular mechanism by which cells can respond and adapt to their environment. Proinflammatory stimuli found within the synovial environment can activate signalling cascades within a cell to alter gene transcription and protein expression. Several signal transduction pathways have been implicated in RA such as mitogen-activated protein kinase (MAPK) and NF- κ B as well as the transcription factors janus kinase (JAK) and signal transducer and activator of transcription (STAT) [688, 689]. Signal transduction pathways, specifically NF- κ B but not PI-3 kinase, have also previously been shown to play a role in the production and secretion of pro-inflammatory cytokines within the cell contact activated macrophages [298]. The results from this DAVID analysis highlight the importance of signal transduction and intracellular signalling in allowing the synovial macrophage to contribute to the pro-inflammatory milieu found in the synovium and also emphasises the ability of the cell contact model of arthritis to replicate this biological process found in the synovial macrophage.

The biological processes of the down-regulated genes in RA synovial fluid and cell contact activated macrophages were metabolic process, T cell differentiation and regulation of IFN- α production. IFN- α is a proinflammatory cytokine that is present within the synovial fluid of RA patients and the macrophage cells may be down-regulating genes associated with its production as an anti-inflammatory regulatory mechanism [690]. The T cell differentiation process is a misleading title as two of the three genes associated with this function (the transcription factor Ikaros and the phosphatase PTPN22/Lyp) have been demonstrated to be expressed in macrophages as well as T cells [691, 692] and the third gene, ZNF278, a zinc finger repressor of gene expression has not been previously demonstrated to be present in T cells. This result highlights the point that the genes associated with each biological process may need to be analysed further to understand why the process is associated with the gene list since genes can appear in several biological process pathways and each gene can be expressed by more than one cell type.

DAVID analysis could not be performed on the shared PsA synovial fluid and cell contact activated macrophages due to the small number of genes within this gene list. However, analysis of the gene list demonstrates that both PsA

macrophages and cell contact activated macrophages are upregulating inflammatory genes such as the cytokine TNF α which has a pronounced role in the pathogenesis of arthritis. These two macrophage types are also upregulating genes such as NRP2 (neuropilin 2) which is a surface receptor originally identified as a factor involved in nerve fibre repulsion during neuronal growth [693]. Neuropilin 2 positive nerve fibres have been detected in the synovium of rheumatoid arthritis patients and soluble neuropilin 2 has also been detected in the synovial fluid of these patients where it was hypothesised to aggravate the loss of sympathetic nerve fibres which is observed in RA [694]. Consequently despite the low numbers of genes that are the same in the PsA and cell contact macrophages, the genes that are shared between the subgroups are of an inflammatory phenotype and have been demonstrated to be involved in the destructive phenotype of arthritis.

The common genes between all three conditions and between cell contact activated macrophages and RA synovial fluid macrophages were revealed to share biological processes such as immune system regulation, which demonstrates that after just 2 hours the Tck contact activation is eliciting an immune response in the M-CSF macrophages similar to that seen in synovial fluid derived macrophages. Nevertheless, this study has also demonstrated that of all the genes differentially expressed between RA PB and RA SF CD14⁺ and between PsA PB and PsA SF CD14⁺ only 11% and 10%, respectively, of those genes are also differentially expressed between the M-CSF and cell contact activated macrophages. This result would indicate that the cell contact model of inflammatory arthritis may not be a suitable *in vitro* model for arthritis since the cell contact activation does not appear to effectively replicate the same level of transcriptional expression within the M-CSF macrophage compared to a synovial fluid macrophage isolated from a patient.

Nonetheless, there are several possible reasons for this discrepancy. Firstly, the FACS ARIA separation suggested that a large proportion of collected events for the macrophage cell fraction was cellular debris which may have been created during the removal of the macrophages from the culture wells after cell contact activation. The presence of this debris would affect the overall purity of the macrophage cells and may have contained cellular components and nuclear material which could have affected the transcriptomic measurement and

analysis. In addition, due to time restrictions only 6 normal donors were able to be recruited for the cell contact activation samples and since one of these samples was the outlier identified at the start of the microarray analysis its removal meant that only 5 matched cell contact samples were compared to 8 matched RA and 8 matched PsA samples. Therefore the full spectrum of the cell contact transcriptome may not have been analysed. The cell contact donors were also only age and sex matched to the PsA patients who had a smaller mean age compared to the RA patients. Many papers have described the effect of aging on epigenetics such as altered DNA methylation and histone modification which in turn impact on chromatin folding [695], women are also more predisposed to rheumatic diseases due to hormonal as well as genetic differences between males and females [696]. Hence not including age and sex matched donors to the RA patients may have automatically discarded genes associated with these two factors.

As noted at the outset of my discussion, the final and most important variable which may have affected the gene expression of the cell contact activated macrophages was the duration of activation. During the cell contact model the macrophages are usually stimulated for 24 hours with the Tcks before analysis, however due to our interest in the early response genes expressed in the macrophages and the variability in the macrophage quality and yield it was decided to analyse the cells after a shorter duration. Consequently trying to compare macrophages which have been activated for 2 hours to macrophages from the synovial fluid which have been activated for an unknown duration presents problems. A recent paper which analysed the stability and temporal changes in inflammatory gene expression in TNF α stimulated murine fibroblasts found three groups of mRNA expression: group 1 genes had a peak mRNA expression at 0.5 hours after stimulation but their expression was unstable and it fell to baseline after 1 hour; group 2 genes had a peak expression at 2 hours however upon the removal of the TNF α stimulus their expression fell to baseline; group 3 genes had peak expression 12 hours after the stimulus, these genes were transcribed at a slow rate but the resulting mRNA was very stable [697]. Subsequently if the same pattern of mRNA expression was occurring within the cell contact activated macrophages then analysing these cells after 2 hours would only detect the group 2 genes as mRNA from the group 1 genes would

probably already be in the process of degradation and the group 3 genes would not be expressed at a detectable level.

In summary, despite the cell contact activated macrophages only sharing 10-11% of the genes expressed in RA or PsA synovial fluid macrophages those genes involve a high proportion of immune system regulation genes indicating that this system is upregulating the types of genes thought to be involved in the perpetual immune activation seen in macrophages of the arthritic joint. However, to fully elucidate the appropriateness of this cell contact system as a model of inflammatory arthritis an extended microarray experiment would have to be set up in which each RA and PsA patient had an age- and sex-matched donor for the cell contact activation and particular care was taken when isolating the macrophage cells from the cell contact to ensure a high cell purity. The cell contact activated cells would ideally be activated over a timecourse of 1-24 hours to determine the different groups of genes being expressed within these cells over time.

Chapter 7 Validation of an *in vitro* live cell imaging system

7.1 Introduction

The macrophage - T cell co-culture experiment is a well established *in vitro* model of inflammatory arthritis. The method of cytokine activation of the T cells for this *in vitro* model confers a pro-inflammatory synovial phenotype which can induce the release of several cytokines and chemokine from the macrophages via direct cellular contact as has previously demonstrated by the vast amount of research performed by our group and several others [298, 308, 309, 408]. Whilst we have obtained a wealth of information regarding several aspects of the *in vitro* model such as the signalling pathways involved in the cytokine and chemokine release from the macrophages as well as the adhesion molecules upregulated by the cytokine activation of the T cells, there are still important facets of this model which have not been explored. One important question which we addressed in the previous chapter is how similar is the transcriptome of the T cell contact activated macrophage compared to the synovial macrophage. However, another important aspect of the cell contact model is determining how the macrophages and T cells in this model interact and how long these interactions last for; this information can be determined using *in vitro* live cell imaging.

Live cell imaging is a widely used technique which has become more sensitive and efficient in recent years with the introduction of new imaging techniques and machines such as two-photon laser scanning microscopy. A large amount of the current work using live cell imaging has focused on the *in vivo* interactions between DCs and T cells within the lymph node [698]. These studies have been very informative in determining the behaviour of the T cell - DC interactions during antigen presentation. Such studies have revealed that when T cells are moving around the lymph node they follow a chaotic trajectory with random movements and are not directed by chemotactic gradients over large distances as was previously thought [699]. Live cell imaging has also demonstrated that T cells may make several contacts of a short duration with antigen loaded DCs before forming a stable cell-cell immunological synapse contact with one DC. The use of live cell imaging has made it possible to visualise the movement of the T cells towards the DCs during antigen presentation and several papers have proposed models for the type of movements and behaviours observed by the T cells before they form stable, long-lived interactions such as “drive by” which is a situation

where the T cell will have a short interaction with the DC but will then move away from the DC and carry on migrating [700].

Despite the wealth of knowledge about the interactions of T cells and DCs gained from live cell imaging there has been far fewer investigation into the interactions between T cells and macrophages either *in vivo* or *in vitro*. One study performed by Underhill et al imaged such interactions using OVA specific murine T cells containing a reporter construct which caused the cell to express green fluorescent protein (GFP) upon productive engagement with OVA presenting macrophages. Using *in vitro* live cell imaging this group demonstrated that 90% of the T cells appeared to move around the surface of individual macrophages and 50% of T cells migrated from one macrophage to another during 3 hours of time lapse imaging. Underhill et al also determined that the T cell – macrophage interactions had to be sustained for 2 hours for the T cell to become fully activated and express GFP however due to the behaviour of the T cells this activation may be the summing up of multiple activatory signals during the numerous interactions with the macrophages [701]. This study was analysing the T cell – macrophage interactions in the presence of antigen and at present there are no studies analysing such *in vitro* interactions in an antigen independent environment.

Histological analysis of the inflamed synovium has shown that macrophages are found in close contact with T cells [286] indicating that direct cell contact may be a mechanism causing the production of pro-inflammatory cytokines. Subsequent studies using the *in vitro* cell contact model have demonstrated this observation to be correct since the incubation of synovial-like Tcks with macrophages induces the production of pro-inflammatory cytokines and chemokines as well as anti-inflammatory cytokines such as IL-10 [269, 308]. Despite the large amount of experimental work on the cell contact model of arthritis there are still gaps in our knowledge regarding the mechanisms of the cell–cell interactions and the behaviour of the cells during the co-culture. Therefore we set out to use an inverted microscope to image these antigen independent interactions in real time. We aimed to determine the number and duration of Tck–macrophage interactions and the overall area of these interactions. Different variables such as using inactivated T cells or fixed Tcks were analysed to visualise their effects on the interaction measurements. Once

the behaviour and movement of the cells during the cell contact model have been fully understood, the live cell *in vitro* imaging system could then be used to analyse the impact that blocking molecules or proteins may have on the Tck–macrophage interaction. It was our intention to bring this imaging system together with the preceding microarray data to visualise the effect of altering key genes, which had identified as being differentially expressed in the synovial macrophages, on the cellular interactions taking place and also whether this may be affecting the cytokine/chemokine profile of the cell contact.

7.2 Real-time imaging

Figure 7.1 demonstrates the experimental set up to allow real time imaging of the cell contact events. The conditions being compared may differ from those in the figure however the general experimental set up was the same. Macrophages were isolated from a healthy donor and stimulated for 5 days with M-CSF before being stained with the red fluorescent dye CMTPIX and plated out onto a specialised imaging culture chamber for 24 hours to allow the cells to adhere. T cells isolated from the same donor which were treated under differing conditions were stained with the green fluorescent dye CFSE and cultured with the macrophages. The cultures were incubated for 2 or 24 hours (as indicated in the text) and then imaged on an inverted microscope. Each condition was imaged for varying lengths of time therefore each co-culture set up was often staggered for each condition to maintain a constant incubation time before being imaged.

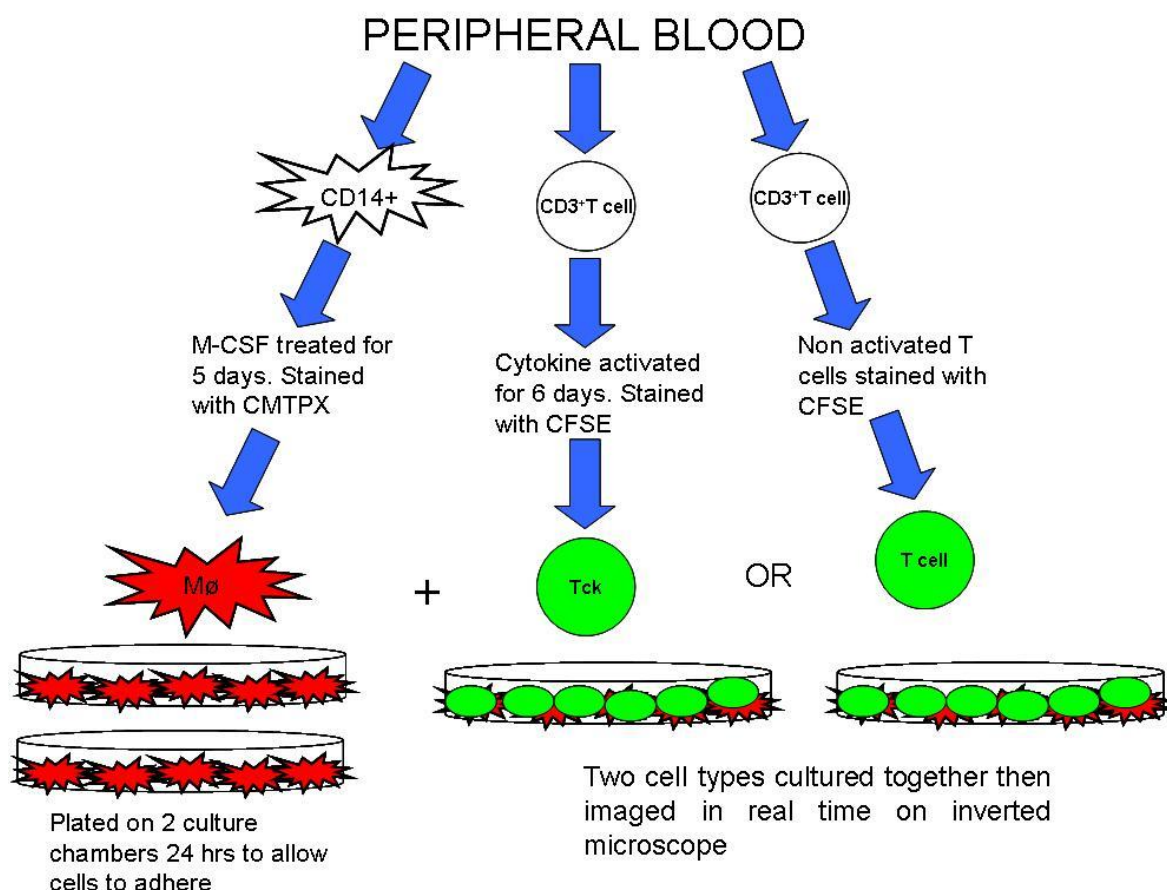


Figure 7.1 Outline of in vitro real time imaging experimental setup

An example of the experimental set up for real time imaging demonstrating how a comparative analysis between a macrophage (Mφ) and Tck culture compared to a macrophage and unstimulated T cell culture would be set up. For this experiment macrophages and T cells are isolated from the donor, T cells are stimulated for 6 day with cytokines to generate cytokine activated T cells (Tcks) and the monocytes are stimulated for 5 days with M-CSF before being stained red with a CMTPIX dye and plated out onto two imaging chamber for 24 hours to allow them to adhere to the chamber. On day 6 T cells are isolated from the same donor and stained green with CFSE and the Tcks are also stained green. Each of the two T cell conditions are then incubated with macrophages and the two cell contacts are incubated for a specified duration before being imaged on the microscope.

7.2.1 Imaging on the microscope

Once the cell contact experiments had been cultured for the specified duration the co-cultures were imaged on an inverted microscope which had a heated stage to maintain the cultures at 37°C. For each imaging condition three movies were taken of three separate areas of the co-culture chamber. Once the area to be visualised had been chosen the microscope was set up to take three images every 12 seconds. The three images consisted of a green fluorescent image, a brightfield image and a red fluorescent image as shown in Figure 7.2. The brightfield image was taken to ensure that the camera remained aligned while the green and red fluorescent images were merged to obtain a single timepoint image. When all of the merged images were played in sequence they produced a real time movie of the *in vitro* cell contact model of inflammatory arthritis.

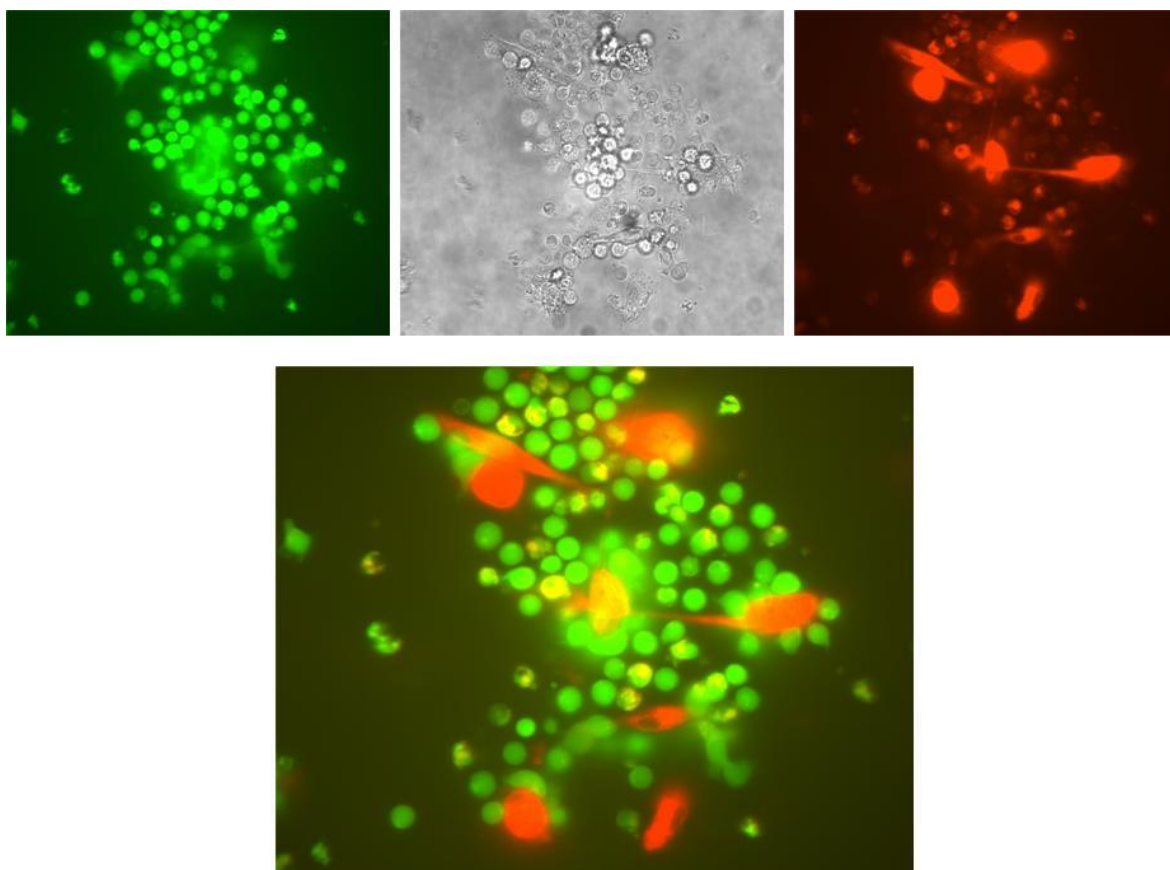


Figure 7.2 Example of the images taken by the inverted microscope for the real time imaging

During the real time imaging the inverted microscope takes three consecutive images. An image of the green fluorescence, a brightfield image and an image in red fluorescence (top three images of panel). The red and the green fluorescent images are then merged to make the single timepoint image (bottom image of panel). The brightfield image is not used in the final timepoint image but is taken to ensure that the microscope remains aligned.

7.2.2 Quantitative measurements of imaging

Once generated, the real-time images and movies were analysed to determine if there is a difference in the interactions between experimental conditions. Volocity is specific imaging software which allows factors such as the number and duration of T cell-macrophage interactions to be examined as well as the colocalization coefficient between the two cell types.

7.2.2.1 Number and duration of interactions

The number and duration of each macrophage-T cell interaction during the course of the real time movies were calculated using the Volocity software. This software has specific functions that can detect red fluorescence (representing the macrophage cells) and green fluorescence (representing the T cells) and then determine where these two fluorescence signals intersect i.e. where the two cell types are interacting. The software measures the interactions taking place in the field of view for the first time frame of the movie and then calculates how long these interactions last for the duration of the imaging. It also measures any new interactions and their duration taking place during the co-culture therefore enabling the user to determine exactly how many interactions took place between the two cell types and how long these interactions last for. The basis for how the software identifies these cell-cell interactions is shown in Figure 7.3.

7.2.2.2 Colocalization coefficient

The Volocity software can also be used to measure the colocalization coefficient of the macrophage-T cell interactions. In the context of digital fluorescence imaging, colocalization can be described as the detection of signal at the same pixel localisation in each of two channels. The two channels are made up of images of two different fluorochromes taken from the same sample area. The Volocity software therefore detects how many yellow pixels are in each time frame image as the yellow pixels are representing the position where the two fluorescent colours (green and red) are overlapping and hence where the two different cell types are interacting. The colocalization coefficient therefore analyses the area of interaction between the two cell types, it has an advantage over the number and duration analysis as statistical analyses can be made on the

colocalization coefficient values between two different experimental conditions to determine if there is a significant difference between the areas of colocalization. In summary there are two different methods of analysis for the real time movies generated of the *in vitro* model of inflammatory arthritis. They are both constructive methods of analysis and enable the amount and duration of interactions as well as the overall areas of interactions to be measured.

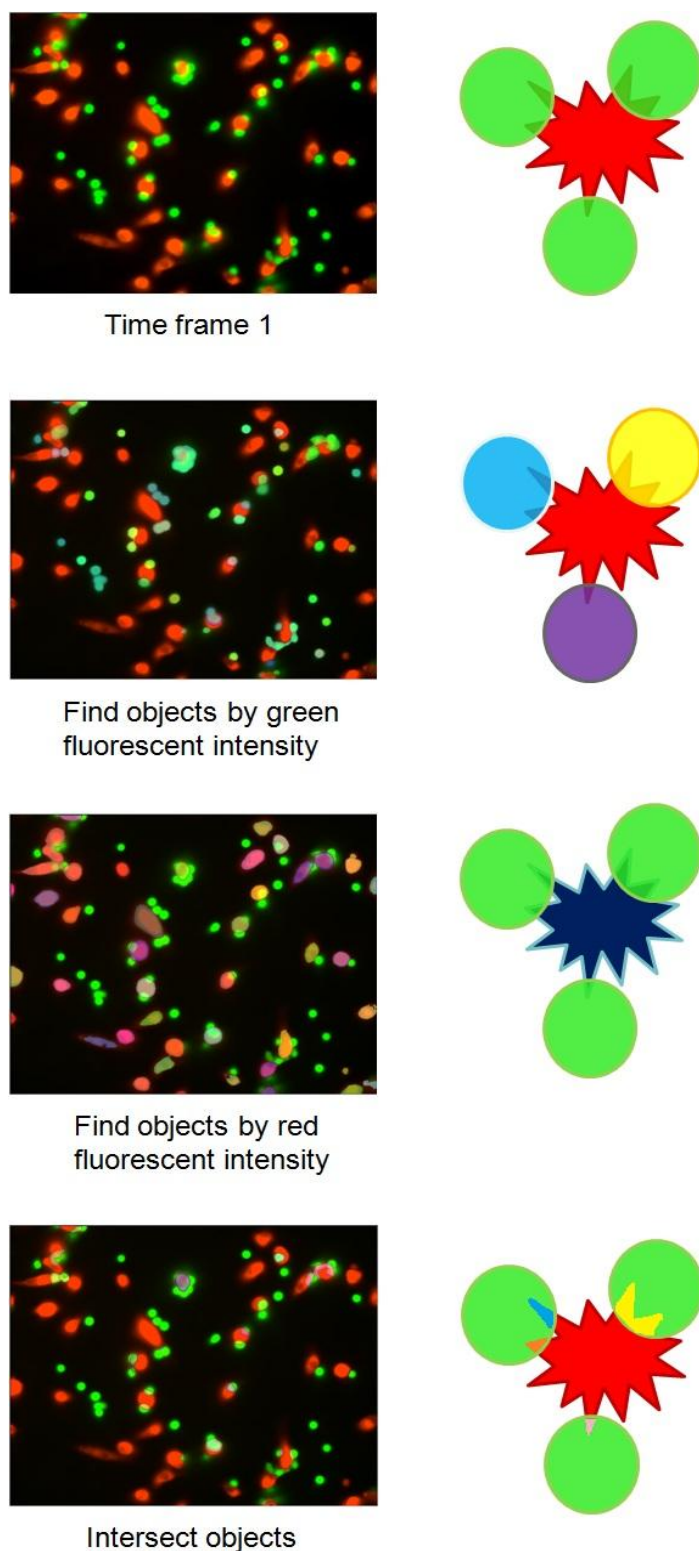


Figure 7.3 Velocity software method for determining the macrophage-T cell interactions Each macrophage-T cell culture movie is made up of individual frames taken by the microscope which, when played in sequence, produces the real time movie. To determine the number and duration of interactions taking place during each movie Volocity begins by analyses the first time frame of the movie. In this image Volocity identifies objects which are fluorescing green (T cells) and identifies objects which are fluorescing red (macrophages). It highlights each new object it identifies in a different colour. The software then identifies the areas of intersection between the red and green objects and each area is classed as an interaction between the two cell types. The software will then track these interactions and any new interactions which form during the movie and determine how long each interaction lasted for. The cartoons on the right hand side of the figure depict how the software views the cells and locates the areas of interaction.

7.3 Comparison of cytokine activated T cells to unstimulated T cells

In the cell contact model of inflammatory arthritis the T cells are stimulated with a specific cytokine cocktail for 6 days, this stimulation induces an activated T cell (Tck) phenotype which resembles T cells isolated from an RA synovial joint. Not only do these Tcks express a similar cell surface ligand and activatory marker phenotype as the synovial T cells they also induce a similar pro-inflammatory cytokine and chemokine profile in macrophages by direct cell contact [269, 298, 308, 309]. To establish whether the cytokine activation of the T cells induced more interactions with the macrophages or a larger area of interactions a comparative analysis of Tcks and inactivated T cells took place.

Figure 7.4 is a representative of three independent experiments performed. Since each co-culture condition was imaged three times in separate areas of the culture chamber panel, Figure 7.4 (A) shows individual time frames of a representative movie for each culture condition taken at $t=0$, $t=60$ seconds and $t=120$ seconds for the macrophage–T cell co-culture (top panel) and the macrophage–Tck co-culture (bottom panel). The white arrows on the macrophage–Tck co-culture images indicate Tck cell movement during the course of the movie. Upon comparison of the two sets of images it can be seen that many Tcks are clustered around the macrophages which is not visualised in the non activated T cells images. Consequently, the cytokine stimulation of the T cells would appear to induce a higher rate of clustering and interaction with the macrophages compared to T cells from the same donor which have not been stimulated.

This pattern of interaction can also be seen in the two real time movies represented by the panel of images in part (A) of Figure 7.4. The supplementary file designated movie 1 demonstrates the inactive T cell – macrophage interactions. It can be seen that there is little T cell movement within the field of view. There are also only a small number of interactions taking place between the T cells and the macrophages which appear to be stable. The movie for the Tck–macrophage co-culture is supplementary file movie 2 and from this short movie it can be seen that the majority of interactions are stable long-lived interactions where many Tcks have clustered around one macrophage indicating

their increased activation status compared to the inactivate T cells. There are only a small number of Tcks which have not formed stable interactions moving though the field of view therefore it is difficult to comment on the migration pattern of the Tcks. It is likely that in the 24 hours prior to the imaging of the co-culture the majority of the Tcks have already formed and established stable interactions with the macrophages therefore a shorter imaging timepoint may be necessary in order to visualise the how the Tcks migrate and behave. I note also that the experiment is not performed under formal flow conditions which might alter the adhesive activities of cells.

Volocity analysis was used to analyse the movies in order to determine the number and duration of interactions which are shown in Figure 7.4 (B). These graphs show the number of interactions on the y axis and the duration of interactions in time frames on the x axis. One time frame is equivalent to a duration of 12 seconds since this is the unit of time between each image being taken on the microscope. The graphs represent the average number and duration of interactions throughout the three movies taken of each co-culture condition. The graph on the left hand side demonstrates that the Tcks (red line) have more sustained interactions with the macrophages. These interaction last for a longer duration which is identified by the peak of interactions just before time frame 200 and at time frame 250 which is not seen in the T cell co-culture (black line). Due to the difficulty in visualising the difference in the number of short duration interactions between the two co-culture conditions all interactions which lasted up to 10 minutes, equivalent to 50 time frames, were plotted on a separate graph (demonstrated by the right hand side graph). Analysing this graph illustrates that as well as having more sustained interactions with the macrophages the Tcks also have a higher number of shorter duration macrophage interactions compared to the T cells. This can be most easily seen at time frames 5 to 10 where the Tcks consistently exhibit a higher number of interactions compared to the T cells. Volocity analysis of the colocalization coefficient in Figure 7.4 (C) reveals that the average area of interaction was significantly higher in the three movies of the Tck co-culture compared to the three movies of the T cell co-culture ($p < 0.0001$).

Hence these results demonstrate that cytokine activation of the T cells does induce more interactions with the macrophages, these interactions are more

sustained and have a significantly larger area. However, analysis of the movies demonstrates that there is little Tck movement towards the macrophages as most of the Tcks have migrated towards and maintained their interactions with the macrophages in the 24 hour period prior to microscope imaging. Since we are also interested in visualising how the Tcks migrate towards and interact with the macrophages it was decided to analyse the co-culture experiments after a shorter 2 hour duration of incubation.

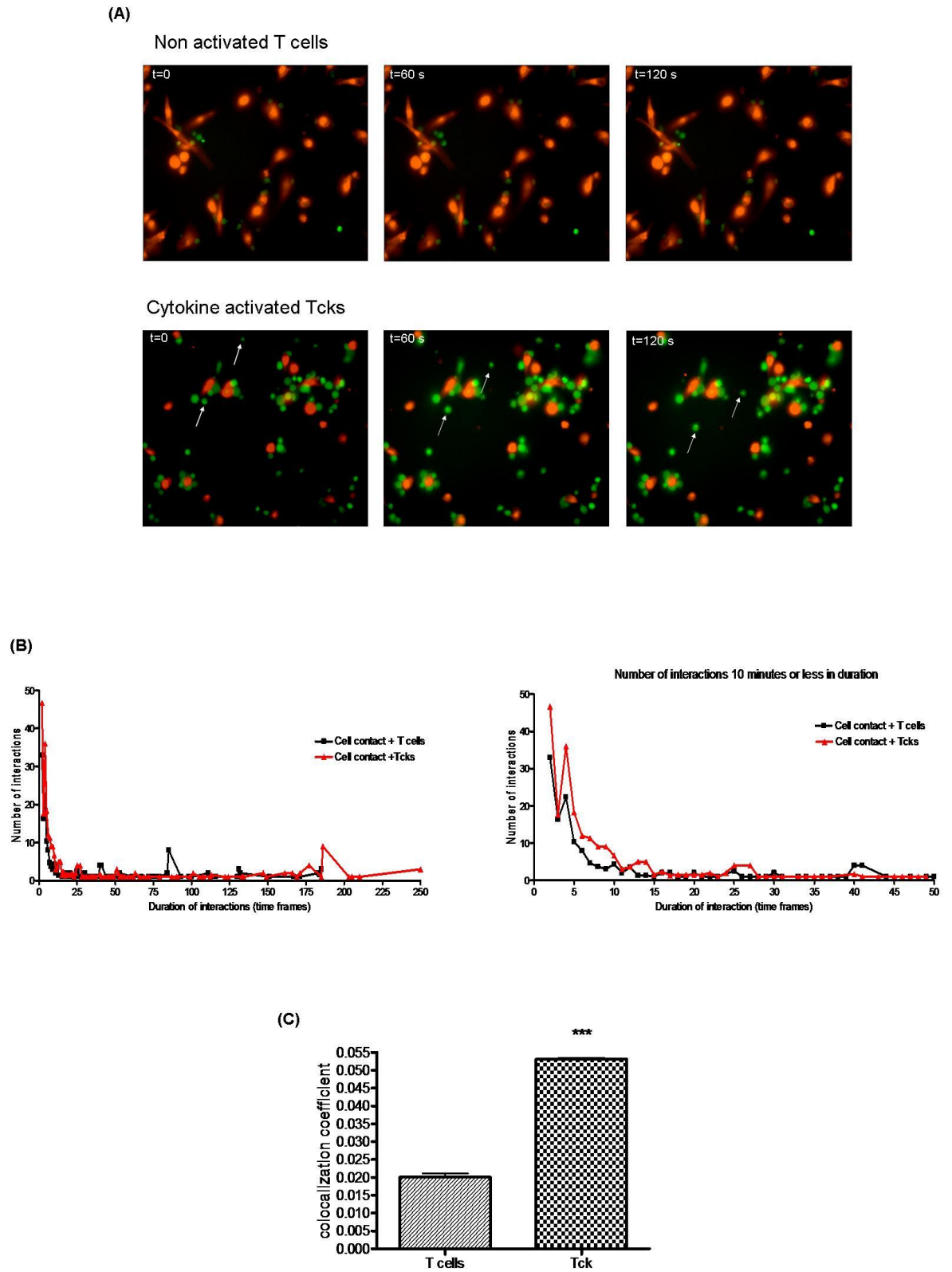


Figure 7.4 Imaging Tcks or non-activated T cells incubated with macrophages for 24 hours
 The two co-culture experiments were analysed after 24 hours of incubation, three fields of view were imaged from each condition. Figure (A) shows single images from the real time movie taken at $t=0$, $t=60$ seconds and $t=120$ seconds from the cell contact containing macrophages and non activated T cells (top panel) and the cell contact containing macrophages and Tcks (bottom panel). The white arrows on the bottom panel of images indicate Tck cell movement during the course of the imaging. These images are a representative of the three movies which were taken of each condition. Figure (B) indicates the number and duration of macrophage-T cell interactions throughout the course of the movies. The duration of interactions is represented in time frames

where one time frame is equivalent to 12 seconds. The data points represent the average value taken from the three movies of the macrophage-T cell co-culture (black line) and the macrophage-Tck co-culture (red line). The graph on the left indicates all the interactions which occur for the duration of the movies. Since the number of short interactions is difficult to visualise on this graph the short duration interactions lasting no longer than 10 minutes (50 time frames) have been plotted on the right hand side graph. Figure (C) demonstrates the average colocalization coefficient of the two co-cultures (mean plus standard deviation). Colocalization coefficient is a measure of the area of interaction between the macrophages and T cells and this result demonstrates that there is a significantly higher amount of interaction taking place in the Tck co-culture compared to the T cell co-culture ($p < 0.0001$).

7.3.1 Comparison of Tcks and T cells after 2 hours of co-culture with macrophages

I next examined cellular contacts between macrophages and T cells/Tcks after a 2 hour duration of incubation. Since we also planned to use this model system to analyse the effect of different proteins/molecules identified from the microarray on this inflammatory model of arthritis, analysing the co-culture system after 2 hours would also allow us to identify early effects of targeting or manipulating these proteins.

Figure 7.5 is a representative of three independent experiments that measured the interactions between macrophages and non-activated T cells or Tcks after 2 hours of co-culture. Figure (A) is displaying screen shots taken from the movie of the macrophages incubated with non-activated T cells (upper panel) or incubated with Tcks (lower panel). Examination of the two image panels revealed that even after 2 hours of incubation the macrophages and T cells are already in contact with one another. This demonstrates that the T cells require only a short duration of time in which to establish their interactions with the macrophages. Upon comparison of the two panels of screenshots, the macrophage-Tck co-culture appear to have substantial clustering of the T cells around the macrophages compared to the non-activated T cell -macrophage co-culture. This clustering of the Tcks around the macrophages is not distributed evenly throughout the field of view and is localised to a subgroup of macrophages. The reason for this is unclear at present but it may be due to an increased activation of the macrophage subset causing increased expression of cell surface activatory markers or increased chemokine or cytokine expression from these cells which could be causing the Tcks to cluster around them.

The representative movie for the macrophage–T cell 2 hour co-culture is designated movie 3 in the supplementary files. Upon observing this real time movie it is clear, especially at the start of the movie, that all of the T cells are moving in the same direction and they all change their direction of movement at the same time. This would indicate that rather than undergoing independent random movement the T cells are all moving throughout the field of view as a result of an external variable which could possibly be the external airflow. Despite this variable, several of the inactive T cells have formed stable contacts with the macrophages indicating that the two cell types can establish stable interactions after just 2 hours of co-culture. Analysing the migrating T cells in the movie demonstrates that these T cells appear to only briefly interact with the macrophages before moving away. These short interactions may be due to the T cells being inactivate and therefore are not expressing the high level of adhesion molecules needed to form strong interactions upon the first encounter of the macrophages. In addition the external air flow could be playing a role in moving the T cells away from the macrophages before the establishment of the stable interactions. This is in contrast to the small number of stable interactions which have formed between the two cell types which may have formed during the 2 hour co-culture in a 37°C incubator without the influence of external air flow.

The representative movie for the macrophage–Tck 2 hour co-culture is designated movie 4 in the supplementary files. Analysis of this movie indicates that the Tcks have formed stable interactions emphasising the observation that the Tcks only need a few hours to form stable cell contacts with the macrophages. In the middle of the field of view in the movie there is a macrophage dense region around which many Tcks have clustered. This clustering of T cells was not seen in the inactive T cell movie indicating that the Tcks have a greater capacity for macrophage cell contact. As was visualised in the previous movie, the external air flow appears to be influencing the movement of the Tcks as all of the Tcks are moving in the same direction though the field of view, making it difficult to distinguish the migration pattern of the Tcks. However, although their movement appears to be influenced by the air flow the Tcks are demonstrating more short duration interactions with the macrophages compared to the inactive T cells. This observation is not due to the

selection of the field of view as three areas were selected at random for this analysis and the same pattern of airflow was visualised in all three fields of view. In addition, the Tcks appear to interact for longer with the macrophages before moving away in comparison to the inactive T cells which appear to only interact briefly.

Panel (B) of Figure 7.5 is showing the number and duration of interactions occurring between the macrophages and the non-activated T cell or the Tcks. The left hand graph indicates the average number and duration of the macrophage-T cell interaction which took place during the two conditions. The interactions between the macrophages and Tcks are indicated by the red line and the interactions between the macrophages and the non-activated T cells are indicated by the black line. On the left hand graph it is difficult to visualise the number of interactions lasting for a short duration however both the Tcks and the non-activated T cells appear to have a similar number of interactions lasting for the maximal duration of 265 time frames (53 minutes). Due to the difficulty in viewing the number of interactions lasting for a short duration, this data was re-graphed specifically analysing interactions which lasted for up to 10 minutes in duration. This graph can be seen on the right hand side of panel (B) and demonstrates that the Tcks (red line) had an increased number of short interactions with the macrophages compared to the non-activated T cells (black line). Volocity analysis of the area of interaction (colocalization coefficient) within each of the movies was also calculated and the results are shown in panel (C). The colocalization coefficient is significantly higher ($p < 0.0001$) when the Tcks are co-cultured with the macrophages compared to the non-activated T cells indicating a higher area of interaction. These results correspond with those that were observed during comparison of the images in panel (A) and supplementary movies 3 and 4 where it was observed that more Tcks appeared to be in contact and clustered around the macrophages compared to the non-activated T cells.

In summary, this 2 hour imaging condition has verified the results of the Tck/non-activated T cell imaging performed after 24 hours of co-culture as it has demonstrated at this timepoint that cytokine activated T cells have a higher number of interactions with the macrophages and these interactions have a larger area. The results of the 2 hour co-culture imaging also inform us that the

T cells interact with the macrophages after a relatively short duration of incubation and it is not necessary to incubate the co-culture for 24 hours before imaging. Performing imaging after such a short incubation has its advantages as the initial movement and interactions of the T cells with the macrophages can be visualised along with the early effects of adding inhibitory molecules or targeting specific genes within the co-culture system.

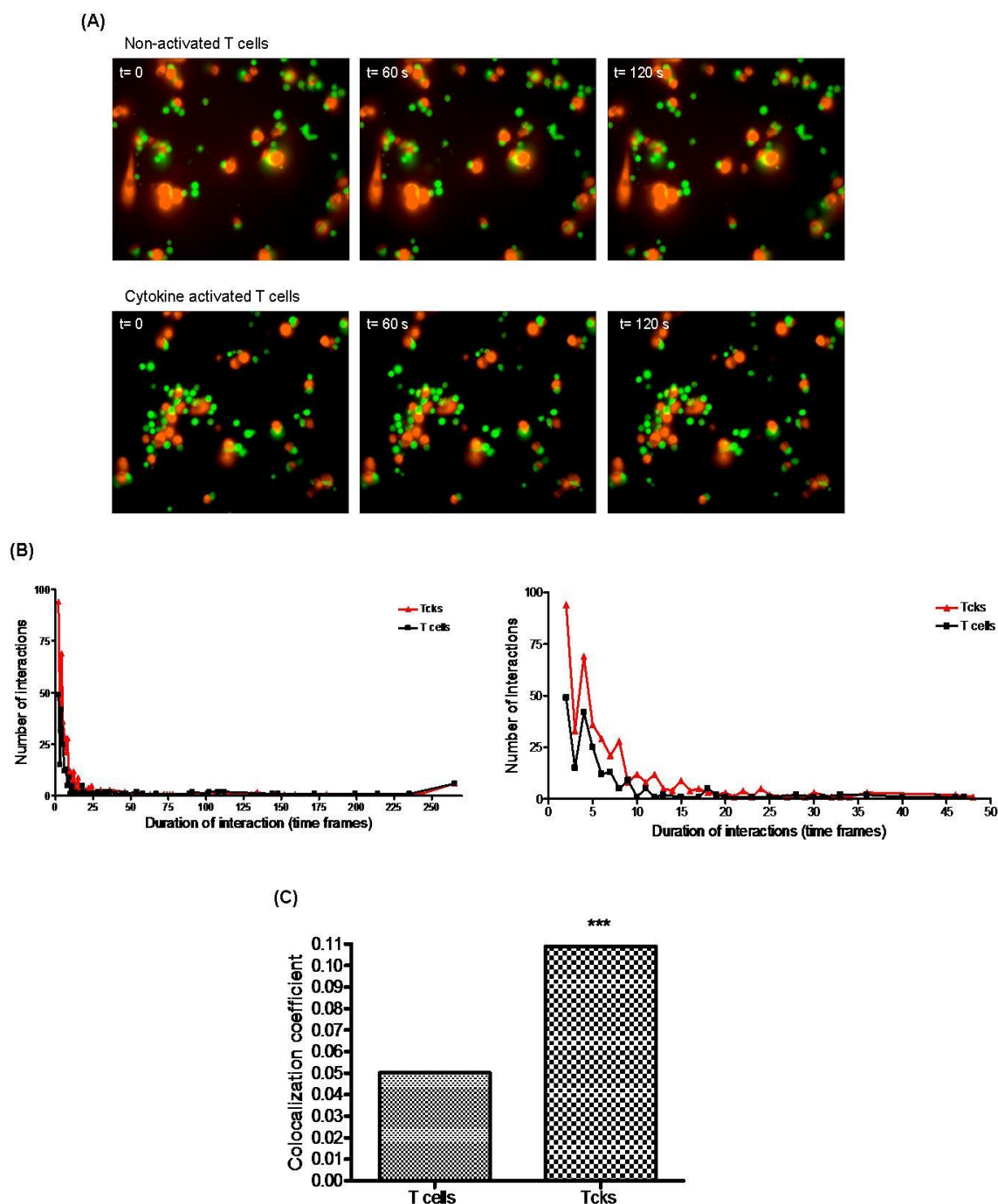


Figure 7.5 Imaging Tcks or non-activated T cells incubated with macrophages for 2 hours

The two co-culture experiments were analysed after 2 hours of incubation. Figure (A) shows single images from the real time movie taken at $t=0$, $t=60$ seconds and $t=120$ seconds from the cell contact containing macrophages and non activated T cells (top panel) and the cell contact containing macrophages and Tcks (bottom panel). Figure (B) indicates the number and duration of macrophage-T cell interactions throughout the course of the movies. The data points represent the values taken from the macrophage-T cell co-culture (black line) and the macrophage-Tck co-culture (red line). The graph on the left indicates all the interactions which occur during the movies. Since the number of short interactions is difficult to visualise on this graph the short duration interactions lasting for up to 10 minutes (50 time frames) have been plotted on the right side graph. Figure (C) demonstrates the average colocalization coefficient of the two co-cultures. Colocalization coefficient is a measure of the area of interaction between the macrophages and T cells and this result demonstrates that there is a significantly higher amount of interaction taking place in the Tck co-culture compared to the T cell co-culture ($p<0.0001$).

7.4 Comparison of fixed and live Tcks after 2 hours co-culture with macrophages

Once it had been established that the Tcks, which have a phenotype similar to RA synovial T cells, have more interactions compared to non-activated T cells we decided to develop and validate the *in vitro* imaging system further by comparing fixed Tcks to live Tcks. We hypothesised that the live Tcks would be able to actively migrate towards the macrophages and therefore would have a larger number of interactions with the macrophages compared to fixed Tcks.

Figure 7.6 is a representative of five experiments which took place to image the fixed and live Tck co-culture conditions with live macrophages. For the fixation process 2% paraformaldehyde was used which fixes the cells through cross linking the proteins. Consequently, the fixed Tcks will still have the cell surface markers and adhesion molecules needed to interact with the macrophages but they will not be able to actively migrate or chemotax towards the macrophages or produce cytokines or chemokines which could activate the macrophages. Panel (A) of Figure 7.6 is showing the screen shots from one of the three fields of view taken for each imaging condition for this experiment. The screen shots are taken at 0, 60 and 120 seconds after the commencement of imaging. Comparison of the two sets of screenshots demonstrates that there are very few interactions taking place between the macrophages and fixed Tcks however the live Tcks have clustered around the macrophages and are interacting with them. This can also be visualised in the real time movies taken of these conditions.

Supplementary file movie 5 is the representative field of view taken of the fixed Tcks—macrophages after 2 hours of co-culture. Upon analysis of this movie it is clear that there are very few interactions taking place between the fixed Tcks and the macrophages. Furthermore, there appears to be a very small number of stable interactions between the fixed Tcks and the macrophages, the Tcks which are moving around the field of view are seen to very briefly interact with the macrophages before moving away. The supplementary file movie 6 represents the live Tcks from the same donor imaged after 2 hours of co-culture. This movie demonstrates that the live Tcks are highly activated and have clustered around and acquired stable interactions with the macrophages. Some of the Tcks are interacting with two macrophages at the same time and can be visualised

spreading over the surface of the macrophage in order to increase the area of interaction. The external air flow does not appear to be playing a large role in the movement of the live Tcks as was seen in previous movies. In addition, it can also be visualised that once a Tck becomes within close proximity of a macrophage it is more likely to interact with the macrophage rather than migrate past it which is what is observed in the fixed Tck movie.

Part (B) of Figure 7.6 reveals the average number and duration of interactions that took place throughout the imaging conditions. Each condition was imaged three times therefore the points on this graph represent the mean number and duration of interactions which took place. These results demonstrate that the live Tcks (red line) had substantially more interactions than the fixed Tcks; the difference was so striking there was no need to display the interactions lasting for up to 10 minutes in duration as was done in the previous figures. The graph also demonstrates that the live cells not only had numerous short interactions with the macrophages but also had interactions lasting for a long duration which is demonstrated by the peak of interactions at approximately 130 time frames.

Panel (C) of Figure 7.6 demonstrates that the mean area of interaction between the macrophages and the Tcks was significantly higher ($p < 0.0001$) in the live cell co-culture compared to the fixed cell co-culture. These results along with the preceding ones verify our hypothesis that the live Tcks would have more interactions with the macrophages compared to the fixed Tcks. Whilst this result was not surprising since fixed Tcks are unable to migrate, this condition was an important one to set up as it ensured that our *in vitro* system was functional and that our methods of analysis were accurate and reliable.

Unfortunately time restrictions meant that no further experiments using our validated *in vitro* real time imaging system could be performed and therefore the imaging method could not be used for its intended purpose of imaging the effect of blocking proteins discovered during the microarray analysis to be upregulated in the synovial fluid macrophages. If time had permitted we would have imaged the cell contact after siRNA treating the macrophages to knockdown plexin A1 production. Plexin A1 is a gene which was highly upregulated in our RA and PsA synovial fluid microarray samples compared to their matched blood samples, it is a surface receptor for semaphorin ligands and

has been demonstrated to have a role in immune regulation and axonal guidance [444, 618]. Hence imaging the effect of siRNA knockdown of plexin A1 could have demonstrated if it played a role in Tck–macrophage interaction and whether silencing plexin A1 in the macrophage had an impact on the number, duration or area of cellular interactions.

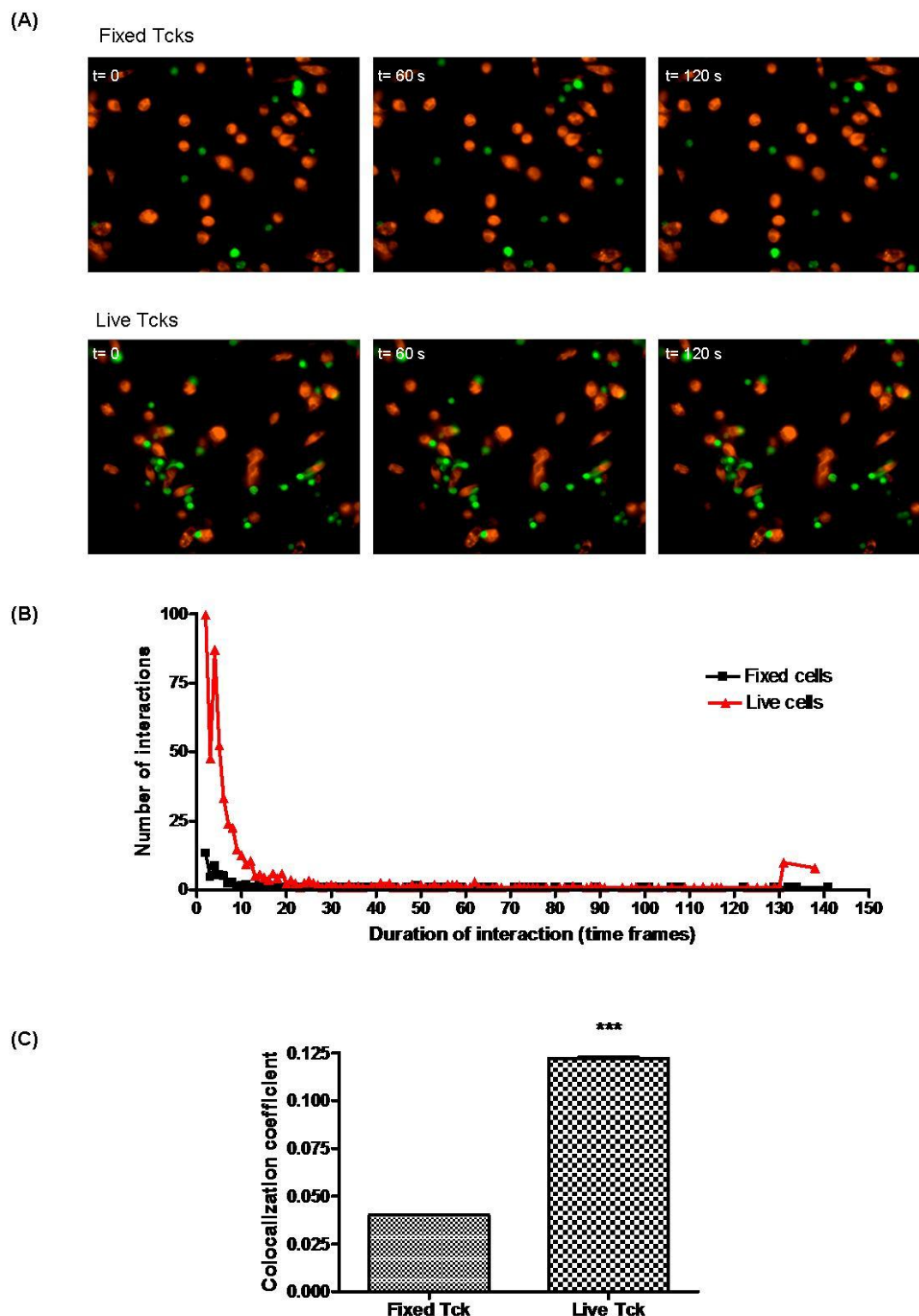


Figure 7.6 Imaging fixed or live Tcks incubated with macrophages for 2 hours

The two co-culture experiments were analysed after 2 hours of incubation. Figure (A) shows single images from the real time movie taken at $t=0$, $t=60$ seconds and $t=120$ seconds from the cell contact containing macrophages and fixed Tcks (top panel) and the cell contact containing macrophages and live Tcks (bottom panel). These images are a representative of the three movies which were taken of each condition. Figure (B) indicates the number and duration of macrophage-T cell interactions throughout the course of the movies. The data points represent the average value taken from the three movies of the macrophage- fixed Tck co-culture (black line) and the macrophage-live Tck co-culture (red line). Figure (C) demonstrates the average colocalization coefficient of the two co-cultures (mean plus standard deviation). Colocalization coefficient is a measure of the area of interaction between the macrophages and T cells and this result demonstrates that there is a significantly higher amount of interaction taking place in the live Tck co-culture compared to the fixed Tck co-culture ($p<0.0001$).

7.5 Discussion

This project has demonstrated the validation work required to verify an *in vitro* imaging model of inflammatory arthritis, and we have illustrated the effects of changes to the model through fixing the Tcks or by adding unstimulated T cells to the culture. In addition, this work has allowed the visualisation of the dynamic nature of the interactions between the cytokine activated Tcks and macrophages. The inflammatory cell contact model has previously been demonstrated to be a robust model of arthritis since the cytokine activation of the T cells induces a phenotype similar to T cells found in the RA synovium [269, 298, 308, 309]. However, previous work has only analysed the cytokine/chemokine profiles of the macrophage–Tck co-culture, the activatory markers present on the Tcks or the inflammatory signalling pathways which are activated in the macrophage as a result of co-culture. Hence, we set out to visualise and measure the interactions that take place within this co-culture model with the eventual aim of imaging the effect of blocking specific proteins shown to be upregulated in the synovial macrophage by combining this work with the microarray results of the preceding chapters.

The first set of experiments which were performed compared the interactions between a macrophage–Tck to a macrophage –T cell co-culture that had been incubated for the standard duration of 24 hours. The results of these experiments revealed that on average the Tcks engaged in a higher number of interactions with macrophages compared to the unstimulated donor matched T cells. These Tck interactions also lasted for a longer duration and had a significantly higher surface area compared to the T cell interactions. Recently, Brennan et al showed that the cytokine activation of T cells results in the upregulation of T cell activation markers such as CD25, CD69 and HLA-DR as well as adhesion molecules such as L-selectin, integrin β -1 (CD29), integrin β -2 (CD18) and CD49d an integrin alpha subunit that in combination with CD29 make up the integrin very late antigen-4 (VLA-4) [309]. These adhesion molecules are known to interact with receptors which are present on the surface of macrophages, such as VCAM-1 which interacts with Tck VLA-4 and ICAM-1 which interacts with Tcks CD18. Consequently, the observations of increased interactions between Tcks and macrophages with a higher surface area compared to those between inactivated T cells and macrophages is unsurprising since the Tcks have

previously been shown to express adhesion molecules which will aid in their establishment and maintenance of interactions with the macrophages. Analysis of the numerous movies taken of the two co-culture conditions demonstrated that while many stable interactions had formed between the Tcks/T cells and the macrophages there were very few Tcks or T cells which were actively migrating or moving throughout the field of view. This result indicated that most of the Tcks/T cells had already established and maintained their stable interactions with the macrophages in the 24 hours prior to the commencement of the real time imaging.

Since part of this investigation was to visualise the movement of the T cells within the cell contact system and visualise the establishment of these interactions it was decided to analyse the co-culture system after a shorter duration of incubation. An incubation period of 2 hours was decided upon before the commencement of the real time imaging. This short incubation period should allow the T cell movement to be observed and the establishment of short and stable interactions as a previous study analysing murine T cell macrophage interactions demonstrated that the T cells move from one macrophage to another after 2 hours of real time imaging [701]. In addition, using a 2 hour incubation period before imaging the co-culture would coordinate well with using this *in vitro* imaging system alongside the microarray results to visualise the function of particular genes. The microarray analysis took place after 2 hours of co-culture to analyse the early response genes that are changed in the macrophages therefore imaging this co-culture system after 2 hours will allow the visualisation of the effect these early response genes have on the interactions between the Tcks and macrophages.

Imaging the Tck–macrophage co-culture in comparison to the T cell–macrophage co-culture after 2 hours of incubation correlated well with the previous analyses of the co-culture after 24 hours. The number of short duration interactions and the colocalization coefficient was higher in the Tck co-culture compared to the T cell co-culture however both co-culture systems had a similar average number of long-duration stable interactions. Hence not only are the Tcks having more short duration interactions with the macrophages but the area of these interactions is also significantly larger, this observation may be caused by the increased expression of adhesion molecules on the surface of the Tcks

after cytokine activation as previously described [309]. Analysis of the movies from these co-culture experiments unfortunately did not reveal a large amount of information regarding the independent movement of the T cells/Tcks due to external factors influencing their movement. In each movie the Tcks/T cells can be observed to all move in a single direction before changing their direction of movement simultaneously which leads to the conclusion that external air flow may be causing the movement of the T cells.

Despite the external air flow, which was not consistent throughout the experiments, patterns of interaction can still be noted from these movies. During the numerous Tck–macrophage co-cultures which were set up the Tcks were seen to cluster around specific macrophages and were not evenly distributed throughout the field of view. Studies analysing murine T cell–DC interactions in the absence of antigen have demonstrated that the rate of contacts is not solely the result of random collision events. Chemokines, such as CCL3 and CCL4, produced at the site of T cell–DC conjugates increases the chance of migrating CCR5-expressing CD8⁺ T cells to contact DCs by a factor of 2 to 4 [702]. In addition *in vitro* observations have revealed that T cell movement and behaviour can be influenced by the presence of surface bound chemokines on the DC. Friedman et al demonstrated that when T cells encounter APCs which have been pulsed with CCR7 ligands in the absence of antigen they remain attached to the APCs for several minutes by a uropodal tether. It was also demonstrated that this attachment was reduced by 62% in the presence of a blocking antibody to ICAM-1 [703]. In the human *in vitro* model of inflammatory arthritis Tcks have been demonstrated to express the chemokine receptor CCR5 and to a lesser extent CCR7 [309] therefore the movement and cellular interaction of these Tcks could be influenced by the expression of chemokines from the macrophages. Since the CCR5 ligands CCL3 and CCL4, otherwise known as MIP-1 α and MIP-1 β , are expressed by activated macrophages [578] the clustering of Tcks around certain macrophages may be the result of these subsets of macrophages expressing a higher level of chemokine ligands which causes the specific migration and interaction of Tcks to these cells. Once these Tcks are in close contact with the macrophages their cellular contacts and maintenance of stable interactions will be mediated by adhesion molecules previously associated with Tcks and macrophages such as L-selectin and ICAM-1. There is no clustering

pattern of inactive T cells around the macrophages seen in the T cell movies which is most likely due to the lack of chemokine receptors expressed on the T cells that ensures these T cells are not moving by a chemotactic gradient towards the macrophages and only interact with the macrophages by random chance.

In conclusion imaging the co-culture after 2 hours of incubation rather than 24 hours showed a similar pattern of colocalization coefficient but the advantage of imaging after such a short incubation allows the movement and brief interactions of the Tcks/T cells with the macrophages to be observed whereas there was very little T cell movement when the co-culture was analysed after 24 hours. However, it must be noted that the Tck/T cell movement may not be entirely random during the real time imaging as the behaviour and motion of these cells may be influenced by the external air flow outside of the imaging chamber.

Once it had been established that the Tcks, which have a phenotype similar to RA synovial T cells, partake in more cellular interactions with the macrophages compared to non-activated T cells it was decided to validate the *in vitro* imaging system further by comparing fixed Tcks to live Tcks after 2 hours of co-culture. Fixation of the Tcks before incubating them with the macrophages results in T cells which express all the surface bound chemokine receptors or adhesion molecules which are known to be upregulated on Tcks [309] but are unable to actively migrate towards the macrophages. Analysis of the graphs in Figure 7.6 which represent one of five experiments clearly indicates that live Tcks have more interactions with the macrophages compared to fixed Tcks and these interactions last for a longer duration and have a significantly higher surface area. These results validated our hypothesis that the live Tcks are able to move towards the macrophages and respond to the chemokines and adhesion molecules present on the surface of the macrophages. Analysis of the representative movies demonstrated that fixed Tcks do form stable interactions with the macrophages, since these cells cannot actively migrate towards the macrophages this would indicate that these interactions are the result of random movement of the fixed Tcks possibly influenced by the external air flow aiding the cells in their movement. Combining these results would indicate that the formation of stable interactions between Tcks and macrophages are the result of

random movement as well as chemotaxis through the production of chemokines from the macrophages. Analysis of the fixed and live Tck co-cultures enabled the validation of the *in vitro* imaging system and ensured that this inflammatory arthritis model produced reliable and reproducible results.

This imaging system had provided us with interesting results regarding the movement and interaction of cells within the cell contact model of arthritis but there are several areas of improvement for future experiments. One such area would be to reduce the influence of the external airflow on the T cell movement through the use of a heated chamber which surrounds the *in vitro* system. This modification would subsequently allow the observation of the independent T cell movement and migration rather than the cell movement being influenced by the air flow. The use of z stacks to take images at different focal depths of the field of view should also be considered as this would enable a 3D image of the cells within the field of view to be acquired. The z stack information would reveal the 3D area of interaction between the Tcks and macrophages, which would be more informative than the current 2D area measured in the preceding experiments. Such z stack information would also enable a more accurate colocalization coefficient to be measured between the Tcks and the macrophages. Also the use of a 3D collagen matrix would make the movement of the cells more physiological and would be representative of the environment within the synovial joint. Several experiments were set up using a collagen matrix (data not shown) but the inability to focus on the cells made it impossible to image their interaction. However, such imaging capacity is available using a confocal microscope which is more advanced than the inverted microscope used for the current study. In summary, the use of a collagen matrix in addition to measuring z stacks on more advanced confocal microscope would greatly enhance the amount of information gained from this *in vitro* imaging system.

In addition, time restrictions meant that this imaging model could not be used for its intended purpose of imaging the effect of blocking genes identified from the microarray analysis performed in this project (see chapter 4) to ascertain whether the interactions between the macrophages and synovial-like Tcks were affected. For example imaging the effect of silencing the plexin A1 gene would be of interest as these experiments may determine whether this cell surface

associated protein, which was found to be highly upregulated in RA and PsA synovial macrophages, has a role in the synovial T cell–macrophage interaction.

Chapter 8: General Discussion

At the outset of my studies many microarray investigations had been performed to analyse the transcriptomic signature of RA and PsA either by whole blood or whole tissue profiling [318-320, 325-327, 329, 330, 340, 341] but very few had analysed the individual cell subsets which drive the pathogenesis of disease. I therefore sought to determine the expressed genetic profiles of matched blood and synovial fluid CD14⁺ monocytes and macrophages from RA and PsA patients to determine how the macrophages change their transcriptome once they migrate from the blood into the inflamed synovial joint. I also sought to compare these profiles to those of healthy blood CD14⁺ monocytes and to cell-contact activated macrophages, representing an *in vitro* model of inflammatory arthritis.

Analysis of the microarray data comparing i) healthy blood, ii) RA blood and iii) PsA blood CD14⁺ cells revealed the unexpected result that the overall genetic profiles of these three monocyte types were remarkably similar and there were very few genes that could distinguish one monocyte subset from another. This result was in contrast to previous microarray analyses which have found a distinct disease signature in RA and PsA blood compared to healthy control blood [326, 329, 330, 340, 341]. The discrepancy between microarray results may be due to differences in sample numbers as my study only included 5 healthy donors and were age- and sex-matched to the PsA patients only. Despite this, my results suggested that the monocytes within RA and PsA are not responsible for the gene expression differences seen in other PBMC microarray studies between healthy and arthritic patients indicating that other cells within the peripheral blood may be causing the diseased genetic signature. Numerous microarray studies have proposed the use of genetic data from patient PBMCs to discover molecular discriminators indicating a response to treatment or disease progression. However, since my data suggest that the RA and PsA blood monocytes do not have a differentiating disease signature compared to healthy controls it may not be appropriate to use this cell subset as a marker of disease or treatment response.

Comparison of matched blood and synovial fluid CD14⁺ cells from the RA and PsA patients also revealed some novel findings. Unsurprisingly, the synovial fluid genetic signature was vastly different to that of the matched blood samples

therefore demonstrating that the monocytes only develop a disease specific signature once they exit the blood and enter the diseased joint. In addition, upon comparison of the differentially expressed genes it was discovered that over 50% of the expressed genes in the synovial fluid CD14⁺ cells compared to the blood cells were the same in both RA and PsA. This result was unexpected given previous analyses by Baeten et al who suggested that the macrophages in SpA and RA were different since they found SpA (including PsA) macrophages expressed higher levels of markers of alternative activation compared to RA macrophages [255] and SpA synovial fluid but not RA was able to polarise blood monocytes to express the alternative activation marker CD163 [253]. However, analysis of the genetic transcriptome generated by my study demonstrated a similar level of alternative activation markers, such as CD204 or mannose receptor, expressed in the PsA CD14⁺ and RA CD14⁺ SF cells. Indeed, I also found similar levels of classical activation markers such as CD86 expressed in both RA and PsA SF cells demonstrating that within this study the RA and PsA macrophages do not have a preferential activation phenotype which contradicts the previous findings. The reasons for this may be due to the Baeten et al study analysing the effects of SF on healthy blood monocytes rather than analysing the diseased macrophages isolated from the source of inflammation as was performed in our study as the effect of the cell-cell contact that takes place within the synovium in addition to the SF environment may play a role in the expression of activation markers and macrophage polarization. However, further studies on the phenotype of the macrophages and protein expression of activation markers are required to test this hypothesis.

Due to the same genes being differentially expressed in the RA and PsA SF macrophages compared to their matched blood monocytes many of the same canonical pathways were significantly associated with these genes lists. Interestingly, the top pathway was LXR activation which has previously been associated with RA but is a novel finding for PsA. Work performed by our research group prior to this observation had demonstrated that LXR agonism exacerbated articular damage in CIA a murine model of arthritis. In addition, *ex vivo* culture of RA PB derived monocytes which were LPS stimulated and treated with two LXR agonists showed an increase in IL-6 and TNF α production, suggesting LXRs have a pro-inflammatory role in arthritis [527]. However in the

present investigation several of the RA and PsA SF macrophage genes associated with this LXR canonical pathway are constituents of the anti-inflammatory lipoprotein HDL. HDL has been previously shown to mediate anti-inflammatory effects within RA since HDL can block the pro-inflammatory cytokine production from monocytes induced by T cell contact through binding to the T cell surface activating factors [558]. Consequently, the net inflammatory effect of the upregulated LXR pathway in the RA and PsA SF macrophages is yet to be defined and should be the focus of further investigations.

Analysis of the highly upregulated genes in both of the RA and PsA SF macrophage subsets compared to the PB monocytes resulted in the identification of two novel genes that had not been previously associated with arthritis. One of these genes was legumain which is a lysosomal cysteine protease that specifically cleaves protein substrates such as MMP2, cathepsins and fibronectin [429, 431] which are all found within the synovium of arthritis patients. My work revealed that legumain was found at the mRNA and protein level in RA and PsA SF CD14⁺ cells and was physiologically active; in addition M-CSF matured macrophages could be induced to express legumain when cultured in RA or PsA SF under hypoxic conditions. Therefore, I hypothesise that legumain is highly upregulated by macrophages once they enter the hypoxic pro-inflammatory environment of the synovium and is required by the cell to activate glycoproteins or enzymes being produced or taken up by the cell which therefore contribute to the destructive inflammation seen in the synovium. Legumain was also seen to be expressed on the cell surface of the synovial macrophages and has also been shown to be excreted from cells [665] and therefore could be activating destructive enzymes such as MMPs within the synovial fluid milieu.

Plexin A1 was the second novel gene discovered by the microarray analysis to be highly upregulated in the RA and PsA SF macrophages compared to the blood monocytes. Plexin A1 is a cell surface receptor which binds to semaphorin ligands and has previously been shown to be important for T cell-DC interactions and subsequent T cell activation [444, 623]. Due to the large body of literature describing macrophage interactions with other cell types in the arthritic synovium causing subsequent activation and pro-inflammatory mediator release I hypothesised that plexin A1 expression on the synovial macrophages may be playing a role in the cellular contacts taking place between these and other

synovial cells. In our study, plexin A1 was shown to be expressed at the protein and RNA level and knocking down the gene using siRNA demonstrated that it may be playing a potential pro-inflammatory role. Nevertheless our studies were hampered by the inability to discover the presence of the most widely known plexin A1 ligand, semaphorin 6D, on activated or synovial T cells despite it previously being detected on activated murine T cells [645]. However, plexin A1 is known to have several semaphorin ligands and whilst our initial studies suggested these may be expressed at the RNA level in synovial membrane and synovial fibroblasts more investigation is required to elucidate what role plexin A1 and its ligands could be playing in arthritic disease.

Analysis of the expressed genetic signature of the Tck activated macrophages, (postulated to represent an *in vitro* model of inflammatory arthritis [298, 309, 314]), in comparison to synovial fluid derived macrophages demonstrated that only 10% of the expressed genes in the cell contact activated macrophages were expressed in RA or PsA synovial fluid macrophages. This would imply that this *in vitro* system may not be a suitable model of arthritis; however my analysis was confounded by several variables particularly the duration of macrophage activation by the Tcks which was restricted to 2 hours. Therefore further investigation especially involving a more extensive timecourse of macrophage activation is required to fully elucidate the relevance of this *in vitro* model to an *in vivo* disease process.

The real time imaging performed on the *in vitro* cell contact model of arthritis provided some interesting observations on the variables influencing the interactions between the T cells and macrophages. It was discovered that the number of individual interactions between the macrophages and T cells was high at 2 hours of co-culture due to the Tcks moving around the field of view and interacting with several macrophages. This observation may help to explain why there were so few genes in the 2 hour cell contact activated macrophages that were shared with the SF macrophages as stable interactions with the Tcks, which would activate the macrophages, were not fully formed after 2 hours of co-culture. Unfortunately time restrictions meant that this imaging system could not be used for its intended purpose which was imaging the effect of blocking genes identified in the microarray analysis. These experiments may have allowed us to visualise whether these genes, i.e. plexin A1, played a role in the

interaction of macrophages with other cell types such as the synovial T cell like Tck.

While this new evidence of the genetic signatures of RA and PsA synovial fluid macrophages has been informative, several avenues of investigation remain to be explored. Future studies must identify the role of plexin A1 within the synovial fluid macrophages and the semaphorin ligand it is potentially interacting with. In addition, understanding the consequences of high levels of active legumain within the RA and PsA synovium through the use of legumain knockout mice and further functional analyses is of paramount importance as it represents an entirely novel enzyme with arthritic disease relevant functions.

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