

Macrophages are critical drivers of synovial inflammation in Rheumatoid Arthritis



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Declaration of Authorship

I, Megan Hanlon, declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work.

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Abstract

Macrophages are an exquisitely plastic pool of innate cells critically involved in directing the immune response in RA. Upon entry into the synovium, peripheral blood monocytes differentiate into the dichotomous M1 (pro-inflammatory) or M2 (anti-inflammatory) macrophage activation states. However, it is now known that macrophages are heterogeneous both in function and origin with many macrophages seeded during embryonic development independently. Despite this, the precise nature and function of infiltrating monocyte-derived macrophages and their precursor cells are poorly defined in RA with even less known about RA synovial tissue macrophages. Therefore, the aims of this thesis are to elucidate the distinct pathogenic functions, bioenergetic demands and activation status of monocytes, monocyte-derived macrophages and synovial tissue macrophage subsets in RA.

In this study we demonstrate that circulating RA CD14⁺ monocytes are primed to produce pro-inflammatory mediators, a phenotype indicative of M1-like macrophage polarisation. Metabolic analysis of RA monocytes using the Seahorse Flux analyser reveals a robust boost in both OXPHOS and glycolysis in RA CD14⁺ monocytes compared to HC. Interestingly, the hyper-inflammatory, hyper-metabolic phenotype of RA monocytes persists following differentiation into *ex vivo* macrophages. RA M1 macrophages replicate the inflammatory memory bias of their precursor cells demonstrating heightened glycolysis and mitochondrial respiration coupled with altered mitochondrial morphology compared to HC. In addition, we demonstrate a consistent upregulation of glycolytic machinery indicating fundamental abnormalities in glucose processing in RA myeloid cells. Furthermore, analysis of polarised RA M1/M2 macrophages reveals divergent inflammatory, bioenergetic and phagocytic functions. Marked transcriptional variance was indicated by RNA-seq, with a key role for STAT3 activation in macrophage polarisation identified. Mechanistically, we demonstrate that the hyper-inflammatory and metabolic phenotype of RA monocytes and M1 macrophages, is mediated through STAT3 phosphorylation whereby inhibition of STAT3 activity switches the pathogenic phenotype of myeloid cells for resolution of inflammation.

Distinct subsets of tissue macrophage in the inflamed RA synovium remain largely unexplored. In this thesis we phenotypically characterise RA synovial tissue macrophages revealing a spectrum of macrophage activation states that don't conform to the binary M1/M2 framework *in vivo*. Within this spectrum we identify for the first time, that the dominant macrophage population residing in the RA synovium is a transitional subset of tissue-resident CD206⁺CD163⁺ macrophages that display elevated CD40 expression. We demonstrate that this subset is enriched in synovial tissue compared to fluid but specifically in RA synovium compared to OA and PsA synovial pathotypes. Furthermore, the CD206⁺CD163⁺ macrophage subset is present in healthy synovial tissue but does not express CD40. CD206⁺CD163⁺ and CD206⁻CD163⁻ macrophage populations were sorted from RA synovial tissue and synovial fluid and RNA-seq analysis performed. We reveal that the CD206⁺CD163⁺ macrophage population is transcriptionally distinct from synovial fluid, double negative CD206⁻CD163⁻ and pure monocyte-derived M1/M2 macrophages, with unique tissue-resident gene signatures. Moreover, we demonstrate differing metabolic demands between CD206⁺CD163⁺ and CD206⁻CD163⁻ macrophage subsets using FLIM analysis. Finally,

CD206+CD163+ macrophages induce enhanced autologous T cell responses and spontaneously secrete high levels of IL-8, IL-6 and TNF α .

Finally, the effect of key macrophage-secreted cytokines, OSM and TNF α on stromal cell (HUVEC/RAFLS) function was examined. OSM differentially regulates pro-inflammatory mechanisms, significantly promotes pro-angiogenic and invasive mechanisms and alters cellular bioenergetics in favour of glycolysis in RAFLS and HUVEC. Moreover, while OSM synergises with TNF α to differentially regulate pro-inflammatory mechanisms in both cell types, synergistic regulation of metabolic reprogramming is observed only in RAFLS, and not HUVEC. Finally, we demonstrate that OSM in combination with TNF α induces transcriptional activity of STAT3 in RAFLS specifically. Taken together, this data provides a greater understanding of the critical role both infiltrating and tissue-resident macrophages play in perpetuating inflammation in RA. Further investigation of the molecular patterns and cues that shape specific synovial macrophage subsets may provide opportunities to reinstate RA joint homeostasis.

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Publications Arising from this Thesis

STAT3 Mediates the Differential Effects of Oncostatin M and TNF α on RA Synovial Fibroblast and Endothelial Cell Function

Megan M Hanlon, Tatsiana Rakovich, Clare C Cunningham, Sharon Ansboro, Douglas J Veale, Ursula Fearon, Trudy McGarry. *Front Immunol.* 2019 Aug 28;10:2056. doi: 10.3389/fimmu.2019.02056.

Altered Metabolic Pathways Regulate Synovial Inflammation in Rheumatoid Arthritis

Ursula Fearon, **Megan M Hanlon**, Sarah M Wade, Jean M Fletcher. *Clin Exp Immunol.* 2019;197(2):170–180. doi:10.1111/cei.13228

Significant Enrichment of Dysfunctional CD206+CD163+ Macrophage Population in Rheumatoid Arthritis Synovial Tissue

Megan M Hanlon, Qingxuan Song, Mary Canavan, Barry Moran, Eamon Breen, Candice Low, Phil Gallagher, Ronan Mullan, Sunil Nagpal, Douglas J Veale, Ursula Fearon.

To be submitted to Journal of Experimental Medicine

Regulation of the Hyper-inflammatory and Hyper-metabolic phenotype in Rheumatoid Arthritis Polarised Macrophages

Megan M Hanlon, Trudy McGarry, Viviana Marzaioli, Candice Low, Kieran Murray, Douglas J Veale, Ursula Fearon.

To be submitted to Annals of Rheumatic Diseases/Cell Reports

Peripheral CD14+ Monocytes from Rheumatoid Arthritis Patients Display Phenotypic and Functional Characteristics of Inflammatory Macrophages and may be a Biomarker of Pre-Clinical Disease

Trudy McGarry, **Megan M Hanlon**, Viviana Marzaoili, Clare C Cunningham, Phil Gallagher, Kieran Murray, Douglas J Veale, Ursula Fearon.

To be submitted to JCI Insight

Published Abstracts

***Distinct Inflammatory and Metabolic Profiles in Rheumatoid Arthritis Monocyte and Monocyte-Derived Macrophages**

Megan M. Hanlon, Trudy McGarry, Viviana Marzaioli, Candice Low, Douglas J. Veale, Ursula Fearon. *European Workshop for Rheumatology Research (EWRR), Leuven (February 2020) (Oral Presentation).*

Significant Enrichment of Dysfunctional CD206+CD163+ Macrophage Population in Rheumatoid Arthritis Synovial Tissue

Megan M Hanlon, Qingxuan Song, Mary Canavan, Barry Moran, Eamon Breen, Candice Low, Phil Gallagher, Ronan Mullan, Sunil Nagpal, Douglas J Veale, Ursula Fearon. *European Workshop for Rheumatology Research (EWRR), Leuven (February 2020) (Poster Presentation).*

***Distinct Macrophage Phenotype and Bioenergetic Profiles in Rheumatoid Arthritis**

Megan M. Hanlon, Trudy McGarry, Mary Canavan, Candice Low, Siobhan Wade, Douglas J. Veale, Ursula Fearon. *Irish Society for Rheumatology, Kildare (September 2019) (Oral Presentation)*

***STAT3 Mediates the Differential Effects of Oncostatin M and TNF α on RA Synovial Fibroblast and Endothelial Cell Function**

Megan M. Hanlon, Tatsiana Rakovich, Clare C. Cunningham, Sharon Ansboro, Douglas J. Veale, Ursula Fearon, Trudy McGarry. *SVHG UCD Translational Medicine Symposium, Dublin (June 2019) (Oral Presentation)*

Distinct Macrophage Phenotype and Bioenergetic Profiles in Rheumatoid Arthritis

Megan M. Hanlon, Trudy McGarry, Mary Canavan, Candice Low, Siobhan Wade, Douglas J. Veale, Ursula Fearon. *SVHG UCD Translational Medicine Symposium, Dublin (June 2019) (Poster Presentation)*

***Distinct Macrophage Phenotype and Bioenergetic Profiles in Rheumatoid Arthritis**

Megan M. Hanlon, Trudy McGarry, Mary Canavan, Candice Low, Siobhan Wade, Douglas J. Veale, Ursula Fearon. *European Workshop for Rheumatology Research (EWRR), Lyon (March 2019) (Oral Presentation).*

Distinct Macrophage Phenotype and Bioenergetic Profiles in Rheumatoid Arthritis

Megan M. Hanlon, Trudy McGarry, Mary Canavan, Candice Low, Siobhan Wade,
Douglas J. Veale, Ursula Fearon. *Metabolism in Cancer and Stromal Cells*, Leuven
(November 2018) (**Poster Presentation**).

***Distinct Macrophage Phenotype and Bioenergetic Profiles in Rheumatoid Arthritis**

Megan M. Hanlon, Trudy McGarry, Mary Canavan, Candice Low, Siobhan Wade,
Douglas J. Veale, Ursula Fearon. *New Horizons in Rheumatology*, Dublin (November
2018) (**Oral Presentation**).

Distinct Macrophage Phenotype and Bioenergetic Profiles in Rheumatoid Arthritis

Megan M. Hanlon, Trudy McGarry, Mary Canavan, Candice Low, Siobhan Wade,
Douglas J. Veale, Ursula Fearon. *American College of Rheumatology (ACR)*, Chicago
(October 2018) (**Poster Tour**).

***Distinct Macrophage Phenotype and Bioenergetic Profiles in Rheumatoid Arthritis**

Megan M. Hanlon, Trudy McGarry, Mary Canavan, Candice Low, Siobhan Wade,
Douglas J. Veale, Ursula Fearon. *Irish Society for Rheumatology (ISR)*, Kildare (September
2018) (**Oral Presentation**).

Distinct Macrophage Phenotype and Bioenergetic Profiles in Rheumatoid Arthritis

Megan M. Hanlon, Trudy McGarry, Mary Canavan, Candice Low, Siobhan Wade,
Douglas J. Veale, Ursula Fearon. *Annual European League against Rheumatic Diseases
(EULAR) Congress, Amsterdam (June 2018)* (**Poster Tour**).

***RA Peripheral CD14⁺ monocytes are Hyper-inflammatory, Hyper-glycolytic and retain a Memory Bias toward M1 Macrophages**

Megan M. Hanlon, Trudy McGarry, Mary Canavan, Candice Low, Siobhan Wade,
Douglas J. Veale, Ursula Fearon. *SVHG UCD Translational Medicine Symposium, Dublin,
(June 2018)* (**Oral Presentation**)

Distinct Macrophage Phenotype and Bioenergetic Profiles in Rheumatoid Arthritis

Megan M. Hanlon, Trudy McGarry, Mary Canavan, Candice Low, Siobhan Wade,

Douglas J. Veale, Ursula Fearon. *SVHG UCD Translational Medicine Symposium, Dublin, (June 2018) (Poster Tour)*

Distinct Macrophage Phenotype and Bioenergetic Profiles in Rheumatoid Arthritis

Megan M. Hanlon, Trudy McGarry, Mary Canavan, Candice Low, Siobhan Wade,

Douglas J. Veale, Ursula Fearon. *British Society of Immunology (BSI), Brighton (December 2017) (Poster Tour)*

***Distinct Macrophage Phenotype and Bioenergetic Profiles in Rheumatoid Arthritis**

Megan M. Hanlon, Trudy McGarry, Mary Canavan, Candice Low, Siobhan Wade,

Douglas J. Veale, Ursula Fearon. *Irish Society for Rheumatology (ISR), Galway (September 2017) (Oral Presentation)*

Oncostatin M induces Inflammation and Differentially Regulates TNF α -induced Pro-inflammatory Mechanisms and Notch Signalling in the RA Joint

Megan M Hanlon, Tatsiana Rakovich, Siobhan Wade, Douglas J Veale, Ursula Fearon, Trudy McGarry. *Annual European League against Rheumatic Diseases (EULAR) Congress, Madrid (June 2017) (Poster Presentation).*

Oncostatin M induces Inflammation, Angiogenesis and Notch Signalling in the RA Joint, and Displays Pleiotropic Effects on TNF α -induced Pro-Inflammatory Effects

Megan M Hanlon, Tatsiana Rakovich, Douglas J Veale, Siobhan Wade, Monika Biniecka, Ursula Fearon, Trudy McGarry. *European Workshop for Rheumatology Research (EWRR), Athens (March 2017) (Poster Tour).*

*** Oral Presentation**

Awards Received

Best Oral Presentation (1st) for:

Distinct Macrophage Phenotype and Bioenergetic Profiles in Rheumatoid Arthritis
Megan M. Hanlon, Trudy McGarry, Mary Canavan, Candice Low, Siobhan Wade,
Douglas J. Veale, Ursula Fearon. *New Horizons in Rheumatology*, Dublin (November
2018) (**Oral Presentation**).

Best Oral Presentation (1st) for:

**RA Peripheral CD14+ monocytes are Hyper-inflammatory, Hyper-glycolytic and retain
a Memory Bias toward M1 Macrophages**
Megan M. Hanlon, Trudy McGarry, Mary Canavan, Candice Low, Siobhan Wade,
Douglas J. Veale, Ursula Fearon. *SVHG UCD Translational Medicine Symposium, Dublin,*
(June 2018) (**Oral Presentation**)

Best Oral Presentation (1st) for:

Distinct Macrophage Phenotype and Bioenergetic Profiles in Rheumatoid Arthritis
Megan M. Hanlon, Trudy McGarry, Mary Canavan, Candice Low, Siobhan Wade,
Douglas J. Veale, Ursula Fearon. *Irish Society for Rheumatology (ISR), Galway*
(September 2017) (**Oral Presentation**)

Best Oral Presentation (2nd) for:

Distinct Macrophage Phenotype and Bioenergetic Profiles in Rheumatoid Arthritis
Megan M. Hanlon, Trudy McGarry, Mary Canavan, Candice Low, Siobhan Wade,
Douglas J. Veale, Ursula Fearon. *Irish Society for Rheumatology, Kildare* (September
2019) (**Oral Presentation**)

Best Oral Presentation (2nd) for:

Distinct Macrophage Phenotype and Bioenergetic Profiles in Rheumatoid Arthritis
Megan M. Hanlon, Trudy McGarry, Mary Canavan, Candice Low, Siobhan Wade,
Douglas J. Veale, Ursula Fearon. *European Workshop for Rheumatology Research*
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Shortlisted for Poster Tour for:

Distinct Macrophage Phenotype and Bioenergetic Profiles in Rheumatoid Arthritis
Megan M. Hanlon, Trudy McGarry, Mary Canavan, Candice Low, Siobhan Wade,
Douglas J. Veale, Ursula Fearon. *American College of Rheumatology (ACR), Chicago*
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Shortlisted for Poster Tour for:

Distinct Macrophage Phenotype and Bioenergetic Profiles in Rheumatoid Arthritis

Megan M. Hanlon, Trudy McGarry, Mary Canavan, Candice Low, Siobhan Wade, Douglas J. Veale, Ursula Fearon. *Annual European League against Rheumatic Diseases (EULAR) Congress, Amsterdam (June 2018) (Poster Tour)*.

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Distinct Macrophage Phenotype and Bioenergetic Profiles in Rheumatoid Arthritis

Megan M. Hanlon, Trudy McGarry, Mary Canavan, Candice Low, Siobhan Wade, Douglas J. Veale, Ursula Fearon. *British Society of Immunology (BSI), Brighton (December 2017) (Poster Tour)*

Travel bursaries granted

Awarded EULAR Travel Bursary for EWRR congress, Leuven; Belgium. 2020

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List of Abbreviations

2-DG - 2-Deoxyglucose
ACL - Anterior Cruciate Ligament
ACPA - Anti-citrillinated Protein Antibodies
ACR - American College of Rheumatology
Ang - Angiopoietin
Anti-CarP - Anti-Carbamylated Protein
APC - Antigen Presenting Cell
A-SAA - Acute Serum Amyloid A
ATP - Adenosine Triphosphate
AVN - Avascular Necrosis
BCR - B cell Receptor
bFGF - basic Fibroblast Growth Factor
BMI - Body Mass Index
CAD - Coronary Artery Disease
CIA - Collagen Induced Arthritis
COX - Cyclo-oxygenase
CPM - Counts per Million
CRP - C-Reactive Protein
CTLA-4 - Cytotoxic T-lymphocyte Antigen 4
CV - Cardiovascular
DAMP - Damage Associated Molecular Pattern
DAS - Disease Activity Score
DC - Dendritic Cell
DEG - Differentially Expressed Genes
DLBCL - Diffuse Large B cell Lymphoma
DMARD - Disease Modifying Anti-Rheumatic Drug
DMSO - Dimethyl Sulfoxide
DNA - Deoxyribonucleic Acid
E. Coli - Escherichia Coli
EAE – Experimental Autoimmune Encephalomyelitis
EBV - Epstein Barr Virus
EC - Endothelial Cell
ECAR - Extracellular Acidification Rate
ELISA - Enzyme-linked Immunosorbent Assay
ESR - Erythrocyte Sedimentation Rate
ETC - Electron Transport Chain
EULAR - European Union League against Rheumatism
FACS - Fluorescence-Activated Cell Sorting
FADH₂ - Flavin Adenine Dinucleotide-2
FCCP - Carbonyl cyanide-p-trifluoromethoxyphenylhydrazine
FCS - Fetal Calf Serum
FcγR - Fc-gamma receptor
FDA - Food and Drug Administration
FDR - False Discovery Rate
FLIM - Two-photon Excited Fluorescence Lifetime Imaging

FLS - Fibroblast-like Synoviocytes
 FMO - Fluorescence Minus One
 G6PD - Glucose-6-Phosphate
 GCA - Giant Cell Arteritis
 GDP – Gross Domestic Product
 GM-CSF - Granulocyte-Macrophage Colony-Stimulating Factor
 GRO α - Growth Regulated Oncogene α
 HA - Hyaluronic Acid
 HAQ - Health Assessment Questionnaire
 HBSS - Hanks' Balanced Salt Solution
 HC - Healthy Control
 HIF - Hypoxia Inducible Factor
 HK2 - Hexokinase-2
 HLA - Human Leukocyte Antigen
 HUVEC - Human Umbilical Vein Endothelial Cells
 IBD - Inflammatory Bowel Disease
 ICAM - Intracellular Adhesion Molecule xviii
 IFN - Interferon
 IgG - Immunoglobulin G
 IL - Interleukin
 iNOS - Inducible NO Synthase
 IPA - Ingenuity Pathway Analysis
 JAK - Janus Kinase
 KIF5A - Kinesin Family Member 5A
 LDH - Lactate Dehydrogenase
 LL - Lining layer
 LPS - Lipopolysaccharide
 MAPK - Mitogen-Activated Protein Kinase
 MCP - Monocyte Chemoattractant Protein
 M-CSF - Macrophage Colony-Stimulating Factor (M-CSF)
 MFI - Median Fluorescence Intensity
 MHC - Major Histocompatibility Complex
 MMP - Matrix metalloproteinase
 MRI - Magnetic Resonance Imaging
 MSD - Meso Scale Discovery
 mTOR - Mammalian Target of Rapamycin
 MTX - Methotrexate
 NADH - Nicotinamide Adenine Dinucleotide
 NF κ B - Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells
 NK - Natural Killer Cell
 NO - Nitric oxide
 NSAID - Non-steroidal Anti-inflammatory Drugs
 OA - Osteoarthritis
 OCR - Oxygen Consumption Rate
 OSM - Oncostatin M
 OXPHOS - Oxidative Phosphorylation
 PAD - Protein Arginine Deiminase

PAMP - Pathogen Associated Molecular Pattern
 PBMC - Peripheral Blood Mononuclear Cells
 PBS - Phosphate Buffered Saline
 PCA - Principal Component Analysis
 PCR - Polymerase Chain Reaction
 PD-1 - Programmed Cell Death Protein 1
 PDGF - Platelet Derived Growth Factor
 PDH - Pyruvate Dehydrogenase
 PFK-1 - 6-phosphofructokinase-1
 PFKFB3 - 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
 PI3K/AKT - Phosphatidylinositol 3-Kinase - Protein Kinase B
 PKM2 - Pyruvate Kinase M2
 PPP - Pentose Phosphate Pathway
 PRKCQ - Protein Kinase C Theta
 PsA - Psoriatic Arthritis
 PTPN - Protein Tyrosine Phosphatase N22
 RA - Rheumatoid Arthritis
 RANKL- Receptor Activator of Nuclear Factor Kappa-B Ligand
 RANTES - Regulated on Activation, Normal T Cell Expressed and Secreted
 RF - Rheumatoid Factor
 RhoA - Ras Homolog Gene Family, Member A
 RIPA - Radio-Immunoprecipitation Assay
 RMD - Rheumatic and Musculoskeletal Diseases
 RNA - Ribonucleic Acid
 RNS - Reactive Nitrogen Species
 ROS - Reactive Oxygen Species
 RTX - Rituximab
 SCFA - Short Chain Fatty Acids
 SCID - Severe Combined Immunodeficiency
 SDH - Succinate Dehydrogenase
 SDS - Sodium Dodecyl Sulfate
 SEM - Standard Error of the Mean
 SFC - Synovial Fibroblast Cell
 SFMC - Synovial Fluid Mononuclear Cells
 SJC - Swollen Joint Count
 SL – Sub-lining
 SNP - Single Nucleotide Polymorphism
 SOCS - Suppressors of Cytokine Signalling
 SPICE - Simplified Presentation of Incredibly Complex Evaluations
 ST - Synovial Tissue
 STAT - Signal Transducer and Activator of Transcription
 SVUH - St. Vincent’s University Hospital
 TB - Tuberculosis
 TBS - Tris-buffered saline
 TCA - Tricarboxylic Acid Cycle
 TCZ - Tocilizumab
 TEM - Transmission Electron Microscopy

TGF β - Transforming Growth Factor β
Th1 - Type 1 helper T cell
Th2 - Type 2 helper T cell
TIMP - Tissue Inhibitor of Matrix Metalloproteinases
TJC - Tender Joint Count
TLR - Toll-like Receptor
TNFAIP3 - Tumour Necrosis Factor α Induced Protein 3
TNFi - Tumour Necrosis Factor α Inhibitor
TNF α - Tumour Necrosis Factor α
TRAF - Tumour Necrosis Factor Receptor Associated Factor
Treg - Regulatory T cell
tSNE - t-Distributed Stochastic Neighbour Embedding
VAS - Vascularity
VCAM - Vascular Cell Adhesion Protein 1
VEGF - Vascular Endothelial Growth Factor
VEGFR - Vascular Endothelial Growth Factor Receptor

Thesis Hypothesis

Do macrophages and their precursor cells display molecular signatures that are associated with disease onset and activity in Rheumatoid Arthritis?

CHAPTER ONE:

General Introduction

1.1 The History of Rheumatoid Arthritis

Guillaume de Baillou is often recognised as the Father of Rheumatology. In 1611, he described rheumatism as an affliction of the joints. His posthumous publication in 1642 clearly distinguished gout as a disease of one joint while rheumatism involves the entire body. However, the initial description of Rheumatoid Arthritis (RA) widely accepted by modern medicine may be attributed to French physician Landre-Beavais in 1800 whereby he documented his treatment of patients with unexplained joint pain, describing the signs and symptoms that we now recognise as RA (Entezami *et al.*, 2011). It wasn't until 1859 that the English physician Alfred Garrod first differentiated gout from other arthritides, in his 'Treatise on Nature of Gout and Rheumatic Gout'. It was in fact his son, Archibald Garrod who re-coined the term 'Rheumatoid Arthritis' in 1890 (Storey, 2001). Rheumatoid comes from the Greek word 'rheuma' meaning that which flows, while arthritis is derived from 'arthros' meaning joint.

However, these are not the earliest references to rheumatologic disease. Evidence for RA dates back as far as ancient Egyptian times in the Ebers Papyrus medical text describing arthritis, in 1500 BC. Hippocrates, the Greek philosopher and often regarded as the Father of Modern Medicine, also described arthritis as far back in 400 BC as "a disease with fever, severe joint pain, fixing itself in one joint now, then in another, of short duration, acute, not leading to death, more apt to attack the young than the old" (Parish, 1963). It was suggested that rheumatic disorders stemmed from an imbalance of humours (meaning juice) whereby the humours would leak into the joint causing pain.

Yet perhaps the most successful depiction of RA may be found in artwork and sculptures. In Ruben's *The Three Graces* (1638), the right-hand of one of the 'Graces' appears disfigured. Rubens was known to strictly adhere to realism in his artwork and so it is quite possible that the model used for this painting suffered from RA (Appelboom *et al.*, 1981). Renaissance painting 'The Temptation of St. Anthony' also hints at RA with striking deformity of the beggar's right hand (Dequeker and Rico, 1992). In addition, exhumed skeletons from North Africa and Europe suggest several forms of rheumatic diseases were in existence thousands of years before (Aceves-Avila *et al.*, 1998; Aceves-Avila, Medina and Fraga, 2001).

1.2 Classification of RA

RA was originally classified in 1987 by Arnett *et al* on behalf of the American College of Rheumatology (ACR) (Arnett *et al.*, 1988). The 1987 criteria was criticised for a lack of sensitivity in diagnosis of early stage RA, due to a reliance on radiological changes (M. M. J. Nielen *et al.*, 2004; M. M. Nielen *et al.*, 2004). Circulating auto-antibodies have been described in the years preceding clinical manifestations of RA (Aho *et al.*, 1991, 1993) and several studies report that earlier intervention improves outcome and may prevent damage (van der Heide *et al.*, 1996). Therefore, a joint ACR/European League Against Rheumatism (EULAR) working group developed the 2010 classification to facilitate the diagnosis and study of early stage RA (Aletaha *et al.*, 2010), which is now routinely used in clinical practice. ACR/EULAR 2010 classifies “definite RA” as confirmed presence of synovitis in at least one joint with no other alternative diagnosis that could better explain the synovitis. ACR/EULAR criteria defines a diagnosis of RA when a score of ≥ 6 across four domains is made, as illustrated in Table 1.1 (Aletaha *et al.*, 2010).

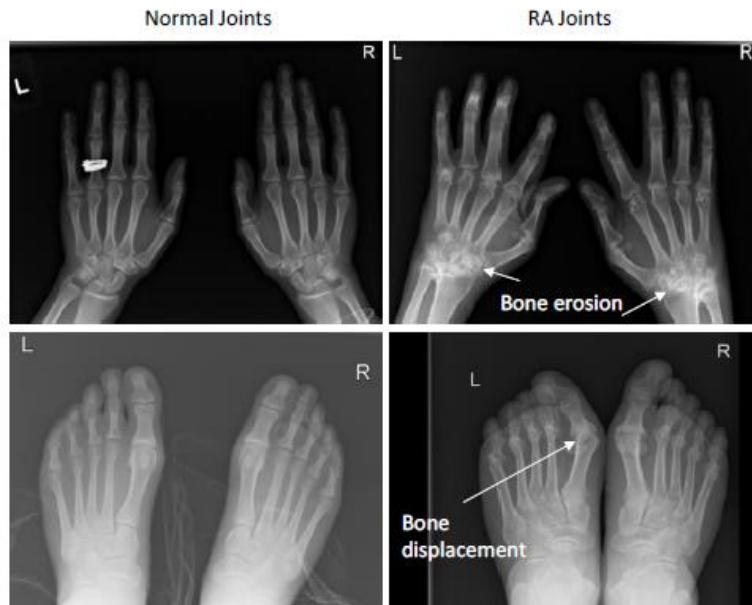


Figure 1.1: Radiographic features of RA joints.

Images depict normal radiographs of hands and feet (left panels) compared to advanced RA joints (right panels). RA radiographs show evidence of peri-articular osteopenia, joint space narrowing, erosive damage and joint displacement as indicated by white arrows. (Archives, St. Vincent's University Hospital Rheumatology Department)

		Score
Joint Involvement	1 medium-large joint	0
	2-10 medium-large joints	1
	1-3 small joints	2
	4-10 small joints	3
	More than 10 small joints	5
Serology	RF (-) and ACPA (-)	0
	RF (+) or ACPA (+)	2
	High RF (+) or ACPA (+)	3
Acute-Phase reactants	CRP and ESR within normal	0
	Elevated ESR	1
Duration of symptoms	< 6 weeks	0
	≥ 6 weeks	1

Table 1.1: ACR/EULAR Classification Criteria, 2010.

Joint involvement refers to any swollen or tender joint upon examination. Large joints refer to shoulders, elbows, hips, knees, and ankles. Small joints refer to the metacarpophalangeal joints, proximal interphalangeal joints, second through fifth metatarsophalangeal joints, thumb inter-phalangeal joints, and wrists. RF: Rheumatoid Factor; ACPA: anti-citrullinated protein antibody; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate. Duration of symptoms refers to patient self-report of the duration of signs or symptoms of synovitis (e.g., pain, swelling, tenderness) of joints clinically involved at time of assessment. (Adapted from (Aletaha *et al.*, 2010))

1.3 Incidence and Prevalence of RA

Accurately assessing the incidence of RA has proven to be quite challenging mainly due to uncertainties surrounding diagnosis and the variability in disease progression among patients. However, RA has an estimated annual incidence of 24–58/100 000 for women with an estimated incidence of 14–30/100 000 for men (Symmons *et al.*, 1994; Doran *et al.*, 2002; Dugowson *et al.*, 2010; Smolen, Aletaha and McInnes, 2016; Littlejohn and Monrad, 2018). Global prevalence of RA is approximately 0.5–1% and is 2–3 times more prevalent in women than men (Helmick *et al.*, 2008; Aletaha *et al.*, 2010). RA presents typically between 40–50 years, and increases with age in both sexes (Puente *et al.*, 1989). Pima Indians and Chippewa Indians have the highest incidence rates of RA with rates of 5.3% and 6.8% reported respectively, while Asians, particularly Chinese and Japanese have the lowest prevalence (0.2%) (Silman and Pearson, 2002; Helmick *et al.*, 2008). A recent estimate from Arthritis Ireland suggests that there are roughly 40,000 people with RA living in Ireland, with approximately 70% of these women.

1.4 Genetic Risk Factors

Epidemiology studies suggest that susceptibility to RA is quite complex, with a combination of environmental and genetic risks along with hormonal factors and the immune system acting as contributing factors (Espinoza and García-Valladares, 2013; Taneja, 2015). The familial nature of RA is well documented, dating back to 1806 where William Heberden suggested ‘some degree of heritability’ in RA (John and Worthington, 2001). Population based genetic studies in recent years have identified over 100 loci associated with increased susceptibility to RA (Kim *et al.*, 2017). Several genes including HLA-DR4, HLA-DR1, a SNP in the PTPN22 (protein tyrosine phosphatase, non-receptor type 22), PADI4, STAT4, PRKCQ, KIF5A, TNFAIP3, TRAF1 and IL2RB genes have all been identified as susceptibility loci for RA (Gregersen, Silver and Winchester, 1987; Nepom, Hansen and Nepom, 1987; Ollier and Thomson, 1992; Begovich *et al.*, 2004; Hinks *et al.*, 2007; Plenge *et al.*, 2007; Remmers *et al.*, 2007; Thomson *et al.*, 2007; Barton *et al.*, 2008). The most important of these genetic risk factors are the human leukocyte antigen (HLA) polymorphisms, responsible for T cell activation. HLA-DRB1 alleles have been associated

with RA in cohorts of all racial and ethnic backgrounds (Furukawa *et al.*, 2015; Liu *et al.*, 2016). Considering the gender imbalance observed in RA, it is of no surprise that female hormonal factors play a role in the development of the disease. Broadly, it has been suggested that oestrogens may enhance immune activation while androgens are considered to be immunosuppressive (Cutolo *et al.*, 2003; CUTOLO *et al.*, 2006; Alpízar-Rodríguez *et al.*, 2017). Specifically studies have demonstrated that androgens can inhibit the differentiation of Th1 and Th17 (Pikwer *et al.*, 2014; Schwinge *et al.*, 2015). In contrast, female hormonal factors have been indicated as risk factors for disease development and thus capable of contributing to RA development. Findings are controversial however with the use of oral contraceptives or hormone replacement therapy found to be both protective and risk factors while other factors associated with low levels of oestrogen such as early menopausal onset are consistently associated with increased risk of RA (Doran *et al.*, 2004; Walitt *et al.*, 2008; Pikwer *et al.*, 2009; Alpízar-Rodríguez *et al.*, 2017).

1.5 Environmental Risk Factors

Although the initial trigger for RA has yet to be elucidated, there are various lifestyle and environmental factors that have been attributed to its development. Furthermore, in those genetically susceptible individuals, subsequent environmental triggers may provoke the development of RA. Smoking has been strongly linked to the pathogenesis of RA. A study in 2010 revealed that the risk of developing RA is twice as likely for smokers compared to non-smokers in men and 1.3 times higher in female smokers compared to non-smoking controls (Sugiyama *et al.*, 2010). Although heavy smokers have an increased risk of developing RA, there is also evidence to suggest that even light smokers or previous smokers also have increased risk (Stolt *et al.*, 2003). Tobacco smoking has been shown to be associated with worse disease prognosis and is strongly linked to anti-citrullinated protein antibodies (ACPA)-positive RA and those who have the HLA-DRB1 shared epitope. A status of ACPA-positivity and the shared epitope coupled with cigarette smoking heightens the risk for RA pathogenesis 21 fold compared to seropositive non-smoking patients with one copy of HLA-DRB1 (Saag *et al.*, 1997; Másdóttir *et al.*, 2000; Lu *et al.*, 2014). Thus, this may shed some light on a possible association between environment and genetic factors.

Diet and obesity have also been reported to increase the risk of RA. There is evidence of a relationship between dietary intake of omega-3 fatty acids, Vitamin D and RA disease activity. Omega-3 and omega-6 fatty acids are metabolised to eicosanoids, lipid mediators of inflammation, yet only omega-3 fatty acids are capable of decreasing pro-inflammatory cytokine production (Naughton *et al.*, 1993). Thus, increasing dietary intake of omega-3 to dampen inflammation is of interest in RA (Volker *et al.*, 2000; Tedeschi and Costenbader, 2016). *In vitro* work has demonstrated that human macrophages treated with two types of omega-3 fatty acids produce lower levels of pro-inflammatory eicosanoids and also inhibits the NLRP3 inflammasome (L'homme *et al.*, 2013). Two trials of the 'Mediterranean diet' (rich in olive oil, fruit and vegetables, fish, reduced quantities of red meat) report improved disease control in the intervention arms (Sköldstam, Hagfors and Johansson, 2003; McKellar *et al.*, 2007). In addition, short chain fatty acids (SCFA) have also been shown to affect macrophage and dendritic cell function resulting in anti-inflammatory effects; decreased production of pro-inflammatory cytokines, paralleled by increased frequency of regulatory T cells (Tregs) (Trompette *et al.*, 2014). SCFA are one of the main metabolites produced by the gut microbiome, thus demonstrating that microbiome metabolites influence immune cell function (Thorburn, Macia and Mackay, 2014).

Obesity and increased cardiovascular risk have been reported in 18-31% of RA patients with one study revealing more than 68% of their cohort classified as overweight (Armstrong *et al.*, 2006; Naranjo *et al.*, 2008). Obesity is associated with co-morbidities in RA such as cardiovascular events and linked to more severe pain, leading to a decrease in quality of life (Wolfe and Michaud, 2007; Ajeganova *et al.*, 2013; Sandberg *et al.*, 2014). Recent data from our research group has shown that BMI and waist circumference correlates with macroscopic synovitis and vascularity (Low *et al.*, 2018). Furthermore, studies have demonstrated that obese RA patients have reduced responsiveness to biological treatments (Gremese *et al.*, 2013). Experimentally, the association between obesity and RA may be mediated through altered expression of specific adipokines, including leptin and adiponectin which are capable of contributing to the pro-inflammatory milieu (Otero *et al.*, 2006; Fantuzzi, 2008). Research in type II diabetes reports decreased adiponectin levels which results in increased levels of circulating TNF α

(Lahiri *et al.*, 2014; Jiang, Li and Li, 2015). Caloric restriction has also been explored and shown to dampen inflammation in RA through inhibition of the NLRP3 inflammasome and IL-1 β both *in vitro* and *in vivo* in animal studies (Vandanmagsar *et al.*, 2011).

Controversially, moderate alcohol consumption has been demonstrated to be protective in RA. A large UK study conducted in 2010 revealed an inverse correlation between alcohol intake and disease severity as indicated by DAS28, CRP, and the Larsen radiograph score. Interestingly however, this inverse relationship appears to be confined to ACPA positive disease (Maxwell *et al.*, 2010; Scott *et al.*, 2013). Mouse models of arthritis have demonstrated that alcohol also reduces NF κ B activation and leukocyte migration (Jonsson *et al.*, 2007).

More recently, links between the gut microbiome and autoimmune disease has been widely explored, whereby studies demonstrate that changes occurring in intestinal microbiome composition are coupled with the onset of autoimmune disease (Ubeda and Pamer, 2012; Luckey *et al.*, 2013; López-Cepero and Palacios, 2015). Our body is colonised by an abundance of commensal microbes that have co-evolved with our immune system, yet with huge changes to modern lifestyles in recent years, the composition of our gut microbiota has dramatically changed. Dysbiosis, or modifications in the diversity and abundances of gut microbiota, can indeed trigger an imbalance of T cell subsets such as Th1, Th2, Th17 and Treg cells (Lee and Kim, 2017). In the context of RA, studies have demonstrated that the microbial composition of early arthritis patients differs from controls with significantly less bacteria from the *Bifidobacterium* and *Bacteroides* family in RA patients (Vaahtovuori *et al.*, 2008). As such, there have been a myriad of studies in recent years highlighting the role of gut microbiota as a crucial environmental factor for RA (Vaahtovuori *et al.*, 2008; Liu *et al.*, 2013; Chen *et al.*, 2016). While no animal model can fully represent RA disease entirely, there have been a significant number of studies demonstrating the induction/exacerbation of RA disease in experimental murine models with the induction of specific microbes (Abdollahi-Roodsaz *et al.*, 2008; Wu *et al.*, 2010). As of yet however, the exact mechanisms underpinning the role of gut microbiota in RA remains unclear.

1.6 Infection

There has been much speculation on the role of pathogens in the establishment of RA, with *Escherichia coli* (*E. Coli*) and Epstein Barr virus (EBV) studied as possible triggers for immune cell response (Kouri *et al.*, 1990; Auger and Roudier, 1997; Blaschke *et al.*, 2000). Similarly, proteus infection (Ebringer *et al.*, 1985), human parvovirus (Ray *et al.*, 2001) and mycobacterium tuberculosis (Kanagawa *et al.*, 2015) have all been proposed as possible triggers, generally via molecular mimicry and may play an aetiological role in individual cases. However, no single organism has been consistently implicated to date (Symmons and Harrison, 2000). Both serological and microbiological studies demonstrate that no one microorganism has been recovered consistently from RA synovial fluid or tissue thus indicating RA is not caused by a single organism. RA has also been associated with periodontal disease, yet the nature of this relationship remains to be truly defined. One study proposes that the bacteria found in periodontal disease, *Porphyromonas gingivalis*, is responsible for promoting aberrant citrullination provoking a local breach of tolerance to citrullinated peptides (Wegner *et al.*, 2010). In addition, studies have demonstrated that patients treated with TNF inhibitors have a greater risk of reactivating latent tuberculosis (TB) (Salgado and Gómez-Reino, 2011). To combat this, TB screening and treatment strategies have been put in place prior to treatment initiation which has led to a reduced incidence of TNFi-associated TB infections (Winthrop, 2006). Taking the evidence from genetic and environmental factors studies together, suggests that RA possibly arises as a result of multiple hits or triggers. That is, in the genetically predisposed or epigenetically modified individual, an initial combination of environmental and stochastic insults results in a breach of immunological tolerance with any additional triggers such as infection, driving the immune response further (Smolen, Aletaha and McInnes, 2016).

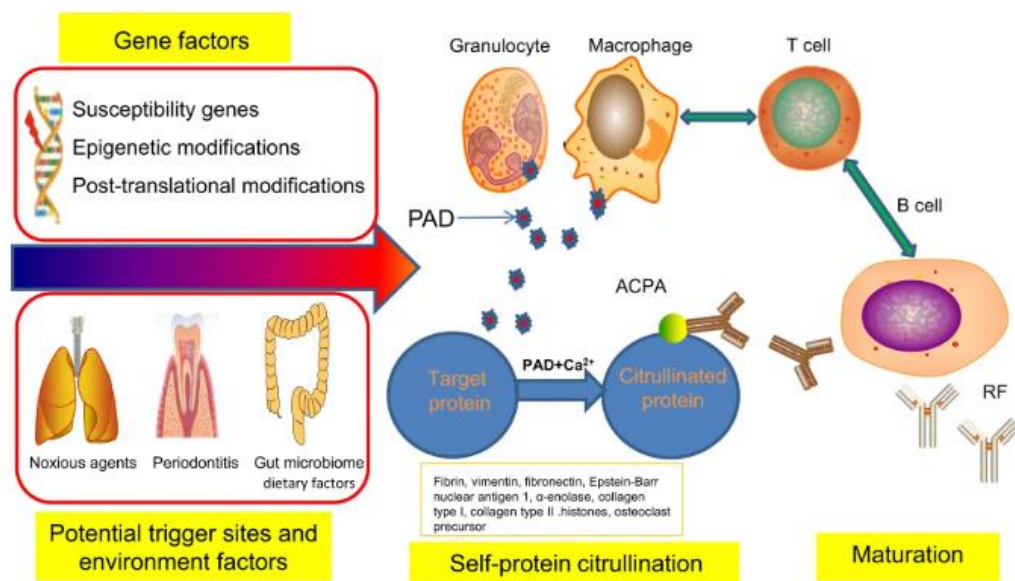


Figure 1.2: Factors contributing to RA.

RA can be triggered by interactions between genetics and environmental factors such as infectious agents, smoking and alterations in the microbiome. This results in the onset of self-protein citrullination catalysed by the calcium dependent enzymes peptidyl-arginine deiminases (PAD) and maturation of anti-citrullinated protein antibodies (ACPA). Subsequently, ACPA activates many immune cells to drive this process further and breach immune tolerance. Taken from (Guo *et al.*, 2018).

1.7 Auto-antibodies

The presence of auto-antibodies are a well-documented phenomenon in RA disease development. Rheumatoid factor (RF), the first autoantibody to be described in RA is present in the sera of 50-80% of patients with RA. RF refers to auto-antibodies directed against the Fc portion of IgG molecules and the presence of this antibody is associated with worsened disease prognosis (Masi *et al.*, 1976; Waaler, 2007). However, there are issues with the specificity of RF, as it is also detected in healthy elderly individuals and those with infectious diseases, thus reducing its predictive and diagnostic value (Schellekens *et al.*, 2000).

The presence of ACPA associated with disease severity is now a well-accepted feature of the disease. It is for this reason that the RA classification criteria was revised with the development of the 2010 ACR/EULAR classification criteria (Aletaha *et al.*, 2010).

ACPA specifically target proteins where the arginine residue has been replaced by citrulline during post-translational modification. This reaction is catalysed by a family of enzymes called peptidyl-arginine deiminase (PAD) (Vossenaar *et al.*, 2003). ACPA are detected in the sera of nearly two thirds of RA patients, and often precede the onset of clinical manifestations, suggesting a central role for citrulline as a diagnostic determinant of this disease (Rantapää-Dahlqvist *et al.*, 2003; M. M. J. Nielen *et al.*, 2004; Alivernini *et al.*, 2017). ACPA positivity is also associated with a more erosive phenotype in RA (Orr *et al.*, 2017), and with increased mortality, mainly associated with cardiovascular events (Gorman *et al.*, 2004; Ajeganova *et al.*, 2016).

Anti-carbamylated protein antibodies (Anti-CarP antibodies) have also been identified in RA patients, and are associated with a more destructive disease phenotype (J. Shi *et al.*, 2011). Anti-CarP antibodies have been identified prior to disease onset and notably can also be present in patients that are seronegative for both RF and ACPA and thus may be an interesting additional biomarker (J. Shi *et al.*, 2011; Shi *et al.*, 2013; Jiang *et al.*, 2014; Gan *et al.*, 2015). Furthermore, in patient serum samples, both ACPA and anti-CarP antibodies are often present together. Despite similarities between these two autoantibody systems however, they target distinct post-translational modifications and at present it is still unclear which autoantibody response is initiated first (Verheul *et al.*, 2018).

1.8 Disability

RA is a chronic and debilitating disease that can leave patients with life-long functional impairment due to irreversible joint damage and an unpredictable disease course. It is a disease characterised by pain, progressive loss of mobility and the ability to care for one's self, thus the impact of RA on patients quality of life can be devastating (Guillemin, 2000). Radiological evidence of joint space narrowing and bony erosion can present as early as in the first 2 years of disease highlighting the urgency for treatment during the so called 'window of opportunity' (Fuchs *et al.*, 1989). Dawes and Symmons first referred to the window of opportunity with regard to RA in 1992 as 'a small window of 2 years in which to get the disease in remission before irreversible damage is done to joints' (Dawes and Symmons, 1992).

Synovial inflammation coupled with progressive joint erosion directly results in loss of function and impaired ability to partake in daily activities (Verstappen, 2013). Indeed, studies have shown that patients with increased joint erosion at first presentation are more inclined to have worse radiographic progression along with subsequent decrease in functional disability compared to those without initial joint damage (Mullan *et al.*, 2007; Verstappen, 2013). Thus, early diagnosis and initiation of therapy are crucial to prevent clinically significant disease. Studies have indicated that the lower the disease activity at 6 months, the more favourable the outcome with a complete halt to joint damage in those who reach clinical remission within the first 3-6 months regardless of treatment utilised (Smolen *et al.*, 2009; Kavanaugh *et al.*, 2013). Therefore, a priority for RA research is diagnosis of preclinical disease with the 'window of opportunity' increasingly referring to preventative therapy rather than interventions in early disease where manifestations of clinical disease are already present (Gerlag *et al.*, 2012; Raza *et al.*, 2012).

Standard disability measures include the Health Assessment Questionnaire (HAQ) score. It has been shown that RA patients have significantly higher HAQ scores compared to the general population which are mainly considered to be associated with high levels of disease activity (Munro *et al.*, 1998). Studies have demonstrated that the initial improvement in the HAQ score upon commencement of treatment is subsequently followed by a progressive decline in function as the articular damage develops (Welsing *et al.*, 2001; BYKERK *et al.*, 2012).

1.9 Cardiovascular Mortality

Since the mid 1900's, various studies have demonstrated an association with increased mortality and morbidity in RA patients compared to the general population, a large portion of which can be attributable to cardiovascular events (Lauper and Gabay, 2017). Studies have indicated that RA patients have a 30-60% increased risk of suffering a cardiovascular (CV) event compared to the general population (Watson, Rhodes and Guess, 2003; Han *et al.*, 2006). CV events are mainly observed early in disease which is in contrast to the general population where such events are generally correlated with age (AJEGANOVA *et al.*, 2012).

CV disease affects RA patients independently of traditional risk factors which suggests that interactions between inflammation and lipid metabolism in the development of atherosclerosis may be implicated. Lipid profiles are directly correlated with an elevated risk of CV disease in the general population, in RA however, effective treatment regimens may act to normalise lipid profiles (Robertson *et al.*, 2013; Purcarea *et al.*, 2014). Mechanisms underpinning the ‘lipid paradox’, i.e. reduced lipid levels in a disease paradoxically related to amplification of CV risk, as yet remains unclear, however studies have suggested a direct role of pro-inflammatory cytokines such as IL-6 (Robertson *et al.*, 2013).

Many studies indicate that inflammation plays a major role in CV events in RA with both ESR and CRP levels associated with a number of CV events and CV mortality (Wållberg-Jonsson *et al.*, 1999; Innala *et al.*, 2011). In addition, it has been demonstrated that high levels of serum acute phase protein Acute Serum Amyloid A (A-SAA) can predict CV events in a long term follow up (Mullan *et al.*, 2010). Seropositivity for RF or ACPA are also associated with elevated CV risk in patients with early disease onset suggesting that auto-antibodies are also a predictor of CV events in RA (Ajeganova *et al.*, 2013).

Considering the important implications between inflammation and the development of CV events, control of disease activity is the main treatment priority. Patients treated with disease modifying anti-rheumatic drugs (DMARDs) or TNF inhibitors have been shown to have decreased risk of CV morbidity and mortality in RA (Micha *et al.*, 2011; Low *et al.*, 2017). Therefore, effective suppression of inflammation can reduce the impact of RA on extra-articular disease complications (Jacobsson *et al.*, 2005; Naranjo *et al.*, 2008).

1.10 Malignancy

Overall, the incidence of malignancy in RA patients is quite comparable to that of the general population, although there is a modest risk of 5-10% (Simon *et al.*, 2015). However, biological DMARDs act by directly modifying immunologic pathways, and thus may alter normal immunosurveillance resulting in an elevated risk of malignancies (Kim *et al.*, 2019). With increasing use of DMARDs in routine management of RA, it is important

to understand the baseline risk of malignancies in RA patients (Singh *et al.*, 2012). With regard to lymphoma, many large cohort studies and meta-analyses have consistently demonstrated that patients who suffer with RA have an increased risk of developing malignant lymphomas (Gridley *et al.*, 1993; Smedby *et al.*, 2006; Baecklund *et al.*, 2014). One such study demonstrates the strong association between RA and diffuse large B cell lymphoma (DLBCL), one of the most aggressive forms of leukaemia, whereby the odds ratio of developing DLBCL dramatically increases with severe disease activity (Baecklund *et al.*, 2006).

In addition, studies also indicate a twofold increased risk of lung cancer associated with RA along with a significantly higher risk of leukaemia (Cibere, Sibley and Haga, 1997; Simon *et al.*, 2015). Smoking is a confounding factor in the relationship between RA and lung cancer. Increasing cumulative doses of smoking heightens the risk of ACPA positive RA while cessation of smoking has been observed to reduce the risk of RA associated with smoking (Stolt *et al.*, 2003; Costenbader *et al.*, 2006; Di Giuseppe *et al.*, 2013). Indeed, smoking increases the risk of developing both lung cancer and RA (Simon *et al.*, 2015), with a recent study proposing that the observed increased risk for lung cancer in RA may not actually be due to the disease itself but rather exposure to smoking (Dagan *et al.*, 2017).

The underlying mechanisms associated with the other increased risk of malignancies are unclear, but studies have shown a decrease in T-suppressor lymphocytes as a result of incessant immunologic stimulation in RA patients who also developed lymphomas (Love and Solomon, 2008). Interestingly, a decreased overall risk of colorectal cancer is reported in RA patients in comparison to the general population (Hashimoto *et al.*, 2015). The observed decreased risk in colorectal malignancy has been attributed to the potential benefits of NSAIDs which are known to decrease this risk (Gridley *et al.*, 1993; Thomas *et al.*, 2000; Hamoya *et al.*, 2016). Indeed meta-analysis of randomised trials indicates that aspirin use for approximately 5 years reduces incidence and mortality due to colorectal cancer by 30-40% (Rothwell *et al.*, 2010).

1.11 Economic Burden

RA poses a significant economic burden comprising both medical and societal costs. Although this disease can present at any age, roughly one-third of RA patients are over 65 years of age. This, coupled with the increasing size of the global aging population, means that the economic burden is quite significant (Firestein *et al.*, 2012). The true extent of this burden is difficult to accurately assess; including medical care expenses but also loss of productivity in the workplace. A study published in 2010 reported that the total annual costs of RA to the US healthcare system, both direct and indirect costs, are estimated to be up to €19.3 billion (Birnbaum *et al.*, 2010). Recent studies have suggested that more than 2.3 million individuals are diagnosed with RA in Europe, generating annual direct and indirect costs of management of over €45 billion. Lundkvist and colleagues report direct medical costs to be €14 billion per annum in Europe (Lundkvist, Kastäng and Kobelt, 2008) while the combined annual cost of RA and OA in Ireland is estimated at €13.6 million (Doherty and O'Neill, 2014). The financial burden is greater in those patients who do not response to biologic or targeted DMARDs as cycling through different biologics increases health care costs and outpatient encounters (Davis, 2019). RA-related absence from work is significant during active disease but can occur even in the early stages of the disease. One study states that the average number of days absent from the workplace due to RA ranges hugely from 2.7-30 days/per annum (Cooper, 2000; Anis *et al.*, 2009). Loss of productivity is also a major issue with many patients unable to work at their full potential known as 'presenteeism' (Li, Gignac and Anis, 2006). In recent years considerable increases in medical costs can be explained by the mass global use of biologic therapies. A 3 year study with over 7,000 RA patients reported that the cost of biologic therapies is nearly three times that of non-biologic agents (Michaud *et al.*, 2003).

1.12 The Normal Synovial Joint

The normal synovial joint, also known as diarthrosis, is a specialised structure that facilitates frictionless and stable movement of two opposing bones. Synovial tissue lines the non-articular surfaces of the joint and it is comprised of 1-3 layers of synoviocytes- specialised columnar cells; interspersed with macrophages (Smith *et al.*, 2003). The articular surfaces of opposing bones are covered by a smooth cartilage layer which is an avascular connective tissue comprised mainly of proteoglycan and type II collagen in a dense matrix (O'Connor, Orford and Gardner, 1988; Martel-Pelletier *et al.*, 2008). The normal function of synovial membrane is to produce synovial fluid to lubricate the inner surface of the joint capsule, deliver nourishment to articular cartilage and ensure frictionless movement.

The synovium itself can be sub-divided into two distinct layers- the intimal/lining layer and the sub-intimal/sub-lining later. The lining layer is generally 1-2 cells thick and consists of two cell types; macrophages (type A synoviocytes) and synovial fibroblast cells (type B synoviocytes). Below this lies the relatively acellular sub-lining layer comprised of scattered blood vessels, loose connective tissue and adipose tissue (Walsh and Pearson, 2001; Smith, 2011). The synovial joint is continually subject to minor trauma and weight bearing forces and so must be a highly dynamic structure. Thus bone undergoes constant remodelling and degradation in order to maintain normal synovial integrity and homeostasis (Iwanaga *et al.*, 2000; Kurowska-Stolarska and Alivernini, 2017).

1.13 The Inflamed Joint

Inflammation of the synovial joint is a fundamental feature of RA pathology, it is the basis of pain, joint damage and systemic manifestations in rheumatic disease. As depicted in Figure 1.1, the inflamed synovium is characterised by increased vascularisation leading to persistent infiltration of immune cells resulting in synovial hyperplasia (Tak and Bresnihan, 2000; Walsh and Pearson, 2001; Smolen, Aletaha and McInnes, 2016; Orr *et al.*, 2017). Although the initiating trigger for RA has yet to be elucidated, one of the earliest events is angiogenesis or new blood vessel formation which facilitates the infiltration of inflammatory leukocytes including T cells, B cells, monocytes, dendritic cells, mast cells and natural killer cells into the sub-lining layer (Pillinger and Abramson, 1995; Tak and Bresnihan, 2000; Fearon *et al.*, 2003; Fearon U, Szekanecz Z, 2020). Thus, the synovium is the primary site of dysregulated immunomodulatory pathways.

It was previously thought that the synoviocytes of the lining layer underwent local proliferation (Qu *et al.*, 1994), however it is now suggested that these cells transform from a relatively quiescent state to an aggressive/invasive phenotype resistant to apoptosis and play a central role in RA disease pathogenesis (Firestein *et al.*, 1990; Müller-Ladner *et al.*, 1996; Ospelt *et al.*, 2004; Filer, 2013). As a result, the hyperplastic synovial lining layer, now 1-8 cells deep, forms a tumour-like mass or 'pannus' which orchestrates articular cartilage degradation and joint destruction. Proliferating synoviocytes produce matrix-metalloproteinases (MMPs) and pro-inflammatory mediators such as cytokines and chemokines to promote destruction of adjacent bone and cartilage (Pap *et al.*, 2000). Furthermore, this perpetuating synovitis facilitates differentiation of infiltrating monocyte progenitors into osteoclasts to expedite bone reabsorption, thus further augmenting loss of function and joint deformity (Mullan *et al.*, 2007).

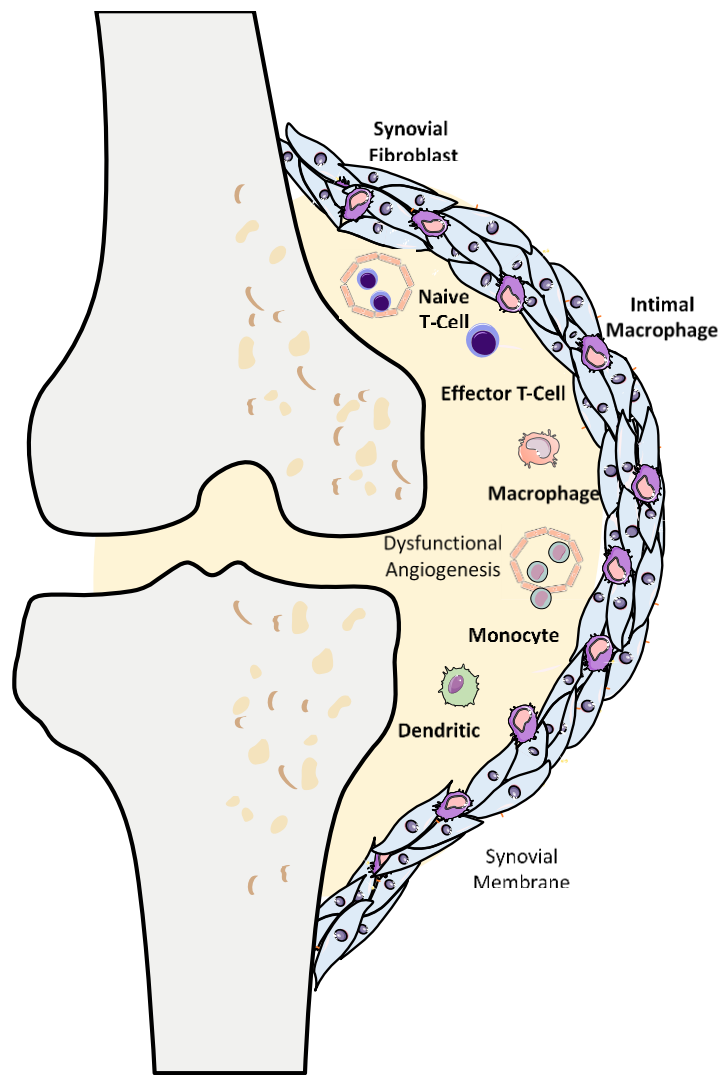


Figure 1.3: The inflamed synovial joint.

In Rheumatoid Arthritis, the synovial membrane becomes hyperplastic, dysfunctional angiogenesis allows for chronic infiltration of inflammatory cells, ultimately developing into a 'pannus which migrates onto and into the articular cartilage and underlying bone. Adapted from (Fearon *et al.*, 2019), Appendix B.

Many of the pathological changes observed in the synovium are reflected in synovial fluid, thus synovial fluid provides an invaluable insight into the processes occurring at the site of inflammation. Inflammation alters the permeability of the synovium, it is no longer capable of selectively allowing/preventing proteins from entering/leaving, thus synovial fluid is protein rich in RA compared to the steady state (Levick, 1981). The lubricant hyaluronic acid (HA) is also altered during inflammation, changing to a lower molecular weight in RA (Dahl *et al.*, 1985), and overall is lost from

the joint. SF is also rich in pro-inflammatory mediators such as cytokines released mainly by macrophages and T cells, thus fluid samples from RA patients have considerably elevated cytokine concentrations (Hui *et al.*, 2012). Specifically, studies have demonstrated elevated levels of IL-6, TNF α , IL-21, GM-CSF and M-CSF amongst others in RA synovial fluid (Santos Savio *et al.*, 2015; Asif Amin, Fox and Ruth, 2017; Lebre *et al.*, 2017). Directly in contact with cartilage, bone and the key cells of the synovium; synovial fluid provides a valuable reflection of the pathophysiological state of the joint.

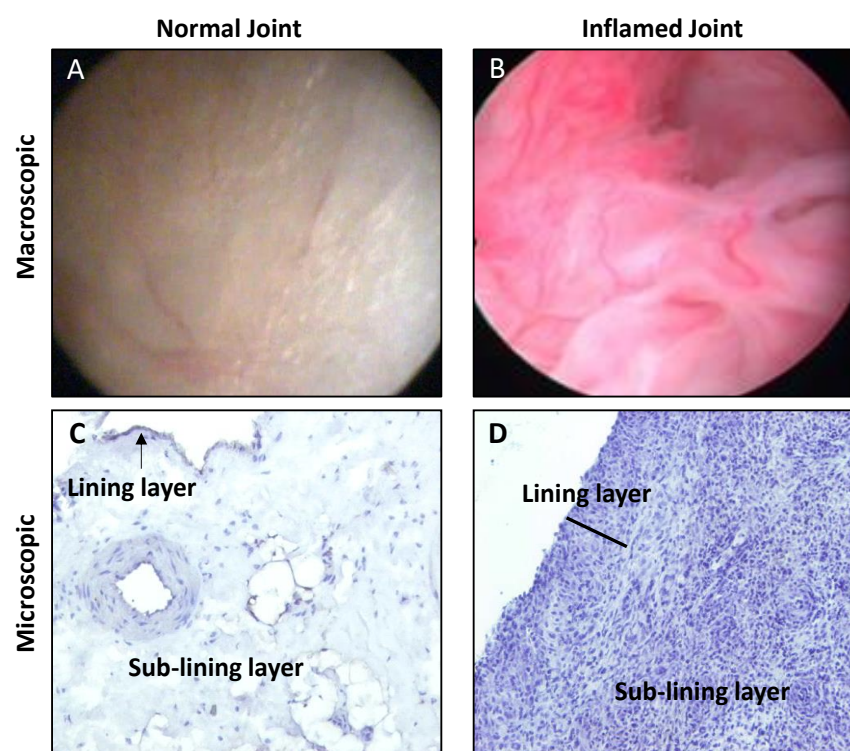


Figure 1.4: Macroscopic and microscopic features of RA joint.

Macroscopic images of the (B) RA joint compared to the (A) normal joint. This is mirrored microscopically by (D) a thickened lining layer (black arrow) and sub-lining leukocyte infiltration in the inflamed joint. (Taken with permission from Prof Fearon's Lab, Molecular Rheumatology/SVUH Rheumatology Department).

1.14 Angiogenesis

Sprouting angiogenesis describes the process where new capillaries sprout from existing blood vessels, and is an early and fundamental event in the pathogenesis of RA (Fearon *et al.*, 2003). This dynamic process is a normal part of physiological mechanisms, however in RA the process is uncontrolled and results in the abnormal sprouting of blood vessels which facilitate increased leukocyte migration, transforming the synovium into an aggressive 'pannus' (Epstein and Harris, 1990; Månsson *et al.*, 1995; Kennedy *et al.*, 2010). Endothelial cells (EC) exist in a quiescent state until exposure to pro-angiogenic stimuli whereby they become activated. This results in secretion of enzymes which degrade the EC basement membrane and extracellular matrix components to allow for EC migration through the connective tissue matrix to form capillary sprouts. This is followed by EC proliferation, tip and stalk cell selection, synthesis of basement membrane and lumen formation (Firestein *et al.*, 2012; Fearon U, Szekanecz Z, 2020).

Angiogenesis is tightly regulated by both pro- and anti-angiogenic growth factors and cytokines. Vascular endothelial growth factor (VEGF) is the master regulator of this process and known as the key 'on' switch, acting at an early stage in vascular morphogenesis. VEGF stimulates EC proliferation and migration (DVORAK, 2005). Other factors that differentially regulate blood vessel activation and maturation in RA include angiopoietins 1 and 2 (Ang1 and Ang2), basic Fibroblast Growth Factor (bFGF), Notch-1, Platelet Derived Growth Factor (PDGF), TNF α , IL-18 and IL-1 (Colville-Nash and Scott, 1992; Fraser *et al.*, 2001; Fearon *et al.*, 2003; Szekanecz, Gáspár and Koch, 2005; Kennedy *et al.*, 2010; Volin and Koch, 2011; Gao *et al.*, 2012). Many of these mediators also play additional roles in inflammation such as activation of other cell types, including macrophages and synovial fibroblasts (McInnes and Schett, 2007; Krausz *et al.*, 2012; García *et al.*, 2014). In addition, chemokines such as CCL2 and CX3CL1 attract immune cells to the site of inflammation to further induce neovascularisation and so indirectly play a role in promoting angiogenesis (Tas *et al.*, 2016).

The stability and maturity of vasculature is critical for the advancement of blood vessels through the synovial membrane. Vascular pericytes provide support and stability to the endothelial cell via complex interactions involving PDGF, Ang1, neural cellular adhesion molecule, RhoGTPases and Notch signalling (Lindblom *et al.*, 2003; Bergers and

Song, 2005; Betsholtz, Lindblom and Gerhardt, 2005; Gerhardt and Semb, 2008). Studies in our research group have demonstrated that a mixture of mature (with pericytes) and immature (without pericytes) vessels exist within the inflamed joint. This is in contrast to non-inflammatory OA joints whereby the majority of vasculature display an intact pericyte layer (Kennedy *et al.*, 2010). This data infers that synovial vessels undergo a dynamic process of simultaneous angiogenesis, maturation and regression, dependent on the growth factors and MMPs in the surrounding microenvironment (Darland and D'Amore, 1999; Bergers and Song, 2005; Gerhardt and Semb, 2008).

Despite this increased vascular supply however, the joint is profoundly hypoxic (Ng *et al.*, 2010). This is due to the fact that while there is an increase in blood vessel formation, this vascular network is highly dysfunctional resulting in abnormal blood flow supplying inadequate nutrients and oxygen to the expanding synovium and thus the metabolic turnover of the pannus outpaces vascular supply, creating a hypoxic microenvironment (Kennedy *et al.*, 2010; Ng *et al.*, 2010; Biniecka *et al.*, 2014; Fearon *et al.*, 2016). Indeed, hypoxia itself is a major trigger of VEGF-mediated angiogenesis and so the hypoxic microenvironment only further serves to promote dysfunctional vasculature which perpetuates the vicious cycle of synovial expansion (Pugh and Ratcliffe, 2003; Szekanecz and Koch, 2008; Fearon *et al.*, 2016; Tas *et al.*, 2016). In addition, complex interactions between many intracellular signalling pathways such as NFκB, phosphoinositide 3-kinase-protein kinase B (PI3K-AKT), MAPK, JAK/STAT and Notch-1 strictly govern angiogenic processes with inhibition of these pathways capable of dampening mechanisms of disease (Gao *et al.*, 2012; Maracle *et al.*, 2017; McGarry *et al.*, 2018).

1.15 Leukocyte Infiltration

The increased vasculature and endothelial cell activation observed in RA facilitates the infiltration of leukocytes into the inflamed synovium. The sequence of adhesive interactions of leukocytes with endothelial cells is termed the 'leukocyte extravasion cascade'. This multi-step process involves a series of adhesive exchanges to allow for cell tethering, rolling, activation, adhesion, crawling and trans-endothelial cell migration (Raza *et al.*, 2006; Schnoor *et al.*, 2015).

The initial step occurs within 1-2 hours and involves weak adhesion or 'rolling' of circulatory leukocytes to activated endothelial cells. This is facilitated by endothelial E, P-selectins, leukocyte L-selectin and their ligands. Subsequently, activation and triggering occur which is mediated by interactions between chemokine receptors on leukocytes and proteoglycans on EC. Firm intracellular adhesion next occurs and is accompanied by secretion of chemokines. Finally, trans-endothelial diapedesis ensues when chemokines bind to endothelial heparin sulphate glycosaminoglycans (Springer, 1994; Szekanecz *et al.*, 2010; Schnoor *et al.*, 2015). Chemokines expressed at the luminal site provide both a chemoattractant gradient and pro-adhesive properties to further facilitate migration of leukocytes across the endothelium (Middleton *et al.*, 2002).

Initially the paradigm of leukocyte extravasation regarded the endothelium as merely a passive substrate for leukocyte diapedesis. However, it is now largely reported that the endothelium is actually an active participant of this multi-step process. Adhesion molecules such as ICAM-1 and VCAM-1 induce significant changes to EC morphology to allow for leukocyte migration (Pober, 2002; Vestweber, 2015). Structures such as endothelial cups which surround adherent leukocytes before engulfing them, act as docking structures to guide leukocytes across the endothelium. Therefore, endothelial adhesion receptors and proteins play a major role in leukocyte transmigration.

1.16 Innate Immune Cells

Cellular infiltrates include immune cells from both the innate and adaptive systems. Among the first responders recruited to the site of inflammation are neutrophils which represent 40-60% of all circulating leukocytes. Mainly found in synovial fluid, neutrophils potentiate synovial inflammation through the production of inflammatory mediators, reactive oxygen species (ROS), reactive nitrogen species (RNS) and proteases (Cascão *et al.*, 2010; McInnes and Schett, 2011). Mast cells comprise approximately 5% of the cellular infiltrate in the inflamed synovium. Through release of their mediators; histamine and tryptase, they increase vascular permeability, promote angiogenesis and recruitment of neutrophils (Walker, Hatfield and Brown, 2012). Monocytes are central players in the initiation of inflammation in RA representing a phenotypically heterogenic and plastic pool of immune cells capable of phagocytosis, pro-inflammatory cytokine production and

differentiation into distinct immune cells. Depending on extracellular cues and their microenvironmental demand, monocytes can differentiate into macrophages, dendritic cells and osteoclasts (Rana *et al.*, 2018). Representing roughly 10% of total leukocytes, monocytes can circulate in the blood for up to 2 days before dying or being recruited to a tissue facing danger (Italiani and Boraschi, 2014).

In the past ten years specific monocyte subsets have been classified according to expression of the markers CD14 and CD16. Three types of monocyte subsets have been characterised as classical monocytes (CD14⁺⁺CD16⁻), intermediate monocyte (CD14⁺⁺CD16⁺) and non-classical monocyte (CD14⁺CD16⁺⁺) (Ziegler-Heitbrock *et al.*, 2010), with many studies indicating that these subsets are transcriptionally distinct (Ancuta *et al.*, 2009; Zhao *et al.*, 2009; Wong *et al.*, 2011). In RA, monocytes tend to skew towards the intermediate subset, producing many pro-inflammatory cytokines such as TNF- α , IL-1 β and IL-6 and are positively correlated with disease activity (Rossol *et al.*, 2012; Klimek *et al.*, 2014; Yang *et al.*, 2014; Tsukamoto *et al.*, 2017). The CD14⁺⁺CD16⁻ compartment of classical monocytes however, are thought to be the circulating precursors of osteoclasts responsible for bone erosion in RA joints (Komano *et al.*, 2006). High levels of monocytes in the periphery could be an important biomarker, with one study demonstrating that circulating monocytes are increased in very early RA, and upon treatment with infliximab a decrease is observed after just 2 weeks (Coulthard *et al.*, 2012).

Macrophages make up the majority of innate immune cells during inflammation and are critically involved in directing and shaping inflammation through a variety of mechanisms. As the major phagocytic cell of the joint, macrophages are charged with the removal of pathogens and cellular debris. Notably, activated macrophages promote a number of pro-inflammatory mechanisms in the synovium such as abundant overexpression of pro-inflammatory cytokines. They are the key producers of the most crucial RA-associated cytokines; TNF α , IL-1 β , IL-6 and Oncostatin M (OSM) (Brennan & McInnes, 2008). In addition to their cytokine production, macrophages secrete high levels of matrix-degrading enzymes, present antigen to T and B cells, phagocytose and drive bone resorption (Szekanecz and Koch, 2007b; Drexler *et al.*, 2008; McInnes and Schett, 2011). Synovial macrophages are also primary targets for angiopoietin growth factor

signalling via the Tie2 receptor to promote the pro-inflammatory activation of macrophages (Krausz *et al.*, 2012).

Studies have demonstrated that CD68+ macrophage expression strongly correlates with the degree of disease activity and is inversely related to *in vivo* synovial Po2 levels (Ng *et al.*, 2010; Kennedy, Ng, Chang, Biniecka, Jacintha N O'Sullivan, *et al.*, 2011; Harty *et al.*, 2012). However, the significant role of macrophages in the pathogenesis of RA is perhaps most strongly demonstrated by the fact that synovial sub-lining (SL) macrophages are the only cell type to date that are consistently reduced in RA responders, regardless of treatment type, suggesting SL macrophage frequency as a potential synovial biomarker to discriminate between responders and non-responders (Mulherin, Fitzgerald and Bresnihan, 1996; Tak *et al.*, 1997; BRESNIHAN *et al.*, 2009; Ng *et al.*, 2010; Kennedy, Ng, Chang, Biniecka, Jacintha N. O'Sullivan, *et al.*, 2011a).

While these innate immune cells are thought to be critically involved in regulating inflammation in RA, very little is known about the phenotype of macrophages, with the majority of studies based on CD68 expression. It is thought that macrophages exist as either one of two distinct phenotypes, the pro-inflammatory (classically-activated) M1 cell and the tissue-resolving (alternatively-activated) M2 cell, which represent two terminal ends of the spectrum. This description of macrophage polarisation was only established in the early 1990's with scientists discovering a role for IL-4 in activating 'alternative' macrophages (Stein *et al.*, 1992). Since then, various genetic markers have been identified as specific signatures for the two poles of macrophage activation states. M1 macrophages are associated with CCR1, CCR2, CCR5, CCR7, TLR4 and IL-6, whilst M2 macrophages are associated with CXCR1, CCL18, KLF4 and CD163 (Galván-Peña and O'Neill, 2014; Xuan *et al.*, 2015; Wang *et al.*, 2017). While this binary model is useful, it fails to reflect the remarkable plasticity and diversity of macrophages; capable of rapidly changing their phenotype in response to environmental cues (Italiani and Boraschi, 2014; Saha, Shalova and Biswas, 2017; Van den Bossche, O'Neill and Menon, 2017). It is now thought that this paradigm should be extended to encompass a wide spectrum of macrophage activation states, the characteristics of which remain largely unexplored.

Dendritic cells (DC) are the major antigen-presenting cell (APC) and after activation, maturation and differentiation, migrate to the lymphoid tissues where they specialise in the cross-presentation of exogenous antigens to naive CD8 T cells (Gierut, Perlman and Pope, 2010). DCs are a heterogeneous population and can broadly be classified into conventional myeloid and plasmacytoid DCs (Ziegler-Heitbrock *et al.*, 2010). Indeed, there are many subsets of DC which can be classified based on functional specialisation, developmental origin or maturity status (Shortman and Liu, 2002). The inflammatory DC subset, most likely monocyte-derived, have been described as the main DC subset present in RA synovial fluid and have been associated with induction of the Th17 response (Segura *et al.*, 2013). In contrast, plasmacytoid DCs in the periphery have been suggested to have a more tolerogenic role in RA (Cooles *et al.*, 2018). A recent paper from our research group identified a significant enrichment of the CD141+ DC subset in the inflamed synovial joint which were significantly more activated and transcriptionally distinct from CD141+ DCs in the periphery (Canavan *et al.*, 2018).

1.17 Adaptive Immune Cells

Prior to disease onset, the sub-lining layer of synovial tissue is relatively acellular, however in active disease the inflamed synovium comprises of approximately 30-50% T cells and 5% B cells (Duke *et al.*, 1982). Recent studies have broadened our understanding of lymphoid tissue heterogeneity in RA patients with the classification of four RA synovial subsets; lymphoid, myeloid, fibroid and low inflammatory (Dennis *et al.*, 2014). T cells are present in all four subsets but abundantly so only in the lymphoid compartment and to a lesser extent in the myeloid subset (Dennis *et al.*, 2014; Asif Amin, Fox and Ruth, 2017). Indeed, extensive RNA sequencing of RA synovium and matched peripheral blood in treatment naïve patients has revealed three distinct pathotypes; a fibroblastic pauci-immune pathotype displaying a distinct lack of immune infiltrate, a macrophage-rich diffuse myeloid pathotype, and a lympho-myeloid pathotype characterized by infiltration of lymphocytes and myeloid cells (Myles J Lewis *et al.*, 2019).

While lymphoid aggregates can be identified in perivascular regions of inflamed synovial tissue, T and B cells are also found scattered throughout the synovial tissue (Iguchi and Ziff, 1986; Baeten *et al.*, 2005; Orr *et al.*, 2017). Many studies have

demonstrated that large follicular lymphocyte aggregates are associated with greater disease severity in comparison with a diffuse infiltrate, and may be a predictor of a reduced response to therapy (Klimiuk *et al.*, 2003; Canete *et al.*, 2009; Orr *et al.*, 2017). Indeed, recent RNA-seq analysis of synovial tissue and blood in early RA has indicated that persistent synovial plasma cell infiltration is associated with rapid disease progression resulting in poor prognosis in terms of radiographic damage (Myles J. Lewis *et al.*, 2019). Effector T cells can be loosely classified into CD8+ cytotoxic T cells or CD4+ helper T cells although a number of T cell subsets have now been identified including Type 1 helper T cells (Th1), natural killer T cells (NKT), regulatory T cells (Tregs) and Th17 helper cells (Th17). Traditionally, RA was believed to be a Th1 mediated disease, however many studies have also now implicated Th17 subsets which produce IL-17A, IL-17F, IL-21, IL-22 and TNF α (Lubberts, Koenders and van den Berg, 2005; Miossec, Korn and Kuchroo, 2009; van Hamburg and Tas, 2018). Indeed, a study in collaboration with our research group identified a specific subset of polyfunctional ex-Th17 cells found to be enriched at the site of inflammation which are resistant to Treg inhibition (Basdeo *et al.*, 2015).

In addition, studies have indicated a significant reduction in CD4+ T cells in response to TNF α inhibitors, with no change to CD8+ T cells. This is consistent with the hypothesis that CD4+ T cells are crucial in initiating and driving specific immune responses (Choy *et al.*, 1996; Veale *et al.*, 1999; Mason *et al.*, 2002). Moreover, a subset of PD-1hiCXCR5-CD4+ T cells have been identified in the RA synovium and periphery which appear to be primed to drive B cell response and antibody production (Rao *et al.*, 2017).

B cells, charged with the production of many pathogenic antibodies such as ACPA, have also emerged as an important target for RA. Studies have indicated that synovial B cells and ACPA positivity may be a predictor of chronic inflammation (Teng *et al.*, 2007). The crucial relationship between synovial B cells, seropositivity and changes in radiographic erosion has been established (Gerlag *et al.*, 2012; Orr *et al.*, 2017). Such evidence has led to the development and success of many B cell targeting therapies which further emphasises the vital role that B cells play in the pathogenesis of RA (Hofmann, Clauser and Manz, 2018). One such B cell targeted therapies is Rituximab, a monoclonal antibody targeting CD20 expressing B cells, which has been shown to be very effective in the treatment RA patients (Porter *et al.*, 2016).

Furthermore, studies analysing the potential prognostic value of the B cell chemoattractant CXC ligand 13 (CXCL13) have shown much promise. CXCL13 plays a role in many autoimmune conditions through redistribution of B cells into inflamed/injured tissues as well as enhancing their BCR-mediated activation (Corsiero *et al.*, 2012). Indeed with respect to RA, CXCL13 appears to be a marker of disease severity, with CXCL13 serum levels shown to be increased in early disease, levels of which correlate with synovial CXCL13 (Meeuwisse *et al.*, 2011; Rosengren *et al.*, 2011; Bugatti *et al.*, 2012; Bugatti, Manzo, *et al.*, 2014; Orr *et al.* 2017).

1.18 Synovial Fibroblasts

Fibroblast-like synoviocytes (FLS) are the resident mesenchymal cells of RA synovial tissue, identified mainly by their expression of vimentin and several types of collagen. FLS are the main stromal cells of the joint, primarily involved in producing extracellular matrix components and in building the structural framework of tissue. In RA however, FLS are transformed into a pathogenic phenotype, capable of interacting with immune cells and thus potentiating the immune response. RAFLS are also active drivers of joint destruction due to their ability to invade collagenous structures including articular cartilage (Mor, Abramson and Pillinger, 2005; Asif Amin, Fox and Ruth, 2017; Veale, Orr and Fearon, 2017).

RAFLS secrete numerous pro-inflammatory mediators that further amplify joint inflammation including VEGF, IL-6, IL-8, MCP-1 and RANKL (Mor, Abramson and Pillinger, 2005; Bottini and Firestein, 2013). In addition, RAFLS have increased cell surface expression of adhesion molecules such as VCAM-1 and Cadherin-11, are potent producers of MMPs and are resistant to apoptosis (Guerne *et al.*, 1989; Bartok and Firestein, 2010; Klein, Ospelt and Gay, 2012). Indeed, MMPs released from RAFLS invade more aggressively through matrigel compared to FLS from OA or avascular necrosis (AVN) synovium (Tolboom *et al.*, 2002). This ability of RAFLS to directly invade and degrade cartilage has also been demonstrated in human RAFLS/SCID mice chimera models (Seemayer *et al.*, 2003; Lefèvre *et al.*, 2009).

In recent years it has become apparent that RAFLS aren't a homogenous cell population as once thought. Studies have now identified that distinct subsets of fibroblasts exist within the inflamed synovium with non-overlapping functions such as joint and cartilage damage and immune-inflammatory regulation, effects that also depend on positional memory (Croft *et al.*, 2019; Mizoguchi *et al.*, 2019). Moreover, synovial fibroblasts from different anatomical locations display distinct transcriptomic and epigenetic signatures resulting in unique site-specific fibroblast phenotypes (Frank-Bertoncelj *et al.*, 2017).

1.19 Cytokines

Cytokines are soluble or membrane bound proteins with molecular weights ranging from 8 to 50 kDa that function to transmit signals between cells. They are capable of mediating a wide range of functions including the regulation of cell differentiation, replication, survival and death, tissue repair and fibrosis (Choy and Panayi, 2001; McInnes and Schett, 2007). As such, pleiotropism (multiple biological properties) is one of the hallmarks of cytokines. Cytokines are functionally classified based on their biologic activity, not structural similarities or amino acid sequence motifs (Dinarello, 2000). Crucial to almost every biological process; cytokines affect embryonic development, disease pathogenesis, response to infection, changes in cognitive function and the ageing process. They have steadfast become a critical frontier in modern medicine with diagnostic, prognostic and therapeutic capabilities in disease (Dinarello, 2007).

Interleukin-1 (IL-1) was the first cytokine to be identified in RA in synovial fluid in 1982 (Fontana *et al.*, 1982). Following this, many studies reported the detection of both TNF α and IL-1 β in RA synovial fluid and synovium (Buchan *et al.*, 1988; Di Giovine, Nuki and Duff, 1988; Hopkins and Meager, 1988; Hopkins, Humphreys and Jayson, 1988; Firestein *et al.*, 1990). To date, many other pro-inflammatory cytokines have been detected in the RA synovium including IL-6 (Houssiau *et al.*, 1988; WOOD *et al.*, 2008), IFN α (Hopkins and Meager, 1988), M-CSF (Firestein *et al.*, 1988), GM-CSF (Xu *et al.*, 1989; Haworth *et al.*, 1991), IL-17 (Chabaud *et al.*, 1999) amongst others.

Cytokines regulate a rich panoply of processes and in recent years have been exploited for their therapeutic potential in RA with the advent of biologic therapies. TNF α and IL-6 have been consolidated as the cytokines of most hierarchical importance due to clear clinical benefits following their inhibition in RA. In particular, TNF α is established to be a 'master cytokine' orchestrating a number of synovial pathological mechanisms including leukocyte activation, endothelial activation and angiogenesis, cytokine expression, stromal cell and osteoclast activation and MMP production (Choy and Panayi, 2001; McInnes, Buckley and Isaacs, 2016). Persistent production of IL-6 and other IL-6-type cytokines such as OSM, has also been demonstrated to be crucial for RA development (Richards, 2013; Li and Zhang, 2017; Narazaki, Tanaka and Kishimoto, 2017).

Many other cytokines are detected within the RA synovium including several members of the IL-1 family; IL-1 α , IL-1 β , IL-1Ra, IL-18, IL-33, and IL-36 (McInnes and Schett, 2011; Garlanda, Dinarello and Mantovani, 2013). However, despite encouraging studies on the functional involvement of IL-1 in synovial processes, IL-1 inhibition has not been successful in treating RA (McInnes, Buckley and Isaacs, 2016). Another cytokine of interest is the Th17-secreted IL-17. IL-17 levels in sera and synovial fluid correlate strongly with many markers of RA disease activity, yet IL-17 blockade has also resulted in disappointing clinical efficacy (Roşu *et al.*, 2012; Robert and Miossec, 2018).

Conversely, anti-inflammatory or immunoregulatory cytokines are also present in the RA synovium, with IL-10 considered the most potent. Recombinant IL-10 is capable of inhibiting TNF α and IL-1 production while IL-10 neutralising antibodies enhance their secretion (Katsikis *et al.*, 1994). However, IL-10 has been reported to have some immune stimulatory effects, as such very few cytokines have exclusively pro- or anti-inflammatory function but instead function in a context and microenvironment dependant manner (Salazar-Onfray, López and Mendoza-Naranjo, 2007; Chen *et al.*, 2019). Other common immunomodulatory cytokines include IL-4, IL-13 and TGF β . TGF β has also been found to have both pro and anti-inflammatory properties. TGF β plays a key role in the suppression of autoimmunity by Tregs yet paradoxically it can induce pro-inflammatory cytokines and MMPs (CHEON *et al.*, 2002; Wahl and Chen, 2005).

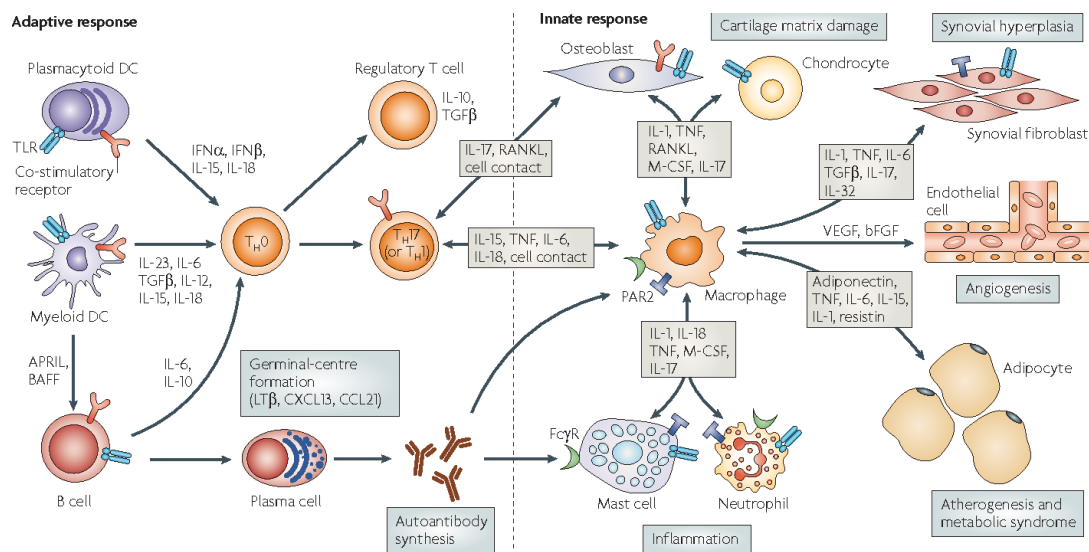


Figure 1.5 Schematic overview of the cytokines involved in RA pathogenesis.

Key cytokines involved in the activation of both innate and adaptive cells of the immune system and the clinical manifestations of these effects. Taken from (McInnes and Schett, 2007).

1.20 Chemokines

Chemotactic cytokines or chemokines are a subset of chemoattractant cytokines involved in leukocyte chemotaxis and migration through the endothelial barrier (Chen, Oppenheim and Howard, 2004). Chemokines can be grouped into four supergene families based on cysteine residue location. This classification system was revised in 2000 with chemokines now considered as chemokine receptor ligands CXCL, CCL, CL and CX₃CL and the corresponding chemokine receptors denoted as follows; CXCR, CCR, CR, CX₃CR (Bacon *et al.*, 2002).

A number of chemokines have been detected in synovial tissue, fluid and sera of inflammatory arthritis patients (Deleuran *et al.*, 1994; Hosaka *et al.*, 1994; Szekanecz *et al.*, 2010). While synovial macrophages are the major source of chemokines, they can also be produced by synovial fibroblasts and endothelial cells (Szekanecz and Koch, 2007a; Moran *et al.*, 2011; Connolly *et al.*, 2012). In addition to pro-inflammatory signalling,

chemokines can also regulate cellular adhesion, cytoskeleton rearrangement and many angiogenic processes (Szekanecz *et al.*, 2010).

Two members of the CXCL group of chemokines CXCL8 (IL-8) and CXCL1 (Gro- α) are considered as key pro-inflammatory chemokines in inflammatory arthritis. Involved in a plethora of cellular functions, they are abundantly expressed in RA synovial fluid, tissue and sera (Koch *et al.*, 1991, 1995; Deleuran *et al.*, 1994). Monocyte chemoattractant protein 1 (MCP-1)/CCL2) and Regulated upon Activation Normal T cell Expressed and Secreted (RANTES)/CCL5) are members of the CCL family and display chemotactic activity towards monocytes, NK and T cells. They have also been detected in the RA synovium and in the serum (Koch *et al.*, 1992; Volin *et al.*, 1998; Szekanecz *et al.*, 2010).

Chemokines mediate their effects via 7-transmembrane domain receptors expressed on target cells (Zlotnik and Yoshie, 2000). Some chemokine receptors such as CXCR2/CCR1/CCR3 have multiple ligands while others are specific for a single ligand (Koch, 2005). As such, redundancy and binding promiscuity is common among chemokine receptors and ligands. Nearly all chemokine receptors have been associated with various types of autoimmunity with CXCR3 and CCR5 particularly associated with RA (Loetscher *et al.*, 1998; Qin *et al.*, 1998). CXCR3 is expressed in the RA synovium and may be one of the most important leukocyte homing receptor in RA (Qin *et al.*, 1998; García-López *et al.*, 2001). In addition, CXCR1 and CXCR2, expressed on macrophages and neutrophils are also abundantly expressed in the RA synovium (Patterson *et al.*, 2002; Szekanecz *et al.*, 2010).

1.21 Overview of Cellular Metabolism

Cellular metabolism can be loosely defined as the sum of all biochemical processes in a cell which encompasses thousands of reactions and metabolites. Core metabolism can be simplified into pathways involving key nutrients such as carbohydrates, fatty acids and amino acids, crucial for energy homeostasis and synthesis of macromolecules (Deberardinis and Thompson, 2012). A new appreciation for metabolic pathways has been driven by the realisation that metabolic perturbations often disrupt normal physiology resulting in disease. The link between metabolic pathways and human pathologies however, can date back thousands of years; Indian physicians observed that

the urine of diabetic patients attracted ants while urine from a healthy individual did not, while Celsus concluded that rich food and drink exacerbated gout attacks over 2,000 years ago (Trowell, 1982; Deberardinis and Thompson, 2012).

Two of the main metabolic pathways at the forefront of research today are glycolysis and oxidative phosphorylation, the utilisation of which is dependent on oxygen availability or environmental stress. The glycolytic pathway is a metabolic pathway involving the step-wise intracellular processing of glucose. Glycolysis begins with the uptake of extracellular glucose from the surrounding environment where it is irreversibly phosphorylated into glucose-6-phosphate (G6PD). Each molecule of glucose is then further oxidised through a series of anaerobic steps to generate two molecules of pyruvate and release of two ATP molecules. Under normoxic conditions in the presence of oxygen, pyruvate is decarboxylated by pyruvate dehydrogenase (PDH) before entering the tricarboxylic acid (TCA) cycle in the mitochondrial matrix. Electron carriers nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide-2 (FADH₂) are major products of the TCA cycle and transfer electrons to the electron transport chain (ETC). The ETC consists of five large protein complexes embedded in the inner mitochondrial membrane: Complex I-VI and ATP synthase (Complex V). The ETC shuttles electrons across these five complexes which generates an electrochemical proton gradient. ATP synthase then uses this proton gradient to produce 36 molecules of ATP in a process called oxidative phosphorylation (OXPHOS) (Chaban, Boekema and Dudkina, 2014; Fearon *et al.*, 2019).

However, in the absence of oxygen, the two glycolysis-derived pyruvate molecules are directed away from the TCA cycle and are instead converted to lactate by lactate dehydrogenase (LDH) in the cytosol, netting two molecules of ATP. Although glycolysis is a relatively inefficient pathway for the production of ATP compared to OXPHOS, anaerobic glycolysis allows for the rapid generation of energy during conditions of low oxygen or environmental stress (Senyilmaz and Teleman, 2015). In addition, glycolysis provides other key benefits to the cell with many glycolytic intermediate metabolites capable of diverging into parallel pathways such as biosynthetic growth pathways to support anabolic growth. For example, G6PD can feed into the pentose phosphate pathway to produce amino acids, NADPH and ribose for nucleotide synthesis (Buchakjian and

Kornbluth, 2010). Glycolysis also allows for the reduction of NAD⁺ to NADH, an essential cofactor, NADH is often recycled back into NAD⁺ to maintain glycolytic flux resulting in the reduction of pyruvate to lactate (O'Neill, Kishton and Rathmell, 2016).

Cells use many different metabolic pathways to generate adequate levels of energy for their specialised functions. The pentose phosphate pathway (PPP) takes place in the cytosol and allows for the diversion of glycolytic intermediates to produce amino acid and nucleotide precursors essential for cell growth and proliferation. Fatty acid synthesis allows for the generation of lipids in cells and is closely linked to mTOR signalling. Fatty acids can then enter the fatty acid oxidation pathway which causes the mitochondrial conversion of fatty acids into acetyl co-A, NADH and other cofactors for further energy generation. Amino acid metabolism is also crucial as many metabolic pathways use amino acids as substrates. Although diverse, these pathways are closely interconnected with metabolites and intermediates from one pathway feeding into alternative pathways. NADPH produced during the PPP and TCA cycle derived citrate is used during fatty acid synthesis, while fatty acid oxidation drives the ETC by producing NADH and FADH₂. Finally amino acid metabolism feeds into the TCA cycle and the amino acid glutamine can be used as a source of citrate for fatty acid synthesis (O'Neill, Kishton and Rathmell, 2016).

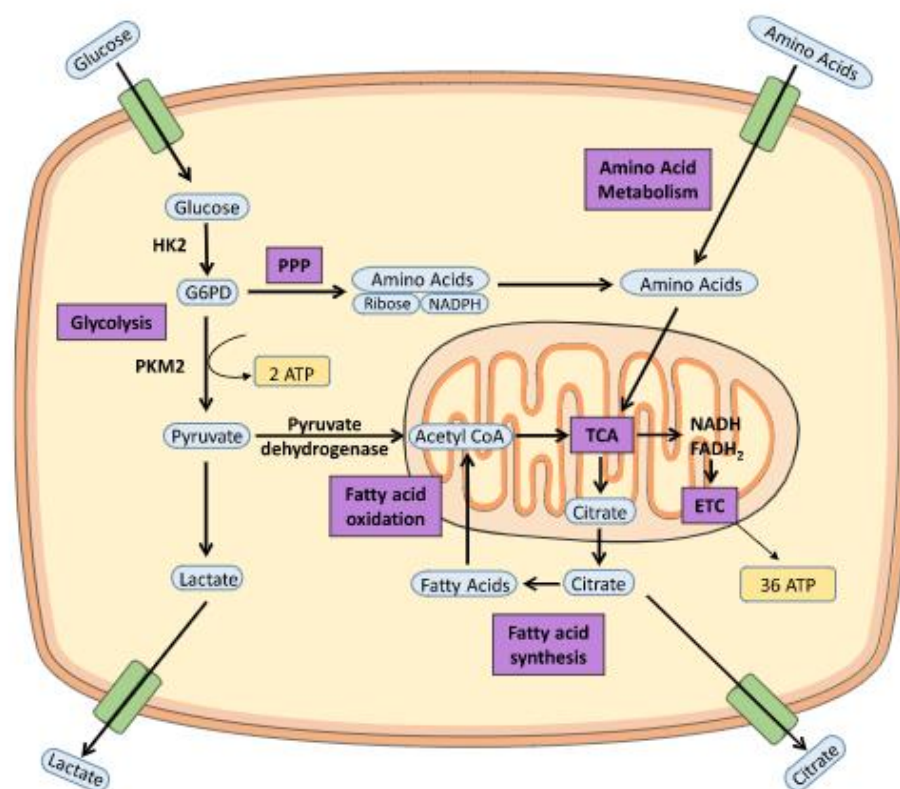


Figure 1.6 Schematic of key metabolic pathways.

Glucose enters the cell via glucose transporters such as GLUT1 and immediately enters glycolysis. Hexokinase 2 (HK2) converts glucose into Glucose 6-phosphate dehydrogenase (G6PD). Glycolysis generates pyruvate from glucose with the help of Pyruvate Kinase M2 (PKM2). This process generates energy in the form of two ATP molecules. Pyruvate is then either converted to lactate and either secreted out of the cell or decarboxylated by pyruvate dehydrogenase and converted to Acetyl CoA which enters the TCA cycle. The TCA cycle generates NADH, FADH₂ to feed into the electron transport chain (ETC) to produce 36 molecules of ATP. Glycolysis also branches off at the level of G6PD to feed into the Pentose Phosphate Pathway (PPP) to produce ribose, NADPH and amino acids. Amino acid metabolism can also feed into the TCA cycle to drive ATP production by the ETC. TCA intermediate citrate drives fatty acid synthesis while fatty acid oxidation drives TCA cycle further by generating Acetyl CoA. Adapted from (Fearon *et al.*, 2019), Appendix B.

1.22 Hypoxia, Metabolism and RA

A fundamental abnormality observed in RA is the inappropriate proliferation of immune cells, a function also observed in the resident stromal cells of the joint that form the invasive synovial pannus. This excessive building of biomass imposes high metabolic demands to generate energy and biosynthetic precursors at a rate which outpaces the dysfunctional oxygen supply creating a hypoxic synovial microenvironment (Kennedy *et al.*, 2010; Ng *et al.*, 2010; Weyand and Goronzy, 2017). Hypoxia, defined as inadequate oxygenation, causes inflammatory cells to switch on invasive mechanisms, and has been significantly implicated in RA pathogenesis (Fearon *et al.*, 2016). Indeed, Ng and colleagues demonstrated that the synovial joint is profoundly hypoxic with pO_2 levels *in vivo* as low as 0.46% (Ng *et al.*, 2010). Subsequent studies have demonstrated that joint hypoxia is inversely associated with increased synovitis, immature and dysfunctional vasculature and immune cell infiltration (Oliver *et al.*, 2009; Kennedy, Ng, Chang, Biniecka, Jacintha N O'Sullivan, *et al.*, 2011; Gao *et al.*, 2012, 2015). The hypoxic synovial microenvironment also involves complex interactions between key pro-inflammatory signalling pathways such as NF- κ B, Notch-1, JAK-STAT and PI3K-AKT (Oliver *et al.*, 2009; Gao *et al.*, 2012, 2015; Li *et al.*, 2013).

Low pO_2 levels stabilises the transcription factor hypoxia-inducible factor (HIF1 α) which translocate to the nucleus to dimerize with HIF1 β subunits, binds to DNA and transcribes hypoxia inducible genes including those encoding pro-inflammatory cytokines, notably IL-1 β (Tannahill *et al.*, 2013; Fearon *et al.*, 2016). HIF1 α also promotes glycolysis by modulating genes for many glycolytic transporters and enzymes. Activity of lactate dehydrogenase (LDHA) is also enhanced in response to HIF1 α (Majmundar, Wong and Simon, 2010). LDHA converts pyruvate to lactate which results in an acidic environment further driving cell proliferation and invasiveness. This is consistent with studies demonstrating elevated lactate levels correlating with diminished glucose levels in synovial fluid of patients with RA, synovial lactic acidosis is also associated with the degree of synovial inflammation (Naughton *et al.*, 1993; Ciurtin *et al.*, 2006; Hitchon, El-Gabalawy and Bezabeh, 2009). Moreover, this acidic microenvironment is known to cause mutations in cells, preventing DNA repair mechanisms which results in the transformation of normal cells and prevention of apoptosis which are key characteristics associated with

the invasive RA synovial fibroblast (Gatenby and Gillies, 2004; Chang and Wei, 2011). Furthermore, glycolytic products such as enolase, lactate, aldolase and pyruvate can act as autoantigens which stimulate abnormal cell proliferation, angiogenesis and pannus formation (Henderson, Bitensky and Chayen, 1979; Akhavani *et al.*, 2009; Chang and Wei, 2011; Biniiecka *et al.*, 2016; Pucino *et al.*, 2017).

Therefore, the hypoxic milieu of the synovial joint coupled with the increased metabolic demands, forces immune cells to adapt to these adverse conditions by switching from a resting state to a highly metabolically active state. Metabolic reprogramming reflects the changes in a cell in response to critical changes in their microenvironment and has become a focus of many research groups.

1.23 Immunometabolism

The fast-moving field of immunometabolism, the concept that metabolic pathways not only provide energy for the cell but also regulate cell phenotype and function, has garnered much attention in recent years. Extrinsic and intrinsic signals resulting in metabolic reprogramming in immune cells provide both energy and the biological intermediates required for rapid and robust immune response. However, the origins of this concept may be rooted in a very significant observation by Otto Warburg in the 1900's. This observation, later termed the Warburg effect, detailed how cancer cells preferentially use glycolysis to generate energy rather than OXPHOS, even in the presence of oxygen (Warburg, 1956). He proposed that this glycolytic switch may provide an explanation for their extraordinary tolerance to extreme local hypoxia along with their ability to compete with normal cells under normoxia.

This metabolic switch has now also been observed in certain immune cells whereby, upon activation, they are capable of switching from OXPHOS to aerobic glycolysis in a manner similar to tumour cells (Garedew, Henderson and Moncada, 2010; Krawczyk *et al.*, 2010; Saha, Shalova and Biswas, 2017; Van den Bossche, O'Neill and Menon, 2017). Despite the poor energy yield gained from glycolysis, it can be rapidly activated. Engaging in oxidative phosphorylation requires mitochondrial biogenesis, a

complex and lengthier process. Ergo, cells that need to swiftly generate ATP, such as activated macrophages, can switch to glycolysis (O'Neill, Kishton and Rathmell, 2016).

1.24 Macrophage Immunometabolism

Metabolic changes and macrophage activation have been linked for many years, indeed altered amino acid metabolism was one of the initial discriminatory markers to define polarised macrophages. LPS/IFN γ pro-inflammatory macrophages (M1) convert arginine to nitric oxide (NO) via inducible NO synthase (iNOS) while alternatively activated IL-4 macrophages (M2) metabolises arginine by arginase-1 (Modolell *et al.*, 1995; Murray *et al.*, 2014). The imbalance between M1 and M2 macrophages, favouring the pro-inflammatory M1 cells is a major driver of RA disease progression.

Early work demonstrated that classically activated M1 macrophage have an ardent appetite for glucose, indicating a reliance on glycolysis (O'Neill, 2015). This reliance, initially counterintuitive, is similar to the Warburg effect observed in cancer cells. It is now known that M1 macrophages switch their metabolism from the more complex oxidative phosphorylation pathway to glycolysis in response to inflammatory triggers such as toll-like receptors (Rodríguez-Prados *et al.*, 2010). In addition, recent work has demonstrated that the TCA cycle is disrupted at two steps in M1 macrophages; one at isocitrate dehydrogenase which leads to an accumulation of isocitrate and the other after succinate (Tannahill *et al.*, 2013; O'Neill, 2015; Lampropoulou *et al.*, 2016).

This cocktail of enhanced glycolytic flux and fragmented TCA cycle, considered a hallmark of M1 macrophage activation, is reflected in many studies of RA macrophages. Macrophage accumulation of succinate causes stabilisation of HIF1 α which subsequently promotes IL-1 β production (Tannahill *et al.*, 2013). Not surprisingly, mice lacking GPR91 the succinate receptor, show reduced macrophage activation and IL-1 β secretion in a model of antigen-induced arthritis (Littlewood-Evans *et al.*, 2016). A metabolic inhibitor of succinate dehydrogenase (SDH), itaconate, has been shown to be elevated in the RA joint, correlates to disease activity and treatment response in arthritis models (Michopoulos *et al.*, 2016). Itaconate also regulates succinate levels and secretion of inflammatory cytokines in activated macrophages (Lampropoulou *et al.*, 2016). RA

macrophages express high levels of the glycolytic enzyme α -enolase, while increased concentrations of glucose promote IL-1 β secretion in RA myeloid cells via NLRP3 (Bae *et al.*, 2012; Ruscitti *et al.*, 2015). In addition, increased levels lactic acid, succinate and citrate have been detected in RA synovial fluid (Kim *et al.*, 2014).

This sustained pro-inflammatory activity of glycolytic macrophages is crucial to RA pathogenesis (McInnes and Schett, 2007). RA synovial tissue macrophages correlate strongly with mitochondrial dysfunction, oxidative stress and joint hypoxia (Ng *et al.*, 2010; Kennedy, Ng, Chang, Biniecka, Jacintha N. O'Sullivan, *et al.*, 2011b; Harty *et al.*, 2012). Such effects are reversed in those who respond to TNFi treatment while a combination of TNF and hypoxic conditions increases macrophage survival (Hamilton *et al.*, 2012). Indeed HIF1 α is abundantly expressed by RA synovial tissue macrophages in comparison to osteoarthritis (OA) and healthy control synovial macrophages (Hollander *et al.*, 2001). *In vitro* studies of CIA (collagen induced arthritis) mouse models have indicated that deletion of HIF1 α in myeloid cells results in decreased macrophage infiltration and joint swelling due to reduced macrophage mobility and invasive capacity (Cramer *et al.*, 2003).

In contrast, anti-inflammatory M2 macrophages are reliant on OXPHOS, have an intact TCA cycle and provide substrates for the electron transport chain (Van den Bossche, Baardman and de Winther, 2015). Studies have demonstrated that glycolysis is also crucial in M2 macrophages as inhibition of glycolysis with 2-deoxyglucose (2DG) inhibits M2 polarisation and function (Tan *et al.*, 2015; Huang *et al.*, 2016). However, conflicting studies have recently suggested that glycolysis is not required for M2 polarisation provided OXPHOS is fuelled by other sources such as glutamine (Wang *et al.*, 2018).

1.25 Metabolites

Once considered arbitrary bystander products of cellular metabolism, lactate and other metabolites have now been identified as active signalling molecules with immunomodulatory functions. The effect of lactate on T cell phenotype has been elegantly described in a study by Haas *et al* whereby high levels of lactate at the site of inflammation acts to entrap CD4⁺ T cells in RA by inhibiting T cell motility. In addition

lactate triggers T cell IL-17 production to drive the inflammatory response further (Haas *et al.*, 2015). In the tumour microenvironment however, accumulation of lactate as a result of the Warburg effect results in acidosis, impairs T cell motility and hinders their cytotoxicity (Brand *et al.*, 2016). Thus, lactate serves to boost pro-inflammatory mechanisms in the inflammatory milieu yet suppresses immunity in the cancer setting.

Differential amino acid metabolism plays a major role in directing cell phenotype and function. The amino acid transporter SLC7A5, induces proinflammatory cytokine secretion in RA monocytes and macrophages through leucine influx, has also been demonstrated to play a key role in tumour survival and growth (Oda *et al.*, 2010; Wang and Holst, 2015; Yoon *et al.*, 2018). Metabolism of arginine to release nitric oxide (NO) by iNOS can have pleiotropic effects depending on cell type. NO production in inflammatory macrophages and DCs acts to inhibit mitochondrial respiration to drive a pro-glycolytic phenotype (Clementi *et al.*, 1998; Everts *et al.*, 2012). However, arginine metabolism in T cells instead promotes a metabolic switch to OXPHOS and exerts anti-inflammatory effects (Niedbala, Cai and Liew, 2006; Geiger *et al.*, 2016).

Finally, accumulation of TCA intermediates citrate and succinate can also shape effector responses of immune cells. Succinate perpetuates the inflammatory response through Hlf-1 α -induced IL-1 β production and generation of ROS in inflammatory macrophages (Tannahill *et al.*, 2013; Mills *et al.*, 2016). Directly, citrate can promote pro-inflammatory mechanisms but also indirectly boosts inflammation through the effects of itaconate. Citrate can be converted to itaconate which inhibits succinate dehydrogenase to drive the pro-inflammatory effects of succinate thus linking the two intermediates (Infantino *et al.*, 2011; Michelucci *et al.*, 2013; Lampropoulou *et al.*, 2016). Enolase is capable of inducing monocyte/macrophage activation in models of RA while promoting regulatory T cell development in cancer models (Bae *et al.*, 2012; Amedei *et al.*, 2013). Thus, nutrients, amino acids and metabolites serve as very active signalling molecules with divergent effects depending on microenvironment and cell type.

1.26 JAK-STAT Signalling Pathway

The Janus kinase (JAK) and signal transducer and activator of transcription (STAT) pathway is one of the major intracellular signalling pathways serving as a critical downstream mediator for a variety of cytokines, hormones and growth factors. JAK proteins, lying downstream of type I and II cytokine receptors, are therefore crucial regulators of cellular responses to inflammation. Indeed, combinations of these proteins allow for site and event specificity, JAK1/2 specific for IL-6, JAK1/Tyk2 for interferon signalling and JAK1/JAK3 for T cell specific cytokines (Shuai and Liu, 2003; O'Shea, Laurence and McInnes, 2013).

Four members of the JAK family have been identified, namely. JAK1, JAK2, JAK3 and TYK2 and seven members of the STAT family: STAT 1-4, STAT5A/B, and STAT6. Upon binding of the specific ligand to its receptor, JAK proteins are recruited to the intracellular domain of the receptor where they dimerize and autophosphorylate. The activated JAKs then recruit and phosphorylate STAT proteins which can then translocate to the nucleus to bind to DNA response elements and modulate transcription of downstream target genes (O'Shea and Plenge, 2012; Dodington, Desai and Woo, 2018). Suppressors of cytokine signalling (SOCS) are capable of negatively regulating JAK/STAT signal transduction.

Ubiquitously expressed, JAK/STAT proteins are involved in a range of fundamental biologic processes including the immune response and inflammation. Not surprisingly, dysregulation of this signal transduction pathway is associated with various immune and inflammatory disorders, including RA (Shuai and Liu, 2003; Boyle *et al.*, 2015; Gao *et al.*, 2015). Immunohistochemical analysis of the inflamed synovium reveals elevated expression of many activated STATs thus implicating the JAK/STAT pathway in RA pathogenesis (Van der Pouw Kraan *et al.*, 2003; Walker *et al.*, 2006). In RA, studies have shown that STAT3 expression is associated with synovitis and mediates RAFLS migration and invasive mechanisms (Ju *et al.*, 2012; Gao *et al.*, 2015). In line with this, studies have demonstrated an association between synovial pO₂ levels *in vivo* and pSTAT3 activation, while hypoxia-induced STAT3 activation boosts HIF1 α activation (Jung *et al.*, 2005; Gao *et al.*, 2015). Inhibition of STAT3 suppresses disease severity in the CIA model of arthritis

(Mori *et al.*, 2011). Tofacitinib, an orally available JAK-inhibitor has been approved for the treatment of RA which has been showing clinical efficacy (Hodge *et al.*, 2016).

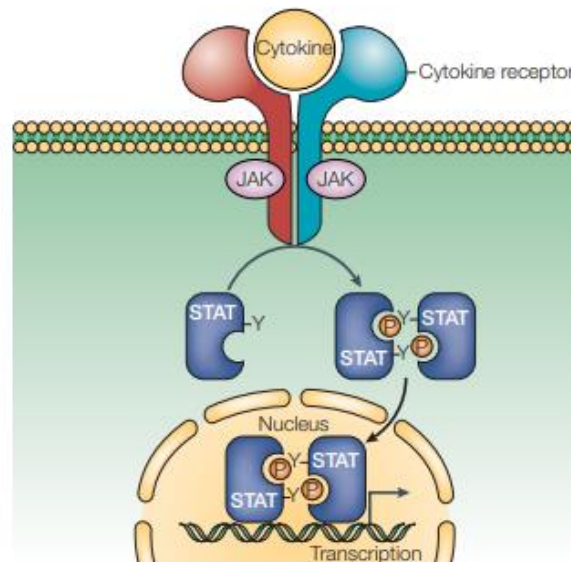


Figure 1.7 Schematic representation of the JAK/STAT pathway.

Cytokine stimulation following receptor-ligand interaction results in the activation of JAKs which can then phosphorylate STATs. Dimerization of STATs results in translocation to the nucleus to activate transcription of target genes Taken from (Shuai and Liu, 2003).

1.27 Treatment for RA

Thirty years ago, a diagnosis of RA would have had devastating consequences resulting in chronic destruction of joints, considerable disability, reduced quality of life and disruptions to employment (Sokka *et al.*, 2009). Prior to the introduction of corticosteroids in the 1950s, treatment options were limited to non-steroidal anti-inflammatory drugs such as aspirin. These therapies, however, do not retard inflammation, merely acting to provide symptomatic relief. The clinical picture from patients with RA has changed dramatically over the past decades with the development of disease modifying anti-rheumatic drugs (DMARDs), capable of altering the course of treatment and management of the disease (Hyndman, 2017). In addition, owing to extensive research, it is now evident that immunological events precede the onset of

clinical manifestations and so early intervention is imperative (Quinn and Cox, 2005). Studies have demonstrated that early treatment is associated with improved clinical outcomes, with the first 3 months after symptom onset representing a therapeutic window of opportunity (Nell *et al.*, 2004; Raza, 2010; van der Linden *et al.*, 2010).

Indeed, a status of remission can be achieved if the disease is recognised early and monitored closely. However, the onset of arthritis is often insidious with patients presenting with one or more swollen joints along with non-specific symptoms such as fatigue and flu-like feelings resulting in challenges in immediate recognition of the disease (Burmester and Pope, 2017). Moreover, no individual treatment is fully effective, and many patients fail to respond or have suboptimal responses. This suggests that despite common pathological mechanisms of disease, the aggressive nature of RA, its concomitant structural damage and its effect on quality of life varies greatly from patient to patient (Furst *et al.*, 2002). Current treatment strategies are outlined below.

1.28 Non-steroidal Anti-inflammatory Drugs

NSAIDs exert their anti-inflammatory, anti-pyretic, and pain-relieving effects by acting on the cyclo-oxygenase (COX) enzyme resulting in the inhibition of prostaglandins at the site of inflammation (VANE, 1971). Two different isoforms of COX have been described (COX I and II) with both non-selective (COX I and COX II) and selective (COX II only) inhibitors approved for the treatment of inflammation (Vane, 1998). COX II is generally undetected under normal physiological conditions but is upregulated upon inflammatory conditions indicating a critical role in inflammation. Four COX II selective drugs collectively called coxibs have been developed: rofecoxib, celecoxib, valdecoxib and etoricoxib, which are used in the treatment of inflammation.

These highly effective analgesics are commonly prescribed to relieve RA patients from pain and inflammation and are one of the cornerstones in pain management for RA (van Walsem *et al.*, 2015). Although widely used, NSAIDs have raised some safety concerns. Merck voluntarily withdrew rofecoxib from the worldwide market due to increased incidence of heart attack and stroke in patients (Mamdani *et al.*, 2004). In 2015, a new safety announcement was issued by the FDA warning that non-aspirin NSAIDs

increase the risk of cardiovascular events. The report also advised that CV events can occur within the first few weeks of NSAID usage and the risk is greater the higher the dose (Bournia *et al.*, 2017). It is now appreciated that all NSAIDs are accompanied with some degree of cardiovascular and gastrointestinal risk which has been reflected in the guidelines (Conaghan, 2012).

Certain NSAIDs have been shown to inhibit glucose metabolism (Gottfried *et al.*, 2013). Of four NSAIDs, diclofenac was shown to be the most potent inhibitor of glycolysis as shown by Sasaki *et al* (Sasaki *et al.*, 2016). Diclofenac has been studied as an inhibitor of lactate transport, interfering with lactate efflux thereby has an inhibitory effect on glucose uptake and glycolysis (Gerthofer *et al.*, no date; Colen *et al.*, 2011; Gottfried *et al.*, 2013). Due to the fact that certain NSAIDs such as diclofenac are monocarboxylates and a recent study has indicated that diclofenac reduces lactate levels by directly targets MCT1 and MCT4, the lactate transporters in a COX-independent manner (Renner *et al.*, 2019). Therefore, treatment with NSAIDs may a role in cellular bioenergetic status of inflammatory cells in RA.

1.29 Disease Modifying Anti-Rheumatic Drugs

Disease modifying anti-rheumatic drugs (DMARDs) are agents that impede both the inflammatory and destructive processes of RA, interfering with the natural course of the disease (Smolen and Steiner, 2003). DMARDs inhibit joint damage, suppress the acute phase response and decrease autoantibody levels (Smolen *et al.*, 2017). Conventional DMARDs include sulfasalazine, gold salts, antimalarial and methotrexate (MTX), biological DMARDs include TNF-inhibitors and IL-6 inhibitors while targeted DMARDs such as JAK inhibitors are also in use.

The most commonly used DMARD is MTX, a folate antimetabolite that inhibits folate dependent enzymes thus interfering with cell proliferation (Purcell and Ettinger, 2003). Originally conceived as a chemotherapeutic agent, MTX confers anti-inflammatory effects by inhibiting cellular proliferation, particularly acting on immune cells central to synovial inflammation such as T lymphocytes (Farber *et al.*, 1948; Chan and Cronstein,

2010). Studies also suggest that anti-inflammatory effects may also be achieved through MTX inhibition of polyamines and the release of adenosine from cells (Chan and Cronstein, 2010).

As an antimetabolite, it is not surprising that MTX affects cellular metabolism. Human FLS treated with MTX displayed significantly reduced HK2 expression and glucose/fructose carriers suggesting that MTX modulates cellular metabolism in FLS (Sanchez-Lopez, Cheng and Guma, no date; Shervington *et al.*, 2018). MTX is also capable of inducing activation of the energy sensor AMPK via AICAR, seen as an imitator of energy deprivation, which correlates with the dampened response in stimulated macrophages (Zheng *et al.*, 2009; Cudrici, Pelletier and Siegel, 2017). MTX may also influence inflammatory and bioenergetic processes by attenuating oxidative stress and scavenging free radicals (Zimmerman *et al.*, 2017; Pålsson-McDermott and O'Neill, 2020). Moreover, a recent study has demonstrated excessive glycolysis in MTX-resistant primary CNS lymphoma-derived cells (Takashima, Hayano and Yamanaka, 2020).

MTX was first approved as a therapy for RA in the 1980s having been previously introduced as a treatment for psoriatic arthritis (BLACK *et al.*, 1964; Weinblatt *et al.*, 1985). Since its approval for RA, it has become the flagship against which all other therapeutic agents have been measured. It is often regarded as the anchor drug for treatment of RA and widely used in combination therapies.

1.30 Biological DMARDs

The clear role of pro-inflammatory cytokines such as TNF α and IL-6 and their surface molecules in the pathogenesis of RA led to the development of highly specific therapeutics targeting these molecules. Biological DMARDs target extracellular mediators of inflammation in RA with high specificity. Currently the approved biological therapies for RA have four mechanisms of actions, inhibition of TNF α , IL-6 receptor inhibition, blockade of T cell costimulation and B cell depletion (Smolen, Aletaha and McInnes, 2016). Interestingly, despite overwhelming evidence implicating IL-1 in the pathogenesis of RA, IL-1 antagonists such as anakinra are relatively less efficacious in comparison to other traditional DMARDs (Scott *et al.*, 2016).

TNF inhibitors (TNFi) were the first of the biological therapies to revolutionise the treatment of RA. The key role of TNF α in RA became clear in studies assessing cytokine levels in the synovium of RA patients, where studies demonstrated that incubation with anti-TNF α antibodies significantly down-regulated the expression of many other key pro-inflammatory cytokines thus implicating it as a master regulator of inflammation (Brennan *et al.*, 1989; Feldmann, Brennan and Maini, 1996; Feldmann *et al.*, 2004). Five TNF α inhibitors are currently approved, infliximab for intravenous use and four subcutaneous compounds (adalimumab, certolizumab pegol, etanercept and golimumab). Etanercept is a TNF-receptor fusion protein, while the others are monoclonal antibodies or fragments of monoclonal antibodies (Law and Taylor, 2019). Indeed, TNFi may soon replace DMARDs as the first line treatment option in RA with studies indicating that TNFi improves bone damage inhibition relative to DMARDs (Veale and Fearon, 2015).

Tocilizumab (TCZ) is an approved recombinant humanized anti-IL-6 receptor monoclonal antibody that targets IL-6, another pivotal cytokine in RA. Tocilizumab, which can be both subcutaneously and intravenously administered, is effective in achieving rapid and sustained improvements in combination with MTX in RA patients who failed TNFi (Emery, Keystone, *et al.*, 2008). Inhibition of IL-6 by TCZ reduces systemic inflammation, RA synovitis and cartilage and bone damage, and may have beneficial cardiovascular effects (Rossi *et al.*, 2015). TCZ has demonstrated efficacy either as a monotherapy or in combination with DMARDs for patients with moderate to severe RA (Al-Shakarchi, Gullick and Scott, 2013). TCZ has paved the way for the development of other IL-6 inhibitors including those that target the cytokine itself such as sirukumab, olokizumab and clazakizumab. Sarilumab is the second IL-6 receptor inhibitor to be approved for RA treatment (Takeuchi *et al.*, 2016; Aletaha *et al.*, 2017).

Abatacept, is a recombinant, dimerised cytotoxic T-lymphocyte antigen 4 (CTLA-4). The endogenous antigen is a natural inhibitor of T cell activation. Abatacept binds to costimulatory molecules CD80/CD86 to prevent CD28-mediated T cell activation (Law and Taylor, 2019). Abatacept is currently the only T cell co-stimulation inhibitor approved for RA, clinical trials have demonstrated its superiority over placebo in those who have failed TNFi and also in TNFi naïve patients (Kremer *et al.*, 2003; Genovese *et al.*, 2005). Interestingly, the efficacy of Abatacept may be as a result of inhibition of myeloid cell

function and not primarily due to the inhibition of T cell activation (Bonelli *et al.*, 2013; Patakas *et al.*, 2016).

Rituximab (RTX) is a B cell targeted therapy available for the treatment of RA following inadequate response to at least one TNFi (Rossi *et al.*, 2015). It is a genetically engineered chimeric monoclonal antibody that binds to CD20 on the surface of B cells (Reff *et al.*, 1994). B cell depletion results in reduced auto-antibody production and inhibition of antigen presentation to T cells (Di Gaetano *et al.*, 2003; Bonelli *et al.*, 2013). Although RTX has demonstrated efficacy in RA, it is less effective in those patients seronegative for ACPA and RF (Edwards, Leandro and Cambridge, 2004; Cohen *et al.*, 2006; Sellam *et al.*, 2011; Isaacs *et al.*, 2013). Another humanised anti-CD20 antibody Ocrelizumab, approved for the treatment of multiple sclerosis, has demonstrated efficacy in RA (Abushouk *et al.*, 2017). In addition, Ofatumumab, which is distinct from other anti-CD20 antibodies as it specifically targets a membrane-proximal epitope on CD20, has shown clinical efficacy comparable to RTX in MTX refractory patients at week 24 (Taylor *et al.*, 2011).

1.31 Non-Biological DMARD Targets

Recent advances in our understanding of the complex interactions between signalling pathways and the immune response have led to a new phase of RA drug development with small-molecule anti-rheumatic agents targeting key nodes in these interactions (O'Shea, Laurence and McInnes, 2013). Intracellular signalling pathways such as the JAK tyrosine kinase family are critical for the signalling of many cytokines involved in driving inflammatory processes in RA such as type I interferons and various interleukins and so have been pushed to the forefront of RA drug development (Hodge *et al.*, 2016). In 2012, Tofacitinib, an orally available pan-(JAK) inhibitor was approved for treatment of patients with RA (Fleischmann, Cutolo, *et al.*, 2012).

Preclinical studies demonstrated that tofacitinib preferentially inhibits JAK1/JAK3-mediated cytokine signalling to modulate the immune response (Meyer *et al.*, 2010; Maeshima *et al.*, 2012). Many clinical studies indicate that tofacitinib given as a monotherapy (Fleischmann, Kremer, *et al.*, 2012; Fleischmann *et al.*, 2017) or in combination with conventional DMARDs (Van Vollenhoven *et al.*, 2012; Kremer *et al.*, 2013; Van Der Heijde *et al.*, 2013), is effective in reducing disease activity. In addition, it has been shown to be effective in MTX naive patients and those with inadequate response to TNFi with tolerability similar to biological DMARDs (Burmester *et al.*, 2013; Lee *et al.*, 2014; Dhillon, 2017; Winthrop, 2017). Other approved JAK inhibitors include Baracitinib, a JAK1/2 inhibitor which was the second JAK inhibitor to be launched for the treatment of RA with significant clinical outcomes shown in Phase 3 trials and approved for mono- or combination therapy in patients with inadequate response to TNFi (Fridman *et al.*, 2010; Kubo, Nakayamada and Tanaka, 2019; Pope *et al.*, 2020). Peficitinib, a pan-JAK inhibitor has been approved for clinical use in Japan and Korea and Upadacitinib a selective and reversible JAK1 inhibitor approved in 2019 have also demonstrated clinical efficacy both alone or in combination with other therapeutics in clinical trials (Pope *et al.*, 2020; Tanaka and Izutsu, 2020). More JAK inhibitors are in development and undergoing clinical trials such as Filogotinib (selective for JAK1) (Genovese *et al.*, 2019) and Decernotinib (selective for JAK3) although the latter has recently been discontinued due to safety concerns (Bechman, Yates and Galloway, 2019; Tanaka and Izutsu, 2020).

In conclusion, JAK inhibition unlike biological treatments which specifically target one cytokine pathway, instigates inhibition of several hubs of key inflammatory cytokines and so is proving to be a novel approach to modulating the inflammatory response (Hodge *et al.*, 2016).

1.32 Personalised Medicine

It is now widely accepted that RA should be viewed as a syndrome or group of diseases consisting of more than one pathogenic entity and clinical phenotypes. This idea is strongly supported by the differences in patients who are, for example, seropositive and seronegative. As such, a treatment that resulted in remission in one patient may in fact be inefficacious for another (Tak, 2012). Therefore, the development of biomarkers to predict treatment response has been a major focus of scientific research, with the goal of personalised medicine to improve therapeutic efficacy while reducing toxicity and cost of treatment (Law and Taylor, 2019). Despite many genome-wide association studies, transcriptomics, proteomics, epigenetics and metabolomics studies, serological status still remains the only applicable means of patient stratification.

Synovial tissue is the key site of pathology in RA. Transcriptomic analysis of synovial tissue is emerging as a clinically significant methodology of identifying disease biomarkers. Advances in arthroscopic biopsy and ultrasound-guided synovial biopsies, with advances in single cell technologies will hopefully allow for detailed profiling of RA synovial tissue to uncover the heterogeneous manifestations of the disease (Romão *et al.*, 2017).

CHAPTER TWO:

Distinct inflammatory and metabolic profiles in Rheumatoid Arthritis macrophages and their precursor cells

2.1 Introduction

Monocytes are crucial innate effector cells central to the initiation and perpetuation of RA disease pathogenesis. Monocytes represent a heterogeneous and plastic pool of immune cells capable of differentiating into macrophages, dendritic cells and osteoclasts subject to the demands of their microenvironment (Rana *et al.*, 2018). In addition to their principal role as precursor cells, monocytes specialize in phagocytosis, secrete many important pro-inflammatory cytokines and chemokines and are involved in the production of reactive oxygen species (ROS) (Cros *et al.*, 2010; Yoon *et al.*, 2014). During inflammation, monocytes have been shown to promote Th17 expansion and preferentially differentiate into inflammatory macrophages (Rossol *et al.*, 2012; Yang *et al.*, 2014). Monocytes are also capable of differentiating into osteoclasts, the main cells responsible for bone erosion in RA, thus firmly consolidating their role in initiation and maintenance of disease activity (de la Rica *et al.*, 2015).

Peripheral blood monocytes may also act as promising biomarkers for the assessment of RA disease activity and treatment response. Studies demonstrate that the proportion of intermediate monocytes positively correlates with RA disease activity and that elevated levels of circulating monocytes in treatment-naïve RA patients are predictive of a reduced or lack of clinical response to treatment with MTX (Chara *et al.*, 2015; Tsukamoto *et al.*, 2017). This may help to guide treatment options for RA.

Upon entry into the synovium, monocytes differentiate into macrophages in response to local M-CSF, inducing the expression of macrophage-associated genes (Merad *et al.*, 2002). This process is particularly enhanced in response to inflammatory conditions and physiological stress (Udalova, Mantovani and Feldmann, 2016). Typically, macrophage functional subsets have been characterized into classical inflammatory M1 and tissue-resolving M2 phenotypes. The M1 macrophages are described as the dominant phenotype in the RA joint. Möttönen and colleagues demonstrated that 68% of RA synovial fluid macrophage-like synoviocytes display a phenotype typical of inflammatory macrophages (Möttönen *et al.*, 1998). A more recent study has confirmed the pro-inflammatory phenotype of *ex vivo* isolated CD14⁺ RA synovial fluid macrophages (Palacios *et al.*, 2015). This is perhaps due to the inflammatory milieu of the synovial microenvironment. Indeed, anti-citrullinated protein antibodies (ACPAs) from RA synovial

fluid skew macrophage polarisation towards a pro-inflammatory M1 phenotype when cultured with peripheral blood monocytes (Zhu *et al.*, 2015). In line with this, studies have demonstrated that M2 polarised macrophages exposed to inflammatory stimuli such as TLR ligands significantly induce the production of pro-inflammatory cytokines and promote Th17 responses, thus indicating that inflammatory signals such as those present in the RA joint, can modify the M2 activation state towards a more pro-inflammatory phenotype (Vogelpoel *et al.*, 2014).

The differing functions of polarised macrophages may be influenced by their bioenergetic status, as recent studies have indicated enhanced glycolytic flux and a concomitant impairment in the TCA cycle is associated with the inflammatory function of M1 macrophages (Saha, Shalova and Biswas, 2017). This is particularly interesting in the context of RA whereby synovial CD68+ macrophages are strongly associated with metabolic activity, mitochondrial dysfunction and oxidative stress *in vivo* (Ng *et al.*, 2010; Biniecka *et al.*, 2011). Moreover, the presence of CD68+ macrophages positively correlates with the degree of disease activity and is inversely related to *in vivo* synovial pO₂ levels (Ng *et al.*, 2010; Kennedy, Ng, Chang, Biniecka, Jacintha N O'Sullivan, *et al.*, 2011; Harty *et al.*, 2012).

The distinct bioenergetic and pro-inflammatory changes observed in M1 macrophages may already be present in circulating monocytes in pathological conditions. A recent study in coronary artery disease (CAD) indicated that monocytes and macrophages, in fact, share a similar metabolic profile. The hyper-metabolic state observed in pathologic macrophages, represented by increased glucose consumption, ATP production and cytokine secretion, was similar to that observed in circulating CAD monocytes, prior to differentiation (Shirai *et al.*, 2016). This suggests that circulating CAD monocytes are already pre-programmed to become inflammatory macrophages. In addition, emerging evidence indicates that monocytes are capable of building immune memory mechanisms via epigenetic reprogramming, a pathway currently being considered as a possible target in atherosclerosis (Neele *et al.*, 2015).

This chapter focuses on the inflammatory and metabolic phenotype of circulating RA monocytes compared to healthy monocytes and whether they have a greater propensity to develop into a pro-inflammatory macrophage phenotype upon activation.

The inflammatory function and metabolic phenotype of *ex vivo* polarised monocyte-derived macrophages in RA is also investigated utilising both functional and transcriptomic analysis. Finally, this chapter assesses whether manipulation of key pathways involved in the inflammatory status of RA monocyte/macrophage can lead to the resolution of inflammation.

2.2 Specific Aims of this Chapter:

- To investigate the inflammatory and metabolic phenotype of RA CD14+ circulating monocytes compared to healthy control
- To examine inflammatory and metabolic differences in RA and healthy control monocyte-derived macrophages
- To investigate if the phenotype of RA CD14+ monocytes persists following differentiation into M1 macrophages
- To conduct functional and bioenergetic characterisation of polarised RA M1 and M2 macrophages
- To conduct in-depth transcriptomic evaluation of RA M1 and M2 macrophages using RNA-seq analysis
- To identify key signalling pathways involved in promoting the resolution of inflammation

2.3 Materials and Methods:

2.3.1 Patient Recruitment

Patients with established RA were recruited from the Rheumatology Department at St. Vincent's University Hospital, Dublin, Ireland. Patients fulfilling the ACR classification criteria were included in this investigation (Aletaha *et al.*, 2010). Patient assessment included same day tender and swollen joint count (TJC/SJC), ESR, CRP and global health visual analogue scale (VAS) (Aletaha *et al.*, 2010). In addition, all patients were assessed for the presence of rheumatoid factor (RF) and ACPAs. All patients were required to give fully informed written consent, approved by the St. Vincent's Healthcare Group Medical Research and Ethics Committee. Healthy blood, used as a comparison, was obtained from anonymous healthy control (HC) donors from St. James's Hospital, Dublin. Ethical approval was obtained by the School of Medicine Research Ethics Committee, Trinity College Dublin.

2.3.2 Peripheral CD14⁺ Monocyte Isolation and Culture

Blood was obtained from RA patients and HC donors and collected in lithium heparin-containing tubes. Peripheral blood mononuclear cells (PBMC) were isolated from blood by Ficoll density gradient centrifugation (Lymphoprep; Stemcell Technologies, Canada). Blood was diluted in a 1:1 ratio with PBS before layering over 15 mL of Lymphoprep (STEMCELL Technologies, Cambridge, UK) and centrifuged at 14000 RPM for 25 min with the brake inactivated. The cloudy PBMC layer was removed at the density gradient interface and washed twice with sterile PBS and centrifuged at 400 g for 5 min. A positive selection of CD14⁺ cells was performed by adding MACS superparamagnetic microbeads (Miltenyi Biotec, France) conjugated with monoclonal anti-human CD14 antibodies to freshly prepared PBMC in MACS buffer (Miltenyi Biotec), according to the manufacturer's instructions. Isolated PBMC were then magnetically sorted and labelled to yield a pure ($\geq 95\%$) population of CD14⁺ monocytes. Cells were cultured in RPMI 1640 supplemented with 10% foetal calf serum (FCS), HEPES (20mM), penicillin-streptomycin (100 units/mL and 100 μ g/mL), Amphotericin B (0.25 μ g/mL) (all Gibco-BRL, UK) and 50 μ g/mL of gentamycin (Sigma-Aldrich). CD14⁺ monocytes were analysed either *ex vivo* or following 3-24 hr stimulations with lipopolysaccharide (LPS; 100 ng/mL) (Enzo Life

Science, UK). For functional experiments, cells were treated with LPS in the presence or absence of the inhibitors; STATTC (STAT-3 inhibitor; 10 μ M), 2-deoxy-D-glucose (2DG) (glycolytic inhibitor; 10 mM) and oligomycin (ATP-synthase inhibitor (oxidative phosphorylation); 2 μ M). For all experiments, inhibitors were added 30 min prior to LPS stimulation.

2.3.3 Isolation and Culture of Monocyte-derived Macrophages

CD14⁺ monocytes from RA/HC PBMC were isolated as above and seeded at a density of 2×10^6 cells/well in a 6 well plate and cultured in complete RPMI supplemented with M-CSF (50 ng/mL) for 8 days to derive *in vitro* macrophage cultures. Macrophages were then polarised for 24 hr to either M1 macrophages using LPS (100 ng/mL) and IFN γ (20 ng/mL) or M2 macrophages using IL-4 (20 ng/mL). For functional studies, macrophages were detached using Accutase cell detachment solution (Sigma-Aldrich, Ireland) for 20 min at 37°C to protect cell viability and surface marker expression. M1-polarised macrophages were also inhibited with STATTC (10 μ M) for 24 hr following polarisation.

2.3.4 Gene Expression Analysis

For gene expression experiments, monocytes were seeded at a density of 1×10^6 cells/well for 3 hr and macrophages were seeded at a density of 2×10^6 cells/well in a 6 well plate on day 0 and polarised on day 8 for 24 hr. Total RNA was then isolated using RNeasy Mini Kit (Qiagen, Germany) according to manufacturer's instructions. To ensure that the integrity of RNA was sufficient for PCR assays, RNA quality was assessed using a NanoDrop 2000 spectrophotometer. Samples with a 260:280 nm ratio of 1.8 and above, and an RNA integrity number (RIN) between 7 and 10 were used in subsequent experiments. 100 ng total RNA was reverse-transcribed to complementary DNA (cDNA) using the high capacity cDNA reverse transcription kit (Applied Biosystems, UK). PCR reaction mixtures contained 1 μ L of cDNA, 10 μ M of specifically designed forward and reverse primers (Table 2.1), SYBR Green II Universal Master Mix (Thermo Fisher Scientific, USA) and RNase-free water. Relative quantification of gene expression was analysed with pre-optimised conditions using the Quant Studio 5 PCR machine (Applied Biosystems, Lewes, UK). All reactions/negative controls were performed in triplicate in a 96 well plate

format. Thermal cycling conditions were as recommended by the manufacturer (Applied Biosystems). Samples lacking multiscribe reverse transcriptase formed negative controls to ensure target-specific quantification. Data was normalized to endogenous housekeeping genes, *RPLPO* and *HPRT1*. Relative changes in gene expression were determined using either the $-\Delta C_t$ or Pfaffl method.

Gene Name	Forward Primer	Reverse Primer
<i>RPLPO</i>	5' GCGTCCTCGTGGAAGTGACATCG 3'	5' TCAGGGATTGCCACGCAGGG 3'
<i>HPRT1</i>	5' ATGGACAGGACTGAACGTCTTG 3'	5' GGCTACAATGTGATGGCCTC 3'
<i>TNFα</i>	5' ACCTCTCTCTAATCAGCCCTC 3'	5' GGTCGAGAAGATGATCTGACTG 3'
<i>IL-6</i>	5' CCCTGAGAAAGGAGACATGTAAC 3'	5' CCTCTTTGCTGCTTTCACACATG 3'
<i>IL-18</i>	5' CTCAAGTGTCTGAAGCAGCCAT 3'	5' CATCATTTCACTGGCGAGCTCA 3'
<i>OSM</i>	5' ACTCCTGGACCCCTATATACG 3'	5' AGTGCTCTCTCAGTTTAGGAACAT 3'
<i>CXCL10</i>	5' TTCAAGGAGTACCTCTCTCTAGAA 3'	5' GGTTGATTACTAATGCTGATGCAG 3'
<i>CXCL11</i>	5' GGCTTCCCATGTTCAAAAGAG 3'	5' TCTCAATATCTGCCACTTTCCTG 3'
<i>IL-27</i>	5' CTTTGCGGAATCTCACCTGCC 3'	5' AGGGAAACATCAGGGAGCTGC 3'
<i>IL-8</i>	5' TTGGCAGCCTTCCTGATTTC 3'	5' TGGCAAACTGCACCTTCAC 3'
<i>PFKFB3</i>	5' ACCAAAGATCACCCACGGATGT 3'	5' AGCGAGTGCAGAATGGACACAA 3'
<i>HK2</i>	5' TTCTTGCTCAGATTGAGAGTGAC 3'	5' TTGCAGGATGGCTCGGACTTG 3'
<i>HIF1α</i>	5' GAAACTTCTGGATGCTGGTGATT 3'	5' GCAATTCATCTGTGCTTTCATGTCA 3'
<i>SOCS3</i>	5' ACTTCGATTCGGGACCAGCC 3'	5' CAGCTGCTTCGCGGCCTC 3'
<i>MCP-1</i>	5' GCTCGCTCAGCCAGATGCAA 3'	5' TGGTGAAGTTATAACAGCAGGTGA 3'
<i>RANTES</i>	5' CATCTGCCTCCCATATTCTC 3'	5' ATGTAGGCAAAGCAGCAGGGT 3'
<i>STAT3</i>	5' TTCACTTGGGTGGAGAAG 3'	5' CGGACTGGATCTGGGTCT 3'
<i>TGM2</i>	5' CAAGTTCATCAAGAACATACCTTGG 3'	5' TAGGATCCCATCTTCAAACCTGCC 3'
<i>KLF6</i>	5' GGAGTACTGGCAACAGACCTG 3'	5' CTCGCTCTGGAGGTAACGTTTC 3'
<i>PPARG</i>	5' TCCCATCATTAAGGAATTCATGTC 3'	5' AGACAACAGACAAATCACCATTTCG 3'
<i>STAB-1</i>	5' TCCTCCAGGACCATGAATGCAT 3'	5' GATGATGTGCTGTCTACAGAGC 3'

Table 2.1: Designed primer sequences used for real-time PCR analysis.

2.3.5 PCR Gene Array

A preliminary human cytokine/chemokine RT2 Profiler™ PCR microarray was performed on monocytes to simultaneously quantify the expression of 34 cytokine/chemokine genes as per manufacturer's instructions. PCR was performed on a LightCycler 480 System (Roche Diagnostics). Relative changes in gene expression were determined using the GeneGlobe Data Analysis Centre (Web resource by Qiagen) using the $2^{-\Delta\Delta C_t}$ method by normalizing the data to four housekeeping genes: *β-Actin*, *B2M*, *HPRT1* and *RPLP0*.

2.3.6 Protein Isolation and Western Blotting

For protein analysis, CD14+ monocytes were seeded at a density of 1×10^6 /well in a 12 well plate and cultured in the presence or absence of LPS (100 ng/mL) for 24 hr. Macrophages were cultured as previously described; seeded at a density of 2×10^6 cells/well in a 6 well plate on day 0 and polarised on day 8 for 24 hr to derive M1 or M2 macrophages, or unpolarised, M0 macrophages. Ice-cold RIPA (Radio-Immunoprecipitation Assay) buffer (Sigma-Aldrich, USA) containing 10 µg/mL phosphatase inhibitor cocktail and 10 µg/mL protease inhibitor cocktail (Sigma-Aldrich) was used to lyse cell suspensions. Total protein concentration and normalisation was performed using a BCA assay (Pierce Chemical Co, USA). Protein (1-2 µg) was resolved by SDS-PAGE (5% stacking, 10% resolving), gels were then transferred onto PVDF membranes (Amersham Biosciences, UK) prior to 1 hr blocking in wash buffer (Tris-buffered saline (TBS) with 0.1% Tween-20) containing 3% bovine serum albumin (BSA) with gentle agitation at room temperature. Membranes were incubated with mouse monoclonal anti-HK2 (Novus Biologicals, USA), rabbit monoclonal anti-PFKFB3 (Abcam, UK), rabbit polyclonal anti-GLUT1 (Abcam), rabbit monoclonal anti-phospho-STAT3 (pSTAT3) and mouse monoclonal anti-total-STAT3 (tSTAT3) (Cell-Signaling Technology, UK), and diluted in 3% BSA in TBS-Tween-20 at 4°C overnight with gentle agitation. β -actin (Sigma -Aldrich) was used as a loading control. The signal was detected using SuperSignal® West Pico Chemiluminescent Substrate (Amersham Biosciences). Band densities were imaged using the ChemiDoc MP Imaging System (Bio-Rad, USA).

2.3.7 Cellular Bioenergetic Function Analysis

To assess the metabolic function of RA and HC CD14⁺ monocytes and macrophages, the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), reflecting oxidative phosphorylation and glycolysis respectively, were measured using the Seahorse-XFe96 Analyser (Agilent Technologies, USA).

RA and HC CD14⁺ monocytes were seeded at a density of 300,000/well in a 96 well Seahorse XFe microplate (Agilent Technologies) and allowed to adhere for 1 hr in RPMI. Following this, cells were then washed and further incubated with Seahorse assay medium (unbuffered DMEM supplemented with 10 mM glucose, pH-7.4; Agilent Technologies) in the presence or absence of LPS (100 ng/mL) for 1 hr at 37°C in a non-CO₂ incubator, before undergoing a Mito Stress test using the Seahorse-XFe96 Analyser. Monocyte-derived macrophages were detached using Accutase cell detachment solution (Sigma-Aldrich, Ireland), reseeded at a density of 50,000 cells/well in a 96 well Seahorse XFe microplate (Agilent Technologies) and allowed to adhere for 1 hr in RPMI. Following this, cells were polarised into M1 and M2 macrophages or unpolarised for M0 conditions and incubated overnight. Cells were then washed and further incubated with Seahorse assay medium for 1 hr at 37°C in a non-CO₂ incubator, before undergoing a Mito Stress test using the Seahorse-XFe96 Analyser

Basal oxidative phosphorylation and glycolysis were calculated from the average of three baseline OCR/ECAR measurements, respectively. The Mito Stress test was performed, using specific metabolic inhibitors; oligomycin (ATP-synthase-inhibitor) (2 µg/mL), trifluorocarbonylcyanide phenylhydrazine (FCCP) (mitochondrial uncoupler) (5 µM), and antimycin A (complex-III inhibitor) (2 µM) (all Agilent Technologies) to assess the metabolic capacity of CD14⁺ monocytes and polarised macrophages. The maximal respiratory capacity and maximal glycolytic capacity were calculated by averaging the three OCR or ECAR measurements following FCCP and oligomycin injection respectively. The spare respiratory capacity was calculated by subtracting the baseline OCR from the maximal respiratory capacity OCR, and ATP synthesis was calculated by subtracting the post-oligomycin OCR from the baseline OCR as depicted in Figure 2.3.1.

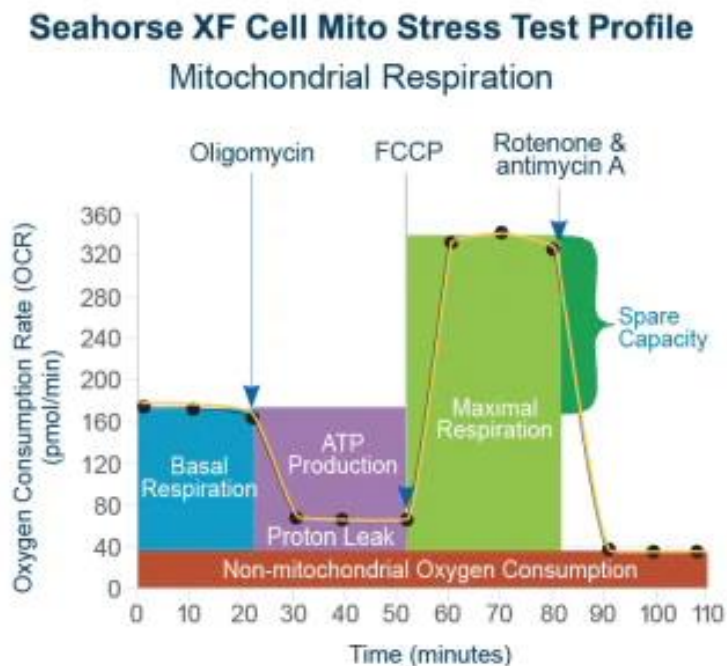


Figure 2.3.1: Representative Seahorse Cell Mito Stress Test assay showing calculable parameters.

2.3.8 Transmission Electron Microscopy

To investigate the morphology and number of mitochondria in RA and HC monocyte-derived macrophages, transmission electron microscopy (TEM) was used. Macrophages were seeded at density of 2×10^6 cells/well in a 6 well plate on day 0 and polarised on day 8 for 24 hr. Following this, cells were fixed in glutaraldehyde (3% in 0.05 M Potassium Phosphate buffer, pH 6.8; Sigma-Aldrich) for 1 hr at room temperature. Samples were processed and analysed using a Jeol JEM2100 LaB6 Transmission Electron Microscope (operated at 100 Kv). Digital images were obtained and analysed using an AMT XR80 capture system and ImageJ software. High-powered images of 5 cells per sample were taken. The number of mitochondria were counted and the average, per sample, was calculated. The number of elongated mitochondria were also counted represented as a percentage of the total.

2.3.9 Measurement of Mitochondrial Mass

Polarised macrophages were washed and incubated with 150 nM MitoTracker Green FM (ThermoFisher) for 30 min at 37°C. Cells were washed in Hanks' Balanced Salt Solution (HBSS) and seeded at 100,000 cells/well in a ViewPlate-96 black (PerkinElmer). Cells were stimulated with 50 µL of LPS solution (200 ng/mL) or HBSS buffer. MitoTracker emission was read using the kinetic mode of the Spectra Max Gemini System with excitation and emission wavelengths of 490 nm and 516 nm. Readings were performed every 15 min during a length of 6 hr.

2.3.10 Flow Cytometry

Expression of cell surface markers was assessed using flow cytometry to validate macrophage polarisation. Polarised monocyte-derived macrophages were stained using the following panel of fluorochrome-conjugated antibodies: CD40, CD68, CD64, CD163, CD206, CD253. Cells were resuspended in 1 mL of PBS with 0.3 µL of Live Dead Red viability dye (Molecular Probes, Thermo Fisher Scientific) for 30 min in the dark at 4°C. Cells were centrifuged and resuspended in 100 µL of FACS Buffer (Dulbecco's PBS without Mg²⁺ or Ca²⁺ (Sigma-Aldrich), 1% heat-inactivated FCS (Biosciences) and 0.05% sodium azide (Sigma-Aldrich), pH 7.4-7.6). To eliminate nonspecific binding of mouse monoclonal antibodies to the Fc-gamma receptor (FcγR), samples were blocked in 5 µL human FcγR-binding inhibitor for 10 min. Fluorochrome-conjugated antibodies against extracellular markers were then added and samples were vortexed and incubated in the dark at room temperature for 30 min. Samples were washed in PBS and centrifuged at 1400 RPM, supernatants were removed and the cells were resuspended in 250 µL of PBS. Samples were acquired using the CyAn Flow Cytometer (Beckman Coulter) and analysed using FlowJo software (FlowJo LLC, Oregon, USA).

2.3.11 Phagocytosis Assay

Receptor-mediated endocytosis was assessed using DQ-labelled OVA (Thermo Fisher Scientific, Massachusetts, USA). Macrophages were previously seeded on day 0 and stimulated on day 8 for 24 hr to generate M1 or M2 polarised macrophages. Cells were then washed, collected in flow tubes and incubated with 4 µL OVA-DQ either at 0°C (passive phagocytosis) or 37°C (active phagocytosis) for 15 min, and subsequently washed

twice. Quantification of the cells' phagocytic profiles was obtained using a BD Accuri™ C6 Plus Flow Cytometer (BD Biosciences, California, USA). Flow cytometric analysis was conducted using FlowJo software.

2.3.12 RNA-Sequencing

High quality RNA was isolated from RA monocyte-derived M1 and M2 macrophages using the RNeasy kit (QIAGEN) and the purity of RNA samples was assessed using a NanoDrop 2000 spectrophotometer (ThermoFisher). RNA samples were reverse transcribed and sequence libraries were generated by Janssen Pharmaceutical, Spring House, PA, USA, using the NuGen Ovation Universal RNA-seq System. The resulting sequencing libraries were analysed using the Cali-per LabChip GX and quantified using KAPA qPCR. Libraries were normalised and pooled. Each pool was then clustered and sequenced on an Illumina NextSeq500 instrument using 2 x 100 bp paired-end reads, following manufacturer's instructions. Raw read quality was evaluated using FastQC before reads were trimmed for adaptors and sequence quality. Trimmed reads were aligned to human GRCh38.84 reference genome using STAR RNA-seq aligner. Aligned reads were quantified for each gene. Aligned data were evaluated for quality using several quality metrics (e.g. mapping rate, coverage) and visually inspected for samples deviating from the population across multiple metrics and principal component analysis (PCA). Statistical analysis of RNA-seq data was performed in R (version 3.5.1).

Differential gene analysis was performed with edgeR package to normalise and identify differentially expressed genes (DEGs) from counts data. Transcripts with zero counts in more than two-thirds of the samples were discarded from downstream analysis to reduce noise in the expression data. Gene counts were converted to log₂ counts per million (cpm), quantile normalised, and precision weighted. RNA-seq gene features were considered differentially expressed if they satisfied a 2-fold change and false discovery rate (FDR) <0.05 cut-off. FDR control was performed with the Benjamini-Hochberg procedure. Heatmaps and Volcano Plots were generated in R with the heatmap.2 and EnhancedVolcano functions in the gplots package. Hierarchical clustering was performed

with Ward's linkage. Ingenuity Pathway Analysis (IPA) was used to identify canonical pathways overrepresented within DEGs.

2.3.13 Statistical Analysis

Statistical analyses were performed using GraphPad Prism 7 software. Wilcoxon Signed Rank test or Mann Whitney U test was used for analysis of non-parametric data. A Student's *t*-test was used for parametric data. P-values of less than 0.05 (* $p < 0.05$) were determined as statistically significant.

2.4 Results

2.4.1 Inflammatory profiling of RA monocytes

In order to compare the expression of pro-inflammatory genes by CD14⁺ monocytes isolated from the blood of RA and HC donors, RNA was extracted from both untreated monocytes and those stimulated with LPS (100 ng/mL) for 3 hr, and a cytokine/chemokine PCR array was performed. Figure 2.1 (A) demonstrates increased expression of 34 cytokine/chemokine in response to LPS stimulation in RA and HC CD14⁺ monocytes. LPS stimulation resulted in a significant increase in 23 genes encoding key cytokines and chemokines, with more than a 1.5 fold increase in RA CD14⁺ monocytes compared to HC CD14⁺ monocytes, suggesting a heightened immune response by RA monocytes (Figure 2.1 (B)).

Selecting those genes with the greatest differences between RA and HC, and those of greater functional interest, 11 of these 23 genes were validated in a larger cohort as indicated by heatmap expression analysis and bar graphs (Figure 2.2 (A-B)). Bar graphs representing 8 of these genes; *TNF α* , *IL-6*, *IL-1 β* , *OSM*, *CXCL10*, *CXCL11*, *IL-27* and *IL-8* demonstrate a significant increase in RA vs HC monocytes (Figure 2.2 (B), $p < 0.05$). This provides a distinct signature of RA CD14⁺ monocyte hyper-inflammation suggesting that RA CD14⁺ monocytes are biased towards pro-inflammatory functions.

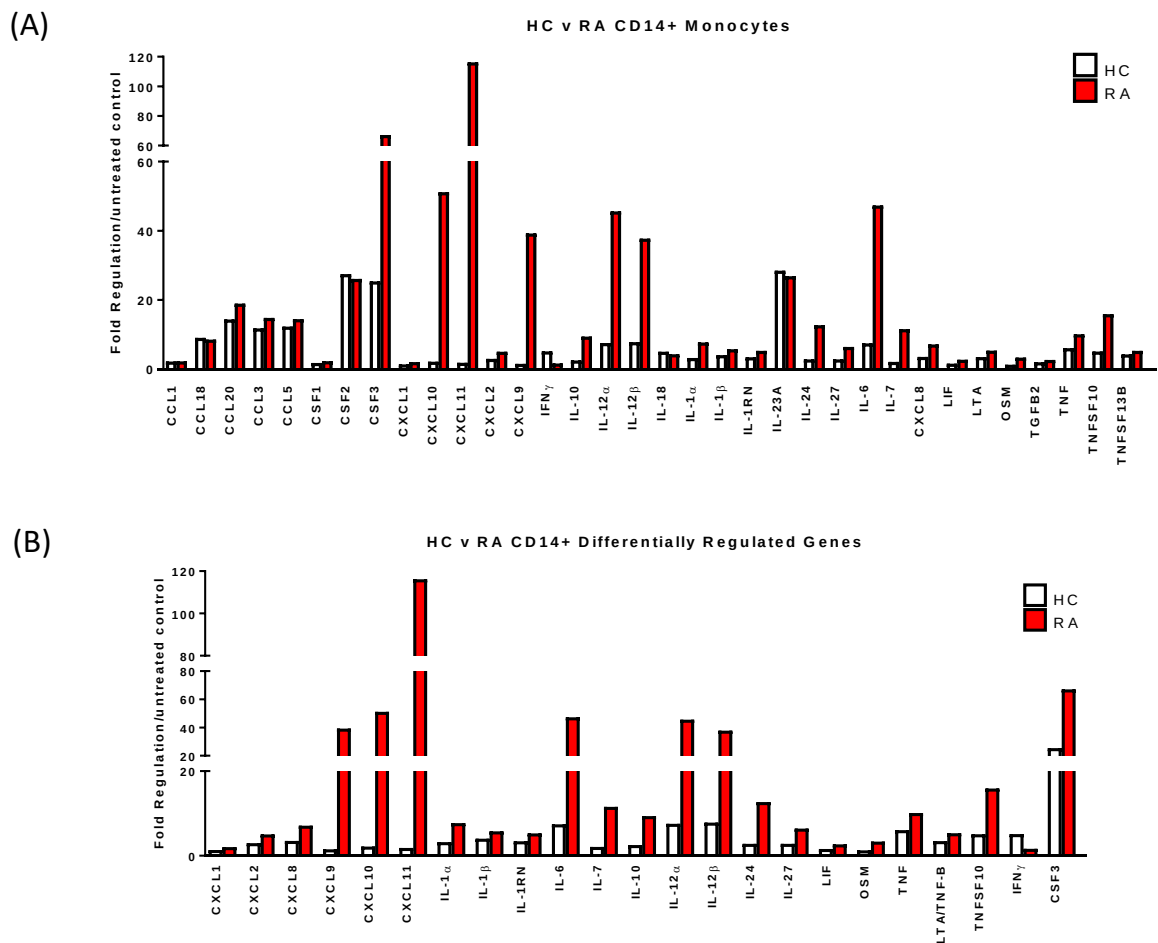


Figure 2.1: CD14+ monocytes from Rheumatoid Arthritis patients are hyper-inflammatory.

(A) Bar graph demonstrating 34 cytokine/chemokine genes upregulated in response to 3 hr LPS stimulation (100 ng/mL) in healthy and RA CD14+ monocytes. (B) Bar graph representing genes that displayed a more than 1.5 fold difference in RA compared to healthy monocytes (n=3). Data expressed as upregulation compared to basal control and normalized to housekeepers *β-Actin*, *B2M*, *GAPDH*, *HPRT1* and *RPLPO*.

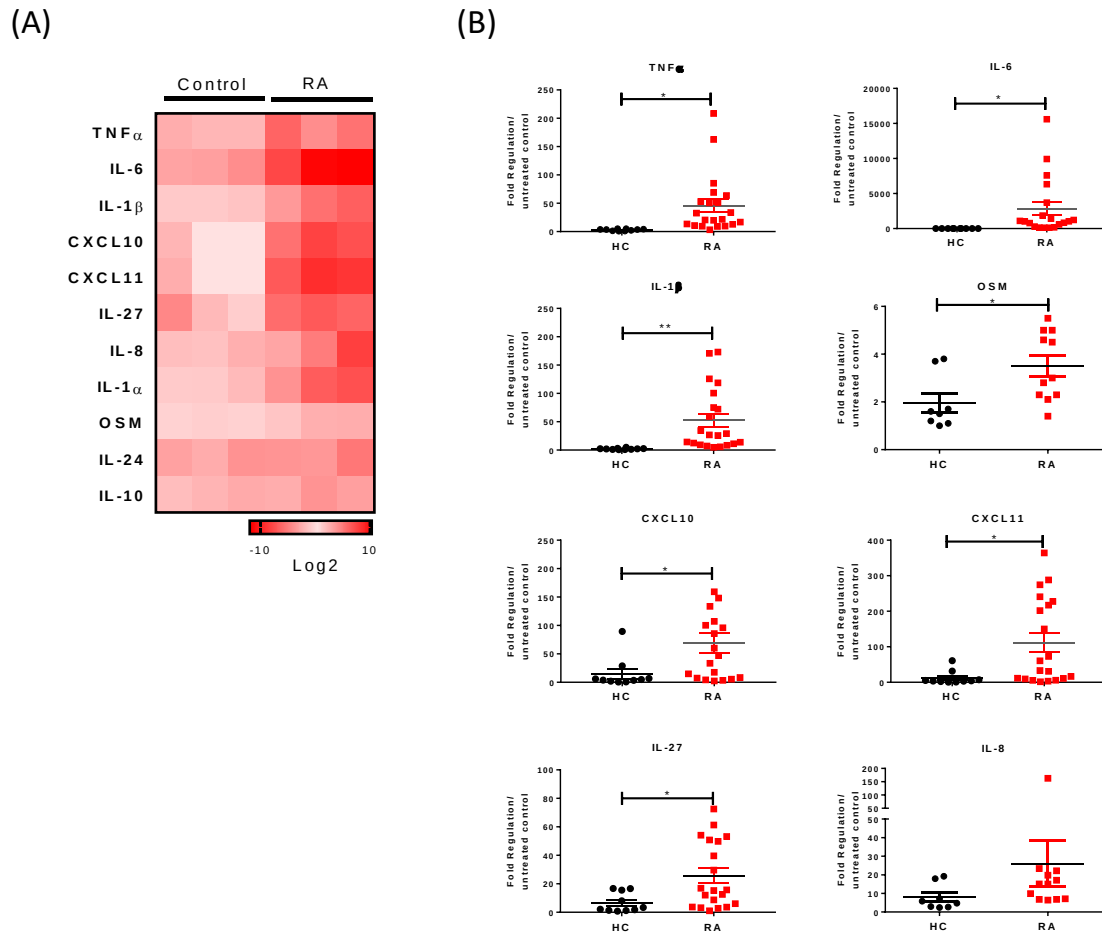


Figure 2.2: Distinct gene signature of hyper-inflammation in RA CD14⁺ monocytes.

(A) Heatmap expression analysis of pro-inflammatory genes differentially expressed between healthy (n=3) and RA (n=3) CD14⁺ monocytes. (B) Further validation of differentially expressed genes between healthy (n=8-10) and RA (n=11-21) CD14⁺ monocytes in response to LPS stimulation (100 ng/mL; 3 hr). Data expressed as mean $\pm$ SEM normalized to housekeeping control using unpaired *t*-test. **p*<0.05, ***p*<0.01, significantly different from basal.

2.4.2 Activated RA monocytes are hyper-metabolic

An increased inflammatory burden is known to be associated with a greater demand for ATP and higher metabolic activity, particularly in inflammatory cells. To investigate if the increased inflammatory phenotype of RA monocytes is due to alterations in energy metabolism, we next measured the two major energy pathways, oxidative phosphorylation and glycolysis using the Seahorse-XFe Flux Analyser. Average bioenergetic profiles of oxidative phosphorylation (OCR) before and after injections of oligomycin, FCCP and antimycin A in basal and LPS-stimulated monocytes are shown in Figure 2.3. Following LPS stimulation, CD14⁺ monocytes display a significant decrease in baseline OCR (Figure 2.3 (B), $p < 0.05$) and ATP synthesis (Figure 2.3 (C), $p < 0.05$) compared to basal control.

Directly comparing *ex vivo* RA CD14⁺ monocytes to HC CD14⁺ monocytes however, a significant increase in mitochondrial respiration (OCR) (Figure 2.3 (B), $p < 0.05$) and ATP synthesis (Figure 2.3 (D), $p < 0.05$) is observed in RA, with no difference observed for maximal or spare respiratory capacity (Figure 2.3 (C) and (E)). Similarly, LPS-activated RA monocytes displayed significantly higher baseline OCR (Figure 2.3 (B), $p < 0.05$), maximal respiratory capacity (Figure 2.3 (C), $p < 0.05$) and ATP synthesis (Figure 2.3 (D), $p < 0.05$) than the LPS-activated HC monocytes. Increased mitochondrial respiration resulted in higher ATP production, thus demonstrating a state of heightened mitochondrial activity.

Average ECAR profiles indicating glycolytic capacity of untreated and LPS-stimulated RA and HC CD14⁺ monocytes are shown in Figure 2.4 (A). Following stimulation with LPS, a significant induction in baseline ECAR (Figure 2.4 (B), $p < 0.05$), maximal glycolytic rate (Figure 2.4 (C), $p < 0.05$) and the ECAR:OCR ratio (Figure 2.4 (D), $p < 0.05$) was observed in both RA and HC CD14⁺ monocytes.

Interestingly, a significant increase in baseline ECAR and maximal glycolytic rate was observed in LPS-stimulated RA CD14⁺ monocytes when compared to LPS-stimulated HC CD14⁺ monocytes (Figure 2.4 (B-C), $p < 0.05$). No significant differences were observed between unstimulated *ex vivo* RA and HC CD14⁺ monocytes (Figure 2.4 (A)-(D)). The metabolic profile demonstrates that, following stimulation, both HC and RA monocytes switch their metabolism to a highly energetic state, an effect that is enhanced in RA

compared to HC (Figure 2.4 (E)). Supporting this concept, a significant increase in gene expression of key glycolytic enzymes is observed in RA monocytes compared to HC. The heatmap in Figure 2.5 (A) demonstrates differential gene expression of *PFKFB3*, *HK2* and *HIF1 α* , in RA (n=3) vs HC (n=3), with no differences observed for *LDHA*, *G6PD* and *SOD-1*. These pro-glycolytic genes were further validated in a larger cohort (Figure 2.5 (B)), demonstrating significant increases in *PFKFB3* and *HK2* ($p < 0.05$). *PFKFB3* is the enzyme which catalyses the conversion of fructose-6-phosphate to fructose-2,6-bisP (F2,6BP), a potent allosteric activator of 6-phosphofructokinase-1 (PFK-1) which is one of the rate-limiting enzymes of glycolysis, while *HK2* is the first enzyme in the glycolysis pathway. Furthermore, expression of the master regulator of metabolism, *HIF1 α* , was significantly induced in RA compared to HC monocytes ($p < 0.05$).

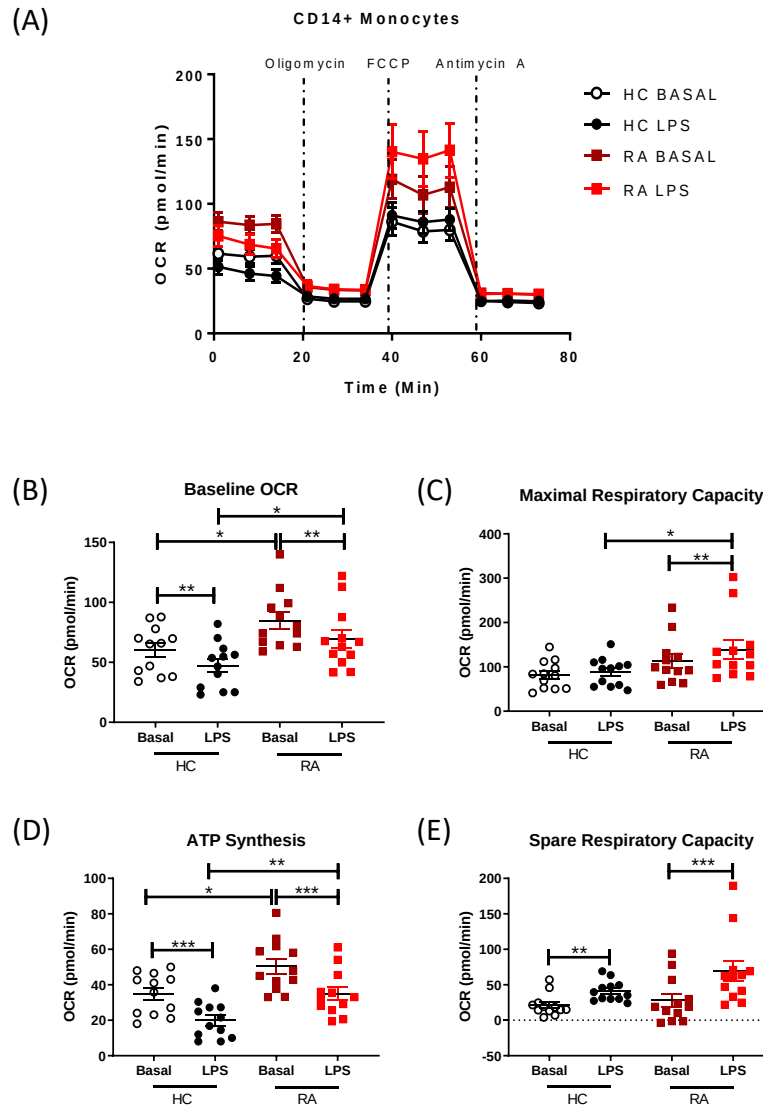


Figure 2.3: Metabolic capacity of RA CD14⁺ monocytes compared to healthy controls.

(A) Average Seahorse bioenergetics profile demonstrating OCR of healthy (n=12) and RA (n=12) CD14⁺ monocytes before and after injections of oligomycin, FCCP and antimycin A in *ex vivo* resting monocytes and LPS-stimulated (100 ng/mL; 1 hr) monocytes. Bar graphs demonstrate (B) baseline OCR, (C) maximal respiratory capacity, (D) ATP synthesis and (E) spare respiratory capacity. Wilcoxon signed rank and Mann Whitney U tests were used where appropriate. Data expressed as mean $\pm$ SEM, *p<0.05, **p<0.01, ***p<0.005 significantly different.

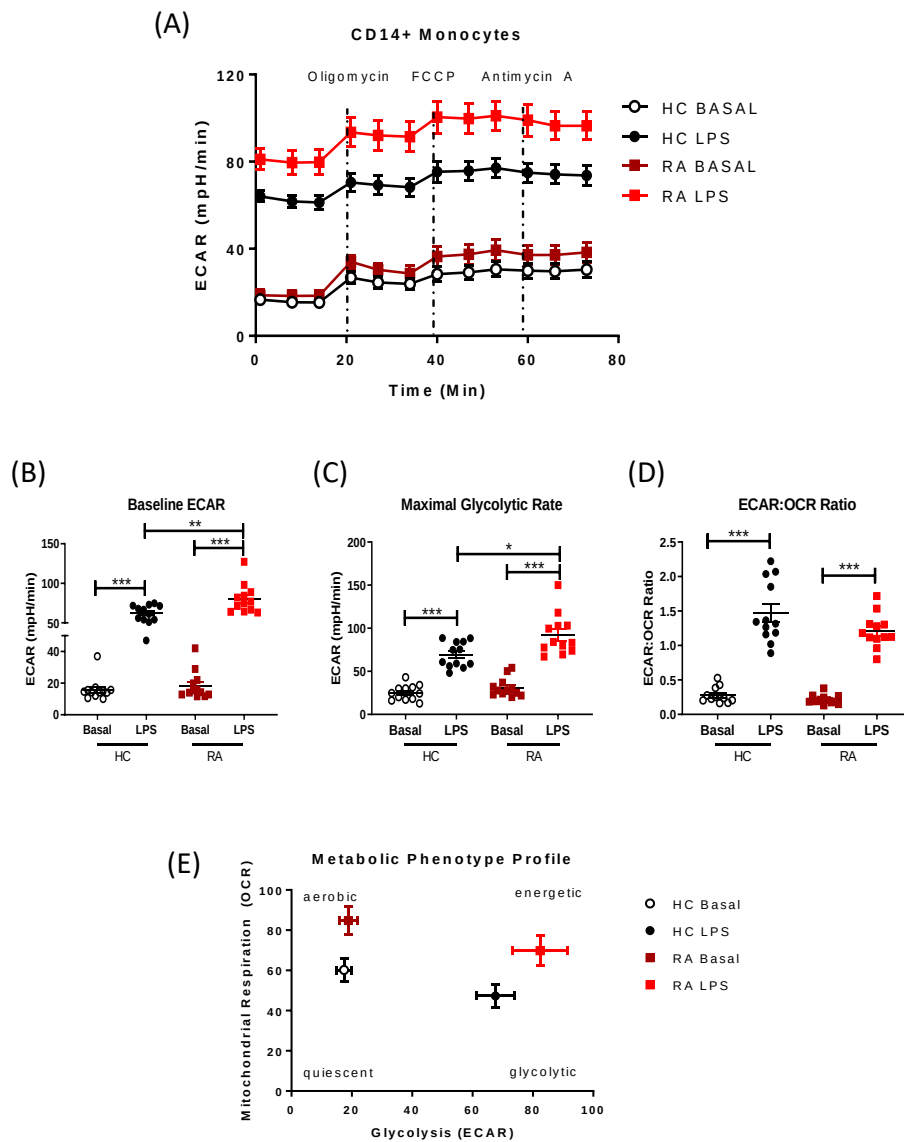


Figure 2.4: CD14⁺ RA monocytes are hyper-glycolytic compared to healthy controls.

(A) Average Seahorse bioenergetics profile demonstrating ECAR of healthy (n=12) and RA (n=12) CD14⁺ monocytes before and after injections of oligomycin, FCCP and antimycin A in *ex vivo* resting untreated and LPS-stimulated (100 ng/mL; 1 hr) monocytes. Bar graphs demonstrate (B) baseline glycolysis, (C) maximal glycolytic rate and (D) ECAR:OCR ratio. (E) The overall metabolic profile of untreated and LPS-stimulated RA and HC monocytes. Wilcoxon signed rank and Mann Whitney U tests were used where appropriate. Data expressed as mean $\pm$ SEM, *p<0.05, **p<0.01, ***p<0.005 significantly different.

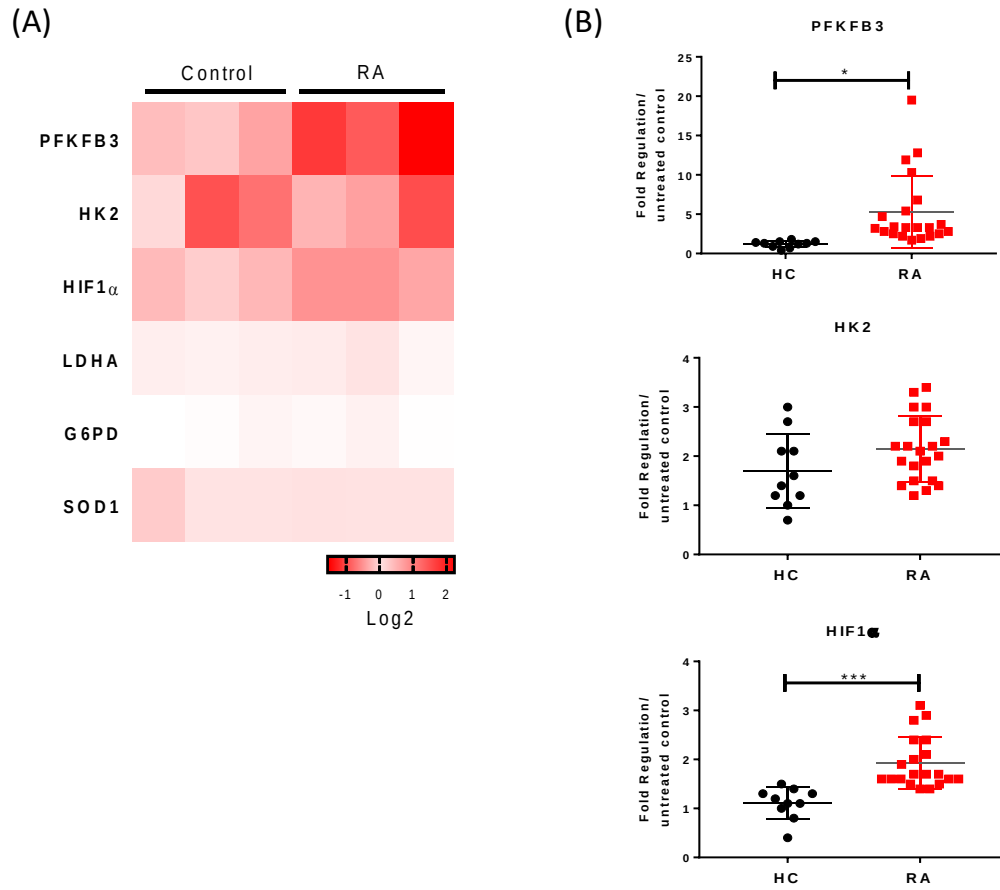


Figure 2.5: RA CD14⁺ monocytes are more glycolytic than healthy controls.

Gene expression profiles of LPS-stimulated (100 ng/mL for 3 hr) HC and RA CD14⁺ monocytes. (A) Heatmap represents expression of metabolic genes in monocytes from HC (n=3) and RA (n=3). (B) Bar graphs represent validation of the increased gene expression of *PFKFB3*, *HK2*, *HIF1 α* in HC and RA CD14⁺ monocytes in (HC n=10) and RA (n=21). Data expressed as mean $\pm$ SEM normalized to housekeeping controls *HPRT1* and *RPLPO* using unpaired *t*-test. **p*<0.05, ***p*<0.01, ****p*<0.005 significantly different.

2.4.3 Glycolysis regulates RA CD14⁺ monocyte inflammatory phenotype

To assess if either energy pathway contributes to the hyper-inflammatory phenotype observed in RA CD14⁺ monocytes, oligomycin, which inhibits oxidative phosphorylation and 2DG, which inhibits glycolysis, were utilized. Blockade of oxidative phosphorylation only partially inhibited the LPS-induced inflammatory burden, with inhibition of *CXCL10*, *CXCL11* and *IL-27*, but no change in *TNF α* , *IL-1 β* or *IL-6* (Figure 2.6 (A)). Blockade of glycolysis with 2DG however, significantly inhibited expression of all 6 signature hyper-inflammatory cytokines/chemokines; *TNF α* , *IL-6*, *IL-1 β* , *CXCL10*, *CXCL11* and *IL-27* (Figure 2.7 (A), $p < 0.05$). This was paralleled by the inhibition of LPS-induced protein expression of key glycolytic enzymes HK2 and PFKFB3 by 2DG treatment (Figure 2.7 (B)), whereas oligomycin inhibition had no effect on the expression of these glycolytic enzymes (Figure 2.6 (B)).

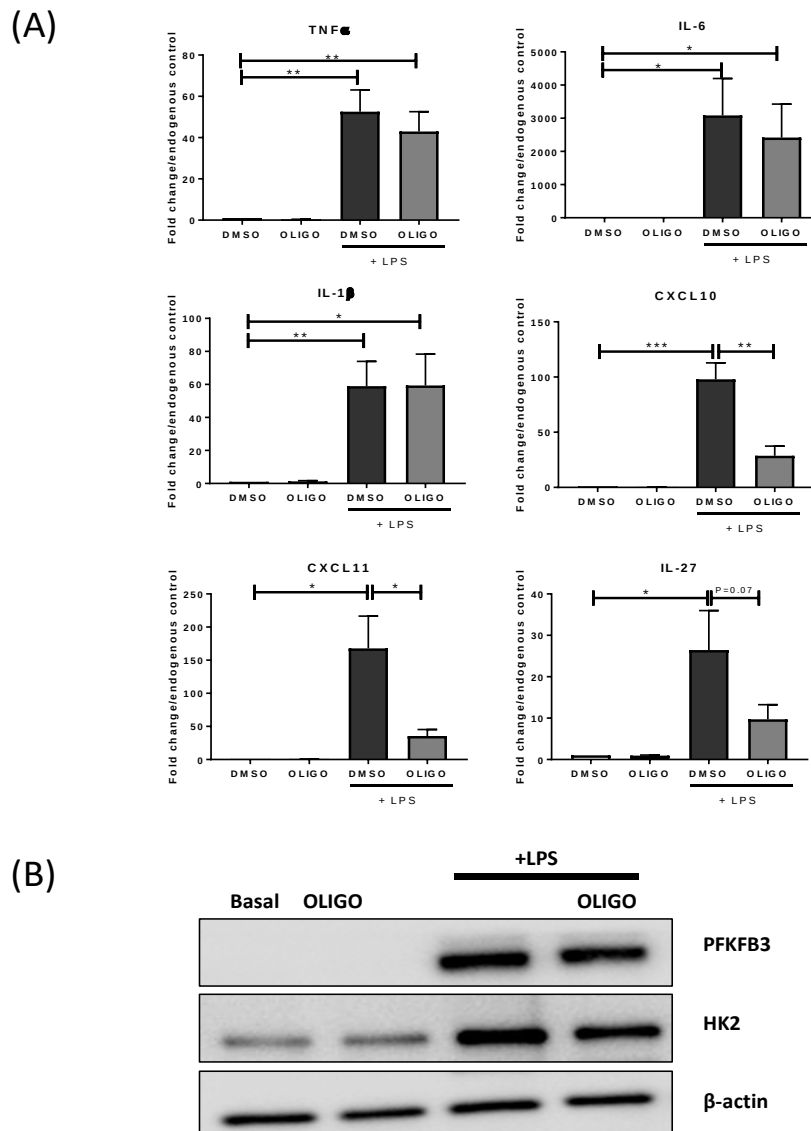
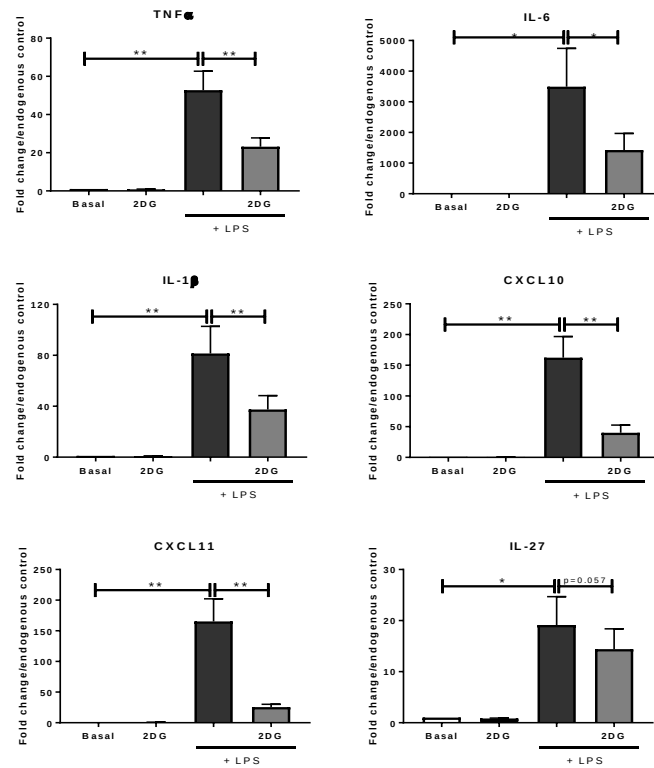


Figure 2.6: RA CD14⁺ monocyte inflammatory phenotype is partially inhibited by oligomycin. RA CD14⁺ monocytes (n=8) were stimulated with LPS (100 ng/mL) for 3 hr +/- oligomycin. (A) Gene expression of *TNFα*, *IL-6*, *IL-1β*, *CXCL10*, *CXCL11* and *IL-27*. (B) Representative western blots showing PFKFB3, HK2 and β-actin protein expression. Data represented as mean ± SEM using paired *t*-test **p*<0.05, ***p*<0.01, ****p*<0.005 significantly different.

(A)



(B)

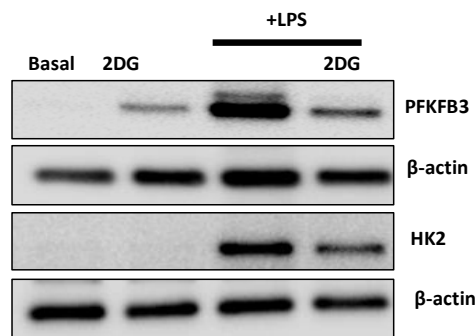


Figure 2.7: RA CD14⁺ monocyte inflammatory phenotype is inhibited by glycolytic blockade. RA CD14⁺ monocytes (n=7) were stimulated with LPS (100 ng/mL) for 3 hr +/- 2DG. (A) Graphs represent gene expression of *TNFα*, *IL-6*, *IL-1β*, *CXCL10*, *CXCL11* and *IL-27*. (B) Representative western blots showing PFKFB3 and HK2 protein expression. Data represented as mean ± SEM using paired *t*-test **p*<0.05, ***p*<0.01, significantly different.

2.4.4 Inflammatory profiling of RA M1 macrophages compared to healthy control

In the inflammatory RA joint, monocytes are recruited from the periphery and differentiate into macrophages in response to local M-CSF and upregulate expression of macrophage-associated genes (Merad *et al.*, 2002). Having observed a hyper-inflammatory, hyper-metabolic phenotype in RA CD14⁺ monocytes compared to HC, we hypothesised that this phenotype persists in *ex vivo* differentiated macrophages. CD14⁺ monocytes were stimulated with M-CSF to produce monocyte-derived macrophages before being polarised into inflammatory M1 macrophages with the addition of LPS/IFN γ or anti-inflammatory M2 macrophages with the addition of IL-4.

Initial experiments analysed specific macrophage markers to validate their polarisation of monocyte-derived M0 macrophages to M1 and M2. As expected, *KLF6* was significantly increased and *PPARG* was decreased in M1 macrophages relative to M0. On the other hand, *TGM2*, *PPARG* and *STAB-1* were significantly induced in M2 macrophages, relative to M0. Importantly, expression of the 4 genes was significantly different between M1 and M2 macrophages (Figure 2.8 (A)). Expression of specific cell surface polarisation markers was also analysed by flow cytometry, which showed an increase in the M2-specific markers, CD206 and CD163 in the M2 macrophages and an induction of inflammatory CD40 and CD253 in the M1 macrophages, thus confirming polarisation (Figure 2.8 (B)).

Following validation of the polarisation protocol, analysis of pro-inflammatory mediator production by RA and HC M1 macrophages was then assessed. RA M1 macrophages demonstrated a stronger induction of the pro-inflammatory mediators; *OSM* ($p < 0.05$), *SOCS3* ($p < 0.05$), *IL-8* ($p < 0.05$), *IL-1 β* ($p < 0.051$) and *MCP-1* ($p < 0.05$) compared to M1 macrophages from healthy control (Figure 2.9). No striking differences were observed for *CXCL10*, *CXCL11* or *TNF α* (Figure 2.9).

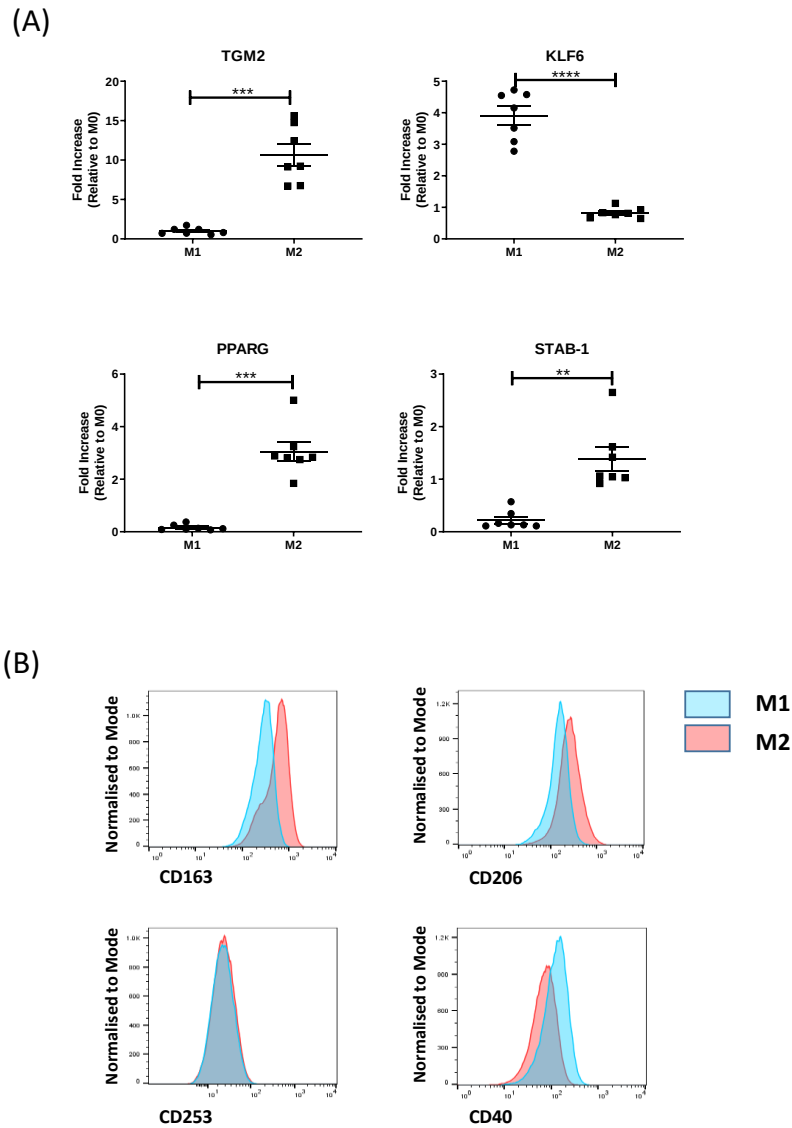


Figure 2.8: Validation of macrophage polarisation.

(A) Gene expression analysis of M1/M2-polarised monocyte-derived macrophages relative to monocyte-derived M0. Upregulation of *KLF6* and down-regulation of *PPARG* denotes M1-like. Upregulation of *TGM2*, *PPARG* and *STAB-1* denotes M2-like. Data is normalised to the housekeeper gene, *HPRT1* and represented as fold change relative to unpolarised M0 macrophages (n=7). Values expressed as mean ± SEM using paired *t*-test **p<0.01, ***p<0.005, significantly different from M1. (B) Representative flow cytometric histograms of cell surface macrophage polarisation markers CD163, CD206 (M2-like) and CD253, CD40 (M1-like).

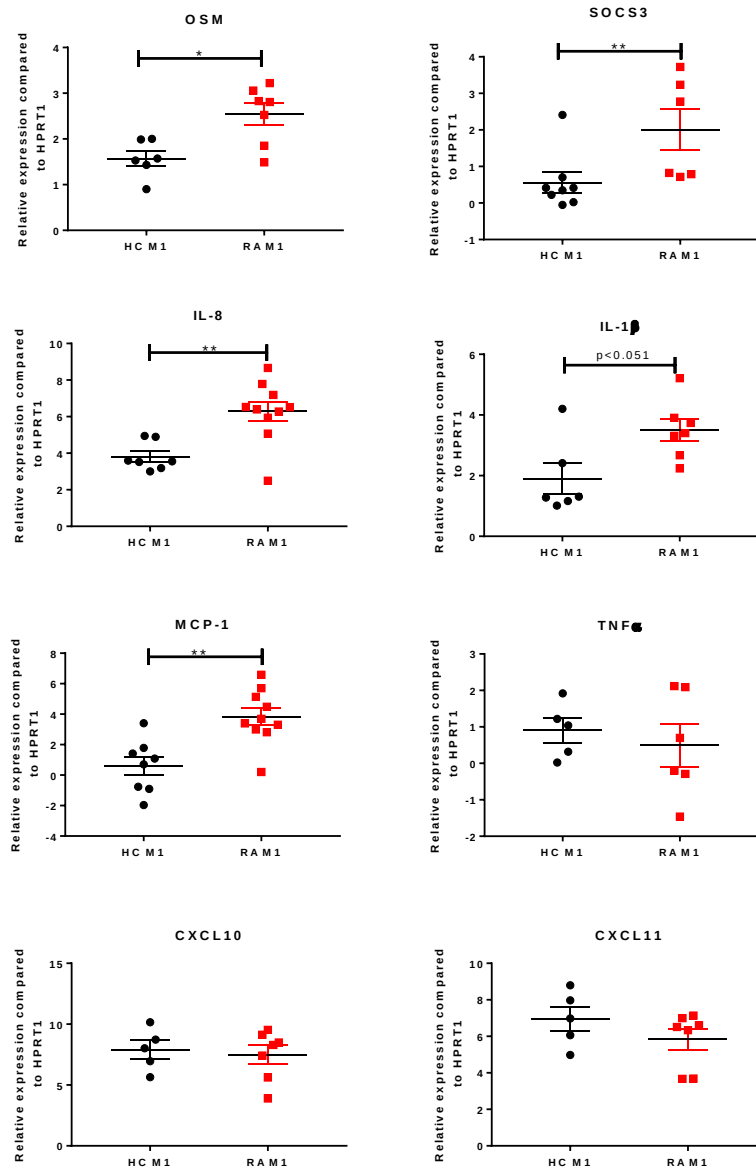


Figure 2.9: Pro-inflammatory gene expression in RA and healthy M1 macrophages.

Graphs represent gene expression of *OSM*, *SOCS3*, *IL-8*, *IL-1 β* , *MCP-1*, *TNF α* , *CXCL10* and *CXCL11* in RA (n=6-10) and HC (n=5-8) M1 macrophages. Data normalized to housekeeping control, *HPRT1* and expressed as mean $\pm$ SEM using Mann Whitney U test *p<0.05, ** p<0.01 significantly different.

2.4.5 Metabolic phenotype of RA M1 macrophages compared to healthy control

Having determined that the enhanced inflammatory phenotype of RA monocytes is coupled with alterations in their energy metabolism, we examined if this was also the case for RA macrophages. As previously described, the Seahorse-XFe Flux Analyser was used to measure the two major energy pathways, in this case, in RA and HC M1 macrophages. Average ECAR profiles demonstrate an increase in baseline glycolysis in RA M1 macrophages compared to HC M1 macrophages (Figure 2.10 (A, B)). This was associated with a significant increase in the expression of key metabolic genes, *PFKFB3*, *HK2* and *HIF1 α* in RA M1 compared to HC M1 (Figure 2.10 (C-E), ($p < 0.05$)). This boost in glycolytic mechanisms is similar to that observed in RA CD14⁺ monocytes.

Indeed, examining mitochondrial activity of RA M1 macrophages compared to HC revealed some striking disparities. The average OCR profile indicates a significant induction of baseline OCR and hence, an increased reliance on oxidative phosphorylation in RA M1 compared to their healthy counterparts (Figure 2.11 (A, B)). This was paralleled by a significant induction of ATP synthesis in RA M1 compared to HC M1 (Figure 2.11 (C), $p < 0.05$). No significant differences were observed in maximal respiratory capacity while a slight decrease in spare respiratory capacity was observed in RA M1 compared to HC (Figure 2.11 (D,E)). Due to both oxidative phosphorylation and glycolysis pathways being boosted in RA M1 macrophages, the ECAR:OCR ratio is similar between RA and healthy control inflammatory macrophages (Figure 2.11 (F)). Elevation of both energy pathways in RA M1 macrophages indicates that they are more metabolically active than HC M1. This is reminiscent of the highly energetic phenotype of their CD14⁺ precursors.

Using TEM, we next examined the ultrastructure of RA and HC inflammatory macrophages. While similar numbers of mitochondria were observed between RA and HC M1 macrophages (Figure 2.12 (B)), examination of mitochondrial morphology revealed that RA M1 macrophages displayed a greater frequency of larger, more dense, elongated mitochondria (red arrows), as shown in Figure 2.12 (A). RA M1 macrophages have significantly more elongated mitochondria compared to HC, which suggests alterations in mitochondrial function (Figure 2.12 (C), $p < 0.05$)).

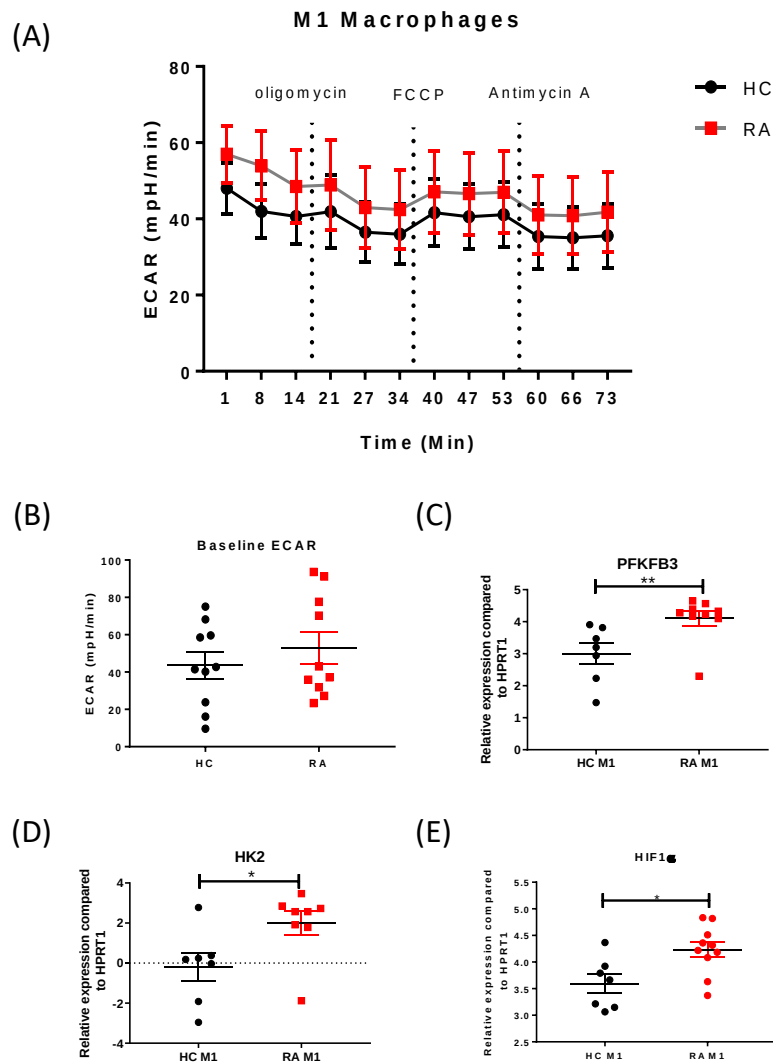


Figure 2.10: RA M1 macrophages are hyper-glycolytic compared to healthy M1 macrophages.

(A) Average Seahorse bioenergetics profile demonstrating ECAR of HC (n=10) and RA (n=10) M1 macrophages before and after injections of oligomycin, FCCP and antimycin A. Graphs demonstrate (B) baseline ECAR quantification, and gene expression of (C) *PFKFB3*, (D) *HK2* and (E) *HIF1α* RA (n=8-10) and HC (n=7). Gene expression data expressed as mean $\pm$ SEM and normalized to endogenous housekeeping control *HPRT1* using Mann Whitney U test, *p<0.05, **p<0.01, significantly different.

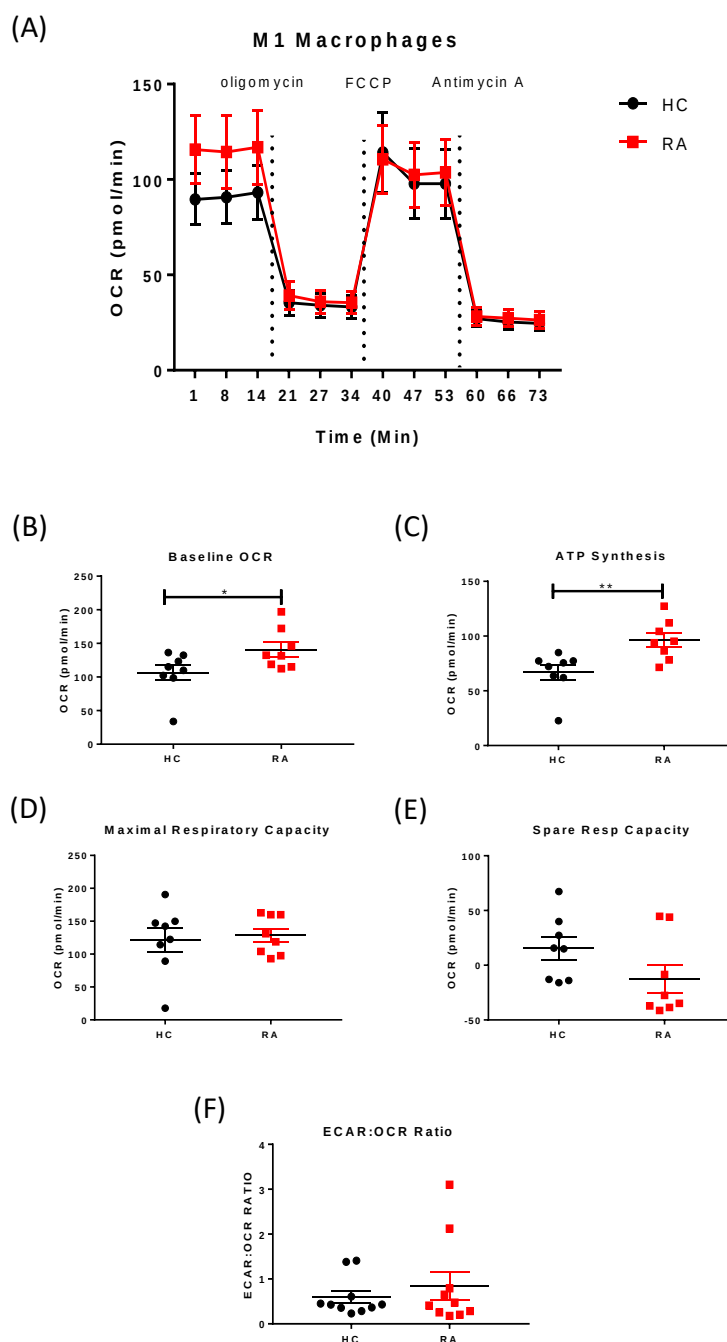


Figure 2.11: RA M1 macrophages are more metabolically active than healthy controls.

(A) Average Seahorse bioenergetics profile demonstrating OCR of HC (n=10) and RA (n=10) M1 macrophages before and after injections of oligomycin, FCCP and antimycin A. (B) Graphs demonstrate (B) baseline OCR, (C) ATP synthesis, (D) maximal respiratory capacity, (E) spare respiratory capacity and (F) the ECAR:OCR ratio. Data represented as mean $\pm$ SEM using Mann Whitney U test, * $p < 0.05$, ** $p < 0.01$, significantly different.

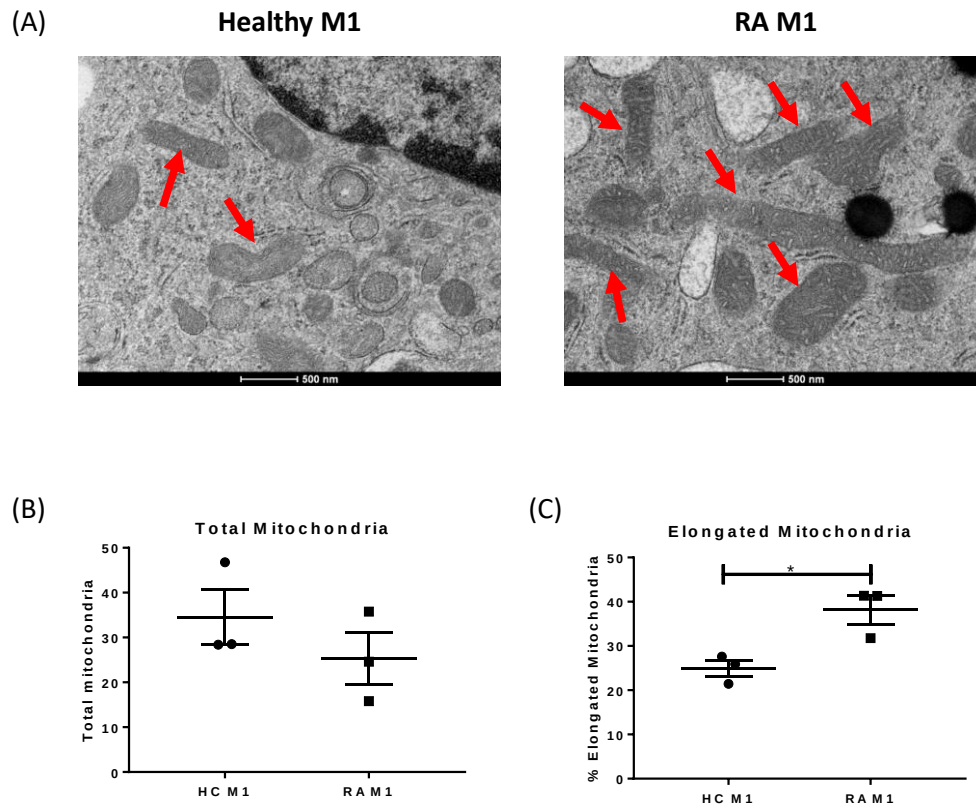


Figure 2.12: Comparison of mitochondrial morphology between RA and healthy M1 macrophages.

(A) Representative TEM images of RA and HC M1 macrophages. Dense, elongated mitochondria are indicated by red arrows. Scale bar represents 500 nm. Graphs represent (B) total numbers of mitochondria, and (C) percentage of elongated mitochondria in HC (n=3) and RA (n=3) M1 macrophages. Data represented as mean $\pm$ SEM using unpaired *t*-test, **p*<0.05, significantly different.

2.4.6 Inflammatory function of RA polarized macrophages

We have demonstrated that RA monocytes, *ex vivo*, display a pro-inflammatory, hyper-metabolic phenotype compared to healthy monocytes. Furthermore, we have shown that this phenotype is maintained when RA monocytes are differentiated into M1 macrophages, suggesting that RA monocytes are already primed to become M1 macrophages once they are exposed to the microenvironment of the inflamed joint. Previous studies have reported differences in the metabolic capacity between macrophage phenotypes, with M1 demonstrating a dependency on glycolysis and M2 showing a preference for oxidative phosphorylation (Rodríguez-Prados *et al.*, 2010; O'Neill, 2015; Van den Bossche, Baardman and de Winther, 2015). However, a direct comparison of the inflammatory and metabolic profile of RA M1 and M2 macrophages has yet to be examined in RA.

Analysis of the expression of key pro-inflammatory mediators in RA M1 and M2 macrophages indicated increased *OSM* ($p < 0.05$), *IL-1 β* ($p < 0.05$), *IL-6* ($p < 0.05$), *IL-8* ($p < 0.05$), *RANTES* ($p < 0.05$) and *SOCS3* ($p < 0.05$) in RA M1 compared to M2 (Figure 2.13). Expression of *IL-27*, *TNF α* , *CXCL10* and *CXCL11* were also increased in M1 compared to M2 but did not reach statistical significance (Figure 2.13).

One of the key effector functions of macrophages is their ability to engulf and destroy cellular debris and foreign material (Hirayama and Nakase, 2017), so we next examined the phagocytic functional capacity of polarised macrophages. Comparison of unstimulated M0, classical activated M1 and alternatively activated M2 macrophage phagocytic function was examined using the OVA-DQ assay. Representative flow cytometry dot plots depicted in Figure 2.14 (A) reveal a blunted phagocytosis of OVA-DQ by inflammatory M1 macrophages compared to M0 and M2. Phagocytic ability was measured by subtracting passive phagocytosis (cells incubated at 0°C) from active, receptor-mediated phagocytosis (cells incubated at 37°C). Representative histograms indicate this shift from passive to active phagocytosis (Figure 2.14 (B)). Further quantification indicates a significant decrease in the phagocytic capacity of RA M1 macrophage populations, relative to RA M0 and RA M2 ($p < 0.05$) as shown in Figure 2.14 (C). These data suggest that the hyper-inflammatory profile of M1 macrophages results in a functional impairment in their ability to phagocytose. Indeed, failure to clear cellular

debris may stimulate other infiltrating immune cells and thus perpetuate the inflammatory response further (Gierut, Perlman and Pope, 2010).

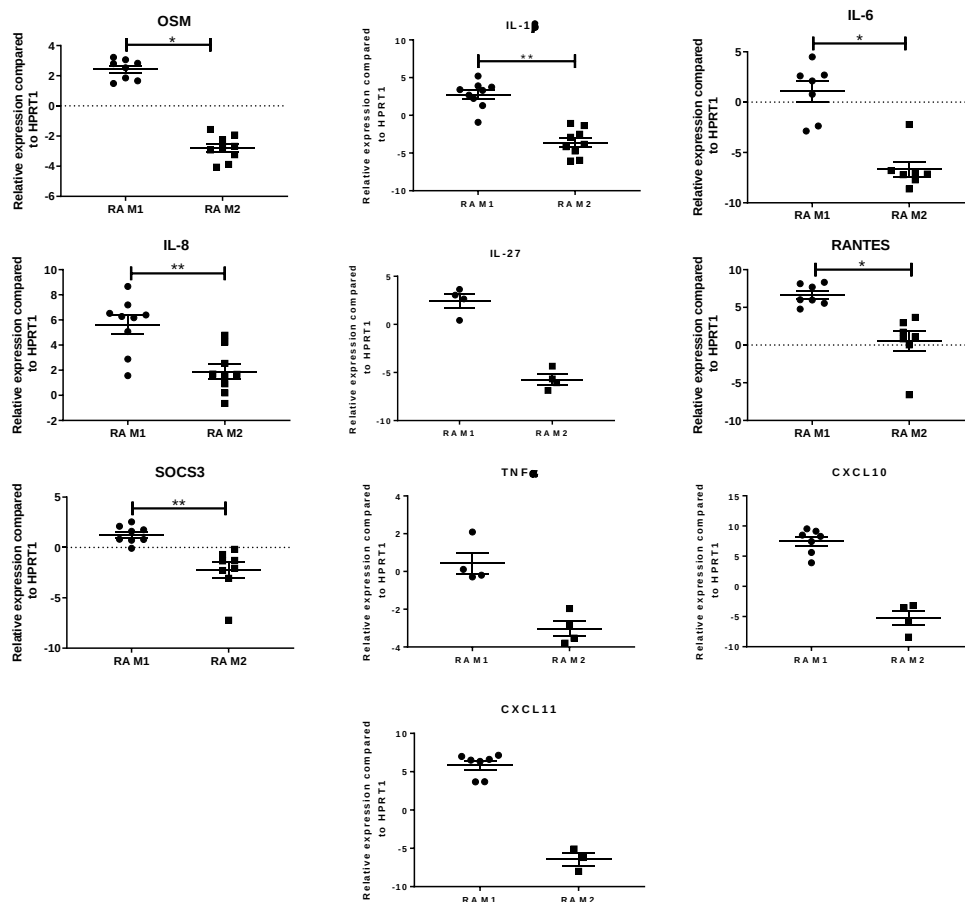


Figure 2.13: Comparison of pro-inflammatory genes in RA polarised macrophages.

Graphs represent pro-inflammatory gene expression of *OSM*, *IL-1 β* , *IL-6*, *IL-8*, *IL-27*, *TNF α* , *RANTES*, *SOCS3*, *CXCL10* and *CXCL11* in RA M1 and M2 macrophages in RA (n=3-9). Data expressed as mean $\pm$ SEM and normalized to housekeeping control *HPRT1* using Wilcoxon signed rank, *p<0.05, **p<0.01 significantly different.

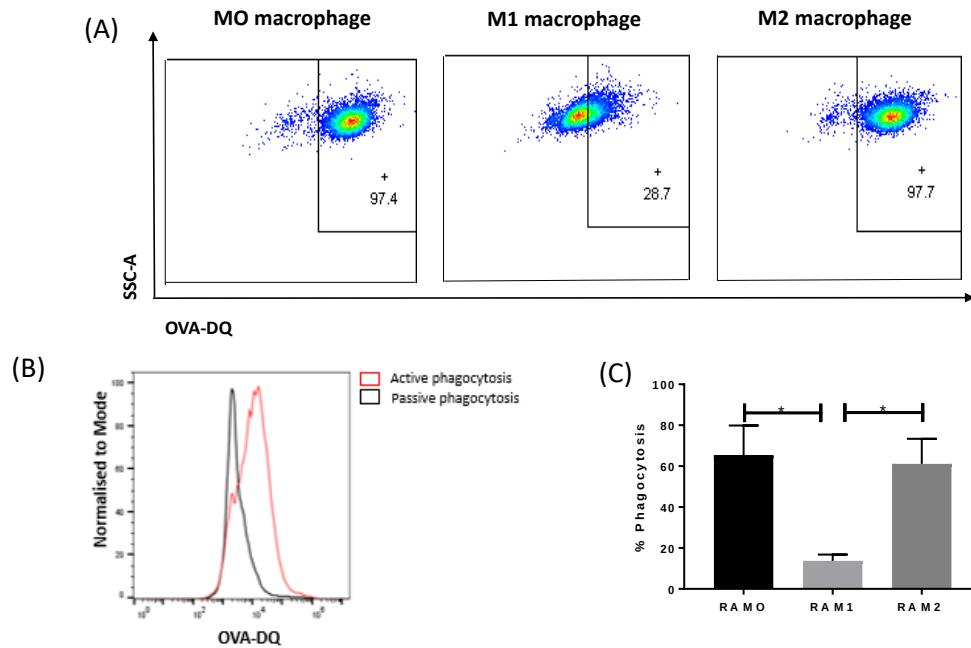


Figure 2.14: Phagocytic function of RA polarised macrophages.

(A) Representative flow cytometry dot plots demonstrating the phagocytic capacity of RA M0, M1 (LPS; 100 ng/mL, IFN γ ; 20ng/mL) and M2 (IL-4; 20 ng/mL) macrophages. Total cell population was initially gated based on side and forward scatter readings before gating on single cells to exclude doublets. Cells were then assessed for OVA-DQ positivity based on the FITC channel. (B) Representative histogram depicting the shift between passive and active phagocytosis. (C) Bar graph representing the difference in phagocytic ability between M0, M1 and M2 macrophages as measured by subtraction of the percent of cells of passively phagocytotic cells from the percent of actively phagocytotic cells (n=10). Data expressed as mean $\pm$ SEM using paired *t*-test, *p<0.05 significantly different.

2.4.7 Comparison of RA polarized macrophage metabolic profiles

To investigate if the distinct inflammatory and functional phenotype of RA M1 macrophages is due to alterations in energy metabolism, we next measured oxidative phosphorylation and glycolysis using the Seahorse-XFe Flux Analyser. Average ECAR profiles are shown in Figure 2.15 (A). A significantly increased baseline ECAR ($p < 0.05$) and maximal glycolytic rate ($p < 0.08$) was observed in RA M1 macrophages compared to their M2 counterparts (Figure 2.15 (B)).

In parallel, we demonstrate a significant increase in the expression of key glycolytic enzymes. A panel of 9 metabolic genes was examined and displayed as a heatmap demonstrating differential metabolic gene expression in M1 compared to M2 macrophages (Figure 2.15 (C)). M1 macrophages display strong expression of pro-glycolytic genes *HIF1 α* , *HK2*, *PFKFB3*, *LDHA* and *PKM2* compared to M2. In contrast, other downstream metabolic genes such as those shunting into the pentose phosphate pathway (*G6PD*), are increased in M2 in comparison to M1; *G6PD*, *PHD3*, and *PDK1/2* (Figure 2.15 (C)). These pro-glycolytic genes were then validated in a larger cohort of patients with significant increases observed for *PFKFB3* ($p < 0.05$), *HIF1 α* ($p < 0.05$) and *HK2* ($p < 0.05$) in M1 compared to M2 (Figure 2.15 (D)). Western blot analysis confirmed an increase in protein expression of *PFKFB3* and *HK2* in RA M1 compared to RA M0 or RA M2, from three individual RA patients (Figure 2.15 (E)).

In contrast to the clear differences in glycolytic ability of polarised macrophages, M1 and M2 macrophages have relatively indistinguishable OXPHOS capacities as indicated by OCR profiles (Figure 2.16 (A)). Polarised RA macrophages have similar baseline OCR, ATP synthesis and maximal respiratory capacity (Figure 2.16 (B-D)). This results in a significant increase in the ECAR:OCR ratio ($p < 0.05$) in favour of glycolysis in RA M1 macrophages (Figure 2.16 (E)). We next stained polarised macrophages with MitoTracker Green dye which accumulates in the mitochondrial matrix and covalently reacts with free thiol groups of cysteine residues to stain total mitochondrial content, regardless of mitochondrial membrane potential. Quantification of the fluorescence intensity of MitoTracker Green staining demonstrates the total mass of mitochondria present in the cytosol (Agnello, Morici and Rinaldi, 2008). Interestingly, we note that RA

M1 macrophages have decreased mitochondrial mass compared to RA M2 suggesting mitochondrial dysfunction (Figure 2.16 (F)).

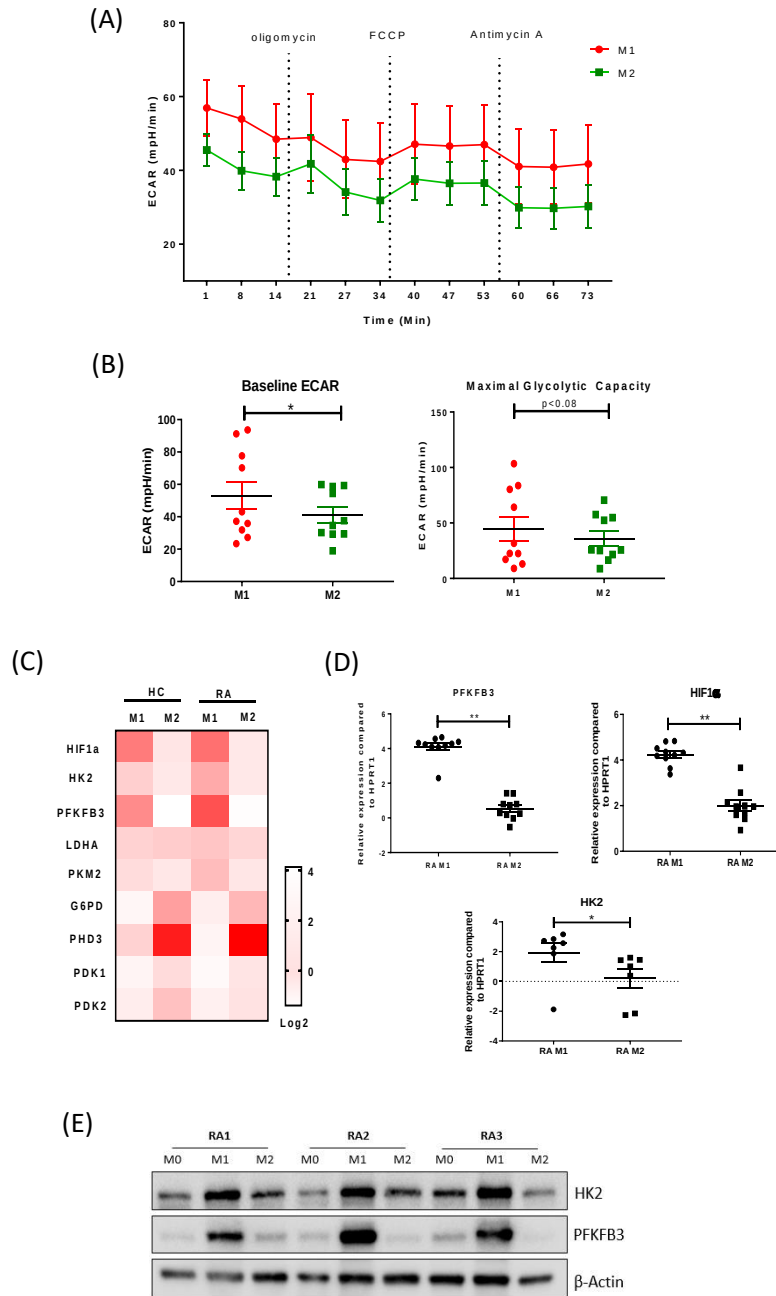


Figure 2.15: RA M1 macrophages are hyper-glycolytic.

(A) Average Seahorse bioenergetics profile demonstrating ECAR of RA M1 and M2 macrophages ($n=10$), before and after injections of oligomycin, FCCP and antimycin A. (B) Graphs demonstrating baseline ECAR and maximal glycolytic capacity. (C) Heatmap representing expression of 9 metabolic genes in M1 vs M2 macrophages ($n=3-6$). (D) Graphs represent gene expression of *PFKFB3*, *HIF1 α* and *HK2* in RA M1 vs M2 macrophages ($n=7-10$). Data expressed as mean $\pm$ SEM, normalized to housekeeping control *HPRT1*, using Wilcoxon signed rank * $p < 0.05$, ** $p < 0.01$ significantly different. (E) Representative western blot demonstrating protein expression of HK2, PFKFB3 and β -actin for RA M0, M1 and M2 macrophages from 3 independent RA patients.

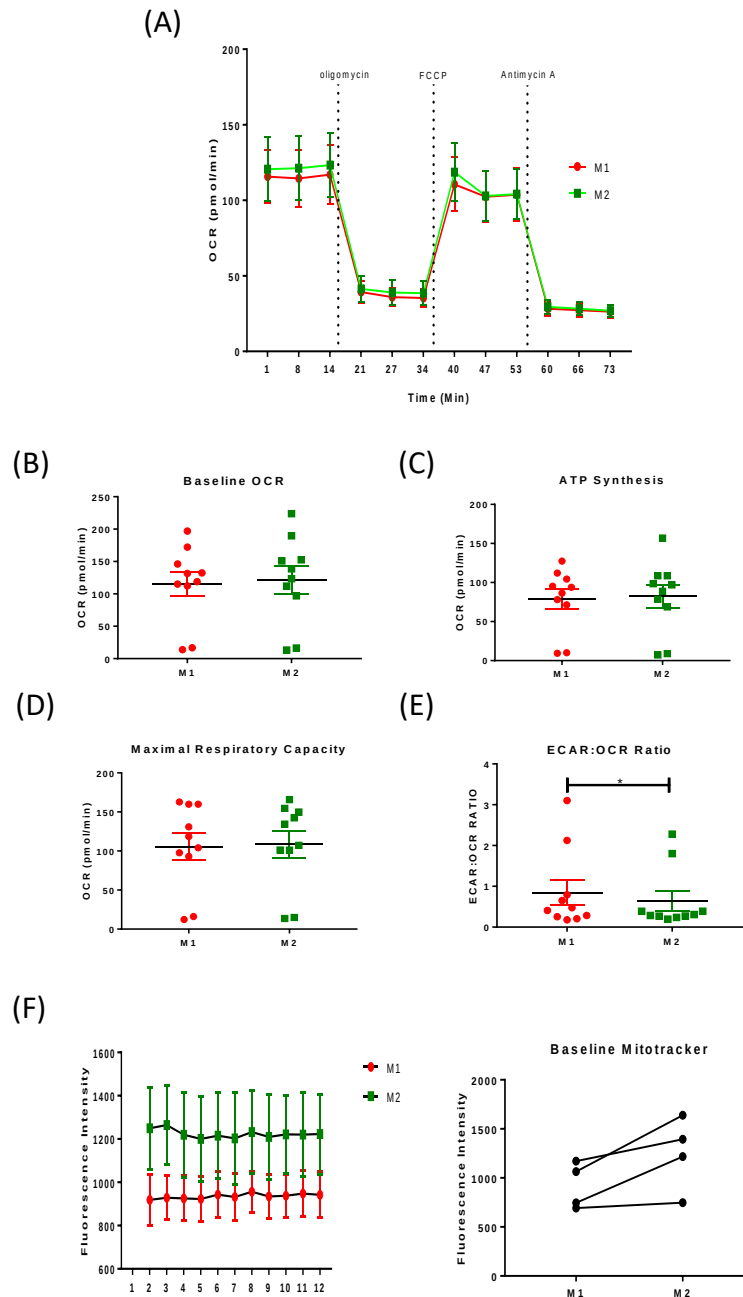


Figure 2.16: Comparison of the metabolic profile of RA M1 vs M2 macrophages.

(A) Average Seahorse bioenergetics profile demonstrating OCR of RA M1 and M2 macrophages (n=10) before and after injections of oligomycin, FCCP and antimycin A. Graphs demonstrating (B) baseline OCR, (C) ATP synthesis, (D) maximal respiratory capacity and (E) the ECAR:OCR ratio. Data represented as mean $\pm$ SEM using paired *t*-test, **p*<0.05 significantly different. (F) Fluorescence intensity of polarised macrophages stained with MitoTracker Green dye to assess total mitochondrial content (n=4). MitoTracker emission was read using the kinetic mode of the Spectra Max Gemini System with excitation and emission wavelengths of 490 nm and 516 nm respectively. Readings were performed every 15 min during a 3 hr time period.

2.4.8 Transcriptional analysis of polarized RA macrophages

This chapter has demonstrated the hyper-inflammatory phenotype of RA CD14⁺ monocytes and differentiated M1 macrophages which is associated with distinct alterations in cellular bioenergetics, a phenomenon that is not observed in RA M2 macrophages. Therefore, to further examine and explore the underpinning mechanisms of these divergent macrophage activation states, we performed RNA-seq analysis on RA monocyte-derived M1 and M2 macrophages.

Principal component analysis (PCA) and unsupervised hierarchical clustering of the total gene expression dataset demonstrates that RA M1 macrophages cluster separately from RA M2 macrophages (Figure 2.17 (A-B)). This indicates that opposing macrophage polarisation conditions results in very distinct transcriptomes that regulate macrophage activation. Next, to interrogate these transcriptional differences in RA M1 and M2 macrophages we analysed the 6,959 differentially expressed genes (DEGs). Volcano plot shown in Figure 2.18 (A) represents the DEGs between RA M1 and M2 with red data points indicating significantly differentially expressed genes (adjusted p -value <0.05), thus further confirming distinct transcriptional signatures between RA M1 and M2 macrophages.

Ingenuity Pathway Analysis (IPA) was performed on DEGs to identify key signalling pathways and potential targets. IPA revealed enrichment of several key inflammatory and metabolic pathways that are over expressed in either M1 or M2 as shown in Figure 2.18 (B). As expected from our previous *in vitro* analysis of polarised RA macrophages, enrichment of many pro-inflammatory signalling pathways such as IL-6, IL-1, OSM and iNOS signal transduction pathways were observed in RA M1 macrophages. In addition, other pathways known to be dysregulated in RA pathogenesis such as JAK/STAT, PI3K/AKT and NF κ B signalling pathways (Fearon *et al.*, 2016), were also enriched in M1 polarised cells. In contrast, enrichment of the TCA cycle and oxidative phosphorylation pathways was observed in anti-inflammatory M2 macrophages.

Further analysis of DEGs focused on those with a significant role in cellular bioenergetics. Selecting metabolism-associated genes that were significantly upregulated in RA M1 macrophages compared to M2, data was input into Reactome database (<https://reactome.org/>) and for subsequent STRING analysis. This database forms

networks of biological interactions extensively referenced from over 100 different online bioinformatics resources including NCBI Gene, Ensembl and UniProt databases (Croft *et al.*, 2011). The resulting pathway diagram depicted in Figure 2.19 (A) allows the honing in on central hubs. Interestingly, many molecules of the JAK/STAT signalling pathway are clustered at the epicenter of this diagram.

This information was of significant interest as the JAK/STAT pathway was one of the top enriched pathways in RA M1 macrophages compared to M2 from the IPA analysis (Figure 2.18 (B)). Of note, the signalling pathway of the transcription factor, STAT3, was also revealed to be enriched in RA M1 macrophages and is at the centre of the Reactome pathway analysis. Gene expression from RNA-seq indicates that RA M1 macrophages have significantly elevated *STAT3* expression compared to M2 (Figure 2.19 (B), $p < 0.05$). This was then confirmed by western blot which showed increased phosphorylation and hence, activation of STAT3 in RA M1 macrophages compared to M0 and M2 from 3 individual patients (Figure 2.19 (C)), suggesting that STAT3 signalling is associated with RA M1 function.

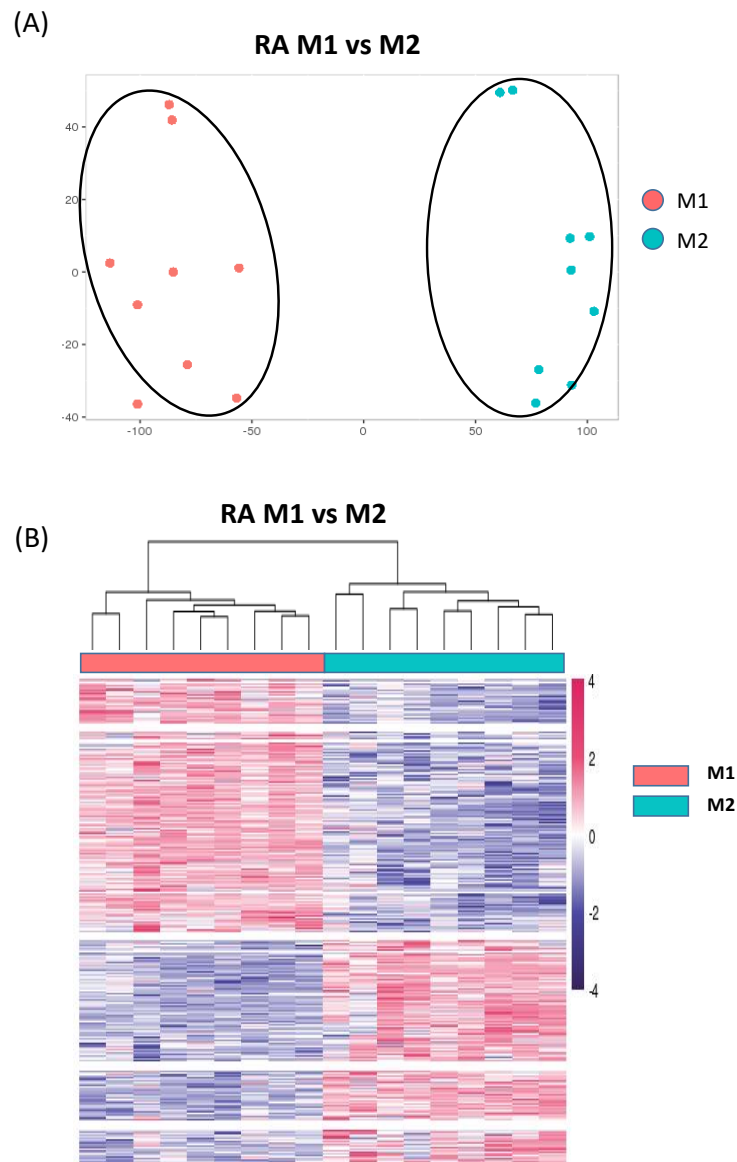


Figure 2.17: RA M1 macrophages are transcriptionally distinct from M2.

High quality RNA was isolated from RA M1 and M2 macrophages and RNA-sequencing was performed. (A) Principal component analysis (PCA) was performed on the total dataset (n=9). (B) Unsupervised hierarchical clustered heatmap of DEGs in RA M1 and M2 macrophages.

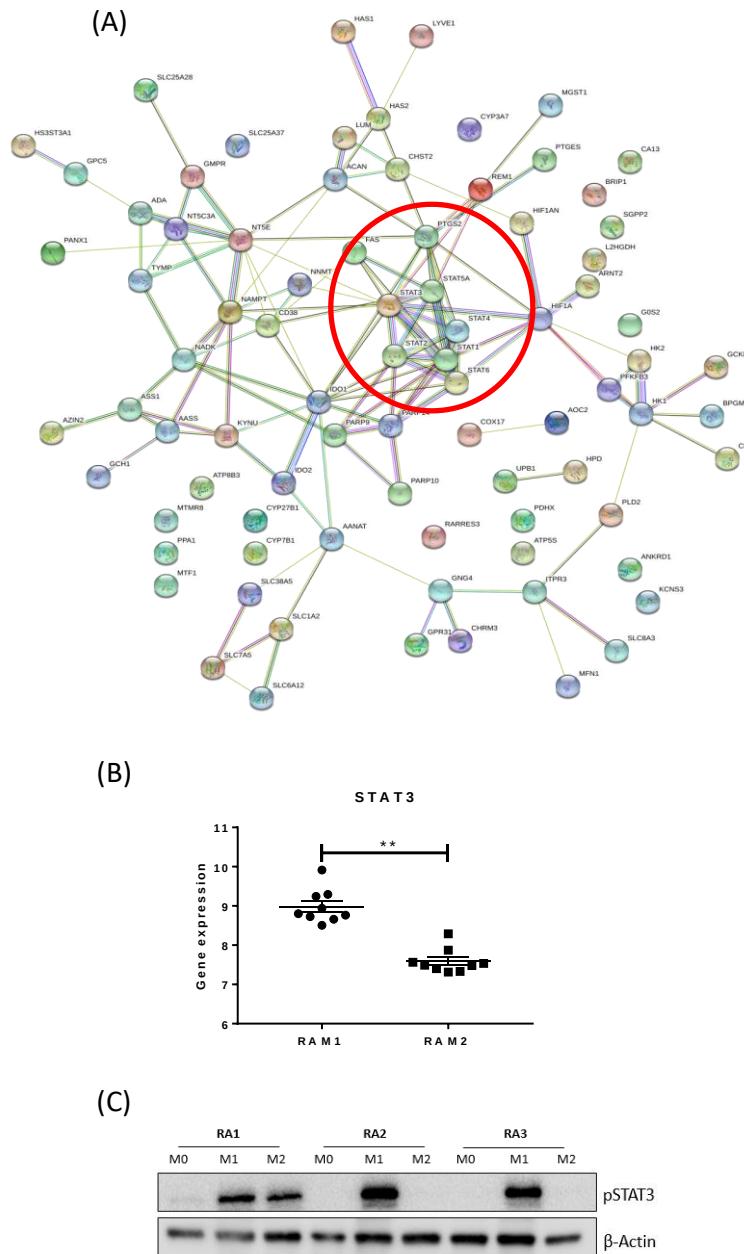


Figure 2.19: STAT3 signalling enriched in RA M1 compared to M2.

(A) Metabolism associated genes enriched in RA M1 compared to M2 input into the Reactome and STRING pathway database and resulting pathway analysis was generated. (B) Bar graphs depicting gene expression of *STAT3* in RA M1 and M2 macrophage populations (n=9). Data expressed as mean $\pm$ SEM using Wilcoxon signed rank statistical analysis, **p<0.01 significantly different from each other. (C) Representative western blot demonstrating protein expression of pSTAT3 and β -actin control for RA M0, RA M1 and RA M2 macrophages from 3 independent RA patients.

2.4.9 STAT3 regulates the inflammatory and metabolic profile in RA monocytes and macrophages

STAT3 has previously been implicated in mediating invasive mechanisms in RA (Ju *et al.*, 2012; Gao *et al.*, 2015), and has recently been linked to the regulation of metabolic activity (McGarry *et al.*, 2018), therefore we next assessed the role of STAT3 in mediating the hyper-inflammatory, hyper-metabolic phenotype observed in RA CD14⁺ monocytes and macrophages.

To investigate whether STAT3 regulation of RA inflammatory macrophages is a disease-specific signature that is imprinted early in the life cycle of macrophages, we analysed STAT3 activation in RA CD14⁺ monocytes compared to healthy control. Indeed, we demonstrate that STAT3 expression was significantly increased in LPS-stimulated RA CD14⁺ monocytes compared to HC (Figure 2.20 (A), $p < 0.05$), consistent with that previously observed for monocyte-derived M1 macrophages (Figure 2.19). Furthermore, selective inhibition of STAT3 using STATiC, resulted in almost complete inhibition of the pro-inflammatory gene signature; *TNF α* ($p < 0.05$), *IL-6* ($p < 0.05$), *IL-1 β* ($p < 0.05$), *CXCL10*, *CXCL11* and *IL-27* ($p < 0.05$) (Figure 2.20 (B)). Treatment with STATiC in various cancer models has displayed much promise however there is a concern that it may trigger off-target effects (McMurray, 2006; Bhola, Johnson and Grandis, 2016). It has been suggested that STATiC is capable of binding to a range of proteins due to its small size and by undergoing intracellular modifications (Kolosenko *et al.*, 2017). Despite these concerns, STATiC is used widely as a small molecule inhibitor for STAT3 activation.

To examine if STAT3 mediates these effects through metabolic pathways, we assessed the effect of STATiC on RA CD14⁺ monocyte oxidative phosphorylation and glycolysis. Figure 2.21 demonstrates average energy profiles, showing that STAT3 inhibition results in a decrease in both OCR (A) and ECAR (B). Additionally, STAT3 inhibition decreased LPS-induced GLUT-1, HK-2 and PFKFB3 protein expression (Figure 2.21 (C)). Interestingly, inhibition of glycolysis using 2DG also showed reciprocal inhibition of STAT3 expression, suggesting bi-directional interplay or a negative feedback loop whereby STAT3 inhibition can reduce glycolysis which, in turn, can reduce STAT3 expression (Figure 2.21 (D)). Finally, inhibition of STAT3 in RA M1 macrophages mirrors the effects observed in RA CD14⁺ monocytes with a significant decrease in baseline OCR,

ECAR, ATP synthesis and maximal respiration in response to treatment with STATTC (Figure 2.22 (A-C), $p < 0.05$).

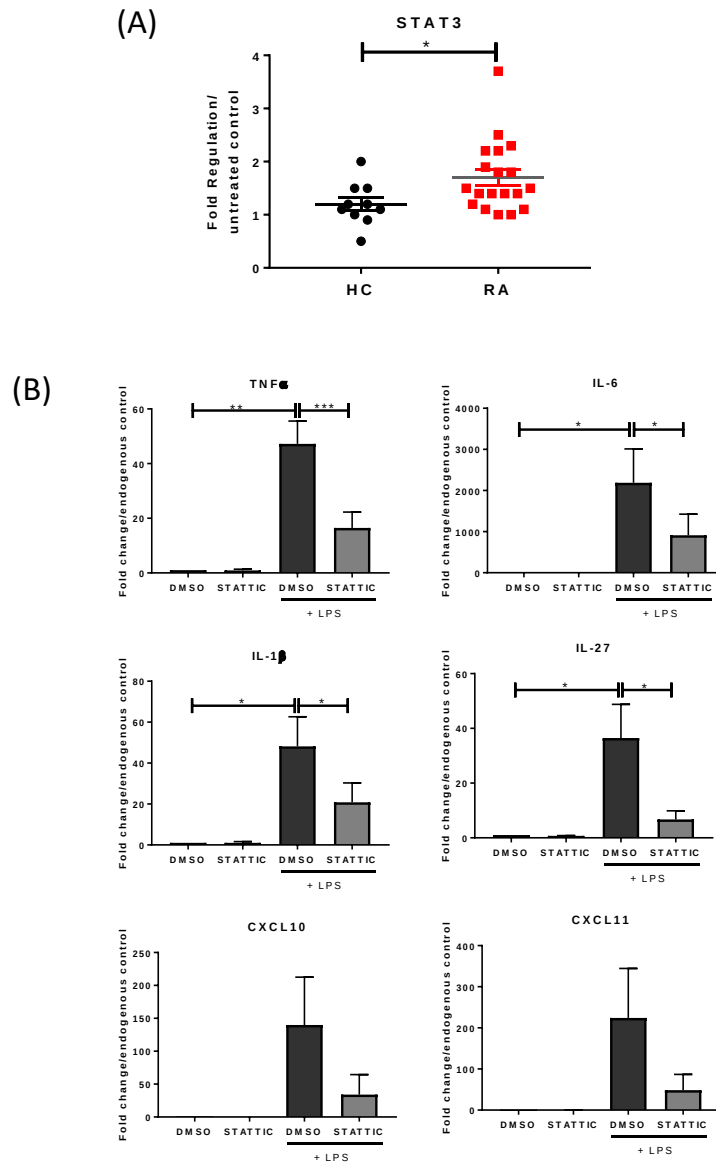


Figure 2.20: RA CD14⁺ monocyte inflammatory phenotype is inhibited by STAT3 blockade.

(A) Graph representing gene expression of *STAT3* in HC (n=10) and RA (n=19) CD14⁺ monocytes in response to LPS (100 ng/mL; 3 hr). Data expressed as mean $\pm$ SEM normalized to housekeeping control using unpaired *t*-test, **p*<0.05, significantly different.

(B) Bar graphs represent *TNF α* , *IL-6*, *IL-1 β* , *IL-27*, *CXCL10* and *CXCL11* in response to LPS +/- STATTIC in RA CD14⁺ monocytes (n=7-8). Data represented as mean $\pm$ SEM using paired *t*-test **p*<0.05, ***p*<0.01, ****p*<0.005, significantly different.

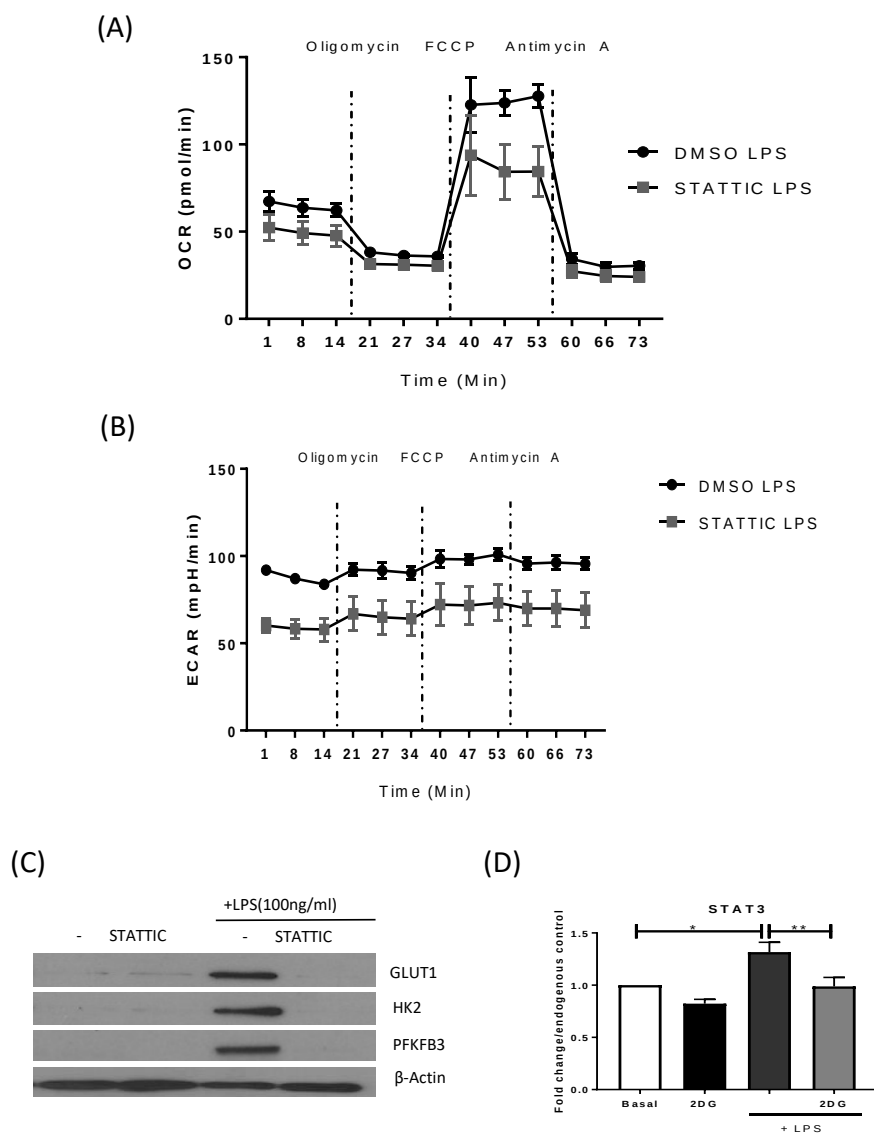


Figure 2.21: STAT3 regulates the metabolic profile of RA CD14+ monocytes.

Average Seahorse bioenergetic profiles demonstrating (A) OCR and (B) ECAR before and after injections of oligomycin, FCCP and antimycin A in LPS-stimulated (100 ng/mL; 1 hr) +/- STATTC RA monocytes (n=2). (C) Representative western blots demonstrating inhibition of LPS-induced GLUT-1, HK2 and PFKFB3 protein expression following incubation with STATTC. (D) Bar graph demonstrates *STAT3* gene expression in response to LPS +/- 2DG in RA CD14+ monocytes (n=5). Data represented as mean $\pm$ SEM, using paired *t*-test, **p*<0.05, ***p*<0.01, significantly different.

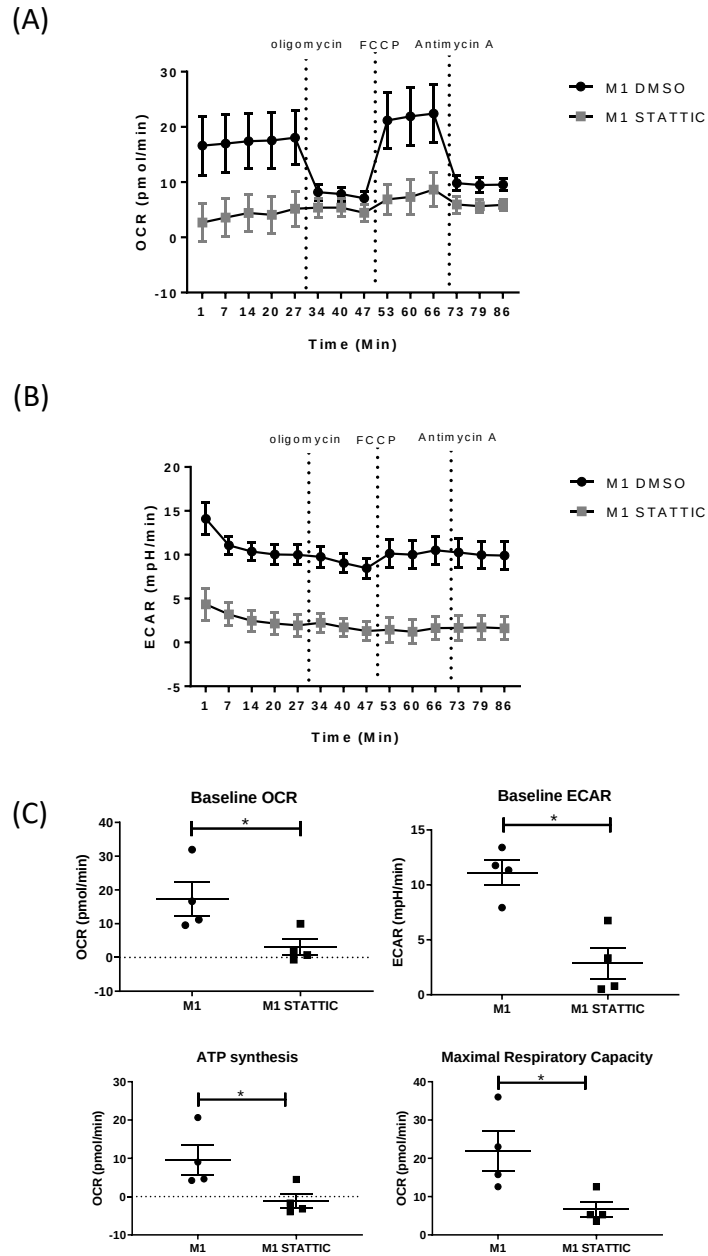


Figure 2.22: STAT3 regulates the metabolic profile of RA M1 macrophages.

Average Seahorse bioenergetic profiles demonstrating (A) OCR and (B) ECAR before and after injections of oligomycin, FCCP and antimycin A in RA M1 macrophages (n=3) +/- STAT3. (C) Graphs demonstrating baseline OCR, baseline ECAR, ATP synthesis and maximal respiratory capacity. Data represented as mean $\pm$ SEM using paired *t*-test, **p*<0.05 significantly different.

2.4.10 Patient demographics

Patient demographics for both monocyte and monocyte-derived macrophage summarised in Table 2.2 and 2.3. For monocyte studies, 51 patients were recruited in total with an average age and disease duration of 58.7 and 12.2 years, respectively. Mean DAS28 score was 2.97 and a mean CRP of 13.5 mg/L indicative of a high disease activity. 56.8% of RA donors were positive for ACPA antibodies and 59% positive for Rheumatoid Factor. Most patients (58.8%) were on MTX therapy either alone or in combination with other treatments with 23.5% receiving NSAIDs and 1% naïve to treatment.

For monocyte-derived macrophage studies, 45 patients were recruited in total. An average age and disease duration of 55.4 and 11.8 years was observed, respectively with 28 female donors (62%). Mean DAS28 score was 3.15 and a mean CRP of 7.4 mg/L indicative of a high disease activity. 55.5% of RA donors were positive for ACPA antibodies and 58% positive for Rheumatoid Factor. 15 patients were on MTX therapy (34%) either alone or in combination with other treatments with 11.4% receiving NSAIDs and 34% naïve to treatment.

All healthy control samples were obtained from either the Irish Blood Transfusion Service St James's Hospital or through Trinity College Dublin healthy blood recruitment and were completely anonymised with no gender and age demographics. For future epigenetic studies, age-matched HC blood will be collected to address this.

The data presented in this chapter highlights stark differences in the inflammatory and metabolic profiles of monocytes and monocyte-derived macrophages from RA and HC individuals, suggesting that RA myeloid cells are primed for inflammation. We also examined the relationship between inflammatory/metabolic markers and measures of disease activity and while all markers were higher in RA disease compared to controls, no correlations were observed for DAS28, CRP, VAS and so not represented in this chapter. The lack of correlations may be due to the heterogeneity of patients or may be due to a lack of significant number of patients. Perhaps with future experiments building on n-numbers, meaningful correlations will become apparent.

Characteristic	Mean	Percentage or Range
<i>Age, years</i>	58.7	28-83
<i>Gender, female</i>	34	66%
<i>RF Positive</i>	30	59%
<i>ACPA positive</i>	29	56.8%
<i>Disease duration, years</i>	12.2	<1-46
<i>DAS28</i>	2.97	0.96-6.58
<i>CRP (mg/L)</i>	13.5	<1-103
<i>On MTX</i>	30	58.80%
<i>NSAIDs</i>	12	23.50%
<i>Treatment naïve</i>	4	1%

Table 2.2: Patient demographics for monocyte studies.

Patient characteristics for isolated monocytes from RA blood (n=51). Values reflect either the group average or the range in each group with given criteria. RF: Rheumatoid Factor, ACPA: Anti-Citrullinated Protein Antibody, DAS: Disease Activity Score, CRP: C-Reactive Protein, MTX: Methotrexate, NSAIDs: Nonsteroidal Anti-Inflammatory Drugs.

Characteristic	Mean	Percentage or Range
<i>Age, years</i>	55.4	24-88
<i>Gender, female</i>	28	62%
<i>RF Positive</i>	26	58%
<i>ACPA positive</i>	25	55.5%
<i>Disease duration, years</i>	11.84	<1-45
<i>DAS28</i>	3.15	1.24-6.64
<i>CRP (mg/L)</i>	7.4	<1-32.3
<i>On MTX</i>	15	34%
<i>NSAIDS</i>	5	11.4%
<i>Treatment naïve</i>	15	34%

Table 2.3: Patient demographics for monocyte-derived macrophage studies.

Patient characteristics for isolated monocytes from RA blood (n=45). Values reflect either the group average or the range in each group with given criteria. RF: Rheumatoid Factor, ACPA: Anti-Citrullinated Protein Antibody, DAS: Disease Activity Score, CRP: C-Reactive Protein, MTX: Methotrexate, NSAIDs: Nonsteroidal Anti-Inflammatory Drugs.

2.5: Discussion

Macrophages are a heterogeneous family of immune cells, critically involved in directing the immune response in RA. They are remarkably plastic which allows them to assume a classically activated, pro-inflammatory 'M1' state or an alternatively activated, immunoregulatory 'M2' state. Deciphering the mechanisms underpinning macrophage polarisation and function may provide insights into the signalling pathways that contribute to the imbalance of M1 over M2 macrophages that has been reported in RA pathogenesis.

In this chapter we demonstrate a distinct inflammatory and metabolic phenotype of RA macrophages and their precursor cells, CD14⁺ monocytes. We report that CD14⁺ monocytes from RA patients are poised to produce pro-inflammatory mediators, a phenotype that persists following maturation into macrophages in the joint. Evaluation of the metabolic competence of monocytes and differentiated macrophages revealed a robust boost in both oxidative phosphorylation and glycolysis in RA compared to HC, coupled with altered mitochondrial morphology. In addition, we demonstrate a consistent upregulation of the glycolytic machinery, both at the gene and protein level indicating a fundamental abnormality in the processing of glucose. Analysis of polarised RA M1 and M2 macrophages revealed striking differences in inflammatory, bioenergetic and phagocytic function along with marked transcriptional variance determined by RNA-seq and identified a key role for STAT3 in macrophage polarisation. Finally, we demonstrate mechanistically that the hyper-inflammatory and hyper-metabolic state of RA monocytes and M1 macrophages, is mediated through STAT3 phosphorylation and that inhibition of STAT3 activity switches the inflammatory and metabolic phenotype of these cells to promote resolution of inflammation.

We have identified an inflammatory signature specific to circulating CD14⁺ monocytes in RA patients compared to healthy individuals. Gene expression analysis revealed that monocytes from RA patients are primed to produce high levels of the key pro-inflammatory cytokines/chemokines, TNF α , IL-6, IL-1 β , OSM, CXCL10, CXCL11 and IL-27. Interestingly a recent review on macrophage polarisation reported that these specific genes are over-expressed and are a hallmark of inflammatory M1 macrophages (Shapouri-Moghaddam *et al.*, 2018). Consistent with this, we demonstrate that RA

monocyte-derived macrophages maintain the hyper-inflammatory memory bias of their precursor cells. Specifically, we demonstrate that monocyte-derived macrophages from RA patients produce excessive levels of IL-1 β , IL-8, OSM, SOCS3 and MCP-1 compared to those from healthy individuals. Thus, indicating that RA monocytes are committed to the production of pro-inflammatory cytokines and this commitment persists once differentiated into mature macrophages.

This is in line with studies assessing monocyte/macrophage function in coronary artery disease (CAD) which suggest that monocyte precursors are primed for inflammation and subsequent differentiation into M1 macrophages (Shirai *et al.*, 2016). Conversely, a recent study indicated that circulating monocytes from giant cell arteritis (GCA) patients display pro-inflammatory signatures similar to healthy individuals, indicating that pre-programming of monocytes is disease-specific (Watanabe *et al.*, 2018). In RA, monocyte and macrophage effector functions may be influenced by citrullinated proteins. ACPA positivity has been shown to influence the inflammatory status of macrophages, with ACPAs from RA synovial fluid initiating a shift in peripheral blood monocytes towards a pro-inflammatory M1 macrophage phenotype *in vitro* (Zhu *et al.*, 2015). Another study reported that ACPAs promote IL-1 β release from macrophages via AKT/NF κ B and subsequent activation of the NLRP3 inflammasome (Dong *et al.*, 2019). Moreover, studies have demonstrated that circulating RA monocytes tend to skew towards the CD14⁺⁺CD16⁺ monocyte subset, which are primed to produce high levels of many proinflammatory cytokines including TNF α , IL-1 β and IL-6, and are thought to be the monocyte subset that predominantly differentiates into M1 macrophages (Rossol *et al.*, 2012; Klimek *et al.*, 2014; Yang *et al.*, 2014; Weldon *et al.*, 2015; Tsukamoto *et al.*, 2017). Finally, elevated levels of circulating monocytes positively correlate with disease activity and are predictive of a poor response to treatment in RA (Chara *et al.*, 2015; Tsukamoto *et al.*, 2017). This underscores the potential role of monocytes as a promising biomarker in informing RA disease activity and treatment response.

In RA inflammatory macrophages, while we do demonstrate a marked increase in the levels of a number of pro-inflammatory mediators compared to HC, similar levels are observed for other key inflammatory cytokines. Most notably, expression of 'the master cytokine' TNF α , which is increased in circulating RA monocytes compared to HC, was

indistinguishable between RA and HC differentiated macrophages. Consistent with this, previous studies examining macrophages differentiated from circulating CAD monocytes, demonstrated elevated expression of IL-6 and IL-1 β , but not TNF α , compared to healthy controls (Shirai *et al.*, 2016). This indicates that the persistence of inflammatory pre-programming is cytokine specific and thus suggests separate regulatory networks for different cytokines. One such mechanism may be STAT3 regulation considering that the promoters for IL-1 β , IL-6 and the IL-6-like cytokine, OSM share binding sites for STAT3, which is not implicated in transcription of TNF α in macrophages (Samavati *et al.*, 2009). Moreover, HIF1 α inhibition strongly reduces macrophage IL-1 β expression, yet has no effect on TNF α production (Tannahill *et al.*, 2013). Further investigation into the complex signalling and metabolic interactions of specific cytokines are needed to fully elucidate this role.

In addition to the inflammatory and metabolic differences in RA and HC monocytes and macrophages, in this study we demonstrate a significant functional impairment in the phagocytic ability of M1 macrophages compared to M0 and M2 macrophages. Despite the crucial role of phagocytosis as a hallmark of macrophage activation, studies do support the concept of a shift in the phagocytic capacity of M1 macrophages during the inflammatory response. Delayed or reduced phagocytosis has been observed in RA mononuclear phagocytes, leading to an increase in ROS generation and resulting in subsequent prioritisation of chemotactic behaviour and cytokine release over phagocytosis (I. Arleevskaya, 2011). Moreover, the observed robust phagocytic activity of M2 macrophages observed in this study is consistent with reports demonstrating increased phagocytosis in alternatively activated macrophages following monocyte co-culture with regulatory T cells (Tregs) (Tiemessen *et al.*, 2007). In the context of RA, the failure of M1 polarised macrophages to efficiently clear cellular debris, coupled with their observed hyper-inflammatory phenotype, may further stimulate the infiltration of other immune cells into the synovium, thus propagating the inflammatory response (Gierut, Perlman and Pope, 2010).

Recent evidence has demonstrated the importance of metabolic rewiring in guiding the functional phenotype of macrophages (Van den Bossche, O'Neill and Menon, 2017). The robust increase in both oxidative phosphorylation and glycolysis observed in

RA monocytes compared to HC demonstrated a state of heightened mitochondrial activity in RA. Moreover, we demonstrate that RA macrophages recapitulate the metabolic profile established in RA monocytes thus indicating they are already metabolically primed once they enter the synovial space. As observed in RA monocytes, RA inflammatory macrophages display a boost in both OXPHOS and glycolysis, compared to HC. Abnormalities in mitochondrial function were further reflected in the ultrastructure of RA macrophage mitochondria which exhibit a dominance of elongated, dense mitochondria compared to healthy macrophages.

This is the first study to demonstrate the priming of circulatory monocytes in RA, though similar mechanisms have been demonstrated in CAD. The hyper-metabolic state as represented by increased oxygen consumption, ATP production and cytokine secretion, in CAD monocytes is similar to that observed in the pathologic RA monocytes in this study (Shirai *et al.*, 2016). Moreover, another study reports a remodelling of mitochondria-associated membranes in RA and CAD macrophages to enhance mitochondrial activity, a phenomenon that is already present in circulating monocytes and dependent on inactivation of glycogen synthase kinase 3b (GSK3b) (Zeisbrich *et al.*, 2018). Interestingly, functional abnormalities of circulating monocytes are not common to all autoimmune disorders as a study comparing monocytes from CAD and GCA demonstrated differential glucose requirements in GCA when compared to CAD circulating monocytes (Watanabe *et al.*, 2018).

The observed concomitant changes in inflammatory function and metabolic outputs are not surprising. A link between immune function and cellular metabolism has been extensively studied in recent years. Indeed, metabolic intermediates have been shown to regulate the expression of many key macrophage-derived cytokines. Succinate-mediated stabilisation of HIF1 α results in IL-1 β secretion and ROS production from macrophages (Tannahill *et al.*, 2013; Mills *et al.*, 2016), while succinate receptor, GPR91-deficient mice display reduced macrophage activation and IL-1 β production (Littlewood-Evans *et al.*, 2016). In addition, elevated glucose concentrations promote IL-1 β production from RA myeloid cells via NLRP3 activation (Ruscitti *et al.*, 2015). As such, the metabolic plasticity of monocytes and macrophages opens the possibility of bioenergetic interference as a therapeutic intervention for RA to direct M1 macrophages away from

inflammatory mechanisms. HIF1 α deletion in myeloid cells of CIA mice has been reported to result in decreased macrophage infiltration and joint swelling due to reduced macrophage mobility and invasive capacity (Cramer *et al.*, 2003). Additionally, silencing of the amino acid transporter, SLC7A5, results in a significant decrease of IL-1 β and glycolysis in RA monocytes and macrophages (Yoon *et al.*, 2018). Indeed, many current treatment options for RA patients are anti-metabolic including traditional DMARDs such as methotrexate as well as biologic and non-biologic therapies (Fearon *et al.*, 2019). Studies have indicated decreased expression of key glycolytic enzymes in the synovium of TNFi responders compared to non-responders (Biniecka *et al.*, 2016). Therefore, the response to such treatment in patients recruited for this study, may have an impact on monocyte metabolic profiles.

Initially, there were concerns that manipulation of metabolic pathways would result in off-target effects clinically. However, it is now appreciated that pathogenic, activated cells are highly dependent on glucose and thus have greater glucose requirements compared to healthy cells. A recent seminal study has indicated that keratinocyte-specific GLUT-1 inhibition does not compromise homeostatic epidermal skin development, with cells capable of adapting their metabolic dependencies to utilise other mechanisms. GLUT-1 deficient pathogenic keratinocytes, however, display oxidative stress and impaired proliferation in a model of psoriasis, thus indicating a selective manipulation of rapidly proliferating cells only, leaving normal cells intact (Zhang *et al.*, 2018). This is interesting in the context of our study whereby glucose restriction using 2DG, a glucose analogue that inhibits hexokinase activity (Ralser *et al.*, 2008), results in significantly diminished expression of pro-inflammatory cytokines. In contrast, interference of mitochondrial respiration using oligomycin resulted in only partial inhibition of pro-inflammatory mediators. Therefore, despite a boost in both metabolic pathways, glycolytic mechanisms are significantly important in shaping the inflammatory effector functions of monocytes and macrophages, manipulation of which may be selective in pathogenic conditions.

The persistence of inflammatory and metabolic pre-programming of monocytes to matured macrophages may also involve altered epigenetic mechanisms. Studies indicate that monocytes are capable of building immunological memory via epigenetic

imprinting (Saeed *et al.*, 2014; Neele *et al.*, 2015). Epigenomic profiling of *ex vivo* monocytes and differentiated macrophages demonstrates that differentiating monocytes undergo substantial epigenetic remodelling, implicating epigenetic mechanisms as a potential target for therapy (Saeed *et al.*, 2014). Furthermore, the epigenetic landscape is crucial in determining macrophage polarisation; Hdac3, a specific histone deacetylase, has been shown to support M1 polarisation while simultaneously blunting M2 activation (Hoeksema *et al.*, 2014). Indeed Hdac3-deficient macrophages display an M2 phenotype and are hyper-responsive to IL-4, thus pharmacological inhibition of Hdac3 may be of therapeutic benefit (Mullican *et al.*, 2011). Moreover, environmental factors such as smoking are associated with DNA methylation (Reynolds *et al.*, 2015) which is interesting in the context of RA considering the strong association of smoking with disease pathogenesis (Sugiyama *et al.*, 2010).

Finally, through RNA-seq analysis, this study identified a key role for the transcription factor, STAT3, in influencing RA macrophage polarisation. STAT3 also mediates inflammatory and metabolic effects in RA monocytes, indicating that this mechanism occurs early in macrophage differentiation. In the context of RA, this is in line with studies linking STAT3 to the activation of other cells involved in the disease process; phosphorylation of STAT3 mediates RA synovial fibroblast migration and invasive mechanisms (Gao *et al.*, 2015). Moreover, activation of STAT3 has been shown to be associated with *in vivo* synovial hypoxia while hypoxia-induced STAT3 activation causes upregulation of HIF1 α (Jung *et al.*, 2005; Gao *et al.*, 2015) and inhibition of STAT3 suppresses disease severity in the CIA model of arthritis (Mori *et al.*, 2011). JAK/STAT signalling is closely linked to altered cellular metabolism, with blockade of the key glycolytic enzyme PFKFB3 resulting in decreased phosphorylation of STAT3 in RAFLS (Biniecka *et al.*, 2016), while STAT3 regulation of glycolysis is achieved through HK2 in cancer cells (Li *et al.*, 2017). Indeed, our group has also recently reported evidence that inhibition of JAK-STAT signalling switches the metabolic and inflammatory profile of synovial cells away from pathogenic mechanisms and towards resolution of inflammation (McGarry *et al.*, 2018). Furthermore, we demonstrate that inhibition of glycolysis with 2DG resulted in a reciprocal inhibition of STAT3 activation indicating a negative feedback loop whereby inhibition of STAT3 blocks glycolysis while inhibition of glycolysis reduces

STAT3 expression. This is consistent with studies demonstrating bidirectional interactions between STAT3 and key glycolytic enzymes HIF1 α , PKM2 and HK2 (Jung *et al.*, 2005; Gao *et al.*, 2015; Shirai *et al.*, 2016; Li *et al.*, 2017).

To summarise, in this chapter we demonstrate that RA monocytes are imprinted with disease-specific hyper-inflammatory and hyper-metabolic signatures and retain this functional commitment, such that differentiation into macrophages conserves this phenotype and results in impaired phagocytic ability. Mechanistically, we have identified a key role for STAT3 in regulating the polarisation of macrophages towards an M1 phenotype, a mechanism which occurs prior to differentiation and is maintained during inflammatory conditions.

CHAPTER THREE:

Significant enrichment of dysfunctional CD206+CD163+ macrophage population in RA synovial tissue

3.1 Introduction

Macrophages are pivotal players in joint destruction. An increase in the numbers of sub-lining macrophages is a hallmark of active disease in RA, which diminish in response to treatment. Indeed, they are the only cell type in the inflamed joint shown to be associated with treatment response, independent of the treatment type, suggesting that they may be a useful synovial biomarker to discriminate between responders and non-responders (Mulherin, Fitzgerald and Bresnihan, 1996; Tak *et al.*, 1997; BRESNIHAN *et al.*, 2009; Ng *et al.*, 2010; Kennedy, Ng, Chang, Biniecka, Jacintha N. O'Sullivan, *et al.*, 2011a). Undeniably, the role of macrophages in the pathogenesis of RA is widely recognised, yet this has been largely based on *in vitro* monocyte-derived studies. The precise nature and function of synovial tissue macrophages in RA is poorly defined as the majority of studies assessing synovial macrophages are descriptive; limited to histological or synovial fluid analysis (Palacios *et al.*, 2015; Kurowska-Stolarska and Alivernini, 2017; Rajasekhar *et al.*, 2017).

As previously outlined, the current dogma is that activated macrophages exist as one of two distinct phenotypical states - M1 or M2, representing two distinct ends of a spectrum. While this binary model is useful, it fails to reflect the remarkable plasticity and diversity of these cells; which are capable of changing their phenotype in response to environmental cues. Indeed, the reliance on one single marker to identify or characterise specific macrophage populations merely provides a snapshot of their activation status. Macrophages *in vivo* are subject to a plethora of stimuli, such that the *realtime* phenotype most likely does not fit this rigid nomenclature (Martinez and Gordon, 2014; Saha, Shalova and Biswas, 2017). Evidence now suggests that this system should be extended to encompass a wide spectrum of macrophage activation states, the characteristics of which remain largely unknown (Xue *et al.*, 2014; Van den Bossche, O'Neill and Menon, 2017). To date, no studies have explored in significant detail macrophage subsets within the inflamed human synovium or their relative contribution to joint destruction, mainly due to the difficulty in isolating these cells.

Recent research has revealed a diversity of macrophage heterogeneity in health and disease, both in function and origin (Udalova, Mantovani and Feldmann, 2016). This has further emphasised the need to explore and characterise the phenotype and ontogeny of macrophage populations in RA. Immunohistological analysis of RA synovial

tissue suggests that macrophages residing in the synovial lining layer differ from those in the sub-lining layer (Tardito *et al.*, 2019). Mature resident macrophages are abundant in the intimal lining layer, displaying an IL-10 phenotype (Ambarus *et al.*, 2012). In contrast, the synovial sub-lining has a more heterogenous phenotype, displaying a mixture of both M1 and M2 markers, possibly due to active infiltration of monocyte-derived macrophages from the periphery (Cauli, Yanni and Panayi, 1997; Ambarus *et al.*, 2012). Moreover, RA disease activity is correlated with increased numbers of synovial macrophages in the sub-lining but not lining layer (Ambarus *et al.*, 2012), thus indicating a distinction between infiltrating (sub-lining) and tissue-resident (lining) macrophages within the synovium.

A conceptual revolution has occurred in recent years regarding the prevailing dogma that all macrophages are derived from circulating monocytes. This linear model, the mononuclear phagocyte system, was coined by van Furth in 1968 and remained a common perception for decades (van Furth and Cohn, 1968; van Furth *et al.*, 1972). However, a series of authoritative lineage tracing studies have significantly challenged this view and our understanding of the origin of tissue-resident macrophages, demonstrating that many macrophages are actually tissue-derived during embryonic development (Ginhoux *et al.*, 2010; Schulz *et al.*, 2012; Yona *et al.*, 2013; Epelman *et al.*, 2014). It is now appreciated that three distinct macrophage precursors are produced in independent waves during embryonic development (Christensen *et al.*, 2004; Ginhoux and Jung, 2014; Gomez Perdiguero *et al.*, 2015). Yolk sac macrophages arising from primitive haematopoiesis comprise the first wave and seed tissues as early as day E9.0. The second wave seeds the fetal liver, where definitive haematopoiesis is established on E11.0 with the third wave coming from haematopoietic stem cells colonizing the bone marrow to produce bone marrow monocytes that seed the blood throughout life (Udalova, Mantovani and Feldmann, 2016).

The relative contribution of these waves of precursors in establishing a pool of mature macrophages differs from one tissue to another. Early fate-mapping studies indicate that microglia in the brain predominantly originate from the yolk sac during embryonic development, while the Langerhans cells of the epidermis derive from the yolk sac and fetal liver (Ginhoux *et al.*, 2010; Hoeffel *et al.*, 2012; Schulz *et al.*, 2012). In fact, the homeostatic contribution of circulating monocytes to macrophages appears to be

restricted to specific tissues including the intestinal lamina propria and the dermis (Tamoutounour *et al.*, 2013; Bain *et al.*, 2014). Instead, many tissue-resident macrophages arise from embryonic precursors prior to birth independent of haematopoiesis and subsequently self-sustain their numbers throughout adulthood (Merad *et al.*, 2002; Hashimoto *et al.*, 2013; Ginhoux and Guilliams, 2016).

This new appreciation for macrophage ontogeny has led researchers to investigate whether monocyte-derived macrophages can fully replicate the phenotype and function of embryonically derived macrophages. An elegant study by van der Laar in 2016 demonstrated that adult monocytes, yolk sac and fetal liver macrophages, when transferred into an empty alveolar niche of *Csf2rb*-deficient mice, indistinguishably differentiated into alveolar macrophages (with the exception of MHCII encoding genes). In contrast, mature macrophages from other tissues did not efficiently colonise the alveolar niche (van de Laar *et al.*, 2016). Therefore, local macrophage differentiation is determined by tissue-specific cues with tissue imprinting a dominant factor in shaping the phenotype and function of macrophages.

Furthermore, given the crucial role that bioenergetics play in macrophage polarisation as demonstrated in Chapter 2, the question arises; to what extent are synovial tissue macrophages metabolically plastic? Studies have indicated that terminally differentiated macrophages from the peritoneum display a certain degree of plasticity, capable of epigenetically and transcriptionally reprogramming in response to their microenvironment (Lavin *et al.*, 2014). Given the cross-talk between metabolic reprogramming, epigenetics and transcriptional regulation in macrophage activation (Baardman *et al.*, 2015; Van den Bossche and Saraber, 2018), it is possible to propose that synovial tissue macrophages should display some degree of metabolic flexibility. Indeed, there has been speculation that tissue-resident macrophages probably exhibit a metabolic profile akin to M2 macrophages, while the more metabolically-plastic recruited monocytes differentiate into M1 or M2 depending on the tissue microenvironment, however this has yet to be formally investigated in RA synovial tissue macrophages (Italiani and Boraschi, 2014; Van den Bossche and Saraber, 2018).

In addition, a recent study has reported that CX3CR1⁺ macrophages in the synovial lining layer form an unusual protective barrier-like layer to shield the joint from inflammation associated with arthritis. Following intricate fate-mapping studies in mice, Culemann and colleagues conclude that these barrier macrophages are not derived from monocytes and act to restrict the inflammatory response by forming this tight-junction shield (Culemann *et al.*, 2019). This study further reinforces the concept that macrophages are exquisitely plastic and adapt to needs of their microenvironment. To add to this complexity, individual subsets of resident macrophages have distinct transcriptional and epigenetic signatures according to their highly specialised functions (Lavin *et al.*, 2014; Okabe and Medzhitov, 2014; Rosas *et al.*, 2014; Chakarov *et al.*, 2019). However, the origin of macrophages in human synovium has yet to be fully elucidated as a clear distinction between tissue-resident macrophages and recruited inflammatory macrophages, and their relative contribution to joint function in health and disease has not yet been possible. Therefore, the aim of this chapter is to explore the role of distinct macrophage activation states within the inflamed synovium, using both functional and transcriptomic analysis.

3.2 Specific Aims of this Chapter:

- In-depth analysis of the spectrum of macrophage activation states within the inflamed RA synovium
- To compare macrophage populations residing in the RA synovium with Osteoarthritis, Psoriatic Arthritis and healthy synovial tissue macrophages
- Phenotypic and transcriptomic comparison of synovial tissue and synovial fluid macrophages
- Comprehensive transcriptomic RNA-seq analysis and bioenergetic characterisation of specific synovial tissue macrophage subsets
- To investigate the effects of synovial tissue macrophages on other immune cells

3.3 Materials and Methods:

3.3.1 Subject Recruitment, Arthroscopies and Sample Collection

Patients with active RA are continuously recruited from outpatient clinics at St. Vincent's University Hospital and The Adelaide and Meath Hospital, Tallaght. Ethical approval to conduct this study was granted by St. Vincent's Healthcare Group Medical Research and Ethics Committee and the Tallaght University Hospital/St. James' Hospital Joint Research Committee. All subjects gave fully informed written consent for use of biological samples and medical information, prior to inclusion in the study. Only patients with an actively inflamed knee joint and those that fulfilled the revised ACR/EULAR criteria were included (Aletaha *et al.*, 2010). Under local anaesthesia, arthroscopies of the inflamed knee were performed using Wolf 2.7 mm needle. RA synovial tissue biopsies were obtained from the site of inflammation under direct visualisation as previously described (Ng *et al.*, 2010), in addition to synovial biopsies from Psoriatic Arthritis (PsA) and Osteoarthritis (OA) patients. Healthy individuals undergoing arthroscopy during anterior cruciate ligament (ACL) reconstruction surgery were included in this study. Healthy subjects were defined as those who had no evidence of any form of arthritis on history or examination and had no cartilage damage or synovitis on knee arthroscopy. Synovial fluid mononuclear cells (SFMC) were also obtained at time of arthroscopy or at rheumatology clinics.

3.3.2 Synovial Tissue Dissociation

Synovial tissue biopsies obtained at the time of arthroscopy were mechanically and enzymatically digested using the GentleMacs dissociator and a soft tumour dissociation kit (Miltenyi Biotech, Germany), according to manufacturer's instructions to yield a single cell suspension of synovial tissue cells. Following arthroscopy, synovial tissue was sectioned into small pieces and added to an enzyme mix composed of 4.7 mL serum-free and antibiotic-free RPMI medium, 200 μ L of enzyme H, 100 μ L of enzyme R and 25 μ L of enzyme A in a gentleMACS C tube. Using the GentleMACS program; m_spleen_4, a mechanical stress was applied to the synovial tissue for 60 sec. Samples were then incubated at 37°C for 30 min under constant rotation using the MACSmix Tube Rotator (Miltenyi Biotech). The samples were subsequently exposed to a second mechanical agitation using the m_brain_03 gentleMACS program and incubated for a further 30 min

at 37°C. A final mechanical agitation was applied for 30 sec using the gentleMACS h_tumor_03 program. The resulting cell suspension was passed through a 70 µm cell strainer to remove any undigested clumps. Red blood cells were removed using Pharmlyse red blood cell lysis buffer (BD) before being washed and pelleted at 1400 RPM.

3.3.3 Isolation of Synovial Fluid Mononuclear Cells (SFMC)

Synovial fluid was obtained from patients undergoing arthroscopy. SFMC were isolated by Ficoll-Metrizoate density gradient centrifugation (Nycomed, Marlow Buckinghamshire, UK). Fluid was diluted in a 1:1 ratio with PBS before layering over 15 mL of Lymphoprep (STEMCELL Technologies, Cambridge, UK) and centrifuged at 1400 RPM for 25 min with the brake inactivated. The cloudy SFMC layer was removed at the density gradient interface and washed twice in sterile PBS and centrifuged at 1400 RPM for 5 min.

3.3.4 Flow Cytometry

Synovial tissue and SFMC samples were stained using a panel of fluorochrome-conjugated antibodies for flow cytometry. The following antibodies were used in a combination of panels to detect macrophage subsets and activation; CD40, CD45, CD68, CD64, CD163, CD206, CD253, CCR4, CCR7, CXCR1, CXCR3 (Table 3.1).

Cells were resuspended in 1 mL of PBS with 0.3 µL of either Live Dead Red or eFluor 450 viability dyes (Molecular Probes, Thermo Fisher Scientific) for 30 min in the dark at 4°C. Cells were pelleted and resuspended in 100 µL of FACS Buffer (Dulbecco's PBS without Mg²⁺ or Ca²⁺ (Sigma-Aldrich), 1% heat-inactivated FCS (Biosciences) and 0.05% sodium azide (Sigma-Aldrich), pH 7.4-7.6). To eliminate nonspecific binding of mouse monoclonal antibodies to the Fc-gamma receptor (FcγR), samples were blocked in 5 µL human FcγR-binding inhibitor for 10 min. Fluorochrome-conjugated antibodies against extracellular markers were then added and samples were vortexed and incubated in the dark at room temperature for 30 min. 100 µL of part A of the cell fixation and permeabilization kit (Biosciences) was directly added to the fluorochrome mix and incubated for a further 15 min in the dark. Cells were then washed, pelleted by centrifugation and resuspended in 100 µL of permeabilization buffer (Part B) containing fluorochrome-conjugated antibodies

against intracellular cytokine markers. Following incubation at room temperature for 30 min, samples were washed in PBS and pelleted at 400 g. Supernatants were removed and the cells were resuspended in 250 µL of PBS. Samples were acquired using the CyAn Flow Cytometer and the LSRFortessa Flow Cytometer (Beckman Coulter) and analysed using Flowjo software (Treestar Inc.).

Antibody	Fluorochrome	Clone
CD3	APC	OKT3
CD40	APC-CY7	5C3
CD45	BV510	H130
CD64	PerCPCY5.5	10.1
CD68*	PE-CY7	Y1/82A
CD163	FITC	GHI/61
CD206	PE	19.2
CD253	APC	RIK-2
CCR4	BV605	L291H4
CCR7	PeCF594	GO43H7
CXCR1	PE-CY7	8FI
CXCR3	BV650	GO25H7

* Indicates intra-cellular stains

Table 3.1: Panel of fluorochrome antibodies used for flow cytometry analysis.

Cells were gated based on forward and side scatter and dead cells and doublets were removed. Fluorescence Minus One (FMO) controls were used to determine gating boundaries. Live Dead Red or eFluor 450 viability dyes (Molecular Probes, Thermo Fisher Scientific) were used to eliminate dead cells. In order to adjust for spectral overlap between detectors, compensation was applied using single stained compensation beads (BD). Synovial macrophages were phenotyped based on the expression of CD45+ CD68/CD64+ and further characterised using the cell surface markers CD40, CD163,

CD206, CD253 as indicated by the gating strategy in Figure 3.1-3.2. Specific macrophage subsets of interest were also sorted using the BD FACSAria Fusion sorter using this gating strategy and total RNA isolated for further RNA-sequencing.

3.3.5 RNA-Sequencing

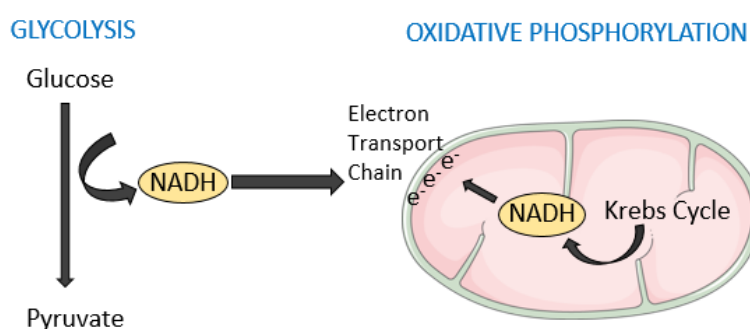
CD206+CD163+ and CD206-CD163- macrophages were sorted from RA synovial tissue and fluid using the FACSAria fusion sorter (BD Biosciences) and high-quality RNA was extracted using the RNeasy total RNA isolation kit (BD Biosciences). High quality RNA was also isolated from patient-matched monocyte-derived M1 and M2 macrophages using the RNeasy kit (QIAGEN) and the purity of RNA samples assessed using the Nanodrop bioanalyser (ThermoFisher). RNA samples were then shipped to Janssen Pharmaceutical, Spring House, PA, USA to be reverse transcribed and sequence libraries generated using NuGen Ovation Universal RNA-seq System according to manufacturer's protocols. The resulting sequencing libraries were analysed using the Caliper LabChip GX and quantified using KAPA qPCR. Libraries were normalised and pooled. Each pool was then clustered and sequenced on an Illumina NextSeq500 instrument using 2 x 100 bp paired-end reads, following manufacturer's instructions. Raw read quality was evaluated using FastQC before reads were trimmed for adaptors and sequence quality. Trimmed reads were aligned to human GRCh38.84 reference genome using STAR RNA-seq aligner. Aligned reads were quantified for each gene. Aligned data were evaluated for quality using several quality metrics (e.g. mapping rate, coverage) and visually inspected for samples deviating from the population across multiple metrics and PCA. Statistical analysis of RNA-seq data was performed in R (version 3.5.1).

Differential gene analysis was performed with edgeR package to normalise and identify differentially expressed genes (DEGs) from counts data. Transcripts with zero counts in more than two-thirds of the samples were discarded from downstream analysis to reduce noise in the expression data. Gene counts were converted to log₂ counts per million (cpm), quantile normalised, and precision weighted. RNA-seq gene features were considered differentially expressed if they satisfied a 2-fold change and false discovery rate (FDR) <0.05 cutoff. FDR control was performed with the Benjamini-Hochberg procedure. Heatmaps and Volcano Plots were generated in R with the heatmap.2 and

Enhanced Volcano functions in the gplots package. Hierarchical clustering was performed with Ward's linkage. Ingenuity Pathway Analysis (IPA) was used to identify canonical pathways overrepresented within DEG.

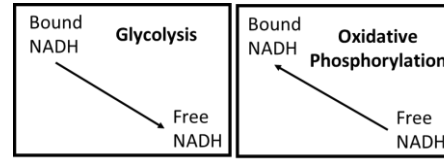
3.3.6 Two-photon Excited Fluorescence Lifetime Imaging (FLIM) of NAD(P)H

In order to assess the metabolic profile of synovial tissue macrophages, we utilised the non-invasive FLIM technique which allows for discrimination between glycolysis and oxidative phosphorylation in live cells. Due to the difficulty in isolating a sufficient number of synovial tissue macrophages for standard metabolic assays such as the Seahorse Flux analyser as outlined in Chapter 2, in this chapter we use FLIM as a metabolic assay as it requires fewer cells. Autofluorescence originating from excited NAD(P)H can serve as a useful redox marker which can be measured non-invasively using two-photon excited FLIM. Cells exhibit mixed pools of enzyme bound NADH and free NADH which have differential emission lifetimes; >1.5 ns and ~400 ps respectively. This enables discrimination between oxidative phosphorylation and glycolysis in cells with free NADH, indicative of glycolysis, and protein bound NADH, a feature of OXPHOS. The principle of this mechanism is extensively outlined in (Okkelman *et al.*, 2019).



Following FACS sorting, CD206+CD163+ and CD206-CD163- macrophages were seeded into Ibidi μ -Slides at a density of 1,000 cells per well in phenol-free RPMI and left to rest overnight. FLIM was performed using a custom upright (Olympus BX61WI) laser multiphoton microscopy system equipped with a Titanium:sapphire laser (Chameleon Ultra, Coherent®, USA), a water-immersion 25x objective (Olympus, 1.05NA) and a temperature controlled stage. The NADH lifetime average (τ_{avg}) was calculated using the following formula:

$$\tau_{avg} = (\tau_1 * \alpha_1) + (\tau_2 * \alpha_2)$$



Lifetimes are indicated by τ_1/τ_2 and the contributions are indicated by α_1/α_2 . The short fluorescence lifetime τ_1 and fraction α_1 relate to free NAD(P)H, while long fluorescence lifetime τ_2 and fraction α_2 correspond to protein bound NAD(P)H. When τ_{avg} is increased, oxidative phosphorylation is dominating, when it is decreased, the cells are preferentially using glycolysis.

3.3.7 Measurement of Pro-inflammatory Cytokines

To assess the release of cytokines from CD206+CD163+ and CD206-CD163- synovial tissue macrophages, sorted macrophages were seeded at a density of 30,000 cells per well of a 48 well plate and cultured overnight. Culture supernatants were then collected and protein concentrations of IFN γ , IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13 and TNF α were measured by multiplex cytokine panel V-PLEX Pro-inflammatory assay (Meso Scale Discovery, USA) according to the manufacturer's instructions. Electrochemiluminescence was measured using the MSD Sector Imager 2400. Cytokine standards ranged from 2 – 10,000 pg/ml.

3.3.8 Autologous Macrophage T cell Co-culture

To further examine the functional role of CD206+CD163+ macrophages in the joint, autologous co-culture experiments with T cells were performed. In addition to antigen presentation, macrophages secrete chemokines that can attract T cells into the joint while macrophage cytokine production can direct T cell polarisation towards Th1/Th17 (Roberts, Dickinson and Taams, 2015). Therefore, an autologous macrophage – T cell co-culture system was conducted to examine the effect of CD206+CD163+ macrophages on T cell activation.

CD206+CD163+ macrophages were sorted from RA synovial tissue along with CD3+ T cells from the same donor. T cells were cultured with CD206+CD163+ macrophages for 5 days

in a ratio of 1:3 in an autologous co-culture system after which T cell cytokine production was assessed by flow cytometry. Following 5 days of co-culture, CD3+ T cells were re-stimulated with PMA (50 ng/mL) and Ionomycin (500 ng/mL) in the presence of Brefeldin A (5 µg/mL) for 5 hours at 37°C. Cells were stained extracellularly for CD3, CD4 and CD8 in addition to Live/Dead Infrared viability dye before being stained intracellularly with antibodies specific for GM-CSF, TNFα, IL-17 and IL-4 and analysed by flow cytometry using the BD LSRFortessa.

Antibody	Fluorochrome	Clone
CD3	BV510	OKT3
CD4	PeCF594	RPA-T4
CD8	PE-CY5	HIT8a
CD161	PE-CY7	HP-3G10
TNFα*	PerCPCY5.5	MAb11
IL-17*	A488	BL168
IL-4*	BV711	MP4-25D2
GM-CSF*	PE	BVD2-21C11

* Indicates intra-cellular stains

Table 3.2: Panel of fluorochrome antibodies used for flow cytometry analysis.

3.3.9 Statistical Analysis

Statistical analyses were performed using Prism 5 software. Wilcoxon Signed Rank test or Mann Whitney was used for analysis of non-parametric data. Student *t*-test was used for parametric data. *p*-values of less than 0.05 (**p*<0.05) were determined as statistically significant.

3.4 Results

3.4.1 Identification of macrophages in RA synovial tissue and fluid

Macrophages are functionally diverse cells that display high plasticity to accomplish their many divergent roles. In this study, we aim to identify specific macrophage phenotypes within the RA synovium, an area which remains largely unexplored. To define the spectrum of macrophage phenotypes that exist within the synovium of RA patients, whole synovial tissue biopsies were obtained at time of knee arthroscopy and digested to yield a single cell suspension (Table 3.3). In addition, SFMC were isolated from RA synovial fluid. Synovial tissue and SFMC were stained using a panel of human macrophage polarisation associated cell surface markers (CD45, CD40, CD68, CD64, CD163, CD206 and CD253) to assess the phenotype and frequency of macrophages.

Following the exclusion of dead cells and doublets, macrophages were identified based on the positive expression of CD45 and positive expression of pan macrophage markers CD68 and CD64 (Figure 3.1 (A)-(E)) and then further analysed based on the co-expression of M1-like markers (CD40 and CD253) (Figure 3.1 (F)) and co-expression of M2-like markers (CD206 and CD163) (Figure 3.1 (G)). Interestingly, macrophages within the RA synovium, in the synovial tissue and the synovial fluid, express markers typical of both M1 and M2 macrophages (Figure 3.1-3.2). Analysis of the frequency and median fluorescence intensity (MFI) of macrophage markers from 9 independent RA synovial tissues and synovial fluids are demonstrated by representative dot plots (Figure 3.3). Quantification of the frequency and MFI of pan macrophage markers CD68 and CD64, (CD64 and CD68 macrophages are identical so both are used as pan markers) were significantly increased in the synovial tissue compared to fluid, indicating that a greater number of macrophages reside in synovial tissue in comparison to the synovial fluid (Figure 3.3 (A-B), $p < 0.05$).

Characteristic	Mean	Percentage or Range
<i>Age, years</i>	58.4	38-77
<i>Gender, female</i>	5	31%
<i>RF Positive</i>	7	44%
<i>ACPA positive</i>	8	50.0%
<i>Disease duration, years</i>	10	<1-41
<i>DAS28</i>	3.51	1.68-6.01
<i>CRP (mg/L)</i>	16.8	<1-53.6
<i>On MTX</i>	7	43.75%
<i>NSAIDs</i>	0	0.00%
<i>Treatment naïve</i>	6	38%

Table 3.3: Patient demographics RA synovial tissue biopsies

Patient characteristics for RA synovial tissue biopsies used for macrophage phenotyping studies (n=16). Values reflect either the group average or the range in each group with given criteria. RF: Rheumatoid Factor, ACPA: Anti-Citrullinated Protein Antibody, DAS: Disease Activity Score, CRP: C-Reactive Protein, MTX: Methotrexate, NSAIDs: Nonsteroidal Anti-Inflammatory Drugs.

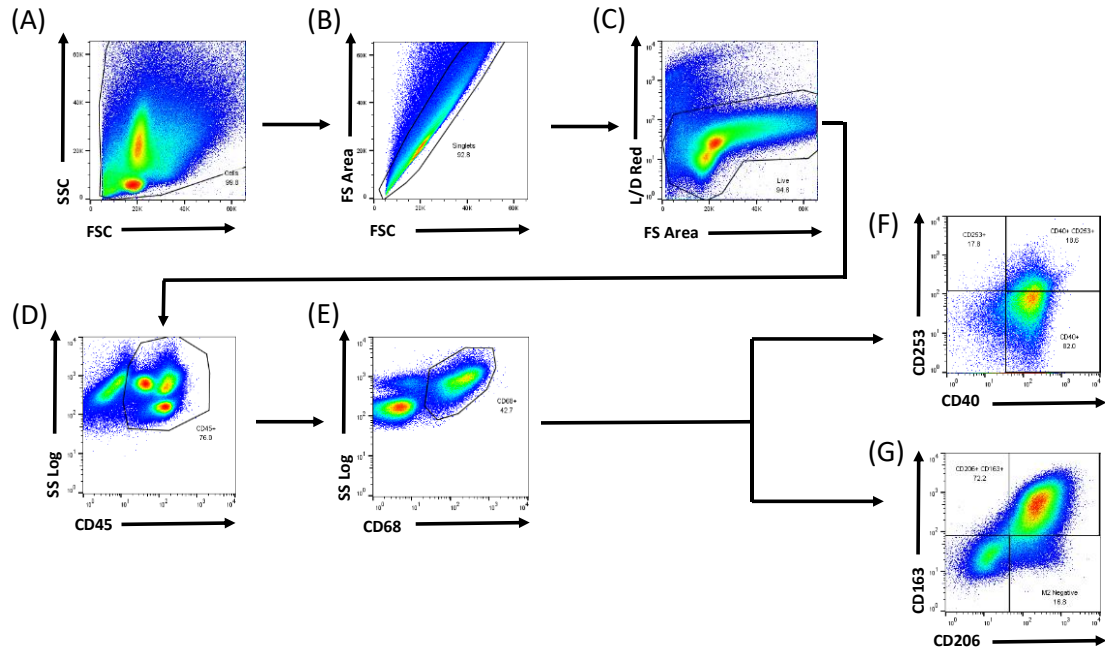


Figure 3.1: CD68/CD64+ synovial tissue macrophage gating strategy.

RA synovial tissue single cell suspensions were stained with a panel of fluorochrome antibodies (CD40, CD45, CD64, CD68, CD163, CD206, CD253). Representative dot plots depicting the gating strategy used to identify and phenotype synovial tissue macrophage subsets. The forward and side scatter parameters of cells were set before doublet exclusion and elimination of dead cells (A-C). Macrophages were then identified as CD45+CD68+CD64+ (D-E) and further characterised into distinct populations based on expression of specific macrophage markers. CD68+/CD64+ synovial macrophages display markers typical of both (F) M1 (CD40+CD253+) and (G) M2 (CD206+CD163+) macrophages.

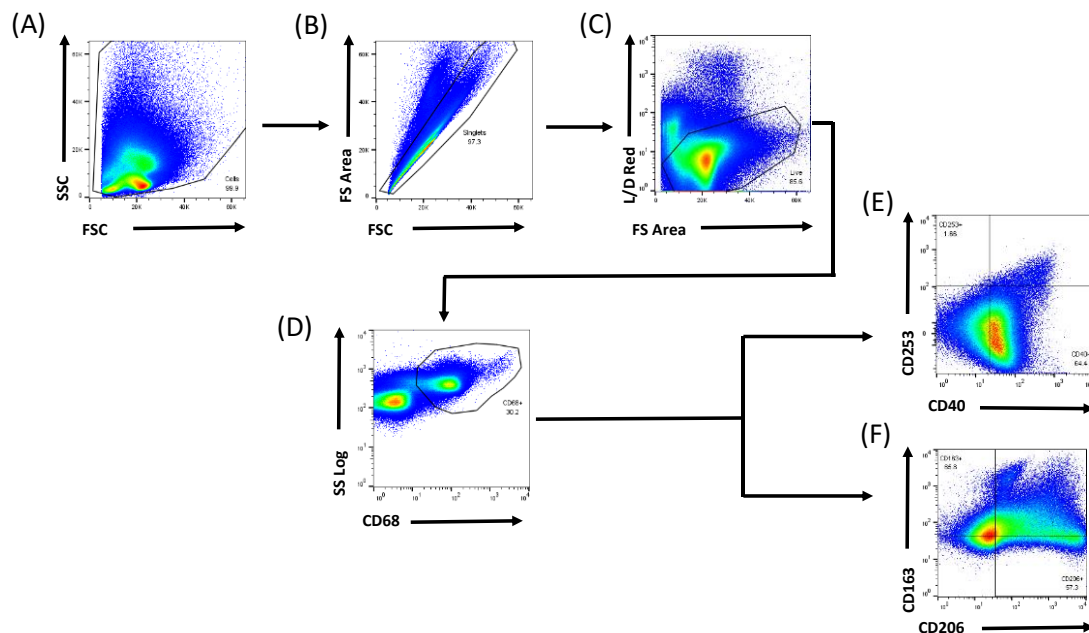
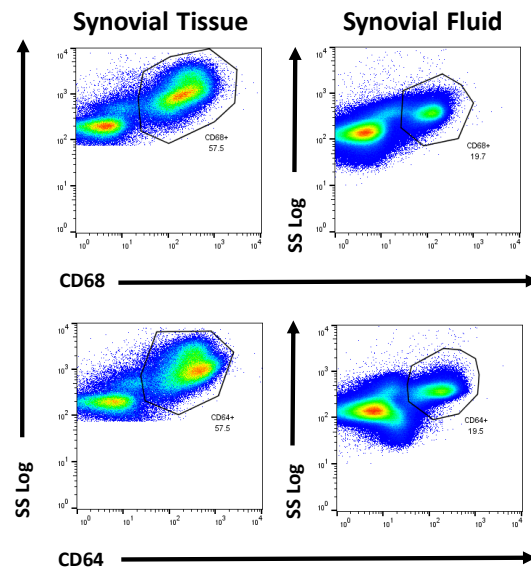


Figure 3.2: CD68/CD64+ synovial fluid macrophage gating strategy.

RA synovial fluid mononuclear cells were stained with a panel of fluorochrome antibodies (CD40, CD64, CD68, CD163, CD206, CD253). Representative dot plots depicting the gating strategy used to identify and phenotype synovial fluid macrophage subsets. The forward and side scatter parameters of cells were set before doublet exclusion and elimination of dead cells (A-C). Macrophages were then identified as CD68+/CD64+ (D) and further characterised into various populations based on expression of specific macrophage markers. Synovial fluid macrophages display markers typical of both (E) M1 (CD40+CD253+) and (F) M2 (CD206+CD163+) macrophages.

(A)



(B)

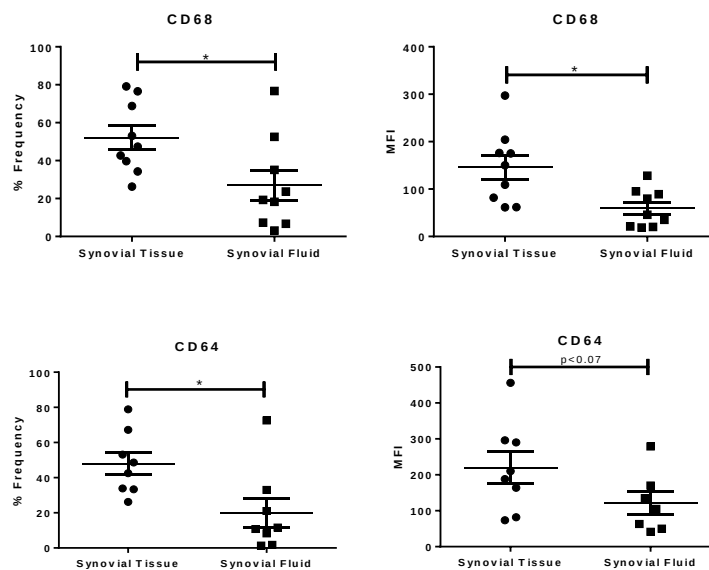


Figure 3.3: Analysis of CD68 and CD64 macrophages in RA synovial tissue vs fluid.

(A) Representative dot plots indicating the differences in the expression of CD68 and CD64 in RA synovial tissue cell suspension and synovial fluid. (B) Percentage frequency and median fluorescence intensity (MFI) of pan macrophage markers CD68 and CD64 in RA synovial tissue (n=9) compared to RA synovial fluid (n=9). Gates were set according to FMO controls. Data represented as mean $\pm$ SEM with each symbol representing a different sample. Statistical analysis was performed using Mann Whitney U test *p<0.05 significantly different from synovial tissue.

3.4.2 Differential macrophage marker expression in RA synovial tissue compared to synovial fluid

The presence of differential macrophage phenotypes becomes more apparent when frequencies of specific macrophage markers are assessed in synovial tissue compared to synovial fluid. The frequency of macrophages expressing the markers CD206, CD163 and CD40 are significantly elevated in RA synovial tissue compared to synovial fluid ($p < 0.05$), with comparable levels of CD253 observed as indicated by dot plots (Figure 3.4 (A)). This is further supported by representative histograms for single stain marker expression, where increased expression of CD206, CD163 and CD40 in synovial tissue compared to fluid was observed (Figure 3.4 (B)).

As described in Chapter 2, macrophages are generally characterised as either classical M1 macrophages involved in driving pro-inflammatory responses or alternatively activated M2 macrophages associated with resolution of inflammation and wound repair. Thus, synovial CD68+ macrophages were characterised into 'M1 like' indicated by double positive expression of CD40 and CD253, or 'M2 like' indicated by double positive expression of CD206 and CD163 to assess the expression of the two major poles of macrophage activation states.

Representative dot plots demonstrate similar expression of M1 markers CD40+CD253+ with comparable mean frequencies in synovial tissue and fluid (Figure 3.5 (C-D)). In contrast, a marked increase in expression of M2-like CD206+CD163+ macrophages were observed in synovial tissue, where the mean frequencies of this population in synovial tissue was 63.5% compared to synovial fluid which was 15.3%. This marked difference in frequency of CD206+CD163+ macrophages was consistent across 9 independent synovial tissues and fluids examined, thus suggesting a role for site-specific macrophage activation states.

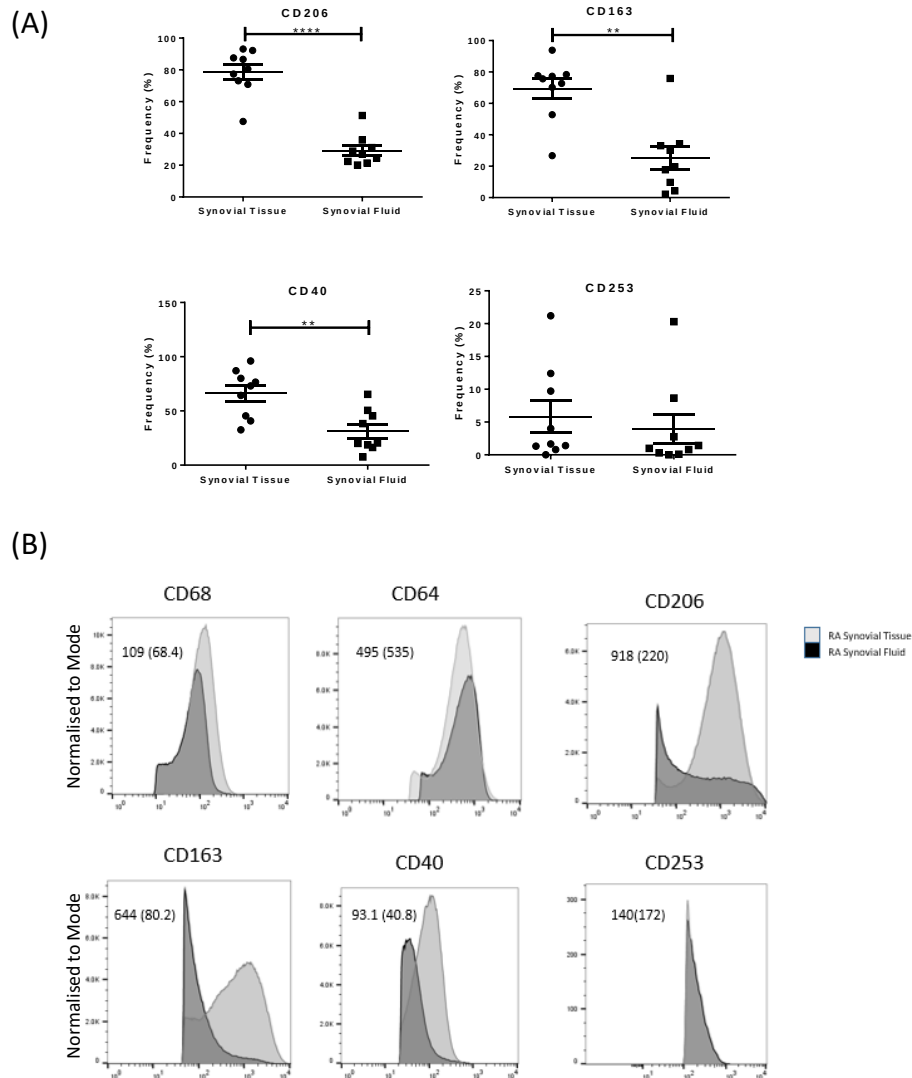


Figure 3.4: Phenotypic characterization of CD68+ RA synovial tissue macrophages compared to synovial fluid.

(A) Frequency of single positive CD206, CD163, CD40 and CD253 on RA CD68+ synovial tissue cell suspensions (n=9) compared to synovial fluid (n=9). Gates were set according to FMO controls. Data represented as mean $\pm$ SEM of 9 individual experiments, each symbol representing a different sample. Statistical analysis was performed using Mann Whitney U test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ significantly different from synovial tissue.

(B) Representative histograms of tissue compared to fluid with median fluorescence intensity (MFI) indicated as either outside or within parentheses, respectively.

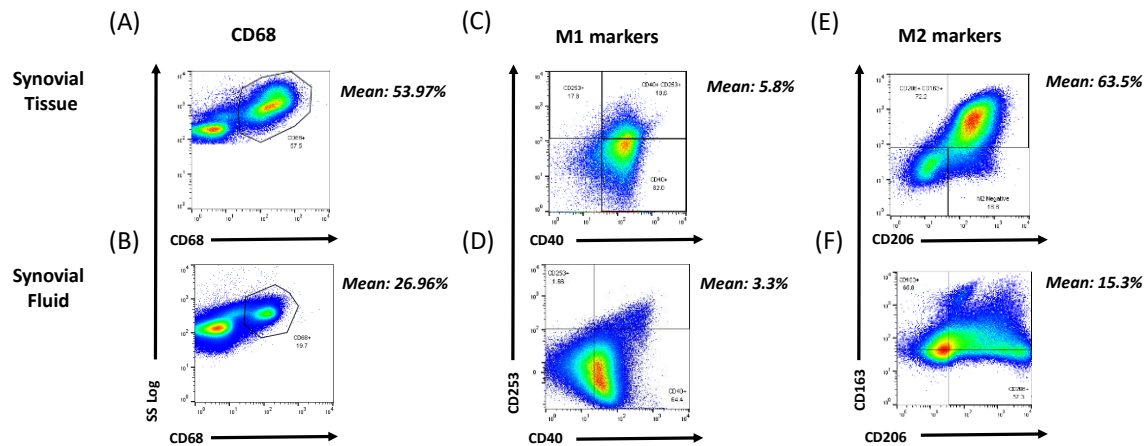


Figure 3.5: Identification of specific CD68+ macrophage subsets the RA synovium.

Representative flow cytometric dot plots for the identification of macrophage subsets in the RA synovium. (A-B) Representative dot plots indicating expression of CD68, (C-D) double positive CD40+CD253+ (markers typical of M1 macrophages) and (E-F) double positive CD206+CD163+ (markers typical of M2 macrophages) in RA synovial tissue and RA synovial fluid. Overall mean percentage frequencies of CD68+, CD40+CD253+ and CD206+CD163+ expression in 9 independent samples with representative shown.

3.4.3 A dominant subset of macrophages exists within a spectrum of synovial tissue macrophages

In the inflamed microenvironment of the joint *in vivo*, macrophages are exposed to a plethora of stimuli, and perhaps don't fully conform to the binary M1/M2 framework. Therefore, we examined both M1/M2 and non-M1/M2 macrophage phenotypes within RA synovial tissue. Analysis of specific macrophage markers identified 7 phenotypically distinct macrophage subpopulations, reflecting more of a spectrum rather than the classic paradigm of M1 and M2 (Figure 3.6 (A)). Based on 9 independent RA synovial tissue samples, we identified for the first time that the dominant population of CD68+ macrophages in the RA synovium express high levels of CD206 and CD163, markers typical of an M2 phenotype (Figure 3.6 (B)). Interestingly however, we note that this double positive phenotype also express high levels of the activation marker CD40 (Figure 3.6 (C)). We therefore hypothesised that this macrophage population are an intermediate or transitional subtype of tissue-specific macrophages, which do not adhere to the traditional dichotomous model of M1 vs M2.

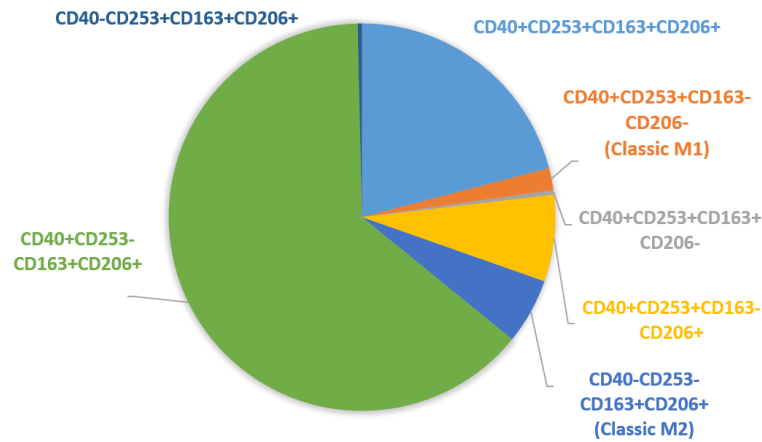
A significant decrease in the frequency of CD206+CD163+ macrophages was demonstrated in RA synovial fluid compared to RA synovial tissue (Figure 3.7 (A), $p < 0.05$). In contrast, the double negative CD206-CD163- subset of macrophages are significantly enriched in synovial fluid in comparison to tissue (Figure 3.7 (A), $p < 0.05$), thus, further highlighting the presence of site-specific macrophage phenotypes.

The dominant macrophage subset of double positive CD206+CD163+ macrophages co-expressing CD40 are specifically enriched in the synovial tissue compared to fluid, with frequency and MFI of CD206+CD163+CD40+ significantly reduced in RA fluid (Figure 3.7 (B), $p < 0.05$). This is further supported by the representative histogram of CD40 expression on the CD206+CD163+ subset in FMO control, fluid and tissue (Figure 3.7 (C)). Representative flow cytometry dot plots demonstrate a shift in CD40 expression from the fluid (mean frequency: 33.7%) to synovial tissue (mean frequency: 82.3%), Figure 3.7 (D).

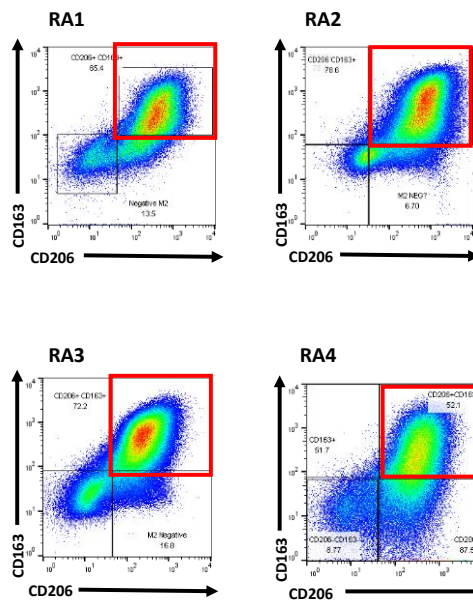
Multiparameter flow cytometry and utilising SPICE analysis facilitated the identification of various synovial macrophage phenotypes. While SPICE analysis visually reveals striking differences in synovial tissue macrophages compared to fluid, it further

confirms that the CD206+CD163+CD40+ macrophage phenotype is dominant and unique to synovial tissue. This population is indicated by the deep red pie segment which is noticeably larger in synovial tissue compared to fluid. In addition, the triple expression of CD206 (light blue arc), CD163 (green arc) and CD40 (red arc) is substantially increased in tissue compared to fluid (Figure 3.8).

(A)



(B)



(C)

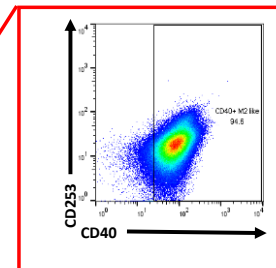


Figure 3.6: Dominant macrophage subset identified in RA synovial tissue.

(A) Pie chart depicting 7 phenotypically distinct macrophage subsets in the RA synovial tissue. (B) Further analysis of RA synovial tissue macrophages revealed the presence of a dominant macrophage population. The dominant macrophage population in the RA synovium are the double positive CD206+CD163+ subset as demonstrated by representative flow cytometric dot plots from 4 different RA patients. (C) This dominant population also expresses high levels of the activation marker CD40.

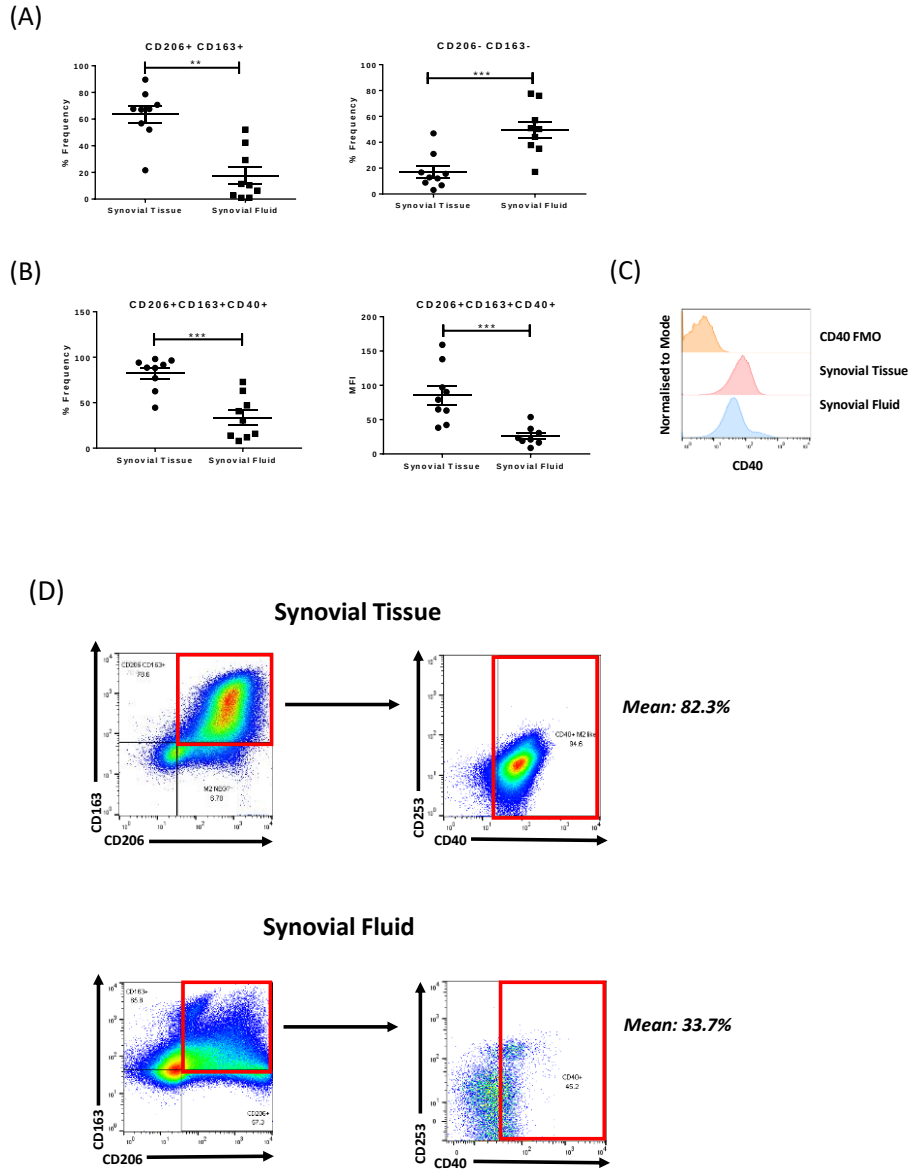


Figure 3.7: Dominant macrophage population enriched in synovial tissue compared to fluid.

(A) Dot plots indicating the significant increase in expression of double positive CD206+CD163+ macrophages in RA synovial tissue (n=9) in comparison to synovial fluid (n=9), with the double negative CD206-CD163- population significantly increased in fluid compared to tissue. (B) Dot plots indicating a significant increase in percentage frequency and MFI of CD40 on the dominant CD206+CD163+ macrophage subset in synovial tissue compared to fluid. (C) Representative histogram of CD40 expression on the double positive CD206+CD163+ macrophage subset in RA synovial tissue, synovial fluid and FMO control. (D) Mean percentage frequency of CD206+CD163+CD40+ macrophage subpopulation in indicated samples (n=9) with representative dot plots shown. Data represented as mean $\pm$ SEM with each symbol representing a different sample. Statistical analysis using Mann Whitney U test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, significantly different from synovial fluid.

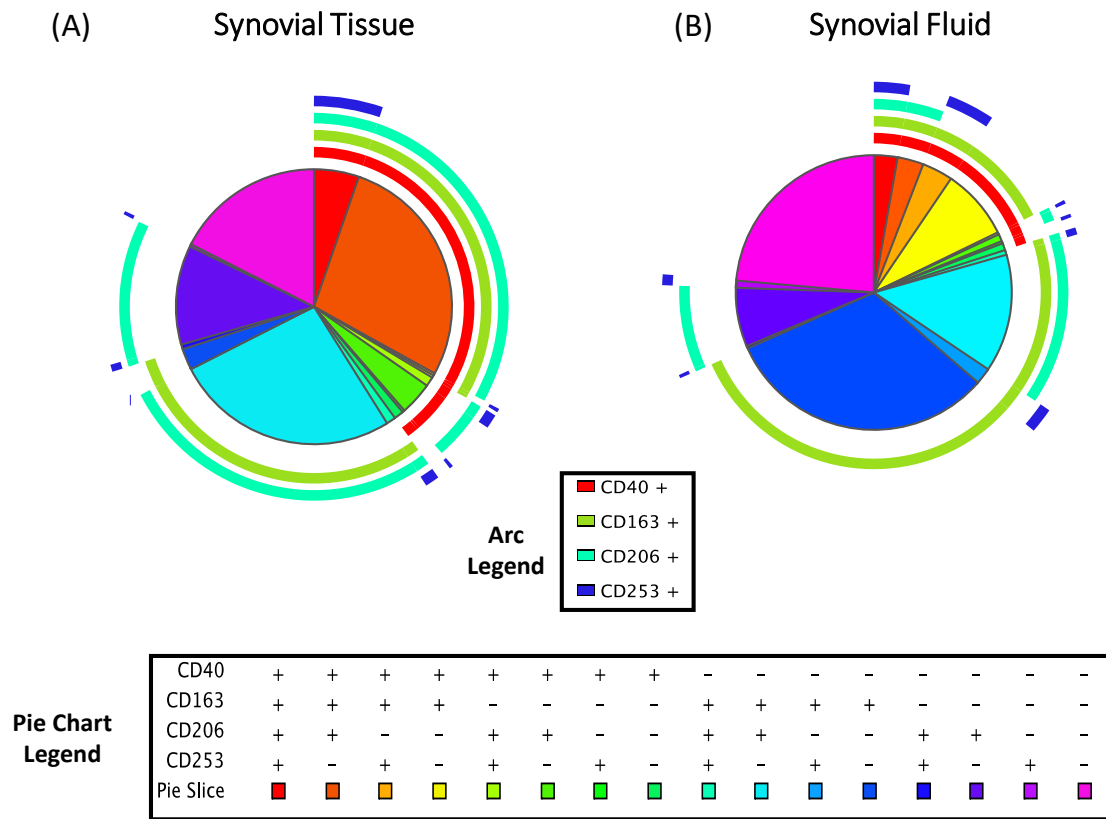


Figure 3.8: Distinct macrophage subsets in RA synovial tissue compared to fluid.

Visual representation of multidimensional flow cytometric data. SPICE analysis was performed for the identification of distinct macrophage subsets in an average of (A) RA synovial tissue (n=9) and (B) fluid (n=6). Each pie segment indicates the different combinations of marker expression as denoted by the legend below. The surrounding pie arcs indicate the specific macrophage markers produced by each pie segment.

3.4.4 The dominant macrophage population in RA synovial tissue is associated with high disease activity

Next we stratified patient samples into high (>2.6) vs low (<2.6) RA disease activity scores (DAS28). Expression of pan macrophage marker CD64 is elevated in patients with high DAS scores (Figure 3.9 (A)). Interestingly, although disease activity was not strongly associated with the double positive CD206+CD163+ macrophage subset alone (Figure 3.9 (B)), when co-expressing CD40, this dominant macrophage population is significantly increased in RA synovial tissue from patients with high disease activity (Figure 3.9 (C), $p<0.05$). In addition, the frequency of the CD206+CD163+CD40+ subset significantly correlates with DAS28 scores ($r=0.6$, $p<0.05$) (Figure 3.9 (C)) supporting their pathogenic role in RA. This is further confirmed in representative flow cytometry dot and histogram plots of CD206+CD163+CD40+ macrophages in patients with high and low disease activity (Figure 3.9 (D)). Taken together, these data indicate a key role of this dominant population in driving RA disease activity and severity.

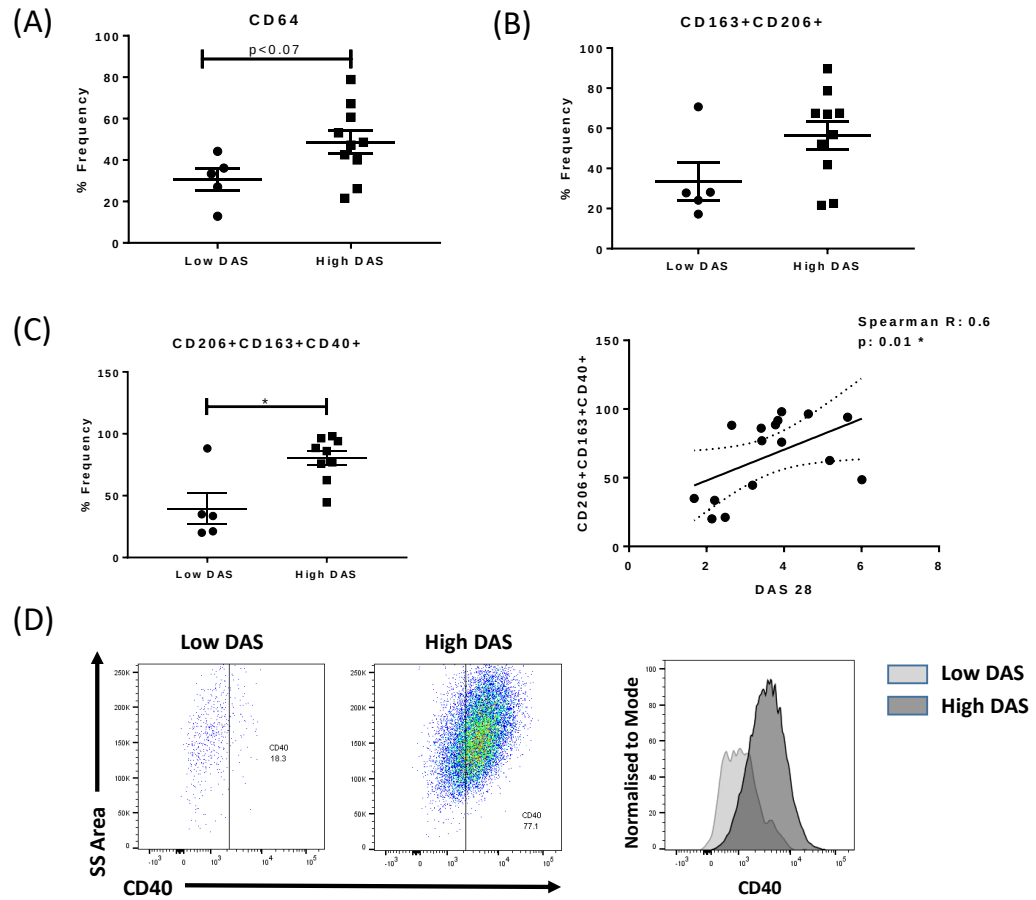


Figure 3.9: Dominant synovial tissue macrophage population is associated with high RA disease activity.

Analysis of (A) CD64 marker expression, (B) double positive CD206+CD163+ and (C) the dominant macrophage subset CD206+CD163+CD40+ based on RA disease activity scores (DAS28). Low DAS indicates a DAS28 score of less than 2.6 with high DAS based on scores of greater than 2.6. (D) Representative flow cytometry dot plots and histogram of CD40 expression on double positive CD206+CD163+ macrophage population in high vs low disease activity. Data represented as mean $\pm$ SEM, with each symbol representing a different sample. Statistical analysis using Mann Whitney U test, * $p < 0.05$ significantly different from synovial fluid.

3.4.5 The dominant macrophage population is enriched in synovial tissue, specifically RA synovial tissue

To investigate if the expression of macrophage subsets in RA synovial tissue is specific to RA or indicative of a general autoimmune state, synovial tissue biopsies were obtained from Osteoarthritis (OA) and Psoriatic Arthritis (PsA) patients undergoing knee arthroscopy. Initially, we demonstrate comparable numbers of macrophages residing in the joints of patients with OA, PsA and RA as indicated by representative dot plots, frequency and MFI of pan macrophage markers CD68 and CD64 (Figure 3.10). However, the composition of macrophage subsets and expression of macrophage polarisation markers, demonstrated distinct differences across disease states. Specifically, a stepwise significant increase in MFI of CD206, CD40 and CD253 in RA compared to PsA and OA tissue was observed (Figure 3.11 (A-B), $p < 0.05$).

When further characterised into 'M1-like' CD40+CD253+ and 'M2-like' CD206+CD163+ macrophage subsets, no obvious differences are observed with comparable mean frequencies between diseases states (Figure 3.11 (A), 3.12 (B)). Interestingly, when gating on CD40 expression, there is a marked enrichment of the dominant CD206+CD163+CD40+ population in RA synovial tissue compared to OA and PsA as demonstrated by significant stepwise increase in CD206+CD163+CD40+ frequency and MFI in RA compared to OA and PsA (Figure 3.12 (B), $p < 0.05$). This increased expression of CD40 on the double positive CD206+CD163+ subpopulation in RA synovial tissue is notably evident in representative dot plots and histogram (Figure 3.12 (C-D)).

Boolean gating and multiparameter flow cytometric analysis were then employed to allow SPICE analysis to be performed. Figure 3.13 highlights the different macrophage phenotypes present in RA tissue compared to OA and PsA. However, of significant interest is the clear stepwise increase in CD40 expression on double positive CD206+CD163+ (red arc) from OA to PsA to RA. Thus, we hypothesise that this dominant CD206+CD163+CD40+ macrophage population is not only significantly enriched in synovial tissue compared to fluid, but specifically RA synovial tissue.

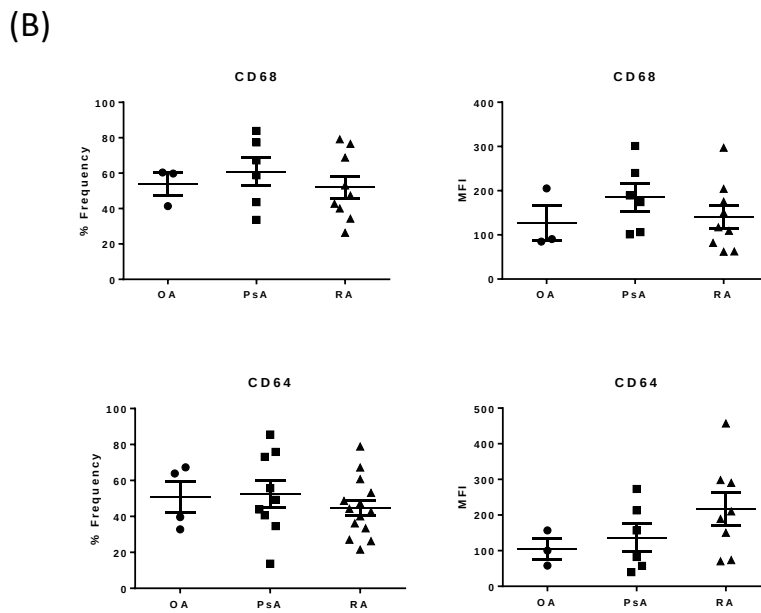
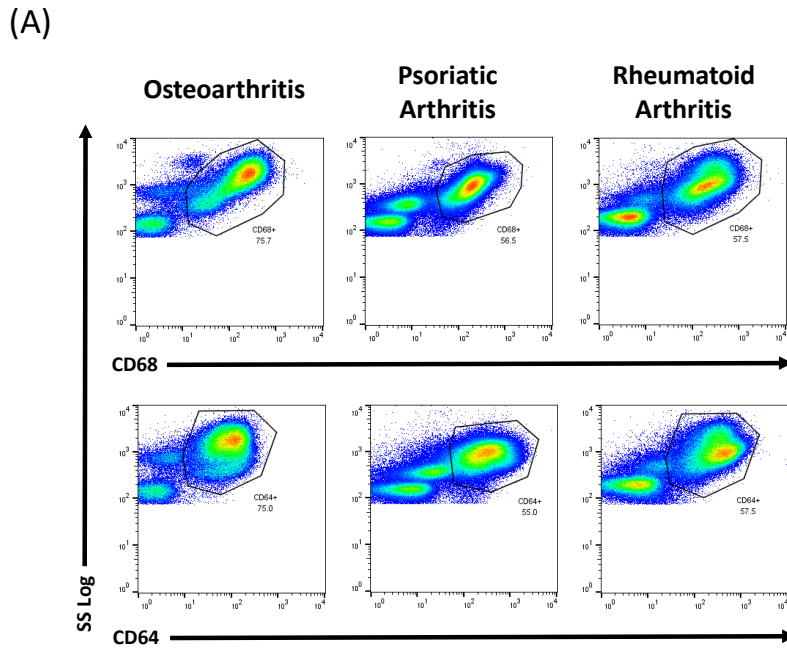


Figure 3.10: Expression of synovial tissue macrophages across disease states.

Synovial tissue biopsies were obtained from RA, Osteoarthritis (OA) and Psoriatic Arthritis (PsA) patients and stained using a panel of fluorochrome antibodies (CD40, CD45, CD64, CD68, CD163, CD206, CD253). (A) Representative flow cytometric dot plots depicting frequency of CD68 and CD64 in OA, PsA and RA synovial tissue. (B) Dot plots representing percentage frequency and MFI of pan macrophage markers CD68 and CD64 in OA (n=3-4), PsA (n=6-9) and RA (n=9-13) synovial tissue, with each symbol representing a different sample.

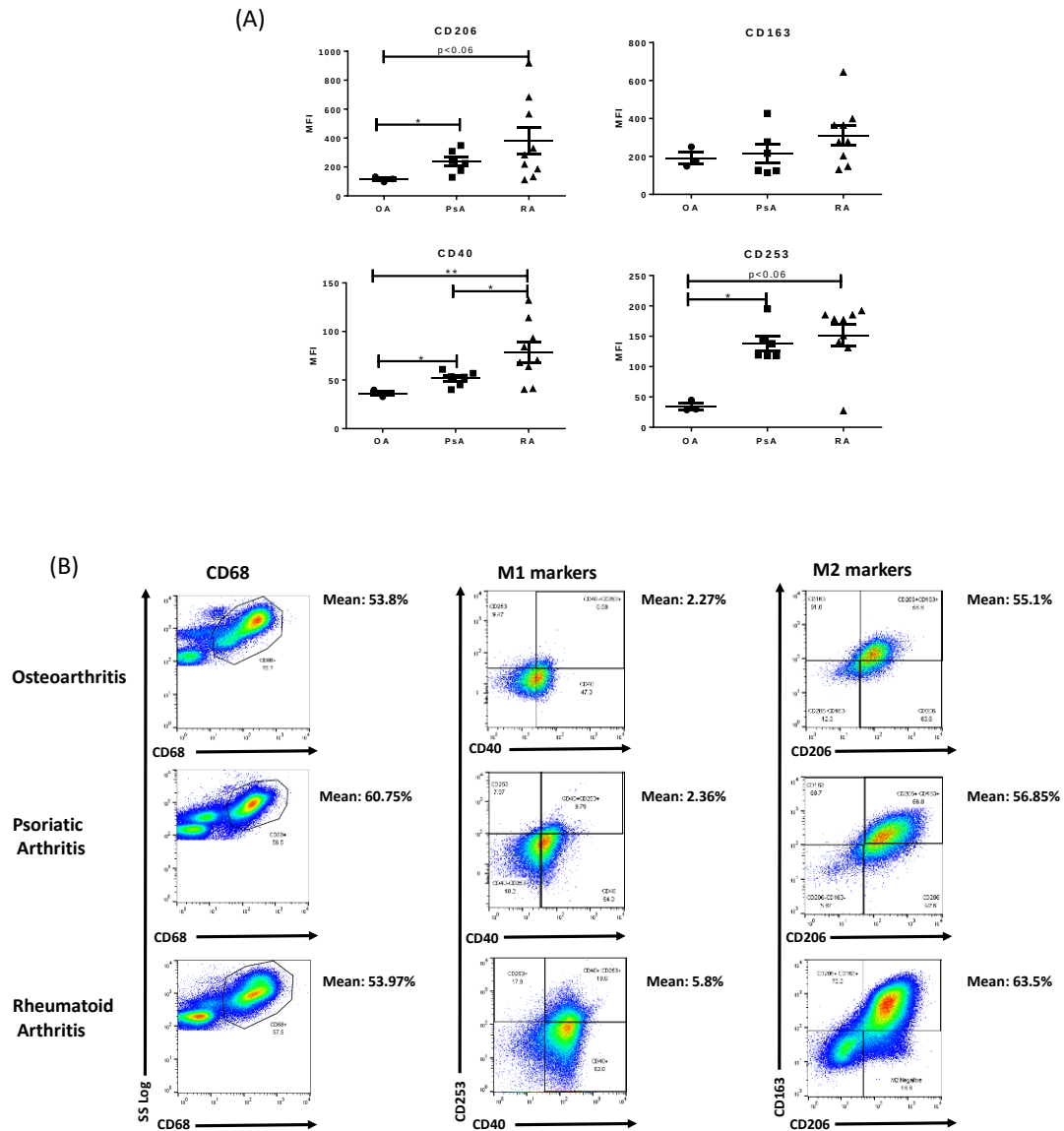


Figure 3.11: Macrophage marker expression differences in OA, PsA and RA tissue.

(A) Dot plots demonstrating the differences in MFI of macrophage markers CD206, CD163, CD40 and CD253 in OA (n=3), PsA (n=6) and RA (n=9) synovial tissue. Data represented as mean $\pm$ SEM with each symbol representing a different sample. Statistical analysis using Mann Whitney U test. * $p < 0.05$, ** $p < 0.01$ significantly different from the indicated diseases groups. (B) Representative flow cytometric dot plots indicating differences of macrophage subsets between OA, PsA and RA synovium. Expression of CD68, double positive CD40+CD253+ (markers typical of M1 macrophages) and double positive CD206+CD163+ (markers typical of M2 macrophages) in OA, PsA and RA synovial tissue. Overall mean percentage frequencies of CD68+, CD40+CD253+ and CD206+CD163+ expression indicated. Gates set according to FMO controls.

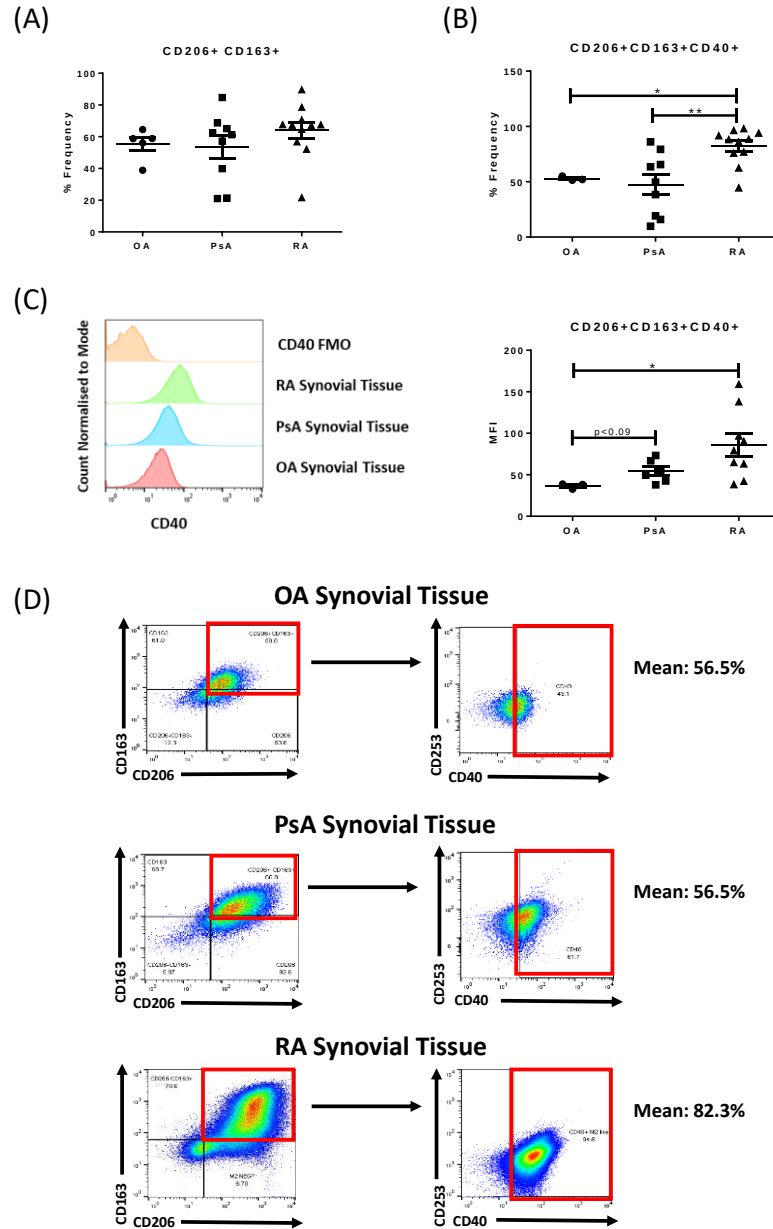


Figure 3.12: Dominant macrophage population enriched in RA synovial tissue specifically.

(A) Dot plots indicating the frequency of double positive CD206+CD163+ macrophages in OA (n=5), PsA (n=9) and RA (n=11) synovial tissue. (B) Dot plots indicating the frequency and MFI of CD40 expression on the dominant CD206+CD163+ macrophage subset in RA synovial tissue compared to OA and PsA disease comparators. (C) Representative histogram of CD40 expression on the double positive CD206+CD163+ macrophage subset in OA, PsA and RA synovial tissue and FMO control. (D) Overall mean percentage frequency of CD206+CD163+CD40+ macrophage subpopulation in indicated disease states. Data represented as mean $\pm$ SEM with each symbol representing a different sample. Statistical analysis using Mann Whitney U test, *p<0.05, **p<0.01 significantly different from indicated disease groups.

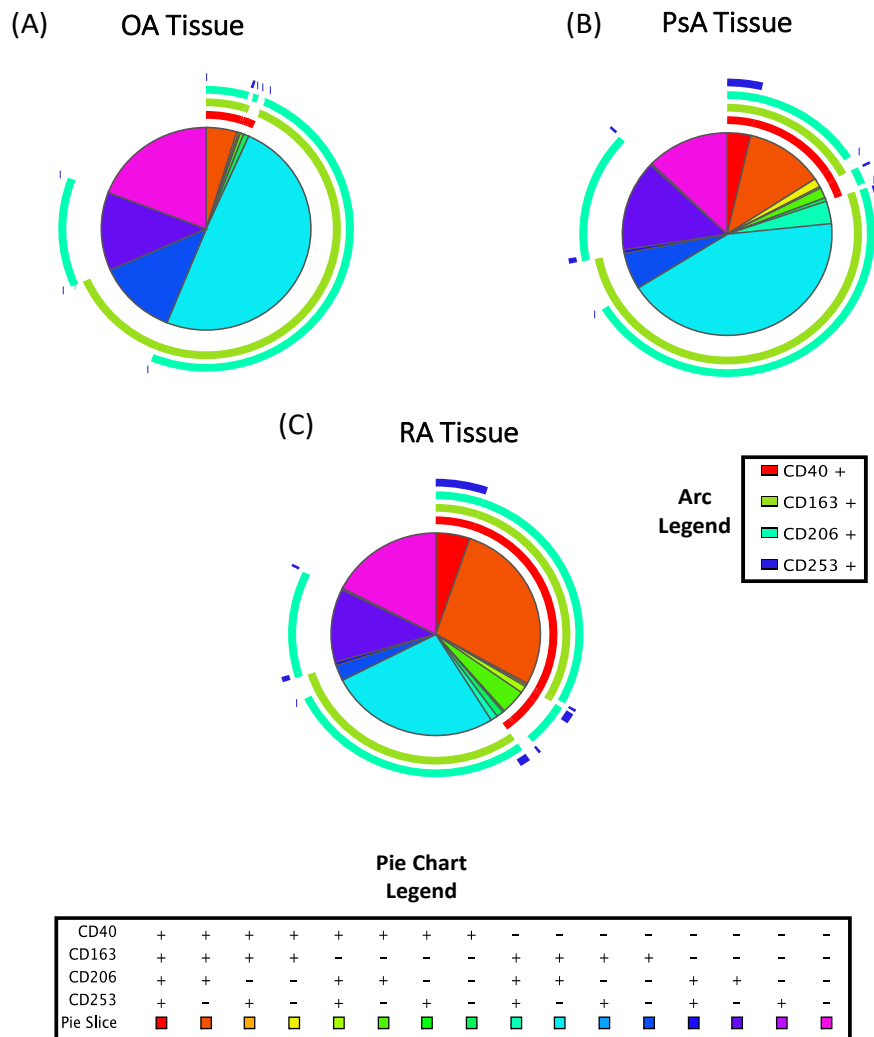


Figure 3.13: Distinct macrophage subsets in OA, PsA and RA synovium.

Visual representation of multidimensional flow cytometric data. SPICE analysis was performed for the identification of distinct macrophage subsets on an average of (A) OA (n=3) (B) PsA (n=6) and (C) RA (n=9) synovial tissue. Each pie segment indicates the different combinations of marker expression as denoted by the legend below. The surrounding pie arcs indicate the specific macrophage markers produced by each pie segment.

3.4.6 The dominant macrophage population enriched in RA synovial tissue is absent in healthy synovium

The identification of a prominent macrophage population primarily comprising of CD206+CD163+, markers typical of an M2 macrophage, in the RA synovium was not expected. M2 macrophages are generally associated with resolution of inflammation and homeostasis. However, this M2 macrophage population in the RA synovium also predominantly express high levels of the activation marker CD40, a marker typical of a pro-inflammatory M1 macrophage. Therefore, we next investigated whether this enriched synovial macrophage population is present in the healthy synovium.

Synovial tissue biopsies from 3 healthy individuals undergoing anterior cruciate ligament (ACL) reconstruction were obtained and assessed for the phenotype and frequency of macrophage subsets. Applying SPICE analysis, we observed a dramatic decrease in the diversity of macrophage subsets in healthy synovium compared to the RA diseased tissue. Specifically, the dominant population of macrophages in healthy synovium are the typically homeostatic M2 like CD206+CD163+ subtype as indicated by the light blue segment of the pie chart (Figure 3.14). Interestingly, co-expression of CD40 on these M2 like macrophages is completely absent in healthy synovial tissue (Figure 3.15 (A-B)). This is further emphasised by representative histogram and dot plots of CD40 expression in CD206+CD163+ macrophages from healthy and RA synovial tissue (Figure 3.15 (C-D)).

Hence, we propose that the double positive CD206+CD163+ macrophage subset dominates the synovium in the steady state, yet upon initiation of active RA disease, this subset transforms to become an activated transitional subset of macrophages capable of expressing high levels of inflammatory CD40.

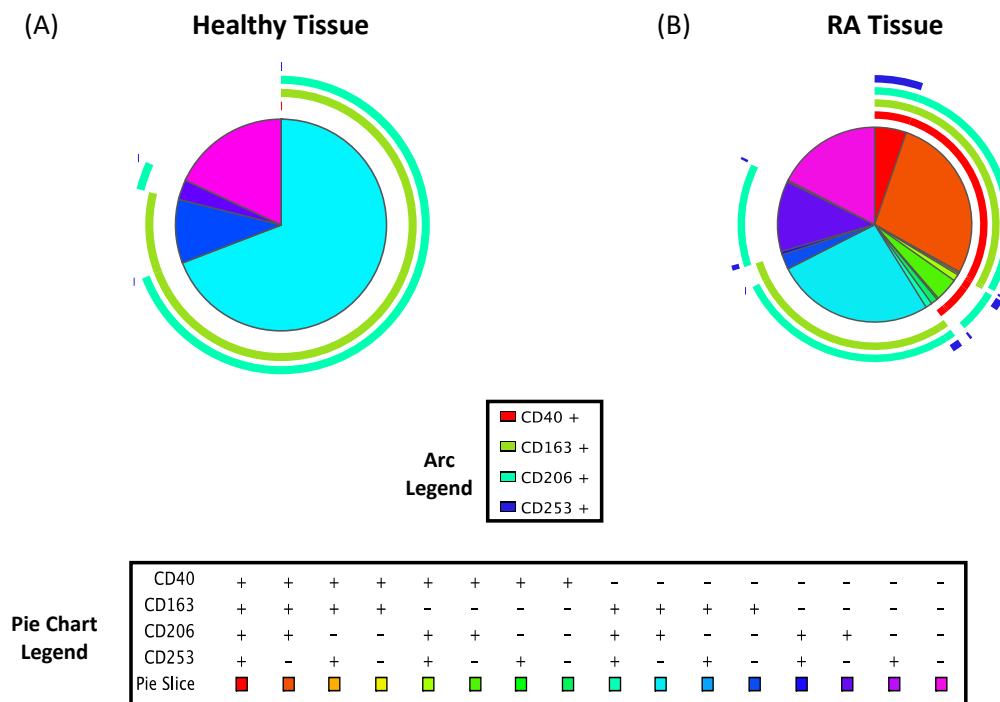


Figure 3.14: Differences in macrophage subsets present in RA and healthy synovium.

Synovial tissue was obtained from healthy control subjects undergoing anterior cruciate ligament (ACL) reconstruction surgery. Healthy synovial biopsies obtained from the knee at time of ACL surgery were digested and stained using a panel of fluorochrome antibodies (CD40, CD45, CD64, CD68, CD163, CD206, CD253). Visual representation of multidimensional flow cytometric data. SPICE analysis was performed for the identification of distinct macrophage subsets in an average of (A) healthy synovial tissue (n=3) and (B) RA synovial tissue (n=9). Each pie segment indicates the different combinations of marker expression as denoted by the legend below. The surrounding pie arcs indicate the specific macrophage markers produced by each pie segment.

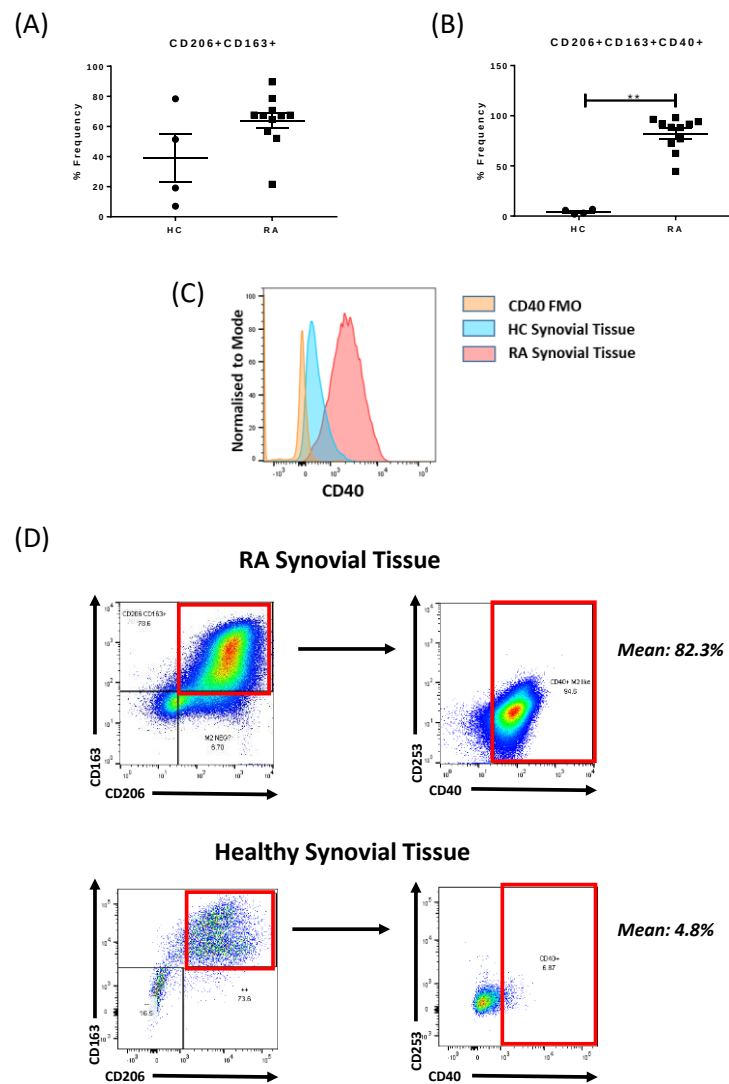


Figure 3.15: Dominant macrophage population enriched in synovial tissue is absent in healthy synovium.

(A) Dot plots indicating the expression of double positive CD206+CD163+ macrophages in healthy (n=4) and RA (n=11) synovial tissue. (B) Dot plots indicating the percentage frequency of CD40 expression on the dominant CD206+CD163+ macrophage subset in RA synovial tissue compared to healthy control tissue. (C) Representative histogram of CD40 expression on double positive CD206+CD163+ macrophage subset in healthy RA synovial tissue and FMO control. (D) Overall mean percentage frequency of CD206+CD163+CD40+ macrophage subpopulation in healthy and RA synovium. Analysis completed in 4 independent healthy tissues and 11 independent RA tissues with representative flow cytometric dot plots shown. Data represented as mean $\pm$ SEM with each symbol representing a different sample. Statistical analysis using Mann Whitney U test, **p<0.01 significantly different from healthy controls.

3.4.7 Analysis of chemokine receptor expression on synovial tissue macrophages

During the inflammatory process in arthritis, leukocytes extravasate through the vascular endothelium into the synovial tissue. This migration is coordinated by a complex network of synovial chemotactic mediators termed chemokines and their receptors. Chemokine receptors can also have functions other than leukocyte recruitment such as macrophage activation, innate immunity and immune regulation depending on the receptor expressed (Szekanecz *et al.*, 2010). Thus, expression of chemokine receptors on the surface of macrophages in the synovium may provide insights into the function of these cells.

Having identified the dominant macrophage population in RA synovium, we initially analysed the expression of specific chemokine receptors in OA, PsA and RA CD206+CD163+ synovial tissue macrophages. Interestingly, while CCR4 expression is comparable between OA and PsA synovial tissue, this expression is diminished in RA synovial tissue macrophages (Figure 3.16 (A)). In contrast, RA macrophages express CCR7, expression of which is decreased in a stepwise manner in PsA and OA tissue macrophages. CXCR1 and CXCR3 expression were similar between the 3 diseases pathotypes with a slight increase in CXCR1 expression in PsA synovial macrophages (Figure 3.16 (A)). This is further reinforced in representative flow cytometric dot plots and histograms of chemokine receptor expression on CD206+CD163+ macrophages from OA, PsA and RA synovial tissue in Figure 3.16 (B-C).

CCR7 is a characteristic marker of pro-inflammatory M1, while CCR4 is typically associated with an immunomodulatory M2 response (Imai *et al.*, 1999; Maolake *et al.*, 2017; Van Raemdonck *et al.*, 2019). The striking decrease in CCR4 on RA synovial tissue macrophages when compared with OA and PsA, coupled with expression of CCR7 compared to OA and PsA may indicate a pro-inflammatory role for RA CD206+CD163+ macrophages.

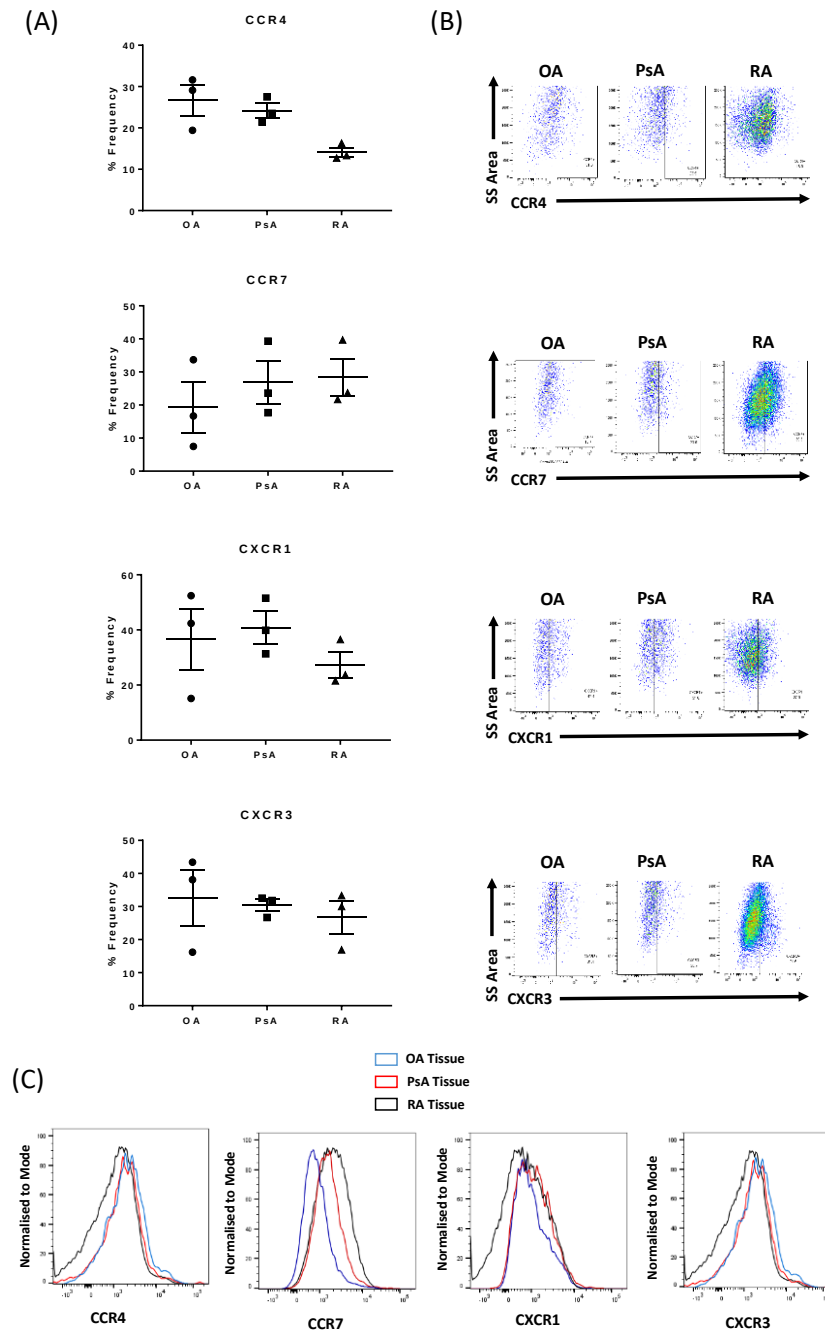


Figure 3.16: Chemokine receptor expression on CD206+CD163+ macrophage subset across diseases.

(A) Dot plots comparing the differences in percentage frequency of chemokine receptors CCR4, CCR7, CXCR1 and CXCR3 expressed on the surface of CD206+CD163+ synovial tissue macrophages in OA (n=3), PsA (n=3) and RA (n=3) patient samples. (B) Representative flow cytometric dot plots of expression of chemokine receptors on macrophages from synovial tissue of indicated disease states. (C) Representative histograms of indicated chemokine receptors expressed on CD206+CD163+ macrophages in OA, PsA and RA synovial tissue.

3.4.8 Expression of chemokine receptors on RA vs HC synovial tissue macrophages

Next we examined the differential chemokine expression on CD206+CD163+ macrophages residing in the synovium of healthy individuals compared to RA patients. Due to the rarity of obtaining healthy control synovial tissue samples along with limited cell yields (with diminished immune cell expression), this analysis is based on 2 healthy control tissues.

Directly comparing the expression of chemokine receptors on the CD206+CD163+ population between RA and healthy synovium, we noted some differences. Figure 3.17 (A-C) depicts the frequency of CCR4, CCR7, CXCR1 and CXCR3 in healthy vs RA synovial macrophages. CCR4 and CXCR1 are minimally expressed in RA macrophages, yet its expression is upregulated on healthy synovial macrophages (Figure 3.17 (A)). This is also clearly reflected in representative flow cytometric dot plots and histograms below (Figure 3.17 (B-C)). In contrast, expression of CCR7 is elevated in RA tissue macrophages compared to healthy controls (Figure 3.17 (A-C)). Comparable expression of CXCR3 was observed between healthy and RA CD206+CD163+ macrophages (Figure 3.17 (A-C)).

As previously stated, CCR4 is typically expressed on M2 macrophages. Upregulation of CXCR1 is also a classic characteristic of alternatively activated M2 macrophages (Gordon and Martinez, 2010; Wang, Liang and Zen, 2014). Therefore, expression of CCR4 and CXCR1 on healthy control CD206+CD163+ macrophages suggests a homeostatic immunoregulatory role for this macrophage population in the steady state. In contrast, CD206+CD163+ macrophages present in the RA synovium lose this homeostatic phenotype and instead upregulate CCR7, a characteristic marker of inflammatory macrophages. Taken together this would suggest that in homeostatic conditions, the dominant macrophage population is the CD206+CD163+ subset of macrophages expressing typical M2-like chemokine receptors; CCR4 and CXCR1. With the onset of RA disease pathogenesis however, this dominant subset, reacting to its surrounding microenvironment, transforms to acquire the activation marker CD40 and upregulates inflammatory CCR7 expression for further leukocyte infiltration.

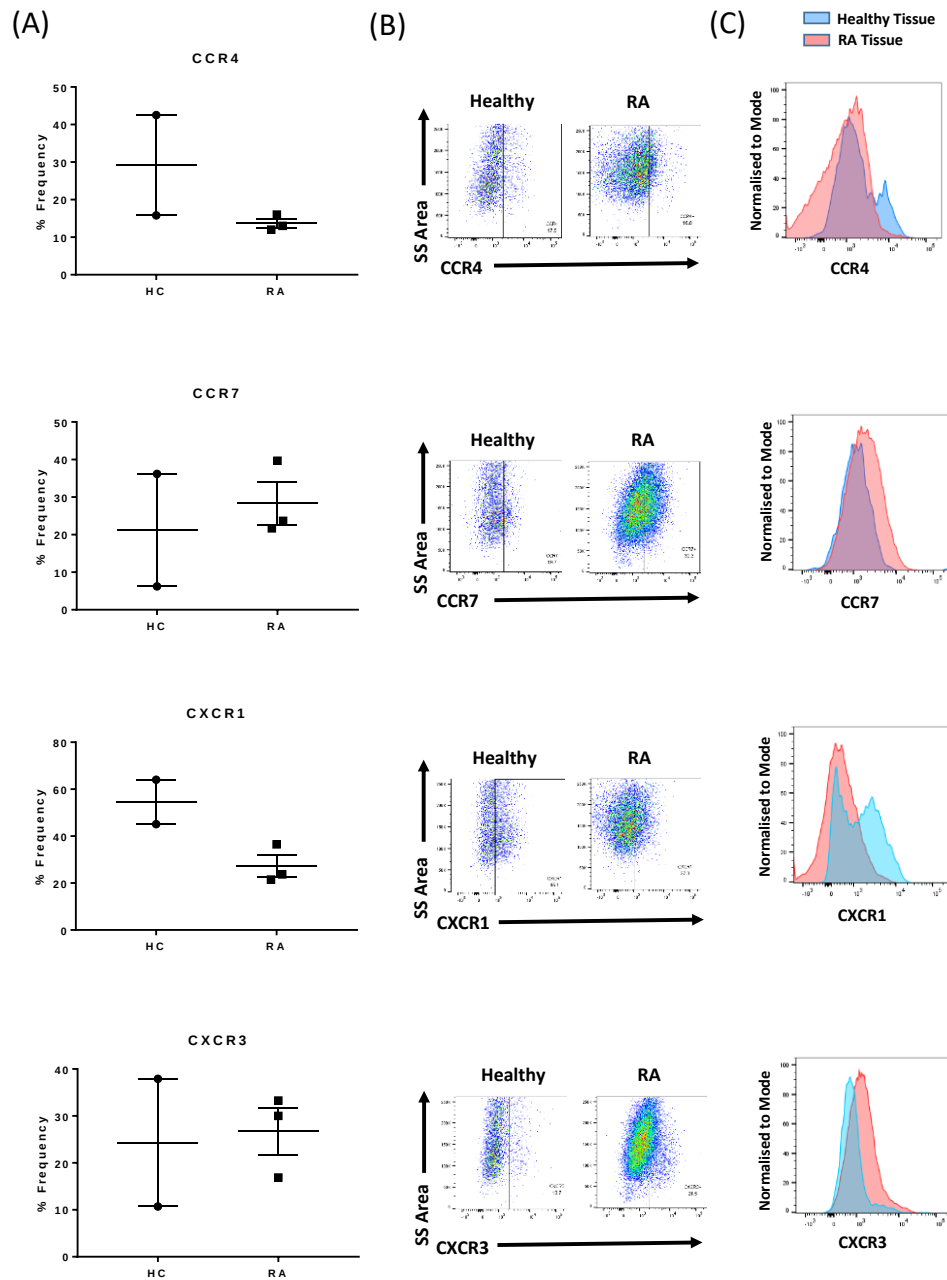


Figure 3.17: Chemokine receptor expression on CD206+CD163+ macrophage subset in RA vs healthy tissue.

(A) Dot plots comparing the differences in percentage frequency of chemokine receptors CCR4, CCR7, CXCR1 and CXCR3 expressed on the surface of CD206+CD163+ synovial macrophages in healthy (n=2) and RA (n=3) tissue. (B) Representative flow cytometric dot plots of expression of CCR4, CCR7, CXCR1 and CXCR3 on macrophages from synovial tissue of indicated disease states. (C) Representative histograms of indicated chemokine receptors expressed on CD206+CD163+ macrophages in healthy and RA synovial tissue.

3.4.9 Chemokine receptor expression distinguishes macrophages between RA synovial fluid and tissue

Although the CD206+CD163+ macrophage population is significantly decreased in RA synovial fluid compared to tissue (Figure 3.7), it is unclear whether this decrease is due to macrophages moving into the tissue from the fluid or whether the CD206+CD163+ macrophages are largely tissue-resident and therefore not present in the fluid.

We hypothesise that if the dominant RA synovial tissue CD206+CD163+ macrophage population are tissue-resident, expression of cell surface markers would differ between this double positive subset in the tissue and synovial fluid. Multiparameter flow cytometry coupled with Boolean gating allowed SPICE analysis to be performed. A marked increase in macrophage chemokine receptor expression on RA synovial tissue macrophages compared to synovial fluid was observed (Figure 3.18 (A-B)). As indicated by the pie segments, an increase in the frequency of macrophages expressing different chemokine receptors, in addition to their combination (pie arcs), was observed in RA synovial tissue compared to fluid.

Analysis of one paired RA synovial tissue and synovial fluid by unbiased, unsupervised, multidimensional analysis of flow cytometric data, allowed cell clustering to be performed using the tSNE algorithm. tSNE is an unsupervised algorithm that performs dimensionality reduction of complex data by clustering cells that have similar expression profiles (Belkina *et al.*, 2018). Therefore, tSNE can be used for data exploration in order to identify novel cell populations. It also helps with data visualization since cell clusters can be more clearly distinguished without the need for multiple “conventional” flow cytometry plots (Foley *et al.*, 2019).

Total CD64 cells were subjected to unsupervised clustering by tSNE algorithm following data concatenation. Data concatenation and relative expression for the indicated parameters are shown (Figure 3.19). On the basis of CD163 expression, two ‘clusters’ or populations of macrophages are identified (Figure 3.19 and 3.20 (A)). Further analysis demonstrated that the two CD163 populations can be distinguished based on the expression pattern of CD206, CD40, CXCR1, CCR4, CCR7 and CXCR3 (Figure 3.20 (B)). ‘Cluster 1’ is of particular interest due to its co-expression of CD206 and high CXCR3

expression (Figure 3.20 (C)). In fact, 'cluster 1' and 'cluster 2' can be completely distinguished based on CD206 and CXCR3 expression (Figure 3.21 (A-C)).

A marked increase in the expression profile of 'cluster 1' (which also expresses CD206 and CXCR3) was observed in synovial tissue compared to fluid (Figure 3.22 (A)). In contrast, the 'cluster 2' CD163 population, appears unique to synovial fluid (Figure 3.22 (A)). Indeed, a clear distinction can be made between synovial tissue (blue) and synovial fluid (red) based on CD163 expression with 'cluster 1' unique to synovial tissue and 'cluster 2' unique to synovial fluid (Figure 3.22 (B)).

This analysis, although exploratory and based on only one patient, does however, highlight the stark difference in macrophage subsets residing the synovial tissue and fluid of RA patients. Therefore, we next decided to transcriptionally profile CD206+CD163+ RA synovial macrophages using RNA-sequencing analysis to gain a greater insight into the phenotype of this population.

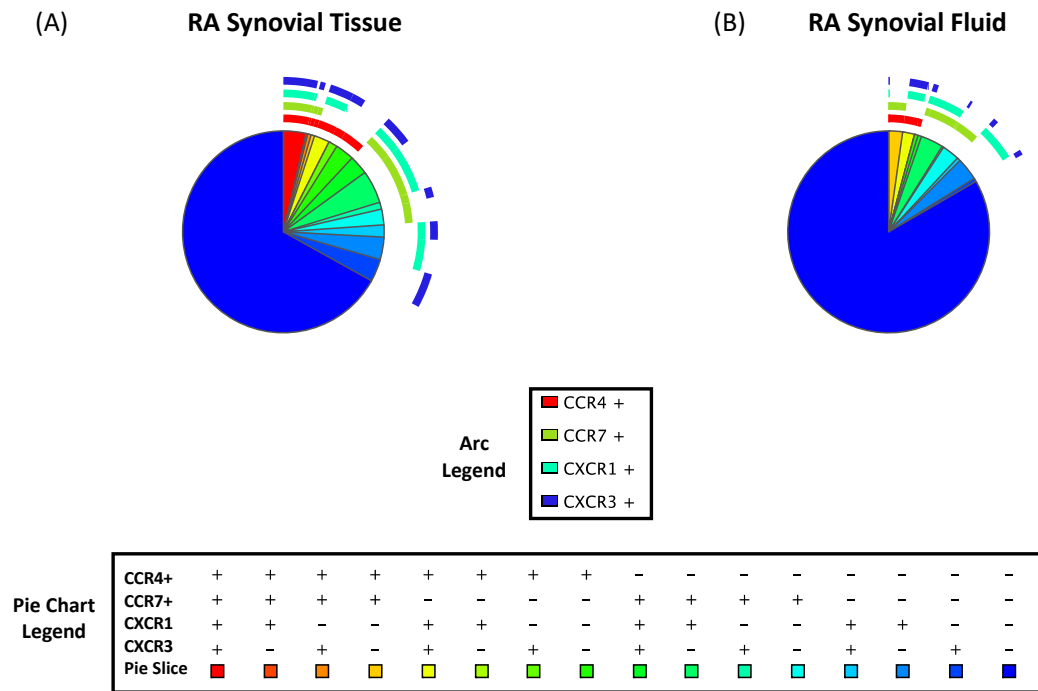


Figure 3.18: Expression of chemokine receptors on RA synovial tissue and fluid macrophages. Visual representation of multidimensional flow cytometric data. SPICE analysis was performed for comparison of chemokine receptor co-expression on CD64+ macrophages in (A) RA synovial tissue (n=3) and (B) RA synovial fluid (n=3). Each pie segment corresponds to a different chemokine receptor combination as denoted by the legend below. The surrounding pie arcs indicate co-expression of specific chemokine receptors on each segment.

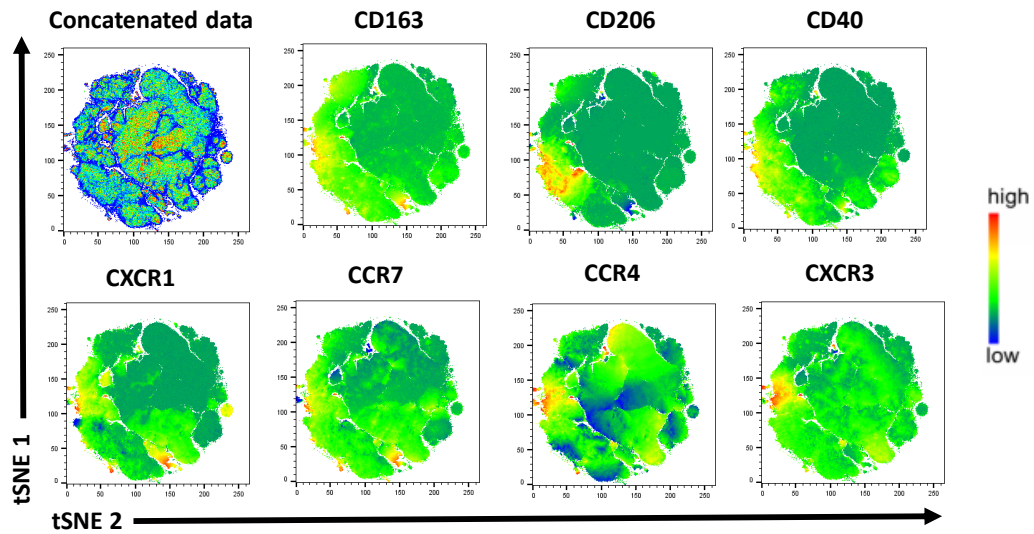


Figure 3.19: Macrophage subpopulation identification by unsupervised clustering.

Multiparametric flow cytometric data of total CD64+ macrophages subjected to unsupervised clustering by tSNE algorithm following data concatenation of RA synovial tissue and matched synovial fluid. Data concatenation and relative expression for the indicated parameters are shown.

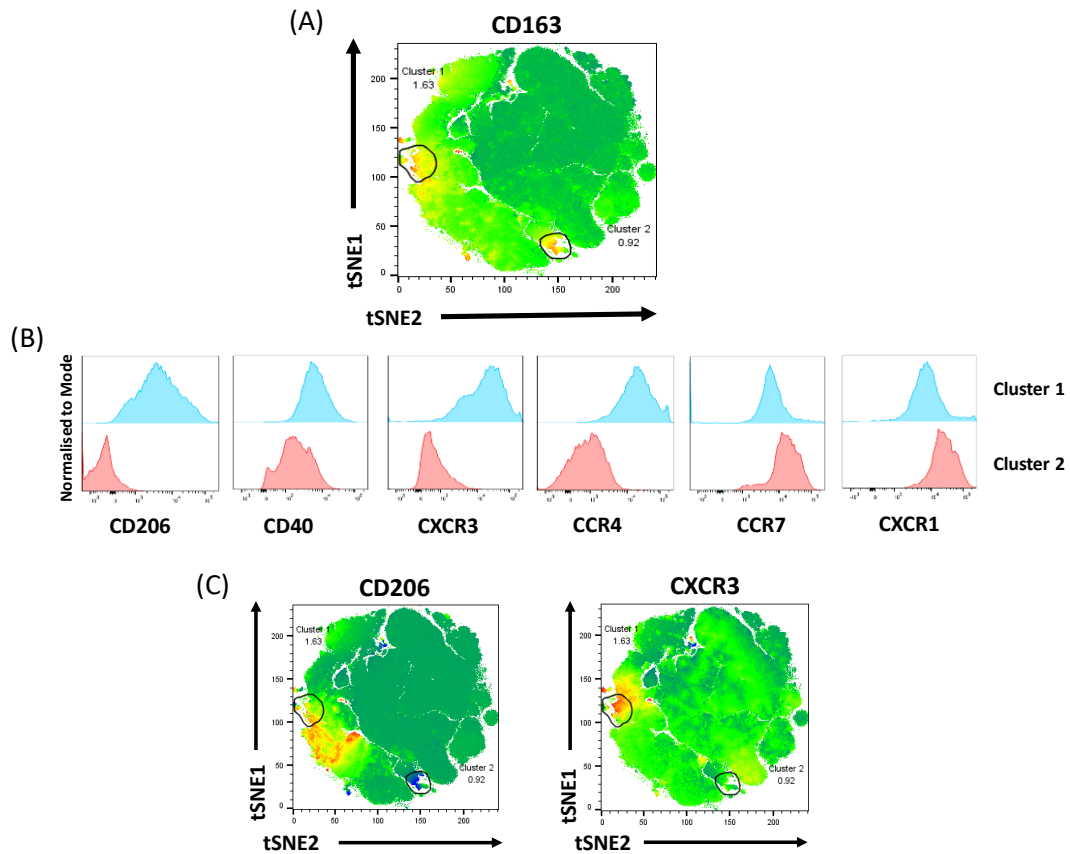


Figure 3.20: CD163 expression defines clusters of macrophages.

(A) Multiparametric flow cytometric data of total CD64+ macrophages subjected to unsupervised clustering by tSNE algorithm following data concatenation of RA synovial tissue and matched synovial fluid. 2 populations of macrophages identified based on CD163 expression with (B-C) differential expression of CD206, CD40, CXCR3, CCR4, CCR7 and CXCR1.

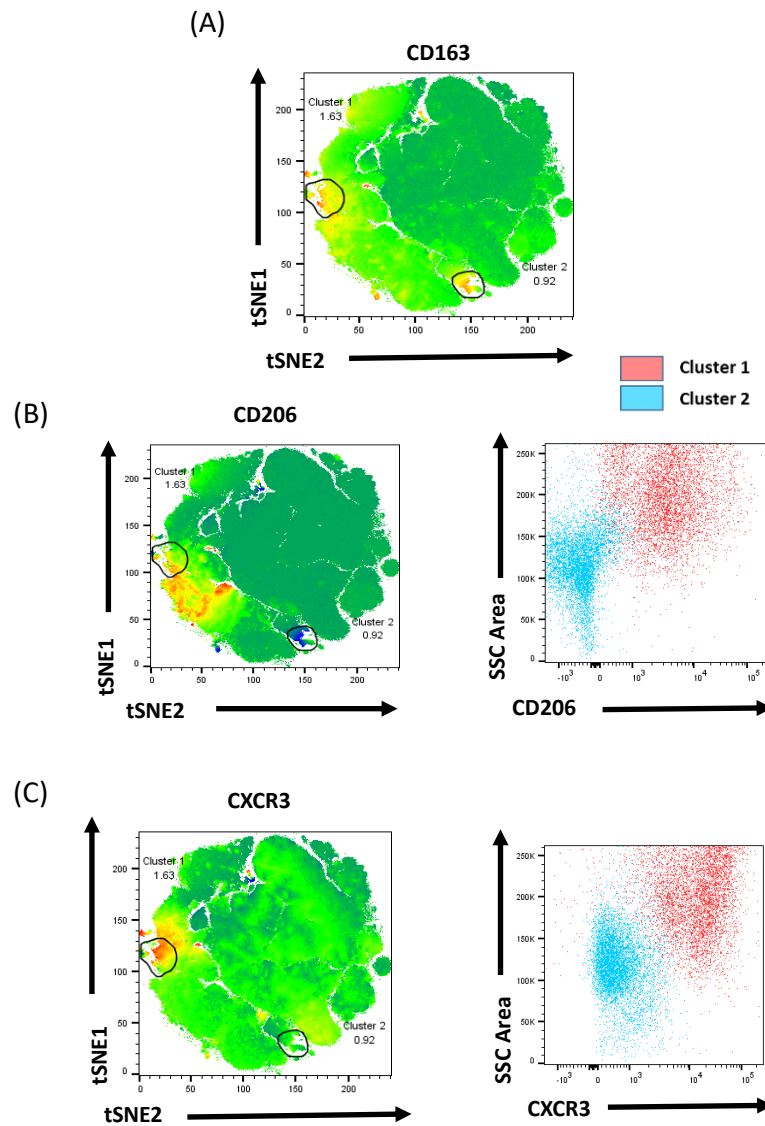


Figure 3.21: Distinct marker expression defines CD163 clusters.

(A) Multiparametric flow cytometric data of total CD64+ macrophages subjected to unsupervised clustering by tSNE algorithm following data concatenation of RA synovial tissue and matched synovial fluid. 2 distinct clusters of CD163 macrophages can be differentiated based on (B) CD206 expression and (C) CXCR3 expression.

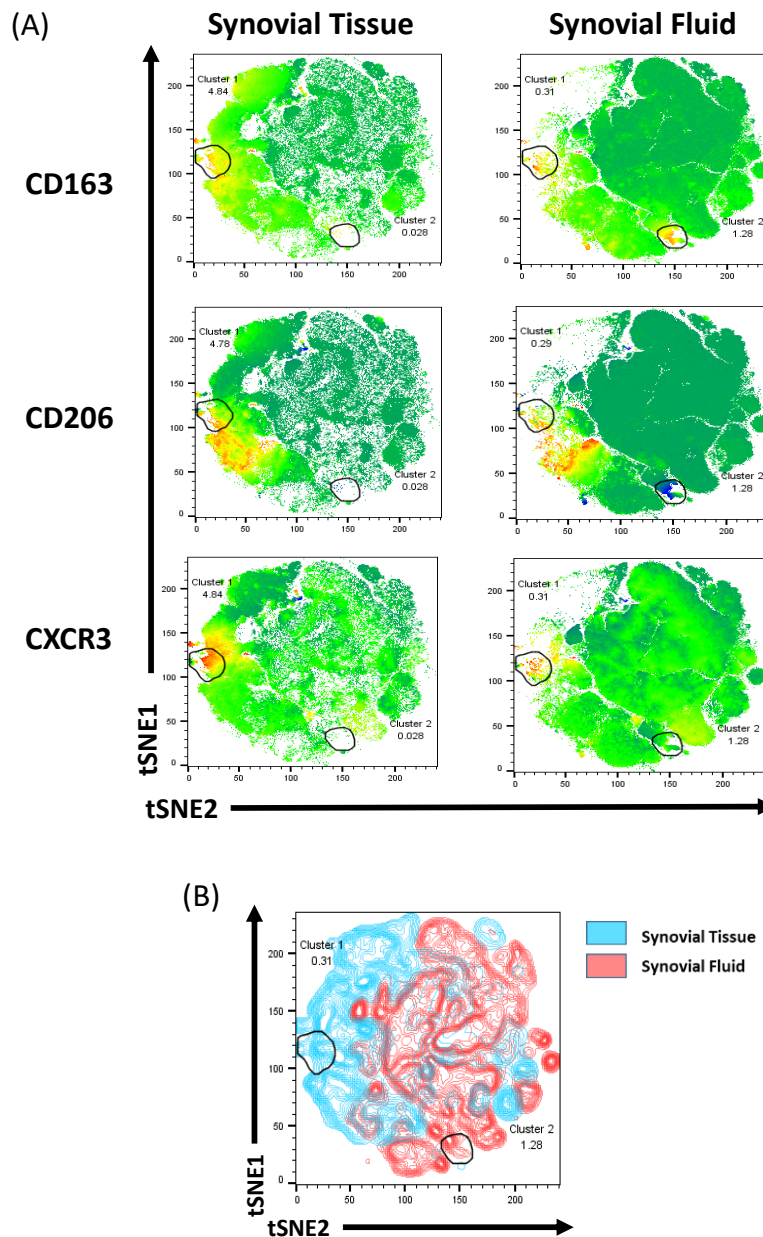


Figure 3.22: Distinct macrophage clusters in RA synovial tissue vs fluid.

(A) The individual synovial tissue and synovial fluid were separated from the concatenated data and CD163 populations assessed based on CD206 and CXCR3 expression. (B) Expression of CD163 from synovial tissue and fluid overlaid for clear separation and macrophage 'clusters'.

3.4.10 FACS sorting of RA synovial tissue macrophage subsets

To further explore the phenotype of CD206+CD163+ RA synovial macrophages, we next decided to transcriptionally profile this population using RNA-sequencing analysis. CD206+CD163+ and CD206-CD163- macrophage subsets were sorted from RA synovial tissue (n=9) and patient-matched RA synovial fluid (n=4). In addition, peripheral blood mononuclear cells were isolated from patient-matched RA donors and monocyte-derived M1 and M2 polarised macrophages differentiated as previously described in Chapter 2 section 2.2.3. High-quality mRNA was isolated from synovial tissue/fluid CD206+CD163+ and CD206-CD163- sorted macrophages and RA polarised macrophages and RNA-Seq was performed.

Figure 3.23 represents the gating strategy used to identify and sort pure populations of CD206+CD163+ and CD206-CD163- macrophages from RA synovial tissue and fluid samples using the FACS Aria Fusion sorter. Table 3.1 summarises the individual yields of CD206+CD163+ and CD206-CD163- populations from each digested biopsy sample. The starting synovial tissue biopsy cell count ranged from 2×10^6 to 6×10^6 cells due to heterogeneity between RA patients.

In total, macrophage populations were sorted from 9 RA synovial biopsies and sent for RNA-seq. Patient demographics are summarised in Table 3.2. The average age and disease duration were 50.6 years and 13.2 years, respectively, with female:male ratio 6:3. Mean DAS28 score was 4.19, and mean CRP 14, indicating high disease activity. 33.33% of RA donors were positive for ACPA antibodies with 44% positive for Rheumatoid Factor (RF).

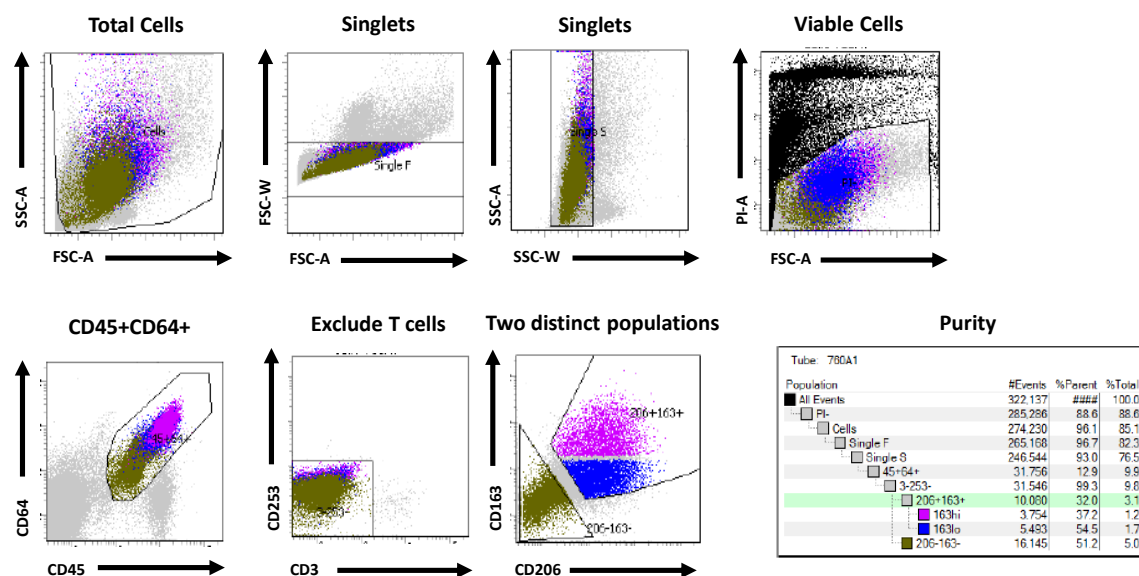


Figure 3.23: Gating strategy for FACS sorting of RA synovial macrophages.

(A) Gating strategy to identify and sort CD206+CD163+ and CD206-CD163- macrophages from RA synovial tissue and synovial fluid mononuclear cells using the FACS Aria Fusion sorter. Following elimination of debris, doublets and dead cells, cells were gated as CD45+CD64+CD3- and further characterised into double positive CD206+CD163+ and double negative CD206-CD163-. Cell sorting purity was also assessed using the FACS Aria Fusion sorter.

	Starting cell count	CD206+CD163+	CD206-CD163-
	3 x 10 ⁶	9,247	16,145
	3 x 10 ⁶	108,000	60,000
	6 x 10 ⁶	823,000	72,000
	6 x 10 ⁶	5,500	6,500
	4 x 10 ⁶	149,000	79,000
	3.3 x 10 ⁶	2,619	1,124
	2 x 10 ⁶	5,500	4,400
	3.5 x 10 ⁶	2,500	5,500
	4 x 10 ⁶	130,000	70,000
Average	3.6 x 10 ⁶	139374	35963.22222

Table 3.4: Yields of sorted macrophages from digested RA synovial biopsy samples.
Cell counts for digested RA synovial biopsies and the subsequent CD206+CD163+ and CD206-CD163- yields.

Characteristic	Average	Percentage or Range
Age (years)	50.67	31-72
Female	6	66.66%
RF Positive	4	44%
ACPA positive	3	33.33%
Disease duration (years)	11.2	<1-35
DAS28	4.19	2.38-6.64
CRP	14	1-31
On MTX	2	25%
On biologic	4	50%
Treatment naïve	3	33%

Table 3.5: Patient demographics sorted RA synovial tissue biopsies.

Patient characteristics for sorted RA synovial tissue biopsies (n=9). Values reflect either the group average or the range in each group with given criteria. RF: Rheumatoid Factor, ACPA: Anti-Citrullinated Protein Antibody, DAS: Disease Activity Score, CRP: C-Reactive Protein, MTX: Methotrexate.

3.4.11 Transcriptional profiling of CD206+CD163+ synovial tissue macrophage

Initial RNA-seq analysis compared the transcriptional differences between double positive CD206+CD163+ and double negative CD206-CD163- RA synovial tissue macrophages. Principal component analysis (PCA) and unsupervised hierarchical clustering of the total gene expression dataset, demonstrates that RA CD206+CD163+ macrophages cluster separately from RA CD206-CD163- macrophages (Figure 3.24 (A-B)). Differentially expressed genes (DEGs) were analysed between the two macrophage populations revealing 164 DEGs. Volcano plot shown in Figure 3.24 (C), represents DEGs with red data points indicating significant DEGs (adjusted p-value<0.05).

Further interrogation of DEGs between the two macrophage populations identified differences in genes associated with adhesion/cell growth, cytoskeletal rearrangement, inflammatory rearrangement, phagocytosis and cellular metabolism (Figure 3.25). Together, these data indicate distinct transcriptional profiles between RA synovial tissue CD206+CD163+ and CD206-CD163- macrophages.

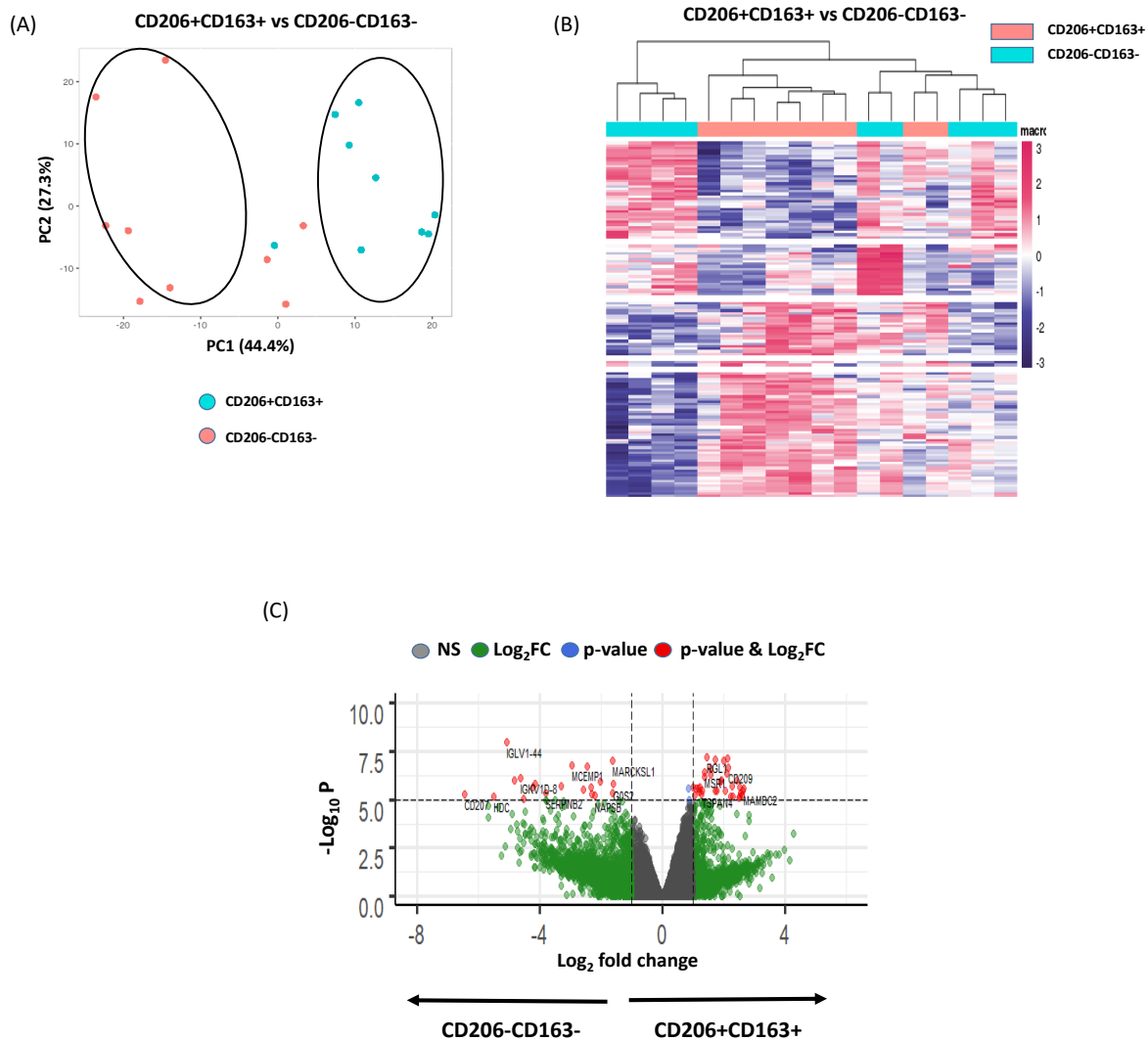


Figure 3.24: CD206+CD163+ macrophages are transcriptionally distinct from CD206-CD163- macrophages.

High quality RNA was isolated from sorted CD206+CD163+ and CD206-CD163- synovial tissue macrophages and RNA-Sequencing was performed. (A) Principal component analysis (PCA) was performed on the total dataset of RA synovial tissue sorted CD206+CD163+ and CD206-CD163- macrophage subsets (n=9). (B) Unsupervised hierarchical clustered heatmap of DEGs in RA synovial tissue CD206+CD163+ and CD206-CD163- macrophages. (C) Volcano plots of the relative difference in expression of all DEGs between CD206+CD163+ and CD206-CD163- macrophages.

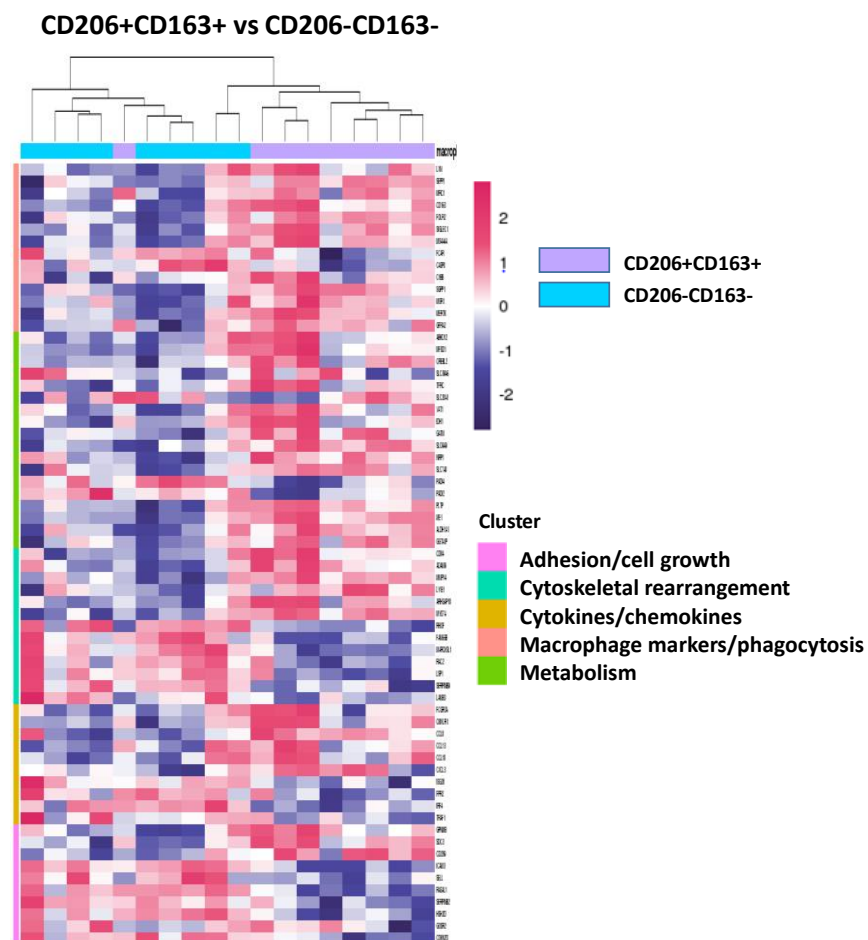


Figure 3.25: Heatmap representation of DEG analysis.

Hierarchical clustered heatmap displaying DEGs involved in adhesion/cell growth, cytoskeletal rearrangement, cytokine/chemokines, macrophage markers/phagocytosis and metabolism in RA synovial tissue CD206+CD163+ macrophages compared to CD206-CD163- macrophages (n=9).

3.4.12 CD206+CD163+ synovial tissue macrophages are transcriptionally distinct from polarized macrophages

Thus far, our extensive analysis of CD206+CD163+ RA synovial tissue macrophages has indicated that this subset is extremely plastic. Despite being classified by two classic M2 markers- CD206 and CD163, this macrophage population does not appear to be a typical M2 macrophage, most notably due to high co-expression of CD40. As previously outlined, the prevailing M1 vs M2 dogma, although useful for simple characterisation, perhaps doesn't capture the true plasticity and diversity of macrophage subsets at the site of inflammation.

We hypothesise that the enriched RA CD206+CD163+ macrophage subset are an intermediate or transitional subset of tissue macrophage, neither M1 nor M2, instead lying somewhere in between a wide spectrum of macrophage activation states. To formally address this, we investigated transcriptional variance between RA synovial tissue CD206+CD163+ macrophages and patient-matched monocyte-derived M1 and M2 polarised macrophages to examine if the CD206+CD163+ macrophages were more M1 or M2 like.

Indeed, unsupervised hierarchical clustering of the total gene expression dataset demonstrates that RA CD206+CD163+ macrophages in fact cluster separately from RA M1 and M2 macrophages (Figure 3.26 (A)). The striking transcriptional differences are further reflected in volcano plots depicted in Figure 3.26 (B-C), whereby CD206+CD163+ synovial tissue macrophages display clear transcriptional differences compared to (A) M1 and, (B) M2, with red data points indicating differentially expressed genes (adjusted p-value<0.05). This confirms our hypothesis that CD206+CD163+ macrophages enriched in the RA synovium are in a transitional activation state and transcriptionally distinct from pure monocyte-derived M1 and M2 macrophages.

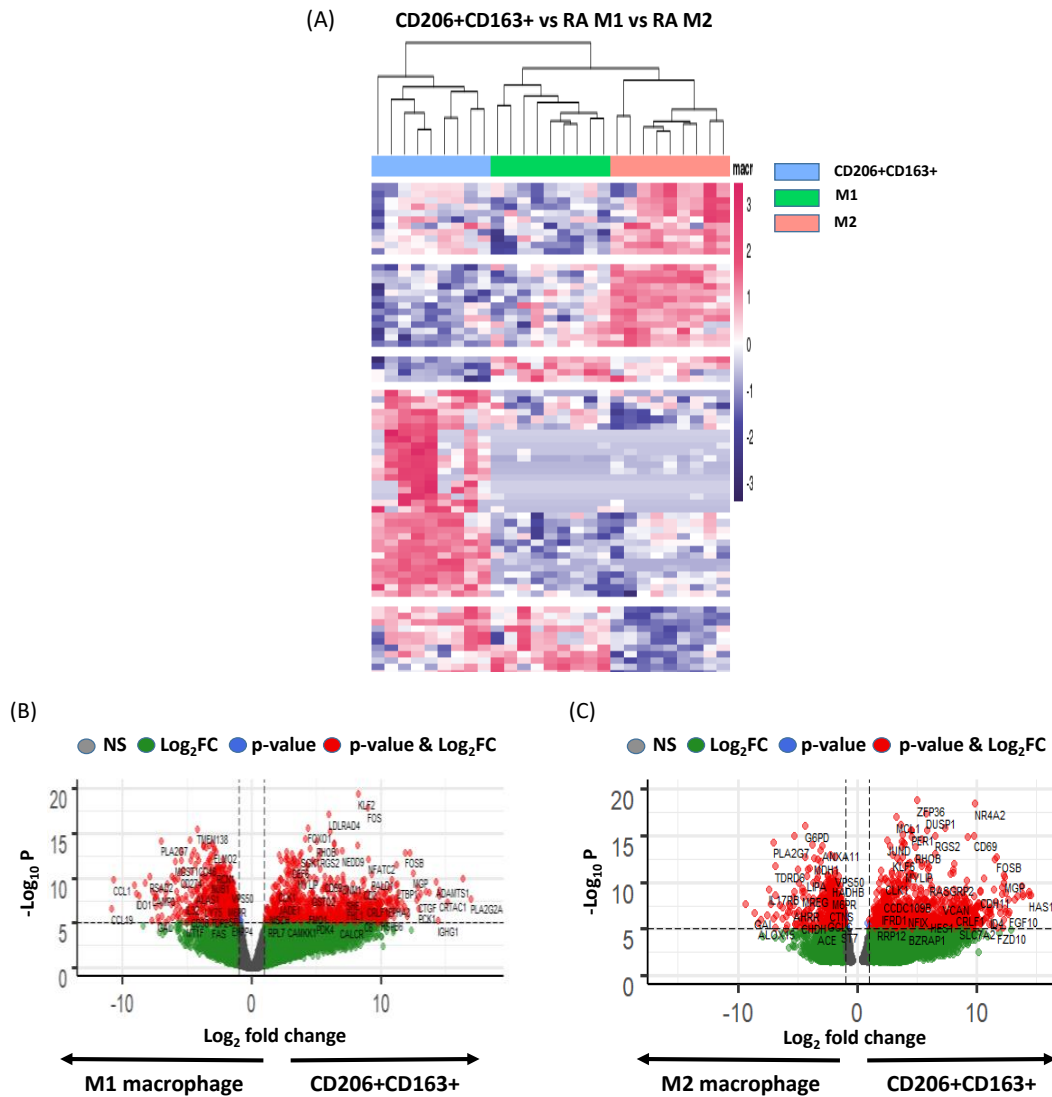


Figure 3.26: CD206+CD163+ macrophages are transcriptionally distinct from M1 and M2.

(A) Clustered heatmap displaying unsupervised hierarchical clustering of genes that are distinct in RA synovial tissue CD206+CD163+ macrophages (n=9) and patient-matched polarised RA monocyte-derived M1 and M2 macrophages (n=9). Volcano plots of the relative difference in expression of DEGs between CD206+CD163+ synovial tissue macrophages and RA monocyte-derived (B) M1 and (C) M2 macrophages.

3.4.13 CD206+CD163+ macrophage population display genetic signatures of tissue residency

One of the key questions in macrophage biology is where are these cells coming from? The origin of macrophages in the human synovium has yet to be fully elucidated as a clear distinction between tissue-resident and inflammatory monocyte-derived macrophages has not yet been possible. However, certain genes are associated with tissue-resident macrophages and certain markers are upregulated in macrophages derived from monocytes. Although not definitive, analysis of these specific signatures may give an insight into the ontogeny of macrophages, specifically those residing in the RA synovium.

Genes associated with tissue residency, *TREM2*, *FOLR2*, *LYVE1*, *C1QA* and *TIMD4*, along with monocyte core genes *CCR2* and *CD48* were analysed. Interestingly, we note a significant increase in all 'tissue-resident' associated genes in synovial tissue CD206+CD163+ macrophages compared to double negative CD206-CD163- macrophages (Figure 3.27, $p < 0.05$). In contrast, monocyte-derived signature genes *CCR2* and *CD48* are reciprocally significantly blunted in CD206+CD163+ synovial tissue macrophages compared to the double negative population (Figure 3.27, $p < 0.05$).

To strengthen this observation, we compared expression of these genes in CD206+CD163+ synovial tissue macrophages to RA M1 macrophages, which are derived from circulating monocytes and therefore not tissue-resident. Figure 3.28 demonstrates significant increased gene expression of *TREM2*, *FOLR2*, *LYVE1*, *C1QA* and *TIMD4* in CD206+CD163+ synovial tissue macrophages compared to monocyte-derived M1 macrophages. These data hint that CD206+CD163+ synovial tissue macrophages are not derived from infiltrating immune cells but are tissue-resident.

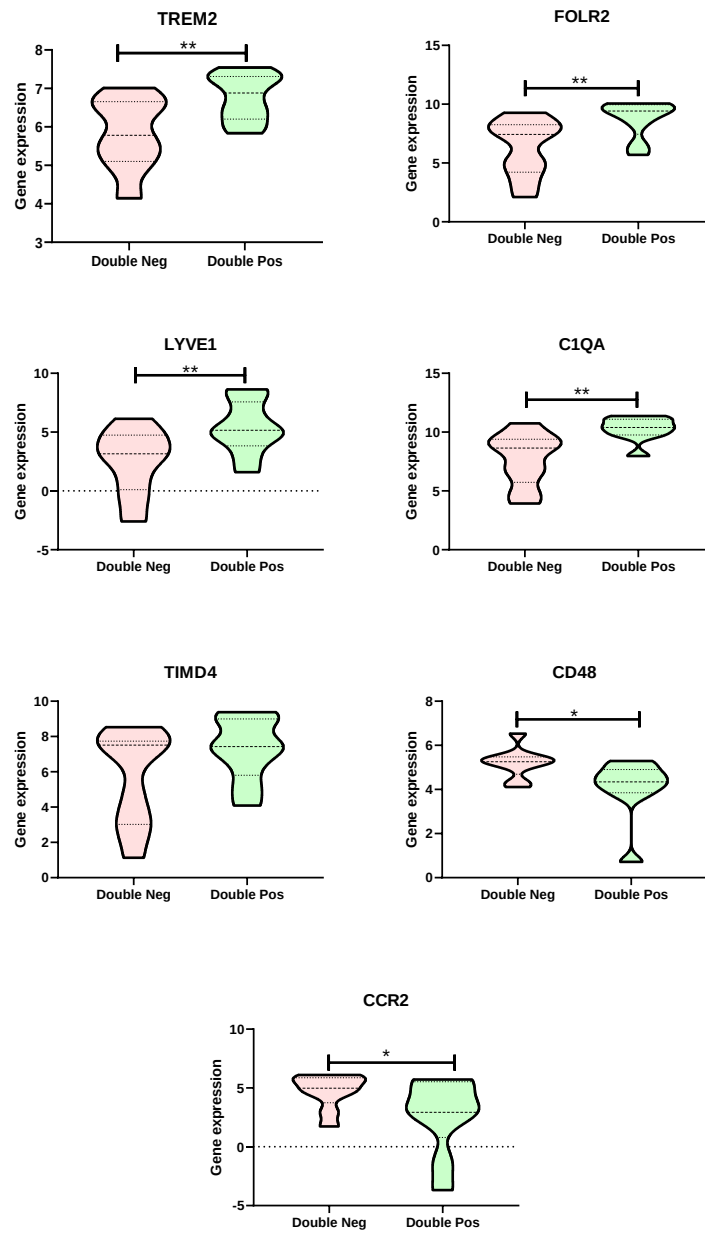


Figure 3.27: CD206+CD163+ macrophages upregulate tissue-resident genes.

Violin depicting gene expression of synovial tissue macrophage associated genes in the CD206+CD163+ (double pos) macrophage population compared to CD206-CD163- (double neg) macrophages (n=9). Data expressed as mean $\pm$ SEM using Wilcoxon signed rank, * $p < 0.05$, ** $p < 0.01$ significantly different from each other.

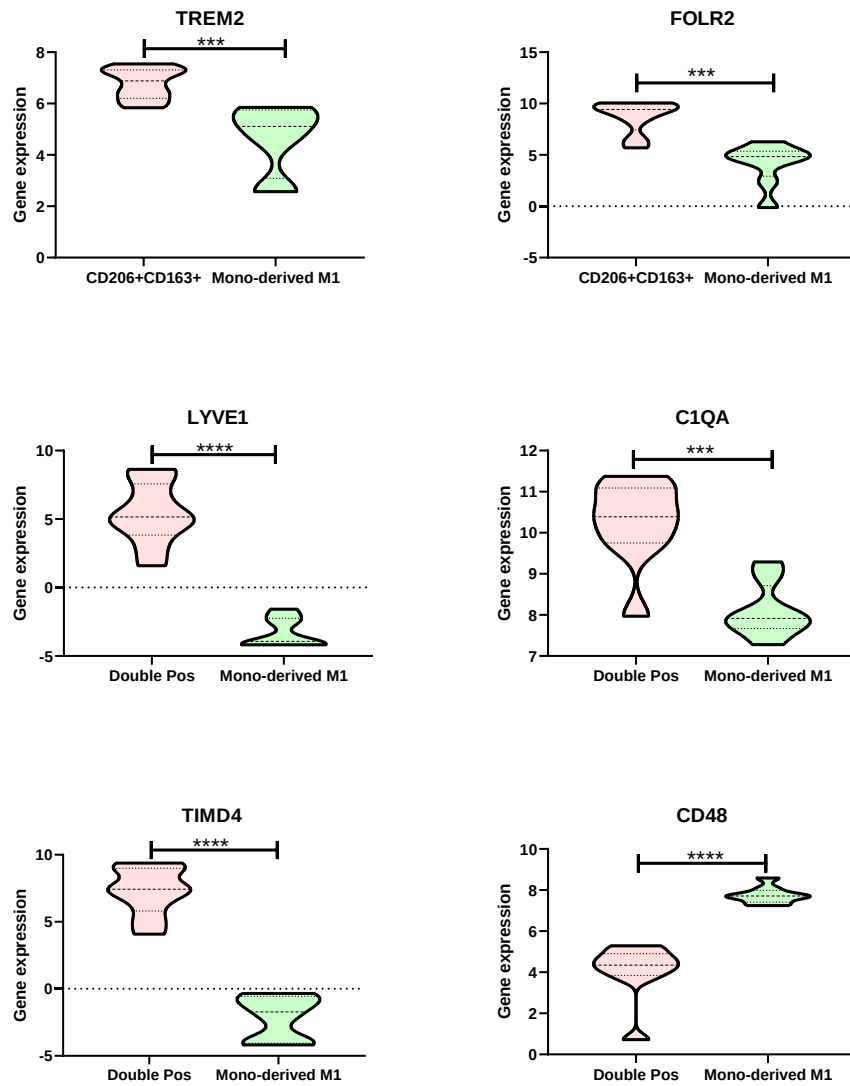


Figure 3.28: CD206+CD163+ macrophages upregulate tissue-resident genes.

Violin plots depicting gene expression of tissue-resident associated genes in the CD206+CD163+ macrophage population (n=9) compared to patient-matched monocyte-derived M1 macrophages (n=9). Data expressed as mean $\pm$ SEM using Mann Whitney U, *p<0.05, **p<0.01, ***p<0.005, ****p<0.001 significantly different from each other.

3.4.14 CD206+CD163+ macrophage subsets are distinct between synovial tissue and synovial fluid

This study has already demonstrated clear differences in CD206+CD163+ macrophage populations residing in the RA synovium compared to the synovial fluid. RNA-seq analysis allowed for further confirmation that synovial tissue and fluid double positive CD206+CD163+ cells are distinct. This is demonstrated by volcano plots of DEGs between synovial tissue and fluid CD206+CD163+ macrophages (Figure 3.29 (A)). Moreover, ingenuity pathway analysis (IPA) allowed for identification of key signalling pathways distinct to synovial tissue double positive macrophages compared to synovial fluid (Figure 3.29 (B)). IPA revealed distinct enrichment of many pro-inflammatory pathways; IL-6, IL-8, NO and ROS signalling in CD206+CD163+ synovial fluid macrophages, indicative of pro-inflammatory infiltrating macrophages (Figure 3.29 (B)). In contrast, enrichment of PPARG signalling and inhibition of matrix metalloproteases is observed in CD206+CD163+ macrophages residing in the synovial tissue, indicative of M2 resolving macrophages (Figure 3.29 (B)). Overall, this confirms distinct site-specific transcripts in CD206+CD163+ tissue macrophages.

In addition, genes associated with tissue-resident macrophages are all significantly decreased in CD206+CD163+ macrophages from synovial fluid compared to synovial tissue (Figure 3.31, $p < 0.05$), with monocyte-derived macrophage signature genes elevated. This information indicates that the phenotypic and functional differences observed between synovial tissue and fluid CD206+CD163+ may be due to differing ontogeny. This information suggests that analysis of synovial fluid may not clearly depict the pathophysiological state of synovial tissue or the processes occurring at the site of inflammation, as once previously thought.

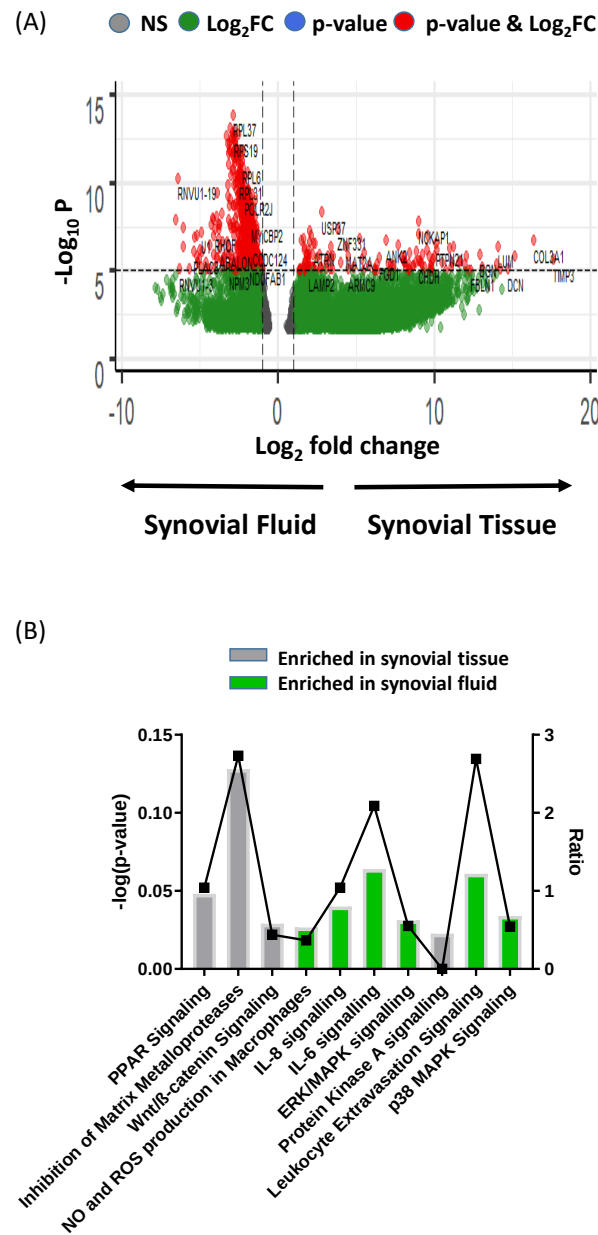


Figure 3.29: RA synovial tissue CD206+CD163+ macrophages are transcriptionally distinct from synovial fluid.

(A) Volcano plot of the relative difference in expression of DEGs between CD206+CD163+ synovial tissue macrophages (n=9) and CD206+CD163+ synovial fluid macrophages (n=4). (B) Ingenuity pathway analysis (IPA) of enriched pathways between CD206+CD163+ synovial tissue macrophages and CD206+CD163+ synovial fluid macrophages. Ratio represents the number of genes from the list that maps to the pathways divided by the total number of genes that map to the same pathway.

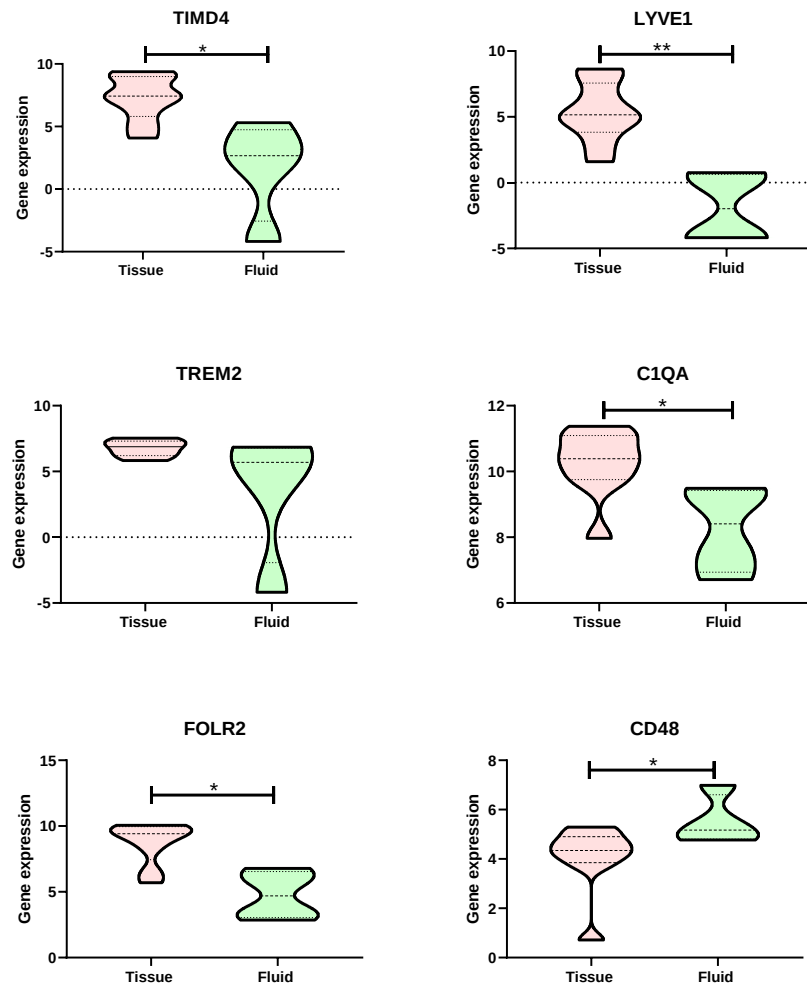


Figure 3.30: CD206+CD163+ tissue macrophages upregulate tissue-resident genes.

Violin plots depicting gene expression of tissue-resident associated genes in CD206+CD163+ tissue macrophages (n=9) compared to CD206+CD163+ fluid macrophages (n=4). Data expressed as mean ± SEM using Mann Whitney U, *p<0.05, **p<0.01 significantly different from each other.

3.4.15 Metabolic analysis of synovial tissue macrophages

While a significant number of studies have demonstrated differences in the metabolic profile of polarised M1/M2 macrophages *in vitro*, the metabolic characteristics of macrophages remains largely unexplored in inflamed tissues. Given the significant role of metabolic rewiring in shaping RA macrophage activation states as demonstrated extensively in Chapter 2, we decided to investigate the bioenergetic profiles of CD206+CD163+ synovial tissue macrophages. RNA-sequencing revealed differential expression of metabolism associated genes between CD206+CD163+ and CD206-CD163- macrophages as indicated by clustered heatmap expression (Figure 3.31 (A)). Further analysis demonstrated significant increases in expression of glycolysis-associated genes *HK2*, *LDHA* and *PKM2* and the amino acid transporter *SLC7A5* in the double negative population compared to the double positive (Figure 3.31 (B)), indicating differences in the bioenergetic function.

To date, no study has assessed the metabolic competence of synovial tissue macrophages. This is mainly due to the difficulty in isolating enough cells for standard metabolic assays such as the Seahorse Flux analyser. However, in this study we conducted a novel metabolic assay which utilises the two-photon excited fluorescence lifetime imaging (FLIM) of NAD(P)H. Auto fluorescence originating from excited NAD(P)H can serve as a useful redox marker which can be measured non-invasively using two-photon excited FLIM. This enables discrimination between oxidative phosphorylation and glycolysis in cells. The FLIM technique requires very few cells to run which allowed us to use sorted synovial tissue macrophages.

Analysis of sorted macrophages from synovial tissue from (n=4) RA patients demonstrated strikingly consistent differences in metabolic preferences between CD206+CD163+ and CD206-CD163- macrophages. CD206+CD163+ synovial tissue macrophages utilised oxidative phosphorylation as their main source of energy, while the CD206-CD163- macrophages preferentially utilize glycolysis (Figure 3.32 (A-B), $p < 0.05$). This is further reflected in representative FLIM images whereby a red/green cell is predominantly using OXPHOS, while a blue cell indicates glycolysis is being used as the main energy source (Figure 3.32 (B)).

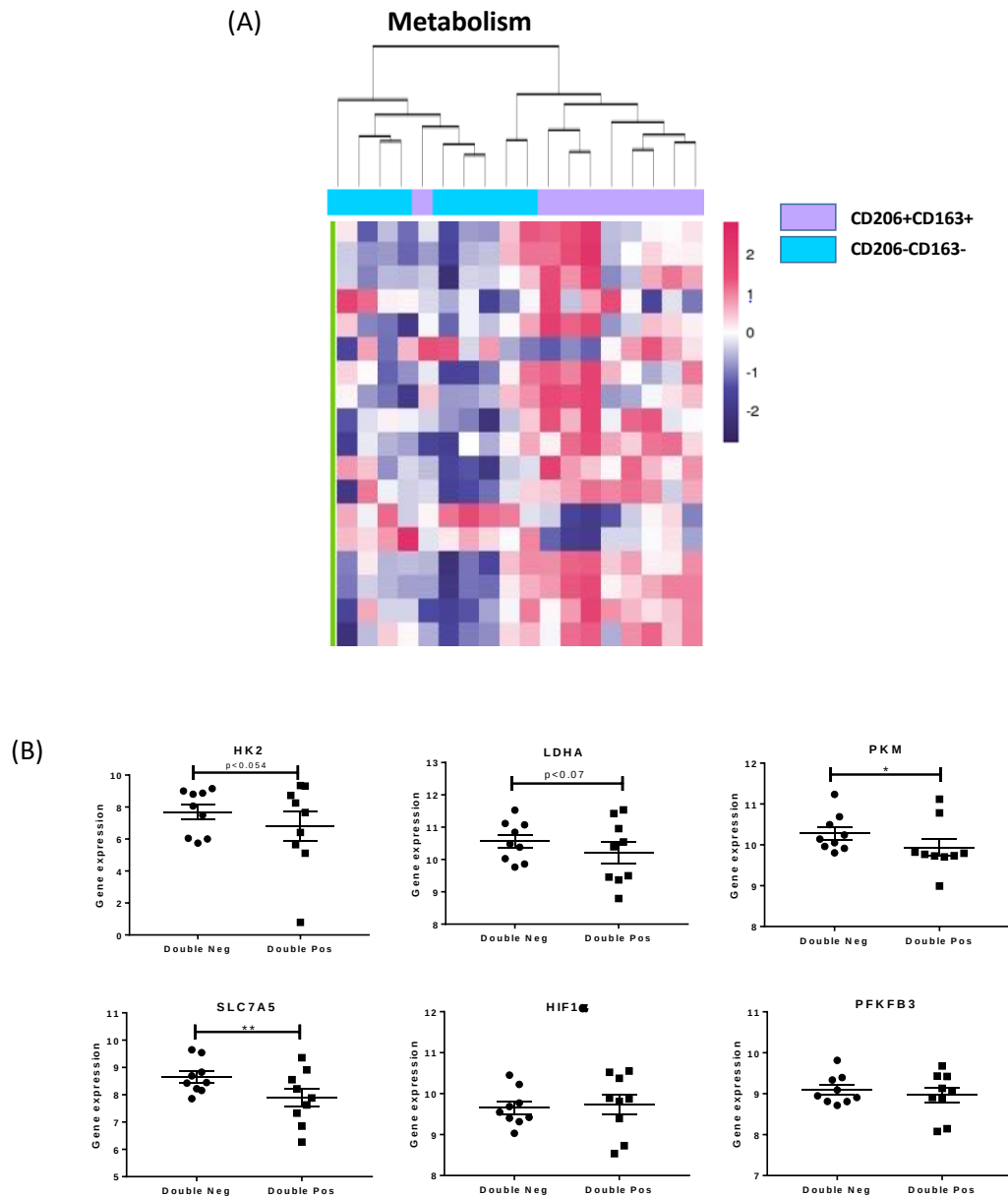


Figure 3.31: Differential expression of metabolic genes in CD206+CD163+ and CD206-CD163- macrophages.

(A) Clustered heatmap displaying metabolism associated DEGs in RA synovial tissue CD206+CD163+ macrophages compared to CD206-CD163- macrophages (n=9). (B) Dot plots depicting gene expression of metabolic genes in the CD206+CD163+ macrophage population compared to CD206-CD163- macrophages. Data expressed as mean $\pm$ SEM using Wilcoxon signed rank, * $p < 0.05$, ** $p < 0.01$ significantly different from each other.

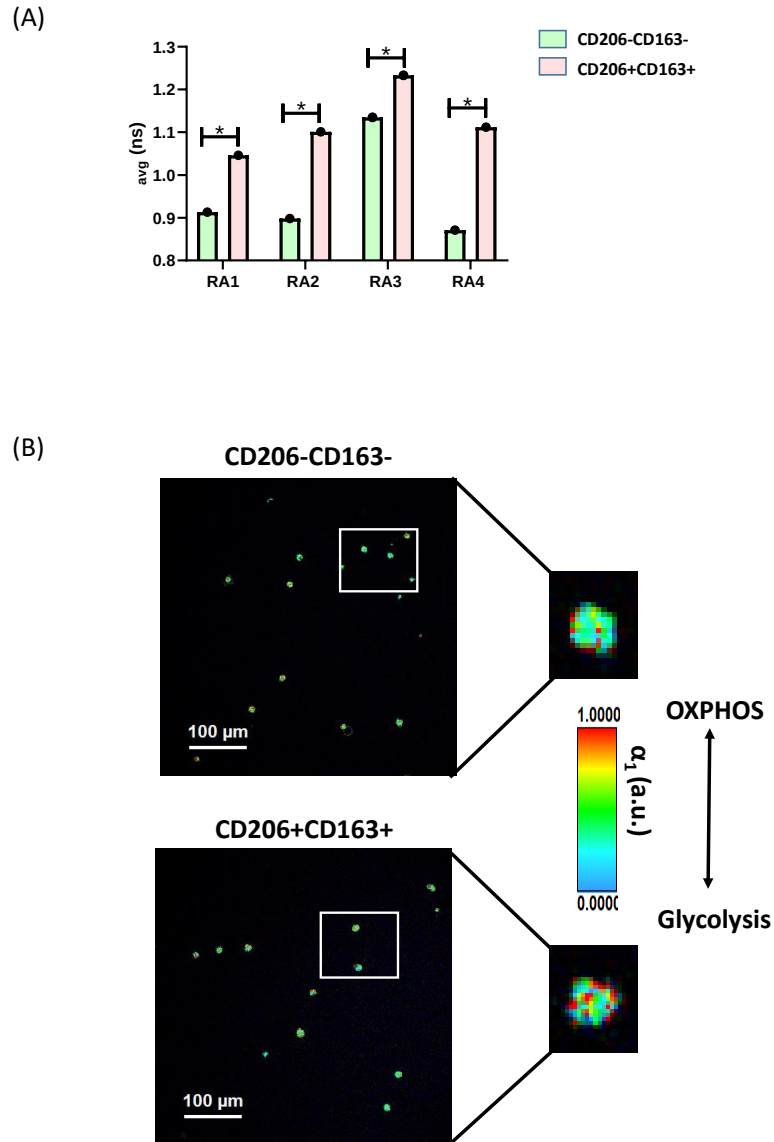


Figure 3.32: NAD(P)H-FLIM imaging to assess metabolic competence of CD206+CD163+ macrophages.

NAD(P)H-FLIM imaging of metabolism in sorted CD206+CD163+ and CD206-CD163- synovial tissue macrophages. (A) Summary of protein bound lifetimes and the fractions of bound NAD(P)H of $n=4$ individual donors, $*p<0.05$ using paired t -test. (B) Representative FLIM images whereby a red/green cell is predominantly using OXPHOS, while a blue cell indicates glycolysis is being used as the main energy source.

3.4.16 Synovial CD206+CD163+ macrophages induce enhanced T cell responses

We demonstrate that the CD206+CD163+ macrophage population spontaneously secrete high levels of pro-inflammatory mediators IL-6, IL-8 and TNF α as measured by Meso Scale Discovery (MSD) assay (Figure 3.33 (A)). To further examine the pathogenic role of CD206+CD163+ macrophages in the joint, we next performed autologous co-culture experiments with synovial T cells. In addition to antigen presentation, macrophages secrete chemokines that can attract T cells into the joint, while macrophage cytokine production can direct T cell polarisation towards Th1/Th17 (Roberts, Dickinson and Taams, 2015). Therefore, we set up an autologous synovial tissue macrophage - T cell co-culture system to examine the effect of CD206+CD163+ macrophages on T cell activation.

CD206+CD163+ macrophages were sorted from RA synovial tissue along with CD3 T cells from the same donor. T cells were cultured with CD206+CD163+ macrophages for 5 days in a ratio of 1:3 in an autologous co-culture system after which T cell cytokine production was assessed by flow cytometry. T cells cultured with CD206+CD163+ macrophages display enhanced activation compared to T cells alone with increased expression of GM-CSF, TNF α and IL-17 (Figure 3.33 (B)).

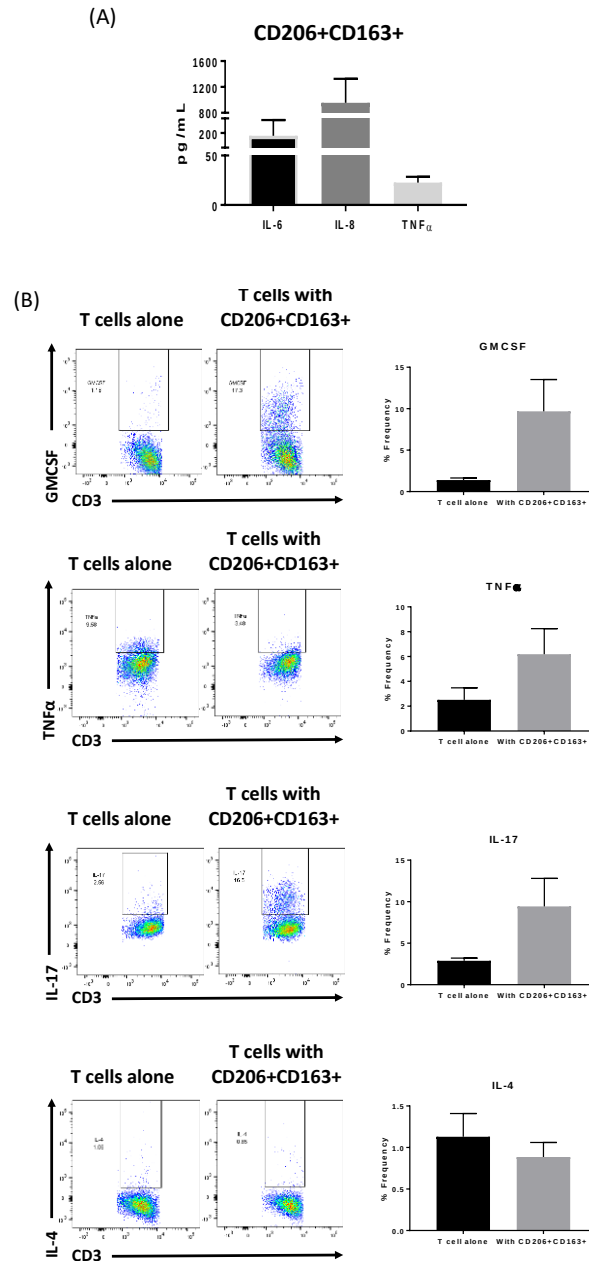


Figure 3.33: Synovial CD206+CD163+ macrophages induce enhanced T cell responses.

(A) Bar graphs represent cytokines secreted from sorted RA synovial tissue CD206+CD163+ macrophages measured by MSD assay (n=5) (B) Sorted RA synovial tissue CD206+CD163+ macrophages and sorted CD3+ T cells from the same donor were cultured for 5 days in a ratio of 1:3 in an autologous co-culture system after which T cell cytokine production was assessed by flow cytometry. Following 5 days of co-culture, CD3+ T cells were restimulated with PMA/Ionomycin in the presence of Brefeldin A: stained intracellularly with fluorochrome-conjugated antibodies specific for GMCSF, TNF α , IL-17 and IL-4 and analysed by flow cytometry. Representative flow cytometry plots showing cytokine staining in CD3+ T cells and dot plots representing the percentage of indicated cytokine within the CD3+ T cell population.

3.5: Discussion

In this chapter we have identified, for the first time, that within the spectrum of macrophage activation states, the dominant macrophage population residing in the synovium of the RA joint are a transitional subset of tissue-resident CD206+CD163+ macrophages co-expressing high levels of CD40. We show that this subset is enriched in synovial tissue compared to fluid and specifically in the RA synovium compared to other inflammatory arthropathies, and positively correlates with higher RA disease activity. Furthermore, we demonstrate that the double positive CD206+CD163+ macrophage subset is dominant in healthy synovial tissue, yet in established RA, this subset transforms into an activated transitional subset of macrophages as indicated by the observed increase in the expression of CD40, which is completely absent in HC. Moreover, we demonstrate differential chemokine receptor expression on CD206+CD163+ synovial tissue macrophages in RA patients compared to other disease states. RNA-seq analysis revealed that the CD206+CD163+ macrophage population is transcriptionally distinct from synovial tissue double negative CD206-CD163-, synovial fluid CD206+CD163+, and from pure monocyte-derived M1 and M2 macrophages, with unique tissue-resident gene signatures. Moreover, we demonstrate differing metabolic capacities between CD206+CD163+ and CD206-CD163- macrophage subsets. Finally, CD206+CD163+ macrophages have the capacity to induce autologous T cell responses and spontaneously secrete high levels of IL-8, IL-6 and TNF α , thus further contributing to the local inflammatory response.

The prevailing presence of a macrophage subset expressing high levels of CD206 and CD163 in the RA synovium was initially unexpected considering these markers typically define an immunoregulatory M2 macrophage phenotype. CD163, a scavenger receptor that binds to the haemoglobin-haptoglobin complex with release of IL-10, is highly phagocytic and exclusively expressed in M2 macrophages (Hogg, Palmer and Revell, 1985; Zwadlo *et al.*, 1987; Kristiansen *et al.*, 2001; Otterbein *et al.*, 2003). Studies have demonstrated elevated levels of soluble CD163 in the RA joint that correlates with RA disease activity and progression in early disease (Matsushita *et al.*, 2002; Greisen *et al.*, 2011). Conversely, other studies indicate that soluble CD163 is only weakly correlated

with measures of disease activity in long standing RA (Jude *et al.*, 2013). The mannose receptor, CD206 is a scavenger receptor expressed mainly by M2 macrophages and dendritic cells (DC) (Martinez-Pomares, 2012). Less is known about the role of CD206+ macrophages in RA although studies have suggested increased soluble CD206 levels in RA patients with high disease activity which are decreased following treatment (Heftdal *et al.*, 2017).

The dominance of an 'M2-like' macrophage is of significant interest; are these macrophages attempting to resolve inflammatory disease? Or have these cells become dysfunctional, similar to that observed in RA for regulatory T cells (Basdeo *et al.*, 2015), and thus fail to constrain the inflammatory response? Indeed, further examination of CD206+CD163+ macrophages revealed strong co-expression of CD40, which is a co-stimulatory activation marker that contributes to pathogenic mechanisms via the CD40/CD40L pathway to sustain chronic inflammation in RA (Valeria Román-Fernández *et al.*, 2019). Moreover, CD40 alleles are associated with risk of developing RA with studies showing that CD40 ligand expression correlates with higher disease activity (Raychaudhuri *et al.*, 2008; Guo *et al.*, 2017). It is now appreciated that tissue macrophages are exquisitely plastic, capable of adapting to the needs of their microenvironment through a combination of tissue-specific cues and transient 'on demand' signals (Kurowska-Stolarska and Alivernini, 2017). In this study we suggest that the inflammatory microenvironment of the RA joint acts as an 'on demand' signal to transform CD206+CD163+ tissue macrophages from a homeostatic M2-like macrophage to a dysfunctional activated state. This is consistent with previous studies demonstrating that the inflammatory milieu of the synovial microenvironment can shape the phenotype and function of monocyte-derived macrophages. Specifically, monocyte-derived macrophages can be skewed towards a pro-inflammatory profile through interactions with RA synovial fibroblasts, citrullinated proteins and danger-associated molecular pattern molecules (DAMPs) (Midwood *et al.*, 2009; Donlin *et al.*, 2014; Zhu *et al.*, 2015). In addition, co-culture with effector T cells induces CD40 expression on monocytes (Taams *et al.*, 2005; Roberts, Dickinson and Taams, 2015). Therefore, perhaps interactions with inflammatory cells such as T cells or exposure to the many soluble mediators that are

skewed towards a proinflammatory profile in the inflamed joint, activate RA synovial tissue CD206+CD163+ macrophages, paralleled by changes to their function capacity.

Despite common clinical features, marked immunological and molecular differences exist between RA and PsA arthritides (Veale and Fearon, 2015). In this study we demonstrate comparable levels of CD68+/CD64+ synovial tissue macrophages in PsA and OA synovium compared to RA. Historical histological analysis however is conflicting with studies indicating increased expression of synovial macrophages in RA compared to PsA (Veale *et al.*, 1993), while other studies demonstrate that the synovial infiltrate of patients with PsA and RA is comparable with regard to numbers of macrophages (Van Kuijk *et al.*, 2006; Laria *et al.*, 2016). Moreover, even less is known about the role of macrophages in OA disease pathogenesis but studies indicate increased CD68+ macrophage expression in RA compared to OA with subsequently lower levels of pro-inflammatory cytokines in OA synovium (Farahat *et al.*, 1993). In addition, increased M1-like cytokine signatures are observed in RA compared to PsA with no changes in M2 cytokine expression (Vandooren *et al.*, 2009). This is in line with our study where comparable expression of the M2-like double positive CD206+CD163+ macrophage subset was observed between RA and PsA synovial tissue. Interestingly however, we noted marked enrichment of the dominant transitional CD206+CD163+CD40+ population in RA synovial tissue compared to PsA and OA. Therefore, we propose that CD206+CD163+ macrophages transition to a highly activated state under the inflammatory microenvironment of the RA synovium specifically.

Furthermore, we demonstrate differential chemokine receptor expression on the surface of RA synovial tissue macrophages compared OA and PsA. Interestingly, although comparable CCR4 expression was observed between OA and PsA synovial tissue, this expression is diminished in RA synovial tissue macrophages. Conversely, RA macrophages express CCR7, which is decreased in a stepwise manner in PsA and OA tissue macrophages. Expression of CCR7 has been identified as a hallmark of RA inflammatory synovial fluid macrophages and polarised M1 macrophages, correlating with RA disease activity (Van Raemdonck *et al.*, 2019). Moreover, the CCR7/CCL21 signalling axis has been shown to promote Th17-mediated osteoclast formation, thus contributing to erosive disease (McHugh, 2019; Van Raemdonck *et al.*, 2019). In contrast, CCR4 expression is

typical of a Th2 immunomodulatory response, highly expressed on tumour associated macrophages exhibiting an 'M2' like phenotype (Imai *et al.*, 1999; Maolake *et al.*, 2017). However, other studies demonstrate that CCR4 deficient macrophages in a mouse model of pulmonary fibrosis exhibit an M2 activation state (Trujillo *et al.*, 2008). The observed decrease in CCR4 expression coupled with increased expression of inflammatory CCR7 in RA synovial tissue macrophages compared to OA and PsA further confirms the dysfunctional activated phenotype of RA CD206+CD163+ macrophages.

Although synovial tissue macrophages have been identified as the most common immune cell present in the healthy synovial membrane (Singh *et al.*, 2004; Smith, 2011), the functions of macrophages in the healthy synovium are poorly described. Previous research on synovial myeloid cells has been limited to analysing monocytes from RA synovial fluid, however monocytes are not present in synovial fluid from healthy individuals (Jepsen, 2009; Kurowska-Stolarska and Alivernini, 2017). Therefore, we obtained synovial biopsies from healthy individuals undergoing ACL reconstruction for a comprehensive analysis of synovial tissue macrophages in the healthy synovium. Phenotypic analysis revealed stark differences in macrophage subsets in the healthy synovium compared to the inflamed RA joint. We observe a dramatic decrease in the diversity of macrophage subsets in healthy synovial tissue biopsies with a dominance of the homeostatic CD206+CD163+ macrophage population. Interestingly, this subset does not exhibit expression of the inflammatory marker CD40 that is significantly expressed in RA. This is consistent with reports of increased CD40 ligand expression in RA synovial tissue compared to healthy synovial biopsies with subsequent increases in CD40L-responsive genes (Guo *et al.*, 2017). Moreover, we observe increased expression of chemokine receptors CCR4 and CXCR1 characteristic of an 'M2' macrophage on healthy synovial tissue macrophages, in contrast to reduced expression of inflammatory CCR7 compared to RA macrophages. Therefore, we propose that CD206+CD163+ 'M2'-like macrophage subset dominates the synovium in the steady state, however, in response to inflammation and initiation of RA disease, this subset transforms to acquire the activation marker CD40 and inflammatory CCR7 and thus becomes dysfunctional.

To investigate site specific macrophage phenotypes, we compared macrophage subsets between RA synovial tissue and synovial fluid. Indeed, striking differences in the

number and composition of macrophage subsets in RA synovial fluid and synovial tissue were observed highlighting microenvironmental and site-specific functions of RA macrophages. SPICE analysis demonstrated that the dominant CD206+CD163+ macrophage subset is significantly enriched in RA synovial tissue compared to fluid along with co-expression of CD40. Using the tSNE algorithm, we clearly observe distinct clusters of CD163+ macrophages unique to RA synovial tissue and patient-matched fluid, respectively. This is the first study to identify significant differences in macrophage subsets between RA synovial fluid and tissue. While studies have examined levels of macrophages-derived cytokines in synovial fluids or synovial tissue in different arthropathies (Gómez-Puerta *et al.*, 2013), no direct comparisons of cellular composition between RA synovial tissue biopsies and fluid have been explored. To further support these findings, RNA-seq analysis of RA synovial tissue and synovial fluid CD206+CD163+ sorted macrophages demonstrated that they are transcriptionally distinct with specific gene signatures, thus suggesting analysis of RA synovial fluid does not clearly reflect the pathophysiological state of the RA synovial tissue.

Moreover, in this study we identified that a spectrum of macrophage activation states exists within the inflamed joint rather than the classic paradigm of the binary M1/M2 polarisation states. Given the striking expression of CD40 on the dominant subpopulation of CD206+CD163+ macrophages we hypothesised that these cells are an intermediate subset of tissue macrophages residing in the middle of a wide spectrum of macrophage phenotypes. To formally address this, we investigated whether CD206+CD163+ macrophages were more similar to M1 or M2 polarised macrophages using RNA-sequencing analysis. Indeed, we reveal that CD206+CD163+ synovial tissue macrophages display a gene signature distinct from both monocyte-derived M1 and M2 macrophages indicating that they are a transcriptionally pure intermediate macrophage subset. Therefore, the M1/M2 dichotomous system is an oversimplification of a more complex reality, and so a broader spectrum of macrophage activation states should be considered for future research.

The concept that all macrophages are derived from circulating monocytes was held in common belief for many years and resulted in a large proportion of research focusing on the functional characterisation of monocyte-derived macrophages and the

binary M1/M2 framework (Davies and Taylor, 2015). However, it is now evident that many tissue macrophage population are seeded during embryonic development independent of haematopoiesis and are capable of self-renewal in the steady state (Merad *et al.*, 2002; Hashimoto *et al.*, 2013; Ginhoux and Guilliams, 2016). This new appreciation of macrophage ontogeny has left a huge chasm in our understanding of the origin and contribution of synovial tissue macrophages. Considering the marked difference in activation status and transcriptional variance observed in CD206+CD163+ macrophage subset residing in the synovial tissue compared to synovial fluid, it is tempting to speculate that this CD206+CD163+ synovial tissue macrophages are in fact tissue-resident. Indeed, CD163+ macrophages are thought to identify macrophages at an advanced maturation stage, such as tissue-resident macrophages (Sánchez *et al.*, 1999; De Rycke *et al.*, 2005). Moreover, histological studies reveal CD163+ macrophages are unaffected by TNFi treatment (De Rycke *et al.*, 2005), this persistence may be a further indication of tissue residency. Elucidating the exact origins of RA synovial tissue macrophages may provide opportunities to preferentially manipulate macrophages from a specific origin. For example, specific targeting of inflammatory infiltrating macrophages may be achieved with minimal impact on homeostatic tissue-resident macrophages (Davies *et al.*, 2013).

To date a clear distinction between tissue-resident and infiltrating monocyte-derived macrophages has not yet been possible. However certain genetic signatures are associated with tissue residency and monocyte-derived macrophages respectively. We demonstrate significant enrichment of genes associated with tissue residency; *TREM2*, *FOLR2*, *LYVE1*, *C1QA* and *TIMD4* were observed in synovial tissue CD206+CD163+ macrophages with monocyte core genes *CCR2* and *CD48* reciprocally significantly blunted in CD206+CD163+ when compared to the double negative CD206-CD163- macrophage subset. Studies indicate that the TIMD4+LYVE+MHCIIloCCR2- macrophage subpopulation are resident in the adult myocardium and are maintained independent of blood monocytes (Dick *et al.*, 2019). Another study indicates that *TREM2* expression is crucial for acquisition of resident macrophage function in the liver (Coelho *et al.*, 2019). *FOLR2* has been identified as a marker for populations of 'resident-like' aortic macrophages (Cochain *et al.*, 2018; Lin *et al.*, 2019). Further strengthening this observation, we

demonstrate increased expression of tissue-resident genes in CD206+CD163+ macrophages compared to macrophages derived from monocytes, thus underscoring the tissue-resident origin of the CD206+CD163+ subset. Moreover, these core tissue-resident genes are especially increased in CD206+CD163+ macrophages from synovial tissue and not synovial fluid, indicating that the phenotypic and functional differences observed between synovial tissue and fluid macrophages may be due to differing ontogeny.

Considering the crucial role that metabolic reprogramming plays in shaping *in vitro* macrophage polarisation, as examined in Chapter 2, the metabolic demands and the extent to which synovial tissue macrophages are metabolically plastic remains to be elucidated. RNA-seq analysis indicated distinct differential expression of metabolism associated genes between CD206+CD163+ and CD206-CD163- macrophages with significant increases in glycolytic genes in the double negative population. Furthermore, using a novel metabolic FLIM assay we were able to assess the metabolic competence of RA synovial tissue macrophages for the first time. We demonstrated a clear distinction in the metabolic demands of synovial tissue macrophage subsets whereby CD206+CD163+ synovial tissue macrophages utilise oxidative phosphorylation as their main source of energy, while the CD206-CD163- macrophages preferentially utilise glycolysis. The metabolic signature of synovial tissue macrophages not only gives an insight into their activation state but may also shed light on their origin. There has been speculation that tissue-resident macrophages may exhibit a metabolic profile akin to M2 macrophages. Microglia, tissue-resident macrophages residing in the brain, have been shown to display low steady-state metabolic demands (Artyomov, Sergushichev and Schilling, 2016). It is though that the infiltrating inflammatory macrophages are short-lived, terminally differentiated cells dependant on glycolysis for rapid immune activation while this short-term metabolic fix would possibly not support the *in vivo* functions and self-renewal capacities of tissue-resident macrophages, most likely requiring a bioenergetically 'healthy' metabolic profile (Italiani and Boraschi, 2014; Van den Bossche and Saraber, 2018).

Finally, we identified a pathogenic role for CD206+CD163+ macrophages in the RA synovium. We demonstrated that CD40 expression on CD206+CD163+ macrophages significantly correlate with increased RA disease severity. In addition, we demonstrate

that this dominant macrophage population spontaneously secrete high levels of pro-inflammatory mediators IL-6, IL-8 and TNF α . In contrast, these macrophages secrete extremely low amounts of anti-inflammatory mediators IL-10, IL-4 and IL-13 (less than 5pg/mL) (data not shown in this thesis). Considering that CD40 is a strong co-stimulatory molecule for T cell activation we next co-cultured CD206+CD163+ macrophages with autologous synovial tissue T cells. Enhanced activation of T cells is observed with increased expression of GM-CSF, TNF α and IL-17. Although this is the first study to examine the effect of specific macrophage subsets on T cell activation, previous *ex vivo* studies have demonstrated that macrophage-like synoviocytes isolated from RA synovial tissue are capable of stimulating T cells in an autologous system (Klareskog *et al.*, 1982). In this study we demonstrate that CD206+CD163+ macrophages can promote IL-17 production in T cells. This is consistent with other studies indicating that synovial fluid myeloid cells can steer infiltrating T cells towards a Th17 response in RA (Egan *et al.*, 2008; Evans *et al.*, 2009; Yoon *et al.*, 2014; Roberts, Dickinson and Taams, 2015).

In summary, we have identified enrichment of a previously undescribed transitional population of tissue-resident macrophages in the RA synovium capable of contributing to disease pathology. Further examination of the cross-talk between tissue-resident macrophages and other pathogenic cells of the inflamed synovium will fully elucidate the role of this population. Moreover, uncovering the molecular patterns and cues that transform this immunoregulatory macrophage population into a dysfunctional inflammatory activation state may provide opportunities to reinstate joint homeostasis in RA patients.

CHAPTER FOUR:

STAT3 mediates the differential effects of Oncostatin M and TNF α on RA synovial fibroblast and endothelial cell function.

4.1 Introduction

The inflamed microenvironment is highly complex, with numerous cells interacting with each other in order to drive the pro-inflammatory response. In the previous chapters we demonstrated that inflammatory macrophages display a hyper-metabolic phenotype coupled with secretion of many key pro-inflammatory cytokines and impaired phagocytic function compared to anti-inflammatory macrophages, effects which are exacerbated when comparing RA to healthy inflammatory macrophages. In addition we have explored the spectrum of macrophage subsets that exist in the inflamed RA synovium. These synovial tissue macrophages interact with many different immune cells at the site of inflammation, such as T cells. Synovial tissue CD3⁺ T cells release pathogenic cytokines TNF α , IL-17 and GM-CSF when co-cultured with the dominant CD206⁺CD163⁺ macrophage subtype in an autologous system. However, macrophages interact with a myriad of cell types, not just immune cells. Stromal cells, which include endothelial cells (ECs) and the invasive synovial fibroblast cells are also regulated by macrophages via the secretion of pro-inflammatory mediators. As demonstrated Chapter 2, when exposed to inflammatory stimuli, macrophages secrete a wide variety of cytokines such as TNF α , IL-1 β , IL-6, IL-8, OSM, CXCL10, CXCL11 and IL-27. This portfolio of cytokines is crucial to the function of macrophages and all of these molecules, in concert, drive the inflammatory response observed in RA (Duque and Descoteaux, 2014). One such cytokine, critically involved in RA pathogenesis, is the IL-6 like cytokine Oncostatin M (OSM). Having previously noted a significant increase in OSM in pro-inflammatory M1 macrophages compared to M2, and interestingly a significant induction of OSM in RA M1 compared to healthy, we decided to further investigate the role of this enigmatic cytokine on stromal cell activation in RA.

OSM was first biochemically characterised as a cytokine released from macrophage differentiated U-937 lymphoma cells that exhibited antiproliferative activity on the A371 human melanoma cells (Zarling *et al.*, 1986). Activated macrophage and monocytes are the primary source of OSM. It is highly expressed in the RA joint, and shares a common receptor signal subunit (gp-130) with IL-6-type cytokines (Gearing *et al.*, 1992). Increased expression of OSM is associated with a plethora of pathologies including atherosclerosis, psoriasis and many cancers (Richards, 2013; Hermanns, 2015). Most recently, OSM has been shown to play a role in inflammatory bowel disease (IBD) with a

study demonstrating heightened expression of OSM and its receptor in the inflamed IBD intestine, correlating with disease severity (West *et al.*, 2017).

OSM signals via the Janus Kinase (JAK) family of receptor-associated tyrosine kinases and is associated with the activation of STAT3 (O'Shea, 1997; Hurst *et al.*, 2002; Fossey *et al.*, 2011; Richards, 2013). Activation of the JAK-STAT signalling pathway is a critical event in the pathogenesis and progression of RA. Indeed, Tofacitinib, an oral JAK1/3 inhibitor, has been approved for the treatment of RA and has demonstrated clinically meaningful improvements in patients with an inadequate response to TNFi and MTX naïve patients (Lee *et al.*, 2014; Strand *et al.*, 2017; Winthrop, 2017). In addition, studies suggest that JAK-STAT signal transduction is at the centre of an intricate interplay between synovial inflammation and cellular bioenergetics with our group demonstrating that synovial hypoxia is associated with pSTAT3 activation (Gao *et al.*, 2015). Furthermore, our group has also recently reported evidence that inhibition of JAK-STAT signalling switches the metabolic and inflammatory profile of synovial cells away from pathogenic mechanisms and towards resolution of inflammation (McGarry *et al.*, 2018).

In the context of RA, overexpression of OSM in synovial fluid and tissue has been observed with levels correlating with joint inflammation (Okamoto *et al.*, 1997). Collectively, studies have demonstrated that OSM plays a critical role in RAFLS activation, promotion of angiogenesis, adhesion molecules and chemokines from RAFLS, altering the matrix metalloproteinases (MMP)/tissue inhibitor of matrix metalloproteinases (TIMP) ratio and inducing RANKL in RAFLS and chondrocytes in favour of joint destruction (Hui *et al.*, 2003; Fearon *et al.*, 2006; Richards, 2013). Blocking OSM in a collagen-induced arthritis mouse model improves joint inflammation and cartilage damage (Plater-Zyberk *et al.*, 2001). Furthermore, recent studies have demonstrated that inhibition of OSM-induced RAFLS pro-inflammatory mechanisms and cartilage degradation are rescued in the presence of JAK-STAT inhibitors (Kjelgaard-Petersen *et al.*, 2018; Diller *et al.*, 2019), effects that are, in part, mediated by a switch in the metabolic profile of the cell (McGarry *et al.*, 2018). Conversely, OSM is a pleiotropic cytokine often displaying divergent effects with both pro- and anti-inflammatory effects depending on the cell type and microenvironment. Previous studies have shown that OSM can inhibit IL-1-induced IL-8 and granulocyte macrophage colony stimulating factor (GM-CSF) and promote TIMP

expression in RAFLS (Richards *et al.*, 1996; Fearon *et al.*, 2006). OSM inhibits TNF α - and IL-17A-induced TIMP-1 while potentiating IL-1 β -induced TIMP-1 expression in RAFLS (Fearon *et al.*, 2006; Moran *et al.*, 2009a). More recently, studies have demonstrated that OSM can inhibit Th17 differentiation in mouse models of arthritis through reciprocal regulation of SOCS3, STAT3, and STAT5 (Son *et al.*, 2017). Therefore, the role of OSM in RA disease pathology is complex, depending largely on cell type and microenvironment.

Adding to this complexity, the effect of these cytokines on the cell may alter the metabolic profile and thus the phenotype of the cell. Indeed, the metabolic milieu of the inflamed joint reflects the chronically active state of immune and stromal cells, with elevated lactate levels and reduced glucose observed in RA synovial fluid, along with increased glycolytic enzyme activity and accumulation of succinate in synovial fluid and tissue (Naughton *et al.*, 1993; Ciurtin *et al.*, 2006; Kim *et al.*, 2014; Biniecka *et al.*, 2016; Bustamante *et al.*, 2017). Thus, in this study we examined the effect of OSM on pro-inflammatory, angiogenic and bioenergetic mechanisms in RAFLS and HUVEC. Furthermore, we investigated the relationship between OSM and the major pro-inflammatory cytokine; tumour necrosis factor α (TNF α), a central player in inflammation and destruction in the RA joint.

4.2 Specific Aims of this Chapter:

- To investigate the effects of macrophage-derived OSM on stromal cell pro-inflammatory and angiogenic function
- To elucidate the role of OSM on cellular bioenergetics in RAFLS and HUVEC
- To investigate the relationship between OSM and TNF α in driving stromal cell pro-inflammatory and metabolic mechanisms
- To examine the role of STAT3 in mediating the synergistic effects of OSM and TNF α

4.3 Materials and Methods

4.3.1 Patient Recruitment and Arthroscopy

RA patients were recruited from the Rheumatology Department, St. Vincent's University Hospital. All patients gave fully informed written consent approved by the St Vincent's University Hospital, Ethics and Medical Research Committee and research was performed in accordance with the Declaration of Helsinki. Synovial tissue biopsies were obtained at arthroscopy under local anaesthetic using a Wolf 2.7 mm telescope (Wolf - Germany) as previously described (Ng *et al.*, 2010). Biopsies were utilized for isolation of primary RA synovial fibroblasts (RAFLS).

4.3.2 Isolation of Primary Fibroblasts

RA synovial biopsies were digested with 1 mg/mL collagenase type 1 (Worthington Biochemical, Freehold, NJ, USA) in RPMI-1640 (Gibco-BRL, Paisley, UK) for 4 hr at 37°C in humidified air with 5% CO₂. Dissociated cells were grown to confluence in RPMI 1640, 10% FCS (Gibco-BRL), 10 mL of 1 mmol/l HEPES (Gibco-BRL), penicillin (100 units/mL; Bioscience), streptomycin (100 units/mL; Bioscience) and fungizone (0.25 µg/mL; Bioscience) before passaging. Cells were used between passages 3–8.

4.3.3 Culture of HUVEC

Human umbilical vein endothelial cells (HUVEC) (ATCC, Manassas, USA) were grown to confluence in endothelial cell basal media (MCDB-131, Gibco) supplemented with L-glutamine (20 mL of 100X solution), hydrocortisone (0.6 µg/mL), hEGF (0.01 µg/mL), penicillin-streptomycin (100 units/mL and 100 µg/mL; Bioscience), fungizone (0.25 µg/mL; Bioscience) and 15% FCS (Gibco-BRL).

4.3.4 Cytokine and Chemokine Measurements

To assess the effects of OSM on pro-inflammatory mediators, RAFLS/HUVEC were seeded in 48 well plates at a density of 3×10^5 and allowed to attach overnight. Cells were incubated in serum-free RPMI-1640 or MCDB-131 for 24 hr and subsequently stimulated with OSM (10 ng/mL). For synergy experiments, cells were also incubated in the presence

or absence of TNF α (0.01 ng/mL, 0.1 ng/mL, 1 ng/mL). Supernatants were harvested and levels of IL-6, MCP-1, IL-8, RANTES and GRO α were measured by specific ELISA (MCP-1: eBiosciences, USA, IL-6, IL-8, RANTES, GRO α ; R&D systems, UK) according to manufacturer's conditions.

4.3.5 Transwell Invasion Assay

Biocoat Matrigel™ Invasion Chambers (Becton Dickinson, UK) were used to assess RAFLS/HUVEC invasion. Cells were seeded at a density of either 3.5×10^4 (RAFLS) or 2.5×10^4 (HUVEC) cells per well in the migration chamber on 8 μ m membranes pre-coated with matrigel. Cells were incubated with OSM (10 ng/mL) for 24 hr (HUVEC) or 48 hr (RAFLS). Non-migrating cells were removed from the upper surface by gentle scrubbing. Migrating cells attached to the lower membrane were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Cells from five random high power fields for each well were counted to assess the average number of invading cells.

4.3.6 HUVEC Tube Formation

Matrigel (50 μ L; BD Biosciences, San Jose, CA, USA) was plated in 96 well culture plates after thawing on ice and allowed to polymerise for 30 min at 37°C in humidified air with 5% CO₂. 2×10^4 cells in supplemented MCDB-131 was added to each well and cells were stimulated with OSM (10 ng/mL) on control medium for 24 hr. EC tubule formation was then assessed using phase-contrast microscopy. Cells were quantified by counting the number of connecting branches formed from five random high power fields as previously described (Fearon *et al.*, 2006).

4.3.7 Adhesion Assay

RAFLS/HUVEC were grown to confluence in 24 well plates, incubated in serum-free RPMI-1640 or MCDB-131 for 24 hr and subsequently stimulated with OSM (10 ng/mL) for a further 24 hr. PBMC from healthy donors were isolated by density gradient centrifugation (Lymphoprep; Stemcell Technologies) according to the manufacturer's recommendations. PBMC were then resuspended in MCDB-131. 7.5×10^4 PBMC were added to each well containing RAFLS/HUVEC and incubated at 37°C with 5% CO₂ for 1 hr.

After the incubation time, supernatants were removed and wells were washed with PBS. Semi-quantification was performed by counting adherent PBMCs as viewed under phase-contrast microscopy (Leica, Germany) at 10X magnification. Cells from five random high power fields for each well were counted to assess the average number of adherent cells.

4.3.8 mRNA Extraction and cDNA Synthesis

To assess the effects of OSM on specific genes, RAFLS/HUVEC were seeded into 6 well plates and allowed to grow to confluence. Cells were incubated in serum-free RPMI-1640 or MCDB-131 for 24 hr and subsequently stimulated with OSM (10 ng/mL). For synergy experiments, cells were also incubated in the presence or absence of TNF α (1 ng/mL). Total RNA was isolated using the miRNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. The purity of RNA samples was assessed using a NanoDrop 2000 spectrophotometer (ThermoFisher). Samples with a 260:280 nm ratio of 1.8 and above and an RNA integrity number between 7 and 10 were used in subsequent experiments. Isolated RNA was stored at -80°C. Total RNA (100 ng) was reverse transcribed to cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, Cheshire, UK).

4.3.9 Gene Expression Analysis

Gene expression data were quantified by RT-PCR using the Quant Studio 5 Thermal Cycler (Applied Biosystem, Lewes, UK). PCR reaction mixtures contained 1 μ L of cDNA, SYBR Green II Universal Master Mix (Thermo Fisher Scientific, USA), RNase-free water and 10 μ M of target mRNA specific primer pairs as observed in Table 4.1. Relative quantification of gene expression was analysed with pre-optimised conditions using the Quant Studio 5 PCR machine (Applied Biosystems, Lewes, UK). All reactions/negative controls were performed in triplicate in a 96 well plate format. Thermal cycling conditions were as recommended by the manufacturer (Applied Biosystems). Samples lacking multiscribe reverse transcriptase formed negative controls to ensure target-specific quantification. Data were analysed using the comparative threshold cycle (Ct) method with normalisation to the expression of *RPLPO* and *HPRT1* as endogenous controls. Relative changes in gene expression were determined using either the $-\Delta$ Ct or Pfaffl method.

Gene Name	Forward Primer	Reverse Primer
<i>RPLPO</i>	5' GCGTCCTCGTGGAAGTGACATCG 3'	5' TCAGGGATTGCCACGCAGGG 3'
<i>HPRT1</i>	5' ATGGACAGGACTGAACGTCTTG 3'	5' GGCTACAATGTGATGGCCTC 3'
<i>VEGF</i>	5' GCAGAATCATCACGAAGTGGTG 3'	5' TCTCGATTGGATGGCAGTAGCT 3'
<i>VCAM-1</i>	5' GTAAAAGAATTGCAAGTCTACATATCAC 3'	5' GATGGATTCACAGAAATAACTGTATTC 3'
<i>ICAM-1</i>	5' AACCAGAGCCAGGAGACACTG 3'	5' GCGCCGAAAGCTGTAGATG 3'
<i>HIF1α</i>	5' GAAACTTCTGGATGCTGGTGATTT 3'	5' GCAATTCATCTGTGCTTTCATGTCA 3'
<i>HK2</i>	5' TTCTTGTCTCAGATTGAGAGTGAC 3'	5' TTGCAGGATGGCTCGGACTTG 3'
<i>LDHA</i>	5' ATGGAGATTCCAGTGTGCCTGT 3'	5' CAGAGAGACACCAGCAACATTC 3'
<i>GLUT-1</i>	5' CTTCCAGTATGTGGAGCAACTGT 3'	5' GCACAGTGAAGATGATGAAGACG 3'
<i>PFKFB3</i>	5' ACCAAAGATCACCCACGGATGT 3'	5' AGCGAGTGCAGAATGGACACAA 3'
<i>PKM2</i>	5' ATTATTTGAGGAACTCCGCCG 3'	5' ATTCCGGGTCACAGCAATGAT 3'
<i>STAT3</i>	5' TTCCTTGGGTGGAGAAG 3'	5' CGGACTGGATCTGGGTCT 3'

Table 4.1: Designed primer sequences used for real-time PCR analysis.

4.3.10 Cellular Bioenergetic Function Analysis

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), reflecting oxidative phosphorylation and glycolysis respectively, were measured using the Seahorse-XFe96 analyser (Seahorse Biosciences) as described in Chapter 2. Specifically, for this chapter, RAFLS/HUVEC were seeded at 15,000 cells per well in a 96 well cell culture XFe microplate (Seahorse Biosciences) and allowed to adhere overnight. Following this, cells were then cultured with OSM (10 ng/mL) for 3hr. For synergy experiments, cells were also incubated in the presence or absence of TNF α (1 ng/mL) for 3 hr. Cells were then washed and further incubated with Seahorse assay medium (unbuffered DMEM supplemented with 10 mM glucose, pH-7.4) for 1 hr at 37°C in a non-CO₂ incubator before undergoing a Mito Stress test using the Seahorse-XFe96 Analyser. Basal oxidative phosphorylation/glycolysis was calculated by the average of three baseline OCR/ECAR measurements respectively, obtained before injection of specific metabolic inhibitors; oligomycin (ATP-synthase-inhibitor), (2 μ g/mL; Seahorse Biosciences, UK)

trifluorocarbonylcyanide phenylhydrazone (FCCP) (mitochondrial uncoupler) (5 μ M; Seahorse Biosciences) and antimycin A (complex-III inhibitor) (2 μ M; Seahorse Biosciences). Oligomycin was injected to evaluate both the maximal glycolytic rate and ATP synthesis, determined by subtracting the amount of respiration left after oligomycin injection from baseline OCR. FCCP was injected to evaluate the maximal respiratory capacity (average of three measurements following injection). Maximal respiratory capacity was determined by subtracting baseline OCR from FCCP-induced OCR and the respiratory reserve (baseline OCR subtracted from maximal respiratory capacity).

4.3.11 Protein Isolation and Western Blotting Analysis

RAFLS/HUVEC were grown to confluence in 6 well plates. Once confluent, cells were incubated in serum-free RPMI-1640 or MCDB-131 for 24 hr and subsequently stimulated with OSM (10 ng/mL). For synergy experiments, cells were also incubated in the presence or absence of TNF α (1 ng/mL). Cells were trypsinized and collected prior to cell lysis. Ice-cold RIPA (Radio-Immunoprecipitation Assay) buffer (Sigma) containing 10 μ g/mL phosphatase inhibitor cocktail and 10 μ g/mL protease inhibitor cocktail (Sigma) was used to extract protein from HUVEC/RAFLS. Measurement of protein concentration was performed using a BCA assay (Pierce Chemical Co, Rockford, IL, USA). Protein (2-5 μ g) was resolved by SDS-PAGE (5% stacking, 10% resolving), gels were then transferred onto PVDF membranes (Amersham Biosciences, Buckinghamshire, UK) prior to 1 hr blocking in wash buffer (Tris-buffered saline (TBS) with 0.1% Tween-20) containing 3% bovine serum albumin (BSA) with gentle agitation at room temperature. Membranes were incubated with mouse monoclonal anti-HK2 (Novus Biologicals, USA), rabbit monoclonal anti-PFKFB3 (Abcam, UK), rabbit polyclonal anti-GLUT-1 (Abcam), anti-pSTAT3 and anti-total STAT3 (Cell-Signalling Technology, UK) diluted in 53% BSA in TBS-Tween-20 at 4°C overnight with gentle agitation. β -actin (Sigma- Aldrich) was used as a loading control. Following three 15 min washes, membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1:5000) for 3 hr at room temperature. The signal was detected using SuperSignal® West Pico Chemiluminescent Substrate (Amersham Biosciences). Band densities were imaged using the ChemiDoc MP Imaging System (Bio-Rad, USA).

4.3.12 Statistical Analysis

Statistical analyses were performed using Prism 5 software. Wilcoxon Signed Rank test or Mann Whitney was used for analysis of non-parametric data. Student *t*-test was used for parametric data. P-values of less than 0.05 (* $p < 0.05$) were determined as statistically significant.

4.4 Results

4.4.1 OSM differentially regulates pro-inflammatory cytokine and chemokine secretion in RAFLS and HUVEC

To initially assess the effect of OSM on pro-inflammatory mechanisms, a range of pro-inflammatory mediators were measured in RAFLS and HUVEC. OSM significantly induced expression of IL-6, MCP-1, and *ICAM-1* in RAFLS and HUVEC (Figure 4.1-4.2, $p < 0.05$), in addition to the main angiogenic growth factor *VEGF* (Figure 4.1-4.2, $p < 0.05$). In contrast, OSM inhibited the secretion of IL-8 ($p < 0.05$) and GRO α from both RAFLS and HUVEC, with no effect observed for RANTES (Figure 4.1-4.2). Interestingly, OSM induced *VCAM-1* in RAFLS (Figure 4.1), but inhibited *VCAM-1* expression in HUVEC (Figure 4.2, $p < 0.05$). This data demonstrates the differential effects of OSM, displaying both pro- and anti-inflammatory effects in different cell types, but also within the same cell type.

4.4.2 OSM promotes adhesion, invasion and tube formation in RAFLS and HUVEC

To further investigate the role of OSM we next assessed cellular function by performing adhesion, invasion and angiogenic assays. OSM stimulation significantly increased the adhesive capacity of RAFLS and HUVEC, resulting in a significant increase in PBMC attachment to the cell surface of RAFLS and HUVEC (Figure 4.3 (A) and 4.4 (A)). Next, to assess the effects of OSM on RAFLS and HUVEC invasion, Transwell Matrigel™ invasion chambers were utilised. Representative images of increased RAFLS and HUVEC invasion following OSM stimulation are shown in Figure 4.3 (B) and 4.4 (B). Quantitatively, RAFLS and HUVEC invasion were significantly induced by OSM compared to basal control (Figure 4.3 (B) and 4.4 (B), $p < 0.05$). Finally, representative images of HUVEC tube formation are shown in Figure 4.4 (C), demonstrating a significant increase in the formation of tube-like structures in response to OSM ($p < 0.05$).

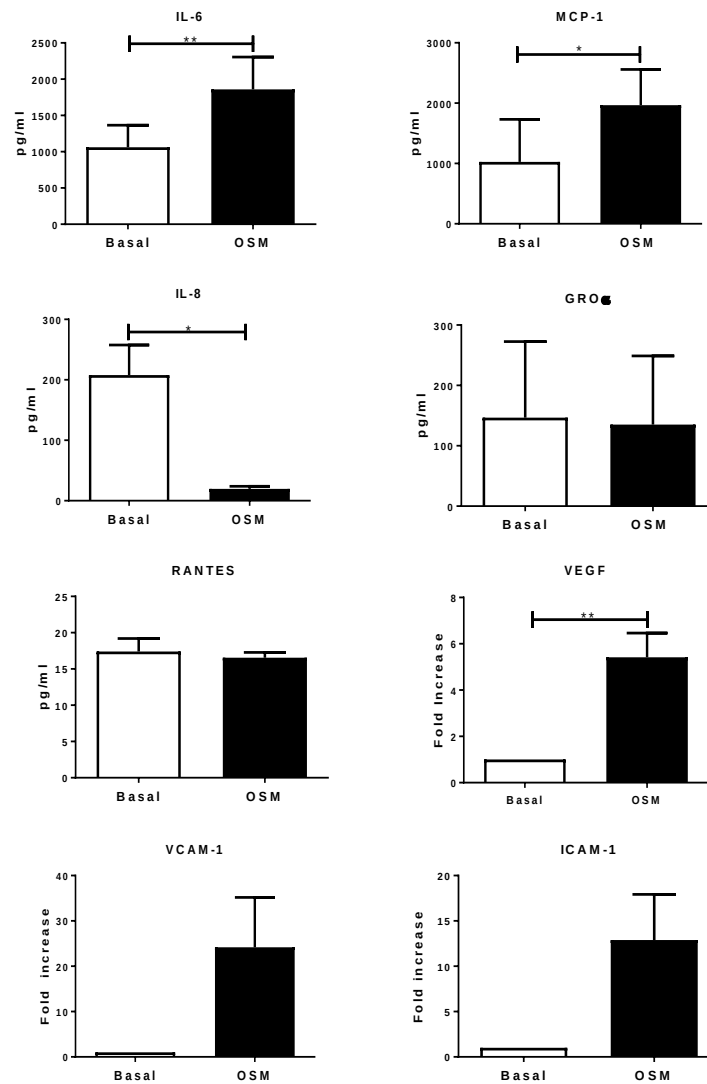


Figure 4.1: The effect of OSM on cytokine/chemokine secretion and angiogenic markers in RAFLS. RAFLS were cultured in the presence of OSM (10 ng/mL) for 24 hr. Bar graphs demonstrating quantification of IL-6, MCP-1, IL-8, GRO α , RANTES secretion in RAFLS, (n=7-10). Gene expression analysis of *VEGF*, *VCAM-1* and *ICAM-1* quantified in RAFLS using Real-time PCR. Fold increase compared to endogenous controls (*RPLPO* and *HPRT1*) (n=6-10). Values expressed as mean $\pm$ SEM using Wilcoxon signed rank and paired *t*-test where appropriate. **p*<0.05, ***p*<0.01, ****p*<0.005 significantly different from basal.

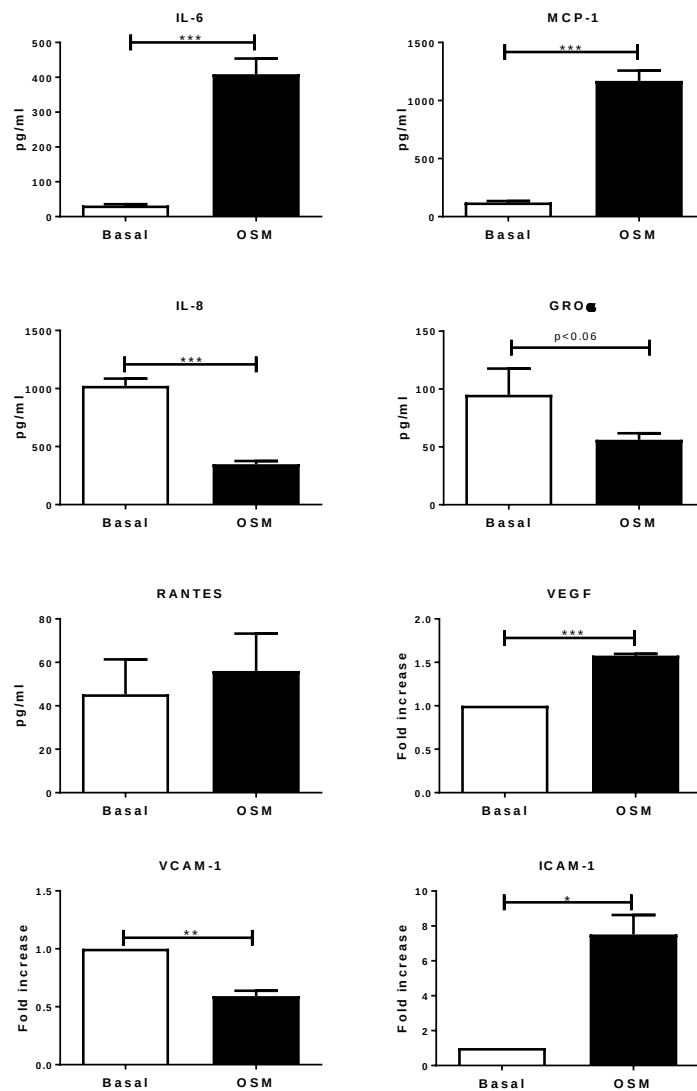


Figure 4.2: The effect of OSM on cytokine/chemokine secretion and angiogenic markers in HUVEC.

HUVEC were cultured in the presence of OSM (10 ng/mL) for 24 hr. Bar graphs demonstrating quantification of IL-6, MCP-1, IL-8, GRO α , RANTES secretion in HUVEC, (n=6-10). Gene expression analysis of *VEGF*, *VCAM-1* and *ICAM-1* quantified in HUVEC using Real-time PCR. Fold increase compared to endogenous controls (*RPLPO* and *HPRT1*) (n=4). Values expressed as mean $\pm$ SEM using paired *t*-test. *p<0.05, **p<0.01, ***p<0.005 significantly different from basal.

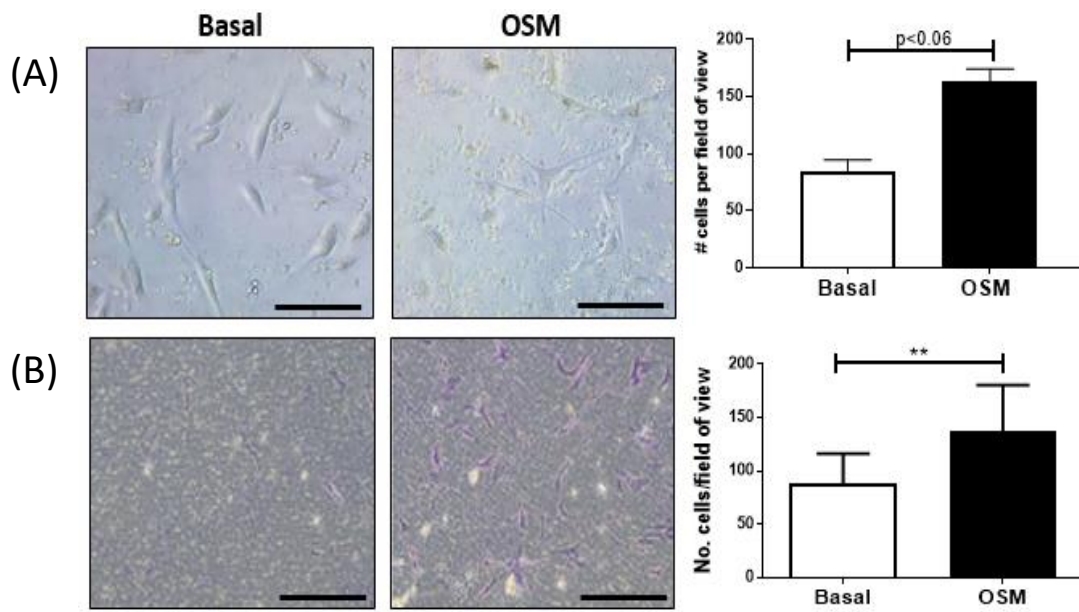


Figure 4.3: The effect of OSM on cell function in RAFLS.

RAFLS were cultured in the presence of OSM (10 ng/mL) for 24 hr. Representative photomicrographs and accompanying bar graphs demonstrating (A) leukocyte adhesion and number of attached cells (n=5), (B) invasion and number of invading cells (n=8) in RAFLS incubated with OSM for 24 hr and 48 hr respectively. Values expressed as mean $\pm$ SEM using Wilcoxon signed rank. *p<0.05, **p<0.01, ***p<0.005 significantly different from basal.

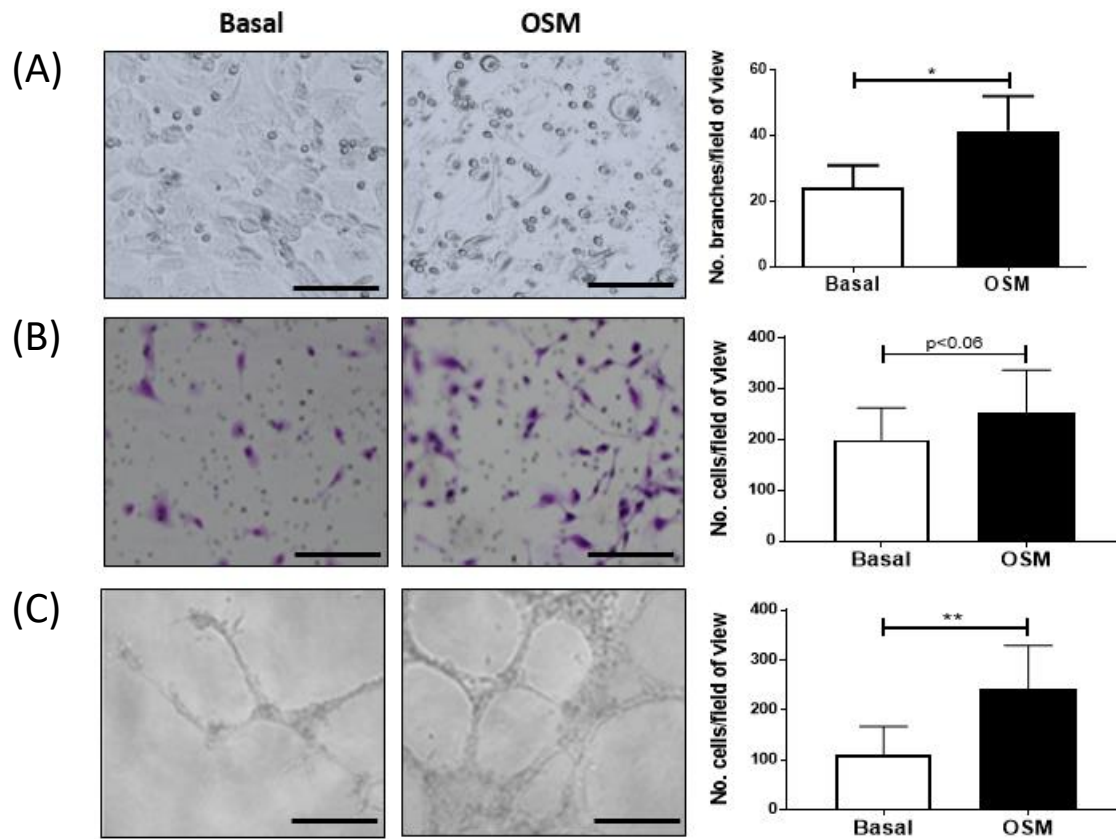


Figure 4.4: The effect of OSM on cell function in HUVEC.

HUVEC were cultured in the presence of OSM (10 ng/mL) for 24 hr. Representative photomicrographs and accompanying bar graphs demonstrating (A) tubule formation and average number of branches (n=3), (B) leukocyte adhesion and average number of attached cells (n=6), (C) invasion and average number of invading cells (n=4) in HUVEC incubated with OSM for 24 hr. Values expressed as mean $\pm$ SEM, paired *t*-test used for HUVEC. **p*<0.05, ***p*<0.01, ****p*<0.005 significantly different from basal.

4.4.3 OSM differentially regulates cellular bioenergetics in RAFLS and HUVEC

To analyse the two major energy pathways, oxidative phosphorylation and glycolysis, in real time the Seahorse XFe-Analyzer was utilized as previously described in Chapter 2. Figure 4.5 (A-B) demonstrates the average bioenergetic profiles for OCR in RAFLS and HUVEC cells before and after injections of mitochondrial inhibitors; oligomycin, FCCP and antimycin A in OSM versus basal control. OSM had no effect on basal respiration in RAFLS, yet increased the maximal respiratory capacity ($p<0.05$), paralleled by a significant reduction in ATP synthesis (Figure 4.5 (A,C), $p<0.05$). OSM had no effect on the OCR profile of HUVEC (Figure 4.5 (B,D)). This was accompanied by a significant shift to a glycolytic profile of both RAFLS and HUVEC, whereby OSM significantly increased baseline glycolysis ($p<0.05$) and maximum glycolytic capacity ($p<0.05$), leading to an overall increase in the ECAR:OCR ratio in favour of glycolysis for both RAFLS (Figure 4.6) and HUVEC (Figure 4.8) (all $p<0.05$). Furthermore, we demonstrated an increase in the glucose transporter GLUT-1 and in HIF1 α (Figure 4.7 and 4.9, $p<0.05$), a master regulator of cellular and systemic homeostatic responses to hypoxia. This glycolytic shift was further supported by the observed increase in key glycolytic enzymes HK2, the first enzyme in the glycolysis pathway, and PFKFB3 (Figure 4.7 and 4.9, $p<0.05$), which is one of the rate-limiting enzymes of glycolysis. *LDHA* and *PKM2* expression were also significantly increased in RAFLS in response to OSM, with no effect observed in HUVEC (Figures 4.7 and 4.9, $p<0.05$).

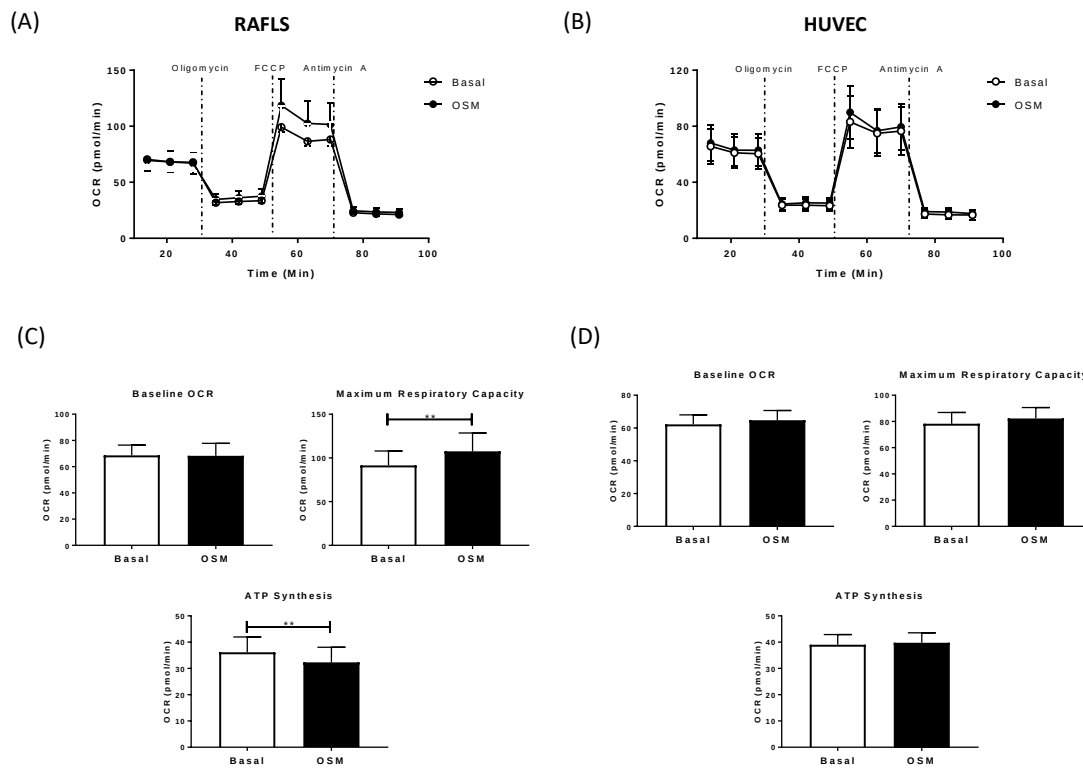


Figure 4.5: OSM has differential effects on mitochondrial respiration in RAFLS and HUVEC.

Average seahorse profiles demonstrating oxygen consumption rate (OCR) (oxidative phosphorylation) in (A) RAFLS (n=8) and (B) HUVEC (n=4), before and after injections of oligomycin, FCCP and antimycin A following 3 hr OSM (10 ng/mL) stimulation. Bar graphs demonstrating baseline OCR, maximal respiratory capacity, and ATP synthesis in (C) RAFLS (n=8) and (D) HUVEC (n=4). Wilcoxon signed rank and paired *t*-test were used for RAFLS and HUVEC respectively. Data is expressed as mean $\pm$ SEM, **p<0.01 significantly different from basal.

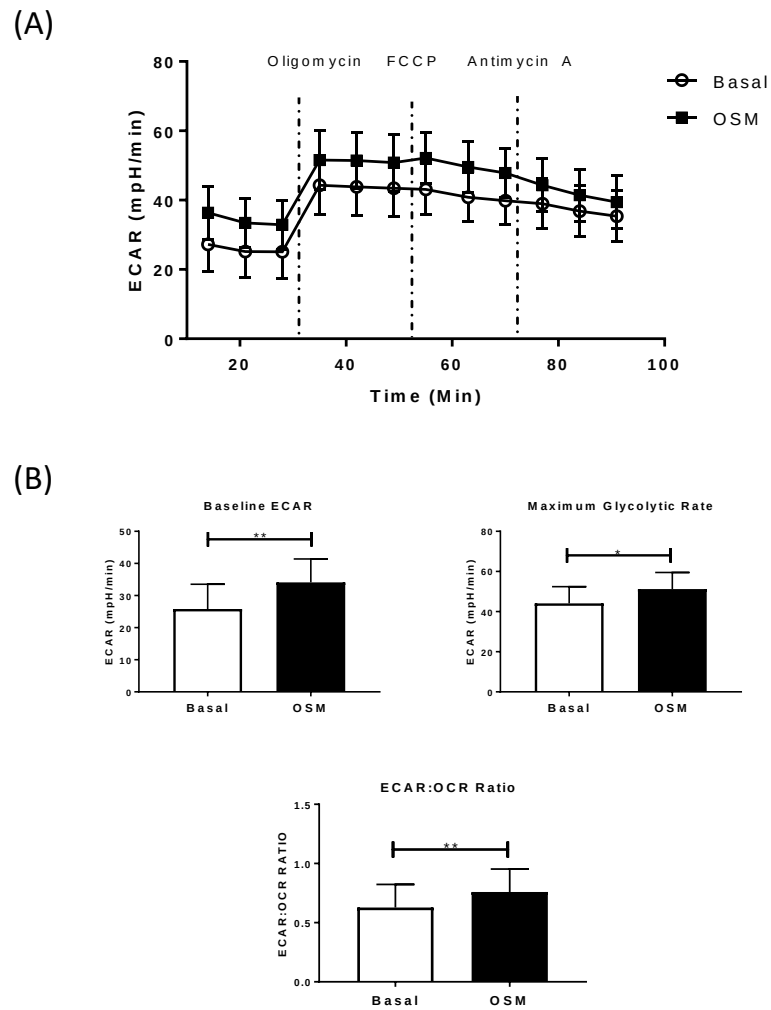


Figure 4.6: OSM induces glycolytic mechanisms in RAFLS.

(A) Average seahorse profiles demonstrating extracellular acidification rate (ECAR) (glycolysis) in RAFLS (n=8), before and after injections of oligomycin, FCCP and antimycin A following 3 hr OSM (10 ng/mL) stimulation. (B) Bar graphs demonstrating baseline ECAR, maximal glycolytic rate and ECAR:OCR ratio in RAFLS. Wilcoxon signed rank used, data expressed as mean $\pm$ SEM, *p<0.05, **p<0.01 significantly different from basal.

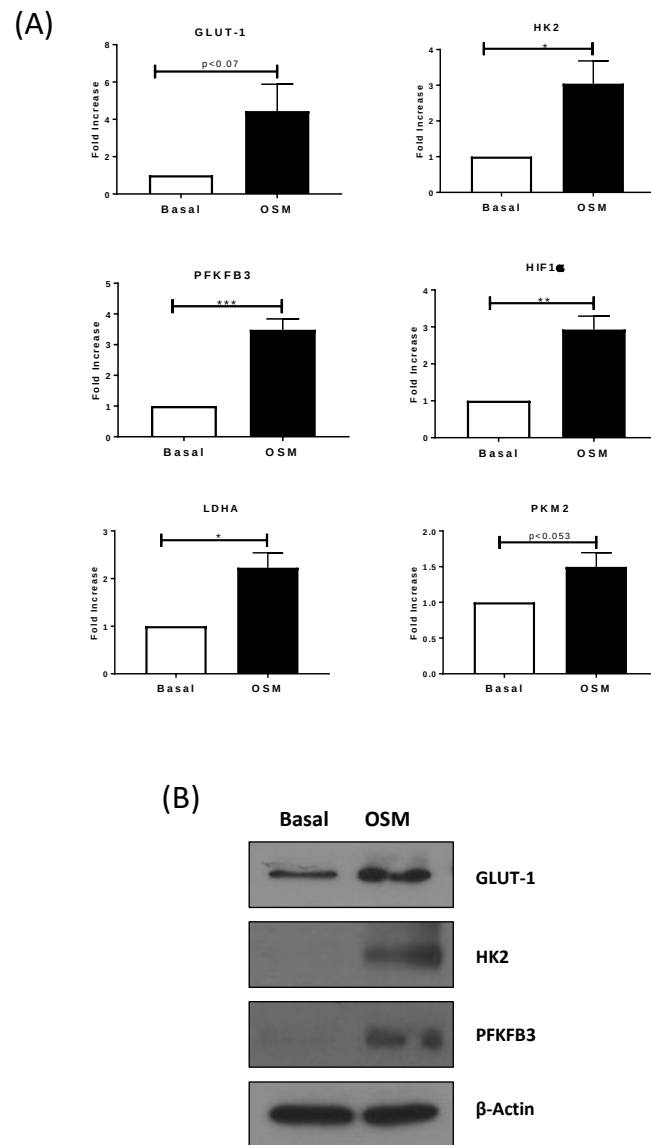


Figure 4.7: OSM induces glycolytic mechanisms in RAFLS.

(A) Bar graphs demonstrating gene expression of *GLUT-1*, *HK2*, *PFKFB3*, *HIF1 α* , *LDHA* and *PKM2* in RAFLS (n=5-6) treated with OSM (10 ng/mL) for 24 hr. Fold increase compared to endogenous controls (*RPLPO* and *HPRT1*). (B) Representative western blot showing GLUT-1, HK2, PFKFB3 in RAFLS, β -actin was used as loading control. Wilcoxon signed rank used. Data expressed as mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ significantly different from basal.

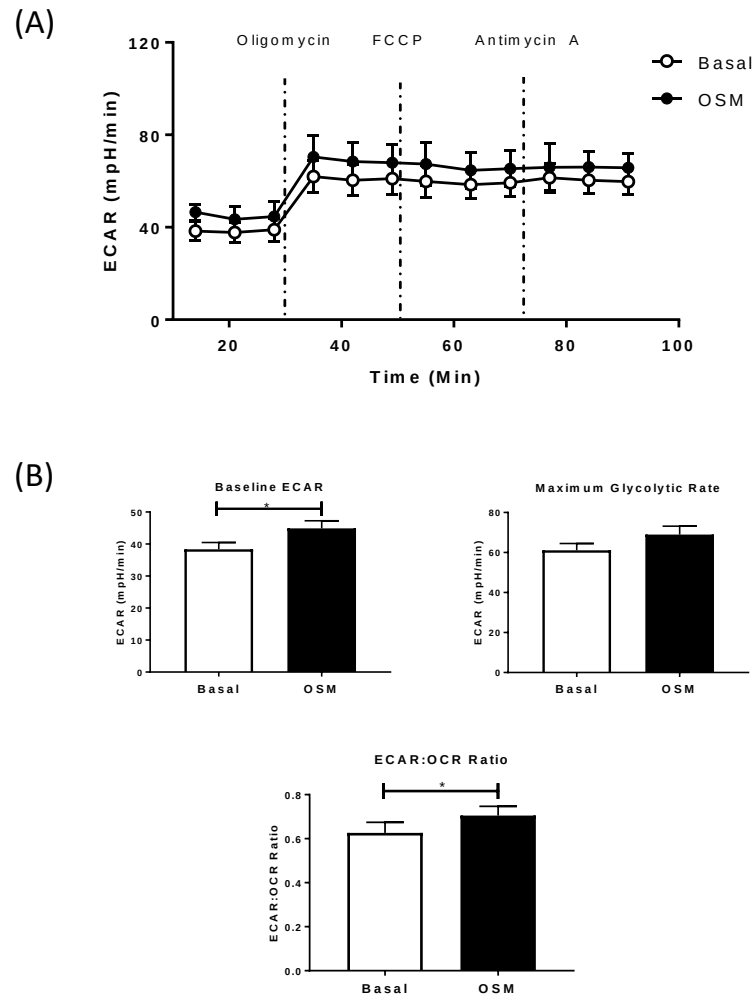


Figure 4.8: OSM induces glycolytic mechanisms in HUVEC.

(A) Average seahorse profiles demonstrating extracellular acidification rate (ECAR) (glycolysis) in HUVEC (n=4), before and after injections of oligomycin, FCCP and antimycin A following 3 hr OSM (10 ng/mL) stimulation. (B) Bar graphs demonstrating baseline ECAR, maximal glycolytic rate and ECAR:OCR ratio in HUVEC. Paired *t*-test used and data expressed as mean $\pm$ SEM, **p*<0.05 significantly different from basal.

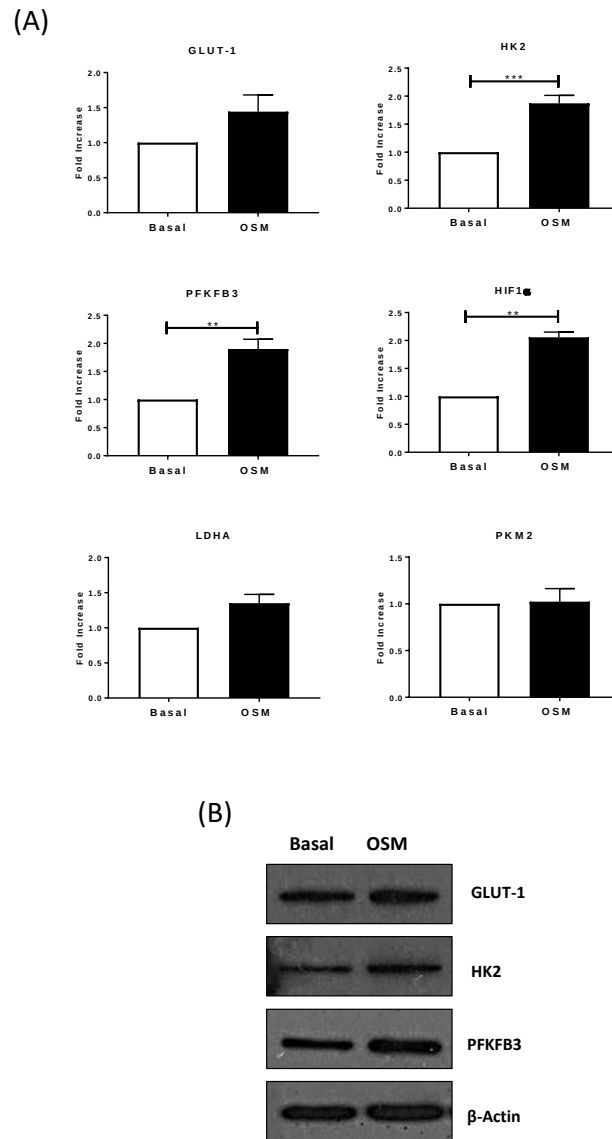


Figure 4.9: OSM induces glycolytic mechanisms in HUVEC.

(A) Bar graphs demonstrating gene expression of *GLUT-1*, *HK2*, *PFKFB3*, *HIF1 α* , *LDHA* and *PKM2* in HUVEC (n=4-8) treated with OSM (10 ng/mL) for 24 hr. Fold increase compared to endogenous controls (*RPLPO* and *HPRT1*). (B) Representative western blot showing GLUT-1, HK2, PFKFB3 in HUVEC, β -actin was used as loading control. Paired *t*-test used and data expressed as mean $\pm$ SEM, **p*<0.05, ***p*<0.01, ****p*<0.005 significantly different from basal.

4.4.4 OSM in combination with TNF α differentially regulates cytokines and chemokines in RAFLS and HUVEC

We have demonstrated that OSM displays differential effects on pro-inflammatory/angiogenic mediators in RAFLS and HUVEC, and shown that in both cell types OSM induces a shift towards glycolysis. Based on previous studies demonstrating the ability of OSM to synergise with other key cytokines within the joint environment, we next examined the effect of OSM in combination with TNF α on these mechanisms. Both RAFLS and HUVEC were cultured with increasing concentrations of TNF α , in the presence or absence of OSM. OSM potentiated the effect of TNF α on both IL-6 ($p < 0.05$) and MCP-1 ($p < 0.05$) at all concentrations in both RAFLS (Figure 4.10) and HUVEC (Figure 4.11). In contrast, OSM inhibited the stimulatory effect of TNF α on IL-8 ($p < 0.05$) and GRO α ($p < 0.05$), with the levels of both chemokines significantly reduced in response to OSM+TNF α compared to TNF α alone (Figure 4.10 and 4.11). Interestingly, OSM alone had no effect on RANTES secretion from both RAFLS and HUVEC, however in combination with TNF α , divergent effects were observed for RAFLS compared to HUVEC. OSM potentiated the effect of TNF α on RANTES secretion (Figure 4.10, $p < 0.05$), however OSM significantly inhibited the effect of TNF α on RANTES in HUVEC (Figure 4.11, $p < 0.05$). These data again show the divergent pro-/anti-inflammatory effects of OSM, and its ability to alter the effects of one of the main cytokines that drives inflammation within the RA joint.

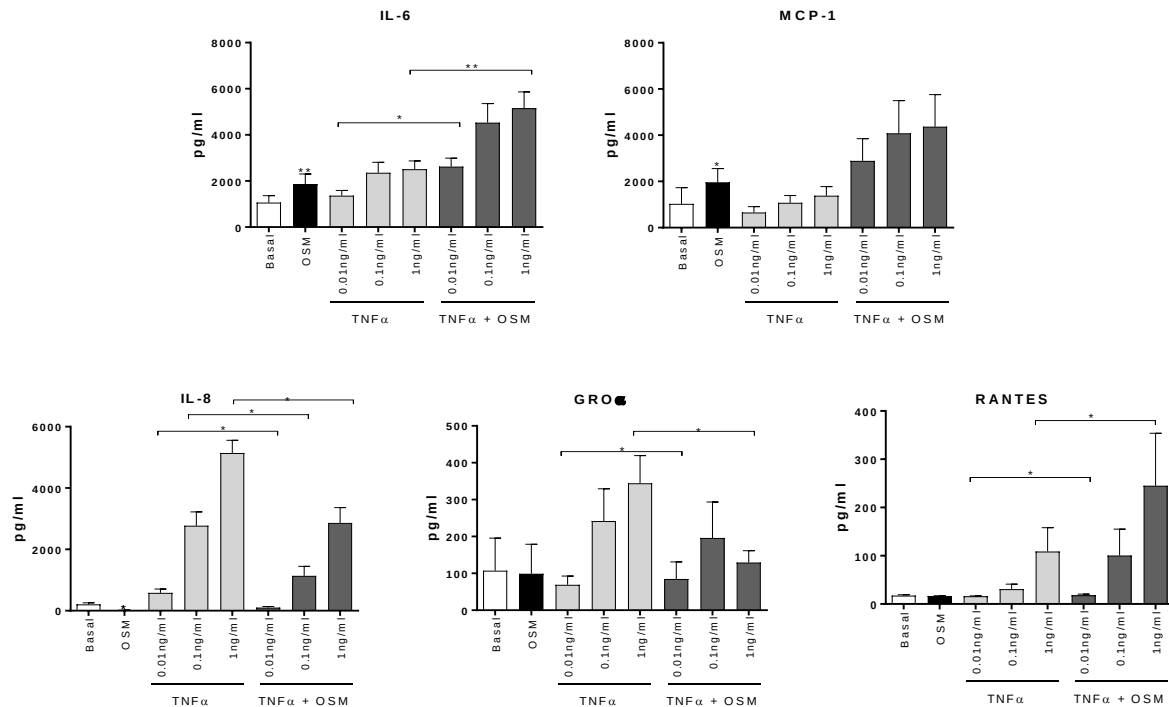


Figure 4.10: OSM in combination with TNFα regulates pro-inflammatory cytokine/chemokine secretion in RAFLS.

RAFLS were cultured in the presence of OSM (10 ng/mL) and increasing concentrations of TNFα (0.01 ng/mL, 0.1 ng/mL, 1 ng/mL) for 24 hr. Bar graphs showing the measured secretion of IL-6, MCP-1, IL-8, GROα and RANTES from RAFLS (n=7-10) following the outlined treatments by ELISA. Values expressed as mean ± SEM, Wilcoxon signed rank used, *p<0.05, **p<0.01, ***p<0.005.

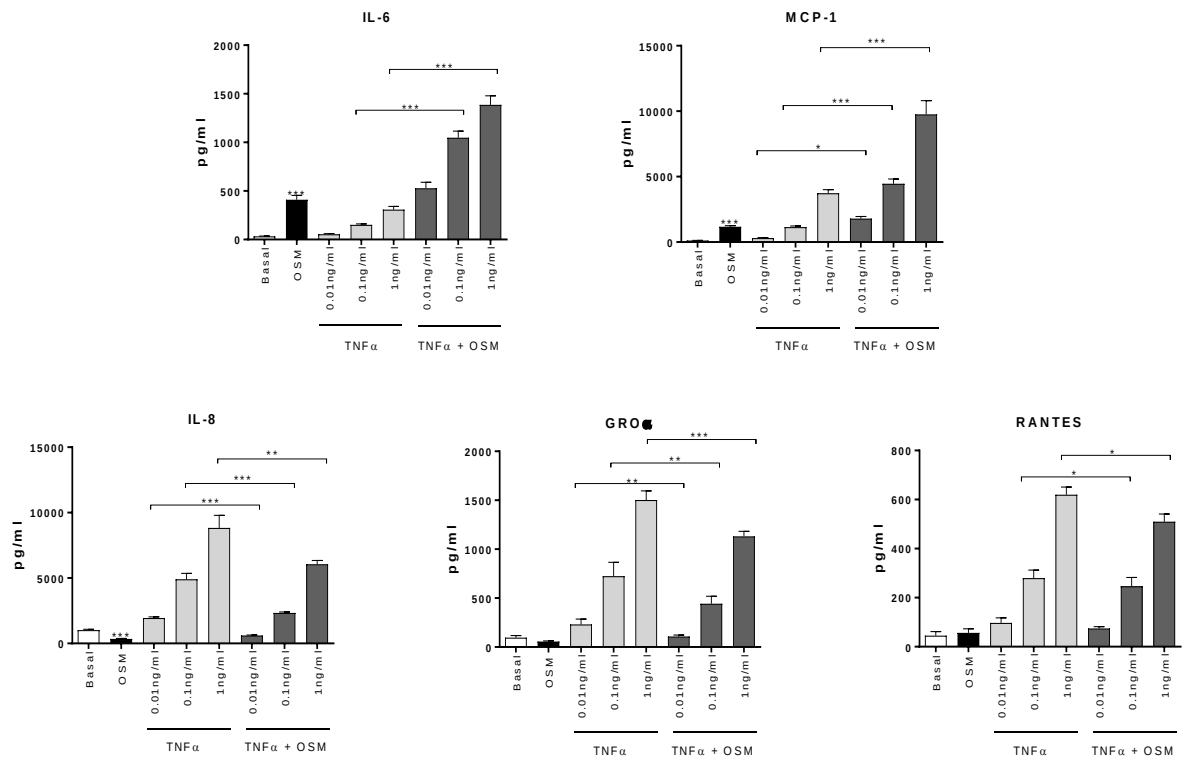


Figure 4.11: OSM in combination with TNFα regulates pro-inflammatory cytokine/chemokine secretion in HUVEC.

HUVEC were cultured in the presence of OSM (10 ng/mL) and increasing concentrations of TNFα (0.01 ng/mL, 0.1 ng/mL, 1 ng/mL) for 24 hr. Bar graphs showing the measured secretion of IL-6, MCP-1, IL-8, GROα and RANTES from HUVEC (n=10-11) following the outlined treatments by ELISA. Values expressed as mean ± SEM, paired *t*-test used, **p*<0.05, ***p*<0.01, ****p*<0.005.

4.4.5 OSM in combination with TNF α regulates metabolic reprogramming in RAFLS, an effect mediated through phosphorylation of STAT3

To further explore the synergistic interaction between OSM and TNF α , we next examined their combined effect on cellular metabolism. While OSM had no effect on baseline OCR in RAFLS, TNF α significantly reduced baseline OCR ($p < 0.05$), an effect further potentiated with the combination of OSM+TNF α (Figure 4.12, $p < 0.05$). Maximum respiratory capacity was significantly reduced in response to the combination of OSM and TNF α (Figure 4.12 (B), $p < 0.05$). Furthermore, the cytokines alone and in combination resulted in a stepwise inhibition of ATP synthesis (Figure 4.12 (B), $p < 0.05$). Conversely, examining glycolytic mechanisms, OSM and TNF α alone and in combination, significantly induced a stepwise progressive increase in baseline glycolysis and the maximal glycolytic rate (Figure 4.13, $p < 0.05$). This resulted in an overall significant increase in the ECAR:OCR ratio in response to both OSM ($p < 0.05$) and TNF α ($p < 0.05$) alone, and further potentiated in response to the combination (Figure 4.13 (B), $p < 0.05$). This metabolic shift was further supported by the increased induction of *GLUT-1*, *HK2* ($p < 0.05$), *PFKFB3* ($p < 0.05$), *HIF1 α* ($p < 0.05$), *LDHA* ($p < 0.05$) and *PKM2* ($p < 0.05$) in response to the combination of cytokines compared to either alone (Figure 4.14). However, no effect was observed for baseline OCR, maximum respiratory capacity or ATP synthesis in HUVEC in response to OSM+TNF α (Figure 4.15 (A-B)). Furthermore, OSM+TNF α resulted in a slight induction in the glycolytic capacity of HUVEC as demonstrated in the ECAR profiles (Figure 4.15 (C-D)) and in the expression of key glycolytic genes (Figure 4.16).

The overall metabolic profile of both RAFLS and HUVEC is shown in Figure 4.17, with a move towards a more glycolytic/energetic profile in response to the combination of OSM and TNF α however there are differences in the mechanisms whereby OCR was inhibited in RAFLS, with no effect observed for HUVEC. Therefore, we next assessed the effect on phosphorylation of STAT3, a key component of the JAK-STAT pathway which mediates OSM signalling. In RAFLS, OSM induced *STAT3* gene expression (Figure 4.18 (A), $p < 0.05$) and STAT3 phosphorylation (pSTAT3) as observed by western blot (Figure 4.18 (C)). TNF α also induced pSTAT3 but to a lesser extent (Figure 4.18). Interestingly, the combination of OSM and TNF α in RAFLS significantly induced *STAT3* gene expression compared to either OSM or TNF α alone (Figure 4.18 (A), $p < 0.05$). Furthermore, the

combination of OSM and TNF α in RAFLS induced activation of STAT3 (Figure 4.18 (C)) in two out three RAFLS. In contrast however, while OSM induced *STAT3* gene expression and protein phosphorylation in HUVEC, the addition of TNF α had no effect either alone or in combination with OSM (Figure 4.18 (B,D)). This suggests that in RAFLS, OSM and TNF α have the ability to act together in the activation of STAT3, an effect that does not occur in HUVEC.

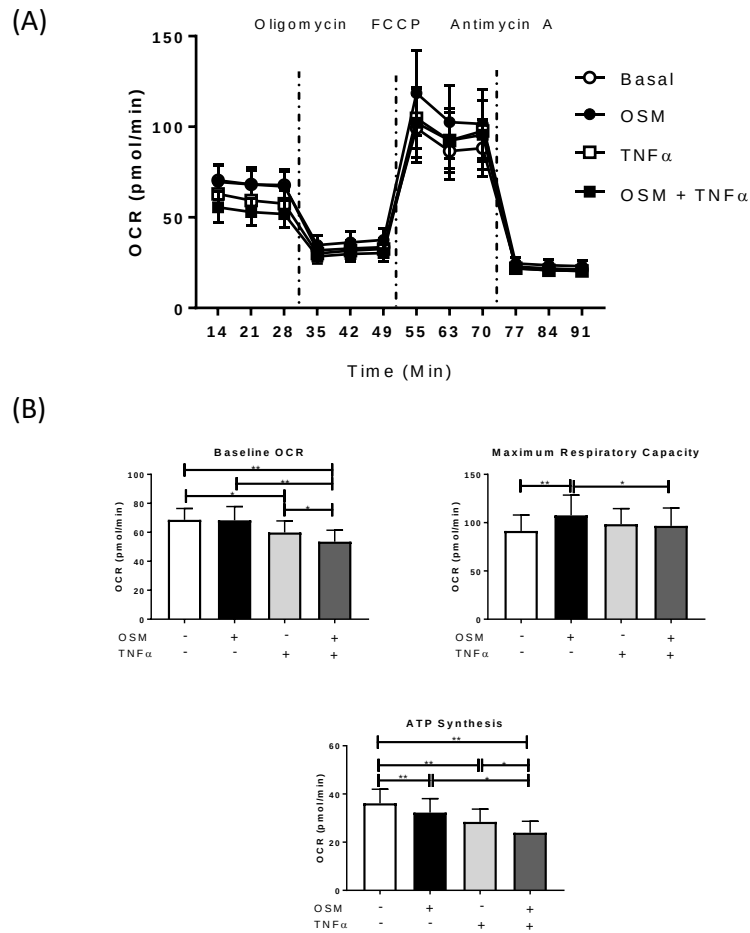


Figure 4.12: OSM in combination with TNF α regulates metabolic reprogramming in RAFLS.

RAFLS were treated with OSM (10 ng/mL) alone or in combination with TNF α (1 ng/mL) for 3 hr. (A) Average seahorse profiles demonstrating oxygen consumption rate (OCR) (oxidative phosphorylation) before and after injections of oligomycin, FCCP and antimycin A in RAFLS (n=8). (B) Bar graphs demonstrating baseline OCR, maximal respiratory capacity, ATP synthesis. Wilcoxon signed rank used for RAFLS. Data expressed as mean $\pm$ SEM, *p<0.05, **p<0.01, ***p<0.005 significantly different.

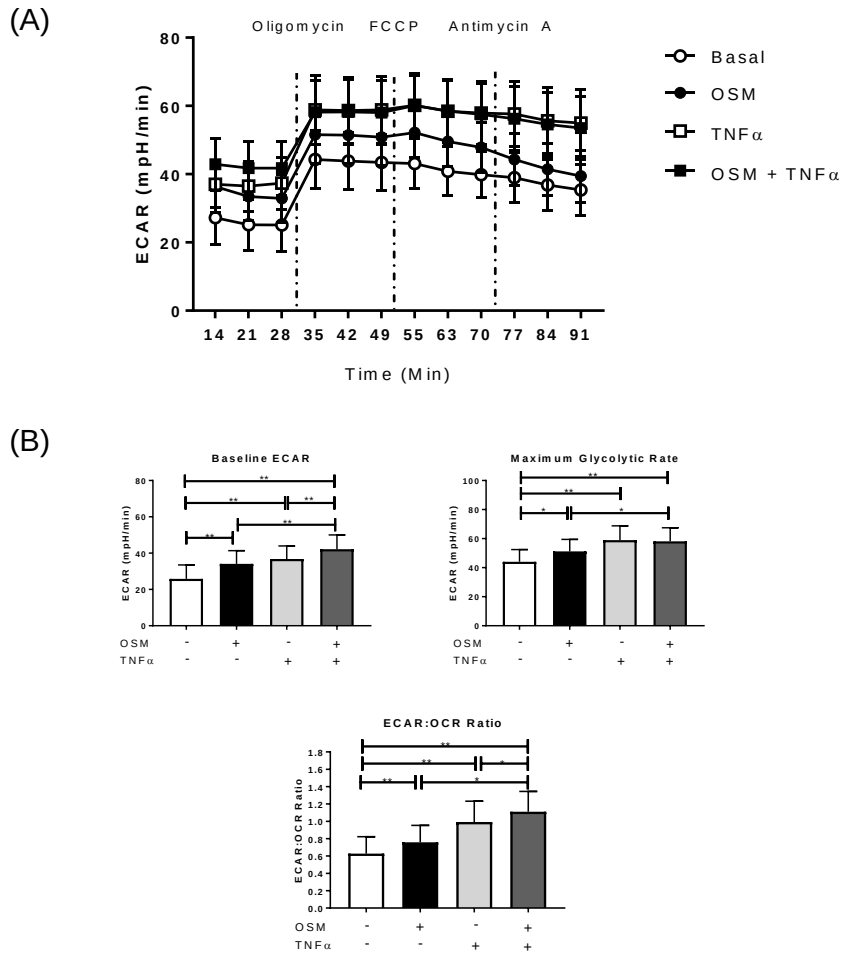


Figure 4.13: OSM in combination with $TNF\alpha$ regulates metabolic reprogramming in RAFLS.

RAFLS were treated with OSM (10 ng/mL) alone or in combination with $TNF\alpha$ (1 ng/mL) for 3 hr. (A) Average seahorse profiles demonstrating extracellular acidification rate (ECAR) (glycolysis), before and after injections of oligomycin, FCCP and antimycin A in RAFLS (n=8). (B) Bar graphs demonstrating baseline ECAR, maximal glycolytic rate and the ECAR:OCR ratio (n=8). Wilcoxon signed rank used for RAFLS. Data expressed as mean $\pm$ SEM, * p <0.05, ** p <0.01, *** p <0.005 significantly different from basal.

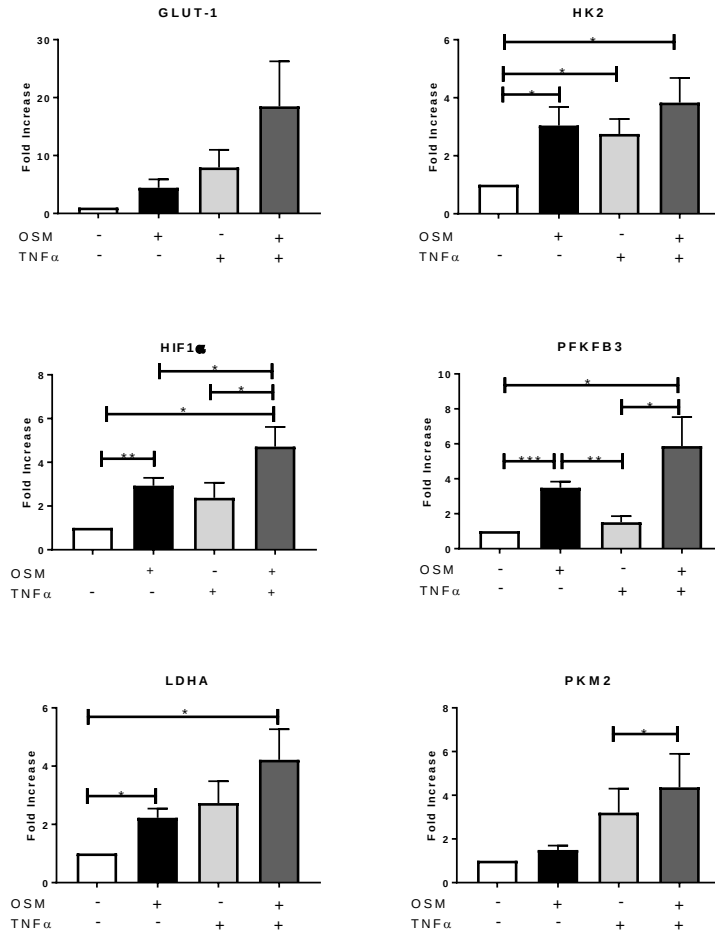


Figure 4.14: OSM in combination with TNF α regulates metabolic reprogramming in RAFLS. Bar graphs demonstrating gene expression of *GLUT-1*, *HK2*, *PFKFB3*, *HIF1 α* , *LDHA* and *PKM2* in RAFLS following treatment with OSM alone or in combination with TNF α for 24 hr (n=6). Fold increase compared to endogenous controls (*RPLPO* and *HPRT1*). Wilcoxon signed rank and paired *t*-test used. Data expressed as mean $\pm$ SEM, *p<0.05, **p<0.01, ***p<0.005 significantly different.

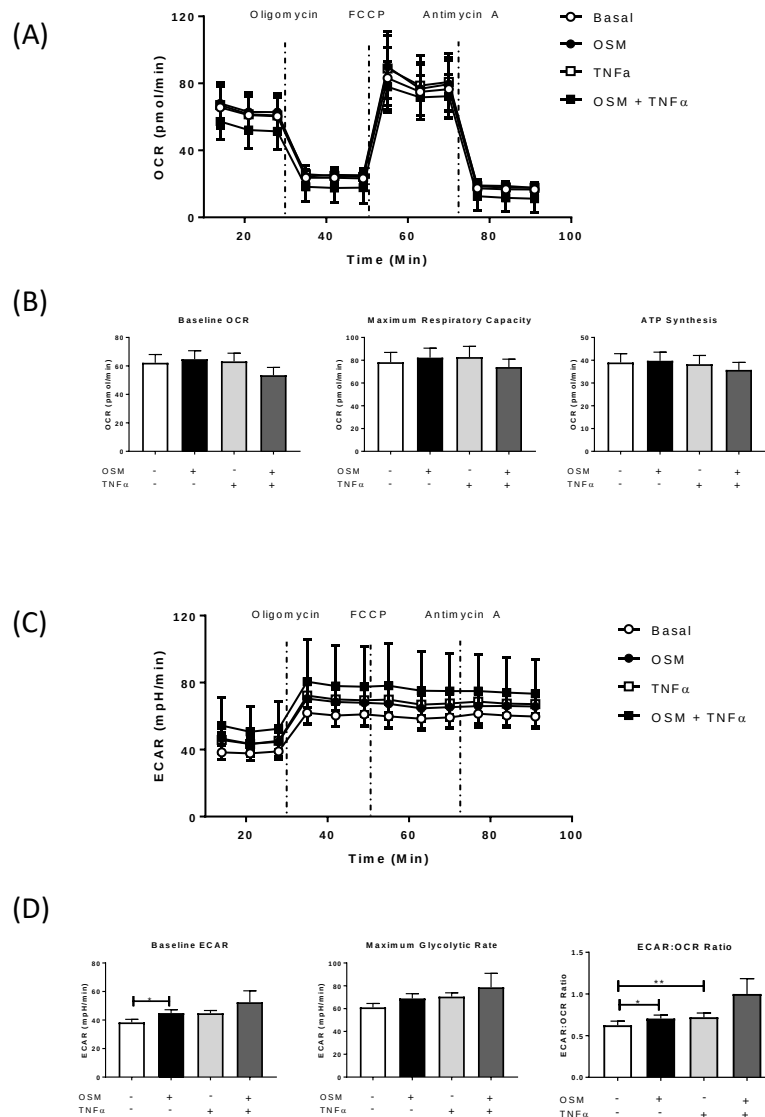


Figure 4.15: OSM does not synergise with TNFα to regulate metabolic changes in HUVEC.

HUVEC were treated with OSM (10 ng/mL) alone or in combination with TNFα (1 ng/mL) for 3 hr. Average seahorse profiles demonstrating (A) oxygen consumption rate (OCR) (oxidative phosphorylation) and (C) extracellular acidification rate (ECAR) (glycolysis), before and after injections of oligomycin, FCCP and antimycin A, (n=4). Bar graphs demonstrating (B) baseline OCR, maximum respiratory capacity, ATP synthesis and (D) baseline ECAR, maximal glycolytic rate and ECAR:OCR ratio. Data analysed using paired *t*-test, data expressed as mean ± SEM, **p*<0.05, ***p*<0.01, ****p*<0.005 significantly different from basal.

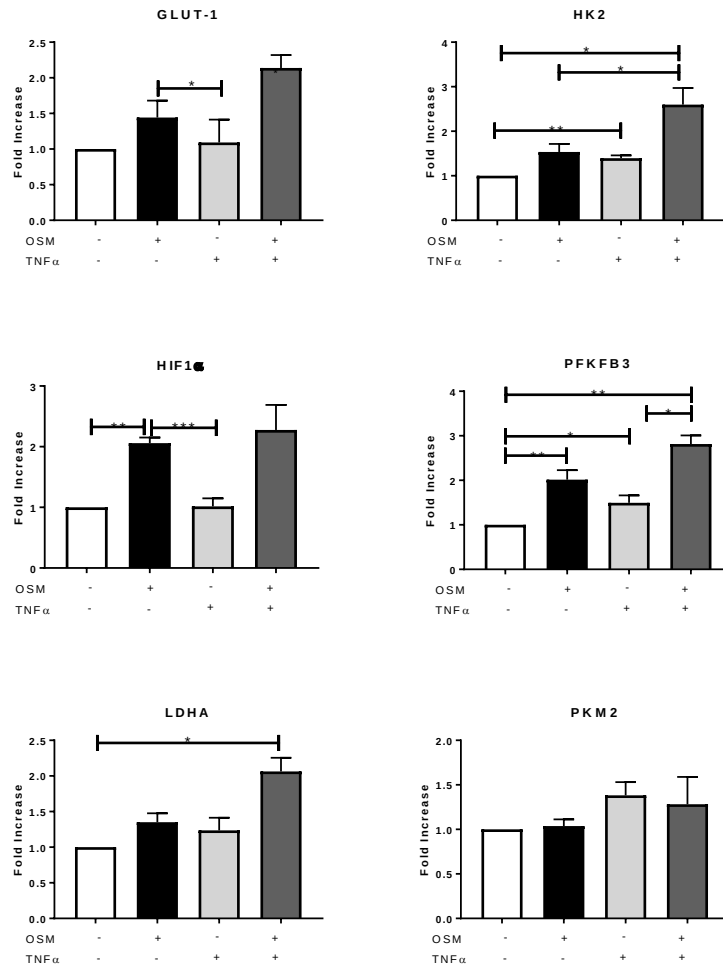
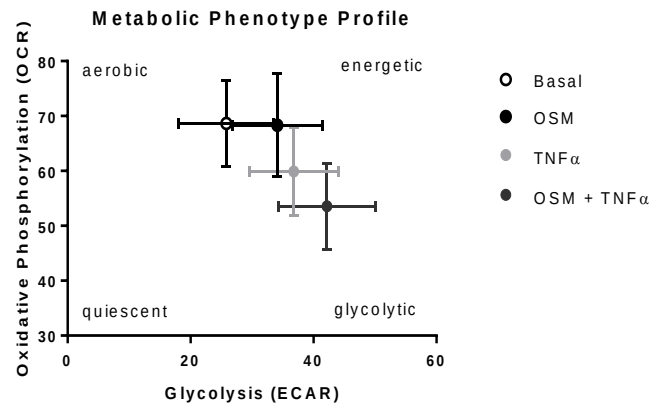


Figure 4.16: OSM does not synergise with TNF α to regulate metabolic changes in HUVEC.

Bar graphs demonstrating gene expression of *GLUT-1*, *HK2*, *PFKFB3*, *HIF1 α* , *LDHA* and *PKM2* in HUVEC treated with OSM alone or in combination with TNF α for 24 hr (n=4-5). Fold increase compared to endogenous controls (*RPLPO* and *HPRT1*). Data analysed using paired *t*-test, data expressed as mean $\pm$ SEM, *p<0.05, **p<0.01, ***p<0.005 significantly different.

(A)

RAFLS



(B)

HUVEC

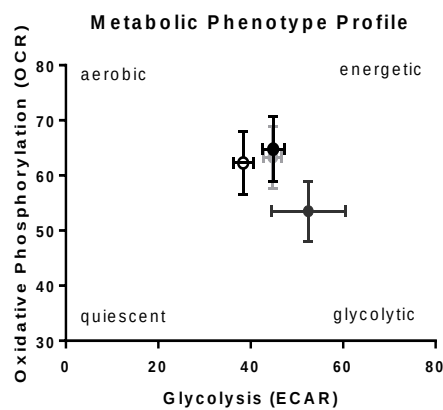


Figure 4.17: Overall metabolic profile of RAFLS and HUVEC.

Metabolic phenotype profiles in (A) RAFLS and (B) HUVEC representing changes in metabolic phenotype in response to OSM (10 ng/mL) and $\text{TNF}\alpha$ (1 ng/mL) alone and in combination.

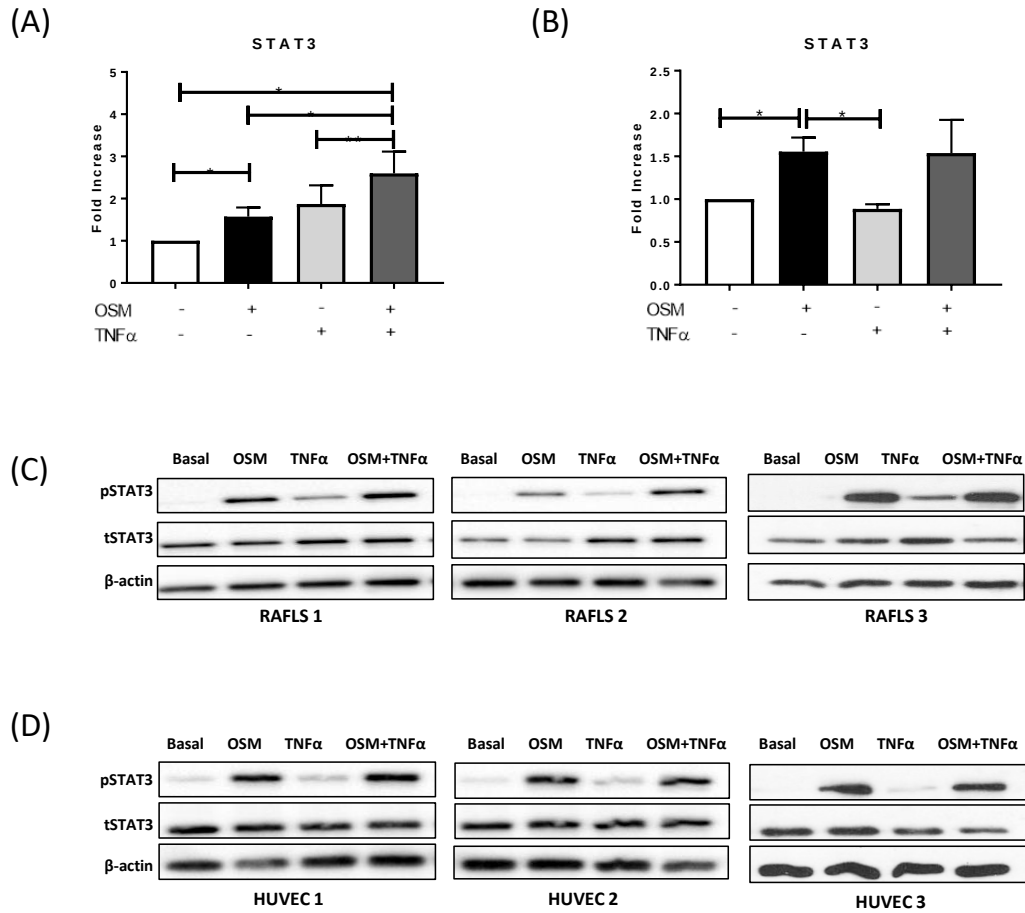


Figure 4.18: OSM in combination TNF α regulates STAT3 expression in RAFLS.

Bar graphs demonstrating gene expression of *STAT3* in (A) RAFLS (n=6) and (B) HUVEC (n=4) following treatment with OSM alone or in combination with TNF α for 24 hr. Fold increase compared to endogenous controls (*RPLPO* and *HPRT1*). Data expressed as mean $\pm$ SEM, using paired *t*-test **p*<0.05, ***p*<0.01. Representative western blot showing phospho-STAT3 (pSTAT3) and total-STAT3 (tSTAT3) in (C) RAFLS (D) and HUVEC. β -actin was used as loading control.

4.5 Discussion

OSM is a crucial player in the pathogenesis of RA, however its relative contribution to specific mechanisms involved in synovial inflammation remain to be fully elucidated, primarily due to the pleiotropic nature of this cytokine. In this chapter, as per our published manuscript (Appendix A) (Hanlon *et al.*, 2019), we demonstrate that OSM differentially regulates pro-inflammatory mechanisms and significantly promotes pro-angiogenic and pro-invasive mechanisms in RAFLS and HUVEC. This is accompanied by a change in the cellular bioenergetic profile of the cells, whereby OSM significantly increases the ECAR:OCR ratio in favour of glycolysis, paralleled by the induction of the glucose transporter GLUT-1 and key glycolytic enzymes (HK2, PFKFB3, HIF1 α). Next, we demonstrate that OSM synergises with TNF α to differentially regulate pro-inflammatory mechanisms in RAFLS and HUVEC. Interestingly, OSM synergises with TNF α to regulate metabolic reprogramming in RAFLS specifically, whereby an induction of glycolytic activity with concomitant attenuation of mitochondrial respiration and ATP activity is observed in RAFLS, but not in HUVEC. Finally, we identify that the combination of OSM with TNF α induces transcriptional activity of STAT3 in RAFLS, with no effect observed in HUVEC. Together, this study indicates that OSM is an important player in orchestrating pro-inflammatory, angiogenic and invasive events in RA, specifically in RAFLS, effects that are mediated by interactions with both TNF α and STAT3.

In this chapter, OSM differentially regulates cytokine and chemokine secretion in both RAFLS and HUVEC, significantly inducing IL-6 and MCP-1, yet inhibiting IL-8 and GRO α , with minimal effect observed on RANTES. While this is the first study to demonstrate the opposing action of OSM on these specific cytokine/chemokines in RAFLS and HUVEC, it is consistent with previous studies demonstrating differential effects in other cell types (Dayer and Burger, 1994; Dumas *et al.*, 2012). Specifically, OSM can induce GM-CSF, IL-6, growth factors VEGF and bFGF, the osteoclastogenic cytokine RANKL and many MMP (Yao *et al.*, 1996; Vasse *et al.*, 1999; Faffe *et al.*, 2005; Fearon *et al.*, 2006; Le Goff *et al.*, 2014). Furthermore, OSM has been shown to differentially regulate chemokines/adhesion molecules, inducing CXCL5, IP10, CCL2, MCP-1 and ICAM-1 in lung fibroblasts, osteoblasts and epithelial cells, with no effect observed for other mediators such as GRO α , MIP-1 and VCAM-1 (Fearon *et al.*, 2006). The effect of OSM on chemokine

expression has also been observed in mouse models of pneumonia (Traber *et al.*, 2015), experimental autoimmune encephalomyelitis (EAE) and cancer (Sugaya *et al.*, 2006; Meares *et al.*, 2014).

Furthermore, we demonstrate that OSM promotes pro-angiogenic mechanisms and leukocyte adhesion, accompanied by induction of *VEGF* and *ICAM-1*. VEGF is a pivotal 'on' switch for angiogenesis, promoting EC proliferation, migration and invasion (Koch *et al.*, 1994; Ferrara and Davis-Smyth, 1997; Koch, 2003; DVORAK, 2005), with numerous studies demonstrating increased expression of VEGF and its receptors in RA synovial tissue (Koch *et al.*, 1994; Fearon *et al.*, 2003; Salvador *et al.*, 2006). This is further supported by studies indicating that OSM can have differential effects on angiogenic mechanisms dependent on STAT activation, with pSTAT1 inhibiting VEGF expression, yet pSTAT3 promoting VEGF expression (RYCHLI *et al.*, 2010; Albasanz-Puig *et al.*, 2012). The effect of OSM on leukocyte adhesion has also been observed in mouse models of arthritis (Bell *et al.*, 1999). OSM has also been shown to upregulate the expression of CCL13 in RAFLS (Hintzen *et al.*, 2009), to induce key chemokines involved in leukocyte chemotaxis (CXCL3, CCL2, CCL5, CCL20), in addition to promoting infiltration of macrophages and neutrophils in mice models of inflammation (Pohin *et al.*, 2016). The observed differential effects of OSM on VCAM-1 expression again highlights its pleiotropic nature in different cell types, possibly influenced by the inflammatory microenvironment.

These striking changes in cellular function are, in fact, mirrored by distinct alterations in the metabolic profiles of OSM-treated cells, resulting in a shift in the ECAR:OCR ratio in favour of glycolysis. This shift is supported by the observed increase in key glycolytic drivers in response to OSM treatment, whereby induction of *HIF1 α* , *PFKFB3*, *HK2*, *LDHA*, *PKM2* and *GLUT-1* was demonstrated. The metabolic switch in HUVEC in response to OSM is consistent with previous studies indicating that active endothelial cells rely heavily on glycolysis. Indeed, 85% of endothelial cell ATP requirements comes from the conversion of glucose to lactate, mechanisms that are crucial for tip cell formation and blood vessel migration (Gao *et al.*, 2013; Stapor *et al.*, 2014; Eelen *et al.*, 2018). This preferential use of glycolysis has also been demonstrated in the inflamed RA joint, with studies showing that glycolytic markers are inversely correlated with synovial pO₂ levels (Kennedy *et al.*, 2010; Biniecka *et al.*, 2016; Balogh *et al.*, 2018). In addition, previous

studies have shown that OSM can promote glycolytic mechanisms in human hepatocyte cell lines in a PDK-1-dependent manner and can induce HIF1 α in different cell types to promote tumour progression in cancer cells (Battello *et al.*, 2016; Shrivastava *et al.*, 2018). Consistent with the observed increase in PFKFB3 in this study, previous studies have reported that blockade of PFKFB3 inhibits angiogenic tube formation, secretion of pro-inflammatory/angiogenic mediators, and key signalling pathways in both RAFLS and endothelial cells (Biniecka *et al.*, 2016). Moreover, blockade of PFKFB3 in animal models of RA, psoriasis and colitis has led to resolution of inflammation (Schoors *et al.*, 2014; Zou *et al.*, 2017).

The inflamed synovial joint is hallmarked by a complex mixture of pro-inflammatory cytokines and chemokines interacting with each other to promote the inflammatory response. In this study we demonstrate that OSM potentiates the effect of TNF α on IL-6 and MCP-1 secretion from HUVEC and RAFLS, inhibits TNF α -induced IL-8 and GRO α , while displaying differential effects on RANTES, with OSM significantly inhibiting TNF α -induced RANTES expression in HUVEC, while potentiating TNF α -induced RANTES in RAFLS. The ability of OSM to cooperate with key pro-inflammatory mediators such as IL-1 β , IL-17 and TNF α has been previously reported (Hui *et al.*, 2003; Barksby *et al.*, 2006; Moran *et al.*, 2009b). In mouse synovial fibroblasts, OSM augments the effects of TNF α and IL-1 β on IL-6 secretion (Le Goff *et al.*, 2014), inhibits IL-1 β -induced IL-8 and GM-CSF expression (Richards *et al.*, 1996), and can synergise with TLR-4 to induce MCP-1 in human aortic adventitious fibroblasts and smooth muscle cells (Schnittker *et al.*, 2013). Furthermore, OSM inhibits TNF α -induced TIMP-1 expression, yet potentiates IL-1 β -induced TIMP-1 and MMP-1 in RAFLS (Cawston *et al.*, 1998; Fearon *et al.*, 2006; Moran *et al.*, 2009a).

Interestingly, we identified that the synergistic effects observed with OSM and TNF α together also differentially altered the metabolic profile of the cells. Specifically, the combination of OSM and TNF α reduced the mitochondrial respiration paralleled by a stepwise induction of glycolysis in RAFLS, an effect not observed in HUVEC. Indeed, we demonstrate that the synergy between OSM and TNF α observed in RAFLS may be STAT3-dependent, an effect that appears to be specific to RAFLS and not HUVEC. In line with this, studies have demonstrated that TNF α is capable of indirectly activating the JAK-STAT

pathway through induction of type I interferons in RAFLS (Rosengren *et al.*, 2012). The mechanisms by which OSM regulates such effects within the inflamed joint however is unclear, yet studies have suggested that differential activation of its receptors gp130/LIF α and gp130/OSM or differential combinations of STATs (whether they form hetero- or homo-dimers) may account for such opposing effects (Gearing *et al.*, 1992; Stahl and Yancopoulos, 1993).

Furthermore, OSM has been shown to regulate STAT1/3 and STAT5/6 in mouse fibroblasts and is also capable of suppressing cell motility via STAT1 activation in lung cancer (Pan *et al.*, 2016). Conversely, a recent study has demonstrated that murine OSM phosphorylates STAT3 via gp130/LIF activation but not STAT1 causing specific regulation of STAT3 responsive genes in primary osteocytes (Walker *et al.*, 2016). Indeed, STAT3 itself is capable of interacting with other STATs; STAT1 for example has been demonstrated to exhibit inhibitory effects against STAT3 signalling in a study on esophageal squamous cell carcinoma (Liu *et al.*, 2018). Thus, a clearer understanding of the various cues directing this complex transcriptional landscape is vitally important.

In this study, we propose that the altered cellular bioenergetics resulting from the synergy between OSM and TNF α may rely on STAT3 activation in RAFLS. Interactions between STAT3 and metabolic enzymes have also been demonstrated previously whereby blocking PFKFB3 causes inhibition of pSTAT3 expression in RAFLS (Biniecka *et al.*, 2016). In cancer cells, STAT3 regulates glycolysis through HK2, (Li *et al.*, 2017; Okano *et al.*, 2017) and mediates HIF1 α -PKM2-interactions (Gao *et al.*, 2013). Furthermore STAT3, has been shown to be localised in the mitochondria, can bind to complex I and, in liver and heart cells, is capable of modulating the electron transport chain by altering activities of complex I and II (L. Z. Shi *et al.*, 2011). Finally, in the context of the RA joint, STAT3 interacts with various other key signalling molecules including Notch, NF κ B and HIF1 α , all of which regulate each other's activation through complex positive and negative feedback loops in the RA joint (Gao *et al.*, 2015).

In conclusion, we have shown that OSM, a macrophage-secreted cytokine, is capable of driving pro-inflammatory and metabolic changes, implicating it as a crucial cytokine in orchestrating the inflammatory response in rheumatoid arthritis. Moreover,

we demonstrate that OSM enhances the destructive effects of $\text{TNF}\alpha$, a key pathogenic factor in disease pathogenesis, effects which are mediated through activation of STAT3.

CHAPTER FIVE:

General Discussion and Future Directions

5.1 General Discussion

Rheumatic and musculoskeletal diseases (RMD) affect up to 60% of the 120 million EU citizens, at an estimated cost of €240 billion with direct costs of 2% EU GDP. Rheumatoid Arthritis is an important RMD, affecting roughly 1% of adults in the developed world (McInnes and Schett, 2017). RA, a chronic autoimmune disease, is typically characterized by synovial inflammation, and structural damage of the cartilage and bone (Emery, McInnes, *et al.*, 2008). If not treated early and aggressively, RA can result in joint destruction with subsequent deformity and loss of function, disability, reduced quality of life and substantial socio-economic costs (Van De Sande *et al.*, 2011; Krishnamurthy *et al.*, 2016; McInnes and Schett, 2017). Fortunately, the clinical picture for RA patients has improved dramatically over the last decade owing to advances in innovative research such as biological DMARDs (Hyndman, 2017). Despite these advances however, no individual treatment is fully effective with many patients failing to respond or displaying sub-optimal responses (Ktak *et al.*, 2016), indicating that the clinical course of RA varies dramatically between patients.

Subtle but very specific differences in the hierarchical control of inflammation at a cellular level, makes the development of individualised treatment regimens extremely difficult (McInnes and Liew, 2005). A major area of interest with RA is personalised or stratified medicine with the goal of personalised medicine to improve therapeutic efficacy while reducing toxicity and cost of treatment (Law and Taylor, 2019). Currently, patients are treated using a 'trial and error' approach which reduces the opportunity of treatment during the therapeutic window of opportunity to avoid irreversible joint destruction. This is largely due to the lack of biomarkers to inform treatment decisions. Despite many genome-wide association studies, transcriptomics, proteomics, epigenetics and metabolomics studies, serological status still remains the only applicable means of patient stratification. Therefore the development of biomarkers will enable rheumatologists to match each patient to their most effective treatments, improve clinical outcomes, limit adverse events and reduce socio-economic costs (Richardson and Isaacs, 2013).

Moreover, the synovium is the main target tissue of RA with transcriptomic analysis of synovial tissue emerging as a clinically significant methodology of identifying new disease biomarkers and therapeutic targets. Improved methods of synovial tissue

analysis and advances in arthroscopic biopsy and ultrasound-guided synovial biopsies, coupled with advances in single cell technologies will enable detailed profiling of RA synovial tissue for classification and stratification and uncover heterogeneous manifestations of the disease (Romão *et al.*, 2017; Veale and Fearon, 2019).

This thesis explores the role macrophages play in regulating the balance between inflammation and homeostasis in the synovial joint. We examine the functional phenotype of circulating monocytes before differentiation into macrophages, polarised monocyte-derived macrophages and synovial tissue macrophages as depicted in Figure 5.1. Furthermore, we examine the effect of macrophage-derived cytokines on stromal cell activation in RA, thus providing a comprehensive study of macrophage biology in RA pathogenesis. The data presented in this thesis addresses the huge chasm in our understanding of the function and origin of macrophages in the inflamed RA synovium, identifying a previously undescribed subset of dysfunctional transitional tissue macrophages. Given the crucial role macrophages play in orchestrating inflammation, understanding the molecular cues that shape macrophage activation states may enable better patient selection for targeted therapies, improve monitoring of treatment efficacy or potentially identify patients with inherently more aggressive disease who require an appropriately more aggressive treatment regime.

Chapter 2 investigated the inflammatory and metabolic profile of RA monocytes to assess whether they are pre-programmed to become M1 pro-inflammatory macrophages. RA circulatory CD14⁺ monocytes displayed higher levels of pro-inflammatory cytokines and homing chemokines compared to healthy controls, a phenotype that is indicative of a M1 like pro-inflammatory macrophage. Moreover, this pathogenic profile persists in differentiated inflammatory M1 macrophages, indicating that RA macrophages memorize the inflammatory bias of their precursor cells. Dysregulation of monocyte function has also been observed in other autoimmune/inflammatory conditions including giant-cell arteritis and CAD patients (Shirai *et al.*, 2016; Watanabe *et al.*, 2018). In addition, myeloid cell immunological memory is possibly due to epigenetic imprinting (Neele *et al.*, 2015), which may be an exciting avenue for therapeutic development.

Another exciting area is the field of immunometabolism, or the concept that metabolic pathways not only provide cellular energy but also dictates cell phenotype and function. Therefore, in this thesis, we examined the metabolic demands of circulating RA monocytes where a committed boost in both oxidative phosphorylation and glycolysis was observed indicating that they are in a hyper-metabolic state. Indeed, RA macrophages recapitulated the metabolic profile of their precursor cells with heightened glycolytic flux and mitochondrial respiration, further reflected by dysfunctional mitochondrial morphology compared to healthy macrophages. These data suggest that in RA, circulating monocytes undergo extensive inflammatory and metabolic rewiring and this phenotype persists following differentiation into mature macrophages. Furthermore, a consistent upregulation of glycolytic mechanisms, including increased expression of key glycolytic enzymes PFKFB3, HK2 and HIF1 α , is observed in RA monocytes and differentiated macrophages indicating a fundamental abnormality in processing of glucose. Blockade of glucose uptake with 2DG, inhibited the secretion of pro-inflammatory mediators from these circulatory *ex vivo* monocytes while interference of oxidative phosphorylation with oligomycin resulted in only partial inhibition of pro-inflammatory cytokines. Thus, suggesting that glycolytic flux plays a greater role in shaping RA myeloid cell functions, despite a boost in both metabolic pathways. This is consistent with previous studies indicating that inflammatory macrophages have an ardent appetite for glucose and maintain high glycolytic flux for rapid immune activation (Rodríguez-Prados *et al.*, 2010; O'Neill, 2015; Van den Bossche, O'Neill and Menon, 2017). Therefore, manipulation of glycolytic mechanisms may be of therapeutic benefit to dampen the immune response in RA.

In this thesis we also demonstrate consistent upregulation of pro-inflammatory mediators and enhanced glycolytic flux coupled with impaired phagocytic ability, in RA M1 macrophages in comparison to M2. RNA-seq analysis revealed distinct gene signatures of these divergent macrophage activation states, enabling exploration of distinct molecular cues and gene signatures that define an M1 macrophage activation state. STAT3 was identified as a key transcriptional factor which is a component of the JAK/STAT signalling pathway. JAK/STAT signalling is currently a target approved for RA and PsA with use of an orally available JAK-inhibitor Tofacitinib demonstrating clinical efficacy

(Hodge *et al.*, 2016). STAT3 inhibition resulted in diminished pro-inflammatory cytokine expression in circulating RA monocytes and reduced the hyper-metabolic phenotype of RA monocytes and mature *ex vivo* macrophages. These data is consistent with previous studies demonstrating that STAT3 activation plays a key role in mediating metabolically driven inflammatory processes (Jung *et al.*, 2005; Gao *et al.*, 2015; Biniecka *et al.*, 2016; McGarry *et al.*, 2018). Moreover, blockade of glycolysis using 2DG resulted in reciprocal inhibition of STAT3 activation indicating a negative feedback loop. This is consistent reports of bidirectional interactions between STAT3 and other key players in metabolism including HIF1 α , PKM2 and HK2 (Jung *et al.*, 2005; Gao *et al.*, 2015; Shirai *et al.*, 2016; Li *et al.*, 2017), indicating complex cross-talk between key signalling pathways within the inflamed synovial microenvironment.

In summary, Chapter 2 provides insight into the inflammatory and bioenergetic functions of RA inflammatory macrophages and circulating monocytes, their precursor cells. Data in this thesis indicates RA CD14⁺ circulating monocytes are primed for inflammation resulting in heightened metabolic demands. Therefore, it is possible to propose that analysis of circulating monocytes may be clinically translatable in terms of early diagnosis, disease progression and response to therapy, underscoring the potential as a promising biomarker to predict RA outcomes and identify new candidate medications for the treatment of arthritis sufferers. Additionally, we demonstrate opposing inflammatory and metabolic functions in RA M1 and M2 macrophages and provide evidence for a mechanistic role of STAT3 in shaping macrophage polarisation and metabolic reprogramming, manipulation of which may result in resolution of inflammation.

Chapter 3 provides in-depth phenotypic characterization of synovial tissue macrophages residing in the RA inflamed joint, identifying a key role for the previously undescribed dysfunctional CD206⁺CD163⁺ synovial tissue macrophage subset. Very few studies have examined distinct subsets of synovial tissue macrophages in the inflamed joint. This is in part due to difficulty in isolating synovial macrophages, but also due to the fact that synovial macrophages at the site of inflammation display remarkable plasticity in response to microenvironmental cues, permanently surveying their environment and rapidly reacting. We demonstrate a wide spectrum of macrophage activation states

existing within the inflamed synovial joint, indeed very few macrophages could be defined as pure M1 or M2, suggesting that this dichotomous system is useful as guide and for ease, but the *in vivo* reality is far more complex.

Specifically, a significant enrichment of a dominant double positive CD206+CD163+ macrophage subtype co-expressing high levels of CD40, was observed in RA synovial tissue compared to fluid. This study also demonstrates that this population is uniquely enriched in the RA synovium with decreased expression in OA and PsA tissue. In addition, synovial tissue macrophages residing in healthy synovial tissue were investigated, an area which remains largely unexplored. Healthy synovial tissue macrophages lack the diversity of macrophage subsets and activation states present in the inflamed RA synovium, with the double positive CD206+CD163+ macrophage subset dominating in the steady state, characteristic of immunoregulatory M2 macrophages. Interestingly, CD206+CD163+ macrophages do not express CD40 in healthy synovium indicating that following initiation of RA, this subset of tissue macrophages, reacting to the surrounding inflammatory microenvironment, acquire the inflammatory activation marker CD40 thus contributing to their dysfunctional state.

Moreover, we demonstrate differential chemokine receptor expression on CD206+CD163+ macrophages in RA synovial tissue compared to healthy tissue and other inflammatory arthropathies. Increased expression of CCR7, which is a hallmark of inflammatory macrophages, was observed in RA CD206+CD163+ synovial tissue macrophages. Indeed, CD206+CD163+CD40+ synovial tissue macrophages are positively associated with increased RA disease severity, indicating a pathogenic role. This is further highlighted by spontaneous secretion of high levels of pro-inflammatory cytokines IL-6, IL-8 and TNF α , in addition to their ability to activate synovial T cell responses in an autologous co-culture system. These data suggest that the dominant macrophage subset in RA synovial tissue contributes to RA disease pathogenesis, with future work aimed at identifying targets that have the potential to restore joint homeostasis.

Finally, in Chapter 3 in-depth RNA-seq analysis was conducted, which revealed differential expression of genes involved in metabolism, cytoskeletal rearrangement, pro-inflammatory mediators and cell adhesion in CD206+CD163+ vs CD206-CD163- synovial tissue macrophages. Furthermore, CD206+CD163+ dominant synovial tissue

macrophages are transcriptionally distinct from monocyte-derived M1 and M2 macrophages, indicating that they are a transitional subset of tissue macrophages. This chapter also highlights distinct discrepancies between RA synovial tissue and fluid CD206+CD163+ macrophage both at a phenotypic and transcriptomic levels, highlighting a role for site specific macrophage subsets, but also suggesting that studying RA synovial fluid does not clearly represent the microenvironment and molecular mechanisms at play in RA synovial tissue. In addition, we propose a hypothesis whereby CD206+CD163+ synovial tissue macrophages are in fact tissue-resident due to increases in core tissue-resident genes when compared to monocyte-derived macrophages, CD206-CD163-macrophages and synovial fluid macrophages. Indeed, metabolic analysis of the double positive CD206+CD163+ macrophages reveals a reliance on oxidative phosphorylation, another clue to suggest that they are indeed tissue-resident macrophages.

This is the first study to examine the precise nature, contribution and origin of synovial tissue macrophages in the inflamed synovium. Further studies are needed to fully elucidate the exact ontogeny of RA synovial tissue macrophages which may provide opportunities to preferentially target macrophages from a specific origin.

The previous chapters of this thesis demonstrate that inflammatory macrophages secrete high levels of many key pro-inflammatory cytokines including IL-1 β , TNF α and OSM compared to that of anti-inflammatory macrophages in RA patients. Following RNA-seq, OSM signalling was demonstrated to be enriched in RA M1 macrophages compared to M2 and in CD206+CD163+ synovial fluid macrophages using IPA analysis. Thus, in Chapter 4 we examined the effect of OSM, a pleiotropic macrophage-derived cytokine, on pro-inflammatory, angiogenic and bioenergetic mechanisms in RAFLS and HUVEC (Appendix A) (Hanlon *et al.*, 2019). OSM differentially regulates pro-inflammatory mediators in stromal cells, with IL-6, MCP-1, ICAM-1 and VEGF all significantly induced, in contrast to the observed inhibition of IL-8 and GRO α , with opposing effects observed for VCAM-1 depending on cell type. Functionally, OSM significantly induced angiogenic network formation, adhesion and invasive mechanisms. This was accompanied by a change in the bioenergetic profile of the cells, whereby OSM significantly increased the ECAR:OCR ratio in favour of glycolysis, paralleled by induction of the glucose transporter GLUT-1 and key glycolytic enzymes (HK2, PFKFB3, HIF1 α). Therefore, OSM has the ability

to activate both endothelial cells, which allow leukocyte influx to the joint, and RAFLS which are primed to invade cartilage, two key stromal cells involved in perpetuating the inflammatory response in RA.

The inflamed microenvironment is highly complex, with numerous cells interacting with each other in order to drive the pro-inflammatory response, through cell-cell interactions or secretion of soluble mediators. Hence, we investigated the relationship between OSM and the major pro-inflammatory cytokine; TNF α . OSM has the ability to synergize with TNF α to differentially regulate pro-inflammatory mechanisms in RAFLS and HUVEC. Interestingly, OSM also differentially synergizes with TNF α to regulate metabolic reprogramming, where induction of glycolytic activity with concomitant attenuation of mitochondrial respiration and ATP activity was demonstrated in RAFLS but not in HUVEC. It is this complex microenvironment, propelled not only by cellular interactions but also intricate cytokine interactions that drives RA disease pathogenesis, thus highlighting the difficulty in obtaining responses and/or full remission in many of these patients. Thus, this chapter provides an insight into the complex landscape of cytokine interactions and redundancy whereby blockade of one pathway may only have an initial or partial effect on inflammation, as another cytokine pathway may be taking over. Targeting OSM or downstream signalling pathways may provide new therapeutic or adjuvant strategies, particularly in those patients with sub-optimal responses to TNFi.

Finally, we identify a key role for STAT3, whereby the combination of OSM with TNF α induces transcriptional activity of STAT3 only in RAFLS, with no effect observed in HUVEC, highlighting the complex molecular mechanisms involved in driving disease. This thesis consolidates the significant position of the JAK/STAT pathway in RA pathogenesis; in differentially regulating cell phenotype and mediating cytokine responses and altering the metabolic profile of the cell.

In summary, the data presented in Chapters 2-4 of this thesis provides a comprehensive investigation into the exact nature and contribution of monocyte-derived and synovial tissue macrophages in perpetuation of inflammation. We have identified that circulatory monocytes are primed for inflammation and maintain a memory bias towards M1 macrophages. A previously undescribed dysfunctional subset of tissue-

resident macrophages in the RA joint has been identified that are associated with disease activity, transcriptionally distinct, and have the ability to further activate immune cells via cell-cell contact. We demonstrated that soluble cytokines secreted from macrophages, further exacerbate the inflammatory process through activation of key stromal cells within the synovial microenvironment. Also, we identify that the inflammatory phenotype of monocytes, macrophages and stromal cells are linked to their metabolic profile, an effect that is mediated in-part by one of the key transcriptional regulators in the joint; STAT3 signal transduction pathway.

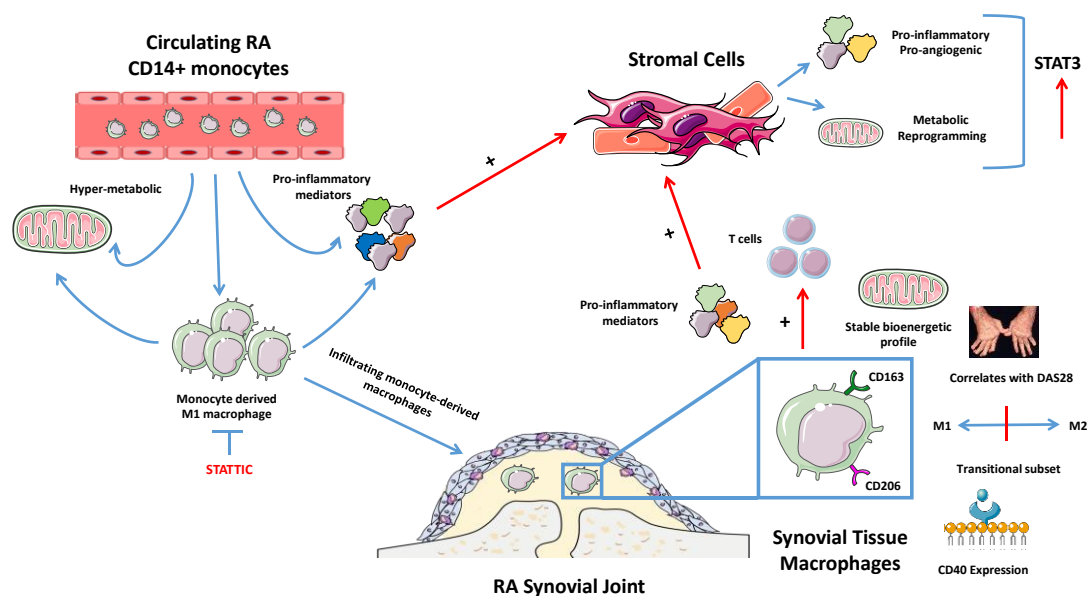


Figure 5.1: Overall schematic of thesis data

Circulating CD14+ monocytes in the RA periphery are hyper-inflammatory and hyper-metabolic, a phenotype that persists upon differentiation into macrophages at the site of inflammation. Inhibition of STAT3 by STAT3IC can rescue this pathogenic phenotype. However, infiltrating monocyte-derived macrophages aren't the only type of synovial tissue macrophage. In this thesis, we conduct an in dept analysis of previously undescribed macrophage subsets present within the inflamed RA synovium. Here we identify an enrichment of the CD206+CD163+ macrophage subset in synovial tissue, specifically RA synovial tissue, a subset that expresses high levels of CD40, is neither a pure M1 or M2 macrophage, correlates with DAS28 and displays a stable bioenergetic profile which may hint to a status of tissue residency. These macrophages can enhance T cell response and spontaneously secrete pro-inflammatory mediators. Finally, we assess the effects of macrophage-derived cytokines Oncostatin M and TNF α on stromal cell activation whereby an increase in pro-inflammatory and pro-angiogenic phenotype and metabolic reprogramming in favour of glycolysis is observed, dependant on STAT3 in RA synovial fibroblasts specifically.

5.2 Future Directions

This thesis provides evidence for the crucial role of macrophages in determining the delicate equilibrium between joint homeostasis and inflammation. Our data suggests that RA circulating monocytes have a greater propensity to develop into inflammatory M1 macrophages and are already primed towards a pro- inflammatory phenotype/function prior to differentiation. These data indicate a potential role of circulating monocytes as promising biomarkers to inform treatment options in RA and aid patient stratification. Hence, in future work phenotypic differences between monocytes and differentiated M1 macrophages at different stages of active RA disease and pre/post therapy will be examined to determine how early in disease onset these functional alterations are present and whether disease specific inflammatory signatures can be reversed following successful treatment. In addition, we aim to investigate if baseline monocyte profiles predict response vs non-response. Given the role of citrullinated proteins in shaping macrophage inflammatory effector functions (Zhu *et al.*, 2015), comparison of monocytes from ACPA+ and ACPA- RA patients will also be examined to investigate if ACPA positivity influences monocyte priming.

Additional investigations into RNA-seq analysis of polarised RA macrophages in this study has identified a NAMPT as novel target in inflammatory M1 macrophages. NAMPT is a rate limiting enzyme in the NAD⁺ salvage pathway and is increased in the RA serum and fluid, positively correlating with disease activity and radiographic progression (Matsui *et al.*, 2008; Šenolt *et al.*, 2011; Franco-Trepát *et al.*, 2019). NAMPT also correlates with induced expression of pro-inflammatory mediators in monocytes and studies have reported that NAMPT inhibition decreases the infiltration and activation of immune cells into arthritic joints in a CIA model (Présumey *et al.*, 2013; Franco-Trepát *et al.*, 2019). Analysis of the differentially expressed genes between RA M1 and M2 macrophages identified NAMPT and several other genes involved in NAD⁺ metabolism (such as IDO1/2, NNMT and KYNU) as central molecules in M1 polarisation, indicated by the STRING pathway analysis (Figure 5.2). Using FK886, a specific NAMPT inhibitor, further studies will be performed that manipulate inflammatory monocyte-derived macrophages in an attempt to skew their profile towards an 'M2' anti-inflammatory phenotype.

As previously discussed, inflammatory and metabolic reprogramming of monocytes to *ex vivo* inflammatory macrophages may involve altered epigenetic imprinting (Neele *et al.*, 2015). In view of this, we have isolated DNA from CD14⁺ monocytes and matched monocyte-derived polarised macrophages from 4 RA patients, 4 arthralgia patients and 4 age-matched healthy control subjects for future epigenetic investigations. DNA methylation and Bisulfite Pyrosequencing assays will be performed to investigate whether epigenetic mechanisms regulate functional alterations in circulating RA monocytes and how early this occurs (arthralgia cohort). Moreover, studies have indicated that certain histone deacetylases are important in maintaining an 'M1' macrophage polarisation state (Hoeksema *et al.*, 2014), so epigenomic profiling of polarised *ex vivo* macrophages may give an indication as to whether distinct epigenetic marks can predict the polarisation state of differentiated macrophages.

It is now appreciated that immunological events precede the onset of clinical manifestations (Quinn and Cox, 2005; Van Der Woude *et al.*, 2010) and so recent studies have focused on defining the different phases of RA disease development, with a particular focus on those 'at risk' of RA (Van Steenbergen *et al.*, 2018). Arthralgia or 'at risk' individuals present with aches and pains and symptoms such as joint pain and autoantibodies but have no clinical signs of arthritis although they are at an increased risk of developing RA (Gerlag *et al.*, 2012; Van Steenbergen *et al.*, 2018). Autoantibodies can be present in the circulation years before disease onset, consequently studying alterations in immune cells of 'at risk' individuals may serve to stratify arthralgia and those at-risk, into those that will or will not develop RA. As such, we have collected blood samples from arthralgia or 'at risk' individuals to investigate if changes identified in established RA monocytes are already evident before the onset of disease.

Preliminary data indicates that circulating monocytes from 'at risk' individuals do display functional abnormalities prior to disease onset. We identified stepwise progression in expression of pro-inflammatory mediators from HC to arthralgia to RA in LPS-activated CD14⁺ monocytes (Figure 5.3 (A)). Furthermore, the metabolic phenotype of arthralgia CD14⁺ monocytes were also altered, showing a similar metabolic phenotype to RA CD14⁺ monocytes, with an increase in both oxidative phosphorylation and glycolysis identified (Figure 5.3 (B)). In addition, to the observed increase in the inflammatory

response, we also demonstrated an increase in *HIF1 α* , *PFKFB3*, *HK2* and *STAT3* in arthralgia CD14⁺ monocytes, compared to HC (Figure 5.3 (C)). These data suggest that the altered phenotype of monocytes actually precedes clinical manifestations and thus circulating macrophage precursors may have potential in identifying ‘at risk’ individuals who will continue to develop RA. So, in future studies, blood monocytes will be isolated from arthralgia patients at 3, 6 and 12 months to investigate whether the dysfunctional inflammatory and metabolic alterations change in concert with RA disease progression. In addition, monocyte-derived macrophages from arthralgia patients will be cultured to elucidate if this commitment to inflammatory mechanisms persists following differentiation, similar to RA monocyte-derived macrophages. Furthermore, experiments will be performed to determine if inhibition of STAT3 reverses this pathogenic phenotype in a similar manner to RA monocytes, thus implicating a key role for STAT3 prior to disease manifestations.

To date, studies of synovial tissue from ‘at risk’ individuals are limited. Given the potential for informing disease severity and treatment decisions, future studies aim to phenotypically characterise synovial tissue macrophage subsets in ‘at risk’ individuals. Indeed, preliminary histological analysis of synovial tissue from pre-RA patients demonstrates the presence of CD68 and CD163 synovial macrophages, suggesting that despite a lack of inflammation on clinical examination there are already early signs that the inflammatory process has initiated (Figure 5.4). CD40-CD40L pathway gene signatures are increased in synovial tissue in early stages of RA disease progression, including arthralgia, compared to healthy controls (Guo *et al.*, 2017). We aim to characterize the CD206⁺CD163⁺ macrophage subset in arthralgia synovial tissue biopsies and potential CD40 co-expression by flow cytometry. Colocalization of CD206, CD163 and CD40 by immunofluorescence in arthralgia and RA tissue will also be performed to assess whether this dysfunctional macrophage subset precedes clinical manifestations.

In this thesis extensive characterisation of RA synovial tissue macrophages, specifically the dominant CD206⁺CD163⁺ macrophage subset, has been performed. To extend these findings, future phenotypic analysis of distinct synovial tissue macrophage subsets will be performed in RA patient’s pre/post therapy and in responder’s vs non-responders to determine if therapeutic interventions results in restoration of tissue

macrophage homeostasis. In-depth analysis of the existing RNA-seq data on the sorted CD206+CD163+ macrophages will allow for the identification of key pathways for interrogation in an attempt to reverse the pathogenic phenotype of RA CD206+CD163+ synovial tissue macrophages, perhaps an upstream regulator of CD40. Subsequently, sorted synovial tissue CD206+CD163+ macrophages will be cultured in the presence of an identified target and their effect on the expression of inflammatory CD40 and CCR7 expression, release of pro-inflammatory mediators and on T cell activation will be determined, to potentially reinstate the immunoregulatory functions of CD206+CD163+ macrophages.

This thesis also reports discrepancies in macrophage frequencies and phenotypes between RA synovial tissue and synovial fluid, possibly due to differing origins as indicated by RNA-seq analysis. We intend to sort CD206+CD163+ macrophages from synovial tissue and synovial fluid and culture, for analysis of spontaneous secretion of pro-inflammatory mediators and for co-culture with autologous T cells. A comparison of the metabolic demands of CD206+CD163+ macrophages from RA synovial fluid and tissue will be performed using the novel metabolic FLIM assay which may further reinforce our hypothesis that CD206+CD163+ synovial tissue macrophages are tissue-resident.

Macrophages and fibroblasts are the resident cells of the inflamed and healthy synovium. A recent study has reported a distinct subset of inflammatory HBEGF+ macrophages in the RA synovium using single cell RNA-seq that is capable of promoting synovial fibroblast invasive mechanisms. In addition, the study demonstrates that synovial fibroblasts are capable of shaping HBEGF+ inflammatory macrophages, thus indicating intricate cross-talk between these two resident cells (Kuo *et al.*, 2019). Given the key role macrophage-secreted cytokines play in inflammatory and metabolic reprogramming of RA synovial fibroblasts as demonstrated in Chapter 4 (Hanlon *et al.*, 2019), we aim to investigate the effects of RA synovial tissue CD206+CD163+ macrophage conditioned media on healthy synovial fibroblasts.

Finally, in these studies we provide evidence for a homeostatic phenotype of CD206+CD163+ macrophages in the steady state as indicated by analysis of healthy synovial tissue biopsies. This is in stark contrast to the dysfunctional and activated CD206+CD163+ macrophage subset observed in RA synovial tissue. Future experiments

aim to sort CD206+CD163+ macrophages from healthy synovial tissue and compare with the same subset from RA synovium to assess pathogenic responses. Furthermore RNA-seq analysis of CD206+CD163+ macrophages from healthy synovium and RA synovium would further highlight potential pathways to perturb for resolution of inflammation. We have already isolated synovial tissue cell suspensions from RA and healthy controls, with 10X genomics and single RNA-seq currently being performed which will further identify distinct macrophage subsets and discern which molecular pathway to target.

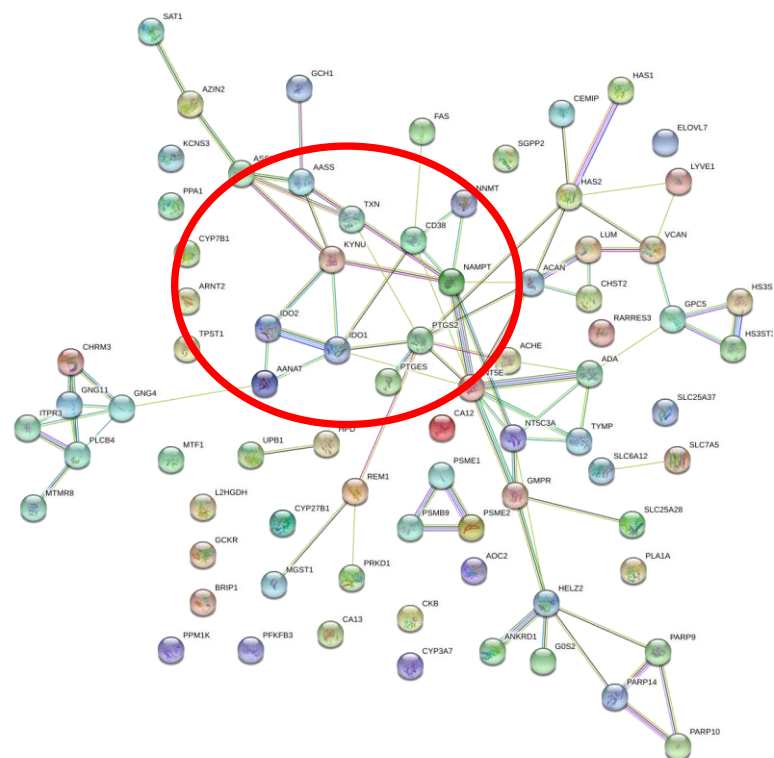


Figure 5.2: Identification of NAMPT as a potential therapeutic target.

Genes enriched in RA M1 compared to M2 input into the STRING pathway database and resulting pathway analysis was generated.

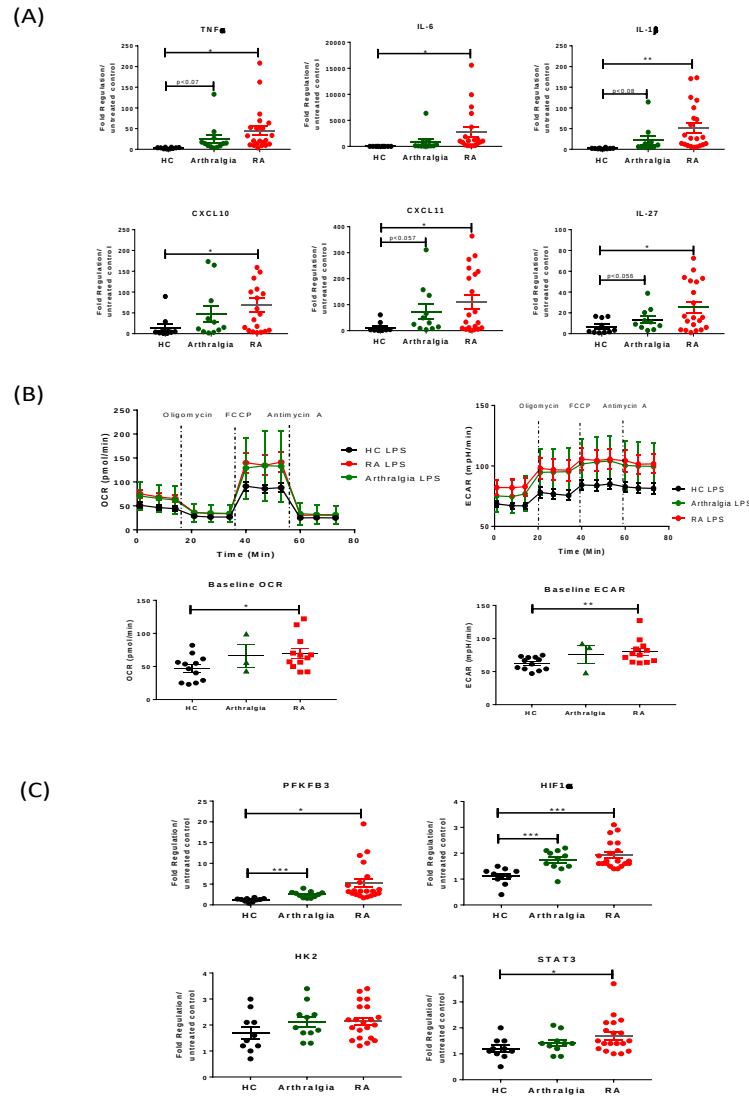


Figure 5.3: Hyper-inflammatory and hyper-metabolic mechanisms precede clinical manifestations.

(A) Bar graphs representing gene expression of pro-inflammatory cytokines in healthy (n=10), arthralgia (n=10) and RA (n=20) CD14⁺ monocytes in response to 100 ng/mL; 3 hr LPS stimulation. (B) Average Seahorse bioenergetics profile demonstrating OCR and ECAR of healthy (n=12), arthralgia (n=3) and RA (n=12) LPS-stimulated (100 ng/mL; 1 hr) monocytes. (C) Bar graphs demonstrating gene expression of metabolic genes in healthy (n=10), arthralgia (n=10) and RA (n=20) CD14⁺ monocytes in response to 100 ng/mL; 3 hr LPS stimulation. Data expressed as mean $\pm$ SEM, normalized to housekeeping control HPRT1, using unpaired *t*-test and Mann Whitney U as appropriate. **p*<0.05, ***p*<0.01, ****p*<0.005 significantly different from basal.

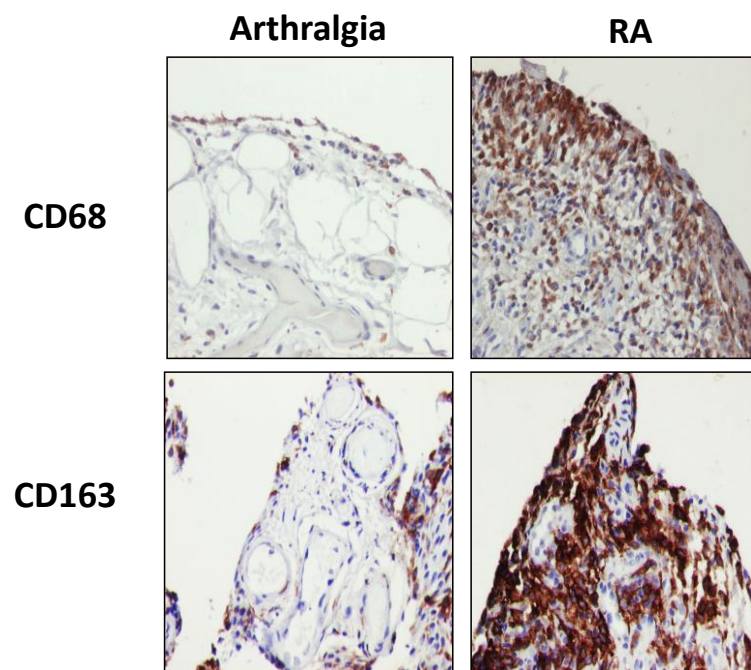


Figure 5.4: CD68 and CD163 staining by immunohistochemistry.

Representative images of immunohistochemical staining for CD68 and CD163 in arthralgia and RA synovial tissue

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CHAPTER SIX:

Appendix



STAT3 Mediates the Differential Effects of Oncostatin M and TNF α on RA Synovial Fibroblast and Endothelial Cell Function

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Objectives: Oncostatin M (OSM), a pleiotropic cytokine and a member of the gp130/IL-6 cytokine family, has been implicated in the pathogenesis of autoimmune diseases. Here we investigate the mechanisms by which its synergistic interactions with TNF α regulate the cellular bioenergetics and invasive function of synovial cells from patients with Rheumatoid Arthritis.

Methods: Primary RA synovial fibroblasts (RAFLS) and human umbilical vein endothelial cells (HUVEC) were cultured with OSM alone or in combination with TNF α . Pro-inflammatory cytokines, angiogenic growth factors and adhesion molecules were quantified by real-time PCR and ELISA. Invasion, angiogenesis and cellular adhesion were quantified by Transwell invasion chambers, Matrigel tube formation assays, and adhesion binding assays. Cellular bioenergetics was assessed using the Seahorse XFe96 Analyser. Key metabolic genes (GLUT-1, HK2, PFKFB3, HIF1 α , LDHA, PKM2) and transcription factor STAT3 were measured using real-time PCR and western blot.

Results: OSM differentially regulates pro-inflammatory mediators in RAFLS and HUVEC, with IL-6, MCP-1, ICAM-1, and VEGF all significantly induced, in contrast to the observed inhibition of IL-8 and GRO α , with opposing effects observed for VCAM-1 depending on cell type. Functionally, OSM significantly induced angiogenic network formation, adhesion, and invasive mechanisms. This was accompanied by a change in the cellular bioenergetic profile of the cells, where OSM significantly increased the ECAR/OCR ratio in favor of glycolysis, paralleled by induction of the glucose transporter GLUT-1 and key glycolytic enzymes (HK2, PFKFB3, HIF1 α). OSM synergizes with TNF α to differentially regulate pro-inflammatory mechanisms in RAFLS and HUVEC. Interestingly, OSM differentially synergizes with TNF α to regulate metabolic reprogramming, where induction of glycolytic activity with concomitant attenuation of mitochondrial respiration and ATP activity was demonstrated in RAFLS but not in HUVEC. Finally, we identified a mechanism, whereby the combination of OSM with TNF α induces transcriptional activity of STAT3 only in RAFLS, with no effect observed in HUVEC.

Conclusion: STAT3 mediates the differential effects of OSM and TNF α on RAFLS and EC function. Targeting OSM or downstream signaling pathways may lead to new potential therapeutic or adjuvant strategies, particularly for those patients who have sub-optimal responses to TNFi.

Keywords: rheumatoid arthritis, cellular bioenergetics, pro-inflammatory cytokines, JAK-STAT signaling, synovial fibroblasts

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by synovial hyperplasia and degradation of articular cartilage and bone, ultimately leading to irreversible disability. Although the initiating trigger for RA is not known, angiogenesis is one of the earliest events in the pathogenesis of this disease. Sprouting angiogenesis allows for a self-perpetuating influx of immune cells into the synovial joint, resulting in expansion of the synovial tissue into an aggressive tumor-like pannus (1). Despite this increased vascular supply, studies have demonstrated that the synovial joint is profoundly hypoxic (2). This is due to the highly dysfunctional and immature nature of the vasculature resulting in abnormal blood flow supplying inadequate nutrients and oxygen to the expanding synovium. Thus, the increasing metabolic turnover of the pannus outpaces vascular supply, rendering the inflamed synovium hypoxic (3–6).

The importance of metabolism in regulating synovial inflammation has recently emerged with many studies indicating that immune and stromal cells undergo a bioenergetic switch to a highly metabolically active state in order to meet the energy demands of the expanding synovium (7, 8). Indeed, the metabolic milieu of the inflamed joint reflects the chronically active state of immune and stromal cells, with elevated lactate levels and reduced glucose observed in RA synovial fluid, along with increased glycolytic enzyme activity and accumulation of succinate in synovial fluid and tissue (9–13). Recent studies have shown that treatment with glycolytic inhibitors dampens cytokine production, invasive mechanisms, and key transcriptional regulators in various synovial cells while also improving disease severity in animal models of arthritis (9, 14, 15).

The cytokine Oncostatin M (OSM) is highly expressed in the RA joint, and shares a common receptor signal subunit (gp-130) with IL-6-type cytokines (16). Produced mainly by macrophages, neutrophils and activated T-cells, OSM signals via the Janus Kinase (JAK) family of receptor-associated tyrosine kinases and is associated with the activation of STAT3 (17–19). Increased expression of OSM is associated with a plethora of pathologies including atherosclerosis, psoriasis, and many cancers (20, 21). Most recently, OSM has been shown to play a role in inflammatory bowel disease (IBD) with a study demonstrating heightened expression of OSM and its receptor in the inflamed IBD intestine, correlating with disease severity (22).

In the context of RA, overexpression of OSM in synovial fluid and tissue has been observed with levels correlating with joint inflammation (23). Collectively, studies have demonstrated that

OSM plays a critical role in RAFLS activation, promotion of angiogenesis, adhesion molecules and chemokines from RAFLS, altering the matrix metalloproteinases (MMP)/tissue inhibitor of matrix metalloproteinases (TIMP) ratio and inducing RANKL in RAFLS and chondrocytes in favor of joint destruction (21, 24, 25). Blocking OSM in a collagen-induced arthritis mouse model improves joint inflammation and cartilage damage (26). Furthermore, recent studies have demonstrated that inhibition of OSM-induced RAFLS pro-inflammatory mechanisms and cartilage degradation are rescued in the presence of JAK-STAT inhibitors (27, 28), effects that are, in part, mediated by a switch in the metabolic profile of the cell (29). Conversely, OSM is a pleiotropic cytokine often displaying divergent effects with both pro- and anti-inflammatory effects depending on the cell type and microenvironment. Previous studies have shown that OSM can inhibit IL-1-induced IL-8 and granulocyte macrophage colony stimulating factor (GM-CSF) and promote TIMP expression in RAFLS (24, 30). OSM inhibits TNF α - and IL-17A-induced TIMP-1 while potentiating IL-1 β -induced TIMP-1 expression in RAFLS (24, 31). More recently, studies have demonstrated that OSM can inhibit Th17 differentiation in mouse models of arthritis through reciprocal regulation of SOCS3, STAT3, and STAT5 (32). Therefore, the role of OSM in RA disease pathology is complex, depending largely on cell type and microenvironment.

Given the pivotal role of metabolism in regulating synovial inflammation, in this study we examined the effect of OSM on pro-inflammatory, angiogenic, and bioenergetic mechanisms in RAFLS and HUVEC. Furthermore, we investigated the relationship between OSM and the major pro-inflammatory cytokine; tumor necrosis factor α (TNF α), a central player in inflammation and destruction in the RA joint.

MATERIALS AND METHODS

Patient Recruitment and Arthroscopy

RA patients were recruited from the Rheumatology Department, St. Vincent's University Hospital. All patients gave fully informed written consent approved by the St. Vincent's University Hospital, Ethics and Medical Research Committee and research was performed in accordance with the Declaration of Helsinki. Synovial tissue biopsies were obtained at arthroscopy under local anesthetic using a Wolf 2.7 mm telescope (Wolf—Germany) as previously described (2). Biopsies were utilized for isolation of primary RA synovial fibroblasts (RAFLS).

Isolation of Primary Fibroblasts

RA synovial biopsies were digested with 1 mg/ml collagenase type 1 (Worthington Biochemical, Freehold, NJ, USA) in RPMI-1640 (Gibco-BRL, Paisley, UK) for 4 h at 37°C in humidified air with 5% CO₂. Dissociated cells were grown to confluence in RPMI 1640, 10% FCS (Gibco-BRL), 10 ml of 1 mmol/l HEPES (Gibco-BRL), penicillin (100 units/ml; Bioscience), streptomycin (100 units/ml; Bioscience) and fungizone (0.25 μ g/ml; Bioscience) before passaging. Cells were used between passages 3–8.

Culture of HUVEC

Human umbilical vein endothelial cells (HUVEC) (ATCC, Manassas, USA) were grown to confluence in endothelial cell basal media (MCDB-131, Gibco) supplemented with L-Glutamine (20 ml of 100X solution), Hydrocortisone (0.6 μ g/ml), hEGF (0.01 μ g/ml), Penicillin/Streptomycin (100 units/ml; Biosciences), Fungizone (0.25 μ g/ml; Biosciences) and 15% FCS (Gibco-BRL).

Cytokine and Chemokine Measurements

To assess the effects of OSM on pro-inflammatory mediators, RAFLS/HUVEC were seeded in 48-well plates at a density of 3×10^5 and allowed to attach overnight. Cells were incubated in serum-free RPMI-1640 or MCDB-131 for 24 h and subsequently stimulated with OSM (10 ng/ml). For synergy experiments, cells were also incubated in the presence or absence of TNF α (0.01, 0.1, 1 ng/ml). Supernatants were harvested and levels of IL-6, MCP-1, IL-8, RANTES, and GRO α were measured by specific ELISA (MCP-1: eBiosciences, USA, IL-6, IL-8, RANTES, GRO α ; R&D systems, UK) according to manufacturer's conditions.

Transwell Invasion Assay

Biocoat Matrigel™ Invasion Chambers (Becton Dickinson, UK) were used to assess RAFLS/HUVEC invasion. Cells were seeded at a density of either 3.5×10^4 (RAFLS) or 2.5×10^4 (HUVEC) cells per well in the migration chamber on 8 μ m membranes pre-coated with matrigel. Cells were incubated with OSM (10 ng/ml) for 24 h (HUVEC) or 48 h (RAFLS). Non-migrating cells were removed from the upper surface by gentle scrubbing. Migrating cells attached to the lower membrane were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Cells from five random high power fields for each well were counted to assess the average number of invading cells.

HUVEC Tube Formation

Matrigel (50 μ l; BD Biosciences, San Jose, CA, USA) was plated in 96-well culture plates after thawing on ice and allowed to polymerise for 30 min at 37°C in humidified air with 5% CO₂. 2×10^4 cells in supplemented MCDB-131 was added to each well and cells were stimulated with OSM (10 ng/ml) on control medium for 24 h. EC tubule formation was then assessed using phase-contrast microscopy. Cells were quantified by counting the number of connecting branches formed from five random high power fields as previously described (24).

Adhesion Assay

RAFLS/HUVEC were grown to confluence in 24-well plates, incubated in serum-free RPMI-1640 or MCDB-131 for 24 h and

subsequently stimulated with OSM (10 ng/ml) for a further 24 h. PBMC from healthy donors were isolated by density gradient centrifugation (Lymphoprep; Stemcell Technologies) according to the manufacturer's recommendations. PBMC were then re-suspended in MCDB-131. 7.5×10^4 PBMC were added to each well containing RAFLS/HUVEC and incubated at 37°C with 5% CO₂ for 1 h. After the incubation time, supernatants were removed and wells were washed with PBS. Semi-quantification was performed by counting adherent PBMCs as viewed under phase-contrast microscopy (Leica, Germany) at $10 \times$ magnification. Cells from five random high power fields for each well were counted to assess the average number of adherent cells.

mRNA Extraction and cDNA Synthesis

To assess the effects of OSM on specific genes, RAFLS/HUVEC were seeded into 6-well plates and allowed to grow to confluence. Cells were incubated in serum-free RPMI-1640 or MCDB-131 for 24 h and subsequently stimulated with OSM (10 ng/ml). For synergy experiments, cells were also incubated in the presence or absence of TNF α (1 ng/ml). Total RNA was isolated using the miRNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. The integrity of RNA samples were assessed using a bioanalyzer (Agilent, CA, USA). Samples with a 260:280 nm ratio of 1.8 and above and an RNA integrity number between 7 and 10 were used in subsequent experiments. Isolated RNA was stored at -80°C . Total RNA (100 ng) was reverse transcribed to cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, Cheshire, UK) and stored at -20°C until further use.

RT-PCR Analysis

Gene expression data were quantified by RT-PCR using the Quant Studio 5 Thermal Cycler (Applied Biosystem, Lewes, UK). Reaction mixtures contained 1 μ l of cDNA, SYBR green I PCR mastermix (Applied Biosystems) and target mRNA specific primer pairs as follows: VEGF for 5' GCAGAATCAT CACGAAGTGGTG 3' VEGF rev 5' TCTCGATTGGATG GCAGTAGCT 3', VCAM-1 for 5' GTA AAA GAA TTG CAA GTC TAC ATATCAC 3', VCAM-1 rev 5' GATGGATTCAC AGAAATAACTGTATTC 3', ICAM-1 for 5' AACCAGAGC CAGGAGACACTG 3', ICAM-1 rev 5' GCGCCGGAAGCTG TAGATG 3', HIF1 α for 5' GAACTTCTGGATGCTGGT GATTT 3', HIF1 α rev 5' GCAATTCATCTGTGCTTTTCATG TCA 3', HK2 for 5' TTCTTGCTCTCAG ATTGAGAGTGAC 3', HK2 rev 5' TTGCAGGATGGCTCGGACTTG 3', LDHA for 5' ATGGAGATTCCAGTGTGCCTGT 3', LDHA rev 5' CAGAGAGACACCAGCAACATTC 3', GLUT1 for 5' CTTCCAGTATGTGGAGCAACTGT, GLUT1 rev 5' GCAC AGTGAAGATGATGAAGACG 3', PFKFB3 for 5' ACCAA AGATCACCCACGGATGT 3', PFKFB3 rev 5' AGCGAGTGCA GAATGGACACAA 3', PKM2 for 5' ATTATTTGAGGAACT CCGCCG 3', PKM2 rev 5' ATTCCGGGTTCACAGCAATGAT 3', STAT3 for 5' TTCCTTGGGTGGAGAAG 3' and STAT3 rev 5' CGGACTGGATCTGGGTCT 3'. Samples lacking multiscribe reverse transcriptase formed negative controls to ensure target-specific quantification. Data were analyzed using the

comparative threshold cycle (Ct) method with normalization to the expression of RPLPO (for 5' GCGTCCTCGTGGAAAGTGA CATCG 3', rev 5' TCAGGGATTGCCACGCAGGG 3') and HPRT1 (for 5' ATGGACAGGACTGAACGTCTTG 3', rev 5' GGCTACAATGTGATGGCCTC 3') as endogenous controls.

Cellular Bioenergetic Function Analysis

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), reflecting oxidative phosphorylation and glycolysis, respectively, were measured using the Seahorse-XFe96 analyzer (Seahorse Biosciences). RAFLS/HUVEC were seeded at 15,000 cells per well in a 96-well cell culture XFe microplate (Seahorse Biosciences) and allowed to adhere overnight. Following this, cells were then cultured with OSM (10 ng/ml) for 3 h. For synergy experiments, cells were also incubated in the presence or absence of TNF α (1 ng/ml) for 3 h. Cells were then washed with assay medium (unbuffered DMEM supplemented with 10 mM glucose, pH-7.4) before incubation with assay medium for 30 min at 37°C in a non-CO₂ incubator. Basal oxidative phosphorylation/glycolysis was calculated by the average of three baseline OCR/ECAR measurements, respectively, obtained before injection of specific metabolic inhibitors; oligomycin (ATP-synthase-inhibitor) (2 μ g/ml; Seahorse Biosciences, UK) trifluorocarbonylcyanide phenylhydrazine (FCCP) (mitochondrial uncoupler) (5 μ M; Seahorse Biosciences) and antimycin A (complex-III inhibitor) (2 μ M; Seahorse Biosciences). Oligomycin was injected to evaluate both the maximal glycolytic rate and ATP synthesis, determined by subtracting the amount of respiration left after oligomycin injection from baseline OCR. FCCP was injected to evaluate the maximal respiratory capacity (average of three measurements following injection). Maximal respiratory capacity was determined by subtracting baseline OCR from FCCP-induced OCR and the respiratory reserve (baseline OCR subtracted from maximal respiratory capacity).

Protein Isolation and Western Blotting Analysis

RAFLS/HUVEC were grown to confluence in 6-well plates. Once confluent, cells were incubated in serum-free RPMI-1640 or MCDB-131 for 24 h and subsequently stimulated with OSM (10 ng/ml). For synergy experiments, cells were also incubated in the presence or absence of TNF α (1 ng/ml). Cells were trypsinized and collected prior to cell lysis. Ice-cold RIPA (Radio-Immunoprecipitation Assay) buffer (Sigma) containing 10 μ g/ml phosphatase inhibitor cocktail and 10 μ g/ml protease inhibitor cocktail (Sigma) was used to extract protein from HUVEC/RAFLS. Measurement of protein concentration was performed using a BCA assay (Pierce Chemical Co, Rockford, IL, USA). Protein (2–5 μ g) was resolved by SDS-PAGE (5% stacking, 10% resolving), gels were then transferred onto nitrocellulose membranes (Amersham Biosciences, Buckinghamshire, UK) prior to 1 h blocking in wash buffer containing 5% non-fat milk with gentle agitation at room temperature. Membranes were incubated with mouse monoclonal anti-HK2 (Novus Biologicals, USA), rabbit monoclonal anti-PFKFB3 (Abcam,

UK), rabbit polyclonal anti-GLUT-1 (Abcam), anti-pSTAT3, and anti-total STAT3 (Cell-Signaling Technology, UK) diluted in 5% non-fat milk containing 0.1% Tween 20 at 4°C overnight with gentle agitation. β -actin (1:5,000, Sigma) was used as a loading control. Following three 15 min washes, membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1:5,000) for 3 h at room temperature. The signal was detected using SuperSignal[®] West Pico Chemiluminescent Substrate (Amersham Biosciences). Band densities were imaged using the ChemiDoc MP Imaging System (Bio-Rad, USA).

Statistical Analysis

Statistical analyses were performed using Prism 5 software. Wilcoxon Signed Rank test or Mann Whitney was used for analysis of non-parametric data. Student *t*-test was used for parametric data. *P*-values of <0.05 (**p* < 0.05) were determined as statistically significant.

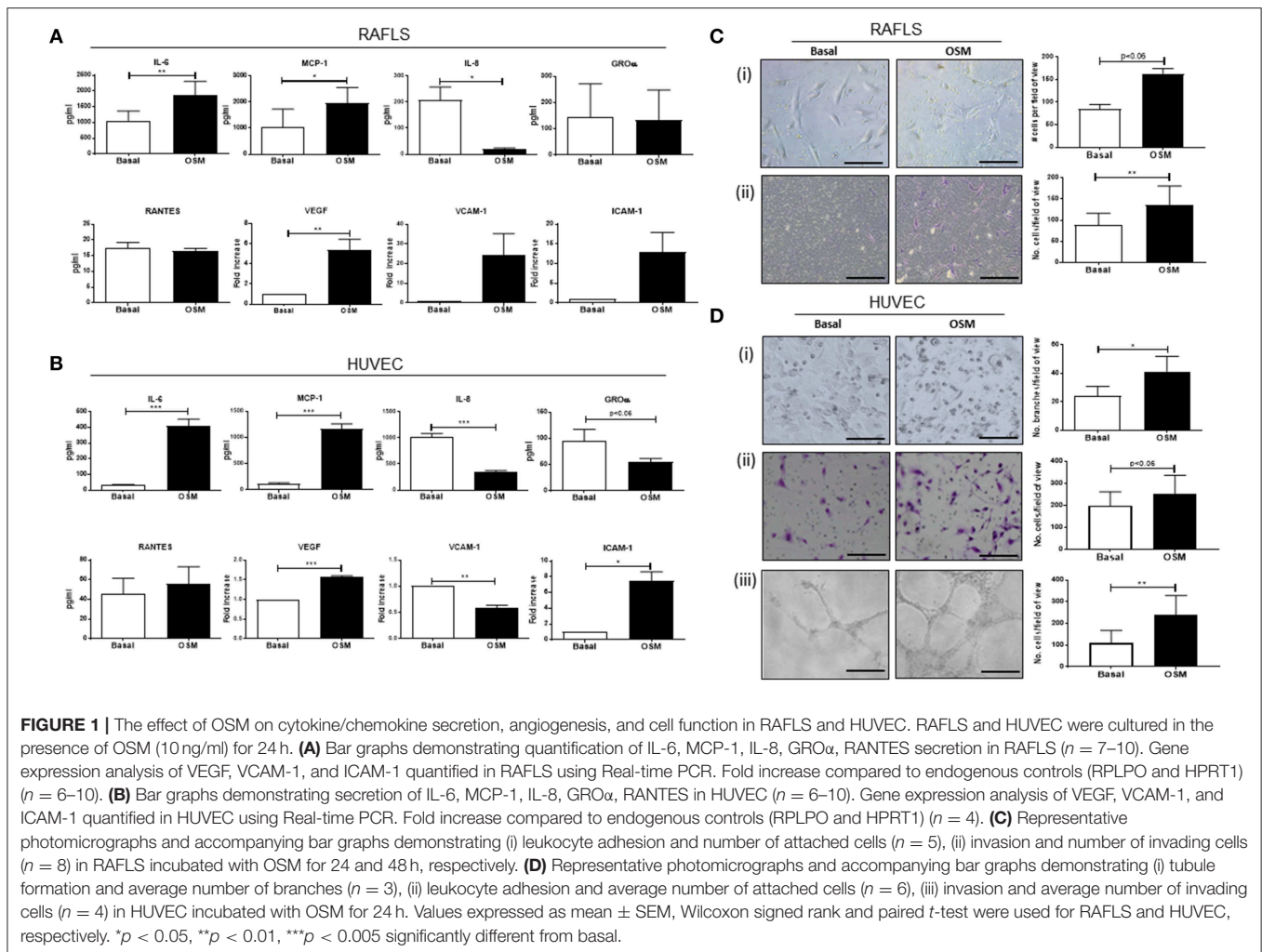
RESULTS

OSM Differentially Regulates Cytokine and Chemokine Secretion in RAFLS and HUVEC

To initially assess the effect of OSM on pro-inflammatory mechanisms, a range of pro-inflammatory mediators were measured in RAFLS and HUVEC (**Figure 1**). OSM significantly induced expression of IL-6, MCP-1, and ICAM-1 in RAFLS and HUVEC (all *p* < 0.05) (**Figures 1A,B**), in addition to the main angiogenic growth factor VEGF (*p* < 0.05) (**Figures 1A,B**). In contrast, OSM inhibited the secretion of IL-8 (*p* < 0.05) and GRO α from both RAFLS and HUVEC (**Figures 1A,B**), with no effect observed for RANTES (**Figures 1A,B**). Interestingly, OSM induced VCAM-1 in RAFLS (**Figure 1A**), but inhibited VCAM-1 expression in HUVEC (*p* < 0.05) (**Figure 1B**). This data demonstrates the differential effects of OSM, displaying both pro- and anti-inflammatory effects in different cell types, but also within the same cell type.

OSM Promotes Adhesion, Invasive, and Tube Formation Mechanisms in RAFLS and HUVEC

To further investigate the role of OSM we next assessed cellular function by performing adhesion, invasion and angiogenic assays. OSM stimulation significantly increased the adhesive capacity of RAFLS and HUVEC, resulting in a significant increase in PBMC attachment to the cell surface of RAFLS and HUVEC (**Figures 1Ci,Di**). Next, to assess the effects of OSM on RAFLS and HUVEC invasion, Transwell Matrigel[™] invasion chambers were utilized. Representative images of increased RAFLS and HUVEC invasion following OSM stimulation are shown in **Figures 1Cii,Dii**. Quantitatively, RAFLS and HUVEC invasion were significantly induced by OSM compared to basal control (both *p* < 0.05) (**Figures 1Cii,Dii**). Finally, representative images of HUVEC tube formation are shown in **Figure 1Diii**, demonstrating a significant increase



in the formation of tube-like structures in response to OSM ($p < 0.05$).

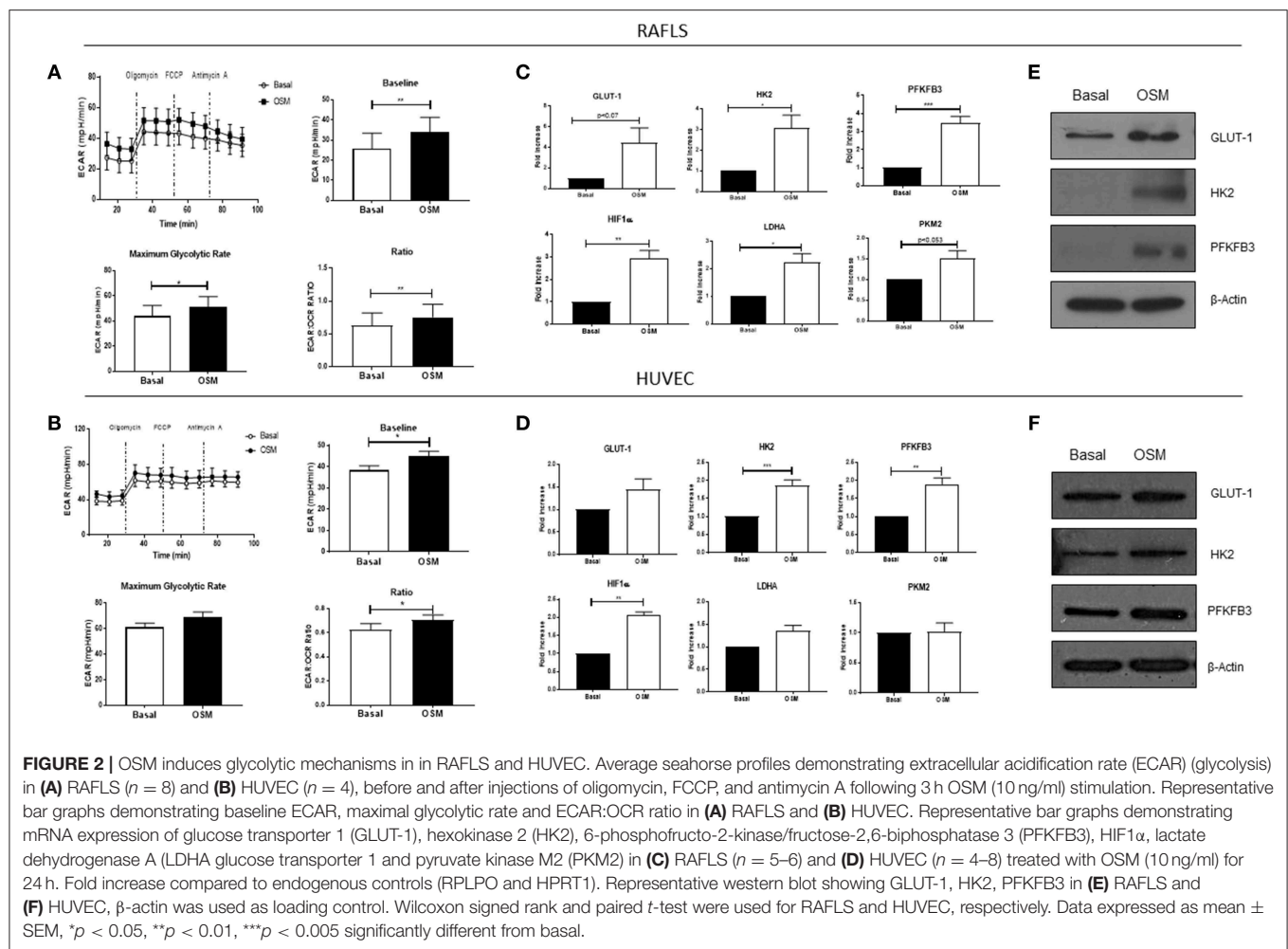
OSM Differentially Regulates Cellular Bioenergetics in RAFLS and HUVEC

To analyse the two major energy pathways, oxidative phosphorylation and glycolysis, in real time the Seahorse XFe-Analyzer was utilized as previously described (29). **Supplementary Figures 1A,B** demonstrates the average bioenergetic profiles for OCR in RAFLS and HUVEC cells before and after injections of mitochondrial inhibitors; oligomycin, FCCP, and antimycin A in OSM vs. basal control. OSM had no effect on basal respiration in RAFLS, yet increased the maximal respiratory capacity ($p < 0.05$), paralleled by a significant reduction in ATP synthesis ($p < 0.05$) (**Supplementary Figures 1A,C**). OSM had no effect on the OCR profile of HUVEC (**Supplementary Figures 1B,D**). This was accompanied by a significant shift to a glycolytic profile of both RAFLS and HUVEC, whereby OSM significantly increased baseline glycolysis ($p < 0.05$) and maximum glycolytic capacity ($p < 0.05$), leading to an overall increase in the ECAR/OCR

ratio in favor of glycolysis for both RAFLS and HUVEC (all $p < 0.05$) (**Figures 2A,B**). Furthermore, we demonstrated an increase in the glucose transporter GLUT-1 (**Figures 2C-F**) and in HIF1 α ($p < 0.01$) (**Figures 2C,D**), a master regulator of cellular and systemic homeostatic responses to hypoxia. This glycolytic shift was further supported by the observed increase in key glycolytic enzymes HK2 ($p < 0.05$) (**Figures 2C-F**), the first enzyme in the glycolysis pathway, and PFKFB3 ($p < 0.01$) (**Figures 2C-F**), which catalyzes the conversion of fructose-6-phosphate to fructose-2,6-bisP (F2,6BP). F2,6BP is a “potent” allosteric activator of 6-phosphofructokinase-1 (PFK-1) which is one of the rate-limiting enzymes of glycolysis. LDHA and PKM2 expression were also significantly increased in RAFLS in response to OSM, with no effect observed in HUVEC ($p < 0.05$) (**Figures 2C,D**).

OSM in Combination With TNF α Differentially Regulates Cytokines and Chemokines in RAFLS and HUVEC

We have shown that OSM displays differential effects on pro-inflammatory/angiogenic mediators in RAFLS and HUVEC, and

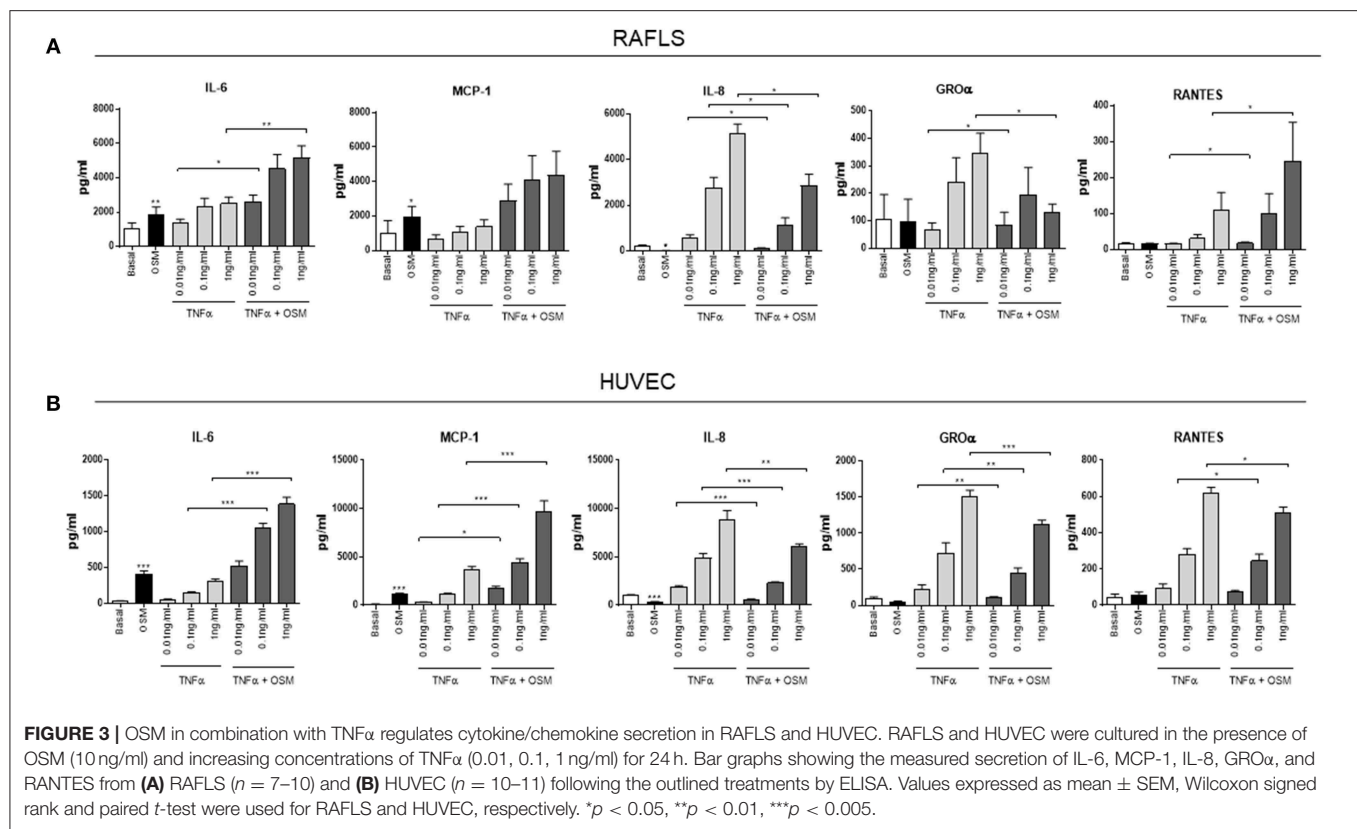


shown that in both cell types OSM induces a shift toward glycolysis. Based on previous studies demonstrating the ability of OSM to synergise with other key cytokines within the joint environment, we next examined the effect of OSM in combination with TNF α on these mechanisms. Both RAFLS and HUVEC were cultured with increasing concentrations of TNF α , in the presence or absence of OSM. OSM potentiated the effect of TNF α on both IL-6 ($p < 0.05$) and MCP-1 ($p < 0.05$) at all concentrations in both RAFLS and HUVEC (Figures 3A,B). In contrast, OSM inhibited the stimulatory effect of TNF α on IL-8 ($p < 0.05$) and GRO α ($p < 0.05$), with the levels of both chemokines significantly reduced in response to OSM + TNF α compared to TNF α alone (Figures 3A,B). Interestingly, OSM alone had no effect on RANTES secretion from both RAFLS and HUVEC, however in combination with TNF α , divergent effects were observed for RAFLS compared to HUVEC. OSM potentiated the effect of TNF α on RANTES secretion ($p < 0.05$) (Figure 3A), however OSM significantly inhibited the effect of TNF α on RANTES in HUVEC ($p < 0.05$) (Figure 3B). These data again show the divergent pro-/anti-inflammatory effects of OSM, and its ability to alter the effects

of one of the main cytokines that drives inflammation within the RA joint.

OSM in Combination With TNF α Regulates Metabolic Reprogramming in RAFLS, an Effect Mediated Through Phosphorylation of STAT3

To further explore the synergistic interaction between OSM and TNF α , we next examined their combined effect on cellular metabolism. While OSM had no effect on baseline OCR, TNF α alone significantly reduced baseline OCR ($p < 0.05$) (Figures 4A–C), an effect further potentiated with the combination of OSM+TNF α ($p < 0.05$) (Figures 4A–C). Maximum respiratory capacity was significantly reduced in response to the combination of OSM and TNF α ($p < 0.05$) (Figure 4B). Furthermore, the cytokines alone and in combination resulted in a stepwise inhibition of ATP synthesis (all $p < 0.05$) (Figure 4B). In contrast, OSM and TNF α alone and in combination, significantly induced a stepwise progressive increase in baseline glycolysis (all $p < 0.05$) (Figures 4C,D) and



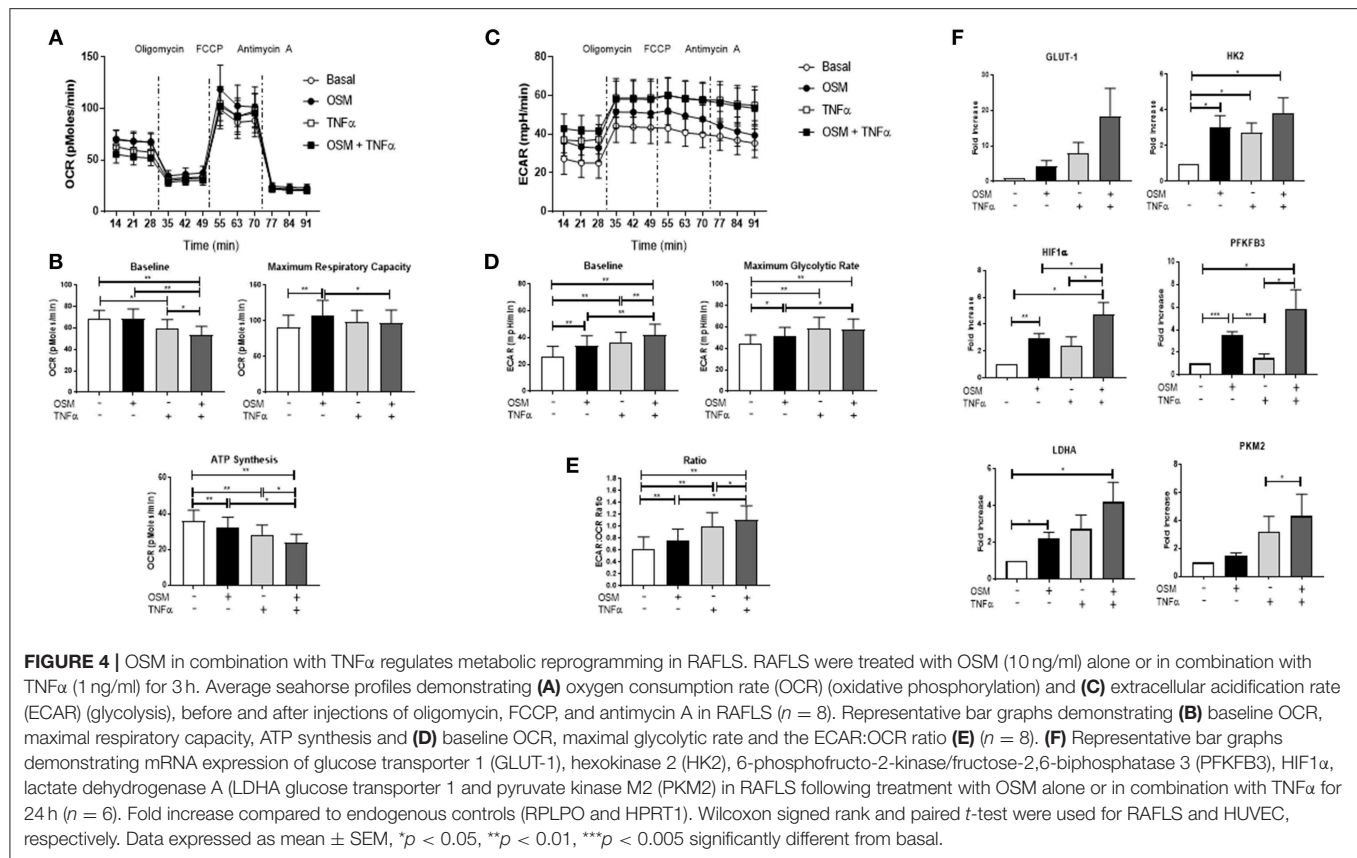
the maximal glycolytic rate (all $p < 0.05$) (Figures 4C,D). This resulted in an overall significant increase in the ECAR/OCR ratio in response to both OSM ($p < 0.05$) and TNF α ($p < 0.05$) alone, an effect that was potentiated in response to the combination ($p < 0.05$) (Figure 4E). This metabolic shift was further supported by the increased induction of GLUT-1, HK2 ($p < 0.05$), PFKFB3 ($p < 0.05$), HIF1 α ($p < 0.05$), LDHA ($p < 0.05$), and PKM2 ($p < 0.05$) in response to the combination of OSM+TNF α compared to either cytokine alone (Figure 4F). In contrast, no effect was observed for baseline OCR, maximum respiratory capacity or ATP synthesis in HUVEC in response to OSM + TNF α (Supplementary Figures 2A,B). However, similar to RAFLS, OSM + TNF α induced a significant induction in the glycolytic capacity of HUVEC as demonstrated in the ECAR profiles (Supplementary Figures 2C–E) and in the expression of key glycolytic genes (Supplementary Figure 2F).

The overall metabolic profile of both RAFLS and HUVEC is shown in Figure 5A, where they move toward a more glycolytic/energetic profile in response to the combination of OSM and TNF α however there are differences in the mechanisms whereby OCR was inhibited in RAFLS, with no effect observed for HUVEC. Therefore, we next assessed their effect on phosphorylation of STAT3, a key component of the JAK-STAT pathway which mediates OSM signaling. In RAFLS, OSM induced STAT3 gene expression ($p < 0.05$) (Figure 5B) and STAT3 phosphorylation (pSTAT3) as observed by western blot (Figure 5C and Supplementary Figure 3). TNF α also induced pSTAT3 but to a lesser extent (Figures 5B–D). Interestingly, the

combination of OSM and TNF α in RAFLS significantly induced both gene expression compared to either OSM or TNF α alone (Figure 5B). Furthermore, the combination of OSM and TNF α in RAFLS induced activation of STAT3 (Figure 5C) in two out of three RAFLS. In contrast, while OSM induced STAT3 gene expression and protein phosphorylation in HUVEC, the addition of TNF α had no effect either alone or in combination with OSM (Figures 5B,D). This suggests that in RAFLS, OSM, and TNF α have the ability to act together in the activation of STAT3, an effect that does not occur in HUVEC.

DISCUSSION

OSM is a crucial player in the pathogenesis of RA, however its relative contribution to specific mechanisms involved in synovial inflammation remain to be fully elucidated, primarily due to the pleiotropic nature of this cytokine. In this study we demonstrate that OSM alone differentially regulates pro-inflammatory mechanisms and significantly promotes pro-angiogenic and pro-invasive mechanisms in RAFLS and HUVEC. This is accompanied by a change in the cellular bioenergetic profile of the cells, whereby OSM significantly increases the ECAR/OCR ratio in favor of glycolysis, paralleled by the induction of the glucose transporter GLUT-1 and key glycolytic enzymes (HK2, PFKFB3, HIF1 α). Next, we demonstrate that OSM synergizes with TNF α to differentially regulate pro-inflammatory mechanisms in RAFLS and HUVEC. Interestingly, OSM synergizes with TNF α to regulate metabolic



reprogramming, whereby an induction of glycolytic activity with concomitant attenuation of mitochondrial respiration and ATP activity is observed in RAFLS, but not in HUVEC. Finally, we identify that the combination of OSM with TNF α induces transcriptional activity of STAT3 in RAFLS, with no effect observed in HUVEC. Together, this study indicates that OSM is an important player in orchestrating pro-inflammatory, angiogenic and invasive events in RA, specifically in RAFLS, effects that are mediated by interactions with both TNF α and STAT3.

In this study, OSM differentially regulates cytokine and chemokine secretion in both RAFLS and HUVEC, significantly inducing IL-6 and MCP-1, yet inhibiting IL-8 and GRO α , with minimal effect observed on RANTES. While this is the first study to demonstrate the opposing action of OSM on these specific cytokine/chemokines in RAFLS and HUVEC, it is consistent with previous studies demonstrating differential effects in other cell types (33, 34). Specifically, OSM alone can induce GM-CSF, IL-6, growth factors VEGF and bFGF, the osteoclastogenic cytokine RANKL, and many MMP (24, 35–38). Furthermore, OSM has been shown to differentially regulate chemokines/adhesion molecules, inducing CXCL5, IP10, CCL2, MCP-1, and ICAM-1 in lung fibroblasts, osteoblasts and epithelial cells, with no effect observed for other mediators such as GRO α , MIP-1, and VCAM-1 (24). The effect of OSM on chemokine expression has also been observed in mouse models of pneumonia (39),

experimental autoimmune encephalomyelitis (EAE), and cancer (40, 41).

Furthermore, we demonstrate that OSM promotes pro-angiogenic mechanisms and leukocyte adhesion, accompanied by induction of VEGF and ICAM-1. VEGF is a pivotal “on” switch for angiogenesis, promoting EC proliferation, migration, and invasion (42–45), with numerous studies demonstrating increased expression of VEGF and its receptors in RA synovial tissue (42, 46, 47). This is further supported by studies indicating that OSM can have differential effects on angiogenic mechanisms dependent on STAT activation, with pSTAT1 inhibiting VEGF expression, yet pSTAT3 promoting VEGF expression (48, 49). The effect of OSM on leukocyte adhesion has also been observed in mouse models of arthritis (50). OSM has also been shown to upregulate the expression of CCL13 in RAFLS (51), to induce key chemokines involved in leukocyte chemotaxis (CXCL3, CCL2, CCL5, CCL20), in addition to promoting infiltration of macrophages and neutrophils in mice models of inflammation (52). The observed differential effects of OSM on VCAM-1 expression again highlights its pleiotropic nature in different cell types, possibly influenced by the inflammatory microenvironment.

These striking changes in cellular function are, in fact, mirrored by distinct alterations in the metabolic profiles of OSM-treated cells, resulting in a shift in the ECAR:OCR ratio in favor of glycolysis. This shift is supported by the observed increase in key glycolytic drivers in response to

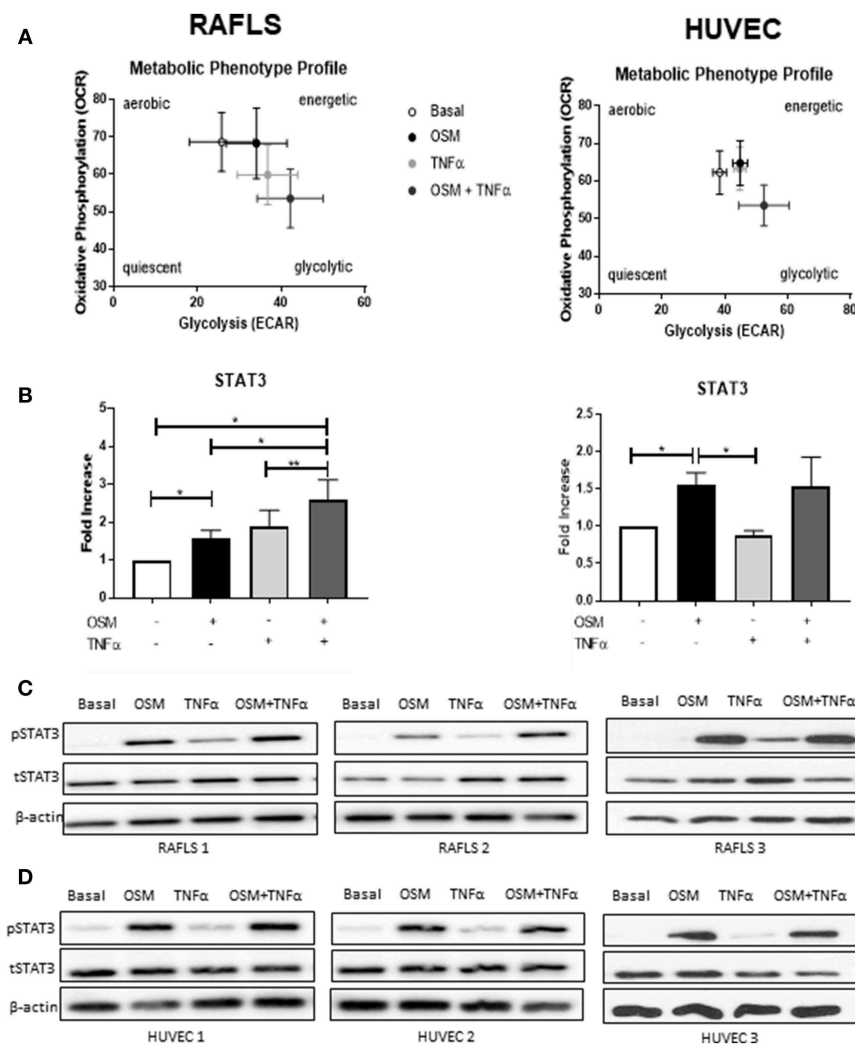


FIGURE 5 | OSM in combination TNF α regulates STAT3 expression in RAFLS. **(A)** Metabolic phenotype profiles in RAFLS and HUVEC representing changes in metabolic phenotype in response to OSM (10 ng/ml) and TNF α (1 ng/ml) alone and in combination. **(B)** Representative bar graphs demonstrating mRNA expression of STAT3 in RAFLS ($n = 6$) and HUVEC ($n = 4$) following treatment with OSM alone or in combination with TNF α for 24 h. Fold increase compared to endogenous controls (RPLPO and HPRT1), data analyzed using paired t -test. Data expressed as mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$. Representative western blot showing phospho-STAT3 (pSTAT3) and total-STAT3 (tSTAT3) in RAFLS **(C)** and HUVEC **(D)**. β -actin was used as loading control.

OSM treatment, where induction of HIF1 α , PFKFB3, HK2, LDHA, PKM2, and GLUT-1 was demonstrated. The metabolic switch in HUVEC in response to OSM is consistent with previous studies indicating that active endothelial cells rely heavily on glycolysis. Indeed, 85% of endothelial cell ATP requirements comes from the conversion of glucose to lactate, mechanisms that are crucial for tip cell formation and blood vessel migration (53–55). This preferential use of glycolysis has also been demonstrated in the inflamed RA joint, with studies showing that glycolytic markers are inversely correlated with synovial pO $_2$ levels (9, 56, 57). In addition, previous studies have shown that OSM can promote glycolytic mechanisms in human hepatocyte cell lines in a PDK-1-dependent manner and can induce HIF1 α in different cell types to promote tumor progression in cancer cells (58, 59). Consistent with the observed

increase in PFKFB3 in this study, previous studies have reported that blockade of PFKFB3 inhibits angiogenic tube formation, secretion of pro-inflammatory/angiogenic mediators, and key signaling pathways in both RAFLS and endothelial cells (9). Moreover, blockade of PFKFB3 in animal models of RA, psoriasis and colitis has led to resolution of inflammation (60, 61).

The inflamed synovial joint is hallmarked by a complex mixture of pro-inflammatory cytokines and chemokines interacting with each other to promote the inflammatory response. In this study we demonstrate that OSM potentiates the effect of TNF α on IL-6 and MCP-1 secretion from HUVEC and RAFLS, inhibits TNF α -induced IL-8 and GRO α , while displaying differential effects on RANTES, with OSM significantly inhibiting TNF α -induced RANTES expression in HUVEC, while potentiating TNF α -induced RANTES in RAFLS. The ability

of OSM to cooperate with key pro-inflammatory mediators such as IL-1 β , IL-17, and TNF α has been previously reported (25, 31, 62). In mouse synovial fibroblasts, OSM augments the effects of TNF α and IL-1 β on IL-6 secretion (38), inhibits IL-1 β -induced IL-8 and GM-CSF expression (30), and can synergise with TLR-4 to induce MCP-1 in human aortic adventitious fibroblasts and smooth muscle cells (63). Furthermore, OSM inhibits TNF α -induced TIMP-1 expression, yet potentiates IL-1 β -induced TIMP-1 and MMP-1 in RAFLS (24, 31, 64).

Interestingly, we identified that the synergistic effects observed with OSM and TNF α together also differentially altered the metabolic profile of the cells. Specifically, the combination of OSM and TNF α reduced the mitochondrial respiration paralleled by a stepwise induction of glycolysis in RAFLS, an effect not observed in HUVEC. Indeed, we demonstrate that the synergy between OSM and TNF α observed in RAFLS may be STAT3-dependent, an effect that appears to be specific to RAFLS and not HUVEC. In line with this, studies have demonstrated that TNF α is capable of indirectly activating the JAK-STAT pathway through induction of type I interferons in RAFLS (65). The mechanisms by which OSM regulates such effects within the inflamed joint however is unclear, yet studies have suggested that differential activation of its receptors gp130/LIF α and gp130/OSM or differential combinations of STATs (whether they form hetero- or homo-dimers) may account for such opposing effects (16, 66).

Furthermore, OSM has been shown to regulate STAT1/3 and STAT5/6 in mouse fibroblasts and is also capable of suppressing cell motility via STAT1 activation in lung cancer (67). Conversely, a recent study has demonstrated that murine OSM phosphorylates STAT3 via gp130/LIF activation but not STAT1 causing specific regulation of STAT3 responsive genes in primary osteocytes (68). Indeed, STAT3 itself is capable of interacting with other STATs; STAT1 for example has been demonstrated to exhibit inhibitory effects against STAT3 signaling in a study on esophageal squamous cell carcinoma (69). Thus, a clearer understanding of the various cues directing this complex transcriptional landscape is vitally important.

In this study, we propose that the altered cellular bioenergetics resulting from the synergy between OSM and TNF α may rely on STAT3 activation in RAFLS. Interactions between STAT3 and metabolic enzymes have also been demonstrated previously whereby blocking PFKFB3 causes inhibition of pSTAT3 expression in RAFLS (9). In cancer cells, STAT3 regulates glycolysis through HK2 (70, 71), and mediates HIF1 α -PKM2-interactions (54). Furthermore, STAT3, has been shown to be localized in the mitochondria, can bind to complex I and, in liver and heart cells, is capable of modulating the electron transport chain by altering activities of complex I and II (72). Finally, in the context of the RA joint, STAT3 interacts with various other key signaling molecules including Notch, NF- κ B, and hypoxia inducible factors (HIF), all of which regulate each other's activation through complex positive and negative feedback loops in the RA joint (73).

In conclusion, we have shown that OSM is capable of driving pro-inflammatory and metabolic changes, implicating it as a

crucial cytokine in orchestrating the inflammatory response in rheumatoid arthritis. Moreover, we demonstrate that OSM enhances the destructive effects of TNF α , a key pathogenic factor in disease pathogenesis, effects which are mediated through activation of STAT3.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript/**Supplementary Files**.

AUTHOR CONTRIBUTIONS

MH designed and performed experiments, analyzed data, and wrote the manuscript. TR, CC, and SA performed experiments. DV recruited the patients and contributed to the discussion. TM and UF supervised the project and co-wrote the manuscript. All authors read, revised, and approved the submitted manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2019.02056/full#supplementary-material>

Supplementary Figure 1 | OSM has differential effects on mitochondrial respiration in RAFLS and HUVEC. Average Seahorse profiles demonstrating oxygen consumption rate (OCR) (oxidative phosphorylation) in (A) RAFLS ($n = 8$) and (B) HUVEC ($n = 4$), before and after injections of oligomycin, FCCP, and antimycin A following 3 h OSM (10 ng/ml) stimulation. (C) Representative bar graphs demonstrating baseline OCR, maximal respiratory capacity, and ATP synthesis in RAFLS ($n = 8$). (D) Representative bar graphs demonstrating baseline OCR, maximal respiratory capacity, and ATP synthesis in HUVEC ($n = 4$). Wilcoxon signed rank and paired t -test were used for RAFLS and HUVEC, respectively. Data is expressed as mean $\pm$ SEM, ** $p < 0.01$ significantly different from basal.

Supplementary Figure 2 | OSM does not synergise with TNF α to regulate metabolic changes in HUVEC. HUVEC were treated with OSM (10 ng/ml) alone or in combination with TNF α (1 ng/ml) for 3 h. Average Seahorse profiles demonstrating (A) oxygen consumption rate (OCR) (oxidative phosphorylation) and (C) extracellular acidification rate (ECAR) (glycolysis), before and after injections of oligomycin, FCCP, and antimycin A ($n = 4$). Representative bar graphs demonstrating (B) baseline OCR, maximum respiratory capacity, ATP synthesis and (D) baseline ECAR, maximal glycolytic rate and (E) ECAR:OCR ratio. (F) Representative bar graphs demonstrating mRNA expression of glucose transporter 1 (GLUT-1), hexokinase 2 (HK2), 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3), HIF1 α , lactate dehydrogenase A LDHA glucose transporter 1 and pyruvate kinase M2 (PKM2) in HUVEC treated with OSM alone or in combination with TNF α for 24 h ($n = 4-5$). Fold increase compared to endogenous controls (RPLPO and HPRT1). Data analyzed using paired t -test, data expressed as mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ significantly different from basal.

Supplementary Figure 3 | OSM in combination TNF α regulates STAT3 expression. Bar graphs representing densitometry quantification of pSTAT3 normalized to β -actin in RAFLS (A) and HUVEC (B). Data expressed as mean $\pm$ SEM.

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Altered metabolic pathways regulate synovial inflammation in rheumatoid arthritis

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Summary

Rheumatoid arthritis is characterized by synovial proliferation, neovascularization and leucocyte extravasation leading to joint destruction and functional disability. The blood vessels in the inflamed synovium are highly dysregulated, resulting in poor delivery of oxygen; this, along with the increased metabolic demand of infiltrating immune cells and inflamed resident cells, results in the lack of key nutrients at the site of inflammation. In these adverse conditions synovial cells must adapt to generate sufficient energy to support their proliferation and activation status, and thus switch their cell metabolism from a resting regulatory state to a highly metabolically active state. This alters redox-sensitive signalling pathways and also results in the accumulation of metabolic intermediates which, in turn, can act as signalling molecules that further exacerbate the inflammatory response. The RA synovium is a multi-cellular tissue, and while many cell types interact to promote the inflammatory response, their metabolic requirements differ. Thus, understanding the complex interplay between hypoxia-induced signalling pathways, metabolic pathways and the inflammatory response will provide better insight into the underlying mechanisms of disease pathogenesis.

Keywords: arthritis (including rheumatoid arthritis), autoimmunity, inflammation

Hypoxia and metabolism in RA

Rheumatoid arthritis (RA) is a chronic progressive autoimmune disease, and is characterized by proliferation of the synovial membrane leading to degradation of articular cartilage and bone, and thus functional disability [1]. An increase in the number of blood vessels with an immature phenotype, displaying incomplete pericyte coverage, has been demonstrated in the RA synovium [2–4]. These blood vessels are dysregulated, and are thought to remain in a ‘plastic state’, thus primed for endothelial cell activation and sprouting, further facilitating immune cell recruitment [2,3,5]. The increased metabolic demand of the expanding synovial pannus leads to oxidative stress, altered cellular bioenergetics and a hypoxic microenvironment, which further promotes

abnormal cell function and synovial invasiveness within the RA joint [6]. The original hypothesis that the inflamed joint is hypoxic was based on studies measuring surrogate markers of hypoxia in RA synovial fluids, where an increase in glycolytic metabolites in the joint was observed [7,8]. Subsequent studies have shown that the inflamed joint is profoundly hypoxic [9–11], levels of which are inversely associated with increased synovitis, dysfunctional vascularity and microscopic inflammation [11,12]. Furthermore, hypoxia-mediated effects within the RA inflamed joint are not only dependent on HIF-1 α but involve complex interactions between key proinflammatory signalling pathways, hypoxia-inducible factor 1- α (HIF-1 α), nuclear factor kappa light-chain-enhancer of activated B cells (NF- κ B),

Notch-1 intracellular domain (Notch-1), Janus kinase/signal transducers and activators of transcription (JAK-STAT) and phosphoinositide 3-kinase-protein kinase B (PI3K-AKT) in synovial tissue and cells [13–16].

Evidence of a key role for metabolism in the regulation of synovial inflammation has recently emerged, where proliferation and rapid activation of immune and stromal cells requires a switch in cell metabolism from a resting regulatory state to a highly metabolically active state, in order to maintain energy homeostasis [6,17]. Under normoxic conditions, one glucose molecule enters the cell and is oxidized by glycolysis, generating two molecules of pyruvate, and in the presence of oxygen, pyruvate is decarboxylated by pyruvate dehydrogenase (PDH) then enters the tricarboxylic acid (TCA) cycle to produce 36 molecules of adenosine triphosphate (ATP) via a process known as oxidative phosphorylation (OXPHOS) (Fig. 1). In the absence of oxygen, pyruvate is diverted away from the TCA cycle, and instead pyruvate is converted to lactate by lactate dehydrogenase (LDH) in the cytosol, generating two molecules of ATP (Fig. 1). However, these metabolic changes can also occur in the presence of oxygen, a

process known as the ‘Warburg effect’. Although glycolysis is less efficient in terms of ATP production compared to the TCA cycle it produces ATP more rapidly, thus better meets the energy demands of activated synovial immune cells. In addition, many glycolytic-derived intermediate metabolites diverge into parallel pathways to promote nucleotide synthesis through the pentose phosphate pathway (PPP) to support cell proliferation and survival [18] and *de-novo* fatty acid synthesis required for the expansion of the endoplasmic and Golgi membranes for the synthesis, trafficking and secretion of proteins [19].

Evidence for this metabolic switch in RA has been demonstrated by several studies showing an increase in mitochondrial dysfunction in the RA synovium [20], along with increased expression of glucose transporters (GLUTs) [21] and glycolytic enzymes, including hexokinase 2 (HK2), pyruvate kinase M2 (PKM2), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), glucose-6-phosphate isomerase (GPI) and LDH [7,8,21–25]. Marked accumulation of metabolic intermediates including lactate, glutamine, succinate [26] and itaconate [27] have been demonstrated in the RA joint which, in turn, can activate synovial cells

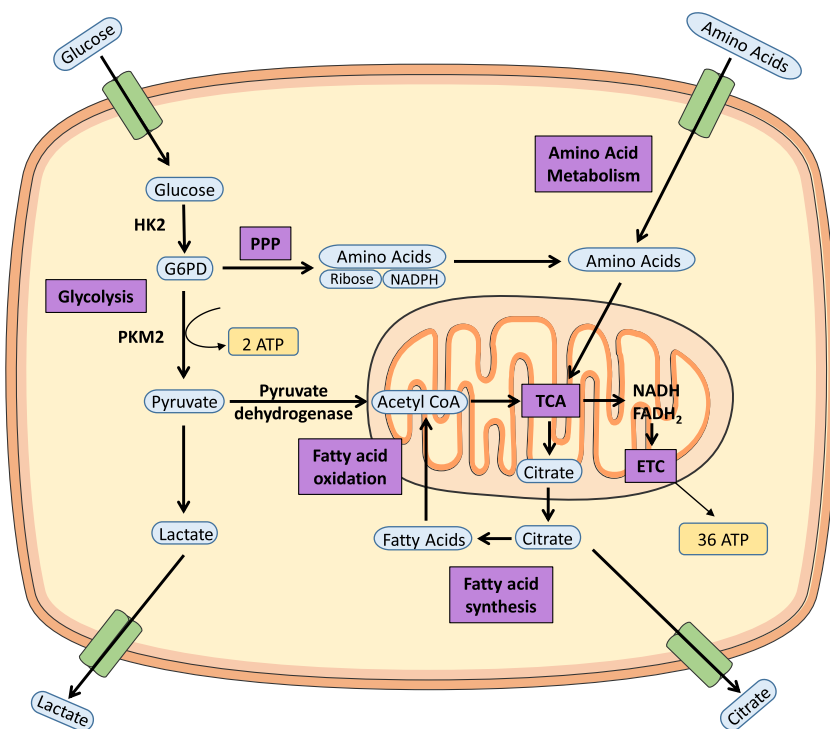


Fig. 1. Schematic illustration of the key metabolic pathways. Glucose enters the cell via glucose transporters and enters the glycolytic pathway. Hexokinase 2 (HK2) converts glucose into glucose 6-phosphate dehydrogenase (G6PD). Glycolysis generates pyruvate from glucose with the help of pyruvate kinase M2 (PKM2). This process generates energy in the form of adenosine triphosphate (ATP). Pyruvate is then either converted to lactate and secreted out of the cell or decarboxylated by pyruvate dehydrogenase and converted to acetyl CoA, which enters the tricarboxylic acid (TCA) cycle. The TCA cycle generates nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide (FADH₂) to feed into the electron transport chain (ETC), which produces 36 molecules of ATP. Glycolysis also feeds into the pentose phosphate pathway (PPP) to produce ribose, NADPH and amino acids. Amino acid metabolism can also feed into the TCA cycle to drive ATP production by the ETC. TCA intermediate citrate drives fatty acid synthesis while fatty acid oxidation drives TCA cycle further by generating acetyl CoA.

[21,28,29], further perpetuating disease. In animal models of arthritis and in *ex-vivo* RA synovial tissue explants, several studies have shown that re-programming of these pathways with specific inhibitors leads to resolution of inflammation [21,30–34].

The RA synovium is a multi-cellular tissue, and many cell types interact to promote the inflammatory response. While synovial cells including T cells, macrophages, dendritic cells and synovial fibroblasts co-exist in this micro-environment, they utilize metabolites differently. Indeed, metabolites produced by one cell can have profound regulatory effects on the function of another cell within the RA synovium. This highlights the need to dissect metabolic pathways and utilization of metabolites in different cell types within the RA synovium, as any potential metabolic therapeutic treatment may be cell type-specific. While there is a significant number of studies examining metabolic pathways in different immune cell subtypes, few studies have investigated these pathways in the context of the individual cells in the RA joint.

The role of T cell metabolism in RA

Growing understanding of the relationship between metabolic processes within T cells and their activation, differentiation and effector functions is emerging. It is now well established that whereas resting T cells primarily utilize oxidative phosphorylation, aerobic glycolysis is promoted within minutes of T cell receptor (TCR) activation independently of transcription or translation via activation of PDHK1 [35], followed by a more sustained induction of the glycolytic machinery [34]. This rapid metabolic reprogramming towards aerobic glycolysis provides the energy and substrates necessary for the proliferation and effector functions of activated T cells. In addition, the differentiation of T helper (Th) into Th17 *versus* regulatory T cells (T_{reg}) cell is profoundly influenced by metabolism. Th17 cell differentiation requires glycolysis and HIF-1 α , whereas HIF-1 α suppresses T_{reg} cell differentiation [31,36]. Despite these recent advances, little is known about how metabolism influences human T cell responses, in particular those of already differentiated memory T cells that are found at sites of inflammation such as the RA joint. There are a number of site-specific factors that are likely to impact upon the metabolism of T cells within the inflamed RA joint, including hypoxia and activation of HIF-1 α , altered nutrient availability and the accumulation of metabolites.

The hypoxic environment of the RA joint might be expected to drive glycolysis in T cells via the induction of HIF-1 α . However, in the circulation there is recent evidence to suggest that this is not, in fact, the case. Rather, naive peripheral blood CD4 T cells from RA patients have been shown to have a defect in

6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), which is the rate-limiting enzyme in the glycolytic pathway [37]. This inhibits their ability to utilize glycolysis upon activation and instead diverts glucose-6-phosphate towards the PPP, which runs in parallel to glycolysis, PPP supporting mainly nucleotide synthesis [37]. The PPP generates the 5-carbon building blocks for nucleic acids in addition to significant reductive power in the form of nicotinamide adenine dinucleotide phosphate (NADPH) [37]. NADPH is required for the process of fatty acid synthesis; indeed, subsequent work by the same group revealed that increased fatty acid synthesis and accumulation of lipid droplets within the cell was a consequence of the reduced glycolytic flux within RA T cells [34]. Furthermore, these conditions resulted in the up-regulation of tyrosine kinase substrate with five SH3 domains (TKS5) expression, which promoted tissue invasiveness of the RA T cells. RA T cell invasiveness could be reversed via inhibition of fatty acid synthesis, suggesting that the fatty acid synthesis pathway may represent a therapeutic target in RA [34]. These studies have provided important insights into the metabolic status of CD4 T cells in RA. The role of CD8 T cells in RA has received less attention than that of CD4 T cells; however, future studies will be important to determine whether CD8 T cells in RA are subject to similar metabolic control.

The inflammatory environment of the RA joint leads to the accumulation of various metabolites [26,28], and it has now become apparent that these metabolites are not simply by-products of metabolism but also exert important immune modulating effects. Interestingly, lactate has been shown to modulate T cell function directly via specific cell surface lactate transporters [28]. Sodium lactate inhibits the migration of CD4 T cells by inhibition of glycolysis via direct inhibition of HK and PFK [28]. In addition, sodium lactate also promotes the expression of interleukin (IL)-17 by CD4 T cells [28]. Furthermore, expression of the lactate transporter SLC5a12 in synovial tissue was shown to correlate with the clinical T cell score, and *in-vivo* blockade of lactate transporters resulted in the release of T cells from the inflammatory site [28]. These data suggest that the elevated lactate concentrations observed in the RA joint inhibit the glycolysis and migration of effector T cells, resulting in their retention at the site of inflammation and also increasing their production of IL-17. These findings provide a rationale for the therapeutic targeting of specific lactate transporters on T cells in autoimmune diseases such as RA (reviewed in [38]). Consistent with this, expression levels of the lactate transporter MCT4 and glycolytic enzymes HK2, GPI, triosephosphate isomerase (TPI), enolase 1 (Eno 1), PKM2 and LDH are significantly reduced in Th17 cells obtained from HIF1 $\alpha^{-/-}$ compared to wild-type (WT) mice [31], demonstrating the dependence of increased glycolytic enzymatic

activity on the oxygen sensing pathway. Furthermore, in animal models of arthritis 3-bromopyruvate (BrPA), a specific HK2 inhibitor, significantly decreased clinical arthritis scores in SKG mice, paralleled by an increase in the $T_{reg}/Th17$ ratio [30]. While the exact underlying mechanisms involved are unclear, it is hypothesized that BrPA may alter the $T_{reg}/Th17$ ratio through differential regulation of their respective transcription factors forkhead box protein 3 (FoxP3) and retinoic acid receptor-related orphan nuclear receptor gamma t (ROR- γ t). Finally, the TCA cycle metabolite succinate has been shown to exert proinflammatory effects on murine macrophages via induction of IL-1 β [39] which, in turn, might be expected to drive Th17 responses. Indeed, a recent study showed that deficiency of the succinate receptor GPR91 attenuates the severity of arthritis in *Sucnr1*^{-/-} mice, reducing expansion of Th17 cells [40].

The role of monocyte and macrophage metabolism in RA

Activated macrophages promote a number of proinflammatory mechanisms in the RA synovium through abundant secretion of proinflammatory cytokines; in addition, macrophages induce nitric oxide synthase, present antigen to T and B cells and drive bone resorption [41]. RA synovial CD68⁺ macrophages correlate strongly with mitochondrial dysfunction and oxidative stress and are inversely related to *in-vivo* synovial pO₂ levels [11,12,20]. HIF-1 α is expressed abundantly by macrophages in the RA synovium, compared to osteoarthritis (OA) and healthy control synovial macrophages [42]. Differential signalling mechanisms in monocytes and macrophages under hypoxic conditions have been observed where monocytes preferentially utilize NF- κ B1, while macrophages utilize HIF-1 α [43]. Furthermore, in collagen-induced arthritis (CIA) models, decreased infiltration of myeloid cells to the joint, reduced paw swelling and disease development was observed in animals with HIF-1 α -deficient macrophages [44].

In the context of metabolic changes, studies have demonstrated comprehensively that classically activated M1 macrophages have an ardent appetite for glucose, indicating a reliance on glycolysis, in contrast to M2-like macrophages which rely on OXPHOS [45,46]. Indeed, recent studies have shown that the TCA cycle is broken at two key steps in M1 macrophages – after citrate and after succinate [39,46]. The majority of research has, however, focused on *in-vitro* monocyte-derived M1 and M2 macrophage models, and not synovial macrophages, due to the difficulty of isolating these cells; however, it is now becoming apparent that the M1 *versus* M2 paradigm may be an over-simplification, and that a spectrum of activation states exists between these two poles within the synovial joint. In the context of RA, studies have shown

increased lactic acid, citrate and succinate in RA synovial fluids [26], consistent with studies showing increased glycolysis and a broken TCA cycle in M1 macrophages. Lactic acid enhances secretion of IL-6 and IL-23 from monocytes and macrophages [47]. Accumulation of succinate in macrophages promotes HIF-1 α activation which, in turn, induces IL-1 β production [39] and in animal models of RA, mice lacking the succinate receptor GPR91 show reduced macrophage activation and secretion of IL-1 β [33]. Itaconate, a metabolic inhibitor of succinate dehydrogenase which has been shown to regulate succinate levels and secretion of inflammatory cytokines in activated macrophages [48], is also increased in the RA joint, and is associated with disease activity and response to therapy in animal models of arthritis [27]. RA macrophages express high amounts of the glycolytic enzyme α -enolase, which through autoantibody recognition induces secretion of proinflammatory cytokines [49]. High concentrations of glucose have also been shown to increase IL-1 β secretion from RA monocytes through an NLRP3-dependent mechanism [50]. Solute carrier family 7 member 5 (SLC7A5), a key amino acid transporter, is increased in RA monocytes and macrophages, silencing of which leads to a significant reduction of IL-1 β [51]. Finally, activation of 5' AMP-activated protein kinase (AMPK) in macrophages inhibits IL-6 production, differentiation of M2 macrophages from synovial fluid monocytes and macrophage expression of IL-6, tumour necrosis factor (TNF)- α and NF- κ B in animal models of arthritis [52,53].

More recent data have shown that macrophages from both RA and coronary artery disease (CAD) patients share metabolic abnormalities to promote inflammation. Disease macrophages appear to be in a hypermetabolic state, addicted to glucose consumption and producing more ATP compared to healthy macrophages [54]. In addition, it has been demonstrated that macrophages from CAD patients are capable of memorizing both the metabolic and inflammatory signatures of their precursor monocytes, indicating that there is a memory bias towards a hyper-inflammatory and hypermetabolic phenotype in disease macrophages [55]. There has been speculation that epigenetic regulation may be the cause of this memorized immune response in both monocytes and macrophages [56–58]. Thus, epigenetic reprogramming is emerging as a key mechanism in macrophage immunometabolism.

The role of metabolism in DC activation in RA

Dendritic cells (DCs) are key players in immunity, and link the innate and adaptive immune response through antigen presentation and cytokine production. Resting DCs, which differ from activated DCs as they are less motile, secretory and immunogenic, rely predominantly on OXPHOS for their energetic needs [59–61]. Activation

of DCs, either by antigen exposure or stimulation by Toll-like receptor (TLR) ligands, results in a two-phased metabolic shift comprised of an initial glycolytic shift with maintained OXPHOS, followed by elevated glycolysis and the cessation of OXPHOS in long-term activated DCs. While the early increase in glycolysis is associated with elevated GLUT1 expression [61,62] and lactate production [59–64], the increased glucose demand by activated DCs is associated with the requirement of glucose-derived metabolic intermediates into the PPP and fatty acid synthesis to facilitate amino acid and protein synthesis for the secretion of proinflammatory mediators associated with DC activation. Inhibition of glycolysis via HK2 blockade or fatty acid synthase in lipopolysaccharide (LPS)-stimulated DCs results in a marked reduction in DC activation and immunogenicity [60]. Molecularly, TBK-IKKe/AKT signalling pathways and downstream target mammalian target of rapamycin (mTOR) have been strongly implicated in early activation of the glycolytic shift, which can be prevented by adhesion-related kinase (ARK) blockade [62]. In the context of RA, while also modulating the T_{reg} /Th17 ratio, BrPA-mediated blockade of HK2 suppressed DC activation and cytokine expression [30], suggesting that the glycolysis pathway may represent a potential therapeutic target in RA.

During the later stages of DC activation, mTOR-mediated induction of inducible nitric oxide synthase (iNOS) and stabilization of HIF-1 α is important for the complete commitment of activated DCs to glycolysis [59,62], with iNOS-derived nitric oxide (NO) blocking the electron transport chain (ETC) and sequestering OXPHOS [59] while elevating HIF1 α -dependent glycolytic gene expression [62,63,65]. While these metabolic changes remain to be studied in the complex nutrient and/or oxygen-deprived microenvironments, such as those found in the inflamed synovium, similar to macrophages, DC can also sense and respond to extracellular metabolites, such as succinate, butyrate and ATP [33,66–69] to potentially trigger a co-ordinated cellular response reflective of the metabolic state of the surrounding microenvironment. A recent study demonstrated that the succinate receptor, GPR91, acts as a chemotactic that facilitates migration of DCs into the lymph nodes, which induces the expansion of Th17 cells and subsequent development of experimental antigen-induced arthritis [40]. This is consistent with previous studies that demonstrated that succinate can promote chemotaxis of DCs through activation of the succinate receptor [66,67].

Role of metabolism in synovial fibroblast activation in RA

Synovial fibroblasts (FLS) are fundamental to disease progression and are active drivers of joint destruction in RA [70]. FLS are characterized by increased proliferation,

resistance to apoptosis and are potent producers of pro-inflammatory cytokines and matrix degradation enzymes, resulting in a highly invasive phenotype [71,72]. Previous studies have demonstrated increased mitochondrial dysfunction, coupled by changes in the ultrastructure of mitochondria in RA-FLS in response to inflammatory stimuli, in addition to altered expression of mitochondrial genes associated with apoptosis, redox balance and mitochondrial protein transport [21,23,73]. Metabolic profiling of FLS demonstrated increases in sugar metabolism (glycolysis and PPP) and amino acid metabolism (tyrosine and catecholamine biosynthesis and protein biosynthesis) [74]. RA-FLS also demonstrate a reliance on glutamine metabolism, with *in-vitro* studies demonstrating inhibition of RA-FLS proliferative and invasive functions under glutamine-deprived conditions [29]. Hypoxia, oxidative stress, TLR2 and TNF- α activation all promote a switch in the metabolic profile of RA-FLS, where an increase in the glycolysis : OXPHOS ratio is observed, paralleled by increases in surrogate markers of glycolysis; PFKFB3, PKM2 and GLUT1 and a more invasive phenotype [21,23,75,76]. Glycolytic inhibitors, including the PFKFB3 inhibitor 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) [21] and HK2 inhibitor, BrPA [76] significantly inhibit RA-FLS invasion and migration capacity, secretion of proinflammatory mediators and activation of HIF-1 α , pSTAT-3, NF- κ B and Notch-1IC. Furthermore, accumulation of metabolic intermediates, including lactic acid, glutamine and succinate in the RA joint, have all been shown to further induce the RA-FLS invasive phenotype [21,76]. Other key pathways involved in FLS activation include the PI3K/AKT1/mTOR, blockade of which resulted in repressed RA-FLS function [77]. Studies have shown that mTOR blockade of RA-FLS invasion is mediated in part through regulation of focal adhesion kinase (FAK) signalling pathways and cytoskeletal rearrangement, a key mechanism involved in RA-FLS movement [78]. Furthermore, a recent study has shown that TNF- α signalling co-opts the mTOR pathway, directing specific signalling pathways that regulate the RA-FLS response to inflammatory stimuli, an effect coupled with nutrient availability of specific amino acids [79]. This is consistent with another study showing that IL-17-induced RA-FLS migration is mediated through the amino acid transporter (LAT1) via mTOR [80].

Metabolomic profiling has shown changes in lipid metabolism of RA-FLS compared to OA cells [76]; however, the role of lipids in the regulation of FLS function has not been studied extensively. Interestingly, studies have shown that molecules in the choline pathway, which can interact with lipids, are highly activated in RA-FLS, with the choline kinase (ChoKa) enzyme [81] and choline transporters [82] increased in RA-FLS. Furthermore, a ChoKa inhibitor suppressed the migrative/invasive

mechanisms of cultured RA-FLS and *in vivo* ameliorated inflammation in the KxBN model [81]. Thus, RA-FLS are transformed from a quiescent state to an aggressive, invasive phenotype in this adverse environment through adaptation of metabolic pathways in order to meet their energy demands, which allows them to resist apoptosis and persist within the inflamed joint.

The above findings in FLS, however, are all performed in RA-FLS which are maintained in culture *in vitro*, so while they provide some understanding of the metabolic pathways activated in RA-FLS in response to specific stimuli, we still lack an understanding of the metabolic demands of RA-FLS subtypes within this adverse inflammatory joint environment. Indeed, it has now become apparent that there are subsets of synovial fibroblasts within the inflamed joint that display pro- and anti-inflammatory phenotypes, with effects also dependent upon positional memory [83–85]. Therefore, further studies are required to examine if different metabolic profiles are observed depending on the RA-FLS subtype and the anatomical location.

Metabolism of synovial endothelium

Endothelial cells (EC) rely heavily on glycolysis, with 85% of the ATP requirements coming from the conversion of glucose to lactate [86]. The three EC subsets (tip, stalk and phalanx cells) differ in their metabolic requirements reflective of their individual functions (migration, proliferation and quiescence, respectively). When ECs are activated, metabolic changes dictate phenotypical differentiation, with tip and stalk cells showing increased glycolytic rates compared to phalanx EC [87]. In the context of RA, few studies have extensively studied metabolic regulation of synovial vessels; however, they display similar dysfunctional morphology to that of the tumour vasculature [2]. RA synovial blood vessel instability and oxidative damage correlate inversely with synovial pO₂ levels [2], and display enhanced expression of the glucose transporter GLUT1 and the glycolytic enzymes GAPDH and PKM2 [21]. Exposure of ECs to hypoxic conditions and oxidative stress induces tube formation, migration and proinflammatory mediators, including vascular endothelial growth factor (VEGF), angiopoietins, monocyte chemoattractant protein 1 (MCP-1), IL-8 and matrix metalloproteinases (MMPs) [2,75,88]. In RA-FLS and EC, hypoxia induces activation of intracellular Notch-1IC and its ligand DLL-4, interaction of which is critical for EC tip cell selection, and lateral inhibition of the trailing stalk cell [13,14]. Blockade of the glycolytic enzyme, PFKFB3, inhibits angiogenic tube formation, secretion of proinflammatory/angiogenic mediators and key signalling pathways in RA-FLS and EC [21]. In addition, enriched expression of G6PI in synovial endothelial cells has recently

been shown, with *in-vitro* G6PI loss-of-function assays demonstrating the requirement of G6PI in mediating hypoxia-induced angiogenesis in RA [89]. Finally, in animal models of arthritis, succinate has been shown to induce synovial angiogenesis through VEGF-dependent HIF-1 α pathways [90]. Therefore, a deeper understanding of the metabolic perturbations in pathological conditions and their cross-talk with immune and stromal cells might offer novel therapeutic opportunities, especially in early disease.

Targeting metabolism in rheumatoid arthritis

As highlighted above, studies examining metabolic pathways in RA have identified specific pathways, enzymes or metabolic intermediates that could potentially be targeted; however, many of our current treatment strategies are already known to alter metabolic pathways. For instance, glucocorticoids which are used routinely as first-line treatment and regulate transcription of many metabolic genes [91] associated with glycolytic, autophagy and mTOR pathways [92,93]. Conventional disease-modifying anti-rheumatic drugs (DMARDs) for RA and psoriatic arthritis (PsA), including methotrexate, leflunomide and apremilast, are anti-metabolic, where they target purine or pyrimidine nucleotide metabolism, the effects of which are known to inhibit both T cells and synovial fibroblast proliferation. Studies have shown that anti-TNF- α treatment decreases expression of GLUT1 and key glycolytic enzymes PKM2 and GAPDH in RA synovium in TNFi responders *versus* non-responders [21]. Tocilizumab, an anti-IL-6 receptor antibody, improved endothelial function and inhibited oxidative stress in RA leucocytes [94]. Tofacitinib, a JAK1 and JAK3 inhibitor, reduces glycolysis in activated synovial fibroblasts, and in RA synovial explants inhibits key glycolytic enzymes, paralleled by reduced expression of key proinflammatory mediators and synovial fibroblast outgrowths [95]. Indeed, interaction of STAT-3 and PKM2 leads to activation of HIF1 α , with subsequent induction of cellular invasive mechanisms creating a vicious PKM2/STAT-3/HIF1 feedback loop [96]. Furthermore, STAT-3 blockade inhibits Notch signalling, which is involved in endothelial tip cell selection and fibroblast invasive mechanisms [1].

Directly targeting specific metabolic pathways has been demonstrated both in *in-vitro* and *in-vivo* models of arthritis where blockade of key glycolytic enzymes inhibits proinflammatory mechanisms [21,30,55,97]. Targeting metabolic intermediates including lactate acid, succinate, citrate, itaconate and lipids are also promising therapeutic avenues, where their cellular accumulation regulates synovial fibroblast invasiveness [21], T cell differentiation and migration [28,31,34], in addition to macrophage polarization [54]. Furthermore, the anti-cancer drug rapamycin,

which targets mTOR, plays a critical role in directing T cell differentiation and function, inhibition of which promotes T_{reg} cell generation [98]. mTOR is also a major repressor of autophagy, and in animal models of arthritis, systemic administration of rapamycin was shown to induce autophagy, paralleled by decreased severity of synovitis and reduction in IL-1 β expression [99]. Finally, metformin, the anti-diabetic drug, which acts in part by indirectly activating the energy sensor 5' AMP-activated protein kinase (AMPK), has been shown to attenuate disease in mouse models of arthritis [100], effects that are mediated by inhibition of mTOR activity, enhanced autophagic flux, suppression of NF- κ B signalling and inflammatory cytokine production [101].

Summary

Lack of nutrients and a poor oxygen supply, paralleled by the increased metabolic demand of the expanding synovial pannus, leads to a bioenergetic crisis. In this environment synovial cells show adaptive survival

responses by switching their utilization of specific metabolic pathways (Fig. 2) in order to satisfy their energy demands. This, in turn, activates key transcriptional signalling pathways which further exacerbates inflammation. However, this environment is complex, with many different cell types co-existing within the inflamed synovium that display different metabolic requirements. Furthermore, metabolites secreted from one cell type have the ability to regulate the pathogenic phenotype of another, thus amplifying the inflammatory response. Understanding the opposing metabolic requirements of the different cell types will provide significant insights into their relevant pathogenic contribution to disease. However, currently the metabolic regulation of specific cell-types/subsets within the inflamed synovium are poorly understood. Metabolic regulation can also have opposing effects depending on the disease settings. For instance, while Haas *et al.* elegantly described that high lactate levels at the site of inflammation act to entrap CD4⁺ T cells in RA by inhibiting T cell motility [28], in the tumour microenvironment accumulation of lactate impairs T cell function and hinders

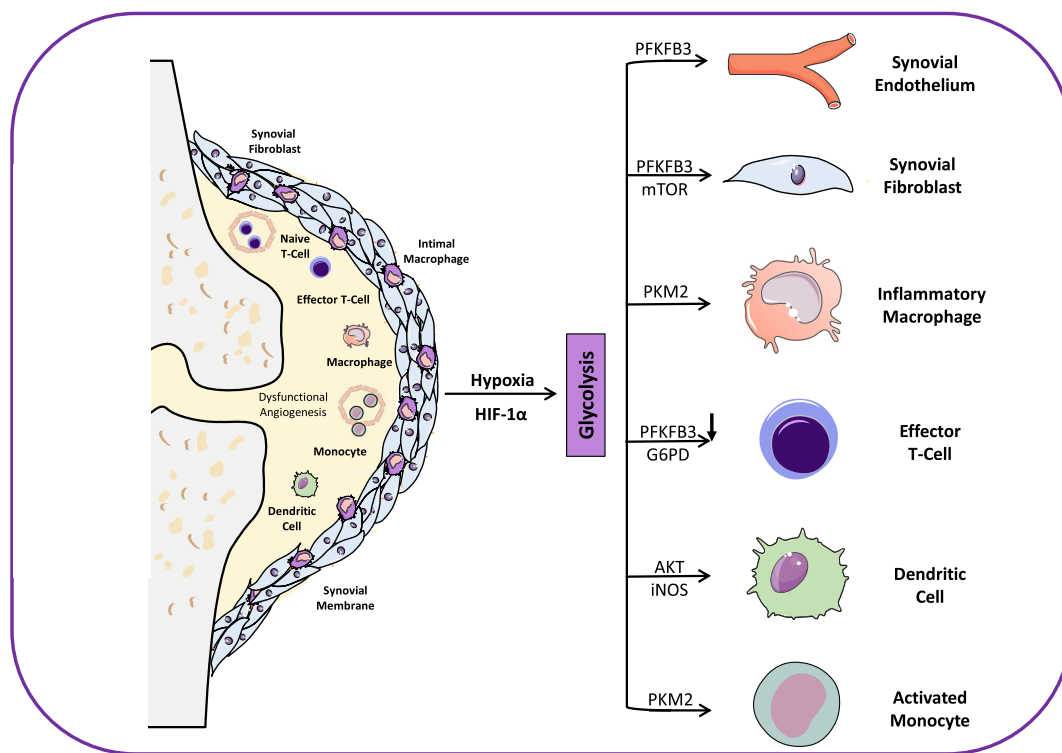


Fig. 2. Schematic illustration of the glycolytic switch in different cell types. The hypoxic conditions of the synovial joint drives hypoxia-inducible factor 1-alpha (HIF-1 α)-induced glycolysis in some of the major cell types of the synovium. HIF-1 α induces expression of some of the key molecular switches to encode proinflammatory and pro-glycolytic mechanisms. Specifically, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) is up-regulated in endothelial cells and synovial fibroblasts in response to hypoxia. The mammalian target of rapamycin (mTOR) pathway is also involved in synovial fibroblast activation. Pyruvate dehydrogenase (PKM2) plays a central role in the metabolic switch observed in inflammatory macrophages and activated monocytes. DC metabolism can be directed by protein kinase B (AKT) signalling pathways in early activation while mTOR-mediated induction of inducible nitric oxide synthase (iNOS) is important during later stages of DC activation, while glucose-6-phosphate dehydrogenase (G6PD) and PFKFB3 are key players in the metabolic switch observed in effector T cells.

their cytotoxicity [102]. Thus, lactate serves to boost pro-inflammatory mechanisms in the inflammatory milieu, yet suppresses immunity in the cancer setting. Enolase has been shown to induce monocyte/macrophage activation in models of RA [49], but promotes T_{reg} cell development in cancer models [103]. Arginine metabolism drives a pro-glycolytic phenotype in macrophages and DCs [60,104]; however, it promotes a metabolic switch to OXPHOS, exerting anti-inflammatory effects in T cells [105,106]. Finally, the amino acid transporter SLC7A5, through leucine influx, induces proinflammatory cytokine secretion in RA monocytes and macrophages via mTORC1-induced glycolytic reprogramming [107], and has also been demonstrated to play a key role in tumour survival and growth [108,109]. Thus, significant additional research is required if therapeutic strategies targeting metabolism are to be successful.

Future research will require in-depth characterization of immune and stromal cells at the site of inflammation. Isolation of single cell suspensions from the synovium for cell sorting, single cell transcriptomics and CyTOF analysis, paralleled by advanced imaging technology of synovial tissue sections, will improve our understanding with regard to which metabolic pathways are driving pathogenic phenotypes at the site of inflammation. Additional characterization of specific subsets, which have been recently described for synovial fibroblasts and macrophages, will further facilitate the development of new treatments that will specifically target pathogenic cell types as opposed to protective phenotypes. Furthermore, analysis of these pathways in pre-RA (arthralgia), early disease RA, established RA and in responders *versus* non-responders to current treatment strategies will also aid in our understanding of metabolism and its role in disease pathogenesis in RA.

Disclosure

None.

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