The Impact of the Synovial Environment and GM-CSF on the Myeloid Compartment in Rheumatoid Arthritis.

Simone Verina Kidger

BSc (Hons)

Submitted in fulfillment of the requirements for the degree of Doctor of Philosophy.

College of Medical, Veterinary and Life Sciences

Institute of Infection, Immunity and Inflammation

University of Glasgow

February 2017

Abstract

The synovial environment in rheumatoid arthritis (RA) is a milieu of Damage Associated Molecular Patterns (DAMPs), cytokines and immune complexes, which can modulate the activation or polarisation of myeloid cells. GM-CSF, which is a pivotal myeloid cell growth factor, is also a pro-inflammatory cytokine that drives aspects of RA immunopathogenesis. Inhibition of GM-CSF signalling has been successful in both mouse models and in clinical trials for RA, however, the specific effect of GM-CSF on myeloid cells in a synovial setting is not well understood. The aim of this thesis was to investigate the impact of the synovial environment and GM-CSF on myeloid cells in RA.

GM-CSF stimulation induced monocytes to secrete substantial amounts of the chemokine CCL17. However, this induction of CCL17 was significantly inhibited upon co-stimulation with RA synovial fluid, but not osteoarthritis (OA) synovial fluid, whilst the expression of other chemokines was unaffected. TLR ligands also inhibited GM-CSF driven CCL17, however, through the use of MyD88/TRIF knockout mouse monocytes, we found RA synovial fluid inhibition of CCL17 was TLR-independent. Small Immune Complexes and IFNα also had the capacity to inhibit GM-CSF induction of CCL17, suggesting multiple mechanisms within the RA synovial fluid to prevent this induction. Despite the consistency of RA synovial fluid causing inhibition of the GM-CSF signalling pathway in comparison to OA synovial fluid, there were no distinct effects on macrophage polarisation. The RA synovial environment has more of an impact on monocyte activation in comparison to macrophage polarisation, as synovial fluid from other arthropathies had the comparable effects on macrophage phenotypes.

This thesis concludes that RA synovial fluid contains several factors that inhibit GM-CSF induction of CCL17. This suggests a regulatory mechanism, preventing the excessive secretion of CCL17 by monocytes, thereby preventing exacerbation of immunopathogenesis.

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Acknowledgements

Firstly, I would like to thank all of my supervisors, but primarily Dr Carl Goodyear for all of the support and guidance you've given me along the PhD journey. You have been exceptionally patient, and I really appreciate all of the time and effort that has gone into both my project and my thesis. Particularly at those times when you clearly were very busy, yet still found time to talk through data or give advice on the next experiments. Without your help and advice, I definitely would not be the scientist I am today and for that I really am grateful. Thank you also to Professor Iain McInnes in Glasgow, and Dr Matthew Sleeman and Dr Matthew Robinson at MedImmune for your input throughout my project to drive the best science out of the project. I really appreciated the opportunity of spending time at MedImmune in Cambridge to learn more about science in industry.

A huge thanks to the Goodyear-McInnes lab of past and present for all their help both in and out of the lab. Everyone has played a part in making my PhD experience what it was and I really appreciate all the moral boosting moments from press-ups in the lab to spontaneous bursts into song as well as the science help towards my project. A special thanks for help in the lab to Cecilia, Pamela and Aysin for all of your FACS knowledge, Jim and Shauna for your IHC help, Derek and Mariola for the siRNA guidance and Felix, Mark, Hussain and Moeed. Thanks also to everyone on level 3 and level 4 who have made my PhD experience what it has been. The lunchtime chats in the common room and drinks after work (which were more frequent when I lived closer) really helped me to switch off.

To Alison, Louise, Kay, Kayleigh and Kenny, you have all made my Scottish PhD experience a truly unforgettable experience. I have such fond memories of many giggles with many at my expense but I really would not have had such a fantastic time in Scotland without you all. A big thanks in particular to the two that sat beside me for my first 2 years, Alison and Louise: Alison, you really know the right things to say! From advice and hugs, to puppies in Christmas hats, during the stressful moments, you really are a great friend. Louise, I can honestly say being sat next to you for the entirety of my PhD has been an experience! I think the bond was sealed after the jointly shed tears when you binned my RNA

samples! You have been an incredible help in the lab from cutting sections, to advice and hugs when they've been required! I will definitely miss the chats about the ridiculous over tea and your unicorn and Disney madness! The next person I sit next to will have a lot to match up to.

Thank you to my friends and family who have all helped without even realising. Lisa, those lunchtime meet ups really were a great excuse to unwind. Claire, thank you for always being at the end of the phone when I needed to get something off my chest. Sarah, it's been great to chat and visit to take my mind off science. To my Nan, your letters really are such a highlight of my week and always make me smile.

I also have to take this opportunity to give a massive thank you to my Mum and Dad. Without your support, love and guidance, I would never have achieved what I have or become the person that I am. You have inspired me to make the most of my life, and I cannot thank you enough for all the help you've given me along the way.

Finally, to my amazing husband, Andrew, you really have been incredible at helping me get to this point. Thank you is just not enough for all that you have done for me, and without you I don't think I would have made it. At least I'd have never been in Scotland, so thank you for making me follow you, as it really has been an unforgettable experience! Thank you for being there through the highs and lows from making me smile on those bad science days to the encouragement through the write-up. Your love has got me through this PhD and for that I am eternally grateful. I love you.

Author's declaration

I declare that this thesis is the result of my own work. No part of this thesis has been submitted for any other degree at The University of Glasgow, or any other institution.

Simone Kidger

Abbreviations

A	ACPA	Anti-Citrullinated Protein Antibody
	ACR	American College of Rheumatology
	APC	Allophycocyanin
B	BM	Bone Marrow
	BSA	Bovine Serum Albumin
C	cDC	Conventional Dendritic Cell
	cDNA	Complementary Deoxyribonucleic Acid
	CIA	Collagen Induced Arthritis
	CRP	C Reactive Protein
	CTLA-4	Cytotoxic T Lymphocyte Associated Protein 4
D	DAMP	Damage-Associated Molecular Pattern
	DAPI	4',6-diamidino-2-phenylindole
	DAS28	Disease Activity Score 28
	DAS40	Disease Activity Score 40
	DC	Dendritic Cell
	DMARD	Disease Modifying Anti-Rheumatic Drug
	DMSO	Dimethyl Sulfoxide
	dNTP	Deoxynucleotide

E	EAE	Experimental Autoimmune Encephalomyelitis
	EDTA	Ethylendiaminetetraacitic Acid
	ELISA	Enzyme-linked Immunosorbant Assay
	ESR	Erythrocyte Sedimentation Rate
	EULAR	European League Against Rheumatism
F	FACS	Fluorescent Associated Cell Sorting
	FBS	Foetal Bovine Serum
	FcR	Fc Receptor
	FcγR	Fc gamma Receptor
	FITC	Fluorescein Isothiocyanate
	FLS	Fibroblast-Like Synoviocytes
	FMO	Fluorescence Minus One
	FOXP3	Forkhead Box P3
G	GATA3	GATA binding protein 3
	GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
	GM-CSFR	Granulocyte Macrophage-Colony Stimulating Factor Receptor
	GM-CSFRα	Granulocyte Macrophage-Colony Stimulating Factor Receptor alpha chain
	GWAS	Genome-Wide Association Study
H	HLA	Human Leukocyte Antigen

I	ICAM	Intercellular Adhesion Molecule
	IFN	Interferon
	Ig	Immunoglobulin
	IHC	Immunohistochemistry
	IKK	IκB Kinase
	IL	Interleukin
	IRAK	Interleukin-1 Receptor-Associated Kinase
	IRF	Interferon Regulatory Factor
J	JAK	Janus Kinase
K	KO	Knockout
L	LAL	Limulus Amebocyte Lysate
	LC	Langerhan Cell
	LPS	Lipopolysaccharide
M	M-CSF	Macrophage-Colony Stimulating Factor
	M-CSFR	Macrophage-Colony Stimulating Factor Receptor
	MAPK	Mitogen Activated Protein Kinase
	MFI	Mean Fluorescent Intensity
	MHC	Major Histocompatability Complex
	MSD	Meso-Scale Discovery

	MTX	Methotrexate
	MyD88	Myeloid Differentiation Primary Response Gene 88
N	n.d	Not Detected
	NFκB	Nuclear Factor Kappa B
O	OA	Osteoarthritis
P	PAD	Peptidylarginine Deiminase
	PAMP	Pathogen Associated Molecular Pattern
	PBMC	Peripheral Blood Mononuclear Cell
	PBS	Phosphate Buffered Saline
	PCR	Polymerase Chain Reaction
	pDC	Plasmacytoid Dendritic Cell
	PE	Phycoerythrin
	PI	Propidium Iodide
	PRR	Pattern Recognition Receptor
	PsA	Psoriatic Arthritis
Q	qPCR	Quantitative Polymerase Chain Reaction
R	RA	Rheumatoid Arthritis
	RANKL	Receptor Activator of Nuclear Factor Kappa-B Ligand
	RF	Rheumatoid Factor

	RNA	Ribonucleic Acid
	RORγT	RAR-related orphan receptor gamma T
	RT	Reverse Transcriptase
S	SD	Standard Deviation
	SIC	Small Immune Complex
	siRNA	Small Interfering Ribonucleic Acid
	SOCS	Suppressor of Cytokine Signalling
	SpA	Staphylococcal Protein A
	STAT	Signal Transducer and Activator of Transcription
T	TAC	Tetrameric Antibody Complex
	TAK	Transforming Growth Factor Beta-Activated Kinase 1
	TBK	Tank Binding Kinase
	TGF-β	Transforming Growth Factor Beta
	Th1	T Helper 1 Cell
	Th17	T Helper 17 Cell
	Th2	T Helper 2 Cell
	TIRAP	Toll-Interleukin 1 Receptor Domain Containing Adaptor Protein
	TLR	Toll Like Receptor
	TMB	3,3',5,5'-Tetramethylbenzidine

	TNFα	Tumour Necrosis Factor Alpha
	TRAF	TNF Receptor-Associated Factor
	TRAM	TRIF-Related Adaptor Molecule
	Treg	T Regulatory Cell
	TRIF	TIR-domain-containing adapter-inducing interferon-ß
	TSLP	Thymic Stromal Lymphopoietin
	TSLPR	Thymic Stromal Lymphopoietin Receptor
V	VCAM	Vascular Cell Adhesion Molecule
	VEGF	Vascular Endothelial Growth Factor
W	WT	Wildtype

Chapter 1 Introduction

1.1 Rheumatoid Arthritis

1.1.1 Clinical symptoms

Rheumatoid Arthritis (RA) is an autoimmune disease with a chronic inflammatory pathology that primarily affects the small joints of the hands and feet (1,2). Clinically, the disease manifests as swelling and pain of multiple joints that can result in irreversible joint deformation (3). Peak onset of disease is between the age of 25 and 55. RA is characterised by: cell infiltration into the joint lining causing severe synovitis; angiogenesis; and bone and cartilage erosion caused by the synovitis (4). The joints are the main affected area, however, RA can also be considered a systemic condition as there are a number of co-morbidities associated in approximately 80% of RA patients (5). These include cardiovascular disease, osteoporosis, periodontal disease, gastrointestinal disorders, pulmonary problems and increased cancer risk (6). RA patients can expect their life expectancy to be 3 to 10 years lower than average (7).

1.1.2 Classification

The diagnosis of RA is made primarily on physical findings. There is no single test that can confirm or exclude diagnosis of RA; therefore a number of criteria are examined to aid diagnosis. These include: the number and location of joints affected; the presence and abundance of rheumatoid factor (RF) or anti-citrullinated protein antibodies (ACPA) in the serum; the levels of C-reactive protein (CRP); erythrocyte sedimentation rate (ESR); and the duration of the symptoms (8). The 1987 American College of Rheumatology (ACR) classification criteria was focused on discerning patients with established RA, which was successful, however, this system led to a failure in identifying patients with early disease (8,9). New identification criteria were defined in the 2010 ACR/EULAR classification criteria created to differentiate patients with synovitis and those patients at more risk of developing erosive RA (Table 1.1). Patients achieving a score of more than 6 were classified as having RA (10). Having the ability to differentiate patients into categories also benefits the identification of patients for clinical trials (8).

Table 1.1 2010 ACR/EULAR RA classification criteria

Symptom	Score
A. Joint Involvement	
1 large Joint	0
2-10 large joints	1
1-3 small joints (with or without involvement of large joints)	2
4-10 small joints (with or without involvement of large joints)	3
>10 joints (at least 1 small joint)	5
B. Serology	
Negative RF and negative ACPA	0
Low-positive RF or low-positive ACPA	2
High-positive RF or high-positive ACPA	3
C. Acute-phase reactants	
Normal CRP and normal ESR	0
Abnormal CRP or normal ESR	1
D. Duration of symptoms	
<6 weeks	0
≥6 weeks	1

1.1.3 Epidemiology

The world prevalence of RA is approximately 0.5-1%, however incidence is declining over time. The annual incidence is now 25-50 cases per 100,000 (11). Interestingly, there is geographical variation in incidence of RA as in China and Japan, the incidence is low (0.2-0.3%) whereas in some native American populations the incidence is high (≥5%) (8,12). In the UK, prevalence of RA is 0.8% with an incidence of 36 per 100,000 women and 14 per 100,000 men (13). There are few prevalence studies in developing countries, however there is some evidence to suggest a significantly lower prevalence (0.1-0.5%) of RA in developing countries in comparison to North America and Northern Europe (14). This suggests there are potential genetic and/or environmental factors that can increase the potential for disease onset. Importantly, in recent years evidence has been generated to support both genetic and environmental risk factors in RA susceptibility.

1.2 Risk Factors for RA

1.2.1 Genetic

In the last couple of decades a substantial amount of work has been done to investigate the link between genetics and RA predisposition. Numerous studies have been undertaken and it is clear that there is no one gene responsible for RA but rather a complicated multi-gene landscape. Genome-Wide Association Studies (GWAS) have identified more than 100 loci associated with RA risk, however the effect of an individual locus is low (15).

Although one gene cannot explain the genetic predisposition in RA, the initial observation in genetic studies revealed that Human Leukocyte Antigen (HLA) was associated with the onset of RA. It remains as the best characterised genetic risk factor (16). The HLA are located in the class II region of the Major Histocompatibility Complex (MHC). Several of the HLA-DRB1 alleles are associated with RA in multiple populations. These alleles share an epitope, which is a short amino acid sequence, located in the hypervariable region of the DRB1 molecule (16-18). The shared epitope hypothesis suggests that the specific antigen-binding groove is affected, supporting the role for T cells in RA (19). Recent studies have also suggested that HLA-DR3 is associated with ACPA negative RA patients, highlighting different molecular signatures for the disease subgroups (20,21).

There are multiple RA risk loci that have been identified from GWAS studies. For example, PTPN22 was identified by GWAS, and is associated with several autoimmune conditions. It encodes lymphoid tyrosine phosphatase (LYP), which in the disease-associated variant has increased activity. T-cell receptor and B-cell receptor signalling was reduced in carriers of this variant leading to the survival of autoreactive T cells. RF and ACPA have also been associated with the PTPN22 polymorphism (15,16). Despite the numbers of loci that have been identified, outside of the HLA loci, these only represent 5.5% of the total risk of developing the disease. Furthermore, there are other risk factors such as environmental risks that have been associated with development of RA (15)

1.2.2 Environmental

Various environmental factors for example smoking, diet and infection have been associated with the onset of RA, however, cigarette smoking the strongest identified factor (22). Smoking attributes to 25% of all RA cases, which increases to 35% for seropositive RA (patients classified as RF and ACPA positive)(Section 1.4.7) (23). Smoking increases susceptibility for RA, with the risk more apparent in men than women, with several studies demonstrating a dose response between heavy smoking and RA, with the risk remaining high even after ceasing to smoke (7,22-25). The mechanism causing the increased risk of RA from smoking is not fully understood, however, there have been some studies to suggest several mechanisms. The cigarette chemical tetrachlorodibenzo-P-dioxin (TCDD) has been found to affect general immunity by upregulating the inflammatory cytokines IL-1β, IL-6 and IL-8 through the stimulation of NFκB and ERK pathways (7,26). Smoking has also been linked to the shared epitope of HLA-DRB1 and the production of ACPAs. HLA-DRB1 is known to be specific to seropositive RA patients, and in smokers, citrullinated proteins are abundant in the lungs. Citrullinated α-enolase is an autoantigen for RA that has linked HLA-DRB1 allele and smoking as risk factors for RA (27).

Several microorganisms have also been implicated in the development of RA due to high titres of antibodies against Epstein-Barr Virus, Mycobacterium tuberculosis or human papillomavirus as well as others in sera of RA patients. However, this is controversial as no specific microbe directly causes RA. It is thought that microorganisms may trigger the development of RA in individuals with a genetic susceptibility (7).

Dietary factors, including vitamin D and omega-3 fatty acids have been shown to have a protective effect for auto-inflammatory diseases including RA. Vitamin D prevents the onset of Collagen-Induced Arthritis (CIA) in mice, and has been shown to prevent the onset of RA in genetically susceptible patients. In already diagnosed RA patients, vitamin D intake has been correlated with lower disease activity. The mechanism is perhaps due to upregulated anti-inflammatory cytokines and inhibited more pro-inflammatory cytokines (23,28). Omega-3 decreases pro-inflammatory mediators through leukotrienes and prostaglandins,

but has had modest effects on RA risk. However, consumption of fish has been shown to improve RA symptoms (29,30).

1.2.3 Gender

Gender plays a part in the susceptibility to RA. Women are 2 to 4 times more likely to develop RA than men, with women less likely to go into remission than men (13,31). In women, RA is also more likely to develop at times of flux in sex hormones. Interestingly, studies have found pregnancy protective, with RA patients going into remission, however relapsing after pregnancy during breast-feeding is high. This is thought to be due to the major changes in hormone levels (32,33). There are many studies to suggest that the Oral Contraceptive Pill is protective in the development of RA, confirming the link between sex hormones and RA (23,34). Innate and adaptive immune cells have the oestrogen receptor, suggesting that oestrogen can have an impact on the immune response. Oestrogen prevents T cell dependent responses and neutrophil adhesion to the endothelium, preventing extravasation into the tissue (35).

1.3 Other Inflammatory Erosive Diseases

1.3.1 Osteoarthritis

Osteoarthritis (OA) is a common chronic musculoskeletal disorder affecting 15% of the world's population. It is a disease associated with ageing, joint trauma and obesity. OA is not an autoimmune disease and has long been thought as a non-inflammatory mechanical disease of weight-bearing joints with destabilisation to the joint causing mechanical damage (36,37). However, more recent findings suggest that OA does have an underlying inflammatory element and that there is synovial inflammation before the structural change in the joint. There are also several immune processes that are ongoing within the synovium in OA, primarily from the innate immune response, with Toll Like Receptor (TLR) signalling playing a major part in immunopathology (37). Currently the only treatments for OA sufferers are pain relief and knee replacements with replacements only viable for approximately 15 years (38,39).

1.3.2 Psoriatic Arthritis

Psoriatic arthritis (PsA), second to RA in incidence of inflammatory arthritis, has less than a 0.1% occurrence in the population (40), but interestingly affects 40% of people with psoriasis (41). Clinically, PsA has very similar manifestations as RA, with small joint arthropathies, however, unlike RA, erosive bone damage is uncommon (42). PsA has a strong genetic link, with 15% of people having a relative with the same condition. CD8 T cells are thought to have a major role in PsA pathology, however autoantibodies are absent from disease (43). The cytokine IL-17 and the IL-17 receptor family members are also crucial to the pathogenesis of PsA. There are increased levels of IL-17 in psoriatic joints, which is thought to be the reason for increased synovial fibroblast proliferation and bone remodelling (44). Anti-IL-17 and anti-IL-17R antibodies are now therapeutics used for the treatment of both PsA and psoriasis (45).

1.4 Immunopathology of RA

In a healthy joint, the synovial membrane consists of few cells, primarily fibroblast-like synoviocytes and macrophages in the intimal lining layer. During inflammation, predominantly $CD4^+$ T cells infiltrate into the synovium (Figure 1.1). Other immune cells for example dendritic cells and B cells are recruited and form tertiary lymphoid follicles with T cells, in the synovium (1). The influx of these immune cells, as well as the production of autoantibodies causes the disease pathology. However, RA is a heterogeneous disease, and currently there is no molecular signature. Dennis et al., 2014, suggest that RA pathologies can be subdivided into homogeneous molecular phenotypes. Within these homogeneous molecular populations, cellular differences have been observed in gene-expression profiles. Through FACS analysis, fibroblasts, macrophages and T cells were observed in all molecular clusters, whereas B cells were found only in lymphoid and myeloid clusters. This suggests that some cell types are more prevalent in some types of disease than others; however, there is as yet no definitive categorisation of RA phenotypes due to the level of heterogeneity. This heterogeneity within RA explains why all patients to do not respond to treatment in the same way. However, the homogeneous molecular phenotypes of RA are caused by specific cellular mechanisms (20,46) (Figure 1.1).

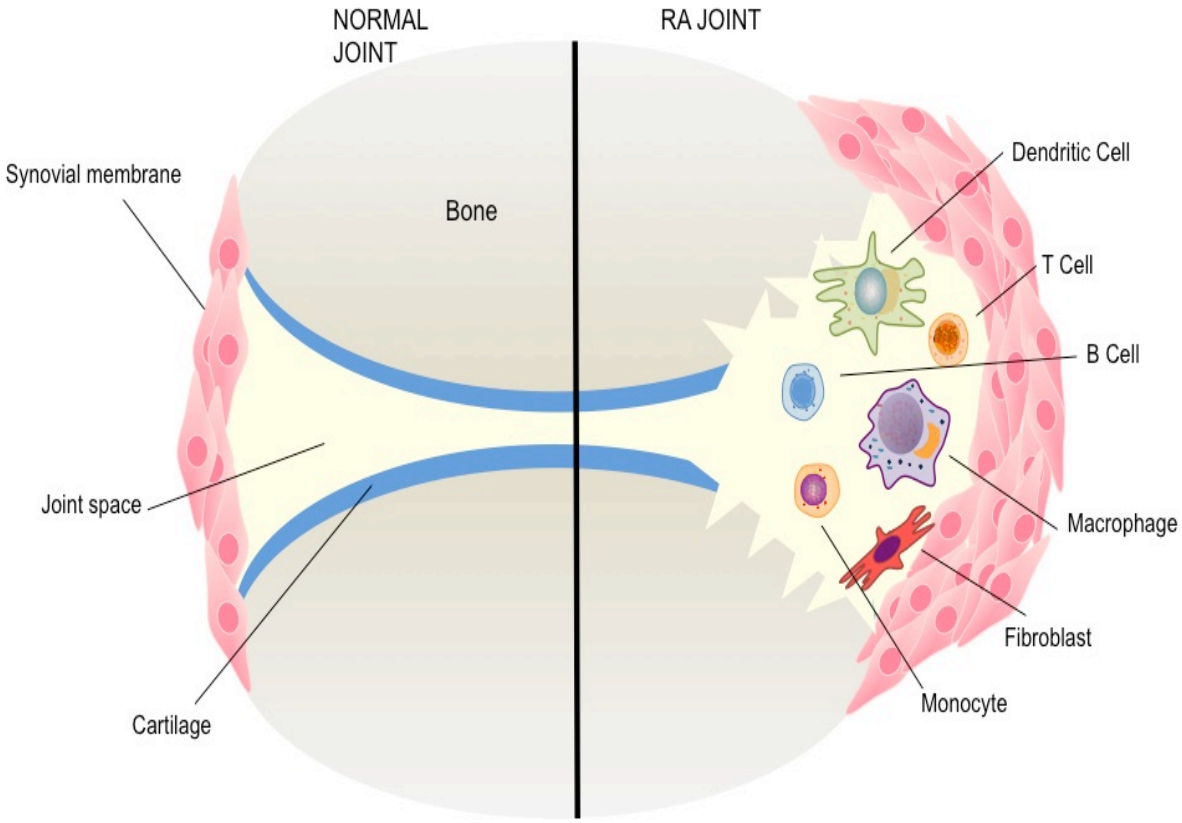

Figure 1.1 Synovial Immunopathogenesis
A normal joint in comparison to the RA joint. In the RA joint, multiple immune cells including monocytes, macrophages, fibroblasts, dendritic cells, T cells and B cells are involved in pathogenesis.

1.4.1 Monocyte and Macrophage lineage in RA

1.4.1.1 Monocytes

Monocytes are a conserved leukocyte population that derive from haematopoietic stem cells in the bone marrow in either the foetal liver or the adult bone marrow (47,48). Monocytes depend on Macrophage Colony Stimulating Factor (M-CSF) for survival, as mice deficient of the M-CSFR had monocytopenia (48,49). After leaving the bone marrow, monocytes circulate for a couple of days in the blood before entering the tissues to receive environmental cues for further differentiation into monocyte-derived cells (50). Monocytes are heterogeneous and can be defined by their CD14 and CD16 expression. CD14 is a co-receptor for TLR4, therefore important in the binding of LPS, whereas CD16, also known as FCγRIII has a low affinity for IgG (51). Monocytes are subdivided into: classical, which are $CD14^{++}CD16^{-}$; intermediate, which are $CD14^{++}CD16^{+}$;or non-classical, which are $CD14^{+}CD16^{++}$. Classical monocytes are the most abundant of the monocyte phenotypes with 10 fold greater numbers than the non-classical monocyte in circulation (52). Classical monocytes are also $CCR2^{high}$ $CXCR1^{low}$ whereas the intermediate and non-classical monocytes are $CCR2^{low}CXCR1^{high}$. This is important as it reflects the chemokines in which they bind. Classical and intermediate monocytes are both pro-inflammatory with classical monocytes responding to CCL2 to migrate into the site of inflammation, and intermediate monocytes binding CXCL8 and CXCL6 (53). The intermediate monocytes normally account for about 10% of the monocytes in the blood in healthy people, however in RA their numbers increase to 11.7% ± 5.6%. This highlights the importance of monocytes in disease pathogenesis (54).

Monocytes, upon inflammatory cues, become immune effector cells that have the ability to migrate into inflamed tissues from the blood. Monocytes produce cytokines and chemokines upon activation, and also have the ability to differentiate into inflammatory monocyte-derived cells (50).

In RA, the CD16 positive, intermediate monocytes are highly represented mainly in the lining layer of the synovial tissue. These monocytes that express CD16 also have a high expression of TLR2 in comparison to monocytes without CD16, with

no difference in the expression of TLR4. They produce cytokines such as TNFα, IL-1β, IL-6 and IL-8 as well as more anti-inflammatory cytokines such as IL-10 and IL-1RA (55). On arrival to the synovium, monocytes are subject to a milieu of many cytokines, chemokines, DAMPs and immune complexes. In response to many synovial factors, monocyte differentiation is initiated. Monocytes can also modify their responses, releasing cytokines and therefore causing the activation of T cells (56). Upon entry into the synovium, a monocyte is exposed to a variety of stimuli. Prior to differentiation, the response of a monocyte is not well understood; therefore this thesis aims to investigate monocyte responses to synovial stimuli.

1.4.1.2 Macrophages

Macrophages are tissue resident cells that maintain tissue homeostasis. In homeostasis, macrophages are involved in the phagocytic clearance of cellular debris and erythrocytes (57). They are termed professional phagocytes as they express receptors such as scavenger receptors and phosphatidylserine receptors that recognise apoptotic or necrotic cells and are not present on all cells (58).There are multiple macrophage lineages and more recently, it has been determined that tissue resident macrophages are not monocyte derived. They are terminally differentiated cells that are embryonic derived (50). Genetic fate-mapping techniques have led to the understanding that macrophages are derived from the yolk-sac and can self renew in the tissue (59). In inflammation both tissue derived macrophages and monocyte derived macrophages have distinctive roles(60).

During inflammation, monocytes are recruited into the tissue where they receive environmental cues and differentiate into cells such as macrophages. Macrophages are heterogeneous as the environmental cues received upon differentiation from monocytes reflect their phenotype (47).

Macrophages or monocyte-derived cells are highly plastic, but have historically been classified into two distinct groups: M1 or M2; M1 macrophages are traditionally differentiated with IFNγ, IFNγ and LPS, or GM-CSF and they have a more pro-inflammatory phenotype; and M2 macrophages are IL-4, IL-4 and IL-13, IL-10 or M-CSF stimulated macrophages, which have a more regulatory

phenotype (61-63). M1 macrophages or classically activated macrophages are considered more pro-inflammatory as upon stimulation, they produce cytokines such as TNFα, IL-1β, IL-6 and IL-12 as well as chemokines and growth factors. They also express high levels of MHC class II, with co-stimulatory molecules to present antigen to T cells, thereby activating the adaptive immune response (64). M2 or alternatively activated macrophages are thought to be more regulatory as they produce IL-10, can antagonise M1 responses and promote the resolution of inflammation (58) (Figure 1.2). However, this classification of macrophage phenotypes is not representative of macrophages *in* vivo as the classifications were determined from *in vitro* monocyte-derived macrophages. In the tissue, the environmental milieu influences differentiation and activation therefore leading to a spectrum of macrophage phenotypes (57). Despite this understanding that macrophages are heterogeneous, there are guidelines for macrophage classification dependent on expression of transcription factors, chemokines, cytokines and scavenger receptors amongst others, which are specifically for *in vitro* derived macrophages (63). These guidelines are useful for clarity between culture conditions that were previously used for M1 or M2 macrophages, however, this does not relate to disease specific macrophages or allow for the spectrum of macrophage phenotypes (Figure 1.2).

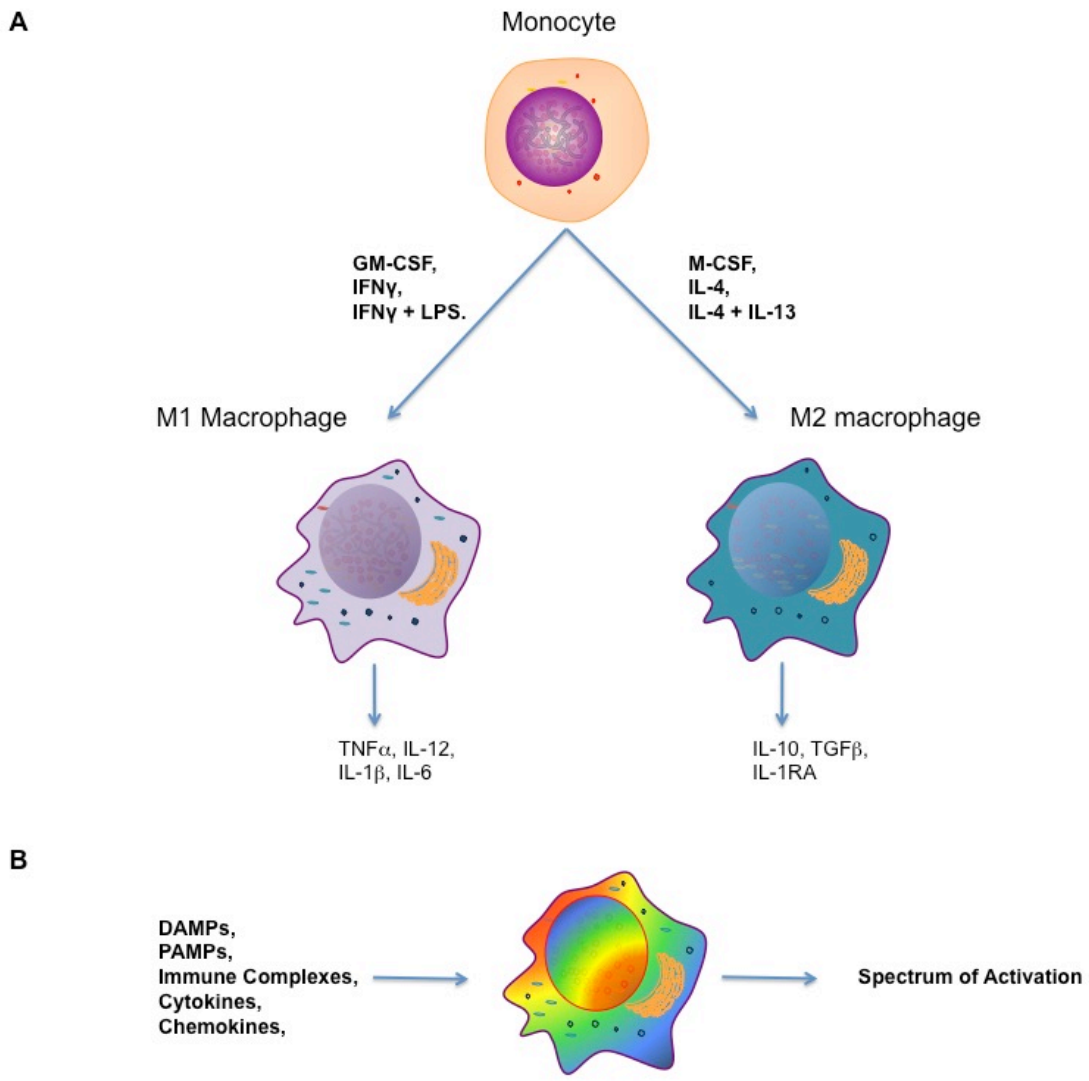

Figure 1.2 Macrophage Differentiation and Polarisation.
A) Traditional terminology for macrophage differentiation. Macrophages differentiated *in vitro* from monocytes with specific identified stimuli, differentiated into either M1 or M2 macrophages. B) The widely accepted concept that due to the large variety of stimuli received in the tissue, macrophages are not easily categorised, but have a spectrum of activation.

In RA, macrophages are responsible for the release of many pro-inflammatory cytokines that influence T cell polarisation (Section 1.4.4). IL-12 is involved in Th1 polarisation (Section 1.4.4), whereas IL-1β, IL-6 and IL-23 are involved in Th17 polarisation. The stimulation by macrophages to skew $CD4^+$ T cells to a Th17 phenotype has been considered to perpetuate inflammation due to a positive feedback loop between the Th17 cells and macrophages (56). Macrophages are present in the intimal lining layer of the synovium, which they share with fibroblast-like synoviocytes (FLS). FLS play a pivotal role in RA pathogenesis and joint destruction through the secretion of Matrix Metalloproteinases, which leads to cartilage destruction and bone erosion (65).

FLS, once activated, secrete TNFα, IL-6 and IL-1, which prolongs inflammation and they also sustain regulatory feedback loops (66). The crosstalk between macrophages and fibroblast-like synoviocytes has been shown to modulate macrophage cytokine expression. Macrophage expression of TNF induced genes, such as type I interferons, were repressed by FLS, and interestingly IL-4 and growth factors such as M-CSF were up-regulated. FLS appear to influence macrophage polarisation to a more M2 phenotype (67). This M2-like phenotype of IL-10 producing macrophages is specific to the intimal lining macrophages, as the sub-lining macrophages have a more heterogeneous phenotype as there are some macrophages that secrete IFNγ and some that secrete IL-10 (68). Despite the variety of macrophages within the synovium, a large proportion secrete pro-inflammatory cytokines such as TNFα, IL-6 and IFNγ, thereby exacerbating inflammation (69). However, it is unknown what the effect of the synovial environment is specifically on the macrophage phenotype. Therefore, this thesis aims to evaluate macrophage phenotypes after stimulation with synovial fluid.

Macrophages are involved with the perpetual inflammation observed in RA due to the cytokines and chemokines they release, and their interaction with the adaptive immune response. Therefore, targeting macrophages has been considered in the treatment of RA. Some therapeutics already in use do indirectly target macrophages such as antibodies targeting TNFα, which is a cytokine that is readily released by activated macrophages (70).

1.4.2 Dendritic Cells in RA

Dendritic cells (DCs) are professional antigen presenting cells, which are recruited to the synovium upon the inflammatory insult (71). DCs can be classified into 2 major categories derived from a common DC precursor (CDP): conventional DCs (cDCs) and plasmacytoid DCs (pDCs). cDCs can then be further divided based on their expression of CD1c and CD141 (72,73). These all have a distinct function to either stimulate or inhibit the immune response (74). DCs are immature in the periphery, however, become activated in response to inflammatory mediators such as: pathogen associated molecular patterns (PAMP) via C type Lectin receptors or Toll Like Receptors (TLR); or by immune complexes (IC) via FCγ receptors (FCγR) (71). Chemokines are involved in the recruitment of DCs to the tissue, where they are exposed to the inflammatory

mediators. Once activated, DCs migrate to the lymph nodes where they present antigen to T cells and release cytokines that lead to T cell maturation (75). In RA, synovial DCs are more differentiated than precursors that are isolated from RA or normal peripheral blood due to the elevated expression of MHC Class II and adhesion molecules (76,77). Synovial fluid contains high numbers of both cDCs and pDCs in comparison to the blood suggesting a role for them in disease pathogenesis in both antigen presentation and through the release of inflammatory mediators (71).

1.4.3 DAMPs in RA

Damage Associated Molecular Patterns (DAMPs) are endogenous danger signals or alarmins that host molecules rapidly release from damaged or necrotic cells. DAMPs are a sterile inflammatory insult that causes activation of Pattern Recognition Receptors (PRRs), such as TLRs or C type lectin receptors (78,79). They have the ability to mediate an inflammatory response by stimulating the recruitment of cells to sites of tissue damage and by activating innate immune cells (80). DAMPs include proteins such as high mobility group box 1 protein (HMGB1), tenascin c, heat shock proteins, extracellular matrix proteins and the S100 group of proteins (81-83). Recent work has suggested that various DAMPS, released during synovial damage, are important in RA (82). One particular class of DAMP that is elevated in RA SF in comparison to OA synovial fluid are the S100 proteins (84). It has also been suggested that the S100A9 protein could be used as biomarker for methotrexate responsiveness (85). The proteome of PBMCs and the serum of methotrexate responders, had higher levels of S100A9 in comparison to non-responders at the baseline before treatment (85). Tenascin C, an extracellular matrix glycoprotein, has also been shown to be a contributing factor to inflammation and joint destruction in the zymosan-induced model of RA in mice. In Tenascin C knockout mice, the inflammation is not sustained in the model, and the mice are protected from joint destruction as cell infiltration and synovial thickening were reduced (86). In RA patients, Tenascin C was identified in circulation and was up-regulated in comparison to controls. After treatment with either Methotrexate or anti-TNFα therapy, circulating levels were decreased (87). High Mobility Box Group Protein 1 (HMGB1) is another DAMP associated with RA pathogenesis. It is a highly abundant and conserved protein that is normally within the cell nucleus. It becomes a DAMP when a cell

undergoes necrosis and it is released from the cell (88). In the mouse model of RA, CIA, inhibition of HMGB1 ameliorated disease, whereas addition of exogenous HMGB1 led to a worsening of disease (89). In RA, HMGB1 is up-regulated in the serum, synovial fluid and synovial tissues, and an inhibitor has been considered as a clinical approach for RA treatment (90). DAMPs are pro-inflammatory as can activate antigen-presenting cells such as monocytes or macrophages by binding to PRRs such as TLRs.

TLRs have been implicated in RA due to their activation upon sensing DAMPS or PAMPs within the synovial environment (91). Upon ligand binding, the signalling cascade is either MyD88-dependent or MyD88 independent (Figure 1.3). MyD88-dependent signalling leads to the activation of NFκB and the transcription of pro-inflammatory cytokines such as IL-6 and TNFα. MyD88-independent signalling, via TRIF, leads to the activation of NFκB and the transcription of type I interferons (92). Both MyD88-dependent and MyD88-independent TLR signalling have a role in RA, and FLS express high levels of TLRs triggering the secretion of multiple pro-inflammatory cytokines and degradative enzymes such as MMPs. Monocytes and macrophages in RA also express high levels of TLR2 and TLR4, which, when activated lead to the secretion of pro-inflammatory cytokines such as TNFα and IL-6 (93).

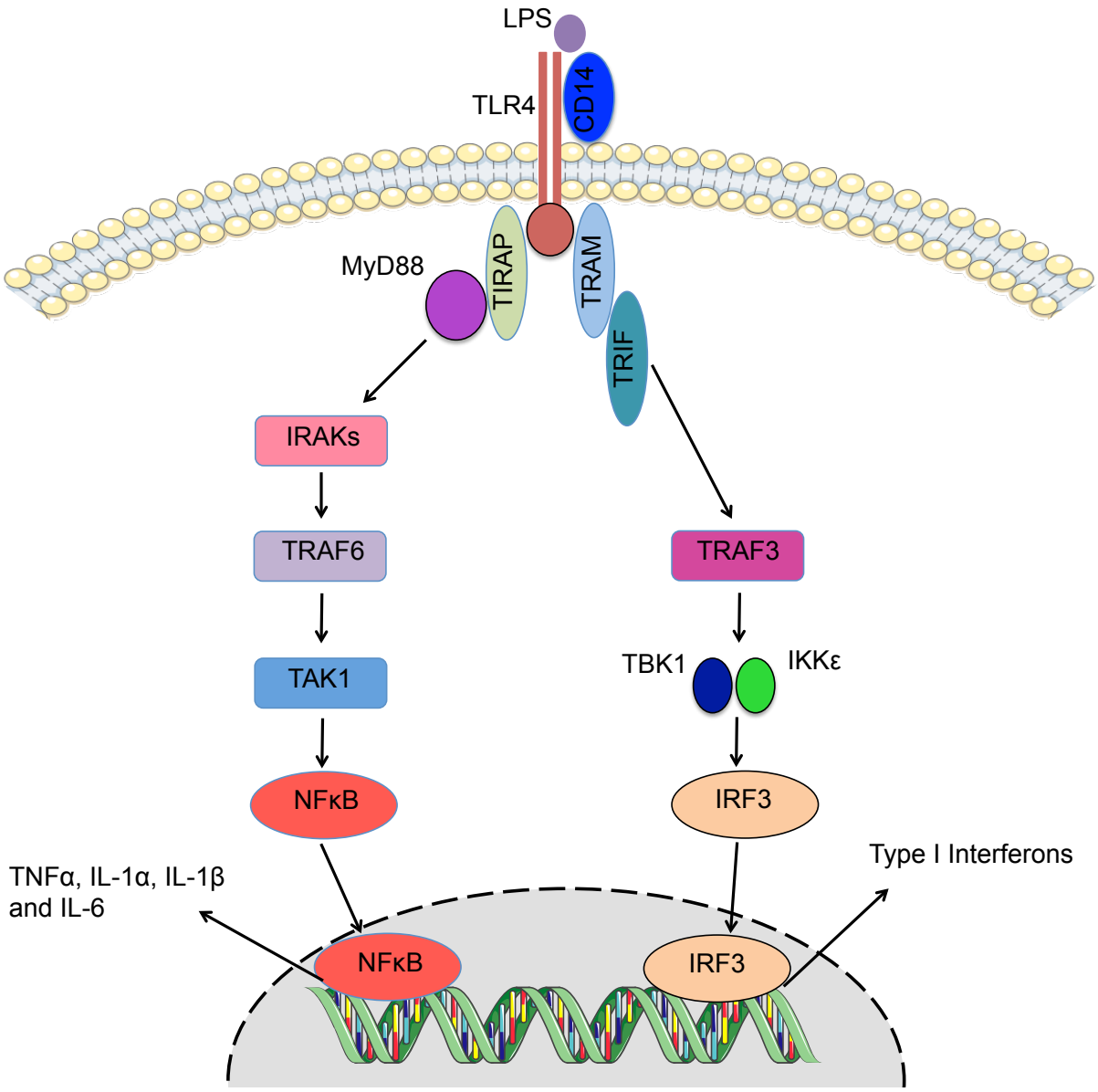

Figure 1.3 TLR Signalling
TLR4 ligand, such as LPS, binds TLR4 with the co-receptor CD14, and downstream cascades signal either via MyD88; leading to a NFkB activation and transcription of pro-inflammatory cytokines, or via TRIF; leading to the activation of IRF3 and the transcription of Type I interferons.

1.4.4 T cells

There are several flavours of T cells, all with different roles, and RA is associated with a Th1 and Th17 immune response (94) (Section 1.4.5). T cells are classified as either $CD4^+$ or $CD8^+$. $CD4^+$ T cells can then be divided into multiple subgroups depending on their cytokine expression profile. Th1 T cells are differentiated from naïve T cells through IL-12 and IFNγ stimulation. Th1 T cells are then identified through the predominance of the transcription factor T-bet, and their production of IFNγ and IL-2. Th2 cells, however, are induced by IL-4 stimulation; they express the transcription factor GATA3 and produce cytokines such as IL-4, IL-5 and IL-13 (95,96). A Th1 immune response evokes cell-mediated immunity, whereas a Th2 response induces an antibody-mediated response, and is therefore associated with allergy (97). Th1 and Th2 T cells were the fore-runners the with regard to T cell polarisation but it is now appreciated that there are alternative T cell subsets or polarisation states that differ based on their cytokine expression. These subsets include Th17 cells, which have been classified due to their production of IL-17A, however they also secrete IL-17F and IL-22 (98). They are induced from naïve T cells through stimulation with IL-1β and IL-23, or TGFβ, IL-6 and IL-23 and they predominantly use RORγT as their transcription factor. Treg cells are a regulatory T cell, that are induced by TGFβ or IL-2. They are identified by expressing FOXP3 transcription factor, and produce TGFβ and IL-10 (95,96,98).

1.4.5 T cells in RA

The role of T cells in RA is much more varied and complex than the classical expansion of effector T cell clones upon recognition of presented antigen (99). In RA, there is not a specific auto-antigen to which T cells are targeted, however, some T cells would recognise autoantigen. T cells are a large proportion of the cells infiltrating into the synovium, and they are implicated in RA pathogenesis. From animal models and previous understanding, RA had been traditionally considered a Th1 driven disease. In the CIA mouse model, cytokine profiles were analysed and IFNγ was abundant as opposed to Th2 cytokines (100,101). Inhibition of IL-12 shown using a knockout mouse and inhibiting antibodies to IL-12, led to reduced disease in mice, suggesting a Th1 driven disease (101,102). However, more recent evidence has shown that Th17 cells

have a more of a role in RA pathogenesis. This became apparent when both IL-6 deficient mice and IL-23 deficient mice had attenuated disease in the CIA model. Both IL-6 and IL-23 are required for Th17 development and the numbers of Th17 cells in the IL-23 deficient mice were reduced (3,103,104). This finding has been confirmed through overexpression of IL-17, which exacerbated disease, or inhibiting IL-17, which improved disease (105-107). A high percentage of Th17 cells have been identified in the synovial fluid of RA patients, compared with peripheral blood. IL-17 has also been found in abundance in the synovial fluid of RA patients (108,109). Despite the strong case for targeting IL-17 in RA, anti-IL-17 therapy has not met primary endpoints in efficacy trials (110). However, trials are still continuing and there is hope for efficacy in combination with other therapies (111).

Interestingly, there are other subsets of T cells found within the RA joint, which, under normal conditions are anti-inflammatory. Treg cells have suppressive functions, through suppressing other T cell subsets. They express high levels of the IL-2R, thereby up-taking IL-2 and depriving other T cells. They also express CTLA-4, which inhibits expression of co-stimulatory molecule CD80. They produce the more immunoregulatory cytokines: IL-10 and IL-35 (112). Treg cells ($CD4^+$, $CD25^+$) are present in the synovium, however they have a decreased ability to control an immune response, as they were unable to regulate pro-inflammatory cytokines released by effector T cells. Interestingly, after anti-TNFα treatment, their function was restored (113).

1.4.6 B cells in RA

B cells are abundantly found in the synovium of rheumatoid arthritis patients. They infiltrate into the synovium and form either diffuse or follicle aggregates (114). They have multiple roles including presenting antigen to T cells and the activation of T cells. The aggregation of B and T cells, in the form of tertiary lymphoid follicles in the synovial membrane, apparent in 60% of RA patients, facilitate their interaction (46,115). B cells also produce pro-inflammatory cytokines such as IL-6 and IFNγ and the chemokine, CXCL13 exacerbating inflammation through the recruitment and stimulation of macrophages and T cells (94,116). The differentiated plasma cells produce autoantibodies such as rheumatoid factor and anti-cilltrullinated protein antibodies (3,117).

There have been studies investigating the role of B cells in RA. μMT mice, which are B cell deficient, were resistant to the onset of CIA, although T cell reactivity to CII was unaffected between wildtype and μMT mice (118). This showed that depleting B cells could be effective as a therapy. Rituximab was initially developed for the treatment of lymphoma, through the targeting of CD20, present on neoplastic B cells, but not plasma cells (119). It was developed as an RA therapy due to the role of B cells in RA (120). Rituximab has been successful in some patients but not in others, highlighting the heterogeneity between RA sufferers and the variety of treatments required (121).

1.4.7 Autoantibodies and Immune Complexes

In RA, there is not a single autoantibody, unlike other autoimmune conditions such as Graves' disease in which antibodies are targeted against the Thyroid Stimulating Hormone Receptor (122). However, in RA, several autoantibodies have been identified such as rheumatoid factor and anti-citrullinated protein antibodies (ACPA) (123,124). In the region of 80% of RA patients are rheumatoid factor positive which is an antibody, that is autoreactive against the antigenic Fc region of IgG (114). Approximately 75% of RA patients are ACPA positive. They have more severe disease than ACPA negative patients (125). ACPA are primarily IgG specific to proteins that have been citrullinated such as vimentin, fibrinogen, collagen, fibronectin and α-enolase (94,126,127). Citrullination is an enzymatic process involving the enzyme Peptidylarginine Deiminase (PAD) 2 or 4, which catalyses the conversion arginine to citrulline in a post-translational modification. These citrullinated proteins are targeted by autoantibodies in RA (128). ACPAs are correlated with a more destructive form of RA, as it has been found that ACPAs can bind to osteoclast surfaces and cause osteoclast differentiation leading to bone resorption through the production of IL-8 (125,129). In mouse studies, the addition of ACPA also causes trabecular bone loss (129). ACPAs form immune complexes and bind Fc receptors causing activation of monocytes and macrophages leading to the secretion of cytokines such as TNFα (127). The binding of immune complexes to cells positive for Fc receptors is also thought to induce the complement system, leading to exacerbated RA pathogenesis (130).

1.4.8 Synovial Fluid

The volume of synovial fluid of a normal joint is minimal, and is thought to be for the transport of nutrients to the cartilage and lubrication of the joint (131). In a diseased joint, the amount of synovial fluid is increased, and contains immune cells. The synovial fluid of an RA patient is a complex milieu, containing inflammatory cytokines, cell debris, metabolites and immune complexes.

There have been multiple studies into the contents of synovial fluid and whether there are potential biomarkers present (46,85,132). Studies have investigated the metabolite profile of RA synovial fluid with non-RA synovial fluid from Behçet's disease, ankylosing spondylitis and gout patients. This analysis identified 22 metabolites that were present and higher in synovial fluid of RA patients compared with non-RA patients. These included succinate, asparagine and citrulline. Interestingly, there were also 10 metabolites that were down-regulated in RA synovial fluid compared with the non-RA synovial fluid, including glycerol and isopalmitic acid. This suggests that the RA synovial fluid metabolite profile has some homogeneity with the potential for metabolic biomarkers (132).

Proteomic analysis of synovial fluid has also been investigated with biomarkers and a greater understanding of disease pathogenesis as the potential outcome. 135 proteins have been identified as differentially expressed at a greater than 3 fold higher level than OA synovial fluid (133). These include the DAMP S100 proteins, S100A8 and S100A9, which have been identified in multiple studies in RA synovial fluid at higher levels than in OA synovial fluid. Both have been suggested as biomarkers to discriminate between RA and other inflammatory arthritis (84,133). Of the proteins identified, the majority were extracellular matrix or cytoplasmic proteins, however proteins of the plasma membrane and nucleus were also identified. Proteins involved in the glycolytic pathway of metabolism were also up-regulated, linking the metabolite profile, and the potential importance of metabolic changes in a diseased joint (133). Interestingly, the proteomic profile is not constant, and changes from early RA to more established RA, suggesting that synovial fluid protein biomarkers could monitor disease progression (134).

PAD enzymes have been identified in RA synovial fluid and are active in 80% of synovial fluids tested (128). The presence of active PAD enzymes in synovial fluid of RA patients suggests that citrullinated proteins would also be present in synovial fluid. Citrullinated fibronectin has been observed in RA synovial fluid, at higher levels than in OA patients. However, the level of citrullinated fibronectin was not correlated with the level of ACPA in synovial fluid (135).

The cytokines in synovial fluid are related to ACPA status, as ACPA positive patients have more T cell derived pro-inflammatory cytokines, including IL-1β and IL-17F, in comparison to ACPA negative patients (136). There are numerous cytokines and chemokines that have been observed in RA synovial fluid, including IFNα, TNFα, IL-1Rα, IL-1β, IL-10, IL-6, CCL17, CXCL8 and CCL5. These have all been found at higher levels than in OA synovial fluid, and some such as TNFα, IL-6 and IL-1β have been found to drive RA pathogenesis. Others such as IL-10 and IL-1Rα are thought to be involved in regulation of disease, whereas the role of the chemokine CCL17, is not as well defined in RA (137-142).

1.5 Cytokine Pathways in RA

Cytokines affect the majority of biological processes including disease pathogenesis, antigen response, specific cognitive changes, embryonic development and ageing (143). In relation to RA, there are cytokines that can be classed as pro- or anti- inflammatory. Cytokines play a major part in the pathogenesis of RA, and have been implicated in multiple aspects of disease processes (144). At each stage of disease from the onset to the resolution of disease, cytokines are involved (3). Cytokines have effects on specific target cells that can promote further immune responses through cell activation, cell localisation and cell longevity in the synovial environment (144). In RA, the pro-inflammatory cytokines TNFα, IL-1, IL-6 and GM-CSF have been found in abundance in the joint at all stages of disease. However, anti-inflammatory cytokines IL-10 and TGFβ have also been found at high levels in the RA joint perhaps suggesting other roles for these cytokines (3,94,123,144).

1.5.1 TNFα

Tumour Necrosis Factor-alpha (TNFα) is a pro-inflammatory cytokine that plays a major role in inflammatory disease, particularly in the pathogenesis of RA. TNFα is produced by a variety of cell types, but primarily by monocytes and macrophages. The secretion of TNFα is a major cause of synovitis in RA, through further induction of pro-inflammatory cytokines such as GM-CSF, IL-1, IL-6 and IL-8 by cells such as fibroblast-like synovial cells or monocytes and macrophages (145) (146). Stimulation of synoviocytes with TNFα leads to an increase in production of IL-6, IL-1 and TNFα, through an autocrine loop (147). The effect of TNFα on fibroblast-like synovial cells leads to the prolonged inflammation in the joint. TNFα has been implicated in cartilage degradation as stimulating chondrocytes with TNFα in *ex vivo* experiments using bovine and porcine cartilage discs, caused cartilage resorption and prevented further synthesis of proteoglycan (148,149). In TNF-receptor 1 deficient mice, incidence of disease was lower than wildtype controls, however the affected joints progressed to the same degree as the wildtype mice, suggesting potentially that it is primarily the onset of disease that is TNFα dependent (150). Due to the involvement in RA pathogenesis, TNFα was investigated as a potential therapeutic target, and is now used as a successful cytokine therapy (Section 1.8.1).

1.5.2 IL-1

IL-1 is subdivided into IL-1α and IL-1β, both of which are expressed in the RA synovial tissue, with IL-1β expressed, both at the mRNA and protein level, at much higher levels than IL-1α (151). IL-1 is produced by monocytes and macrophages and has wide ranging effects on synoviocytes, chondrocytes, B and T cells through binding to type I IL-1 receptors (152). IL-1β has also been found in the serum of RA patients, with levels correlated to disease activity. Interestingly, in mouse models of RA using IL-1 deficient mice, there was no change in paw swelling or disease incidence. Cartilage erosion was however greatly reduced, leading to an implication of IL-1 in the cartilage resorption and bone erosion phenotype of RA through the activation of osteoclasts and matrix-enzyme production by chondrocytes (3,150,152). IL-1 was subsequently

investigated as a potential therapeutic, however, it had limited success as a cytokine therapy in RA (Section 1.9.1)

1.5.3 IL-6

IL-6 is a pro-inflammatory cytokine that has its effects on multiple cell types. This is in part due to having both membrane bound and soluble receptors (sIL-6R), thereby increasing the number of responsive cells (153). IL-6 can induce the development of osteoclasts and in conjunction with other cytokines such as TNFα can result in bone erosion (154,155). IL-6 also stimulates neutrophil migration, and the differentiation of B cells into plasma cells. This is important as IgG, IgA or IgM rheumatoid factors as well as anti-citrullinated protein antibodies are increased in RA patients and therefore the increase in IL-6 in RA patients would lead to increased B cell activity further influencing synovial inflammation (153). Mice treated with a monoclonal antibody to IL-6 during the CIA model, showed reduced disease scores compared with a control treatment. Also, IL-6 knockout mice had significantly lower disease incidence than wildtype controls (103). In patients, IL-6 and sIL-6R are upregulated in RA synovial fluid in comparison to OA (156,157). The level of IL-6 in the synovial fluid correlates with CRP, a measure of inflammation and therefore disease suggesting an important role for IL-6 in RA pathogenesis (157). The IL-6 signalling pathway has therefore been targeted, with antibodies generated against the IL-6R for RA therapy (Section 1.8.2).

There are other cytokines that are important in RA pathogenesis. This includes GM-CSF, which will be discussed in Section 1.10.

1.6 Chemokines in RA

1.6.1 Chemokine structure and function

Chemokines, or chemotactic cytokines, are small proteins that direct the migration of leukocytes (158). There are four conserved cysteines with intramolecular disulphide bonds, which dictate the tertiary structure of the chemokine. The nomenclature of chemokines is determined by the position of the first two cysteines. The cysteines of CC chemokines are adjacent, whereas in CXC chemokines, the cysteines are separated by one amino acid. (159).

Chemokines have multiple functions but their main role is to direct leukocyte migration in homeostasis and inflammation as well as to stimulate cells to release further pro-inflammatory mediators such as cytokines (160).

To fulfil their function, chemokines bind specific chemokine receptors, which are G protein-coupled receptors (GPCRs), and cause rapid structural change within the cell through the conformational change of actin and activation of integrins (159). The length of the N-terminal domain determines ligand specificity as truncation of this domain can change receptor specificity, which is the case for CXCL7 and CCL2 (161). Chemokine receptors are classified according to the chemokine ligands in which they bind (162,163). However, not all receptors bind chemokines within their own class, as there are chemokine receptors that bind multiple chemokines (Table 1.2). There are 10 CC chemokine receptors and 6 CXC chemokine receptors as well as a single CX3CR and an XCR (162-164). The ability of chemokine receptors to bind multiple chemokines allows the system to have a redundancy mechanism (165).

Multiple chemokines are involved in the immune response, however there are some that are considered to have important roles in RA pathogenesis. CXCL8, CXCL10 and CCL2 are chemokines that are secreted by innate immune cells such as monocytes and macrophages (158). CCL17 currently is not associated specifically with RA pathogenesis, however this thesis aims to investigate a potential role for this chemokine in disease.

Table 1.2 Chemokine Receptors and their chemokine ligands

Chemokine Receptor	Ligands
CC Receptors	
CCR1	CCL3, CCL3L1, CCL5, CCL7, CCL8, CCL14, CCL15, CCL16, CCL23
CCR2	CCL2, CCL7, CCL8, CCL13, CCL16
CCR3	CCL5, CCL7, CCL8, CCL11, CCL13, CCL15, CCL24, CCL26, CCL28
CCR4	CCL17, CCL22
CCR5	CCL3, CCL3L1, CCL4, CCL4L1, CCL5, CCL8
CCR6	CCL20
CCR7	CCL19, CCL21
CCR8	CCL1
CCR9	CCL25
CCR10	CCL27, CCL28
CXC Receptors	
CXCR1	CXCL6, CXCL8
CXCR2	CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8
CXCR3	CXCL9, CXCL10, CXCL11
CXCR4	CXCL12
CXCR5	CXCL13
CXCR6	CXCL16
CX3C Receptor	
CX3CR1	CX3CL1
XC Receptor	
XCR1	XCL1

1.6.2 CXCL8 (IL-8)

High levels of CXCL8 have been found in multiple areas of RA synovial tissue, including the synovial fluid and synovial membrane (166). Interestingly, the presence of CXCL8 in RA is not just confined to the inflamed joint, but systemically, as serum levels have been found at elevated levels. The level of CXCL8 in the sera correlated with the levels of CXCL8 in the synovial fluid suggesting a strong inflammatory response (166,167). The role of CXCL8 in RA is not well defined; however, there is evidence to suggest CXCL8 is involved in the recruitment of neutrophils to the inflamed joint and angiogenesis (160,167-169). The use of an anti-IL-8 antibody in the LPS induced arthritis model in rabbits, showed a reduction in neutrophil infiltration thereby leading to reduced inflammation in the synovial membrane. However, this was only at the early

stage of inflammation, as in a later phase, there was mononuclear cell infiltration and inflammation of the tissue, due to other chemokines such as CCL2 (170). Therapies targeting CXCL8 with an anti-CXCL8 antibody have not been published but there was no clinical improvement in RA patients and there was a rise in total CXCL8 levels in circulation (160).

1.6.3 CXCL10

CXCL10, also known as IP-10, is induced by stimulation of monocytes with IFNγ and binds CXCR3 (Table 1.2) leading to the migration of activated T cells. Neutralisation of CXCL10 leads to an inability of effector T cells to be recruited, which was shown in a model of Experimental Autoimmune Encephalomyelitis (EAE) (171,172). CXCL10 is upregulated in the synovial fluid of RA patients compared to OA patient synovial fluid. In rheumatoid factor positive patients, serum levels of CXCL10 are also elevated (166). Macrophages and fibroblasts of the RA synovial tissue express high levels of CXCL10 (173). There is also evidence to suggest that CXCL10 induces osteoclastogenesis through the positive feedback loop with RANKL. RANKL induces CXCL10 in osteoclast precursors, which thereby upregulates RANKL expression in T cells in the synovium (174). A neutralising CXCL10 antibody has been used in the mouse model of CIA, which led to significantly inhibited infiltration of CD4+ T cells and macrophages into the synovium of the joint. Furthermore this led to decreased levels of RANKL and therefore attenuated bone erosion (174,175). Current studies, therefore, suggest that CXCL10 is important in rheumatoid arthritis pathogenesis, and the crosstalk between CXCL10 and RANKL drives the infiltration of inflammatory cells into the synovium and subsequent bone erosion (174).

1.6.4 CCL2

CCL2, also known as MCP1, is a chemokine that binds CCR2 and directs migration of monocytes. CCL2 is expressed by multiple immune cells and therefore plays an important role in the inflammatory response. In rheumatoid arthritis, CCL2 and CCR2 are highly expressed in the synovial tissue (176). In a mouse model of CIA, using an anti-CCL2 monoclonal antibody during the initiation of CIA showed significantly lower disease scores, and significantly lower histological scores at multiple sites within the joint. Interestingly, when the anti-CCL2 antibody was

used as a therapeutic, during the progression of the disease, there was exacerbation of disease, with mice showing an increase in clinical and histological scores (177). This suggests that the infiltration of leukocytes into the joint is not specifically reliant on CCL2. Furthermore, a human clinical trial evaluating the blockade of CCR2, revealed that blockade had no positive effects and also led an increase in the circulation of bioactive CCL2: somewhat similar to the effect of blocking CXCL8 (160,178).

1.6.5 CCL17 and CCR4

CCL17 also known as Thymus and Activation-Regulated Chemokine (TARC) is found in abundance in the thymus and is released by $CD1c^+$ dendritic cells upon stimulation with primarily thymic stromal lymphopoietin (TSLP). TSLP is induced by multiple cytokines such as TNFα, IL-1β and IL-4 in multiple cell types and is thought to play a role in RA. Mouse models of RA in TSLPR null mice have shown a decrease of severity and less incidence of disease in comparison to wild type mice suggesting a role for TSLP signalling in RA (179,180). After TSLP induces CCL17, it binds CCR4, and causes migration of CCR4 positive T cells.

CCR4 is the receptor not only for CCL17 but also for CCL22. CCL22 is more potent at inducing CCR4 internalisation than CCL17 and has also been implicated in disease (181). Internalisation of the chemokine receptor is important for receptor desensitisation and the ability of cells to continuous changes in chemotactic gradients (181). CCR4 is primarily, but not exclusively, found on Th2 cells, and therefore there are several studies implicating CCL17 and CCL22 in Th2 driven disease (180). Atopic dermatitis is an example of a Th2 specific disease and high levels of CCL17 have been found in the inflamed skin. In a mouse model, treatment with a steroid ointment led to a very large decrease in CCL17 expression and reduction in disease severity. This involvement of CCL17 in atopic dermatitis has led to its use as a biomarker (182).

CCR4 is also expressed on Th17 cells (Section 1.4.4), which have been implicated in the pathogenesis of several autoimmune diseases such as multiple sclerosis as well as RA. Th17 cells have been found in abundance in the joints of RA patients, and positively correlated with disease progression. Synovial Th17 cells express CCR4, suggesting a role for CCR4 and its ligands in RA (183).

Interestingly, Treg cells also express CCR4, suggesting a more regulatory role for CCR4, CCL17 and CCL22. $CCR4^+$ Treg cells have been shown to migrate towards the chemokine ligand CCL22 in lymphoid infiltrates surrounding breast cancer tumours (184). Tregs also migrate to inflamed lungs in allergic inflammation, attenuating inflammation (185).

The role of CCL17 in RA is not as well understood. However, CCR4, the chemokine receptor for CCL17 has been studied. In the mouse model of CIA, CCR4 null mice had significantly reduced incidence of arthritis and significantly lower disease scores than the wildtype control(186). This suggests that there is a role for CCR4 positive cells in RA. There is limited data linking CCR4 to human disease, and there is no correlation linking CCR4 positive CD4 T cells with disease activity. However, CCR4 is expressed on PBMCs at significantly higher levels in RA patients than in healthy controls. As CCL17 is one of the 2 ligands for CCR4, potentially CCL17 is also involved in RA pathogenesis. CCL17 has been investigated in the RA patient sera, however there was no significant difference compared with controls (187). However, in RA synovial fluid, CCL17 is much more highly expressed than in OA synovial fluid (137).

1.7 Treatment of RA

The aim of therapy in RA is to maximise quality of life through the control of RA symptoms and prevention or joint erosion. The most effective way of enabling this control of symptoms is to dampen the inflammation in order to reach clinical remission whereby there is an absence of disease activity signs and symptoms (188). To measure the effectiveness of therapies, disease activity scores such as DAS28 and DAS40 as well as CDAI and SDAI are used as measures of clinical remission (189).

1.7.1 DMARDs

Disease Modifying Anti-Rheumatic Drugs (DMARDS) such as sulfasalazine, hydroxychloroquine and azathioprine are the first method of treatment for RA, with Methotrexate (MTX) the most commonly used in the first instance (190). The mode of action of methotrexate in RA is still not completely understood, however there are multiple areas in which it could be having its effects. MTX

was initially used as a specific antagonist to folic acid to have anti-proliferative effects on malignant cells by inhibiting synthesis of purines and pyrimidines. Low dose MTX was initially used as a treatment for RA as it was assumed the anti-proliferative properties it possessed, would be beneficial to inhibit the proliferation of lymphocytes (191). This led to an improvement in disease and with the addition of folic acid to treatment, this prevented some of the toxicity associated with MTX (191). However, after multiple trials, it was determined that to dampen inflammation, MTX, had to have more wide-ranging effects than just an anti-proliferative effect on leukocytes (192). MTX has inhibitory effects on neutrophils as well as monocytes and macrophages, all of which impact upon synovial inflammation. At a biochemical level, MTX causes an overproduction of adenosine which prevents production of multiple inflammatory cytokines such as TNFα, IL-8, IL-12 and IFNγ from lymphocytes (192). These are just a few of multiple effects that have been discovered, and explain some of the mechanisms of MTX action in RA treatment. However, approximately only a third of patients respond well enough to MTX, and if there is not a great enough effect, biologic therapies are the next method of treatment (193,194). Interestingly, MTX has been described as the 'anchor drug' as the majority of clinical trials for new therapies, compare responses to MTX (193,195).

1.7.2 Therapeutics

Development of targeted biologic therapies were the next major advance in RA therapy as these drugs specifically target molecules responsible for disease pathogenesis (196). There are now multiple cytokine therapies that have been developed both with and without success, for example TNFα inhibitors such as etanercept, the IL-1 inhibitor anakinra, anti-IL-17 inhibitors such as Secukinumab and the anti-IL-6R Tocilizumab. There are other therapies such as abatacept which is an immunomodulatory drug that inhibits T cell activation through the CD80/86-CD28 costimulatory signal (196,197). There has also been specific cell targeted therapy such as rituximab, which is a monoclonal antibody that binds to CD20, on B cells, and leads to B cell depletion (196). Small molecule inhibitors have also been developed, targeting signalling pathways that are important in the pathogenesis of RA such as Tofacitinib which inhibits JAK1 and JAK3, thereby preventing STAT1 activation, and preventing secretion of cytokines such as IL-6 and IFNγ in naïve $CD4^+$ T cells (198).

1.8 Successful cytokine therapies

There has been varying success with the cytokine therapies that have been developed. TNFα inhibition was the first cytokine therapy to be developed and has been very successful; showing that targeting the cytokine network and disrupting the inflammatory communication mechanism can have a positive impact on disease outcomes (199).

1.8.1 TNFα Inhibition

TNFα was initially determined as a good cytokine to target in RA as: several other pro-inflammatory cytokines depend on TNFα; both TNFα and the TNF-R are upregulated in diseased synovium; and an anti-TNFα antibody prevented joint destruction and decreased disease severity in the mouse model CIA (200). In the CIA model of RA, addition of an anti-TNFα antibody for a week preceding the induction of disease, and during the course of disease showed that the clinical score was significantly lower in the anti-TNFα treated animals in comparison to PBS treated controls. The histopathological analysis also showed less severe arthritis (201). In a CIA model using an anti-TNFα antibody as a therapeutic, 2-3 weeks after induction of disease, there was less TNF in the joint of immunised mice and significantly reduced footpad swelling at 1 and 3 months post induction of disease (202). A neutralising, chimeric anti-TNFα monoclonal antibody was first tested in man in 1993 and showed that the antibody was safe and positive for efficacy, with a 97% improvement of morning stiffness (203). In further clinical trials, it was established that inhibition of TNFα was effective in treating patients who were not responders to MTX (204). The mechanism of action of anti-TNFα therapy is due to the downregulation of cytokine cascade as cytokines such as IL-6, IL-1 and GM-CSF are downstream of TNFα stimulation. Also, the migration of leukocytes into the joint is decreased due to the downregulation of endothelial adhesion molecules such as ICAM-1, E-selectin and VCAM-1. Anti-TNFα also reduces angiogenesis via downregulation of VEGF (200,205). The success of TNFα inhibition has led to the development of multiple therapeutics targeting TNFα including etanercept, infliximab and adalimumab (206). These biologics all target TNFα, however, infliximab and adalimumab are

both antibodies against TNFα, whereas etanercept is a construct involving two p75 TNFα receptors coupled to the Fc portion of human IgG (207).

1.8.2 IL-6R blockade

After the success of inhibiting TNFα, blockade of IL-6 was investigated due to its role in RA pathogenesis (see 1.8.2). Initially, in the CIA mouse model, a monoclonal antibody to the IL-6R was administered at the initiation of disease and led to significantly decreased disease scores over the course of the disease period. Interestingly, in this model when the anti-IL6R antibody was tested at multiple different days after induction of disease, only when administered at day 0 or day 3 showed a reduction in disease (208,209). After the success of inhibition of IL-6R in mouse, this was translated into the clinic. Tocilizumab is a humanised monoclonal antibody that binds to both IL-6R and sIL-6R, and was developed for treatment of RA. In clinical trials, inhibition of the pro-inflammatory effects by IL-6 through blockade of both soluble and membrane bound IL-6R by Tocilizumab showed significant improvements in disease with significantly more patients attaining ACR20, ACR50 and ACR70 than the placebo control (210). This has led to more treatment options, due to the numbers of patients that are non-responders or intolerant to DMARDS and anti-TNFα therapy (211).

1.9 Unsuccessful cytokine therapies

1.9.1 IL-1 inhibition

IL-1RA is produced by synovial tissue and it localised primarily to the perivascular macrophages at both an mRNA and protein level. It binds to IL-1 and acts as a competitive inhibitor of IL-1 to its membrane bound receptor (212,213). As the addition of IL-1β directly induces arthritis when injected into joints, and blockade of IL-1 in CIA led to decreased bone resorption and degradation, IL-1 became a cytokine to target for the treatment of RA (213,214). Due to IL-1RA already endogenously inhibiting IL-1, recombinant IL-1RA (Anakinra) was investigated as a therapy in RA patients (215). In safety and efficacy trials, Anakinra caused 41% less radiologic joint damage compared with placebo receiving patients (213). However, a subsequent trail in patients who

were unresponsive to TNFα inhibition was unsuccessful, as after 3 months of therapy, only 8% of patients received an ACR20 response with none receiving an ACR50 or ACR70 response. There was also an increase in the mean level of C-reactive protein and tender swollen joints showing that patients unable to respond to TNFα treatment, will also not respond to IL-1 blockade (216). Despite Anakinra not showing success in RA, it is a successful treatment for auto-inflammatory conditions such as Juvenile Idiopathic Arthritis, gout, Cryopyrin Associated Periodic Syndromes and Adult Onset Still's Disease (2,215,217,218).

1.9.2 Unmet Need

Despite the new cytokine therapies that have revolutionised RA treatment strategies, there are still a number of patients who do not respond to TNFα inhibition or IL-6R blockade. Only 60-70% of patients respond to TNF blockade regardless of DMARD status (219). Therefore, there are still a large percentage of patients who have no further treatment options and are living with a debilitating disease. It is for this reason, that there is still ongoing research into other biologics including other cytokine therapies. This project aims to look at the role of GM-CSF in RA with regards to Mavrilimumab, an anti-GM-CSFRα antibody that has been developed as a therapeutic for the treatment of RA.

1.10 GM-CSF

GM-CSF has traditionally been considered a growth factor important in the maintenance and differentiation of bone marrow derived monocytes. However, GM-CSF has since been shown to have multiple functions in the regulation and activation of granulocytes and macrophages during inflammatory insults, differentiation and proliferation (220,221). GM-CSF is therefore considered a pro-inflammatory cytokine due to the effects it has on cell activation.

1.10.1 Biology

GM-CSF is a cytokine that is secreted from multiple cell types including fibroblasts, T cells, B cells, endothelial cells, epithelial cells and macrophages (222). Stimulation from sources such as LPS or TNFα can lead to the secretion of GM-CSF from these cells (223). The GM-CSF receptor is heteromeric and formed

of an alpha and beta chain. Myeloid cells and non-haematopoietic cells such as alveolar epithelial cells and endothelial cells express the GM-CSFR, but it is not expressed on T cells (222-224). The β chain of the GM-CSFR constitutively associates with JAK2, however, both cytoplasmic domains are crucial for receptor activation (222,225). The binding of GM-CSF initiates JAK2 auto-phosphorylation and activation of STAT5, MAPK or PI3K signalling pathways (Figure 1.4). JAK2 has potent signalling effects and therefore suppressor of cytokine signalling (SOCS) inhibits JAK2 by targeting it for degradation (221).

GM-CSF has a range of functions on mature cells including polarisation of macrophages to what is considered an M1, pro-inflammatory phenotype, stimulating them to produce cytokines such as TNFα and IL-6 (226). GM-CSF primes neutrophils to become more phagocytic and upregulates the integrin CD11b causing more adhesion to the vascular endothelium and entry into the tissue (227). Mature dendritic cell cross-presentation is increased by GM-CSF stimulation and it can influence differentiation of monocytes (228). Elevated levels of GM-CSF have been associated with inflammatory conditions including RA (229).

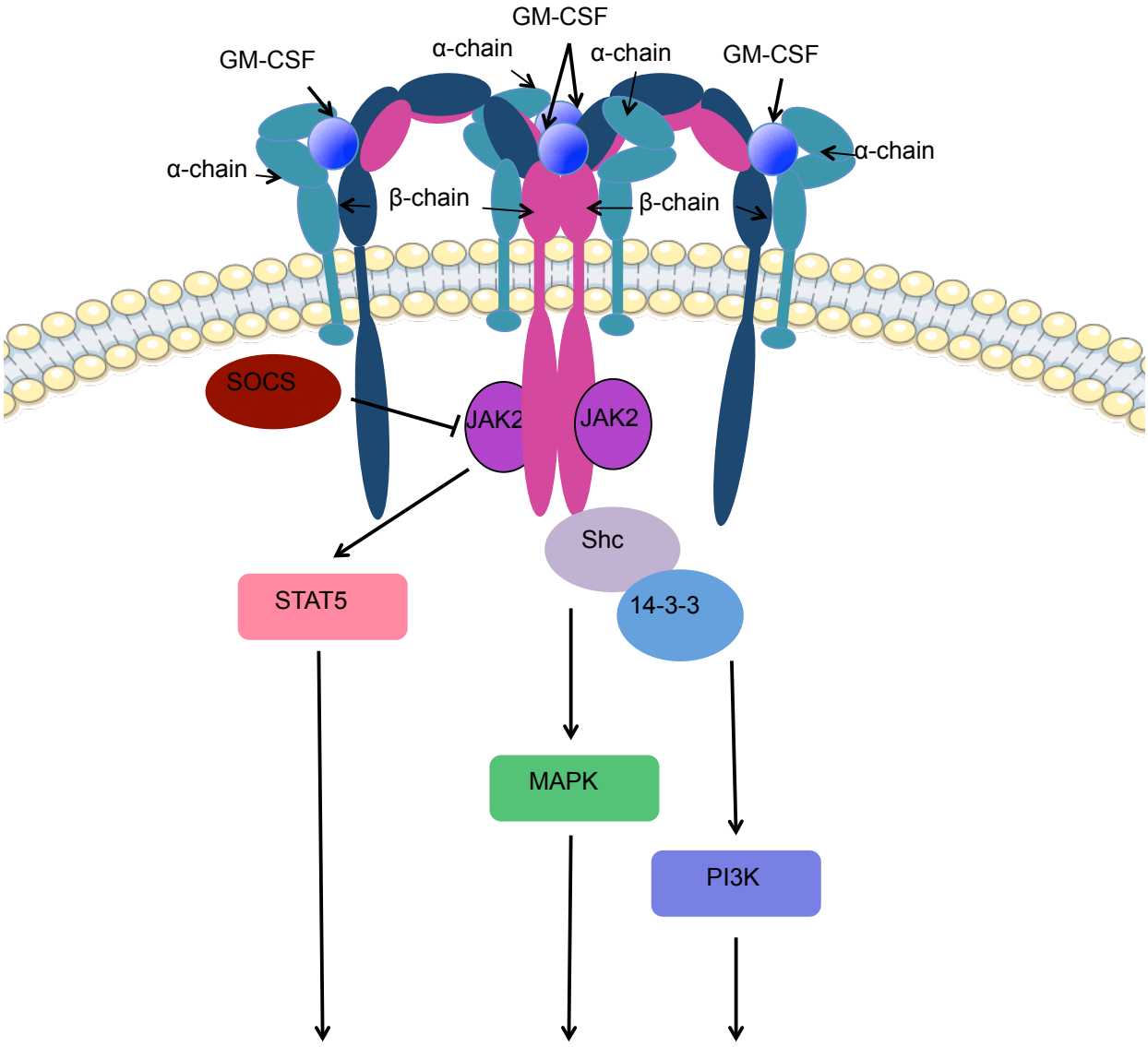

Figure 1.4 GM-CSFR signalling
The α and β chains of the GM-CSFR form a dodecamer with multiple GM-CSF binding sites. JAK2, which is constitutively associated with the β chain becomes activated upon GM-CSF signalling. The signalling cascades are via STAT5, MAPK or PI3K. SOCS is an inhibitor of JAK2.

1.10.2 Clinical Relevance

GM-CSF is considered to play a role in multiple inflammatory conditions. In RA there have been many studies to elucidate how GM-CSF exacerbates inflammation. It was initially thought that as GM-CSF causes the secretion of many pro-inflammatory cytokines, GM-CSF depletion could ameliorate disease by reducing TNFα and IL-1 (220,230). Interestingly, in GM-CSF null mice, there was no defect in myeloid cell development, however, in the lungs of these mice there was defective alveolar macrophage maturation and an accumulation of surfactant (231). The effect of the loss of GM-CSF in mice was studied in mouse models of RA, including the CIA model and mBSA/IL-1 experimental arthritis model. In both of these models of arthritis, the GM-CSF null mice had reduced disease scores, reduced incidence and improved histological scores (232,233). This was confirmed with both prophylactic and therapeutic treatment with an anti-GM-CSF antibody in the acute streptococcal wall arthritis model, where disease was significantly improved after antibody treatment (234,235). The opposite effect occurred when GM-CSF was overexpressed in the CIA model of arthritis. Recombinant GM-CSF treatment of mice led to significantly worse disease and histological scores in comparison to the vehicle controls (236). This mouse data both using a GM-CSF knockout and therapeutic treating with an anti-GM-CSF antibody confirmed that GM-CSF has a pathogenic role in murine models of RA. These studies led to further investigation and the initiation of targeting GM-CSF with monoclonal antibodies in human disease.

1.10.3 Targeting GM-CSF

GM-CSF and its receptors are found in abundance in the synovial membrane and synovial tissue of patients with RA in comparison to healthy controls and OA patients (237). Co-localisation of the GM-CSFRα with CD68 and CD163 was also observed suggesting expression of the GM-CSFRα on monocytes and macrophages (238). Translating the data observed in mice, into human began with *in vitro* studies, demonstrating that PBMCs stimulated with GM-CSF could produce TNFα in a dose dependent manner (239). Inhibition of GM-CSF signalling was possible via blockade of GM-CSF itself through neutralising antibodies or specific binding of antibodies to the receptor. This has led to two therapeutics, Mavrilimumab and MOR103 that are currently being tested in clinical trials (229).

Mavrilimumab (CAM3001) is a fully human monoclonal antibody, which targets the α chain of the GM-CSFR. In an initial mouse model, Mavrilimumab showed a dose dependent reduction in clinical score in already established CIA (238). Acceptable safety profiles were observed in cynomolgus monkeys, and the phase I clinical trial using a single, escalating intravenous dose of Mavrilimumab in patients with RA demonstrated an appropriate safety profile (240,241). Phase IIa clinical trials showed improved signs and symptoms of RA patients on methotrexate treatment but with active disease. This was demonstrated as the DAS28-CRP was improved at the highest doses of 50 and 100mg at week 2, which continued throughout the 12 week treatment. The phase IIb clinical trial was also successful with all the primary endpoints achieved. The AR50, ACR70 and DAS28 remission score was also significant at the highest dose of 150mg in comparison to the placebo (242).

MOR103 is a fully human, high affinity monoclonal antibody targeted to GM-CSF, which was initially developed for the treatment of multiple sclerosis (MS). This has now reached phase IIb trials for the treatment of MS, however for the treatment of RA, the phase Ib and phase IIa trials examined safety in a dose escalation study. Preliminary evidence suggested that MOR103 was efficacious with improvements in DAS28 scores at both 1.0 and 1.5mg/kg groups (229,243).

The results of targeting both GM-CSF and its receptor in clinical trials are positive. Inhibiting GM-CSF signalling prevents pro-inflammatory cytokine secretion, which attenuates pathogenesis (229). However, the downstream effects of GM-CSF blockade are still unknown.

1.11 Hypothesis and Aims

The effect of GM-CSF on cells of the myeloid compartment in rheumatoid arthritis is not well understood, therefore the aim of this project has been to unravel the impact of GM-CSF on myeloid cells in the synovial environment. We hypothesised that GM-CSF up-regulates chemokines in monocytes and that the synovial environment impacts myeloid cell phenotypes leading to an increase in inflammatory mediators.

GM-CSF exacerbates the mouse model of RA, CIA, through the induction of pro-inflammatory cytokines such as TNFα and IL-1β (234). Mavrilimumab has also shown success in clinical trials through the inhibition of GM-CSF (244). However, the mechanisms of GM-CSF on RA pathogenesis have not been investigated in a human system. The purpose of this project is to create an *in vitro* system to mimic disease, to further analyse myeloid cells, specifically monocytes and macrophages. The secretion of chemokines from monocytes will then be examined after GM-CSF stimulation, in synovial stimuli. Also the effect of the synovial environment on macrophage phenotypes will be assessed to determine whether the macrophage changes its phenotype when polarised with disease stimuli and whether Mavrilimumab treatment alters this.

We hypothesise that GM-CSF will be involved mechanistically in leading to an inflammatory monocyte in the synovial environment. Also, that the synovial environment will exacerbate the secretion of inflammatory mediators from monocytes. The monocyte-derived cells or macrophages derived with synovial fluid, we hypothesise to have a distinct inflammatory phenotype that respond to Mavrilimumab treatment. The following aims will specifically be addressed:

- How do monocytes respond to GM-CSF stimulation and does the synovial environment impact this?

- How is the secretion of CCL17 regulated in monocytes in an inflammatory environment?

- Can synovial fluid be used to create a disease-like macrophage that can be used as a robust tool for analysing phenotypic changes in response to therapeutics?

Chapter 2 Materials and Methods

2.1 Patients and Controls

All samples were obtained from buffy coats, healthy volunteers or RA patients. The RA patients were identified from Rheumatology clinics in Glasgow Royal Infirmary and fulfilled the 2010 ACR/EULAR criteria for RA. For the peripheral blood samples, patients were not taking biologic therapies and they had active disease. Samples were obtained after written consent, with ethical approval granted by the West of Scotland Research Ethics Committee.

2.2 Primary Human Cell Culture

2.2.1 Isolation of PBMCs from buffy coats and peripheral blood

Peripheral blood mononuclear cells (PBMCs) were separated from human buffy coats (Scottish National Blood Transfusion Service) by density gradient centrifugation. Buffy coats were diluted in dPBS (Gibco) at a 1:1 ratio whereas peripheral blood was not diluted. 5-8ml was layered onto 3ml Histopaque-1077 (Sigma-Aldrich) in 15ml centrifuge tubes. This was centrifuged at 400g for 30 minutes, at room temperature with no brake. The PBMC layer was removed, transferred into a 50ml falcon tube and washed twice in sterile dPBS (Gibco), centrifuging between washes (300g, 1x 10 minutes, 1 x 5 minutes). Cells were then resuspended in 10ml cell separation media (Appendix). In order to count viable cells, 50μl cell suspension was added to 200μl dPBS. This was further combined at a 1:1 ratio with trypan blue (Sigma) for dead cell exclusion, counted using a haemocytometer and visualised using a light microscope. The value obtained from one main square was equal to the number of cells x 10^6 in 10ml. After a further centrifuge at 300g for 5 minutes, cells were resuspended at 5 x 10^7 per ml in cell separation media (Appendix) and added to a capped 6ml polystyrene tube (BD falcon).

2.2.2 Purification of T cells from PBMCs

$CD4^+$ T cells were separated using the EasySep $CD4^+$ enrichment kit (Stem Cell Technologies), to negatively select for CD4 cells. Initially, the antibody cocktail was added to the PBMCs at a concentration of 50μl/ml for 10 minutes at room

temperature. The magnetic beads were added at a concentration of 100μl/ml for 5 minutes at room temperature. The volume was made up to 2.5ml before placing in a purple magnet (Stem Cell Technologies) for 5 minutes. The required $CD4^+$ cells were negatively selected by inverting the magnet, with the tube inside. These cells were counted (2.2.1), divided into 3 equal samples and centrifuged. Of these $CD4^+$ samples, 2 were further processed to generate $CD45RA^+$ and $CD45RO^+$populations. These separations were also using negative selection kit, therefore to separate $CD45RA^+$ cells, a CD45RO Tetrameric Antibody Complex (TAC) depletion kit (Stem Cell Technologies) was used. Cells, at 5 x 10^7/ml, were incubated with the antibody, at a concentration of 20μl/ml for 15 minutes. 100μl/ml of Anti-biotin TAC was added for 10 minutes, prior to 5 minutes with 50μl/ml magnetic beads, and 5 minutes in the magnet before inverting the magnet and isolating the $CD45RA^+$ cells. For the CD45RO selection, a CD45RA TAC depletion kit (Stem Cell Technologies) was used. The CD45RA TAC was added to the cells for 15 minutes, prior to the addition of 50μl/ml magnetic beads for 5 minutes. The tube was placed in the magnet for 5 minutes, inverted and the CD45RO cells collected.

2.2.3 T cell stimulation

The $CD4^+$, $CD45RA^+$ and $CD45RO^+$ T cells were cultured at 1 x 10^5 cells per well in T cell media (see appendix) in 96 well plates with or without 5 x 10^4 αCD2/CD3/CD28 beads (Miltenyi Biotech) per well and human recombinant CCL17 (Peprotech) at 100ng/ml or 500ng/ml. Cells were cultured for 24 or 48 hours, where supernatants were collected and analysed for GM-CSF secretion by ELISA (2.7.2)

2.2.4 Purification of Monocytes from PBMCs

Monocytes were separated using the EasySep Human monocyte enrichment kit (Stem Cell Technologies), to negatively select for $CD14^+$ cells without depleting $CD16^+$ cells, as per manufacturer's instructions. Firstly, the antibody cocktail was added to PBMCs (Section 2.2.1) at a concentration of 50μl/ml, incubating on ice for 10 minutes. The addition of the magnetic beads at a concentration of 50μl/ml followed this for 5 minutes on ice. The tube was then made up to 2.5ml with cell separation media and the cap removed before adding to a purple

EasySep magnet (Stem Cell Technologies). After 2.5 minutes incubation, the magnet, with the tube inside, was inverted, and these negatively selected cells collected. The cells remaining in the tube were resuspended in a further 2.5ml cell separation media, and placed back in the magnet a further 2 times for maximum return. Enriched cells were counted as specified in 2.2.1, washed, centrifuged (300g, 5 minutes) and resuspended in complete RPMI media (see appendix) at the desired concentration for each experiment.

2.2.5 Macrophage differentiation

Recombinant human M-CSF (Peprotech) was added at 100ng/ml and 1ml of cells at $1x10^6$/ml were seeded in 24 well plates (Corning, Sigma Aldrich) and incubated at 37°C, 5% CO_2 for 7 days. Cells were washed twice with warm sterile dPBS, and the complete media replaced with 300μl media with 100ng/ml M-CSF for M0 phenotype, 20ng/ml IFNγ (Sigma-Aldrich) for M1 phenotype, 100ng/ml IL-4 (R&D systems) for M2 phenotype, and 100ng/ml GM-CSF (R&D systems) for M-GM phenotype.

2.2.6 Monocyte stimulation

Monocytes and macrophages were stimulated with 2.5%, 5% or 10% synovial fluid in complete media (detailed in figures) from Rheumatoid Arthritis, Osteoarthritis or Psoriatic Arthritis patients for 24 hours at 37°C. LPS (Invivogen) was used at 15.63ng/ml or 10ng/ml (detailed in figures). CAM3001 (Mavrilimumab) and CAT004 (Isotype) were used from a top concentration of 10μg/ml in monocyte stimulations. Tofactitinib (LC Labs), using DMSO as the vehicle, was used at a top concentration of 1000nM in 0.001% DMSO. All pre-treatments were for 30 minutes prior to initiation of the assay.

2.2.7 Monocyte genetic manipulation

NTER transfection reagent (Sigma Aldrich) was used according to manufacturer's instructions. In brief, control siRNA (Dharmafect) and SOCS3 siRNA (Dharmafect) were pre-incubated with the NTER peptide at a concentration of 50nM for 15 minutes at 37°C. $1x10^5$ monocytes were cultured in RPMI with 10% human serum in 300μl in a 24 well plate. The siRNA-NTER peptide complex was added drop-wise into each well and cultured for 6 or 24 hours (detailed in figures) prior to a

media change and further stimulation for 24 hours. Transfection efficiency was confirmed by FACS (Section 2.9) using labelled, control siRNA. Knockdown was confirmed by qPCR (Section 2.6.3) using Taqman primers for SOCS3 (Life Technologies, Hs02330328_s1), and beta-actin as the housekeeping control (Life Technologies, Hs01060665_g1).

2.3 Immune Complexes

2.3.1 Small Immune Complex Formation

Using the previously established method (245), Staphylococcal Protein A (SpA) immunoglobulin complexes were formed using human IgG and SpA. 25μg/ml human IgG (Jackson Immunochemicals) was centrifuged at 8000g to remove large IgG complexes, and incubated with 1.56μg/ml SpA (Repligen) (1:4 molecular weight ratio) for 1 hour at 37°C. This allowed the formation of complexes with 4 IgG molecules and 2 SpA molecules. After the formation of the complexes, dPBS was added to dilute the complexes, for a final 1 in 20 dilution for use in assays. Equivalent amounts of Human IgG, and SpA were used alone as controls for SIC.

2.3.2 Depletion of Immune Complexes from Synovial Fluid

Protein A (Life Technologies), Protein G (Life Technologies) or Protein A/G/L (Biovision, Cambridge Biosciences) magnetic beads were placed in an eppendorf, washed with the appropriate buffer, according to manufacturer's instructions, and placed on a magnet. The supernatant was removed, and 2 further washes with dPBS were performed, with vortexing in between. 500μl synovial fluid was added directly to the beads at the recommended concentration (supplier dependent), and incubated at room temperature with end over end mixing for 1 hour. The eppendorfs were placed back on the magnet, and the depleted synovial fluid removed. This synovial fluid was placed back onto a magnet to ensure all magnetic beads had been removed. The depleted synovial fluid was then used to stimulate monocytes.

2.4 Fluorescent labelling

2.4.1 Pacific blue labelling of antibody

Using the pacific blue monoclonal antibody labelling kit (Life Technologies), CAT004 (MedImmune) was fluorescently labelled. The conjugation reaction was performed by adding 10μl 1M sodium bicarbonate buffer to 1mg/ml antibody (diluted in PBS). 100μl of the protein solution was added to the vial of reactive dye and inverted several times to fully dissolve the dye. This was incubated at RT for 1 hour with inversions every 15 minutes. To prepare the spin column, the resin was added to the top of the column allowed to settle and then centrifuged for 3 minutes at 1100g. 100μl of the reaction volume was added drop-wise to the centre of the spin column and allowed to absorb into the gel bed. This was centrifuged at 5 minutes at 1100g and the labelled protein collected in the spin column. The labelled CAT004 was then used for FACS (Section 2.9)

2.5 Mice

2.5.1 Extraction of bone marrow monocytes

Bone marrow was extracted from either WT C57BL/6 mice or MyD88/TRIF knockout mice (courtesy of Prof Rick Maizel, Edinburgh). Mice were euthanized and the hind legs including both the femur and tibia were removed. Bone marrow was flushed from the bones using complete MEM-α in a 5ml syringe. To break any cell clumps, the cell suspension was pipetted up and down before passing through a 70μm cell strainer (VWR). The cell suspension was washed in complete MEM-α, centrifuged at 300g for 5 minutes before red blood cell lysis using ACK lysis buffer (Life Technologies). Cells were then counted as previously described (2.2.1) and resuspended at 1 x 10^8 per ml in cell separation buffer in 6ml capped polystyrene tube (BD falcon). Monocytes were separated from the bone marrow using a mouse monocyte enrichment kit (Stem cell). In brief, cells were incubated on ice with normal rat serum at 50μl/ml of cells and enrichment cocktail at 50μl/ml of cells for 15 minutes. Cells were then washed by topping up the sample tube with cell separation buffer and centrifuged at 300g for 10 minutes. The cells were resuspended at 1 x 10^8 per ml and incubated with biotin selection cocktail at 60μl/ml of cells for 15 minutes at 4°C. Following this

incubation, the magnetic particles were added at 150μl/ml of cells for 10 minutes at 4°C. The total volume was then brought to 2.5ml before placing the tube in a purple magnet (Stemcell Technologies) for 5 minutes. The magnet, with the tube inside, was inverted into a falcon and as it was a negative selection, the cells in the falcon were counted and washed at 300g for 5 minutes.

2.5.2 Stimulation of monocytes

Monocytes were cultured in 200μl in 96 well plates at a concentration of 5 x 10^5/ml for 24 hours. All conditions had 1ng/ml recombinant mouse GM-CSF added, with or without 10ng/ml LPS, or 5% RA synovial fluid from 3 patients. Supernatants were collected after 24 hours and analysed by ELISA (Section 2.7.2).

2.6 Transcript Analysis

2.6.1 RNA extraction

Supernatant was removed from monocyte or macrophage cell cultures and cells were lysed using 350μl RLT buffer from the RNeasy mini kit (Qiagen). The lysates were transferred to eppendorf tubes, vortexed to break up clumps, transferred to QIAshredder tubes (Qiagen) and centrifuged at full speed for 2 minutes. 70% ethanol was then immediately added to the flow through and transferred to the top of a spin column from the RNeasy mini kit (Qiagen). The manufacturer's instructions for the RNeasy kit were then followed. In brief, the flow through from the QIAshredder plus 70% ethanol was spun through the spin column at 8000g for 15 seconds. The flow through was discarded and the column washed with RW1 buffer at 8000g for 15 seconds. An on-column DNase digest was performed at this point by adding 80μl DNase incubation mix (10μl DNase in 70μl RDD buffer) for 15 minutes at room temperature. Following the DNase digest, columns were washed with RW1 buffer at 8000g for 15 seconds followed by a wash at 8000g for 15 seconds with RPE buffer. The flow through was discarded and RPE was added to the column again for a 2 minute centrifuge at full speed. The collection tube was then changed and the column spun at 8000g for 1 minute to dry out the membrane. The column was transferred to an eppendorf

tube and the RNA eluted in 30μl RNase free water by centrifuging at 8000g for 1 minute. The RNA concentration, and quality or potential contamination was measured using a Nanodrop 1000 (Thermo Scientific).

2.6.2 cDNA synthesis

To make cDNA, 10μl of a specified concentration of RNA (dependent on experiment) was added to 3ul random primers from the Affinity Script cDNA synthesis kit (Agilent) and 2.7μl RNase free water. The samples were mixed and incubated at 65°C for 5 minutes, then at room temperature for 10 minutes. 2μl of 10x affinity script RT buffer, 0.8μl dNTP mix, 0.5μl RNase block ribonuclease inhibitor and 1μl Affinity script multiple temperature RT were added to each sample before mixing and put in a PCR machine (Applied Biosystems 2720 Thermal Cycler) (Table 2.1)

Table 2.1 cDNA synthesis

Temp	Time
25°C	10 minutes
42°C	5 minutes
55°C	60 minutes
70°C	15 minutes

2.6.3 qPCR

2.6.3.1 Primer Design

Primers (forward and reverse)(Table 2.3) were either used from the literature, from primer bank, or were designed using primer 3 software by using the mRNA sequence from NCBI (National Centre for Biotechnology Information). Primers were designed with an amplicon size of 100-150 base pairs, the primer size of 18-23 base pairs in length, with 40-60% GC nucleotide content, a maximum 3' complementarity of 1, a maximum self-complementarity of 2 and a melting temperature between 59.5 and 61°C. NCBI blast was used to confirm the efficiency of primer designs and amplicon size was measured using Ape – A plasmid editor v1.17.

2.6.3.2 Primer Validation

2ul cDNA, 1μl of forward and reverse primer (Table 2.3), 25μl Red Taq (Bioline) and 21μl nuclease free water were added to PCR tubes, to run on an end point PCR (Table 2.2).

Table 2.2 End Point PCR

Temp	Time	Cycles
94°C	5 minutes	1
94°C	15 seconds	35
55°C	30 seconds	
72°C	30 seconds	
72°C	10 minutes	1

Samples were run on a 1.8% agarose gel with 0.01% ethidium bromide at 110v until the 1kb+ ladder (Invitrogen) had migrated the full length of the gel. The gel was visualised with ethidium bromide using a UV transluminator. The primers were confirmed by checking the observed band size with the known amplicon size.

2.6.3.3 Primer Sequences

Table 2.3 Primer Sequences

Gene	Forward sequence 5'-3'	Reverse sequence 5'-3'
APOL1	TTCGAATTCCTCGGTATATCTTG	CACCTCCAGTTATGCGTCTG
APOL2	ATGATGAAGCCTGGAATGGA	TCAGAGCTTTACGGAGCTCAT
APOL3	GCCTGGAAGAGATTCGTGAC	CTTCAGAGCTTCGTAGAGAGCA
APOL6	AAGTGAGGCTGGTGTTGGTT	CGTCTTGTAGCTCCACGTCTT
CCL17	GCAAAGCCTTGAGAGGTCTTGA	CGGTGGAGGTCCCAGGTAGT
CCL22	GTCCTGTTCCCATCAGCGAT	CAGGCTGGAGACAGAGATGGA
CD23	GGGAGAATCCAAGCAGGAC	GGAAGCTCCTCGATCTCTGA
CD36	CTGAAACACAATGCACAGAGAGA	AAAGATGCGAAATGTCACAACAC
CD64	TGGGAAAGCATCGCTACAC	GCACTGGAGCTGGAAATAGC
CLEC4E	GGCTGTGACCGGAACTGTG	CCGAGTGAAGATCCCCTTTTTA
CSF1R	CTGCCCAGATCGTGTGCTC	AGGTTGAGGGTCAGGACTTTTT
CXCL8	AGAAGTTTTTGAAGAGGGCTGAGA	CAGACCCACACAATACATGAAGTG
LILRA2	CAGCCACAATCACTCATCAGA	AGGGTGGGTTTGCTGTAGG
LILRB3	GGAGATACCGCTGCCACTAT	GGTGGGTTTGCTGTAGGC
IRF4	ACCCGGAAATCCCGTACCA	GGCAACCATTTTCACAAGCTG
IRF5	TGTCAGTGCAAGGTGTTCTGG	TTTGCGGTCAGGCCATTCT
IRF7	CCCAGCAGGTAGCATTCCC	GCAGCAGTTCCTCCGTGTAG
MRC1	GGGTTGCTATCACTCTCTATGC	TTTCTTGTCTGTTGCCGTAGTT
TNF	CCCTGTGAGGAGGACGAAC	TGAGCCAGAAGAGGTTGAGG
GUSB	CTCATTTGGAATTTTGCCGATT	AGGTCTCCAACTGGCATTAGAA
GAPDH	AATCCCATCACCATCTTCCA	TGGACTCCACGACGTACTCA

2.6.3.4 qPCR

Transcripts were analysed by qPCR in triplicate in 384 well plates (Starlabs) by adding to each well 1μl cDNA, 0.15μl primer pair, 5μl SYBR green (Invitrogen, Life Technologies) and 3.85μl nuclease free water (Life Technologies, UK). A non-template control (NTC) was used in place of cDNA for each primer set to ensure no contamination. Plates were sealed using adhesive plate sealers (Starlabs) and centrifuged at 400g for 1 minute. Plates were analysed using the Applied Biosystems 7900HT Sequence Detection System with a 50°C start followed by 94°C for 10 minutes, then 40 cycles of 94°C for 15 seconds and 60°C for 30 seconds. Data was exported from RQ manager as amplification data. All genes were normalised to the housekeeping GUSB after initial experiments, which were normalised to GAPDH (detailed in figures). ΔCt was generated for each sample through the subtraction of the gene of interest from the corresponding house keeping Ct value. The $2^{-\Delta Ct}$ value was then calculated and the relative quantitation further calculated in comparison to a control, using the $2^{-\Delta\Delta Ct}$ formula.

2.6.4 Taqman Low Density Array

TLDAs were custom designed (Life Technologies) initially with 32 genes (Table 2.4) and then 2 plates of 16 genes (Table 2.5). Plates were at room temperature for 30 minutes prior to starting the assay. cDNA samples (300ng) were diluted to 50μl and 50μl Taqman Universal Mastermix II No UNG (Life Technologies) was added. The sample was then added to the designated port on the plate, centrifuged twice (1200rpm, 1 minute) using the Sorvall/Hereaus centrifuge with the specified TLDA buckets. Plates were sealed using a low density array sealer.

Table 2.4: Macrophage TLDA card

APOL1	APOL2	APOL3	APOL6	CCL13	CCR7	CD36	CLEC4E
CSF1	CSF1R	CSF2	CSF2RA	CXCL11	CD23	CD64	FGL2
FN1	IRF4	IRF5	IRF7	LILRA2	LILRB3	MRC1	MSR1
PPARG	TLR1	TLR2	TLR4	TLR6	UBC	GUSB	GAPDH

Table 2.5: Monocyte TLDA cards

CCL2	CCL22	CD36	GAPDH	MRC1	CREB1	IL8	CXCL10
CCL17	CD1C	IL6	IL12B	CSF2	CCL3	CCL4	UBC

CLEC4E	CSR2RA	TLR4	GAPDH	IRF5	TNF	CD1A	CD200R1
CCL20	CD209	CHI3L1	STAB1	CXCL2	CD163	CCR2	UBC

2.6.4.1 TLDA Analysis

Plates were analysed using the Applied Biosystems 7900HT Sequence Detection System using SDS2.4 and RQ manager before exporting the results data and using Applied Biosystems Data Assist to generate the ΔCt and RQ for each sample.

2.7 Cytokine and Chemokine Analysis

2.7.1 Sample Preparation

Cell culture supernatants were centrifuged (500g, 5 minutes) to pellet the cell debris. The supernatants were removed and diluted in assay buffer accordingly. Plasma samples were centrifuged at full speed for 10 minutes to eliminate histopaque contamination.

2.7.2 ELISA

ELISAs were performed as per the manufacturer's instructions, which had slight variations depending on supplier (Table 2.6). In brief, half volume, high-binding plates (Fischer Scientific) were coated with capture antibody in PBS or recommended coating buffer overnight at 4°C. Plates were washed 4-5 times in PBS + 0.5% Tween (PBST) and blocked in assay buffer for 1 hour to prevent non-specific antibody binding. The block was aspirated and the samples or standards added either with or without the detection antibody for 2 hours (RT with shaking). If the detection antibody was not added with the samples, this was added after the samples were aspirated and washed a further 4-5 times in PBST, for 1 or 2 hours (RT with shaking). After the detection antibody incubation, the plates were washed 4 times in PBST. The streptavidin-HRP was diluted in assay buffer and added to plates for 30 minutes (RT with shaking). A further 4 washes were performed after this step and Tetramethylbenzidine (TMB) substrate

solution (eBioscience) added until the standard curve had developed, which ranged between 5 and 30 minutes depending on the analyte. Once developed, 4N sulphuric acid was used to stop the reaction, and plates read at 450nm using Tecan Sunrise Absorbance reader. Data was exported to Excel and analysed ensuring the points of the standard curve were on the logarithmic phase, straight line of the curve. The equation for the straight line was used to calculate the unknown values. Any points outside the parameters of the standard curve were not included and re-run with further dilutions or if the sample was run neat, these samples were labelled as not detected.

Table 2.6: ELISA kits

Cytokine/Chemokine	Species	Supplier	Detection Range
IL-6	Human	Life Technologies	15.62-1000pg/ml
IL-8	Human	Life Technologies	15.62-1000pg/ml
TNFα	Human	Life Technologies	15.62-1000pg/ml
CCL17	Human	Biolegend	3.9-250pg/ml
CCL17	Human	R&D Systems	7.81-500pg/ml
CCL22	Human	R&D Systems	7.81-500pg/ml
GM-CSF	Human	R&D Systems	15.62-1000pg/ml
CCL17	Mouse	R&D Systems	31.2-2000pg/ml

2.7.3 Luminex

A 10-plex Human chemokine panel (Life Technologies) and a base kit (Life technologies) were used to quantify chemokine levels as per the manufacturer's instructions. In brief, standards were prepared using assay diluent. Beads were prepared by vortexing and sonicating. 95μl of the bead solution was added to each well and washed. 50μl incubation buffer, followed by 100μl standards or blanks were added to the plate. 50μl assay diluent was added to the remaining wells, followed by the addition of 50μl of samples leading to a 1 in 2 dilution of samples. Plates were incubated at room temperature for 2 hours in the dark with shaking. Plates were then washed twice, followed by the addition of 95μl biotinylated antibody solution. They were incubated in the dark at room temperature for 60 minutes. The plates were washed twice after the incubation and 95μl Streptavidin-RPE solution was added to the plates, they were covered and incubated at room temperature with shaking for 30 minutes. The plates were then washed 3 times and wrapped in foil and stored at 4°C overnight in buffer. The following day, the wash buffer was replaced in the wells, and the

plates were incubated at room temperature with shaking for 10 minutes prior to running on the Bio-Rad Bio-Plex machine. The Bio-Plex Manager 4.1 programme was used, and machine instructions were followed. The data was exported as a Microsoft Excel file and analysed using Microsoft Excel and Graphpad Prism 6.0c.

2.7.4 Meso-Scale Discovery (MSD) Multi-Array

The chemokine 10 plex and cytokine 9 plex both using the 10 spot plate format were used and manufacturer's instructions were followed. In brief, supernatants were diluted 1 in 2 for the cytokine plates and 1 in 4 for the chemokine plates. The cytokine plates were then blocked in blocking solution for 1 hour at room temperature with shaking, followed by 3 washes in PBS + 0.05% Tween. Standards and samples were diluted and added for 2 hours at room temperature with shaking. The detection antibody solution was added on top of the samples and standards for 1 to 2 hours at room temperature with shaking. After 3 more washes in PBS + 0.05% Tween, read buffer was added to each well and the plates read using an MSDSECTOR Imager. Data was analysed using Microsoft Excel and Graphpad Prism 6.0c.

2.8 Immunohistochemistry (IHC)

2.8.1 Antigen Retrieval

Paraffin Embedded synovial membranes which had been previously sectioned were heated at 60°C for 35 minutes to soften the wax. The slides were then deparaffinised and hydrated through a xylene and graded alcohol series. Briefly, slides were added to xylene for 2 x 5 minutes, 100% ethanol, 90% ethanol and 70% ethanol each twice for 3 minutes. The final hydration step was putting the slides in running water for 5 minutes. Following the hydration, slides were washed in Tris Buffered Saline with 0.05% Tween (TBST) for 5 minutes before antigen retrieval in citrate buffer. The citrate buffer (Appendix) was preheated for 5 minutes in a microwave before the addition of the slides for a further 8 minutes. After cooling, the slides were washed in TBST before the endogenous peroxidase activity was quenched by incubating the sections in 0.5% H_2O_2 in methanol for 30 minutes. The sections were then washed for 5 minutes in TBST and sections ringed with a wax pen (Vector Laboratories).

2.8.2 Primary Antibody and ImmPress™ Secondary

After the initial preparation of the sections, the slides were blocked for non-specific binding by incubating sections with 2.5% normal horse blocking serum for 30 minutes in a humidified chamber. The serum was tapped off and slides were incubated overnight at 4°C with primary antibody (Table 2.7) in 2.5% horse serum/2.5% human serum in TBST. The slides were brought to room temperature and washed in TBST for 5 minutes. They were then incubated in ImmPRESS reagent specific to the host species of the primary antibody for 30 minutes. After a 5 minute wash in TBST, sections were incubated in Impact DAB chromagen solution until the stain intensity develops for a maximum 2 minutes.

Table 2.7: IHC Primary Antibodies

	Raised in	Supplier	Clone	Isotype	Supplier	Concentration
GM-CSFRα	Rabbit	Genetex	Polyclonal	DA1E	New England Biolabs	10μg/ml
CCR4	Rabbit	Novus Biotechne	Polyclonal	DA1E		2μg/ml
CCL17	Rabbit	Abcam	Polyclonal	DA1E		2.6μg/ml
CD3	Mouse	Vector	PS1	IgG2a	Dako	1μg/ml
CD68	Mouse	Dako	PGM1	IgG1b	Dako	1μg/ml

2.8.3 IHC scoring

Rheumatoid arthritis and Osteoarthritis sections were scanned using an Aperio ScanScope XT, followed by analysis using Aperio ImageScope software.

2.8.3.1 Percentage of positive staining

The sections were analysed as initmal lining, sub-lining, interstitium and aggregates. For each compartment, 3 areas for each section were scored and averaged. The scoring was analysed as percentage of positive cells, with <25% = 1, 26-50% = 2, 51-75% = 3 and >76% = 4. The areas scored were matched for each tissue for CCL17, CCR4, GM-CSFRα, CD3 and CD68.

2.8.3.2 Positive cell count

The lining layer was scored at a 40x magnification, counting all positively stained cells in the field of view. Five areas were scored for each tissue and each antibody (CCL17, CCR4, GM-CSFRα, CD3 and CD68). This was repeated in RA and OA sections, and the number of positive cells from the 5 counted areas was averaged for analysis.

2.9 Flow Cytometry (FACS)

2.9.1 Assessment of cell purity - Human

CD14+ cells were analysed for purity after separation from buffy coats. 1 x 10^6 cells were transferred to 6ml tubes, the volume brought to 1ml with FACS buffer (Appendix) and centrifuged at 300g for 5 minutes. The supernatant was removed and antibodies were added for CD16-PE, CD3-APC, CD14-APC-Cy7, CD19-FITC (Table 2.8) for 20 minutes at 4°C in the dark. After this incubation, the cells were washed once more with 500μl FACS buffer and then resuspended in 300μl FACS buffer. Samples were analysed on the MACS QUANT (Miltenyi). All FACS data were analysed using FlowJo 10 software (TreeStar).

2.9.2 Assessment of cell purity – Mouse

Enriched monocytes were analysed for purity by using 5 x 10^5 cells in 6ml tubes (BD, Falcon). These cells were washed in 1ml PBS at 300g for 5 minutes followed by a wash in FACS buffer (300g, 5 minutes). The supernatant was removed and antibodies were added for CD11b-V450, Ly6C-PerCP-Cy5.5 and GR1-APC (Table 2.8) for 20 minutes at 4°C in the dark. Following this, excess antibody was washed off in FACS buffer (300g, 5 minutes) and cells were resuspended in 250μl FACS buffer before analysis using the MACS QUANT (Miltenyi Biotech)

2.9.3 Apoptosis analysis

Monocytes were analysed for apoptosis by transferring the cultured cells into eppendorfs, centrifuging at 400g for 5 minutes and then washed with 1ml PBS. The cells were resuspended in 1ml Annexin V binding buffer (BD) and centrifuged once again. The cells were then resuspended in 100μl Annexin V binding buffer and 5μl Annexin V-FITC (BD) added per sample for 15 minutes at room temperature in the dark. There was a further wash in 1ml Annexin V Binding Buffer and resuspended in 200μl and 5μl PI (BD) added before analysis on the MACS QUANT (Miltenyi Biotech).

2.9.4 Phospho FACS

Separated monocytes (2.2.4) were incubated with 5% synovial fluid or 10ng/ml LPS in complete RPMI for 4 hours in capped 6ml tubes (BD, Falcon) 37°C, 5% CO_2. After incubation, cells were dissociated with cold dPBS (300g, 5 minutes) followed by a wash in warm (37°C) 0.1% BSA in dPBS (300g, 5 minutes). Washed cells were fluorescently labelled with surface markers CD14–PE-CF594, CD16–PE, CD19–AF700, CD3–V450 (Table 2.8) in 50μl 0.1% BSA in dPBS for 5 minutes. For antibody concentrations, see Table 2.8. For unstimulated tubes, 0.1% BSA in dPBS was added to the tubes and for stimulated cells, 1ng/ml GM-CSF in 50μl 0.1% BSA in dPBS was added for 10 minutes at 37°C, 5% CO_2. This incubation time was set after a trial at 10,15 and 20 minutes with 10 minutes the optimum time. Cytofix was added directly to the cells to stop any further signalling, for 15 minutes at room temperature. Cells were washed in cell wash (BD) followed by a wash in 0.1% BSA in dPBS (300g, 5 minutes). Cells were then permeabilised with

400μl Perm III buffer (BD) for 30 minutes at 4°C. This permeabilisation buffer was extensively washed (3 times) initially with cell wash (BD), followed by 0.5% BSA in dPBS. Cells were resuspended in 50μl 0.5% BSA in dPBS and stained with STAT1-PerCP-Cy5.5, STAT3-AF488 or STAT6-AF488 and STAT5-AF647 (Table 2.8) for 30 minutes at 4°C. The excess antibody was washed off in 0.5% BSA in dPBS (300g, 5 minutes) and cells were resuspended in 200μl 0.5% BSA in dPBS. Cells were analysed using the LSR II analyser (BD).

Table 2.8 FACS antibodies

Specificity	Reactive Species	Clone	Fluorochrome	Supplier	µl/Test
Annexin V	Human		FITC	BD	5
CD3	Human		APC	BD	10
CD3	Human	UCHT1	V450		0.5
CD14	Human	MφP9	APC-Cy7	BD	5
CD14	Human		PE-CF594	BD	2
CD16	Human	3G8	PE	BD	1
CD19	Human		FITC	BD	10
CD19	Human		AF700	BD	2
PI			PI	BD	5
STAT5 (pY694)	Human	47	AF647	BD	2
STAT 3 (pY705)	Human	4/P-Stat3	AF488	BD	2
STAT1 (pY701)	Human	4a	PerCP-Cy5.5	BD	2
STAT6 (pY641)	Human	18/P-Stat6	AF488	BD	2
CD11b	Mouse	M1/70	V450	BD	5
Gr1	Mouse	RB6-8C5	APC	BD	5
Ly6C	Mouse	AL21	PerCP-Cy5.5	BD	5

2.10 Limulus Assay

Immune complex depleted synovial fluids and neat synovial fluids were analysed for endotoxin contamination and 10ng/ml LPS was used as a positive control. The endotoxin was analysed using the Limulus Amebocyte Lysate (LAL) Kinetic kit (Lonza). The manufacturer's instructions were followed, but briefly, the endotoxin standard was vortexed for 15 minutes before serial dilutions were made with the LAL reagent water, in endotoxin free glass tubes, for the standard curve. The top standard was a concentration of 5.0EU/ml and 4 following 10 fold dilutions. The standards, samples, and controls were added to the microplate for 10 minutes at 37°C. Immediately prior to addition to the plates, the Kinetic QCL reagent was reconstituted with LAL reagent water and added to the wells. The plate was then measured at a wavelength of 405nM as a kinetic using the WinKQCL software.

2.11 Statistical Analysis

All statistical analysis was performed using GraphPad Prism 6 software (GraphPad). Different statistical tests were used depending on the data being

analysed. For the analysis of 3 or more groups of data with only one variable, a One-way ANOVA or non-parametric equivalent was used. The Bonferroni's post test was used if the overall analysis detected significance. For the comparison of 3 or more groups of data but with 2 variables (i.e dose response of various stimulations) the two-way ANOVA was used. Figure legends detail the statistical test used for each of set of data. A p value less than 0.05 was considered significant.

Chapter 3 GM-CSF/CCL17 axis in RA

3.1 Introduction

GM-CSF was traditionally identified as a maturation factor for macrophages (1.3.1.2), however, this role was broadened once it was discovered at sites of inflammation. It is now appreciated that GM-CSF has pro-inflammatory properties, which are important in RA pathogenesis (133,236,246,247). The initial arthopathy associated observation came from studies that were using human recombinant GM-CSF as a post-chemotherapy treatment. Joint pain was observed after 1 day of GM-CSF treatment, which disappeared gradually after GM-CSF treatment was halted (159,233,248). Following this observation, manipulating GM-CSF in animal models of RA further supported the importance of this molecule in rheumatic disease pathogenesis. In brief, daily injections of GM-CSF into mice with Collagen Induced Arthritis (CIA) significantly increased disease severity (236). In accordance with this finding, GM-CSF knockout mice have significantly reduced disease severity when compared to WT mice, and interestingly heterozygous mice have an intermediate score (233). A therapeutic treatment regime, using a GM-CSF antibody (234), and more recently an anti GMCSFR alpha antibody (238), also significantly reduced CIA disease scores. This has now been translated into humans, with Mavrilimumab: a human monoclonal antibody to GM-CSFRα. Clinical trials have shown promising results, with an improvement in DAS28-CRP when compared to placebo (244,249). These data have shown that GM-CSF has a role in RA pathogenesis and inhibition attenuates signs and symptoms of disease.

In addition to a GM-CSF associated pathology in RA, there has also been significant work done to evaluate the role of TLRs in RA pathology. TLRs are expressed at high levels on both peripheral blood derived monocytes, and tissue resident macrophages from RA synovial membranes (241,250-252). PAMPs and DAMPs are ligands that can activate TLRs and these have therefore been implicated in synovial inflammation. Each TLR recognises specific ligands such as TLR4, which is a receptor that recognises, amongst others, the PAMP Lipopolysaccharide (LPS) and the DAMP Tenascin C (80,86,252). In disease, multiple TLRs can be activated, and it has been suggested that targeting more than one could be a potential for therapeutic treatment (80,252,253). TLRs

signal through the adaptor molecules MyD88 or Mal/TIRAP and TRAM/TRIF, but ultimately activate the transcription factor NFκB, through the phosphorylation and degradation of IκB from the NFκB-IκB complex (234,253-255). NFκB then transcribes genes for pro-inflammatory cytokines and chemokines such as TNFα, IL1 and CXCL8 (IL-8) (235,253,256,257). Consequently TLRs are thought to be important regulators of inflammatory cytokine and chemokine expression (250).

In RA, many chemokines have been studied, such as CCL2, CXCL8 and CXCL10 (section 1.5), however there is limited research into CCL17, despite its receptor, CCR4, being expressed in RA tissue (258). In RA, synovial tissue has significantly higher levels of CCL17 mRNA than OA or healthy donor tissue (259). CCL17, previously known as Thymus and Activation-Regulated Chemokine (TARC), was discovered in 1996 to be constitutively expressed in the thymus and to induce T cell migration (260). Thymic Stromal Lymphopoietin (TSLP) stimulated dendritic cells secrete CCL17 and co-stimulation of TSLP with TLR ligands leads to an increase in CCL17 expression (261,262). CCL17 binds to CCR4, with CCL22 the only other common ligand for this receptor (263). More recently, CCL17 via CCR4 has been shown to chemoattract Th17 and Treg cells (186,264). Studies into CCR4 and its role in RA show that in CIA, CCR4 null mice have significantly lower disease scores over time (186). This suggests that CCR4 ligands (CCL17 or CCL22) could play a role in RA pathogenesis.

Many *in vitro* studies have used GM-CSF to differentiate monocytes into macrophages or DCs. However, the early effect of GM-CSF on monocyte biology, in particular the production of chemokines and cytokines has not yet been fully investigated. When monocytes enter the inflamed joint, they enter a milieu of cytokines, including GM-CSF, DAMPs and PAMPs. The aim of this study was initially to understand what effect GM-CSF stimulation has on monocyte functional outcome: including cytokine / chemokine secretion, before they further differentiate. The additional co-stimulation of TLR ligands, mimicking the activation upon entry into the synovium was also investigated, with the hypothesis that further cytokines and chemokines would be secreted. This relationship between chemokines, the GM-CSFRα, monocytes/macrophages and T cells in the synovium were also examined to try and understand the immunopathology.

Chapter Aims:

- To investigate the effect of GM-CSF with and without TLR ligand co-stimulation on the cytokine and chemokine production by monocytes.

- To determine how CCL17 production by monocytes influences T cell activation.

- To evaluate the spatial relationship in the RA synovial membrane tissue of CCL17, its receptor CCR4, GM-CSFRα and monocytes/macrophages and T cells.

3.2 Results

3.2.1 Chemokine secretion in GM-CSF stimulated monocytes.

In vitro, GM-CSF is classically used to differentiate monocytes into macrophages and/or dendritic cells (265,266). However, little is known about the initial impact of GM-CSF on monocyte biology. In order to determine the influence of GM-CSF on monocytes before differentiation, monocytes were isolated from human buffy coats using a monocyte enrichment kit (StemCell Technologies)(Section 2.2.3), negatively selecting for $CD14^+$ monocytes without depleting $CD16^+$ monocytes. Cell purity was analysed by flow cytometry. Typically, the composition of PBMCs after histopaque separation was 52% $CD14^+$, $CD16^+$ or $CD14^+CD16^+$ monocytes, 34.9% were $CD3^+$ T cells and 4.58% were $CD19^+$ B cells (Figure 3.1A & B). After separation monocyte purity was 82 ±10% (mean ± standard deviation)(Figure 3.1C). Importantly, contaminating populations were also assessed, revealing that there was only minor contamination of purified monocytes was T and B cells (Figure 3.1D). The monocyte purity was a limitation of the separation, however as predominantly monocytes and macrophages are GM-CSFR positive, stimulation with GM-CSF ensured more specific targeting for chemokine analysis.

To determine the effect of GM-CSF stimulation on monocytes, monocytes were stimulated with 0.1ng/ml or 1ng/ml GM-CSF for 24 hours and supernatants were analysed for CCL17 secretion by MSD (Section 2.7.4). For a broad view of chemokine secretion in GM-CSF stimulated monocytes, other chemokines were simultaneously evaluated. With increasing concentrations of GM-CSF stimulation, monocytes upregulated their secretion of CCL17 (Figure 3.2). This was the most consistently up-regulated chemokine of those analysed with little variation between the buffy coat donors. With no GM-CSF stimulation, very little (<20pg/ml) CCL17 was secreted; however, with 0.1ng/ml GM-CSF stimulation, this increased by approximately 100 fold (mean ± SD). At 1ng/ml, there was significant up-regulation in CCL17 production in comparison to no GM-CSF stimulation (Figure 3.2). There was also a dose dependent increase in CCL22 secretion with GM-CSF stimulation, however, this was not a significant increase due to the variation observed between monocyte donors. CXCL8 production was also significantly increased after GM-CSF at 1ng/ml in comparison to no

stimulation. There was no difference in CCL3, CCL4, CCL11 and CXCL10 production after GM-CSF stimulation, however, there was a large level of variability between monocyte donors. This suggests these chemokines were not reliably regulated by GM-CSF in monocytes. However, CCL17 and CCL22, the ligands for CCR4, were both up-regulated by GM-CSF, highlighting the importance of both monocytes and these chemokines in RA pathogenesis, prior to monocyte differentiation. This is potentially an important process in exacerbation of inflammation, as infiltrating monocytes that are exposed to GM-CSF, can secrete CCL17 leading to the recruitment of $CCR4^{+}$ T cells (Section 3.1).

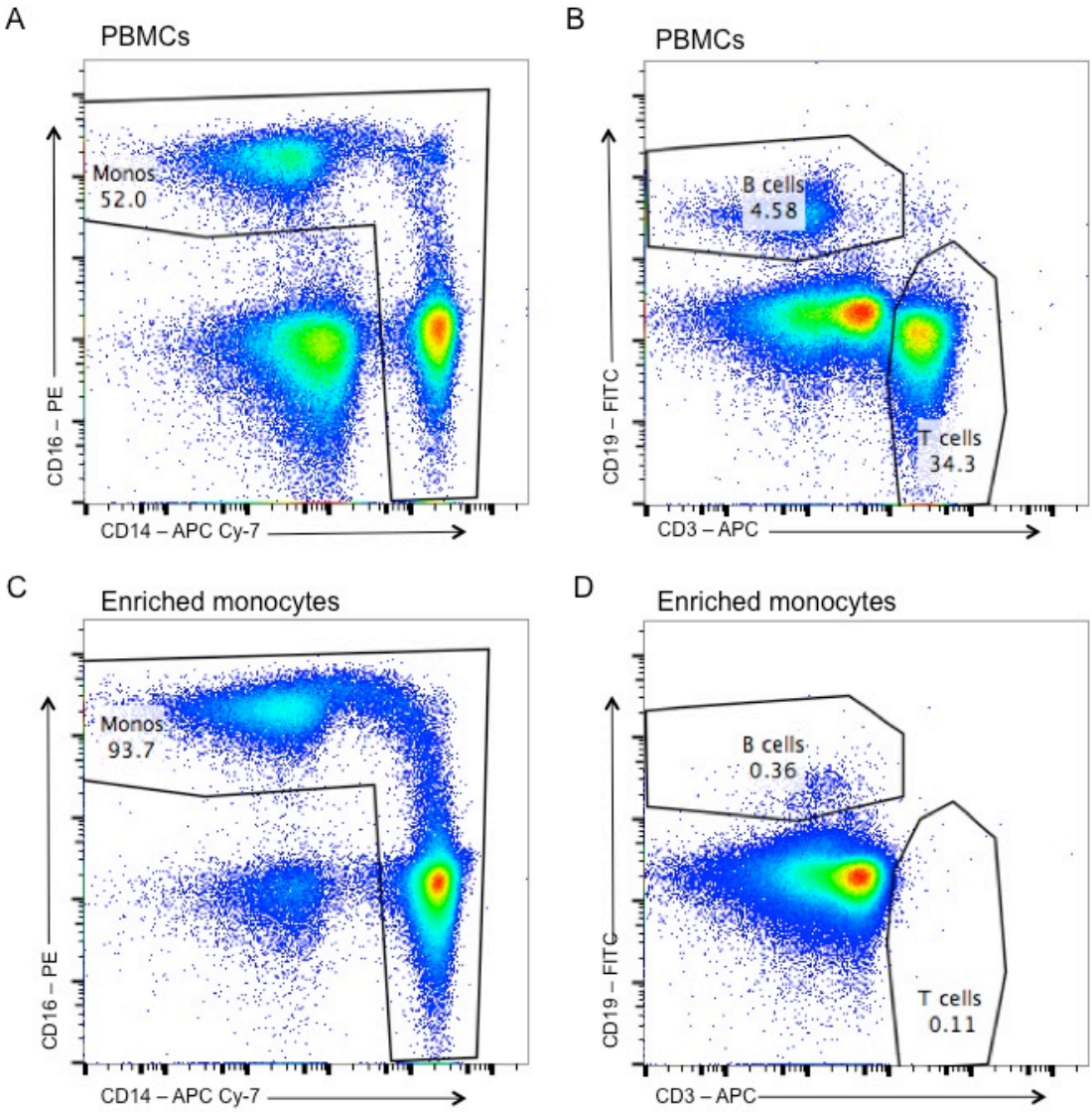

Figure 3.1 Human Monocyte Purity Check
An example of monocytes separated from human buffy coats analysed for purity by flow cytometry. A) Percentage of monocytes in total PBMCs. B) T cell and B cell percentage of PBMCs. C) Monocyte purity after enrichment from PBMCs. D) T cell and B cell contamination in monocyte separation.

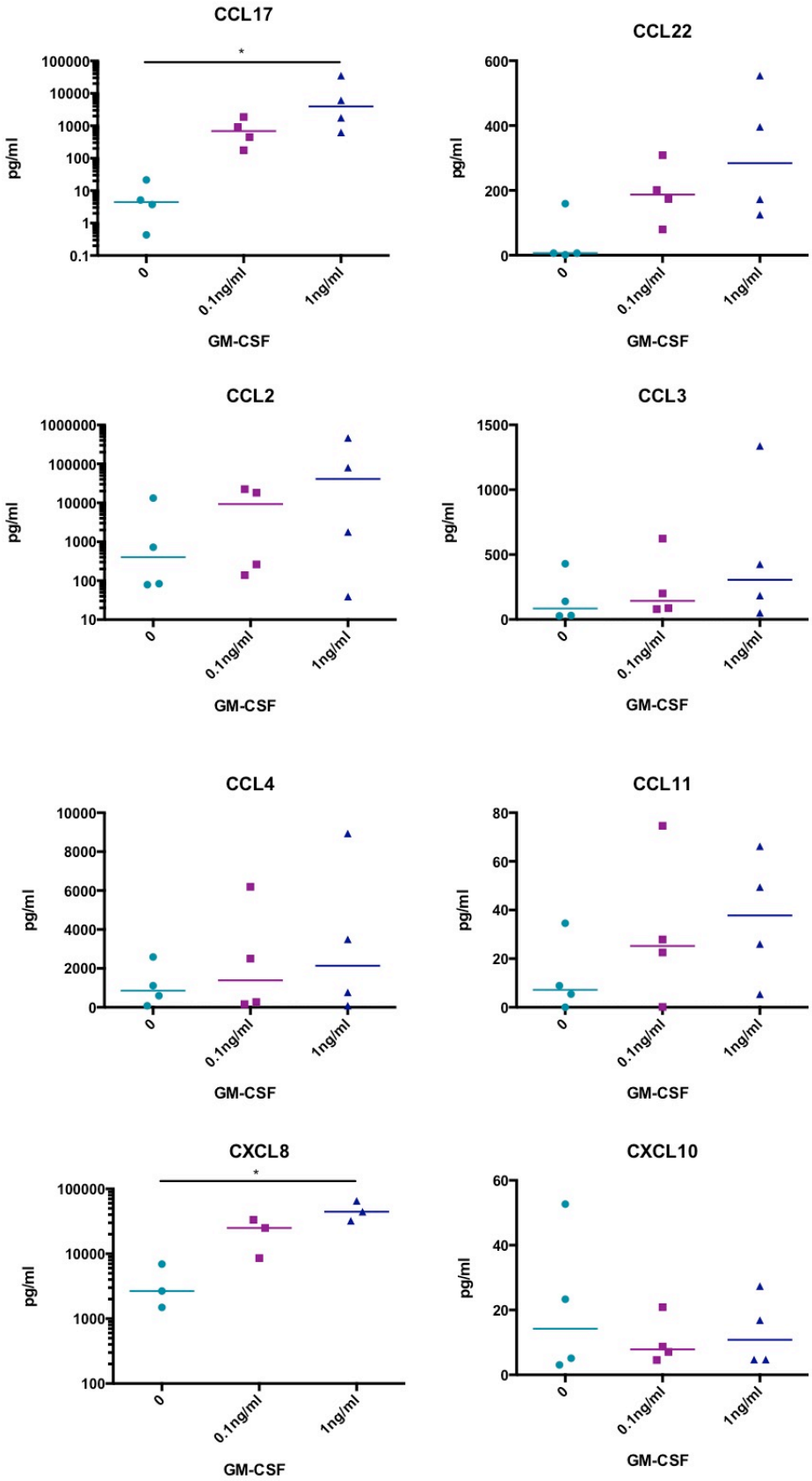

Figure 3.2 Chemokine Induction after GM-CSF stimulation of monocytes
Monocytes separated from human buffy coats (n=4) were stimulated in complete media or with the addition of human recombinant GM-CSF at 0.1ng/ml or 1ng/ml for 24 hours at 37°C. Supernatants were collected and analysed by MSD for chemokine secretion. Horizontal line indicating the median. Statistically analysed using Kruskal-Wallis non-parametric test, followed by Dunn's post test. * = $p<0.05$

3.2.2 CCL17 secretion in RA monocytes.

The data described above shows that CCL17 is produced by monocytes after GM-CSF stimulation. To elucidate whether monocytes from RA patients, have the same capacity, or even an enhanced capacity, to produce CCL17 when stimulated with GM-CSF, monocytes derived from the peripheral blood of RA patient (n=7) and healthy controls (n=7) were isolated, and compared. In brief, monocytes were stimulated with increasing concentrations of GM-CSF for 24 hours, after which, supernatants were collected and analysed for CCL17 by ELISA. In both RA and healthy monocytes, GM-CSF stimulation induced equivalent secretion of CCL17 (Figure 3.3A). A bell shaped response was observed, where there was an increase in secretion of CCL17 with increasing GM-CSF stimulation peaking between 1ng/ml and 10ng/ml GM-CSF, whilst at 100ng/ml GM-CSF stimulation, the induction of CCL17 decreased. This potentially suggests that at higher concentrations, monocytes are insensitive to GM-CSF or the monocyte phenotype changes with greater GM-CSF stimulation and monocytes respond differently. These results demonstrate that RA monocytes have the same capacity as healthy monocytes to secrete CCL17, which suggests that any dysregulation leading to RA pathogenesis is not via the monocyte response to GM-CSF.

The production of CCL17 is not only restricted to monocytes and previous work has demonstrated that T cells stimulated with IL-4 (267) or dendritic cells (DCs) stimulated with Thymic Stromal Lymphopoietin (TSLP) can also secrete CCL17 (268). As monocytes and DCs are both haematopoietic stem cell derived, and it is not known whether TSLP will have similar effects on monocytes. Monocytes were separated from human buffy coats and stimulated with increasing concentrations of TSLP or GM-CSF. Consistent with previous data, GM-CSF (in a dose-dependent manner) induced CCL17 (Figure 3.3B). TSLP was unable to induce CCL17 at any concentrations, however a positive control for TSLP stimulation would be required to confirm this finding. GM-CSF can be used in the differentiation of monocyte derived cells such as inflammatory DCs (73). Therefore, GM-CSF could up-regulate TSLPR in during the differentiation process.

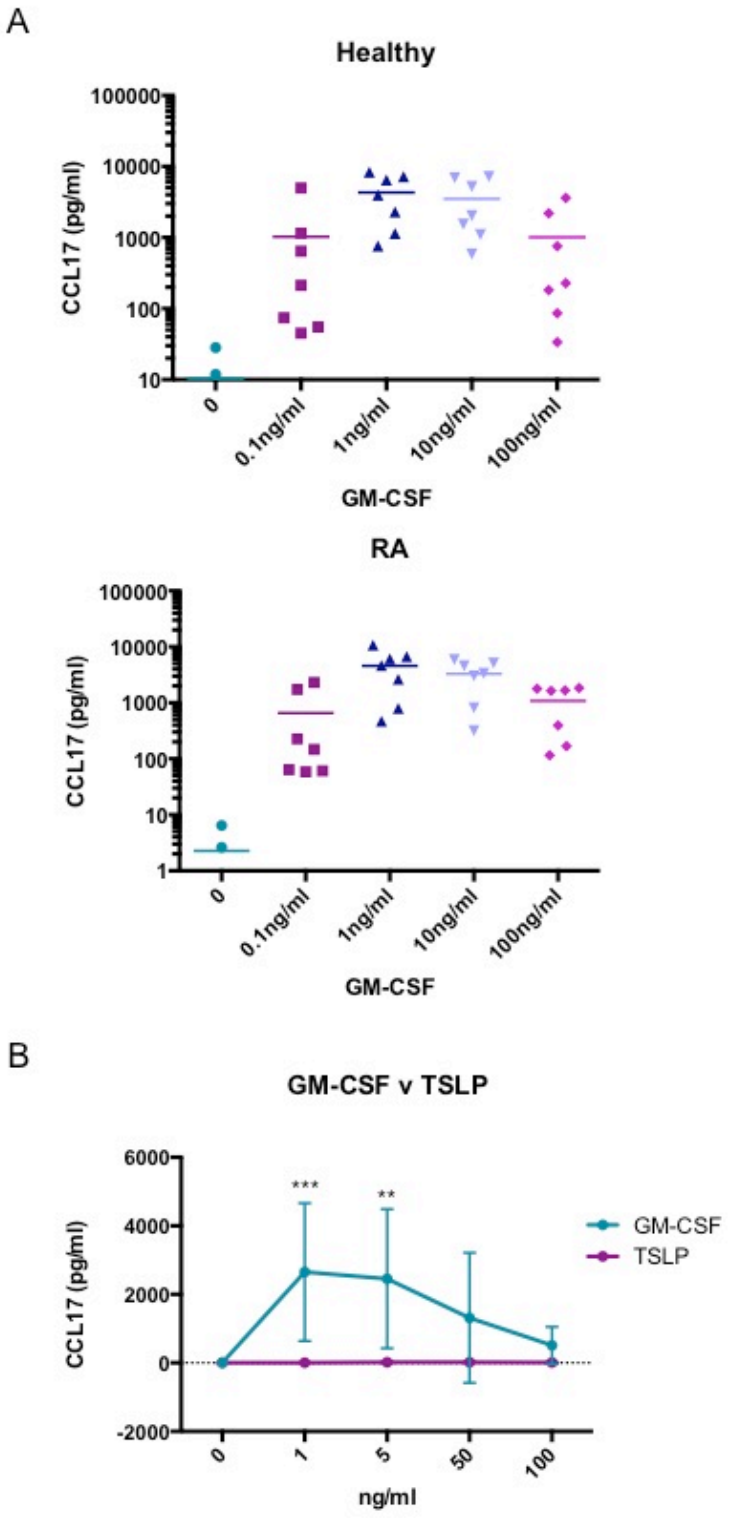

Figure 3.3 CCL17 induction in RA monocytes and TSLP inability to induce CCL17.
A) Monocytes were separated from human peripheral blood from RA patients (n=7) or healthy controls (n=7) and stimulated with recombinant human GM-CSF at increasing concentrations for 24 hours. Supernatants were collected and analysed by ELISA for CCL17. At 0ng/ml, 5 donors had undetectable levels of CCL17. Horizontal line indicates the mean. B) Monocytes were separated from human buffy coats (n=4) and stimulated with increasing concentrations of GM-CSF or TSLP for 24 hours. Supernatants were collected and analysed for CCL17 by ELISA. Statistics used: 2-way ANOVA performed on the dataset in B) followed by Bonferroni's post test ** = $p<0.01$, *** = $p<0.001$.

3.2.3 TLR ligands inhibited GM-CSF induction of CCL17 in monocytes.

The data described in sections 3.2.1 and 3.2.2 demonstrated that monocytes from RA patients and healthy controls produced CCL17 under GM-CSF stimulation. The synovial milieu contains PAMPs and DAMPs, many of which are TLR ligands. TLR ligands have previously been shown to up-regulate cytokine and chemokine responses such as CXCL8 and TNFα, in monocytes (269) Therefore, to determine the effect of TLR ligands on GM-CSF driven CCL17 induction in both healthy and RA patients, monocytes were co-stimulated with LPS. RA and healthy monocytes were co-stimulated with increasing concentrations of GM-CSF in the presence or absence of 10ng/ml LPS.

In both healthy and RA monocytes, LPS prevented the induction of GM-CSF driven CCL17. With GM-CSF stimulations of 1ng/ml and 10ng/ml, there was significant inhibition of CCL17 observed in both healthy and RA monocytes (Figure 3.4A). The TLR4-mediated inhibition of CCL17 was unexpected, as LPS is known to induce the secretion of other pro-inflammatory cytokines and chemokines in monocytes.

To determine whether the inhibition of CCL17 by LPS on GM-CSF stimulated monocytes was specific to TLR4 alone, multiple TLR ligands were used to assess specificity. The following TLR ligands were evaluated as they covered a range of receptors. In brief, we chose FSL1 (a TLR2/6 ligand), Clo97 (a TLR7/8 ligand) and Poly I:C (a TLR3 ligand). Consistent with the previous data (Section 3.2.1), GM-CSF was able to induce CCL17 and this was significantly inhibited by LPS. Importantly, this induction of CCL17 was significantly inhibited by all TLR ligands analysed (Figure 3.4B). This suggests that regardless of the TLR ligand used, the underlying TLR-mediated signalling cascade was activated, resulting in inhibition of GM-CSF-mediated CCL17 induction.

In order to confirm that the GM-CSF-mediated signalling led to an up-regulation of transcript that would subsequently be translated into protein, CCL17 transcriptional analysis was undertaken. Furthermore, the impact of TLR4-mediated signalling on transcript and protein levels was also evaluated. Monocytes stimulated with GM-CSF alone or GM-CSF with LPS were cultured for

0, 6, 18 and 24 hours with cells lysed at each time point for transcriptional analysis. The transcripts of CCL17, CCL22 and CXCL8 were analysed. GM-CSF stimulation caused an induction of CCL17 transcript between 6 and 18 hours, which continued until 24 hours. The monocytes co-stimulated with LPS were unable to induce CCL17 transcript, with a significantly lower level of CCL17 transcript at 18 and 24 hours (Figure 3.5). Suggesting that TLR4-mediated signalling events prevent transcription of CCL17 rather than post-translational inhibition.

CCL22 is the only other common ligand with CCL17 for CCR4 (263). The CCL22 protein was not significantly up-regulated by GM-CSF stimulation (Figure 3.2), however, we aimed to determine whether TLR-mediated signalling had any effect on the CCL22 transcript. The transcript for CCL22 was not significantly induced by GM-CSF at 18 and 24 hours, unlike the other CCR4 ligand, CCL17 (Figure 3.5). The co-stimulation of GM-CSF with LPS also prevented CCL22 induction. This is a potentially interesting finding, alluding to a mechanism in which TLR-mediated signalling inhibits GM-CSF induction of CCR4 ligands.

CXCL8 was analysed as a positive control, as LPS would be expected to cause an increase in CXCL8 transcript expression (82,270). The data showed that GM-CSF alone induced low levels of CXCL8 transcript (Figure 3.5). However, at 6 hours after co-stimulation, LPS caused an increase in transcript expression. At 18 and 24 hours after LPS co-stimulation, the level of transcript had returned to the same level as GM-CSF alone.

The transcriptional analysis of CCL17 confirmed that up-regulation of the transcript directly led to the increase in protein (Figure 3.2). Inhibition of CCL17 protein was caused by TLR-mediated signalling that impacted CCL17 transcription rather than protein secretion (Figure 3.5). CCL22 transcript induction by GM-CSF, was also inhibited by LPS, suggesting a CCR4 ligand specific phenomenon, as CXCL8 transcript was up-regulated with LPS co-stimulation.

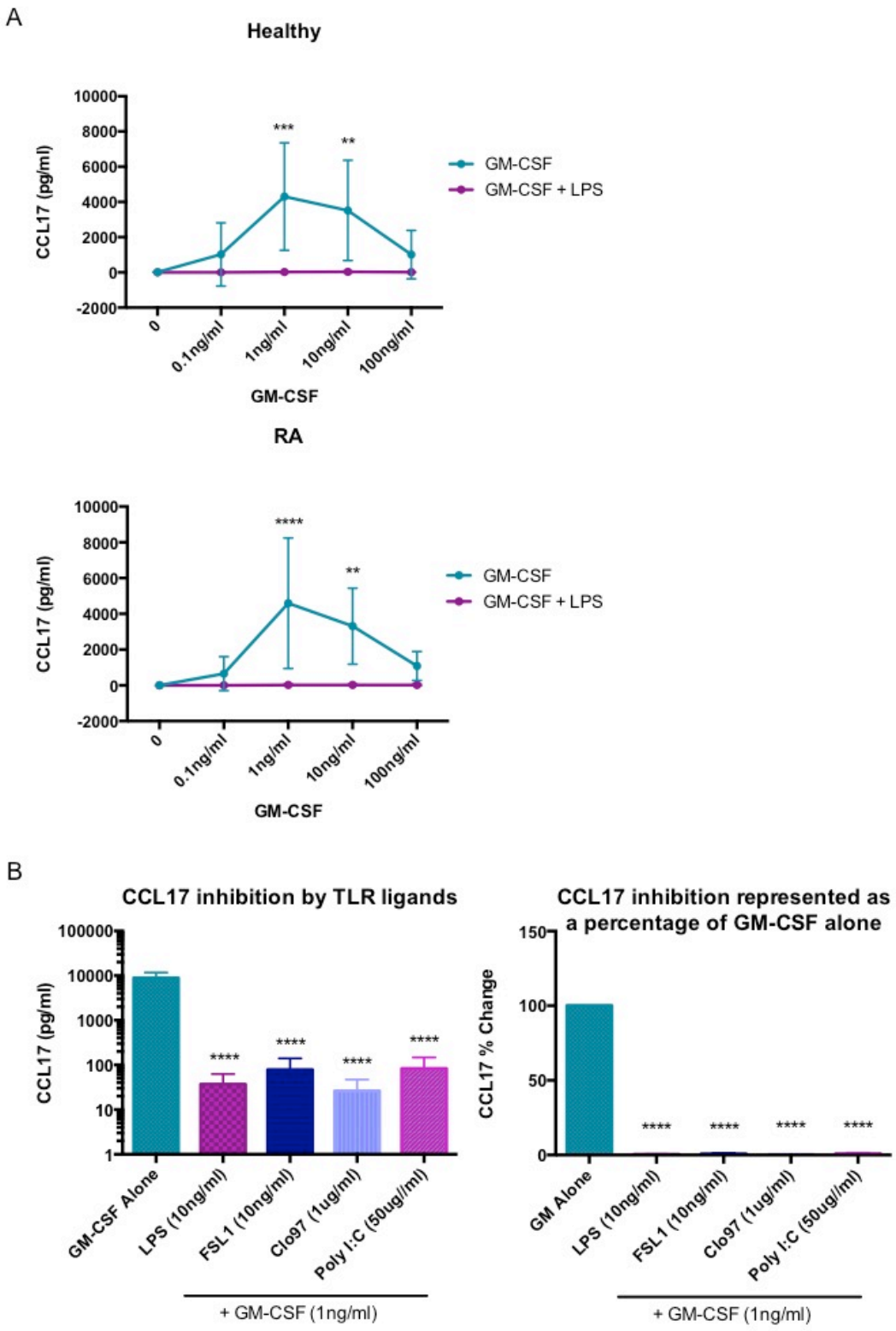

Figure 3.4 LPS or TLR ligand inhibition of CCL17 in GM-CSF stimulated monocytes
A) Monocytes were isolated from RA peripheral blood (n=7) or healthy peripheral blood (n=7) and were stimulated with increasing concentrations of GM-CSF or co-stimulated with GM-CSF and 10ng/ml LPS for 24 hours. Supernatants were collected and analysed for CCL17 by ELISA. Statistically analysed using 2-way ANOVA followed by Bonferroni's post test. ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$. B) Monocytes isolated from buffy coats (n=3) stimulated with 1ng/ml GM-CSF with or without 10ng/ml LPS, 10ng/ml FSL1, 1μg/ml Clo97 or 50μg/ml Poly I:C for 24 hours. Supernatants were collected and analysed for CCL17. Left panel: raw concentrations, right panel: % change relative to GM-CSF alone condition. Statistically analysed using one-way ANOVA followed by Bonferroni's post test **** = $p<0.0001$.

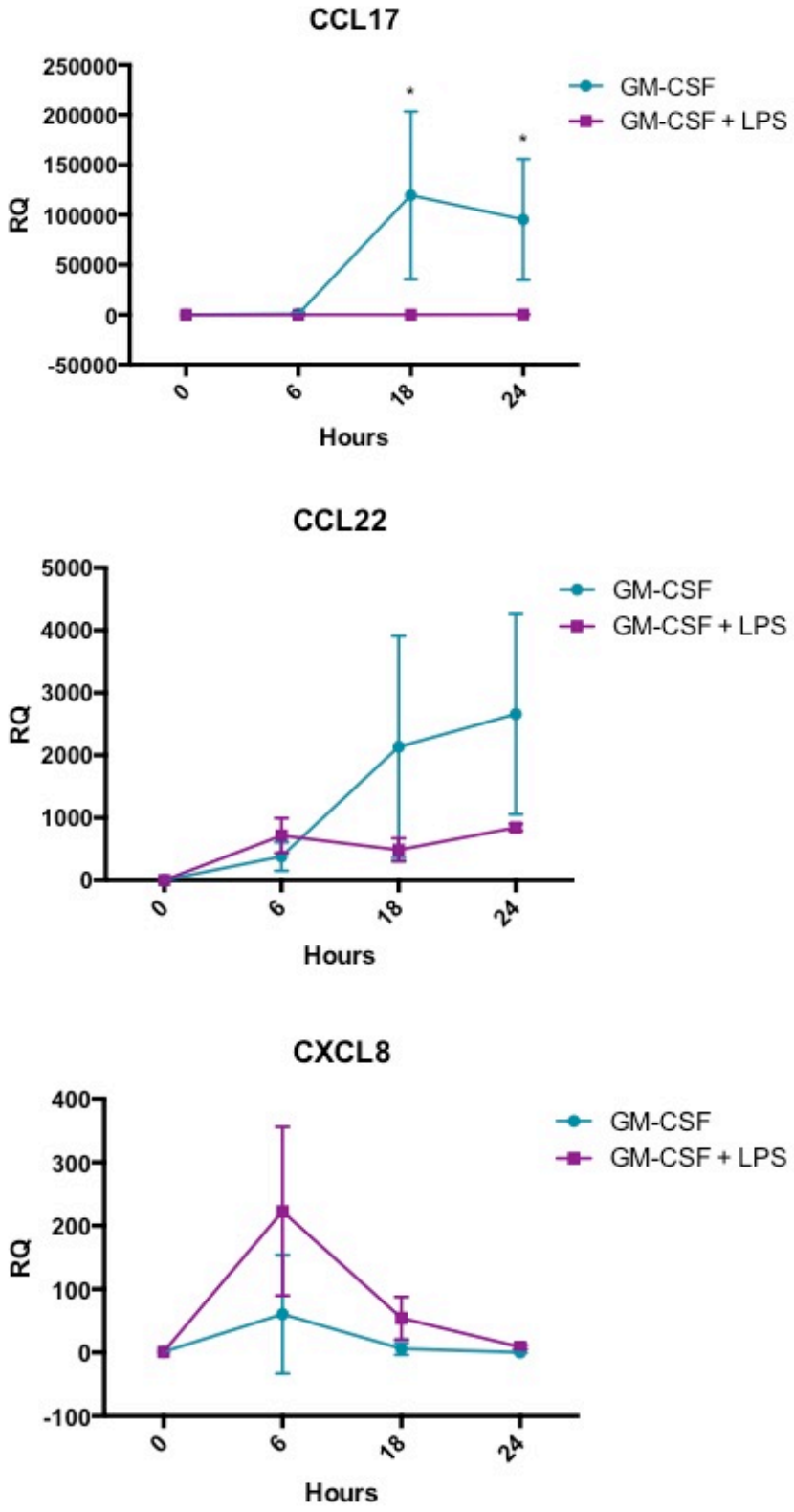

Figure 3.5 Transcriptional analysis of CCL17, CCL22 and CXCL8 after GM-CSF and LPS co-stimulation.
Monocytes isolated from human buffy coats (n=3) were stimulated with 1ng/ml GM-CSF in the presence or absence of 10ng/ml LPS. Cells were lysed at 0, 6, 18 and 24 hours after incubation. RNA was extracted and CCL17, CCL22 and CXCL8 transcripts analysed by qPCR. Results analysed in relation to housekeeping (GUSB) and Relative Quantification (RQ) calculated by $2^{-\Delta\Delta cT}$ relative to time 0. Statistically analysed using 2-way ANOVA followed by Bonferroni's post test. * = $p<0.05$.

3.2.4 Investigation into the mechanism of LPS inhibition of CCL17

The finding that LPS can significantly inhibit the induction of CCL17 in monocytes upon GM-CSF stimulation was an unexpected and novel finding, as LPS exacerbates secretion of other pro-inflammatory cytokines and chemokines such as TNFα and CXCL8 (270). In parallel to the work undertaken in this thesis on-going studies performed by collaborating scientists at MedImmune investigated the mechanism underlying LPS-mediated inhibition. From that data (*personal communication*), it was hypothesised that Suppressor of Cytokine Signalling 3 (SOCS3) was essential for inhibition by LPS. SOCS3 is downstream of GM-CSFR signalling, as a negative regulator of cytokine signalling (Figure 1.2)(221). Therefore, in order to establish whether SOCS3 was involved in the process of inhibition by LPS, SOCS3 was down-regulated using siRNA technology. Initial experiments were performed to maximise transfection efficiency and knock down of the gene. To maintain monocytes in a setting that was supportive of survival and which would not lead to overt maturation, cells were cultured in human serum (271). To determine whether monocytes would still respond to GM-CSF and secrete CCL17 after initial culture in human serum, monocytes were cultured for 24 and 48 hours before stimulation with GM-CSF in the presence or absence of LPS. This was in comparison to monocytes that were stimulated with GM-CSF and LPS at 0 hours (Figure 3.6). Monocytes that were stimulated with GM-CSF at 0 hours, produced approximately 1000pg/ml of CCL17, which was almost completely inhibited by LPS. After 24 hours of culture in 10% serum before stimulation with GM-CSF for 24 hours, monocytes secreted about 125pg/ml CCL17, which was inhibited by LPS. After 48 hours of culture in 10% serum, before stimulation with GM-CSF, the ability of monocytes to produce CCL17 was effectively inhibited, with levels in the region of 40pg/ml. Therefore, in order to allow sufficient time for siRNA knockdown, prior to initiation of the assay, but still with the ability for CCL17 to be induced by GM-CSF, the 24 hour culture in human serum was used for further experiments.

The 24 hour period of culture in human serum allowed time to transfect the cells with siRNA prior to initiation of the assay. In order to establish the optimum conditions for siRNA transfection and knockdown, the initial transfection was for 24 hours to maximise time for the knockdown. Transfection efficiency was determined by transfecting the cells with AF-488 labelled control siRNA (Figure

3.7A). FACS analysis showed that 77% of the cells were still viable after 24 hours, and that of the live cells, 51.8% took up the labelled siRNA. Multiple concentrations of SOCS3 siRNA were tested in comparison to the control siRNA for knockdown of SOCS3 after 24 hours of transfection. 6.25nM was variable between the 2 monocyte donors, whereas 12.5nM, 25nM and 50nM were all successfully able to decrease the SOCS3 transcript (Figure 3.7B). However, despite successful knockdown of SOCS3, monocytes stimulated with GM-CSF, in both the control and SOCS3 siRNA, were unable to produce CCL17 (Figure 3.7C). The level of CCL17 secreted in the negative siRNA control was variable between donors but was still induced. The initial data suggests that transfection alone affects the monocyte's ability to secrete CCL17 upon GM-CSF stimulation. In order to maximise monocyte viability, the transfection time was decreased to ensure a recovery period. The aim was to ensure delivery of siRNA into the cell, but also to remove the transfection reagent, which may have been having an inhibitory effect on the cell. In an exploratory experiment (n=1), a 6 hour transfection with 50nM siRNA, SOCS3 siRNA successfully knocked down the SOCS3 transcript (Figure 3.7D). Monocytes recovered for 18 hours, prior to stimulation with GM-CSF in the presence or absence of LPS. The negative control, which was not exposed to siRNA or transfection reagent, produced 130pg/ml CCL17 with stimulation (Figure 3.7E). However, exposure to the control siRNA and SOCS3 siRNA prevented induction of CCL17.

In summary, transfection of monocytes (regardless of target) results in an inability to respond to GM-CSF stimulation and therefore secretion of CCL17. The current data suggests that this is due to interference from the transfection reagent that prevented GM-CSF signalling.

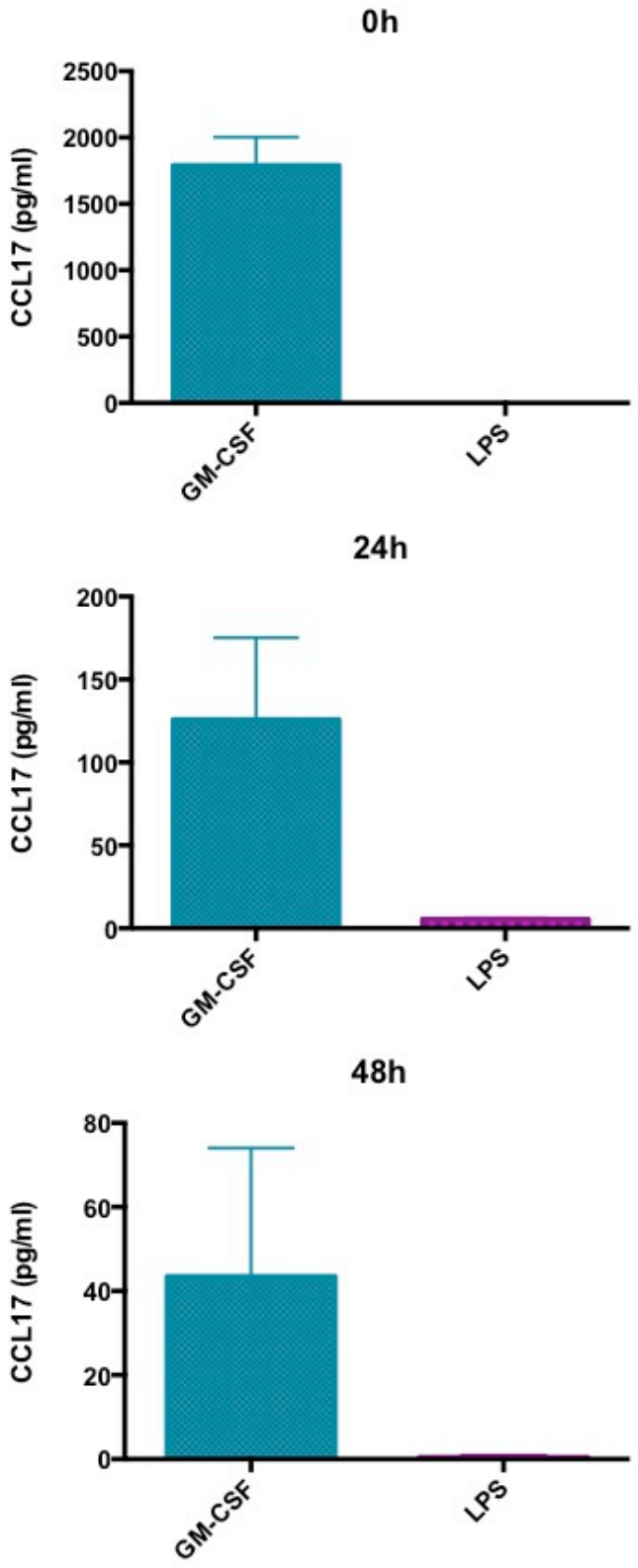

Figure 3.6 Delayed assay start with monocytes cultured in human serum.
Monocytes were separated from human buffy coats (n=3) and cultured in 10% human serum opposed to FBS. Monocytes were either stimulated at 0h with GM-CSF with or without 10ng/ml LPS, or cultured in human serum for 24 or 48 hours before the initiation of the assay stimulation of GM-CSF with or without 10ng/ml LPS. For the monocytes cultured before the initiation of the assay, the media was changed at the same time as the stimulations. After the addition of GM-CSF with or without LPS, monocytes were cultured for 24 hours, supernatants were collected and analysed for CCL17 by ELISA.

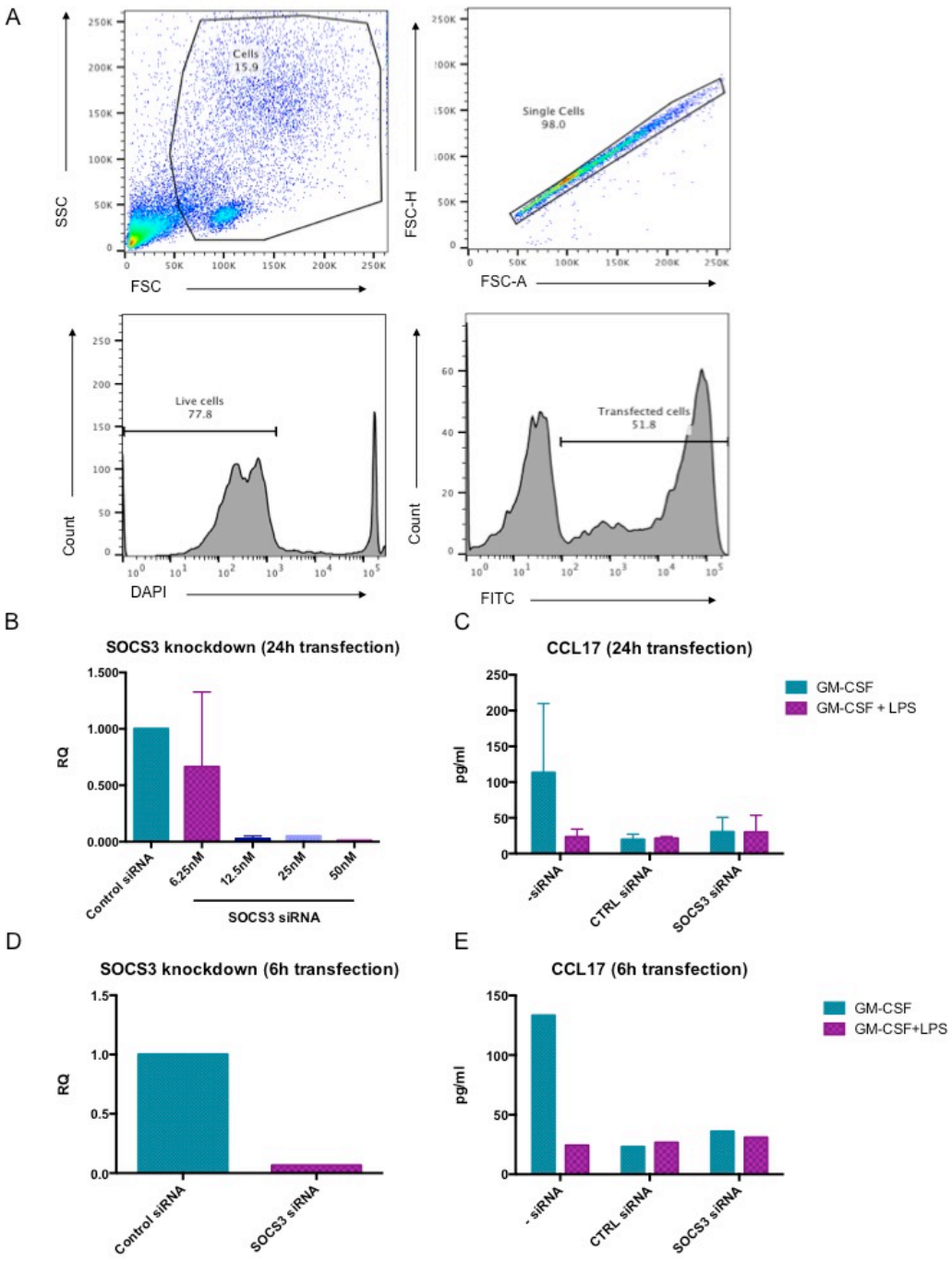

Figure 3.7 siRNA knockdown of SOCS3 in monocytes.
Monocytes were cultured in media containing 10% human serum and transfected with the siRNA-NTER peptide complex, which had been pre-incubated together for 15 minutes, for 24 hours (A, B and C) or 6 hours (D and E). A) Using labelled control AF488-siRNA, transfection efficiency was analysed by FACS. Cells were also stained with DAPI prior to analysis on a MACS QUANT to assess cell death. B) Control siRNA, and 4 concentrations of SOCS3 siRNA were used to determine the most effective concentration for use in further experiments (n=2 for 6.25nM and 12.5nM, n=1 for 25nM and 50nM). C) 50nM siRNA (control or SOCS3) was used to transfect monocytes, after 24 hours of transfection, media was changed and monocytes were stimulated with GM-CSF with or without LPS. D) and E) 50nM siRNA (control or SOCS3) was used to transfect monocytes for 6 hours (n=1). Media was changed, and at 24 hours, monocytes were stimulated with GM-CSF with or without LPS for 24 hours. After culture, cells were lysed for transcriptional analysis of SOCS3 knockdown by qPCR (D) and supernatants were collected for analysis by ELISA for CCL17 (E).

3.2.5 Activated T cells produced GM-CSF, but CCL17 does not exacerbate GM-CSF secretion.

The ability of monocytes to produce CCL17 suggests that they may have a role in recruiting $CCR4^+$ T cells to the inflamed tissue. We hypothesised that monocytes producing CCL17 upon GM-CSF stimulation, would not only be able to potentially recruit $CCR4^+$ T cells to the particular area/tissue, but also induce secretion of GM-CSF in a positive feedback loop. In order to assess this hypothesis, $CD4^+$ T cells were divided into memory and naïve T cells via the presence of CD45RA and CD45RO. Naïve T cells would not be expected to express CCR4 as expression increases upon differentiation into memory T cells (272). The T cells were then cultured with or without anti-CD2/CD3/CD28 beads in the presence or absence of increasing concentrations of CCL17.

After 24 hours, total CD4 T cells produced approximately 500pg/ml GM-CSF when activated with anti-CD2/CD3/CD28 beads (Figure 3.8A). Anti-CD2/CD3/CD28 bead stimulation of total CD4 T cells was essential for the production of GM-CSF. Importantly, CCL17 had no impact (be it negative or positive) on GM-CSF secretion in total CD4 T cells. The $CD45RA^+$ T cells produced about 300pg/ml GM-CSF and the $CD45RO^+$ T cells produced in the region of 400pg/ml GM-CSF.

After 48 hours, all analysed subsets of T cells failed to induce GM-CSF without stimulation from anti-CD2/CD3/CD28 beads (Figure 3.8B). However, the activated CD4 T cells produced approximately 1000pg/ml GM-CSF, with the $CD45RA^+$ T cells secreting about 600pg/ml GM-CSF and the $CD45RO^+$ T cells secreting about 1000pg/ml. The increasing concentrations of CCL17 made no difference to the level of GM-CSF produced. This data suggested that CCL17 had no impact on any of the T cell subsets, however if there was the opportunity, future work could include analysis of CCR4 expression in relation to the ability of T cells to produce GM-CSF.

Although we do not show that CCL17 is involved in a positive feedback loop, these data demonstrate that activated $CD4^+$ T cells secrete GM-CSF. This suggests that recruitment of $CCR4^+$ T cells into the tissue would lead to exacerbation of of GM-CSF secretion when activated, through contact with

antigen-presenting cells or potentially inflammatory cytokines, thereby stimulating monocytes to further secrete CCL17.

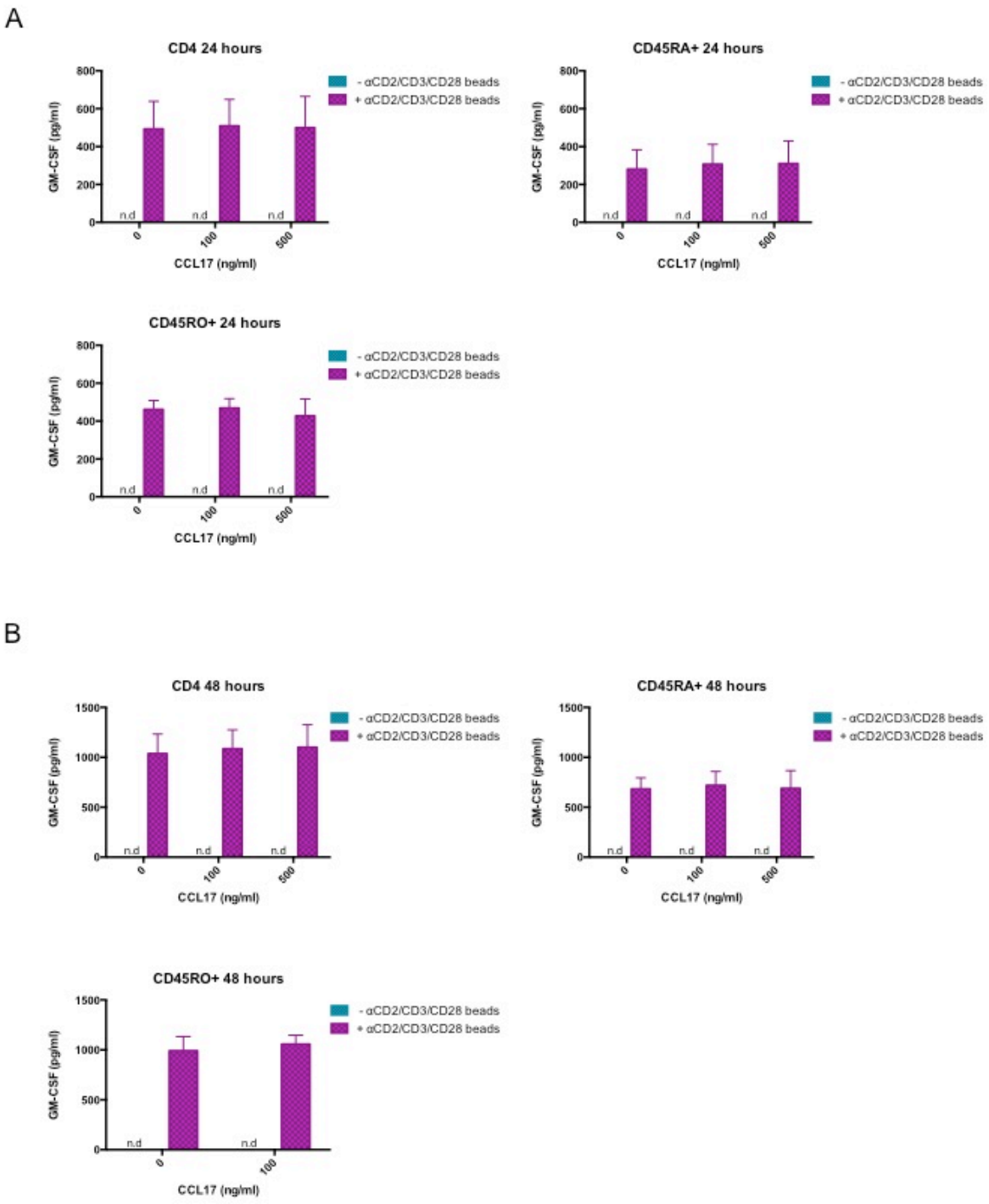

Figure 3.8 GM-CSF production in T cells after CCL17 stimulation
CD4 T cells were isolated from buffy coats (n=3) and further separated into $CD45RA^+$ T cells and $CD45RO^+$ T cells. $CD4^+$, $CD45RA^+$ and $CD45RO^+$ T cells were cultured in the presence or absence of anti-CD2/CD3/CD28 beads and with 0, 100 or 500ng/ml CCL17 for 24 hours (A) or 48 hours (B). Supernatants were collected after culture and analysed for GM-CSF by ELISA. n.d = not detectable.

3.2.6 Immunohistochemistry analysis of RA synovial membranes highlighted the spatial distribution of CCL17, CCR4, GM-CSFRα with CD3 and CD68.

The relationship between GM-CSF stimulated monocytes, CCL17 and CCR4 T cells has been analysed in an *in vitro* system (Section 3.2.5). In order to understand the relationship between these cells in the RA tissue, synovial membranes were analysed by immunohistochemistry. CCL17 was examined to determine whether there was a relationship between the locality of CCL17 and CCR4 positive cells or GM-CSFRα positive cells. CD68 and CD3 were analysed to assess the spatial relationship between CCL17, CCR4, GM-CSFRα with CD68 and CD3.

Synovial membranes from RA patients (n=8) were sectioned consecutively. They were then stained with primary antibodies towards GM-CSFRα, CCR4, CCL17, CD3 and CD68. The sections were scanned using an Aperio ScanScope XT microscope and the intimal lining layer, sub-lining layer, interstitium and aggregates or follicles were analysed and scored for each specific stain in the individual compartments (Figure 3.9). The staining was imaged (Figure 3.10) and scored (Figure 3.11) for each compartment of the RA synovial membrane. CCL17 staining was variable between the RA synovial membranes, however it was relatively highly expressed across all compartments of the section. CCR4 staining was similarly expressed across all compartments of the synovial membrane, but to a lesser degree. GM-CSFRα was highly expressed in the intimal lining layer, with a high percentage of cells expressing GM-CSFRα in the sub-lining and interstitium. The aggregates had significantly lower levels of expression than the intimal lining and the interstitium. Similarly, CD68 was highly expressed in the intimal lining and interstitium, with expression in the sub-lining and aggregates significantly lower. The lowest CD68 expression was in the aggregates with a significantly lower percentage of positive cells than in all other areas of the synovial membrane. Interestingly CD3 was not as highly expressed as CCR4, as there was minimal detection in the intimal lining, and high levels in the aggregates, implying in this context that CCR4 is expressed on cells other than $CD3^+$ cells. The GM-CSFRα staining profile was similar to CD68 expression in the intimal lining suggesting that they could be co-expressed.

Based on the observed staining, it is justifiable to surmise that there are potentially interactions between GM-CSFRα positive cells, CCL17 and CCR4 positive cells as they are similarly located within the tissue. This proposes that the *in vitro* finding observed could be relevant within the RA synovial membrane.

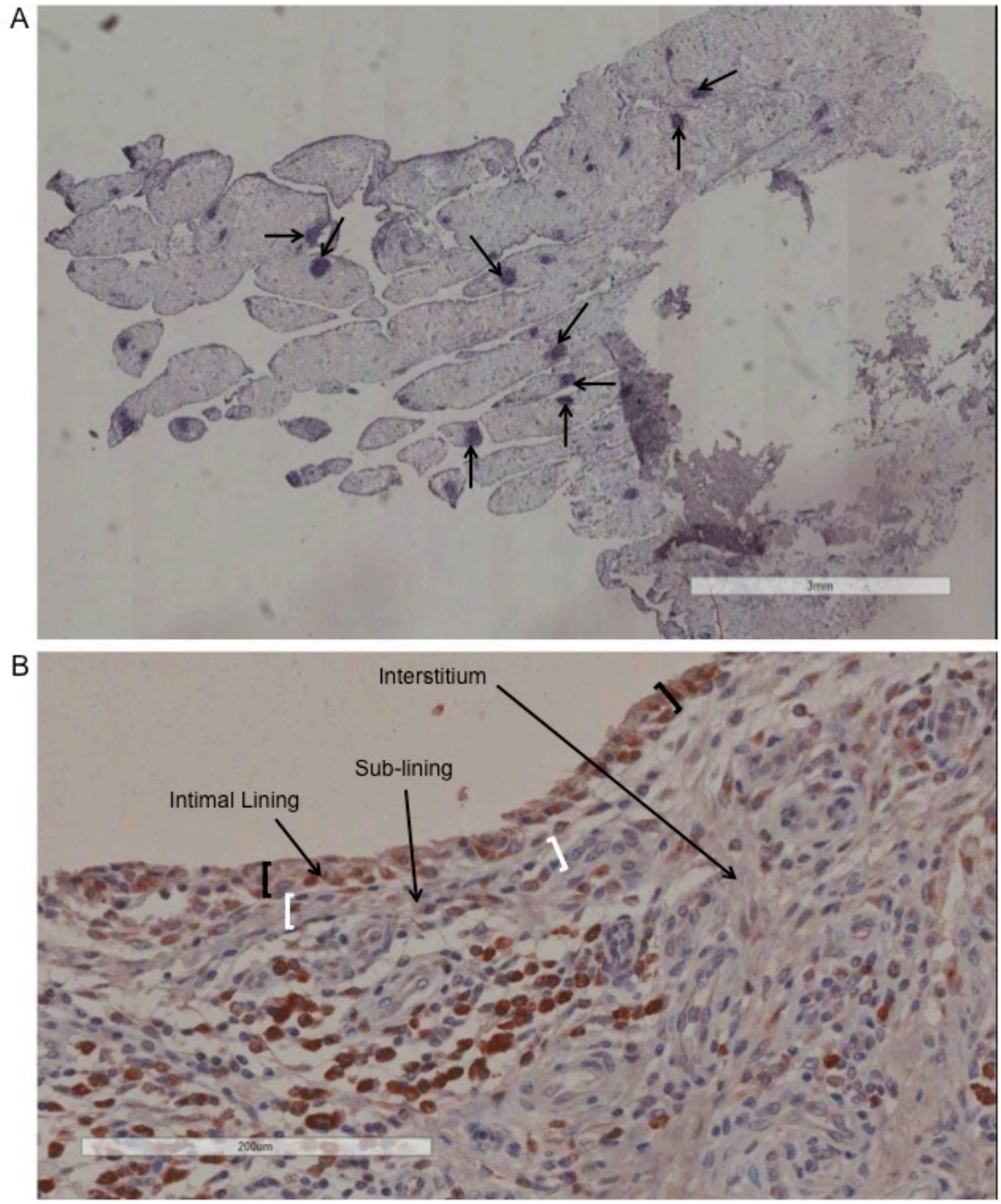

Figure 3.9 RA synovial membrane pathology
Representative images of RA synovial membrane indicating the compartments scored in further figures. A) Rabbit polyclonal IgG stained section with haematoxylin counter stain. 1.5 times magnification with arrows indicating aggregates or follicles. B) GM-CSFRα stained section with haematoxylin counter stain. 20 times magnification with black brackets indicating intimal lining and the white brackets indicating the sub-lining.

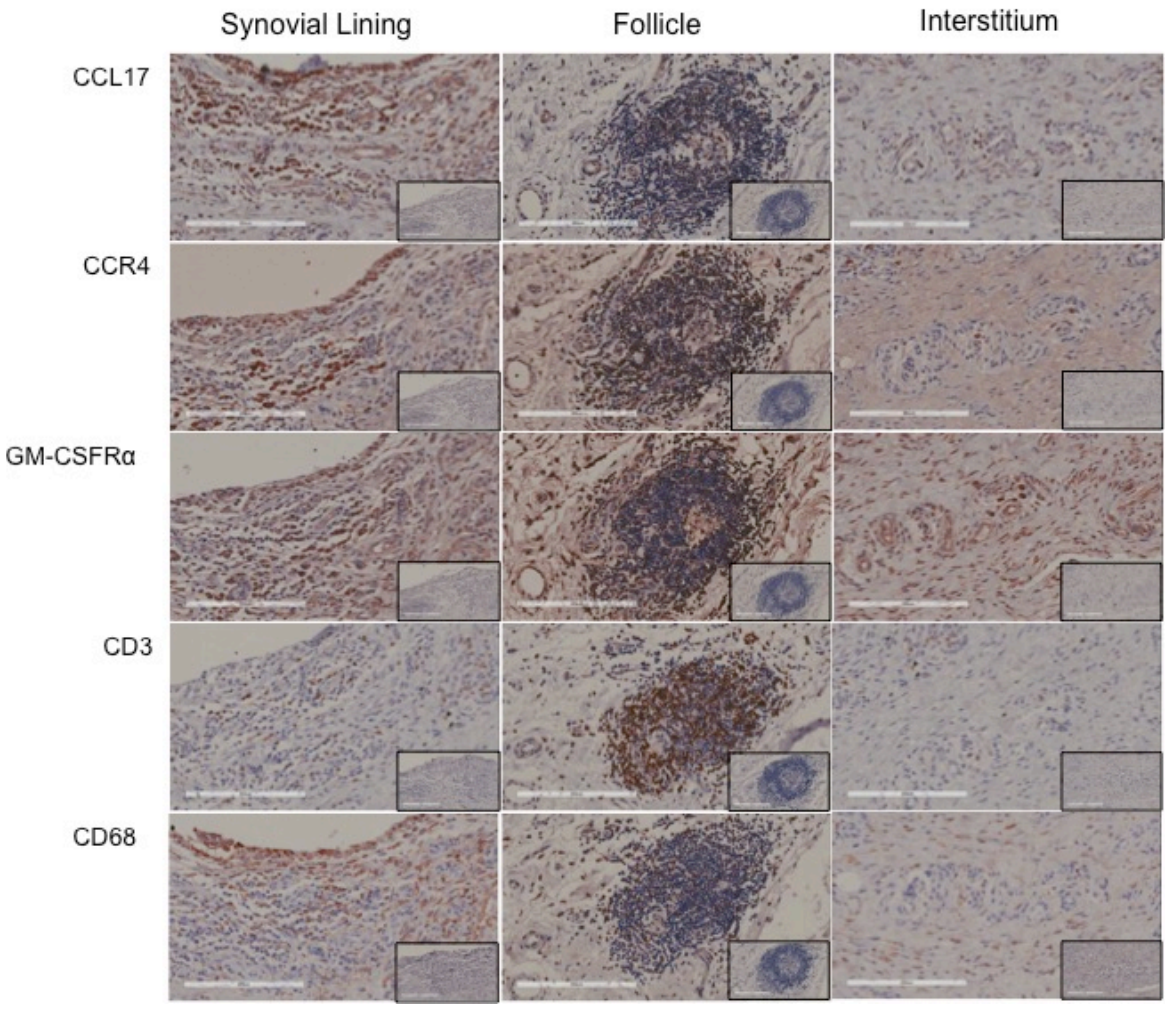

Figure 3.10 Immunohistochemistry of RA synovial membranes of CCL17, CCR4, GM-CSFRα, CD3, CD68.

Sections were stained for CCL17, CCR4, GM-CSFRα, CD3 and CD68. They were scanned using an Aperio ScanScope XT microscope and analysed using Aperio Imagescope software. Representative images of lining layers (intimal and sub-lining), follicle and interstitium of the 8 RA synovial membranes analysed. Relevant isotype inset.

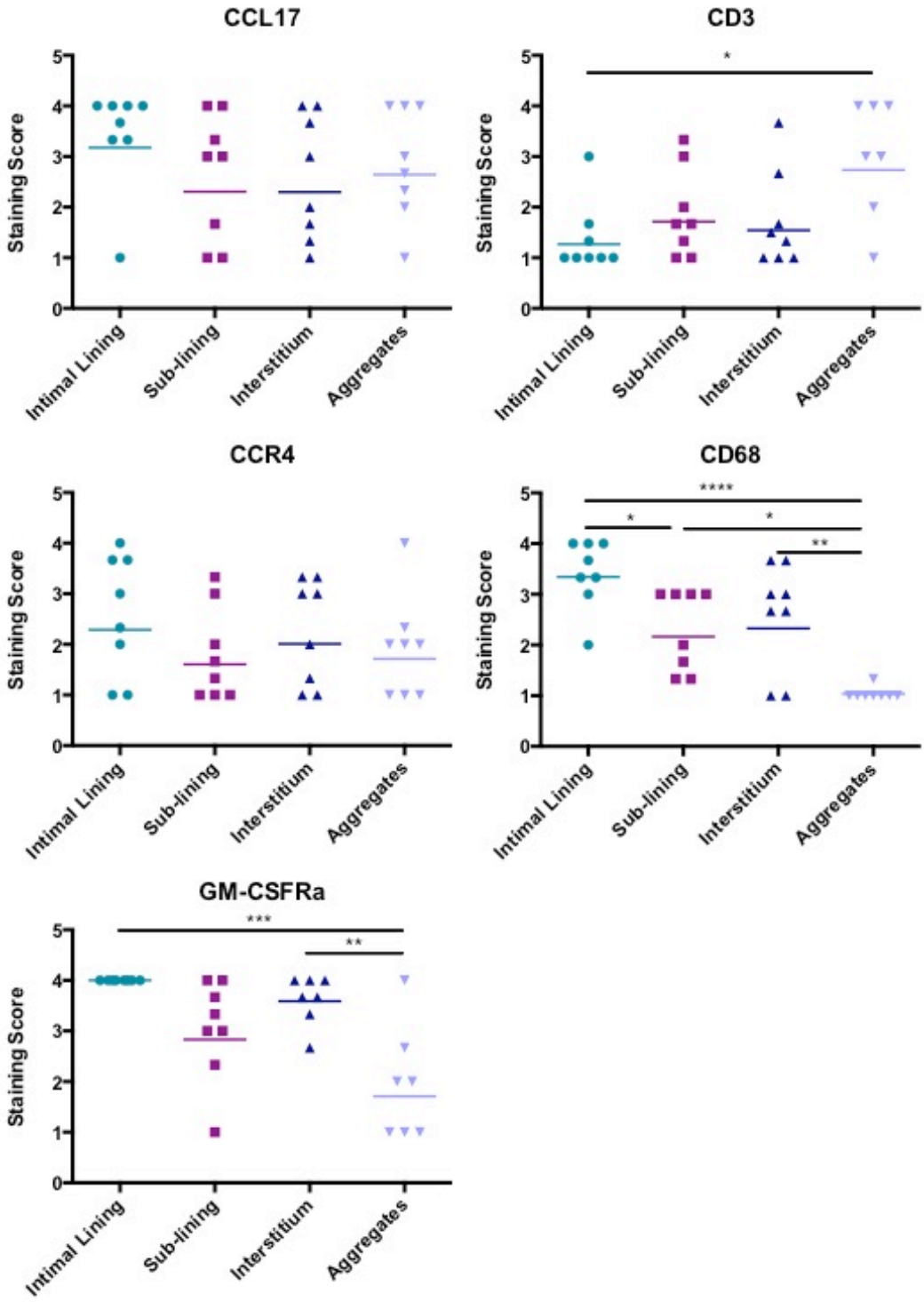

Figure 3.11 RA synovium scoring compared intimal lining, sub-lining, interstitium and aggregates.
Sections stained in Figure 3.10 were scored for the percentage of cells positive for CCL17, CCR4, GM-CSFRα, CD3 and CD68. The staining score was: <25% of positive cells = 1, 26-50% = 2, 51-75% = 3, >75% = 4. 3 areas for each tissue of each area (intimal lining, sub-lining, interstitium and aggregates) were scored and averaged for each section (n=8). This was repeated for each antibody analysed. Horizontal bar indicates the mean. Statistically analysed using one-way ANOVA followed by Bonferroni's post test. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$.

3.2.7 Histological comparison between RA and OA synovial membranes.

The RA synovial membranes that were analysed showed the abundance of the GM-CSFRα in the intimal lining layer, coinciding with CD68 expression. CCL17 was observed in all compartments of the synovial membrane in a similar pattern with CCR4. CD3 had low expression in the lining layers but significantly more in the aggregates. To understand whether this was specific to the RA synovial membrane, OA synovial membranes were analysed as a comparison arthropathy (Figure 3.12). OA sections were scored in the same way as the RA sections. 3 areas of the intimal lining layer and the sub-lining layer were scored for each synovial membrane (Figure 3.13). The representative images in Figure 3.12 show a difference between the RA and OA synovial lining layer pathology. The OA synovial membrane had a much thinner lining layer with far fewer cells than in the RA sections. The architecture of the RA synovial membrane lining was much more inflamed with more aggregates and immune cells. CCL17, GM-CSFRα and CD68 were highly expressed in both RA and OA synovial lining layer, with CCR4 at a slightly lower level and CD3 barely present at all. The scoring showed no difference between RA and OA in CCL17, CCR4, GM-CSFRα, CD3 or CD68 in the intimal lining, and only a difference in the CD3 of the sub-lining showing significantly less staining in the OA sections than the RA (Figure 3.13). This scoring did not represent the staining observed, as clearly the RA synovial membranes were much more inflamed and had a greater cellular infiltrate, enlarging the synovial lining. To account for this, the sections were re-scored by counting the number of positively stained cells in a 40x magnification field of the synovial lining (Figure 3.14). This scoring with absolute numbers of positively stained cells, was much more representative and showed significantly fewer positively stained cells for CCL17, CD3 and CD68 in the OA sections. However, GM-CSFRα and CCR4 appear to be slightly lower in OA synovial membranes than RA synovial membranes, but there was no significant difference. The interstitium was also compared between RA and OA synovial membranes. Only CD68 was significantly lower in OA than RA in the interstitium, with CCL17, CCR4 and GM-CSFRα at comparable levels. CD3 was low in both RA and OA, which was why there was no difference.

These results show that the staining pattern and overall pathology should inform the method of scoring to give a true and accurate representation of the observed staining. As the RA synovial membranes were more inflamed, and therefore had a greater cell number, they were pathologically different to OA. Furthermore, the greater cell number observed in RA synovial membranes translated to a greater number of positively stained cells in comparison to OA, suggesting a more inflamed environment. Due to the increased level of CCL17 in RA synovial membranes in comparison to OA, this suggests that CCL17 could have a greater role in RA than OA.

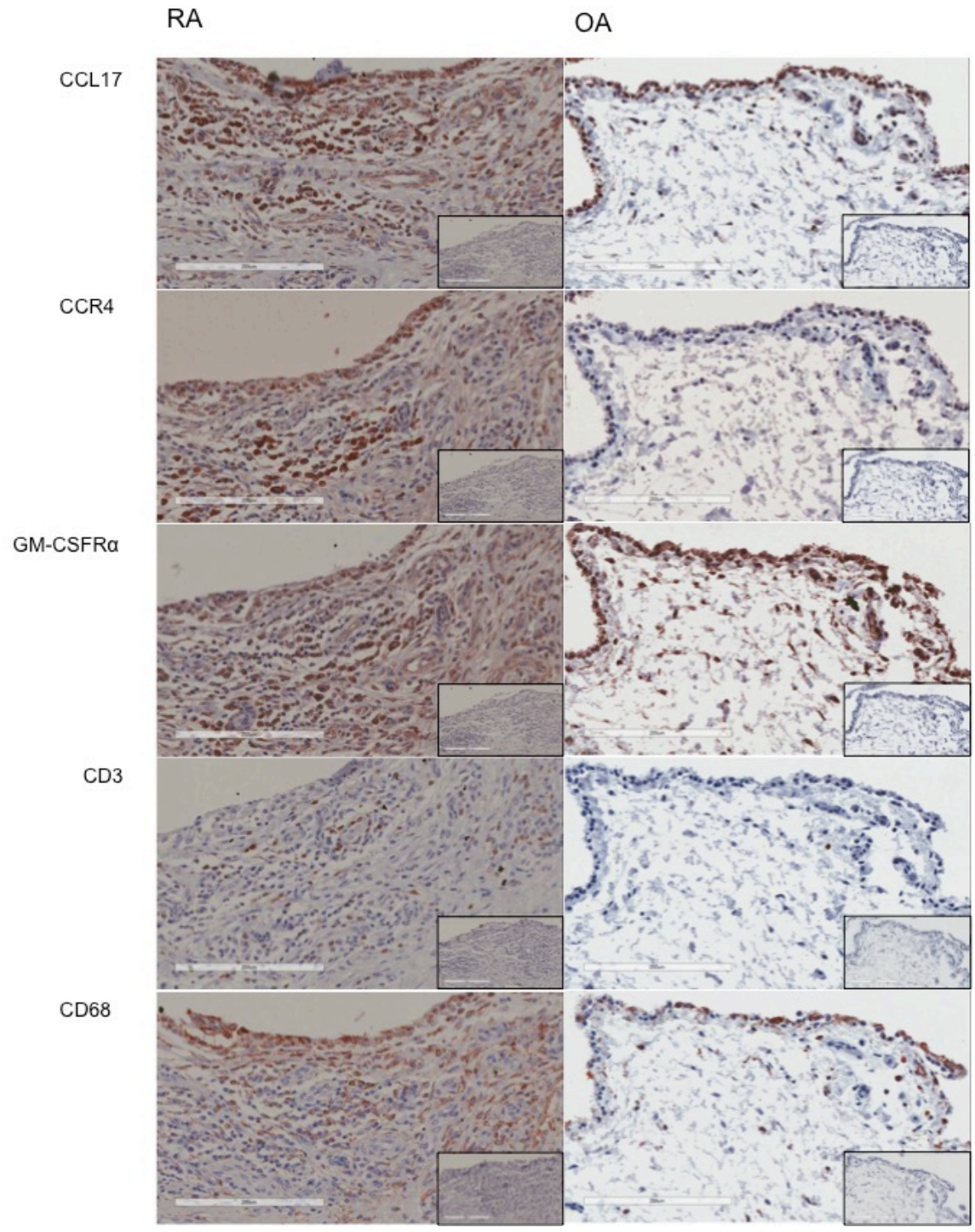

Figure 3.12 IHC of synovial lining of RA and OA sections
OA synovial membranes were histologically stained for CCL17, CCR4 GM-CSFRα, CD3 and CD68 to directly compare with the RA synovial membranes (Figure 3.10). This figure represents the RA (n=8) and OA (n=3) synovial membranes analysed. The same scoring system as the RA synovial membranes was adopted. The relevant isotype inset.

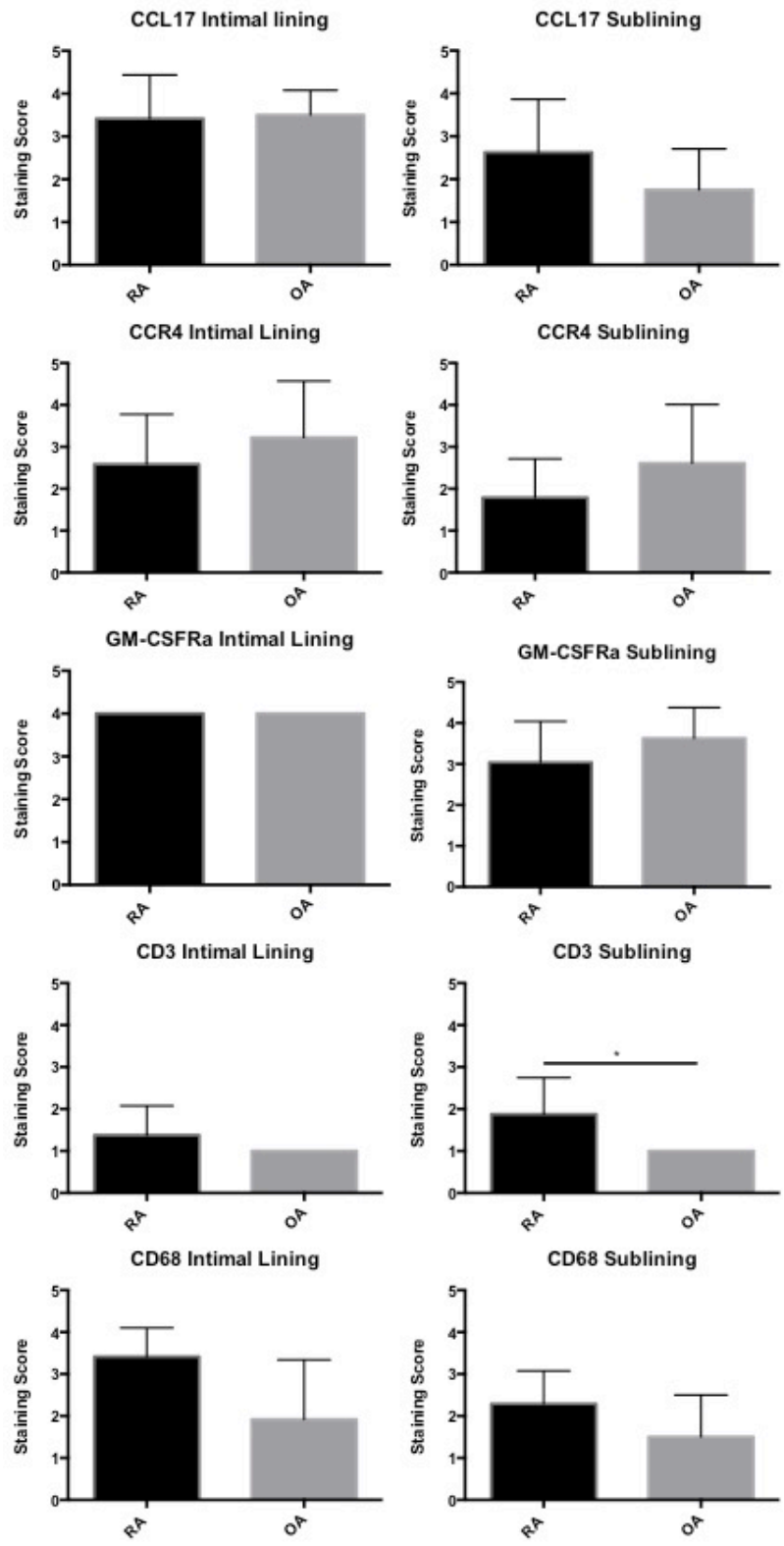

Figure 3.13 RA histological scoring compared with OA in intimal lining, and sub-lining. Histological scoring of the RA and OA synovial membrane intimal lining and sub-lining layers. The scores are averaged from 3 areas of each section and for each antibody stain. <25% of positive cells = 1, 26-50% = 2, 51-75% = 3, >75% = 4. For RA, n=8 and for OA, n=3. Statistically analysed using an unpaired T-Test with * = $p<0.05$.

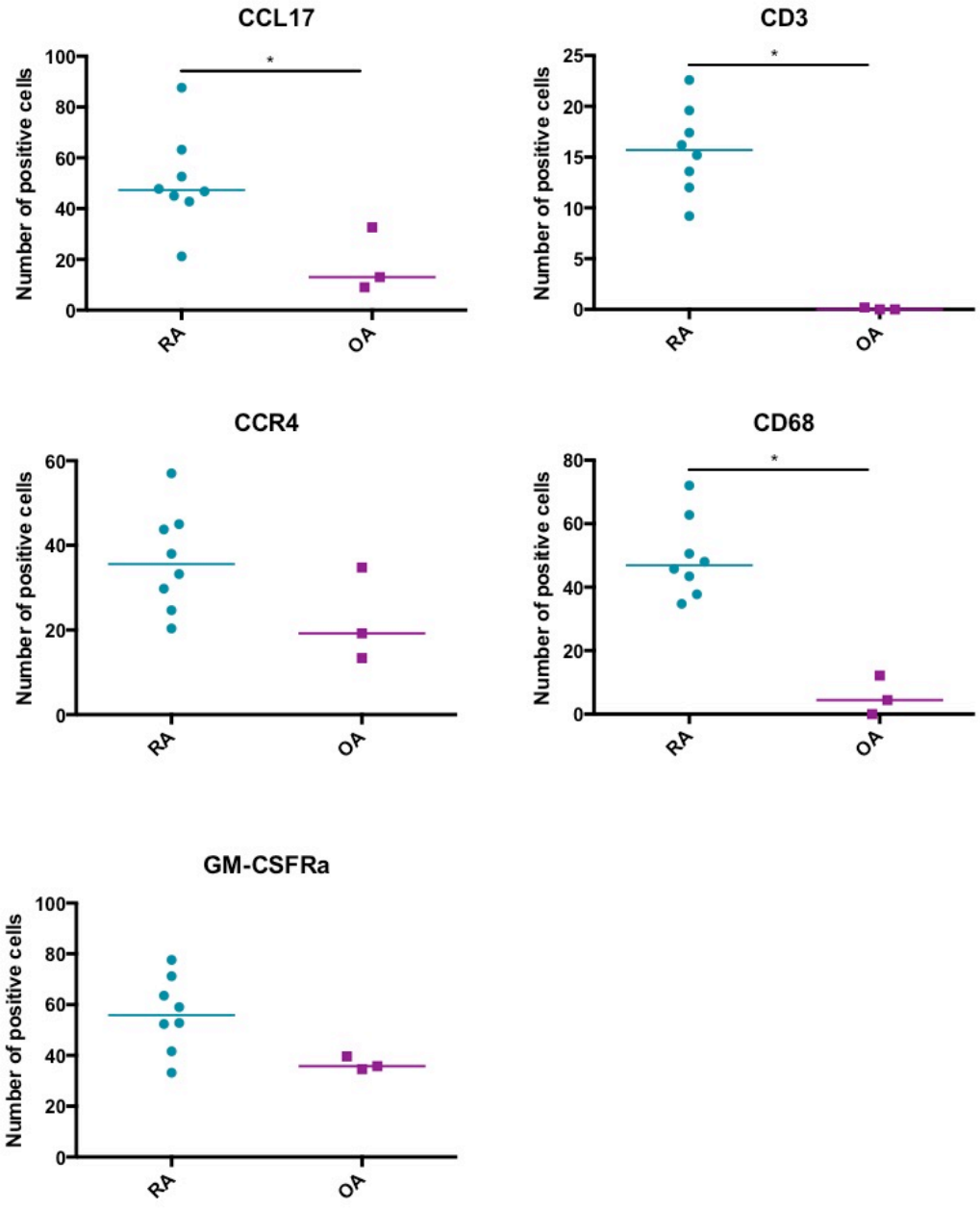

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Figure 3.14 Positive cell count of RA and OA synovial lining.
Score comparing RA (n=8) and OA (n=3) synovial membranes by counting the number of positively stained cells in the field of view for 5 areas of synovial lining at 40x magnification. To account for differences between RA and OA lining, thickness, the whole field of view was counted, including the intimal and sub-lining layers. Absolute cell numbers graphed with the horizontal bar indicating the median. Statistically analysed with a Mann-Whitney Test. * = $p<0.05$.

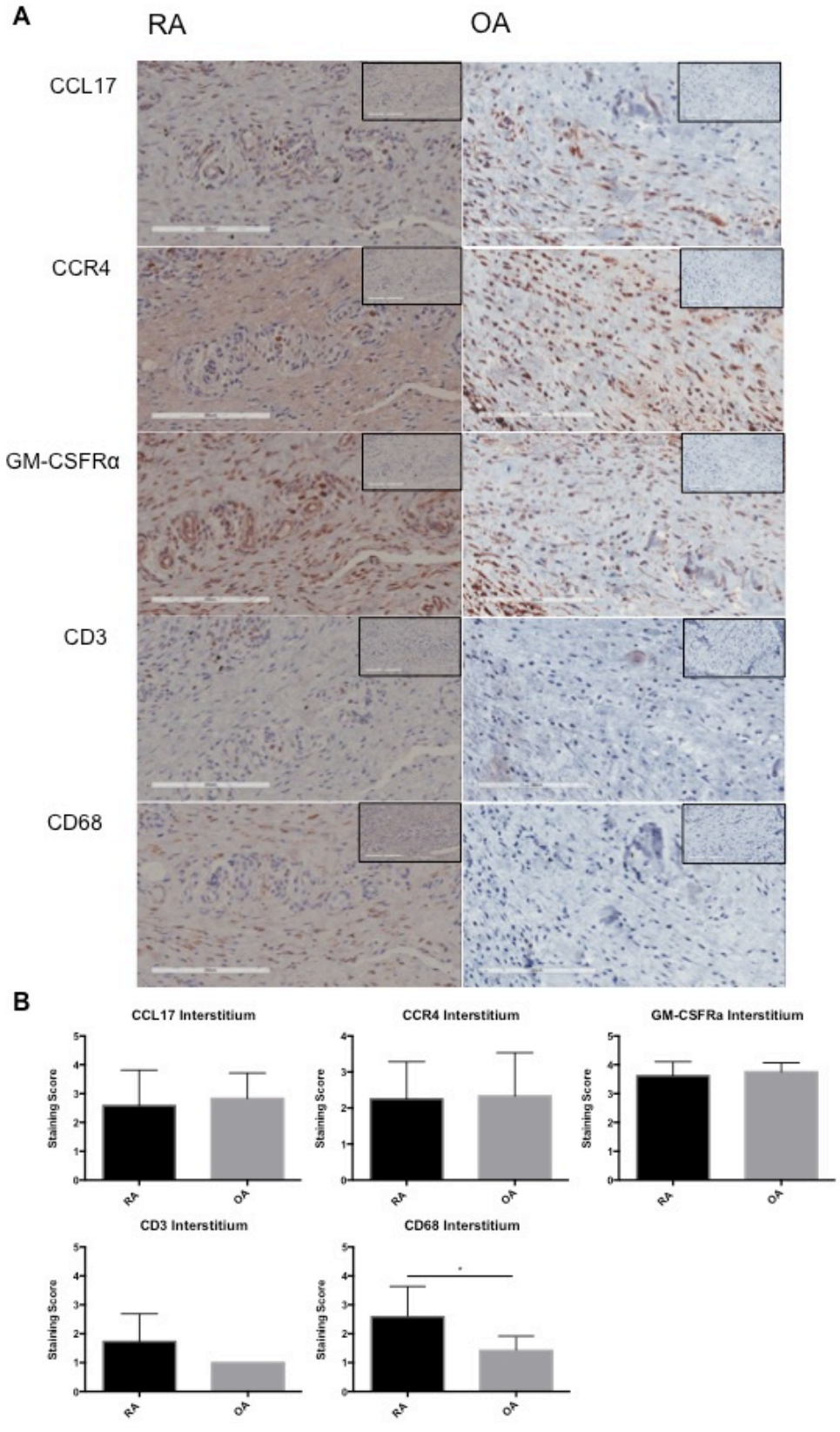


Figure 3.15 Interstitium of RA compared with OA
Histological comparison between the interstitium of RA patient synovial membranes and OA patient synovial membranes. (A) Representative images of staining in the interstitium of RA (n=8) and OA (n=3) patients. (B) Score of an average of 3 counted areas of each section in (A) <25% of positive cells = 1, 26-50% = 2, 51-75% = 3, >75% = 4. For RA, n=8 and for OA, n=3. Statistically analysed using an unpaired T-Test with * = $p<0.05$

3.3 Discussion

There has been an increasing body of evidence supporting the role of GM-CSF in RA. GM-CSF has been reported at high levels in the RA joint (228,273), and monocytes are one of the major infiltrating cells to the inflamed tissues. This chapter has evaluated the effect of GM-CSF on monocytes, as these cells traffic into the inflamed tissue and are subject to many inflammatory stimuli including GM-CSF. GM-CSF caused monocytes to secrete multiple chemokines, rising with increasing GM-CSF concentrations (Figure 3.2). CXCL8, also known as IL-8, was secreted at high levels upon GM-CSF stimulation, which is in accordance with previous literature (274). The variation observed between the different monocyte donors was large, particularly with the production of CCL3, CCL4 and CCL11, as some monocyte donors responded to GM-CSF, and secreted large levels of chemokine whereas others had no response at all. This variation made it hard to interpret the data, however the consistency of CCL17 secretion in all monocyte donors upon GM-CSF stimulation, led us to further evaluate this chemokine.

In RA, there is dysregulation of the immune system causing chronic inflammation (94). We hypothesised the response to GM-CSF in RA patients could be exacerbated, as it is thought that circulating cells are pre-primed, and could therefore be hyper-responsive (275). Therefore we sought to elucidate whether monocytes from RA patients had a greater capacity to secrete CCL17 upon GM-CSF stimulation. RA monocytes secreted comparable levels of CCL17 as the healthy monocytes suggesting that these monocytes were not hyper-responsive to GM-CSF (Figure 3.3A). Interestingly, with higher concentrations of GM-CSF (100ng/ml) the level of CCL17 produced did not saturate as might be expected but decreases. The reason for this is unknown, however, it has been found that macrophages upon GM-CSF stimulation do not produce CCL17 (MedImmune, unpublished), therefore it is possible that the monocytes after 24 hours with high concentrations of GM-CSF are already committed to a differentiated macrophage, and therefore are no longer required to producing as much CCL17. Upon entering the rheumatoid joint, the migrating monocytes would be subject to GM-CSF and as we have found, they would produce CCL17. CCL17 has previously been described to be a chemokine secreted by dendritic cells in the inflamed lung and skin in response to TSLP (262,268). TSLP has recently been

implicated in RA pathogenesis, with significantly higher levels of the protein in synovial fluid of RA patients compared with OA patients. There was also a significant decrease in the level of TSLP as disease improved upon treatment with anti-TNFα therapy, and in a TNFα dependent mouse model, neutralising TSLP led to improved disease scores. (268,276). Furthermore, monocytes are known to expresses the TSLP Receptor (TSLPR) (276), so we aimed to define whether monocytes, like dendritic cells, could produce CCL17 upon TSLP stimulation. Monocytes were unable to secrete CCL17 when treated with TSLP despite expressing the TSLP receptor (277) (Figure 3.3B), suggesting discrete, separate mechanisms of CCL17 production by monocytes and dendritic cells. However, a positive control, using dendritic cells to confirm activity of TSLP, would be required for any conclusions.

The synovial milieu, that monocytes enter, contains DAMPs and PAMPs, and therefore we investigated whether co-stimulation of monocytes with PAMPs would effect the CCL17 secretion. RA monocytes were compared with healthy monocytes to determine whether they both have the same response to LPS. Surprisingly, the co-stimulation of monocytes with GM-CSF and LPS caused inhibition of CCL17 (Figure 3.4A). This was consistent across RA and healthy monocytes. To elucidate whether this was just a TLR4 specific phenomenon, we used several TLR agonists, which all caused inhibition of the CCL17 pathway (Figure 3.4). This was an unexpected finding, as LPS is known to exacerbate the production of cytokines and chemokines in monocytes such as IL-6, TNFα and CXCL8 (IL-8) (255). We therefore aimed to confirm these findings by examining the transcript expression over time with GM-CSF and LPS stimulation. Upon GM-CSF stimulation, the CCL17 transcript became more highly expressed between 6 and 18 hours until 24 hours, whereas LPS prevented any CCL17 transcript upregulation (Figure 3.5). This suggests LPS prevents GM-CSF induced CCL17 transcription, either directly or indirectly. Intriguingly, the CCL22 transcript was also up-regulated by GM-CSF, however this was much more variable between the monocyte donors. The transcript also appeared to be inhibited by LPS. CCL22 was not as consistent as CCL17, and would need to be analysed for a greater time to see a difference. Despite not seeing an upregulation in CCL22 protein after 24 hours of GM-CSF stimulation (Figure 3.2), collaborators have found CCL22 to be highly up-regulated after 48 hours (data not shown). Therefore, the

kinetics of this transcript would need to be analysed for a longer period of time. The inhibition of CCL17 and CCL22 by LPS is interesting as they are both ligands for CCR4, suggesting a CCR4 ligand specific phenomenon. Furthermore, as CCR4 is a chemokine receptor on Th17 cells (186), TLR-mediated inhibition of CCR4 ligands is potentially a regulatory mechanism to prevent excessive migration of Th17 cells into the inflamed tissue. Due to multiple TLR ligands in the synovial fluid, it would also be interesting to confirm the effect of the synovial fluid on GM-CSF driven CCL17. Therefore we are going to further investigate this in Chapter 4. To confirm that the LPS was effective and the observations with CCL17 and CCL22 were correct, we analysed the CXCL8 transcript, and as expected saw an up-regulation in the transcript after 6 hours with GM-CSF and LPS co-stimulation. Therefore, the CCL17 inhibition by LPS was a true and novel observation, leading us to question the mechanism causing this inhibition.

A parallel investigation into the cause of TLR-mediated inhibition of GM-CSF driven CCL17 transcription suggested inhibition could be regulated by SOCS3. We attempted to investigate whether SOCS3 was involved but whilst we could successfully reduce SOCS3 expression in cells using siRNA, the transfection reagents significantly modified the behaviour of primary monocytes and therefore we were unable to draw any conclusions from these experiments. Further experiments of negative regulators of inflammation should be considered.

To assess the impact of GM-CSF induction of CCL17 in monocytes, we analysed the effect of CCL17 on non-activated and activated T cells to determine whether they up-regulated GM-CSF secretion. We hypothesised that CCL17, produced by monocytes under GM-CSF stimulation, caused CCR4 positive T cells to up-regulate GM-CSF in a positive feedback loop. Total CD4 cells were analysed and they were also divided into naïve ($CD45RA^+$) and memory ($CD45RO^+$). The $CCR4^+$ T cells would be in the memory compartment, as previous studies have identified high levels of CCR4 expression in memory T cells (278,279), and naïve T cells were used as a comparator. All categories of T cell, when activated, secreted GM-CSF, with memory T cells producing more than the matched naïve T cells (Figure 3.8). T cells have previously shown to secrete GM-CSF (222,280), however the effect of CCL17 on this secretion was unknown. Increasing concentrations of CCL17 over 24 or 48 hours had no effect on the level of GM-

CSF produced. This suggests that CCL17 in this system, acts purely as a chemokine, instigating the migration of $CCR4^+$ T cells to the tissue, where upon activation by antigen, produce GM-CSF (Figure 3.16). Previous studies also support this hypothesis, as CCL17 causes $CCR4^+$ T cells to migrate towards the chemokine (281). Interestingly, GM-CSF has been shown to be produced by Th17 cells, and an RORγT binding site is present in the GM-CSF promoter, suggesting Th17 cells are the main producers of GM-CSF (228,282,283). This supports our hypothesis that $CCR4^+$ T cells such as Th17 cells produce GM-CSF. However, there is also evidence to suggest that other T cells can produce GM-CSF. These include cells that are Th1, Th2 and Th17 independent as they do not express their master transcription factors or their signature cytokines, however they secrete GM-CSF (228,280,284). Some propose that these GM-CSF producing T cells are in fact a distinct, pathogenic subset (280,284,285). Our data also supports this theory as naïve T cells, which were not expected to produce GM-CSF, also secreted GM-CSF, however, this was at lower levels than memory T cells. Despite the subset or classification of T cells, (whether they are Th17, or a specific GM-CSF producing T cell) they migrate to the inflamed tissue in response to CCL17, and secrete GM-CSF when they receive activation cues.

This data was analysed *in vitro,* therefore, to determine the relevance to the RA synovial tissue, synovial membranes were analysed by immunohistochemistry. This allowed us to evaluate the relationship between GM-CSFRα and CCL17 with CCR4 and in relation to CD68 and CD3. To gauge where these proteins were located within the tissue, they were scored in the intimal lining, sub-lining, interstitium and the aggregates or follicles (Figure 3.10,Figure 3.11). These areas have been well defined in the RA synovial membranes for scoring (286-288). This analysis therefore initially defined that CCL17 was in all areas of the tissue, CCR4 was not specific to one area whereas CD3 was not highly expressed in the lining layers but was more apparent in the follicles. CD3 and CCR4 would be expected to be co-expressed as most of the literature indicates CCR4 is a chemokine receptor on T cells, specifically Th2, Th17 and Treg cells(186,264,281). However, there have been some studies indicating that CCR4 is also present on other cells. CCR4 has been identified on monocytes at higher levels on RA peripheral blood monocytes than on normal peripheral blood monocytes (289). CCR4 has also been found on platelets (290), but is in the main

expressed on T cells. Therefore, the unexpected, differing expression of CCR4 with CD3, could potentially be explained by Katschke et al., with CCR4 expression at higher levels on RA monocytes. This therefore could also be the case in the synovial membrane. The CCR4 expression in the synovial lining was high and this theory could therefore be explained by the high level of CD68 also present in the intimal and sub-lining. CD68 was high in the lining layers, and significantly lower in the aggregates. This was consistent with previous analysis (291). GM-CSFRα had a similar distribution as the CD68, which was also consistent with the literature, as they have been shown to be co-expressed (238). Finding CCL17 in all areas of the synovial membrane suggests that it is playing a part in RA pathogenesis. It was variable between RA donors, but we also found the responsiveness of monocytes to produce CCL17 after GM-CSF stimulation quite variable, some with as much as 10 fold differences (Figure 3.3A). To improve the understanding of how CCL17, CCR4 and GM-CSFRα interact, double fluorescent microscopy would have allowed us to examine the specific cell types expressing CCR4 and GM-CSFRα. It would also shown the interaction of CCL17 with the cells of the synovial membrane. Double fluorescent microscopy was attempted (data not shown), however there were issues with spectral overlap that were proving too difficult to overcome in the time period. For this reason, consecutive sections were analysed to give the greatest understanding possible without co-staining. As these proteins were all found in the RA synovial membrane, and potentially support our hypothesis outlined in Figure 3.16, we wanted to assess whether these observations were specific to RA synovial membranes.

OA synovial membranes were used as a comparator arthropathy, as the mechanism of disease is recognised to be less inflammatory with different factors driving disease in comparison to RA. The ideal comparator would be healthy synovial membrane, however, this tissue was unavailable. The OA tissue was able to provide insight into whether or not the expression of the proteins we analysed in RA, was disease specific or an aspect of synovial pathology. The initial observation of the OA synovial membranes was how the synovial lining layer was much thinner in comparison to RA. Also, the cells were much more widely distributed in the interstitium and there were no aggregates. There is heterogeneity between OA synovial membranes, as aggregates have been

observed in other OA tissues, however there were none in the sections analysed in this study. Furthermore, in the 3 OA synovial membranes I examined, the intimal lining layers were similar in their thickness, however in more inflamed OA tissues, this can be much thicker and it is in the inflamed tissues where aggregates are more likely to occur (292,293). The initial scoring system we used was the same that was used with the RA synovial membranes, by dividing the tissue into compartments (Figure 3.12,Figure 3.13). This showed no difference between the RA and OA synovial membranes in the intimal lining layer, which we decided was not representative of the sections. Therefore we used a different scoring system that involved counting the positive cells in a 40x magnification field. This method was much more representative of the inflammatory status of the tissue, and took into account the thickness of the lining layer. This led to significant differences in CCL17, CD68 and CD3 expression between RA and OA synovial lining. There was still no statistical difference in GM-CSFRα and CCR4 expression between RA and OA, however there was a slight decrease in the numbers of positive cells in the OA sections. The low number of analysed OA synovial membranes potentially explains this, and to improve the reliability of the results, increasing the number of sections analysed would allow a more accurate representation. GM-CSF has also been implicated in OA pain and it could have a role in the inflammatory aspects of OA, suggesting a role for GM-CSFRα expression in OA tissue (294). Despite not having statistical significance in all of the analysed proteins, this data begins to reveal that there is a difference between RA and OA synovial membranes. This was particularly apparent with the increased immune cell population in RA and the number of cells positive for CCL17. The immunohistochemistry supported our rationale, that CCL17 is involved in RA pathogenesis, the RA synovium is highly inflamed with CD68 and CD3 cells and that GM-CSFRα is highly expressed.

In summation, these findings have found a novel aspect of biology, in which monocytes under GM-CSF stimulation, produce CCL17. CCL17 is unable to activate $CCR4^+$ T cells to produce GM-CSF, however activated CD4 T cells will produce GM-CSF and CCL17 as a chemokine, attracts more $CCR4^+$ T cells to the tissue. The GM-CSF signalling pathway can be inhibited by TLR ligands, via an as yet unknown mechanism, proposing a unique TLR/GM-CSFR signalling interaction. To give this work context to RA disease pathology, RA synovial

membranes were analysed and show CCL17, GM-CSFRα and CCR4 expressed in the same compartments of the tissue, suggesting interactions. Interestingly, the circulating levels of CCL17 and CCL22 were reduced in patients following treatment with anti-GM-CSFRα (Mavilimumab) suggesting a role for these chemokines in RA (295). Therefore, further investigation is required to evaluate monocytes under synovial environment conditions.

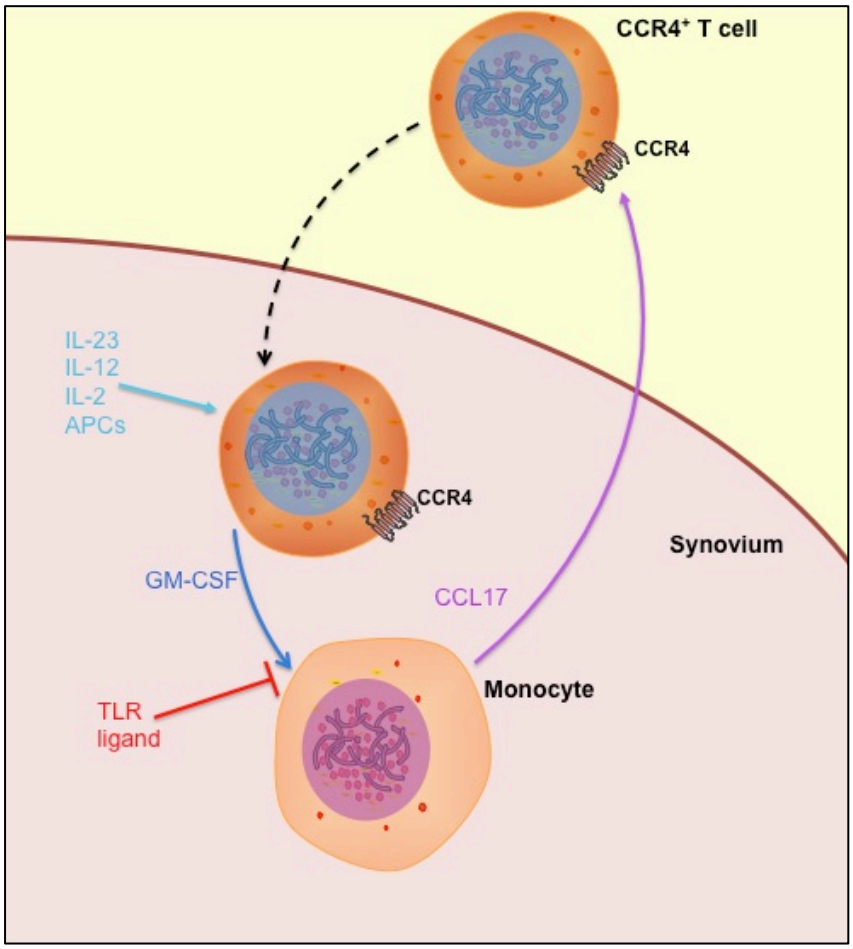


Figure 3.16 Schematic detailing our hypothesis.
Monocytes secrete CCL17 upon stimulation with GM-CSF. This chemoattracts CCR4 T cells to the tissue, where they are activated by antigen presenting cells (APCs), or receive cues from cytokines such as IL-23, IL-12 and IL-2 to secrete GM-CSF. This GM-CSF then stimulates the monocytes to secrete CCL17 in a positive feedback loop. The initial factor driving this loop is unknown, as without the monocyte, there would be no CCL17, therefore no T Cell recruitment. Without T cells, there would be no GM-CSF and therefore no stimulation of the monocytes. However, TLR ligands that are autologous to the synovial fluid can inhibit this pathway by preventing GM-CSFR signalling in the monocyte thereby preventing CCL17 secretion.

Chapter 4 The impact of the synovial environment on the GM-CSF/CCL17 axis

4.1 Introduction

The previous chapter analysed the effect of GM-CSF on monocytes. After identifying GM-CSF as a potent cytokine that induced monocytes to secrete CCL17, the capacity for this pathway to be inhibited by TLR ligands was also identified.

The synovial environment is a complex milieu that contains endogenous TLR ligands such as HMGB1 and S100 proteins. These endogenous proteins can be cytosolic proteins released as result of cell damage or necrosis. They are also known as Danger Associated Molecular Patterns (DAMPs) and are involved in perpetuating inflammation due to the downstream TLR pathways (91). TLR activation leads to the secretion of pro-inflammatory factors such as IL-1β, TNFα and MMPs, which are known to further activate immune cells or lead to tissue degradation (80,255). The synovial milieu, that contains DAMPs, is heterogeneous between RA donors, however, it has been profiled proteomically and metabolically to try and identify biomarkers (132,133).

Proteomic analysis of RA synovial fluid and OA synovial fluid identified several up-regulated proteins in RA synovial fluid in comparison to OA. In particular, several DAMPs including S100A8, S100A9 and S100A12 were up-regulated 9-fold, 29-fold and 33-fold respectively in comparison to OA (133). They were also found to be highly expressed in RA synovial fluid in comparison to OA synovial fluid in a previous study (84). The increase in S100 protein expression in RA synovial fluid has led to research on their role. They can bind TLRs and lead to monocyte activation (269). There were other proteins that were up-regulated in RA synovial fluid such as Coronin A, actin binding protein 1, and fibrinogen-like 2 amongst several others, highlighting its complexity and the number of mediators that could exacerbate inflammation (133).

DAMPS in the synovial milieu are not the only factors that are associated with RA pathogenesis. There are also immune complexes that form in the synovial tissue such as rheumatoid factor and ACPA immune complexes that are present in the

majority of RA patients and are associated with a more destructive disease phenotype (296). ACPAs are auto-antibodies to citrullinated proteins such as fibrinogen, fibrin and vimentin (94,297,298). Immune complexes activate monocytes and macrophages through FcγRs, leading to up-regulation in the secretion of pro-inflammatory cytokines such as TNFα (299,300). There is also evidence to suggest that immune complexes engage both FcγR and TLRs. In macrophages stimulated with citrullinated fibrinogen (cFib) alone or citrullinated fibrinogen immune complexes (cFib-IC), the cFib activated TLR4 and the MyD88 pathway, and cFib-IC engaged both FcγR and TLR4. Inhibiting TLR4 significantly reduced the amount of TNFα secreted from macrophages, highlighting the exacerbated inflammatory response (298).

To add to the DAMPs and immune complexes in the synovial milieu that can activate cells, there are also cytokines that enhance inflammation. Cytokine profiles of RA synovial fluid has indicated an increase in TNFα, IL-1α, IL-2 and IFNα in comparison to OA synovial fluid (301-303). IFNα is a member of the type I interferon family and was highly up-regulated in sero-positive RA (302). Type I interferons have increasingly been implicated in RA pathology as an up-regulation of interferon stimulated genes have been identified in RA patients. IFNα increases MHC expression and activation of macrophages and lymphocytes (3). Interestingly, type I interferon responses may identify a clinically distinct subset of RA, by a distinct molecular phenotype, with increased activity from the innate immune system (304).

The heterogeneity of RA synovial fluid has made the understanding of its impact on cells difficult to interpret. The previous chapter analysed the effect of GM-CSF on monocytes and the impact on monocytes of TLR agonist co-stimulation. To understand the effect of the GM-CSF stimulation on monocytes in a synovial environment, this chapter aims to dissect the effect of synovial fluid on GM-CSF stimulated monocytes.

Chapter Aims:

- To assess the cytokine and chemokine content of RA synovial fluid.

- To analyse the impact of synovial fluid co-stimulation on GM-CSF stimulated monocytes.

- To elucidate the underlying mechanism causing the observed effect by synovial fluid.

4.2 Results

4.2.1 RA synovial fluid contains a higher concentration of cytokines and chemokines in comparison to OA.

The synovial milieu, despite several studies analysing its content, is a heterogeneous environment that is incompletely defined. To assess the cytokine and chemokine content of synovial fluids, RA and OA synovial fluids were analysed by MSD (Section 2.7.4). RA synovial fluids can interact with antibody based assays due to rheumatoid factor leading to up-regulated readings, whereas MSD technology contains a buffer that prevents this. The RA synovial fluids had variable levels of cytokine (Figure 4.1). 2 of the 4 RA synovial fluids had elevated levels of GM-CSF, IFNγ, IL-12p70 and IL-10. All 4 RA synovial fluids had higher levels of IL-6 and TNFα compared to OA synovial fluids, however, there was no significant difference between the cytokine levels of RA and OA synovial fluids. The OA synovial fluids had very little of all analysed cytokines apart from IL-6 where there were slightly increased levels, however this was variable between the fluids.

The OA synovial fluids had a greater chemokine content than cytokine, and in some instances comparable with the RA synovial fluids. This was the case for CCL3, CCL4, CCL13, CCL17 and CCL22, suggesting that these synovial fluids could be from OA patients with a more substantial inflammatory component in their disease (305)(Figure 4.2). The RA synovial fluids had slightly increased levels of CCL2, CXCL8 and CXCL10, however this was not to a significant level potentially due to the small numbers analysed.

The comparison of synovial fluids from RA and OA patients showed that there was a large level of variability between donors, but on the whole the RA synovial fluids had more inflammatory mediators than the OA synovial fluids. Several studies have shown up-regulated levels of cytokines and chemokines in RA synovial fluid in comparison to OA synovial fluid (137,301,302). Therefore in order to compare with the literature, it would be important to increase the number of analysed synovial fluids, as heterogeneity between synovial fluids makes them difficult to conclusively analyse.

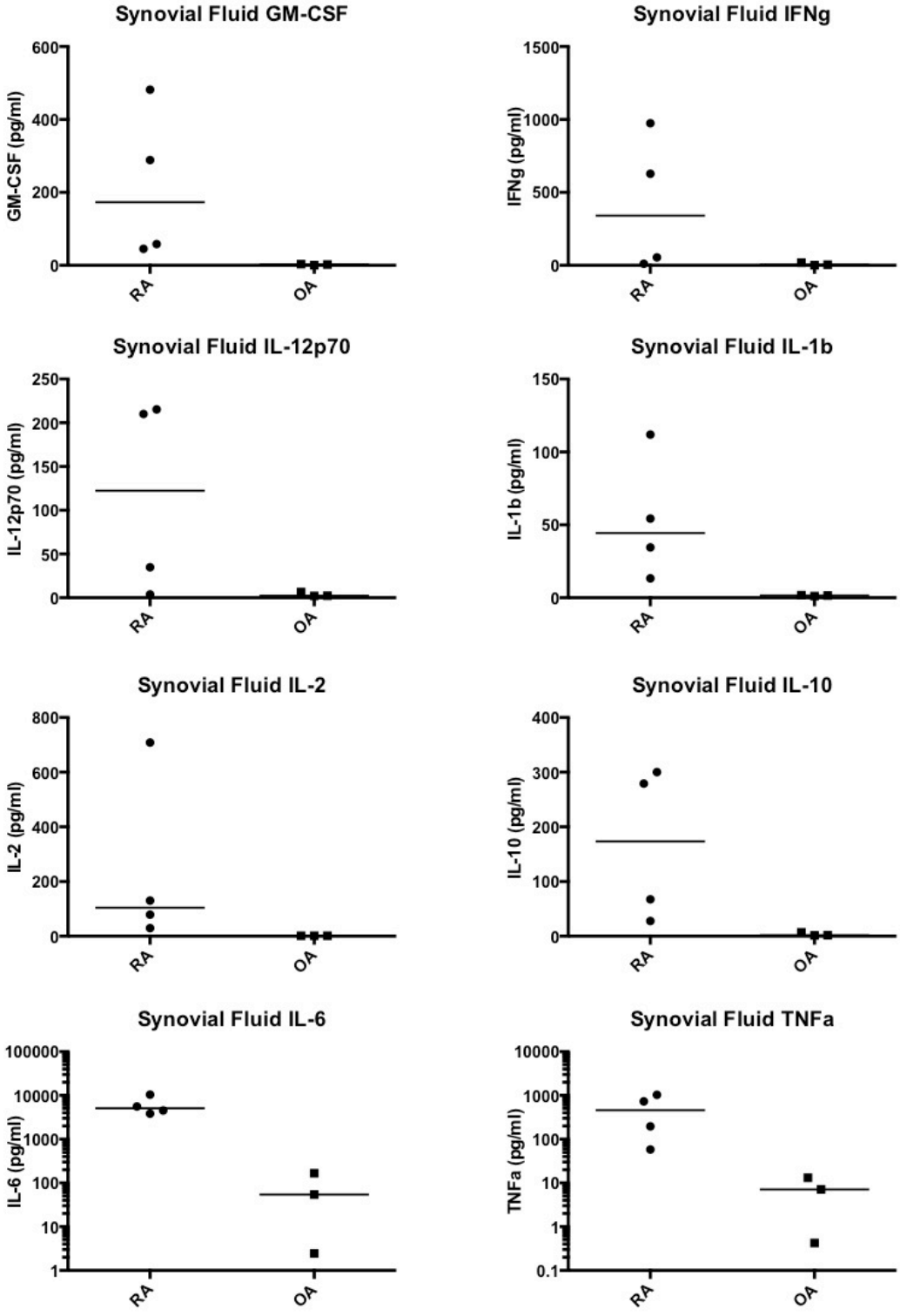


Figure 4.1 Cytokines in Synovial Fluid
RA synovial fluids (n=4) and OA synovial fluids (n=3) were analysed using cytokine multiplex MSD technology. Horizontal bars represent median. Statistical analysis using Mann-Whitney Test. No statistical differences observed.

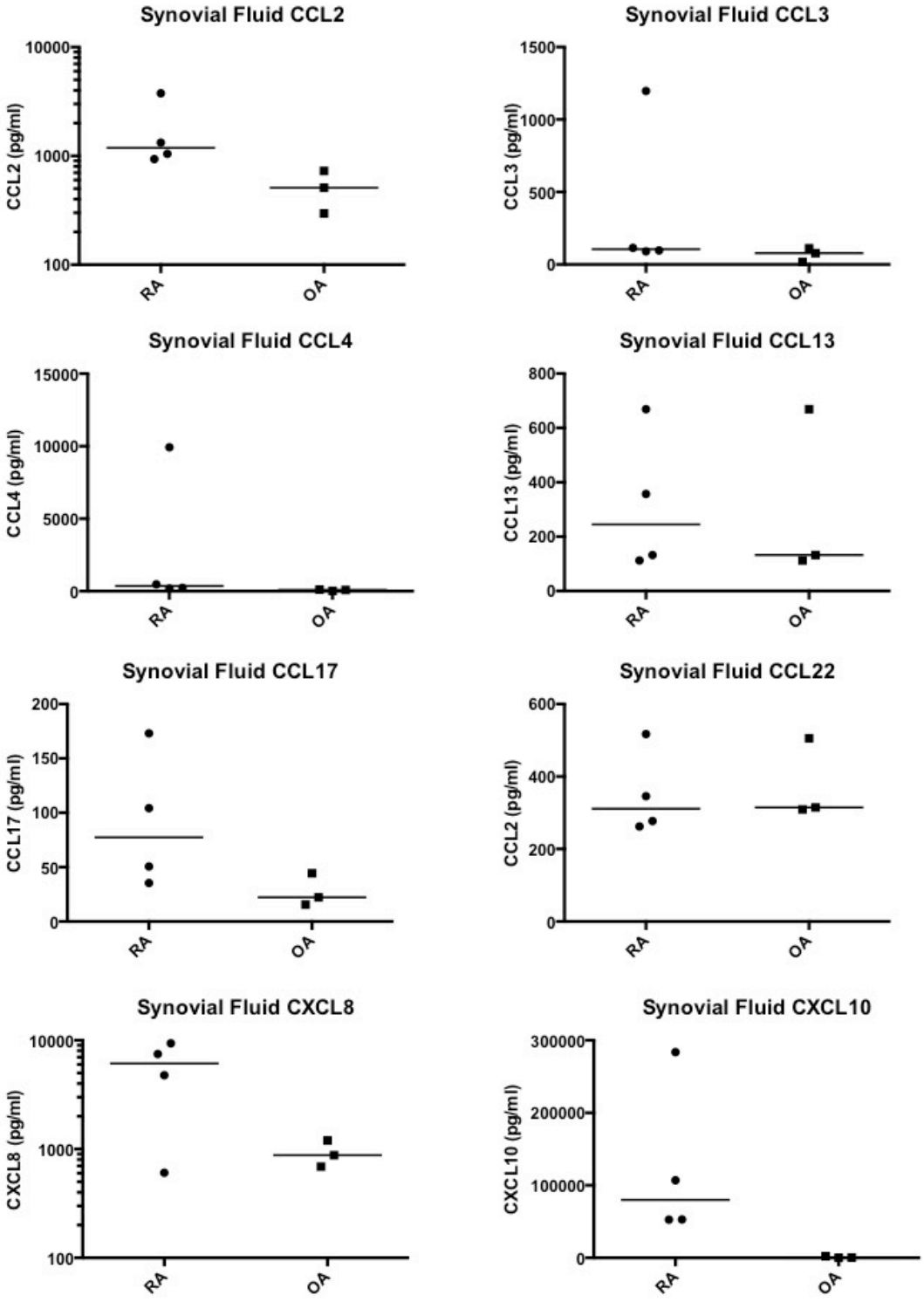


Figure 4.2 Chemokines in Synovial Fluid
RA synovial fluids (n=4) and OA synovial fluids (n=3) were analysed using chemokine multiplex MSD technology. Horizontal bars represent median. Statistical analysis using Mann-Whitney Test. No statistical differences observed.

4.2.2 Synovial fluid impacts GM-CSF stimulated monocyte CCL17 secretion.

RA synovial fluid contains cytokines and chemokines (Figure 4.1, Figure 4.2), however there variability was observed between different patients. Chapter 3 assessed the ability for TLR ligands to inhibit GM-CSF induced CCL17. Therefore, as RA synovial fluid is known to contain many endogenous TLR ligands or DAMPS, the effect of RA synovial fluid on GM-CSF stimulated monocytes was investigated. Furthermore, the evaluation of synovial fluid on GM-CSF stimulated monocytes mimicked the impact of the synovial environment on monocytes upon entry into the synovium in RA. Monocytes were co-stimulated with GM-CSF (1ng/ml) and synovial fluid (2.5% and 5%) for 24 hours, after which supernatants were analysed for chemokine expression. Co-stimulation of GM-CSF with RA or OA synovial fluid had no effect on CCL2 and CCL11 secretion in comparison to GM-CSF alone (Figure 4.3A). RA synovial fluid had varying effects on CXCL8 induction when co-stimulated with GM-CSF, however, there was no significant induction above the level of GM-CSF alone. Co-stimulation of GM-CSF and RA synovial fluid caused no showed no significant inhibition CCL22, but it did appear to have a slight decrease in induction. OA synovial fluid had no impact on CCL22 induction. Interestingly, co-stimulation of GM-CSF with OA synovial fluid, at both 2.5% and 5% caused a significant inhibition of CCL3 and CCL4, whereas RA synovial fluid co-stimulation had no effect on these chemokines.

CCL17, which was induced by GM-CSF, and inhibited by TLR ligands (Figure 3.4), was significantly inhibited by RA synovial fluids at both 2.5% and 5% (Figure 4.3B). OA synovial fluid had a much more variable effect on GM-CSF driven CCL17. Each synovial fluid caused a different response, as co-stimulation with GM-CSF in some instances caused exacerbation of CCL17 in comparison to the GM-CSF alone control, whereas in others caused inhibition or no change. This suggests that there is a factor consistently present in RA synovial fluid, but not in OA synovial fluid, that causes inhibition of GM-CSF induced CCL17.

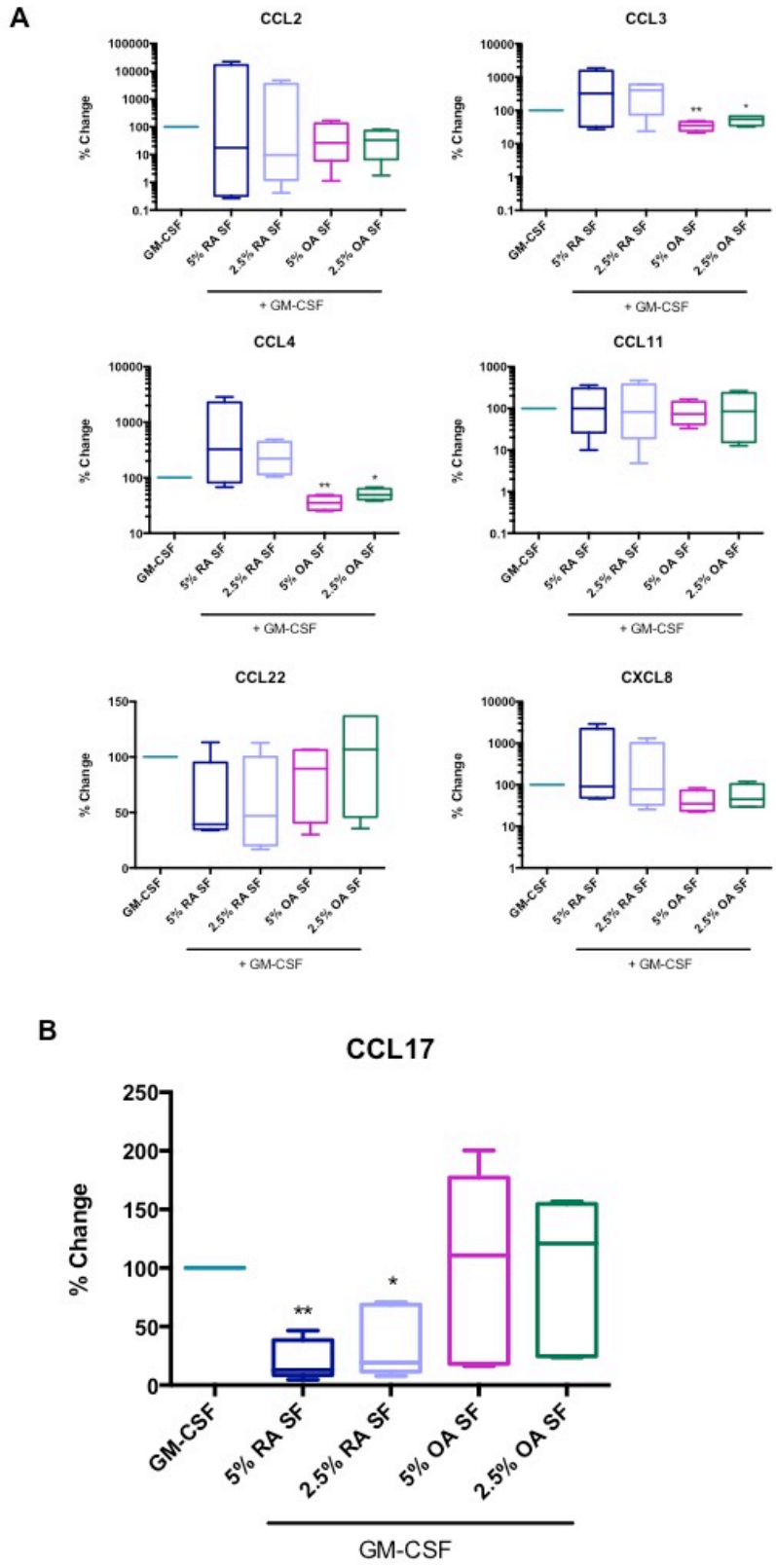


Figure 4.3 Synovial Fluid impact on GM-CSF induced chemokines.
Monocytes isolated from buffy coats (n=4) were stimulated with 1ng/ml GM-CSF with or without 2.5% / 5% synovial fluid from RA patients (n=4) or OA patients (n=4) for 24 hours. Supernatants were collected and analysed by MSD for multiple chemokines (A) and CCL17 (B). Data analysed as percentage change due to the large variation between monocyte donors, with GM-CSF alone made to 100% and all other conditions compared to this. GM-CSF alone absolute values ranged between 300 and 6000pg/ml. Synovial fluid stimulated monocytes analysed as induction, to discount any chemokine already in the synovial fluid. Statistically analysed using one-way ANOVA and bonferroni's post test. * = $p<0.05$ and ** = $p<0.01$.

4.2.3 Transcript analysis of the kinetics of synovial fluid inhibition.

RA synovial fluid caused significant inhibition of CCL17 protein induction by GM-CSF. There was heterogeneity between the OA synovial fluids, leading to varied capacities to inhibit CCL17. Therefore to investigate the mechanism of synovial fluid inhibition of CCL17, RA synovial fluids were used due to their robust inhibition. LPS prevented CCL17 transcript induction (Figure 3.5), so it was investigated whether RA synovial fluid also affected CCL17 transcription. A time course analysing the CCL17 transcript was undertaken, where monocytes were co-stimulated with GM-CSF and RA synovial fluid, and transcripts were analysed at 6, 18 and 24 hours relative to 0hr. The CCL17 transcript was induced by GM-CSF stimulation between 6 and 18 hours (Figure 4.4). The RA synovial fluids analysed both prevented induction of the transcript. The monocyte donors were variable in their induction of CCL17 transcript, however there was still a significant difference at 18 and 24 hours between the GM-CSF stimulated monocytes and co-stimulated monocytes with GM-CSF and RA synovial fluid. The CCL22 transcript was also up-regulated at 18 and 24 hours after GM-CSF stimulation. Similarly, the RA synovial fluids prevented induction of this transcript, as there was significantly lower CCL22 transcript at 18 and 24 hours. The CXCL8 transcript was induced by GM-CSF at 6 hours, but RA synovial fluids were not able to inhibit the induction of this transcript. However, the mRNA peak for CXCL8 was potentially before the 6 hour time point and therefore missed. The transcript and protein data were similar, suggesting that the inhibition of CCL17 protein by RA synovial fluid was due to inhibition of transcription (Figure 4.3B).

These data data shows that the observed induction of the CCL17 protein was directly due to the increase in transcript expression, as expected, however the inhibition by synovial fluids, observed in the protein, can be attributed to the failure of transcription of CCL17. The CCL22 transcript induction did not correlate with the protein observation although it was similar, as the transcript was significantly inhibited by RA synovial fluid, but only a slight decrease in CCL22 protein. This was not significant or as robust as the effect of RA synovial fluid on the transcript. However, this analysis suggests a CCR4 ligand specific phenomenon, whereby RA synovial fluid inhibits GM-CSF stimulated monocyte

induction of these chemokines in a similar manner to LPS as observed in Chapter 3.

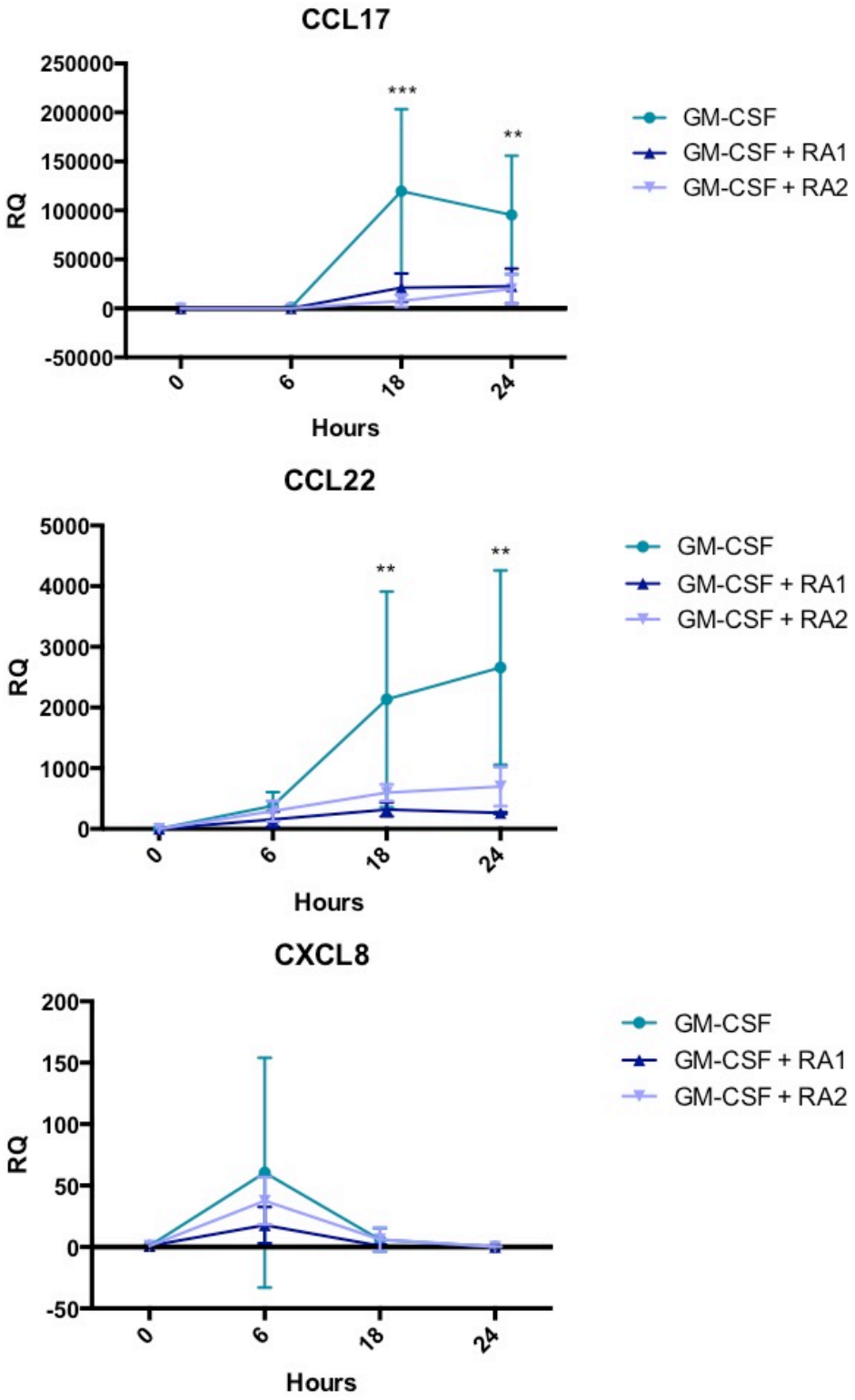


Figure 4.4 Time course assessing synovial fluid effect on transcripts.
Monocytes isolated from buffy coats (n=3) were stimulated with GM-CSF or co-stimulated with 5% RA synovial fluid (n=2). Transcripts (CCL17, CCL22 and CXCL8) were analysed at time 0, 6, 18 and 24 hours after stimulation. Analysis in comparison to GUSB housekeeping and RQ calculated in relation to time 0. Statistically analysed using 2-way ANOVA with Bonferroni's post test. ** = $p<0.01$, *** = $p<0.001$.

4.2.4 Synovial fluid inhibition of CCL17 is not via extracellular inhibition of a secreted factor.

The CCL17 transcript was up-regulated after GM-CSF stimulation between 6 and 18 hours (Figure 4.4). This was an extended period of time between GM-CSF stimulation and CCL17 transcript up-regulation. Therefore it was hypothesised that GM-CSF could be inducing the secretion of an extracellular factor, that further stimulated the monocytes and led to CCL17 transcription. The synovial fluid could then potentially inhibit this extracellular secreted factor.

To evaluate whether a secreted factor, via GM-CSF signalling, led to CCL17 secretion, conditioned media was used. Conditioned media was made by incubating monocytes, from 3 individual buffy coat donors, for 4, 6 and 8 hours with 1ng/ml GM-CSF. The media was then pooled before addition to further monocytes. Conditioned media alone caused CCL17 induction in monocytes, which was potentially due to presence of GM-CSF in the media (Figure 4.5). Therefore, to overcome this issue, monocytes were pre-treated with Mavrilimumab (CAM3001) to prevent residual GM-CSF in the media causing an up-regulation in CCL17. Monocytes pre-treated with mavrilimumab were unable to induce CCL17 secretion after conditioned media treatment.

This data suggested that the up-regulation of CCL17 transcription after GM-CSF signalling was not indirect via a secreted factor. Therefore, this data supports the theory that RA synovial fluid inhibition of CCL17 is via intracellular signalling inhibition rather than extracellular inhibition. GM-CSF leads to the transcription of the CCL17 gene via JAK2 and STAT5 (Figure 1.4). It has been shown that STAT5 leads to the transcription of the transcription factor IRF4 and it is IRF4 that induces transcription of the CCL17 gene (345). Therefore, synovial fluid could be inhibiting either transcription of IRF4 or the transcription of CCL17 by IRF4.

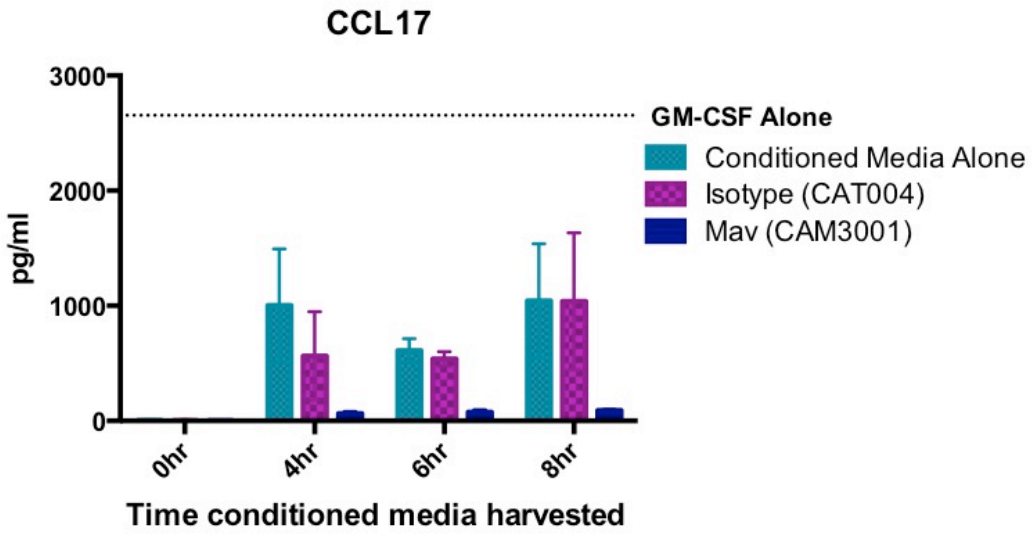


Figure 4.5 Conditioned media from GM-CSF stimulated monocytes was unable to induce CCL17.
Monocytes isolated from buffy coats (n=3) were stimulated with 1ng/ml GM-CSF for 4, 6 and 8 hours whereby supernatants were collected and pooled from the donors and frozen. Monocytes from 3 further buffy coats were pre-treated with CAM3001 or CAT004 for 30 minutes before the addition of the conditioned media for 24 hours. 1ng/ml GM-CSF used to stimulate control monocytes. Supernatants were collected and analysed for CCL17 by ELISA.

4.2.5 Delaying the co-stimulation with RA synovial fluid still inhibited GM-CSF induced CCL17.

The transcriptional time course showed how the CCL17 transcript was not up-regulated by GM-CSF until between 6 and 18 hours, however the delay from GM-CSF signalling to CCL17 transcription was not due to an intermediate extracellular secreted molecule. Therefore, inhibition of CCL17 by RA synovial fluid was intracellular, but the time at which the inhibition was occurring was unknown. To assess when the inhibition by synovial fluid occurred, a further time course was performed. This time course involved adding synovial fluid at multiple time points after the monocytes were stimulated with GM-CSF, with the level of CCL17 protein analysed after 24 hours. With the addition of RA synovial fluid at 0 hours, CCL17 was significantly inhibited (Figure 4.6). However, when the RA synovial fluid was added at up to 4 hours after the initial stimulation with GM-CSF, the CCL17 transcript was still inhibited. At 6 hours after GM-CSF stimulation, the RA synovial fluid was less able to inhibit CCL17 and there was an increase in the variability between the monocyte donors. When the synovial fluid was added to the GM-CSF stimulated monocytes after 8 hours, there was no longer any inhibition and much more variable responses from each monocyte donor.

This data implies that the signalling cascade downstream of GM-CSF receptor resulting in CCL17 induction is complex, allowing the synovial fluid to have ability to inhibit CCL17 transcription despite addition 4 hours after GM-CSF stimulation. The synovial fluid potentially inhibits factors that are much further downstream of GM-CSF receptor signalling, or leads to promoter silencing or transcript degradation.

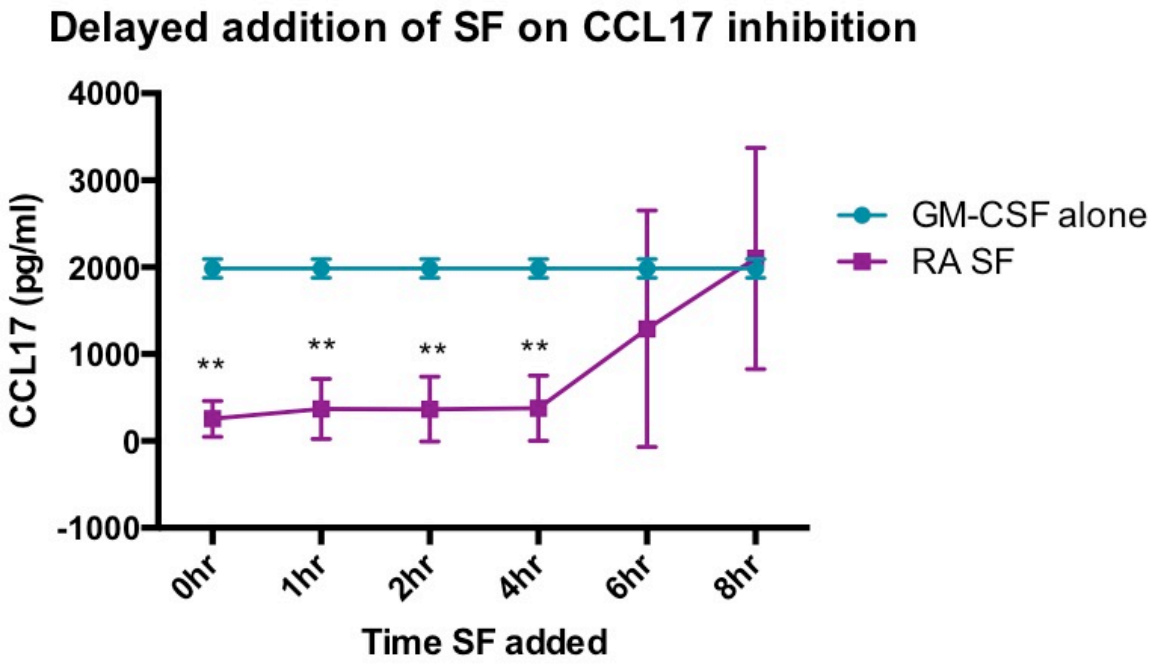


Figure 4.6 Time course analysing when synovial fluid inhibited CCL17
Monocytes isolated from buffy coats (n=3) were stimulated with 1ng/ml GM-CSF. RA synovial fluid (n=1) was added at 0, 1, 2, 4, 6 or 8 hours after the initial GM-CSF stimulation. Supernatants were taken at 24 hours after GM-CSF stimulation and analysed for CCL17 by ELISA. Statistically analysed using a 2-way ANOVA with Bonferroni's post test. ** = P<0.01.

4.2.6 Synovial fluid inhibition of CCL17 is TLR independent.

RA synovial fluid inhibition of GM-CSF induced CCL17 was a robust finding that occurred in all analysed synovial fluids to all monocyte donors. To have such a consistent effect suggests a factor present within all RA synovial fluids. The mechanism of this inhibition was unknown, but as the synovial milieu contains many DAMPs that bind TLRs, it was hypothesised that TLRs could be mediating this inhibition. The previous chapter analysed the TLR induced inhibition of CCL17, therefore the synovial fluid could be influencing the GM-CSF induction of CCL17 in the same mechanism. Initially, it was investigated whether TLR2 or TLR4 were involved, so inhibiting antibodies were used. Initially a dose response was tested to determine the concentration of antibodies to use in the assay. The inhibiting anti-TLR2 and anti-TLR4 were pre-incubated with monocytes for 30 minutes prior to the addition of the relevant TLR ligand (LPS or FSL1). This pre-incubation was to allow the antibodies to bind to the receptor to prevent competition with the ligand. With no antibody, after GM-CSF stimulation, both LPS and FSL1 inhibited CCL17, as predicted (Figure 4.7A). The IgG1 isotype had no effect on the inhibition of CCL17 by LPS or FSL1, which was to be expected. The anti-TLR4 antibody successfully prevented LPS from inhibiting GM-CSF induced CCL17 secretion at all concentrations analysed. The anti-TLR2 antibody was not as successful at preventing the binding of FSL1 to TLR2. At 1μg/ml, there was no difference between the FSL1 alone and the pre-treated with the antibody. At 3μg/ml and 10μg/ml there was a slight increase in the level of CCL17 secreted, suggesting that it had partially inhibited TLR2. For the following assay, both antibodies were used at 10μg/ml.

To examine whether the anti-TLR2 or anti-TLR4 antibodies could reverse the inhibition of CCL17 observed with RA synovial co-stimulation with GM-CSF, they were used both individually or in combination. Monocytes were pre-treated with the anti-TLR2, anti-TLR4 or both for 30 minutes prior to the co-stimulation with RA synovial fluid. Stimulation with GM-CSF and RA synovial fluid significantly inhibited the ability of monocytes to secrete CCL17, as previously observed (Figure 4.3). The anti-TLR2 and anti-TLR4 antibodies individually or in combination were unable to reverse the inhibition by RA synovial fluid. However, as the anti-TLR2 antibody did not have good inhibition, this was potentially only anti-TLR4 antibody that was unable to reverse the inhibition by RA synovial fluid

(Figure 4.7). This suggests that RA synovial fluid inhibition of GM-CSF driven CCL17 is either via a different TLR or another distinct mechanism.

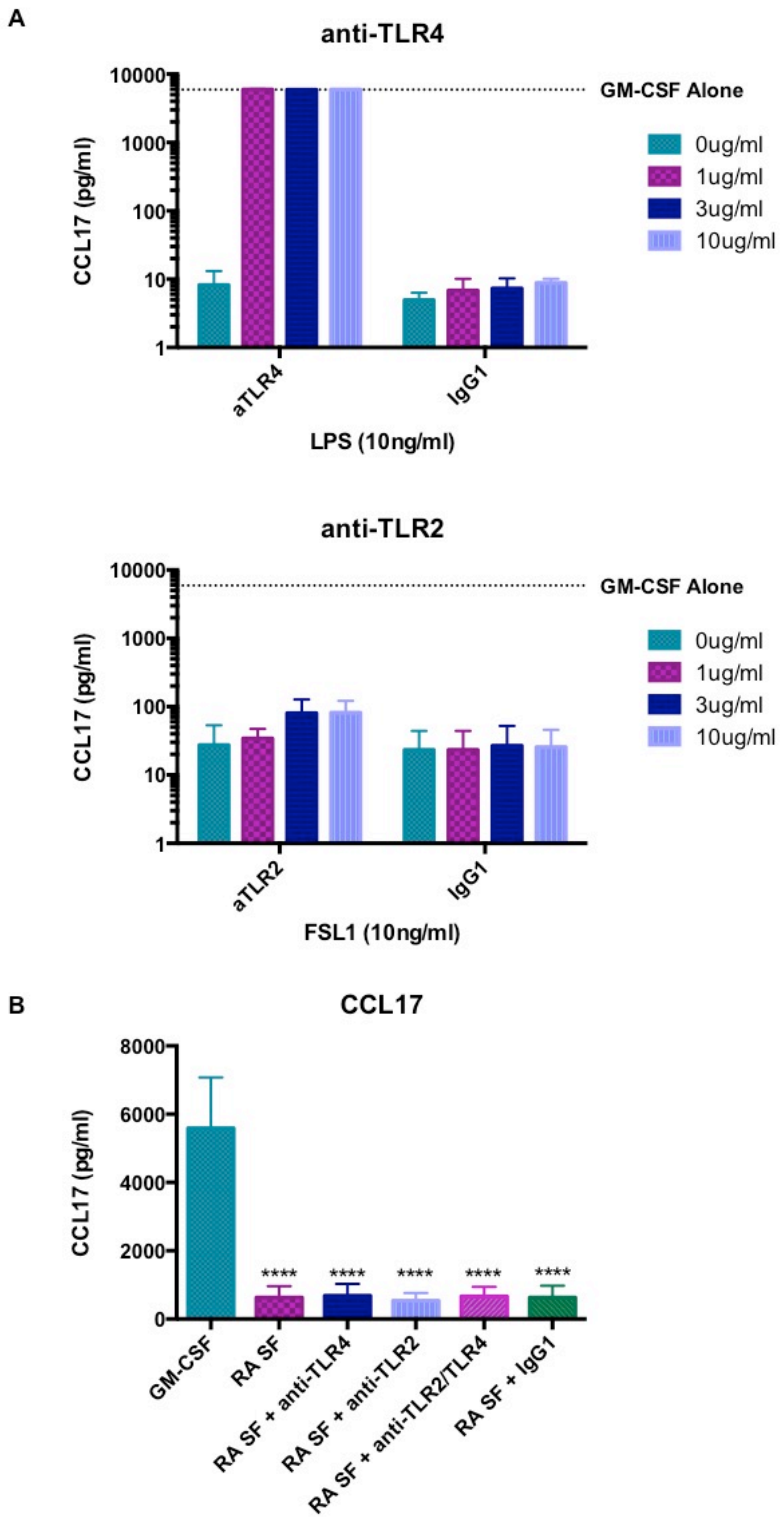


Figure 4.7 TLR2 and TLR4 inhibiting antibodies could not reverse the CCL17 inhibition by RA synovial fluid.
A) Monocytes isolated from buffy coats (n=2) were pre-treated with increasing doses of anti-TLR4, anti-TLR2 antibodies, or with the relevant isotype, for 30 minutes. Monocytes were then stimulated with 1ng/ml GM-CSF with either 10ng/ml LPS (TLR4) or 10ng/ml FSL1 (TLR2) for 24 hours. B) Monocytes from buffy coats (n=3) were pre-treated with 10μg/ml anti-TLR4, anti-TLR2 antibodies, in combination or with relevant isotype, IgG1 for 30 minutes. Monocytes were further stimulated with 1ng/ml GM-CSF or co-stimulated with 5% RA synovial fluid (n=3). The supernatants were collected and analysed for CCL17 by ELISA. B) statistically analysed by 2-way ANOVA with Bonferonni's post test. **** = $p<0.0001$.

The RA synovial fluid inhibition of CCL17 was not reversed using anti-TLR2 or anti-TLR4 antibodies, either individually or in combination. Therefore, to determine whether inhibition of CCL17 by RA synovial fluid was due to multiple TLR agonists within the synovial fluid, MyD88/TRIF double knockout mice were used, which are unable to signal through any TLR pathway. MyD88/TRIF knockout mouse monocytes were isolated from the bone marrow and stimulated with GM-CSF and RA synovial fluid. The purity of the monocytes isolated from the bone marrow, was analysed by flow cytometry to ensure the cultured cells were pure. The total bone marrow contained 14.6% cells that were CD11b, Ly6C positive and the isolated monocytes were 83.9% pure, which was within the expected range (Figure 4.8A-D).

Upon GM-CSF stimulation, the wildtype monocytes secreted CCL17, which was comparable with human monocytes. In the wildtype monocytes, GM-CSF induced CCL17 secretion was inhibited by RA synovial fluid (Figure 4.8E). LPS was used as a control as it also inhibited CCL17 in wildtype monocytes. GM-CSF stimulation of the MyD88/TRIF double knockout monocyte caused secretion of CCL17, however, this was at lower levels in comparison to the wildtype. LPS was unable to inhibit the induction of CCL17, confirming that these monocytes were unable to signal via TLRs. Interestingly, RA synovial fluid caused inhibition of CCL17 in the MyD88/TRIF knockout monocytes. Although, this was not a significant inhibition due to the variation in the GM-CSF alone condition, the level of CCL17 secreted in the RA synovial fluid co-stimulation was lower than the wildtype control.

RA synovial fluid still had the ability to inhibit GM-CSF induced CCL17 in MyD88/TRIF double knockout monocytes. This suggests the mechanism causing inhibition of CCL17 by RA synovial fluid is TLR independent.

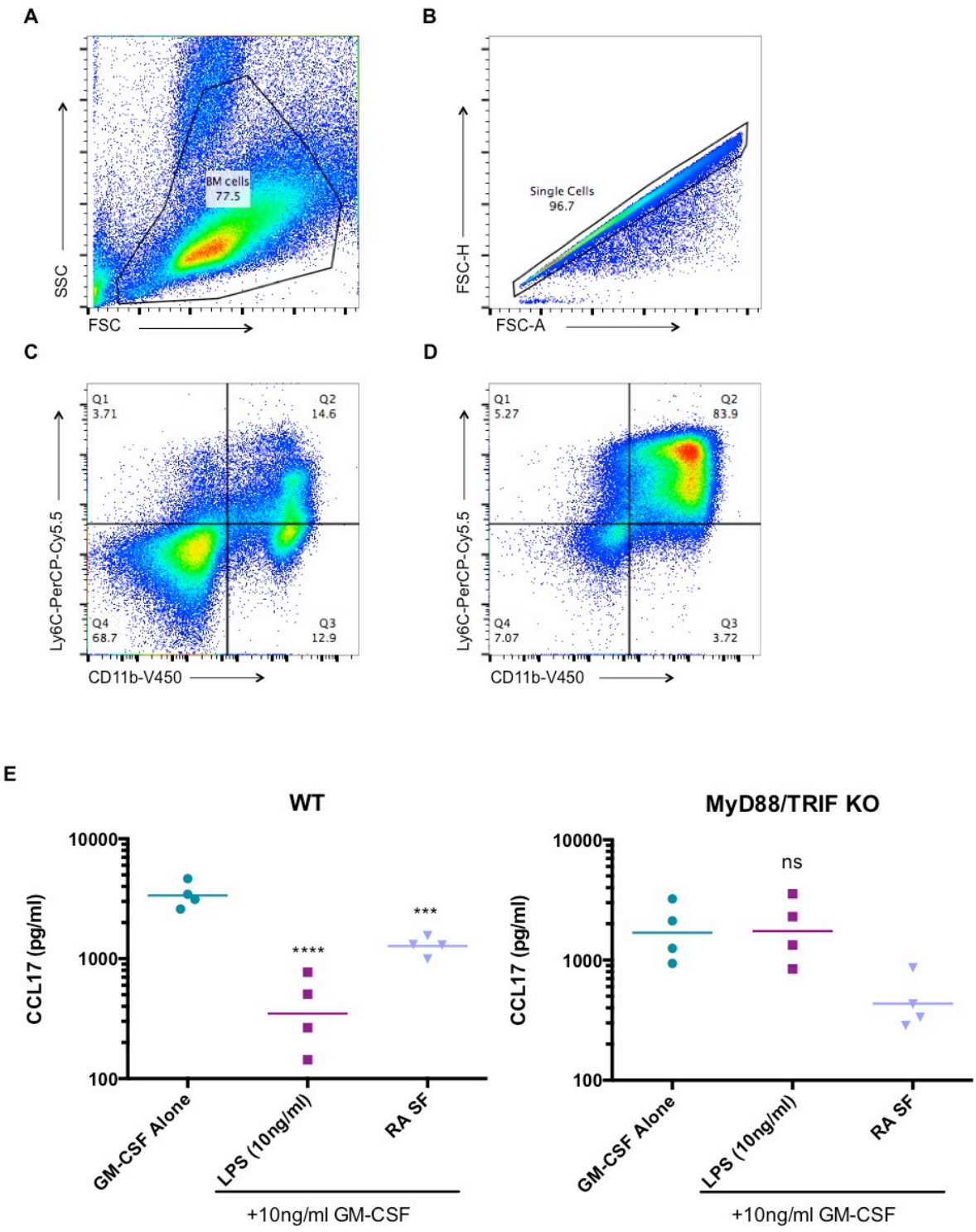


Figure 4.8 MyD88/TRIF knockout mouse monocyte GM-CSF induced CCL17 induction inhibited by RA synovial fluid.
Monocytes isolated from bone marrow were assessed for purity. A) Bone marrow cells which were further gated for single cells (B). The CD11b and Ly6C positive cells were analysed in total bone marrow (C) and the isolated monocytes (D). Wildtype (C57BL/6) (n=4) and MyD88/TRIF double knockout (n=4) mouse monocytes were isolated from bone marrow and stimulated with 10ng/ml GM-CSF or co-stimulated with 10ng/ml LPS or 5% RA synovial fluid (n=1) (E). Statistically analysed using one-way ANOVA with Boferroni's post test. *** = $p<0.001$, **** = $p<0.0001$, ns = not significant.

4.2.7 IFNα and small immune complexes can inhibit the induction of CCL17 by GM-CSF stimulation.

Synovial fluid not only contains endogenous TLR ligands, but also high levels of immune complexes and type I interferons, and it was hypothesised that other factors could lead to the inhibition of GM-CSF induced CCL17. Therefore, we investigated small immune complexes and IFNα and the impact on CCL17, when co-stimulated with GM-CSF. Small immune complexes (SIC) were formed by making an IgG-SpA complex, at a ratio of 2 IgG to 4 SpA molecules, using a pre-defined method from the group (Section 2.3.1). Monocytes co-stimulated with SIC had a significant down-regulation in the level of GM-CSF induced CCL17 produced, comparable with the level of inhibition observed in the RA synovial fluid (Figure 4.9A). The IgG and SpA alone controls were unable to inhibit CCL17, suggesting the molecules had to form immune complexes in order to inhibit CCL17. This suggests that Fc Receptor-mediating signalling, via immune complexes, can also contribute to the inhibitory milieu that results in the inhibition of GM-CSF driven CCL17.

To establish whether immune complexes are a component of the inhibitory factor in synovial fluid causing the down-regulation of CCL17, immune complexes were depleted from RA synovial fluid. Using magnetic protein G, protein A or protein A/G/L beads, synovial fluids were depleted and compared to their matched, non-depleted synovial fluid to assess their ability to inhibit CCL17 in monocytes after GM-CSF stimulation. There was no difference between protein G or protein A depleted RA synovial fluid in comparison to complete synovial fluid, despite the non-significant difference between GM-CSF alone and protein G depleted synovial fluid (Figure 4.9B). This was in relation to their ability to inhibit CCL17. Protein A/G/L depleted synovial fluid caused a greater inhibition of CCL17 than the non-depleted synovial fluid. This was an unexpected finding as depleting the synovial fluid of immune complexes would be expected to potentially reverse the inhibition. The level of inhibition was comparable to the LPS inhibition, therefore it was suggested that there was potential endotoxin contamination in the depleted synovial fluid, through the reagents used for the depletion. To assess whether this was the case, the depleted and complete synovial fluids were analysed for endotoxin using the kinetic limulus assay. The results of this assay showed that there was no contamination, compared with the

LPS positive control (Figure 4.9C). Therefore, the greater inhibition observed in the protein A/G/L depleted synovial fluid was not due to endotoxin contamination.

To elucidate whether another major component of RA synovial fluid could cause inhibition of GM-CSF induced CCL17 that would not have been removed after depletion of immune complexes, type I interferons were analysed. To investigate this, monocytes were co-stimulated with GM-CSF and 10ng/ml or 100ng/ml IFNα. LPS was used as a positive control. At both 10ng/ml and 100ng/ml, IFNα inhibited the GM-CSF driven CCL17 (Figure 4.10). At 100ng/ml, the level of inhibition was significant and at a similar level to that of LPS. The ability of IFNα to cause inhibition of CCL17 supports the concept that there are multiple factors within synovial fluid causing the inhibition as TLR ligands also have this ability.

These data suggest that there are multiple methods of causing inhibition of GM-CSF induced CCL17 including by IFNα and small immune complex co-stimulation. This suggests that as synovial fluid contains many of the factors that have been found to cause inhibition, there are several mechanisms in which synovial fluid can employ to cause inhibition of CCL17 after GM-CSF stimulation of monocytes.

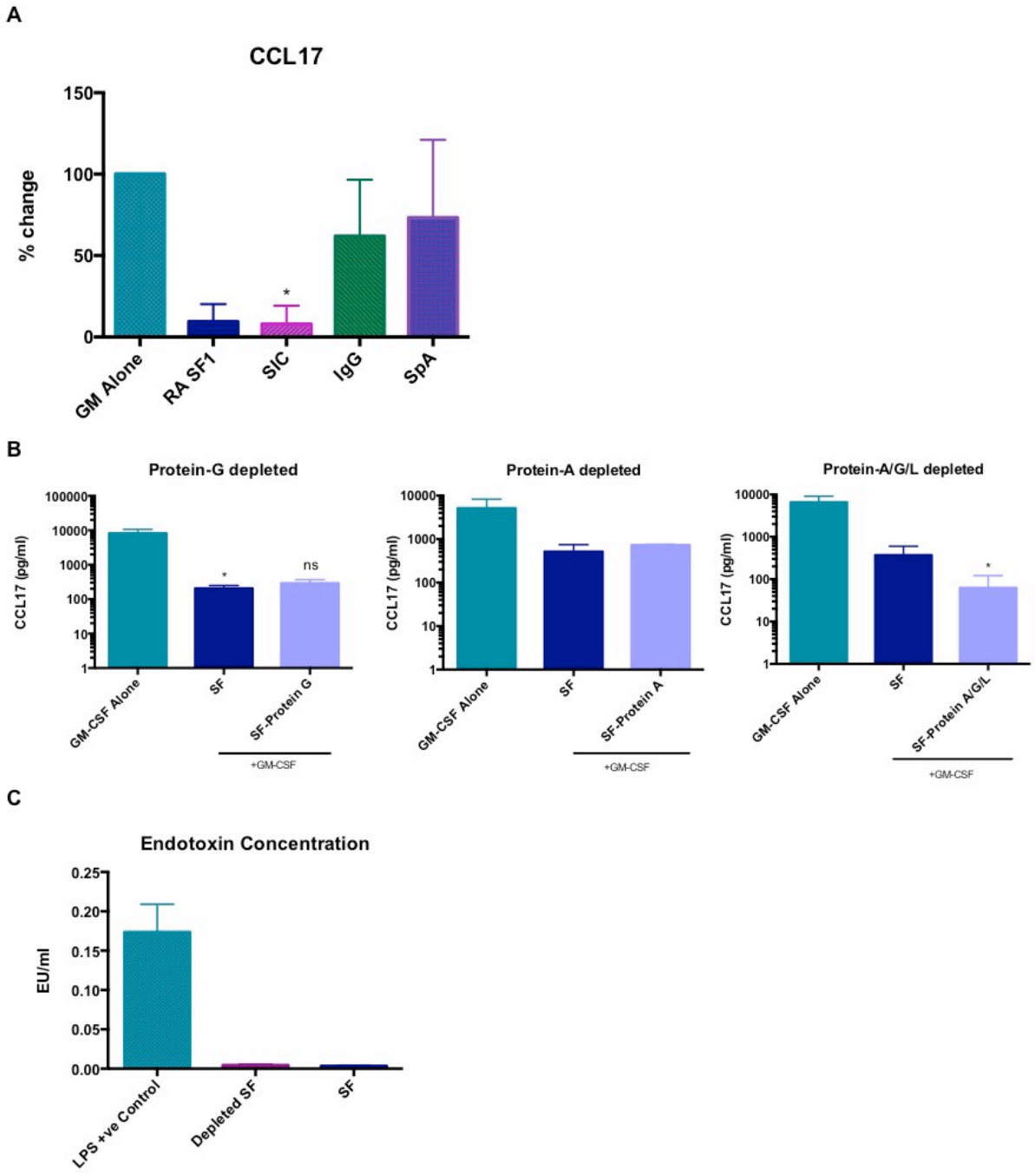


Figure 4.9 Immune Complexes inhibit CCL17, but depletion was unable to reverse the inhibition.

A) Monocytes isolated from buffy coats (n=3) were stimulated with 1ng/ml GM-CSF, with or without RA synovial fluid (n=1) or small immune complexes formed from complexing 2 human IgG molecules with 4 SpA molecules. IgG and SpA used alone as controls. B) Protein A, Protein G or Protein A/G/L magnetic beads were used to deplete RA synovial fluids (protein A n=3, protein G n=2, protein A/G/L n=5). Monocytes isolated from buffy coats (n=3) were stimulated with 1ng/ml GM-CSF and RA synovial fluids with matched depleted synovial fluids. Supernatants were collected after 24 hours and analysed for CCL17 by ELISA (A and B). C) Depleted and non-depleted synovial fluids were analysed for endotoxin contamination by the kinetic limulus assay. 10ng/ml LPS was used as a positive control. Statistically analysed using Kruskal-Wallis non-parametric test with Dunn's post test. * = $p<0.05$.

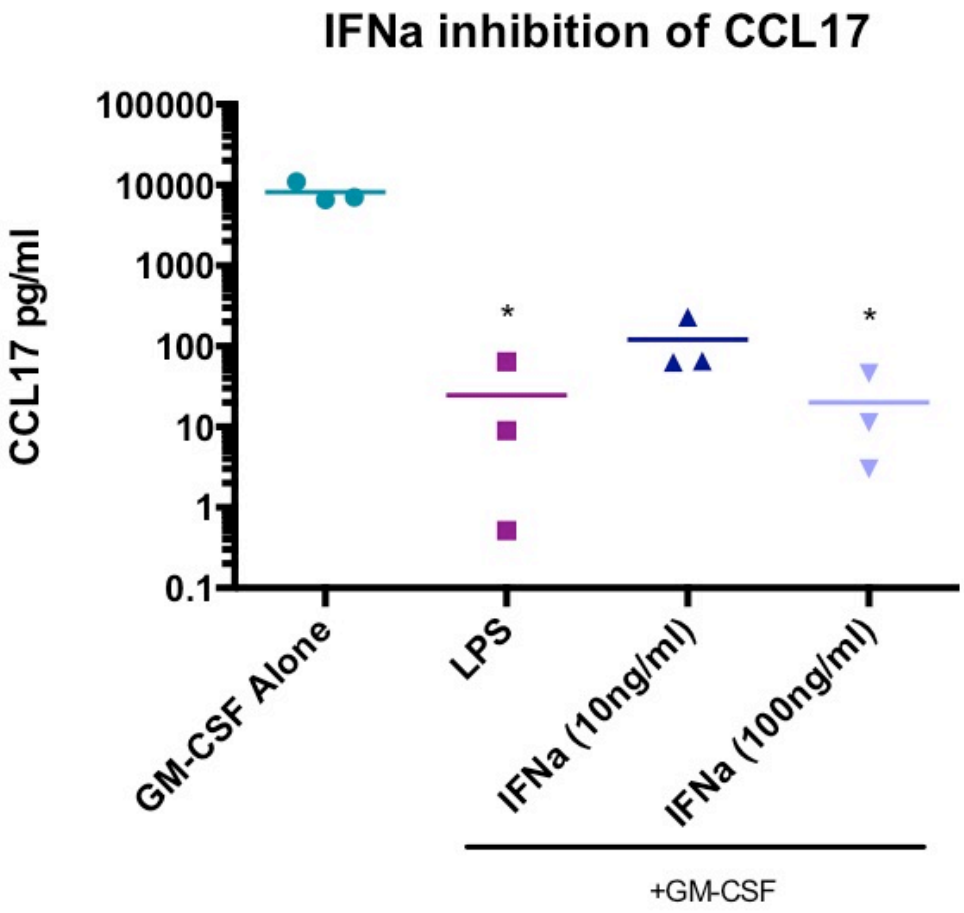


Figure 4.10 IFNα inhibited GM-CSF induced CCL17 in monocytes.
Monocytes isolated from buffy coats (n=3) were stimulated with 1ng/ml GM-CSF with or without 10ng/ml LPS, 10 or 100ng/ml IFNα for 24 hours. Supernatants were then analysed for CCL17 by ELISA. Statistically analysed using Kruskal-Wallis non-parametric test with Dunn's post test. * = $p<0.05$.

4.2.8 Investigation into GM-CSF and CCL17 associated signalling.

The previous data demonstrated that RA synovial fluid was able to inhibit GM-CSF driven CCL17 and multiple factors within synovial fluid could potentially cause this inhibition. To investigate the underlying mechanism causing synovial fluid-mediated inhibition of this pathway, associated signalling pathways were investigated. Despite the ability of synovial fluid to inhibit CCL17 4 hours after initial GM-CSF stimulation, STAT5 which is downstream of GM-CSFR signalling (306) was assessed to confirm that it was not involved. The binding site for the transcription factor, STAT6, has been identified on the CCL17 locus, and therefore could have the potential to be inhibited by synovial fluid (307). In order to elucidate the signalling pathway involved in the inhibition of CCL17 by RA synovial fluid, pSTAT5 and pSTAT6 were analysed.

Monocytes were incubated with synovial fluid for 4 hours prior to stimulation with GM-CSF for 10 minutes. With no GM-CSF stimulation, the pSTAT5 Mean Fluorescent Intensity (MFI) was 115 ± 17 (Mean ± SD) in $CD14^+$ monocytes, however with GM-CSF stimulation, there was a significant increase in pSTAT5 expression to 233 ± 76 (Mean ± SD) (Figure 4.11). Pre-incubation with RA synovial fluid had varying effects on the MFI of pSTAT5 in $CD14^+$ monocytes. Interestingly, this was not due to the synovial fluid used, but the monocyte donor that caused the variability, suggesting that monocytes from different donors respond differently to the stimuli in RA synovial fluid. The $CD16^+$ monocytes were much more variable when stimulated with GM-CSF as some increased expression of pSTAT5, however this was not a consistent finding. The pre-incubation with RA synovial fluid also made no change to the level of pSTAT5. The analysis of pSTAT6 in monocytes showed that unstimulated monocytes have a residual level pSTAT6 (Figure 4.12). In $CD14^+$ monocytes this was an MFI of 433 ± 8 (Mean ± SD) however, in $CD16^+$ monocytes this was much lower at 185 ± 8 (Mean ± SD). There was a slight increase in pSTAT6 expression in GM-CSF stimulated $CD16^+$ monocytes, however this was very small and there was no change to the $CD14^+$ monocytes. Interestingly, both RA synovial fluids analysed caused a down-regulation in pSTAT6, below the unstimulated level in the $CD14^+$ monocytes but not the $CD16^+$ monocytes. This could potentially suggest that STAT6 is involved in the pathway of inhibition, however this was only investigated in 2 monocyte

donors, so more work needs to be performed to investigate the validity of this finding.

Analysis of signalling pathways associated with GM-CSF signalling and CCL17 transcription suggest that STAT5 is not involved in RA synovial fluid inhibition of CCL17 transcription. STAT6, which is potentially the transcription factor for CCL17 after GM-CSF stimulation, could be involved in the down-regulation of CCL17 transcription after RA synovial fluid stimulation, however further work is required to evaluate this.

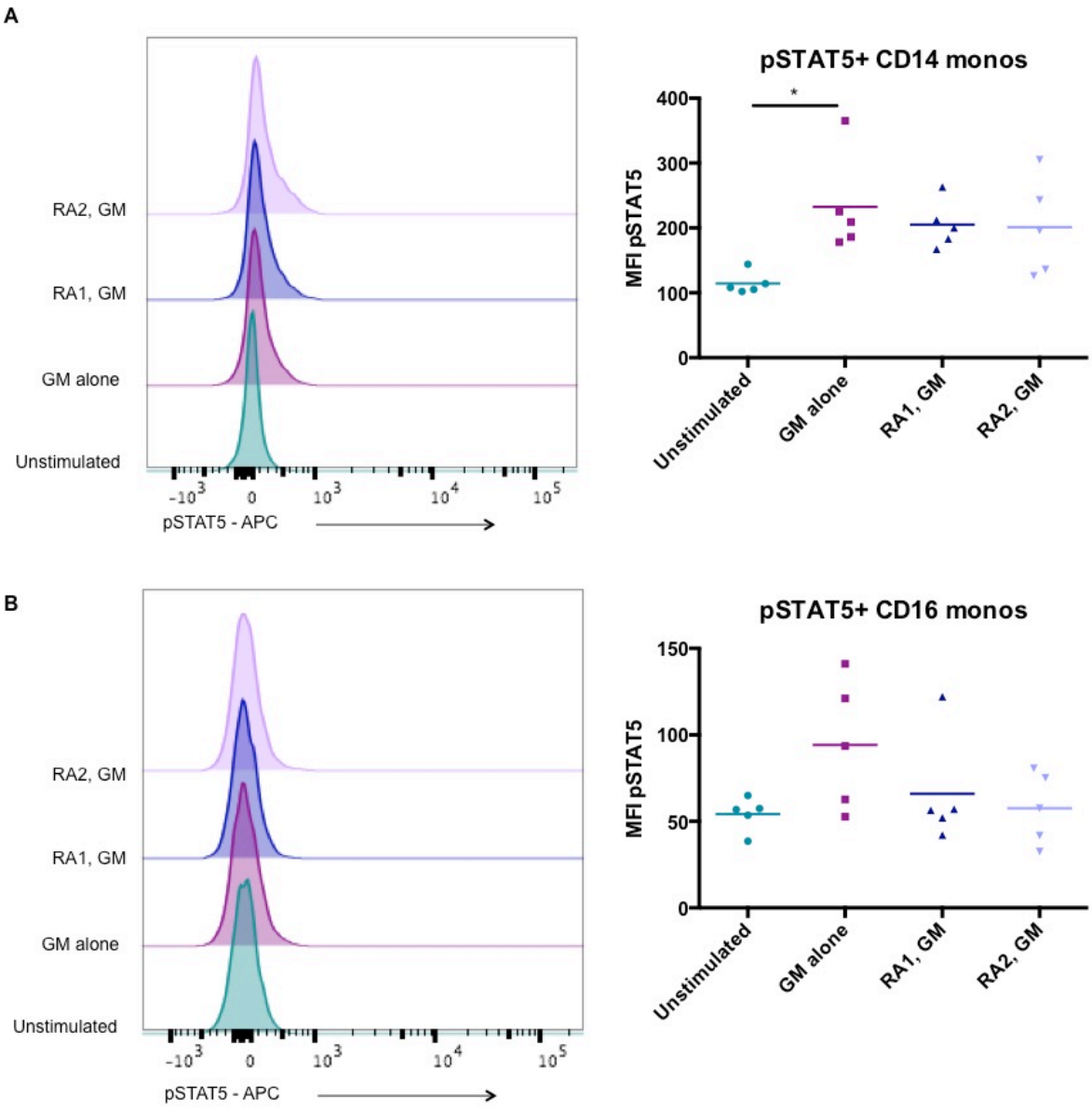


Figure 4.11 pSTAT5 analysis after RA synovial fluid and GM-CSF stimulation.
Monocytes isolated from buffy coats (n=5) were incubated either in media alone or with 5% RA synovial fluid for 4 hours. Monocytes were cell surface stained, and stimulated for 10 minutes with GM-CSF before immediate fixation. After pSTAT5 staining, samples were analysed on the BD LSRII. A) Representative histograms of pSTAT5 gated on CD14 positive cells. Pre-gating on PBMCs and $CD3^{-}CD19^{-}$. Graphically represented in right hand pane. B) Representative histograms of pSTAT5 gated on CD16 positive cells. Pre-gating on PBMCs and $CD3^{-}CD19^{-}$. Graphically represented in right hand pane. Gating determined by FMOs. Statistically analysed using One-way ANOVA with Bonferonni's post test. * = $p<0.05$.

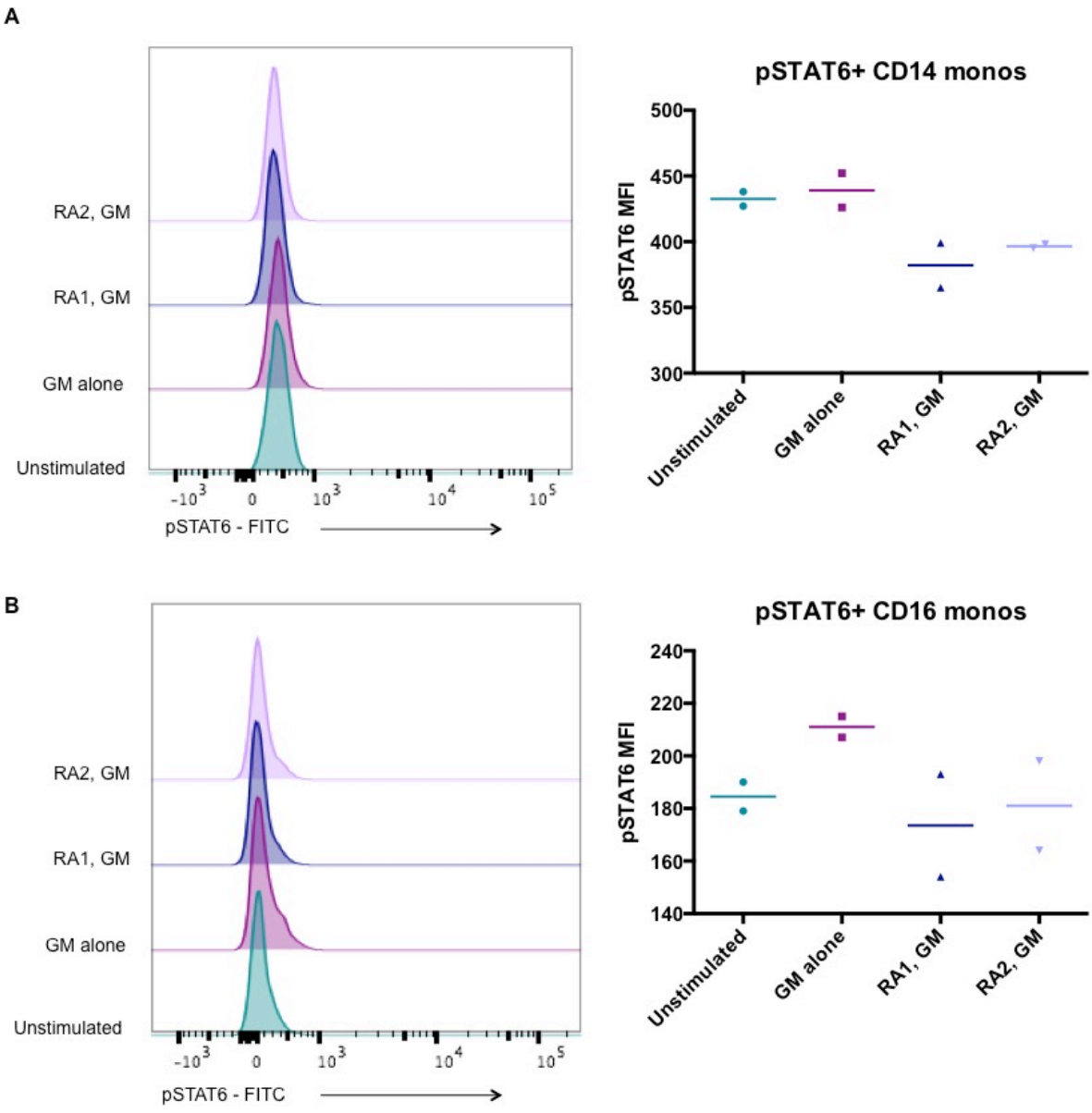


Figure 4.12 pSTAT6 analysis after RA synovial fluid and GM-CSF stimulation.
Monocytes isolated from buffy coats (n=2) were incubated either in media alone or with 5% RA synovial fluid for 4 hours. Monocytes were cell surface stained, and stimulated for 10 minutes with GM-CSF before immediate fixation. After pSTAT6 staining, samples were analysed on the BD LSRII. A) Representative histograms of pSTAT6 gated on CD14 positive cells. Pre-gating on PBMCs and $CD3^{-}CD19^{-}$. Graphically represented in right hand pane. B) Representative histograms of pSTAT6 gated on CD16 positive cells. Pre-gating on PBMCs and $CD3^{-}CD19^{-}$. Graphically represented in right hand pane. Gating determined by FMOs.

4.2.9 Investigation into CCL17 inhibition by RA synovial fluid through the inhibition of JAK1 and JAK3.

The analysis of STAT5 and STAT6 phosphorylation supported the theory that the factor in synovial fluid causing inhibition was not directly interfering with GM-CSFR signalling upstream of STAT5. However, STAT6, a transcription factor known to bind to the CCL17 promoter, is potentially down-regulated by RA synovial fluid. It is unknown how the factor within RA synovial fluid is causing inhibition of CCL17; therefore the mechanism was further investigated. Therefore, in order to further dissect potential signalling pathways involved, Tofacitinib, a JAK1/3 inhibitor was used to pre-treat monoyctes. JAK1 is upstream of STAT6 signalling (308), therefore, this inhibitor would give us a potential indication as to whether JAK signalling was involved in the inhibition. However, Tofacitinib is known to have off-target effects by slight inhibition of JAK2. As GM-CSFR signals via JAK2, the effect of Tofacitinib was analysed by measuring CCL17 after increasing doses of Tofacitinib and GM-CSF stimulation.

With no pre-treatment with Tofacitinib, GM-CSF was able to induce between 600 and 6000pg/ml CCL17 (Figure 4.13). This was variable due to the monocyte donors. With the vehicle control (DMSO) pre-treatment, there was no difference to the level of CCL17 secreted. However, there was a dose dependent decrease in CCL17 produced with an increase in concentration of Tofacitinib. This confirmed that there were some off-target effects of Tofacitinib on JAK2. Therefore, to reduce the impact of Tofacitinib on GM-CSF signalling and to ensure CCL17 induction after GM-CSF stimulation, the 2 lower doses of Tofacitinib were used for further experiments. Previous data have shown that 100nM and 300nM both effectively inhibited JAK1/3 pathways in T cells and DCs, therefore, these doses were assumed viable to use in this experiment (198,309).

In order to establish whether Tofacitinib was able to reverse the observed inhibition by RA synovial fluid, monocytes were pre-treated with 100nM, 300nM Tofacitinib or vehicle control (0.001% DMSO) and GM-CSF for 30 minutes. Following treatment, monocytes were further stimulated with RA synovial fluid or LPS. Monocytes pre-treated with the vehicle control secreted 3088pg/ml ± 3194 (Mean ± SD) CCL17 (Figure 4.14). There was a large level of variability, therefore to compare each individual monocyte donor to the GM-CSF alone

control, percentage change was also calculated with GM-CSF alone at 100% and the other conditions relative to this. LPS co-stimulation caused a 90% (mean ± SD) reduction in CCL17 secretion and RA synovial fluid co-stimulation led to greater than 60% (mean ± SD) inhibition. After monocytes were pre-treated with 100nM Tofacitinib, GM-CSF caused the secretion of 1045pg/ml ± 1159 (mean ± SD), which was lower than the vehicle control and also variable between monocyte donors. LPS co-stimulation with 100nM Tofacitinib caused 80% inhibition (mean ± SD) of CCL17 secretion and RA synovial fluid also inhibited CCL17 by greater than 60% (mean ± SD). Pre-treatment of monocytes with 300nM Tofacitinib led to a reduction in GM-CSF stimulated CCL17 as monocytes only secreted 184pg/ml ± 100 (mean ± SD). LPS only caused a 38% (mean ± SD) reduction in CCL17 secretion, whereas RA synovial fluid inhibited CCL17 production by 50% (mean ± SD).

This analysis suggests that pre-treatment of monocytes with Tofacitinib at 300nM caused less inhibition of CCL17 when monocytes were co-stimulated with GM-CSF and LPS. Therefore JAK1 and/or JAK3 could potentially have a role in LPS inhibition of CCL17. However, the actual level of CCL17 after LPS stimulation was similar across all pre-treatment conditions, so the outcome of this experiment is difficult to conclude.

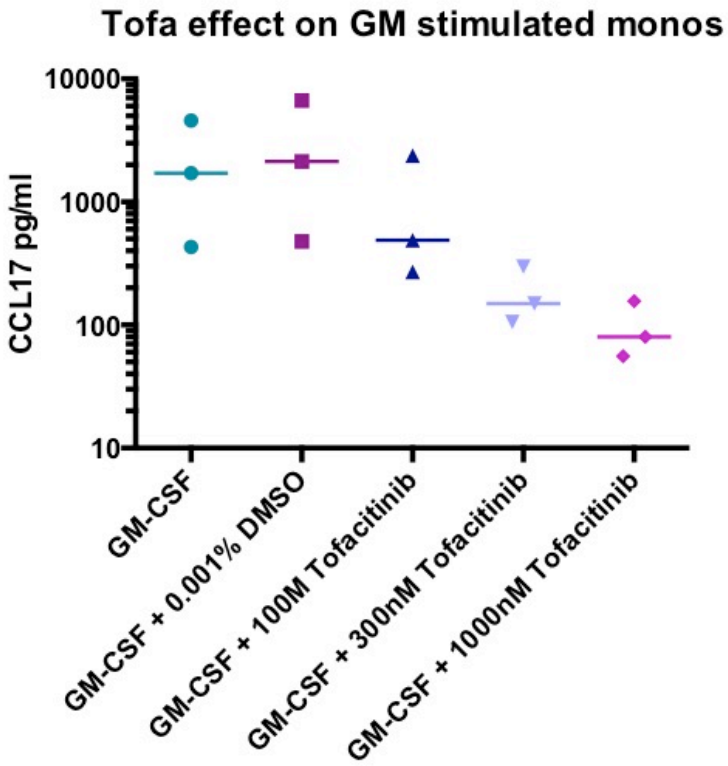


Figure 4.13 Tofacitinib effect on GM-CSF induction of CCL17
Monocytes isolated from buffy coats (n=3) were stimulated with Tofacitinib at 100nM, 300nM or 1000nM in the vehicle control (0.001% DMSO) and 1ng/ml GM-CSF for 24 hours. Supernatants were collected and analysed for CCL17 by ELISA. Horizontal Bar represents the median. Statistically analysed using Kruskal-Wallis non-parametric test. No significance identified.

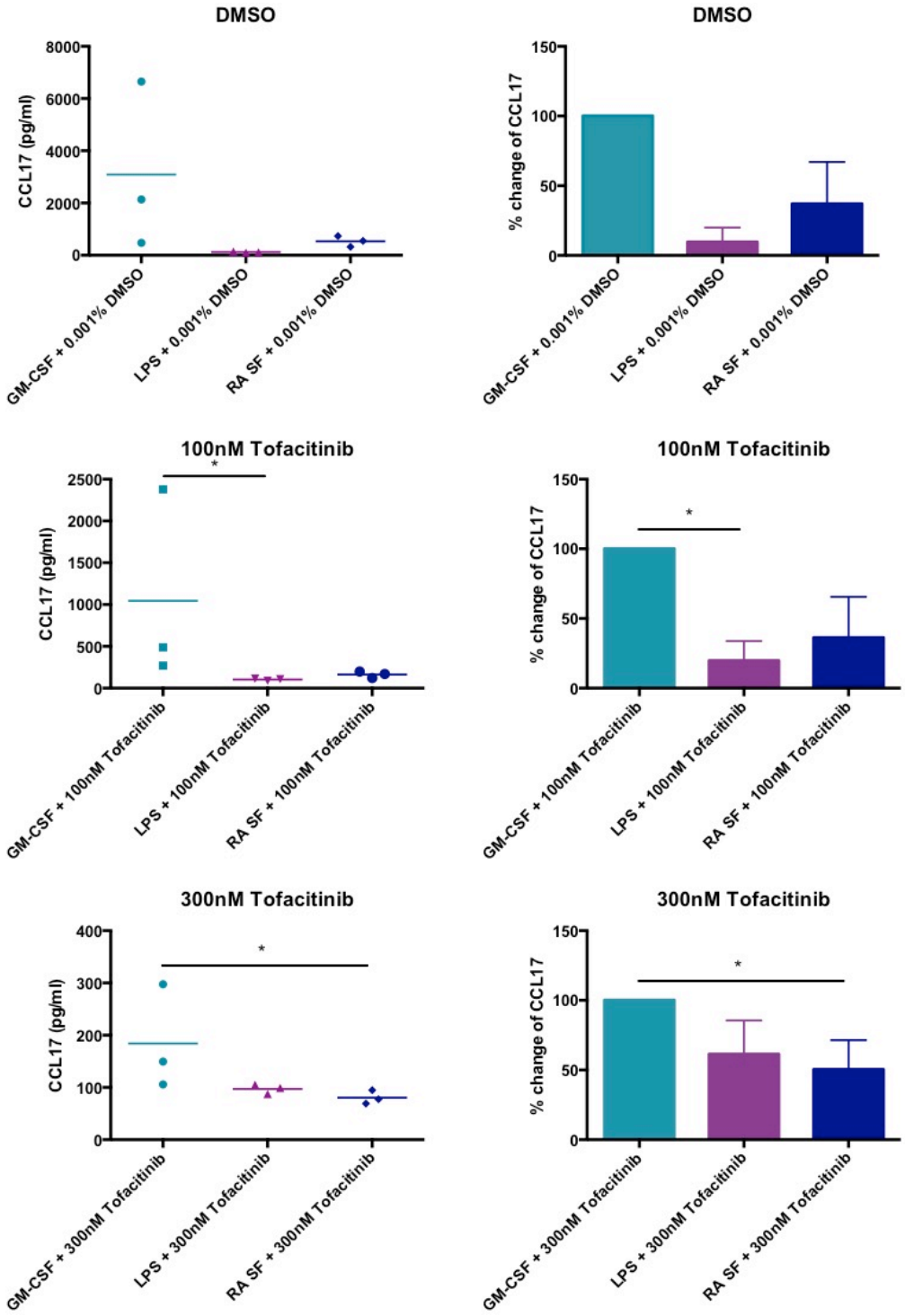


Figure 4.14 CCL17 inhibition analysed using Tofacitinib
Monocytes isolated from buffy coats (n=3) were stimulated with 1ng/ml GM-CSF and treated with Tofacitinib at 100nM, 300nM or 1000nM in vehicle control (0.001% DMSO) for 30 minutes. DMSO used as a control. Monocytes were then treated with 10ng/ml LPS or 5% RA synovial fluid (n=3). After 24 hours, supernatants were collected and analysed for CCL17 by ELISA. Data analysed as actual values, and percentage change in relation to GM-CSF alone for each donor. Horizontal Bar represents the median. Statistically analysed using Kruskal Wallis non-parametric test with Dunn's post test. * = $p<0.05$

4.3 Discussion

There have been several studies profiling the contents of synovial fluids from proteomics to metabolomics, to try and detect whether there are any synovial biomarkers (84,132,133). The analysis of synovial fluid aimed to assess the level of cytokines in comparison to OA. Most inflammatory cytokines and chemokines were present in RA synovial fluid and in some cases in OA synovial fluid (Figure 4.1 and Figure 4.2). There were no statistically significant differences between RA and OA synovial fluids, which was due to the low numbers of synovial fluids analysed, and the heterogeneity between different fluids. To improve this, more RA and OA synovial fluids would have to be profiled. This analysis clarified the large differences between individual patient RA synovial fluids. This suggested that the effect of the RA synovial environment on GM-CSF induced monocytes had the potential to be extremely variable dependent on the stimuli present in the synovial fluids.

We investigated monocytes stimulated with GM-CSF in a synovial environment to determine whether the stimuli within the synovial fluid such as cytokines, DAMPs or immune complexes would exacerbate the secretion of chemokines. Analysing the secretion of chemokines after GM-CSF and RA synovial fluid or OA synovial fluid co-stimulation showed that there were great differences between the response of monocytes to stimuli as well as the differences between the individual synovial fluids. The only robust response from RA synovial fluid was in its inhibition of CCL17 both at 2.5% and 5% (Figure 4.3B). OA synovial fluid was variable in its ability to inhibiting CCL17. Interestingly, co-stimulation of GM-CSF with OA synovial fluid, caused differing effects on CCL17 production. This suggests that the factor within the synovial fluid causing the inhibition of CCL17 is robustly found in RA synovial fluids, and only occasionally in OA synovial fluid. However, the OA synovial fluids significantly inhibited GM-CSF induced CCL3 and CCL4, which RA synovial fluid had no impact on. This suggests an OA specific phenomenon and disease specific factors in synovial fluid that impact monocyte inflammatory secretions. However, more work needs to be done to interrogate the properties of OA SF that can lead to inhibition of CCL3 and CCL4. In this study, we were particularly intrigued by the inhibition of CCL17 by RA synovial fluid. This was interesting as the previous chapter analysed how CCL17 was inhibited by TLR ligands.

Similarly to LPS in the previous chapter, RA synovial fluid significantly inhibited the induction of CCL17 transcript at 18 and 24 hours, suggesting a comparable mechanism (Figure 4.4). The other ligand for CCR4 is CCL22, and interestingly the CCL22 transcript is also inhibited by RA synovial fluid. This suggests a CCR4 specific effect that could impact the contribution of monocyte driven recruitment of $CCR4^+$ cells into the synovium. The up-regulation of the CCL17 transcript after GM-CSF stimulation was between 6 and 18 hours. This is an extended time period from stimulation to transcription, suggesting a potential indirect signalling pathway either intracellularly or via a secreted factor that further stimulates the cell. To investigate whether GM-CSF stimulates the secretion of a factor that then induces the monocyte to secrete CCL17, conditioned media was used to analyse this (Figure 4.5). Several time points were used to take media after the initial GM-CSF stimulation of monocytes. As residual GM-CSF would still be in the media, Mavrilimumab was used to pre-treat monocytes and within the media itself to prevent any residual GM-CSF causing the up-regulation of CCL17. This experiment showed, however, that at all analysed time points, the conditioned media with Mavrilimumab was unable to induce CCL17. Therefore, the GM-CSF signalling pathway to CCL17 transcription must be intracellular.

To begin to understand the ability of synovial fluid to intracellularly inhibit the transcription of CCL17, we investigated stimulating monocytes with synovial fluid up to 8 hours after the initial GM-CSF stimulation. Interestingly, synovial fluid was able to inhibit GM-CSF induced CCL17 when added to monocytes up to 4 hours after the initial GM-CSF stimulation (Figure 4.6). This supports the transcriptional data, which showed the induction CCL17 after GM-CSF stimulation was potentially an indirect intracellular pathway due to the delay in transcription. Therefore, the synovial fluid must inhibit a downstream signalling molecule that prevents CCL17 transcription.

Due to the results of the previous chapter where TLR agonists inhibited CCL17, we hypothesised that endogenous DAMPs in the synovial fluid that bind TLRs were causing this effect. DAMPs have previously been shown to activate TLRs, for example the S100 proteins that are up-regulated in RA synovial fluid (84). S100A12 activates TLR4 in monocytes and induces inflammatory gene expression (82). Tenascin C is also a ligand for TLR4 and leads to the production of

inflammatory cytokines. In Tenascin C knockout mice, there was significantly reduced CIA disease scores and an abrogation in the cytokines produced (86). DAMPs are involved in RA pathogenesis, therefore we questioned whether DAMPs within synovial fluid were causing the inhibition in CCL17 through the binding of TLRs.

To determine whether the inhibition of CCL17 was due to TLR binding DAMPs within the synovial fluid, anti-TLR2 and anti-TLR4 antibodies were used (Figure 4.7). Initially, a dose response was performed to determine the concentration of the antibodies to use in further assays. The anti-TLR4 antibody successfully reversed the LPS inhibition of CCL17 at all concentrations, however the anti-TLR2 antibody was less successful at reversing FSL1. At the higher concentrations, the anti-TLR2 antibody made a slight impact on the FSL1 inhibition, however the antibody was not as effective as the anti-TLR4 antibody. To improve this experiment, a number of concentrations of FSL1 should have been used to assess the competition and whether the anti-TLR2 antibody had the ability at lower concentrations of FSL1 to reverse the inhibition of CCL17. However, the anti-TLR2 antibody was still used to determine whether the inhibition by RA synovial fluid could be reversed. The anti-TLR2 and anti-TLR4 antibodies, both individually and in combination were unable to reverse the inhibition by RA synovial fluid inhibition on GM-CSF induced CCL17. Despite the less effective anti-TLR2 antibody, in comparison to the anti-TLR4 antibody, there was no difference in the level of inhibition by synovial fluid. This led us to question whether it was a redundancy mechanism, whereby the inability of signalling via TLR2 or TLR4 led to the other TLRs being activated. Previous experiments showed signalling via other TLRs could also inhibit CCL17 therefore to test this theory, all TLRs had to be inhibited.

In order to assess this, MyD88/TRIF knockout mice were obtained and monocytes isolated from their bone marrow. MyD88 and TRIF are the adaptor molecules for signalling pathways downstream of TLRs (310). Inhibiting MyD88 prevents the majority of TLR signalling, however TRIF allows residual signalling via TLR3 and TLR4 in MyD88 knockout mice, therefore a double knockout of MyD88 and TRIF prevents signalling via TLRs (310,311). C57BL/6 mouse monocytes were used as a control, with LPS and RA synovial fluid significantly inhibiting GM-CSF induced CCL17 (Figure 4.8). This was a surprising, but useful finding that human RA

synovial fluid had the ability to inhibit CCL17 in mouse monocytes as it suggested cross-reactivity of the inhibitory factor within RA synovial fluid. Furthermore, LPS, which inhibited wildtype monocyte CCL17, would be expected to be ineffective in MyD88/TRIF double knockout mouse monocytes. This was the case as there was no inhibition of CCL17 by LPS in these monocytes, confirming that these mice lacked the ability to signal via downstream molecules from TLRs. However, surprisingly, the RA synovial fluid still inhibited the GM-CSF induction of CCL17 in MyD88/TRIF knockout monocytes. This showed that the factor causing inhibition of CCL17 in synovial fluid was not acting via TLRs and therefore had a different mechanism causing the inhibition than the TLR ligands. Synovial fluid is known to contain a whole milieu of inflammation inducing factors, from DAMPs, to immune complexes and cytokines (84,134,137).

Identifying that RA synovial fluid had the ability to inhibit GM-CSF induced CCL17, independently of TLR signalling, we analysed whether the inhibition could be replicated by other means. Initially small immune complexes were investigated as a high proportion of RA synovial fluids contain immune complexes (296). Small immune complexes also significantly inhibited GM-CSF stimulated secretion of CCL17 (Figure 4.9). Although these were manmade immune complexes and not the immune complexes found in the RA synovial fluid, it supports the hypothesis that immune complexes can also inhibit this pathway. Immune complexes bind to Fcγ receptors and previous studies with SIC show that through Fc mediated pathways, macrophages can be primed towards more regulatory and anti-inflammatory pathways (245). The inhibition of CCL17 by SIC suggests that the down-regulation caused by synovial fluid, could in fact be a regulatory mechanism, preventing the migration of $CCR4^{+}$ cells into the synovium. To determine whether eliminating immune complexes from the synovial fluid would reverse the observed inhibition of CCL17, protein A, protein G and protein A/G/L magnetic beads were used (Figure 4.9). We undertook studies to determine the efficacy of the depletion, however, the assay was inconclusive and we were unable to repeat due to time restraints and sample availability. Due to the nature of the technique, we were confident that depletion occurred, however, this is an uncertainty. There was no effect on the inhibition by synovial fluid when protein A or protein G were depleted, however we were unable to assess the level at which the immune complexes were

removed. Interestingly with the depletion of protein A/G/L, there was an even greater inhibition of RA synovial fluid to the level of LPS inhibition. This suggested that endotoxin contamination could be a possibility, but after a limulus assay, the synovial fluids were not contaminated. This showed that by removing more of the immune complexes, there was a larger inhibition of CCL17. The reasons for this are unknown, however we confirmed that immune complex depletion did not reverse the inhibition of CCL17. This suggests that there are other factors in synovial fluid that would be unaffected by immune complex depletion, but that also have the ability to inhibit CCL17. Type I interferons, specifically IFNα, is known to be highly expressed within RA synovial fluid but not in OA synovial fluid, which we know had less ability to inhibit CCL17 (302). This cytokine would be unaffected by depletion of immune complexes, therefore could explain why no reversal of inhibition was observed. Using recombinant IFNα, there was inhibition of CCL17 to comparable levels as LPS inhibition (Figure 4.10). This highlighted that there are multiple factors, commonly found within RA synovial fluid, which had the ability to inhibit GM-CSF driven CCL17. To investigate the intracellular mechanism leading to inhibition of CCL17, the downstream signalling of GM-CSFR was analysed.

GM-CSF binds its receptor GM-CSFR (formed of α and β chains) and signals via JAK2 and STAT5. To determine whether STAT5 signalling was impacted by synovial fluid, we investigated its phosphorylation status after pre-stimulation with synovial fluid, and a short 10 minute stimulation with GM-CSF (Figure 4.11). This analysis showed that in comparison to unstimulated monocytes, GM-CSF stimulation caused an upregulation in pSTAT5 MFI in $CD14^+$ monocytes. There was a difference between $CD14^+$ and $CD16^+$ monocytes in the induction of pSTAT5 after GM-CSF stimulation as this was more apparent in the $CD14^+$ monocytes than $CD16^+$ monocytes suggesting different responses to GM-CSF. Interestingly in some monocytes donors there was no effect on the MFI of pSTAT5 in monocytes with the RA synovial fluid pre-treatment. However, some monocyte donors had fewer pSTAT5 positive monocytes after RA synovial fluid pre-treatment. This suggests that there are perhaps multiple mechanisms leading to the inhibition of CCL17.

Transcription of CCL17 was not induced when monocytes were co-stimulated with GM-CSF and RA synovial fluid (Figure 4.4), and the signalling directly downstream of GM-CSFR through STAT5 was not consistently targeted (Figure 4.11). Therefore to determine whether RA synovial fluid was affecting the direct transcription of CCL17, we analysed a known transcription factor. Binding sites for STAT6 have been identified in the promoter region of the CCL17 locus through reporter assays examining IL-4 induction of CCL17 in T cells (267,307). As STAT6 had been identified as a transcription factor for CCL17 in T cells, we aimed to analyse whether its phosphorylation was potentially inhibited by RA synovial fluid, thereby preventing the transcription of CCL17. Monocytes expressing pSTAT6 were present even in unstimulated conditions suggesting a basal level of signalling through STAT6. There was a very slight increase in the percentage of pSTAT6 positive monocytes after GM-CSF stimulation, however due to the small number analysed, this was difficult to interpret. Furthermore, phosphorylation is indicative of an involvement of STAT6 after GM-CSF signalling, however it does not confirm whether STAT6 translocated to the nucleus, or whether it is the transcription factor for CCL17 after GM-CSF stimulation. For nuclear translocation of STAT6, it must be phosphorylated but also dimerized, therefore analysis of dimerization would be required to assess the involvement of STAT6 (312,313). Interestingly, both RA synovial fluids analysed appear to inhibit pSTAT6 to below the basal level in $CD14^+$ monocytes, however this was not the case in $CD16^+$ monocytes. The difference between the response of $CD14^+$ monocytes and $CD16^+$ monocytes suggests their differing phenotypes could impact their response to GM-CSF. However, this experiment required further repeats to be conclusive. Another caveat to this finding was that if GM-CSF did not induce a high level of pSTAT6 positive cells, it suggests that this was not the transcription factor response for inducing CCL17 in response to GM-CSF. However, the previous transcript analysis showed a lag time between GM-CSF stimulation and CCL17 transcription (Figure 4.4). Therefore, the 10 minute GM-CSF stimulation of monocytes to analyse the potential transcription factor for CCL17 could have been extended to multiple later time points. The RA synovial fluid had a 4 hour pre-stimulation with the monocytes, which could explain why there was an inhibition observed below the basal level of the unstimulated monocytes as there was enough time for this transcription factor to be inhibited.

Therefore, further analysis of STAT6 is required to elucidate whether it has an involvement in RA synovial fluid inhibition of GM-CSF driven CCL17.

Tofacitinib is a small molecule inhibitor that preferentially inhibits JAK3 and JAK1 signalling (198,314). Inhibition of this pathway, it had the potential to shed light onto the signalling pathway in which the synovial fluid was inhibiting CCL17 transcription as the JAK molecules are upstream signalling molecules of STATs. Specifically, JAK1 is an upstream of STAT6 signalling (308). Initially, we analysed whether Tofacitinib had any off-target effects by assessing the impact of treatment on the CCL17 production by GM-CSF stimulated monocytes (Figure 4.13). There was a dose dependent decrease in the level of CCL17 secreted with increasing concentrations of Tofacitinib, suggesting that the GM-CSF signalling molecule JAK2 was also partially inhibited. This has been observed before despite the major target molecules of JAK1 and JAK3 (314,315). Due to this targeted inhibition of GM-CSF induced CCL17, the effect of Tofacitinib on LPS or RA synovial fluid inhibition of CCL17 was analysed in comparison to the GM-CSF with matched pre-treatment for a more accurate comparison (Figure 4.14). At 100mM, there was no reversal of the RA synovial fluid inhibition of CCL17, however at 300mM, there was an increase in the percentage change in the LPS inhibition of CCL17. This appears to show a slight reversal in the inhibition observed, however, when the specific concentration of CCL17 was analysed, the level of CCL17 secreted with LPS co-stimulation was comparable across all conditions. This was therefore difficult to interpret, as there were too many variables that affected the result. Therefore, as we have been unable to determine the precise intracellular signalling pathway leading to CCL17 inhibition, we analysed the potential factors within the synovial fluid that could be causing the inhibition.

Initially, after establishing that RA synovial fluid inhibited GM-CSF induced CCL17, we investigated the potential factor within the synovial fluid that was causing this inhibition. However, these studies identified that there were multiple factors that had the ability to cause inhibition all of which are present in RA synovial fluid. These ranged from the cytokine IFNα, to immune complexes and TLR binding DAMPs (Figure 4.15). The ability of GM-CSF driven CCL17 induction to be inhibited in several ways suggests a regulatory mechanism to

prevent the infiltration of CCR4 positive cells into the joint. GM-CSF is abundant in the joint, and therefore there is huge potential for high levels of CCL17 to be secreted. The ability of the inflamed synovial milieu to dampen the secretion of CCL17 shows a mechanism to prevent further excessive inflammation through the migration of Th17 cells into the joint. There are other regulatory mechanisms within the joint such as the secretion of IL-10 from macrophages, which is highly expressed in the synovial fluid (136) and in the blood there are significantly higher levels of IL-10 in RA blood compared with healthy controls (316). IL-10 is classed as a more anti-inflammatory cytokine that classically dampens the immune response through suppressing interactions between macrophages and T cells and down-regulation of macrophage inflammatory cytokine secretion (317,318). However, as IL-10 as well as other anti-inflammatory cytokines are not at high local concentrations, they are unable to mediate a response against the more abundant pro-inflammatory cytokines. Therefore, despite their presence, other pathogenic factors prevent resolution, and drive inflammation. Potentially, synovial fluid inhibition is a regulatory mechanism that is too little, too late to alter disease pathogenesis.

The ability of multiple factors within the RA synovial fluid to inhibit GM-CSF induction of CCL17 in monocytes is potentially another regulatory mechanism in RA, to try to dampen the immune response. The inhibition of chemokines that would chemoattract Th17 cells suggests a preventative mechanism, without which, hugely exacerbated inflammation of the joint would exist. The synovial milieu has the capacity to influence cell responses that are exposed to it, making the understanding of the synovial environment and cellular responses hugely important.

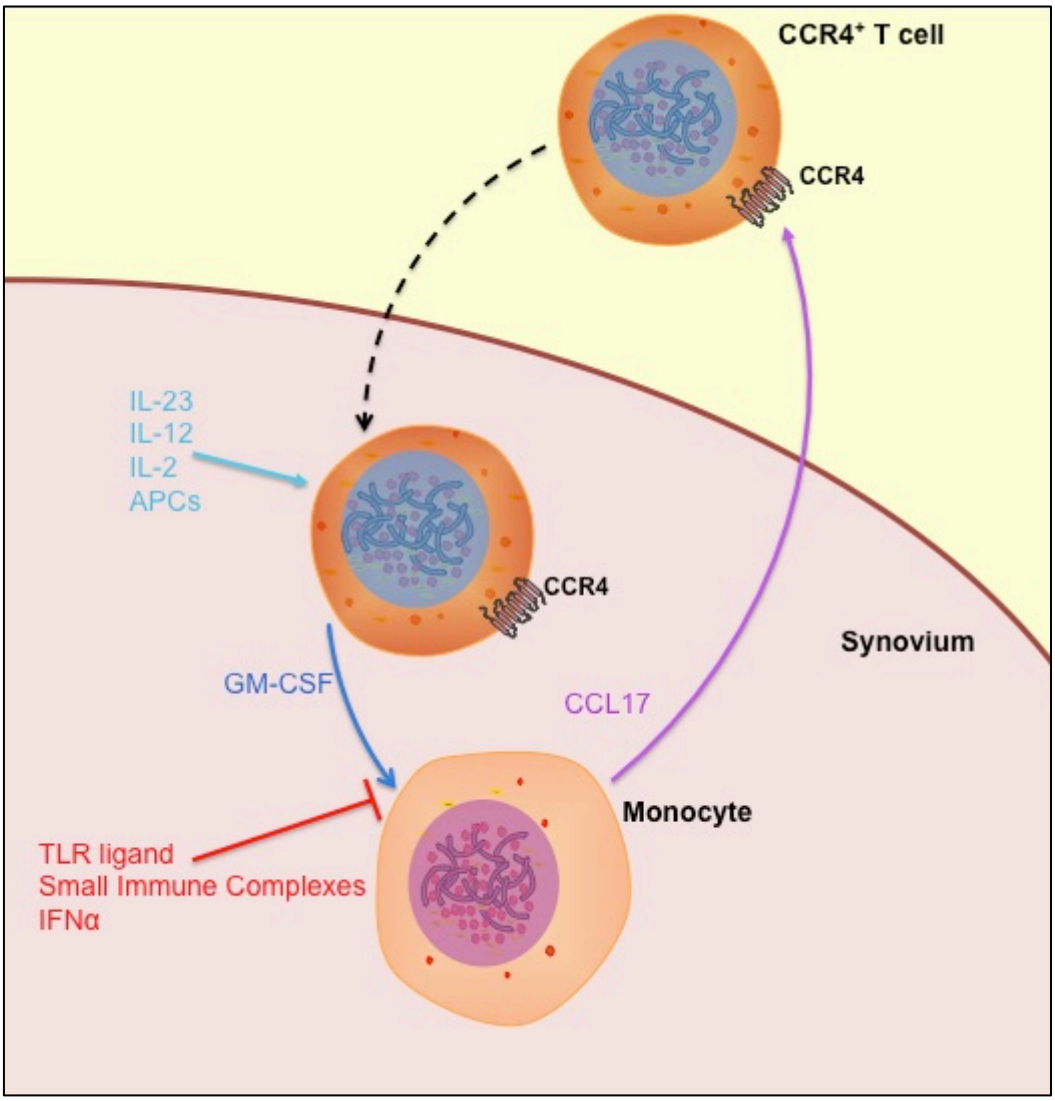


Figure 4.15 Schematic of updated hypothesis
Upon stimulation with GM-CSF, monocytes secrete CCL17, which leads to the recruitment of $CCR4^+$ T cells to the synovium. The $CCR4^+$ T cells upon activation, produce GM-CSF in a positive feedback loop. Synovial fluid, or specifically: TLR ligands; Small Immune Complexes; and IFNα can inhibit GM-CSF induction of CCL17 in monocytes.

Chapter 5 Macrophage and monocyte phenotypes in the synovial environment

5.1 Introduction

The previous chapter assessed how GM-CSF induced CCL17 in monocytes, but also how the RA synovial milieu inhibited this induction. Multiple components within the synovial fluid had the ability to cause inhibition of CCL17, suggesting the synovial fluid can influence the cellular secretome and have an impact on cellular phenotype.

Human monocytes can be classified into subsets by their expression of CD14 and CD16 (319). The majority of monocytes lack CD16 expression and are classified as classical monocytes ($CD14^{++}CD16^{-}$). They are present in blood at 10 times greater levels than other subsets (52). The subset that are $CD16^{+}$, are classed as non-classical monocytes ($CD14^{+}CD16^{++}$), however some state these monocytes are pro-inflammatory as they respond to LPS and secrete TNFα. Even under non-inflammatory conditions, non-classical monocytes can migrate into tissues (320). Monocytes are not only classified by their expression of CD14 and CD16 but expression of chemokine receptors such as CCR2 or the presence of specific TLRs (Section 14.1.1). Cytokine and chemokine secretion can also allow the classification of the populations.

The traditional viewpoint is that when monocytes receive specific environmental cues, they differentiate into monocyte-derived cells, however, there have also been studies to suggest that there are tissue resident monocytes that do not differentiate into macrophages (321). When macrophages differentiate, they are classified into distinct categories, depending on specific transcriptional markers, surface receptors or cytokines released. Until recently, the nomenclature of macrophage classification has ranged from M1 and M2 macrophages to alternatively activated, classically activated and regulatory macrophages. These terms have been generated in the main from *in vitro* cultured human blood monocyte derived macrophages or mouse bone marrow macrophages (63). Traditionally, monocytes were differentiated into macrophages using growth factors such as GM-CSF for an M1 macrophage or M-CSF for an M2 macrophage (265). Alternatively activated macrophages were differentiated in IL-4 with or

without IL-13 and classically activated macrophages were differentiated in IFNγ. However, there are several other *in vitro* stimuli that are used to differentiate macrophages such as IL-10, immune complexes and LPS (62,63).

There are specific transcriptional markers that are used to define *in vitro derived* macrophage subsets to form a transcriptional signature, such as CD64, which is an M1 marker, and CD23, which is an M2 marker (322). However, the spectrum of macrophages isolated from tissue, or differentiated *in vitro* under different conditions, has led to cells that do not fit into a distinct category. There is still dispute over macrophage classifications, however, it is now widely accepted that tissue resident macrophages are embryonic derived. Monocytes that infiltrate into a tissue in response to inflammatory cues, differentiate dependent on the specific stimuli in the milieu, or interactions with other cells, into a broad spectrum of monocyte-derived cells, which do not necessarily fit into a distinct category (50). They may have aspects of an M1 or an M2 but are unique (57). Monocyte-derived cells can be classified with transcriptional markers, scavenger receptors, metabolic profiles cytokines and chemokines (63,323).

Macrophages within the RA synovium reside primarily in the intimal lining layer, where they interact with synovial fibroblasts and are exposed to the synovial milieu (67). Monocytes increase in number in the synovium upon inflammatory insult, and as they differentiate into macrophages, the 30-40% increase in macrophage numbers in the RA patient synovium suggests that monocyte infiltration directly contributes to this (255,324,325). In general, macrophages within the RA synovial lining are positive for GM-CSFRα, as there was a significant up-regulation of GM-CSFRα positive macrophages in RA patients in comparison to OA patients(238). The presence of increased levels of GM-CSFRα in the RA synovial tissue suggests that there is the capacity for more GM-CSF signalling to occur. Therefore, inhibition of this receptor via Mavrilimumab could alter the phenotype of macrophages or their monocyte precursors, particularly within the synovial milieu, which was shown in the previous chapter to influence the secretion of chemokines (Fig 4.3).

The RA synovial environment contains many stimuli that could impact monocyte and macrophage phenotypes. Understanding the effect of these stimuli on polarisation of macrophages and the activation of monocytes is not well understood. Therefore, this chapter aims to identify the impact of the synovial environment on macrophage polarisation and monocyte activation.

Chapter Aims:

- To assess the impact of synovial fluid on macrophage polarisation.
- To determine how monocyte activation markers are affected by synovial fluid.
- To establish whether Mavrilimumab affects monocyte phenotypes.

5.2 Results

5.2.1 The effect of *in vitro* stimuli on macrophage polarisation

Transcriptional signatures for macrophage polarisation states have been well defined for M1 and M2 macrophages. Investigations were undertaken to determine whether a transcriptional profile could be generated that were comparable with previously reported studies, to then be used in further experiments. The effect of disease-specific stimuli on these transcriptional profiles could then be evaluated. Differentiation of macrophages has traditionally been with individual stimuli such as M-CSF or GM-CSF for 7 days (265). These experiments investigated whether macrophages differentiated with M-CSF could then be skewed with alternative stimuli, to create different polarisation states. This would aim to understand macrophage plasticity after stimulation with differing environmental factors. Therefore monocytes isolated from buffy coats were differentiated using M-CSF for 7 days for an M0 macrophage. From this M0 macrophage, further stimulations with IFNγ, IL-4 or GM-CSF were used to create different polarised macrophages.

There are multiple methods described to determine macrophage polarisation state, however in initial studies, transcriptional profile was interrogated. Specific transcriptional markers were chosen from a particular paper from Beyer et al., (322) in which transcriptional profiles were analysed, specifically in M1 and M2 differentiated human macrophages. The majority of available data has only looked at specific polarisation states; therefore this was expanded to interrogate alternative polarisation states. This was achieved by identifying transcriptional profiles for 4 experimentally differentiated macrophage phenotypes.

Therefore, investigations into macrophages polarised with M-CSF followed by IFNγ (M1), IL-4 (M2) or GM-CSF (M-GM) were compared to M-CSF (M0) differentiated macrophages. The evaluation of transcriptional signatures in M1, M2 and M-GM macrophages revealed that M1 macrophages have a different transcriptional signature in comparison to other macrophage phenotypes. M1 macrophages had significantly higher transcript expression of APOL2, APOL3, APOL6 and IRF7 compared with M0 macrophages (Figure 5.1). Macrophages

differentiated with IL-4, or M2 macrophages, also differed from M0, M1 and M-GM macrophages. M2 macrophages had significantly elevated expression of CD23 with 10 fold greater expression than M0 macrophages. However, M2 macrophages had significantly lower expression of CD64 with greater than M0 and M1 macrophages. IRF4 and IRF5 were also significantly higher, with a greater than 3 fold increase in expression in M2 macrophages in comparison to M0 macrophages. Macrophages differentiated with GM-CSF, or M-GM macrophages, had no specific marker that was more highly expressed than other macrophages. Despite this, M-GM macrophages had greater than 2 fold increase in expression of IRF4 and IRF5 in comparison to an M0 macrophage.

This transcriptional analysis confirmed that experimental stimuli, in this case, IFNγ, IL-4, GM-CSF or M-CSF have differing effects on macrophage phenotypes. Dependent on the stimuli received, the polarisation of these macrophages was altered, even after initial differentiation with M-CSF showing their plasticity. This suggests that these macrophages could be altered by their micro-environment.

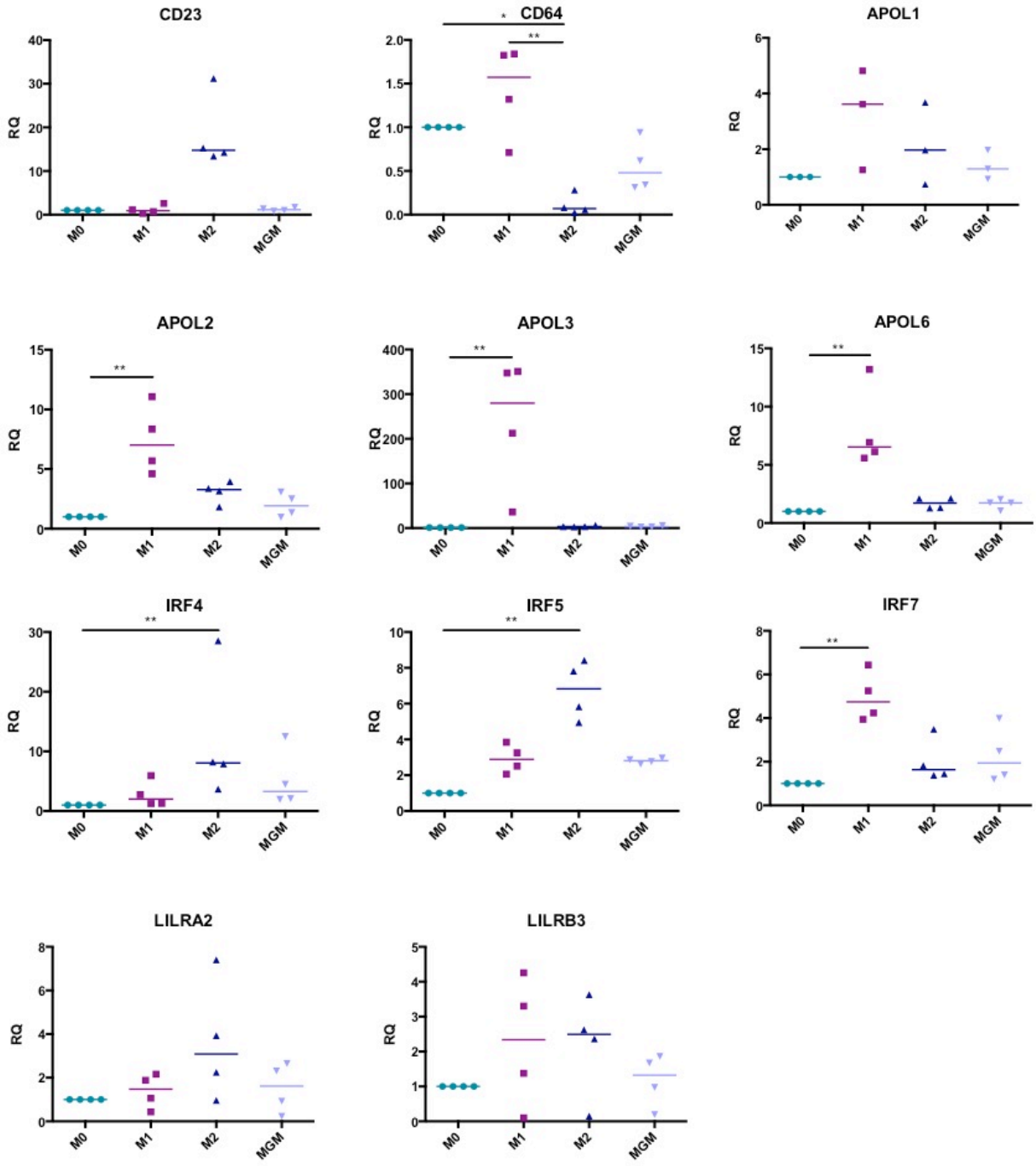


Figure 5.1 Experimental macrophage transcriptional profiles.
Monocytes isolated from human buffy coats (n=4) (n=3 for APOL3) and cultured in 100ng/ml M-CSF for 7 days. Media was replaced with further stimulations of 100ng/ml M-CSF for M0, 20ng/ml IFNγ for M1, 100ng/ml IL-4 for M2 or 100ng/ml GM-CSF for M-GM for 24 hours. Cells were lysed and RNA extracted for analysis of specific transcripts by qPCR using SYBR green. Transcripts were normalised to GAPDH housekeeping. RQ was calculated for each experimental macrophage transcript in relation to an M0 macrophage was analysed. Horizontal bar indicates the median. Statistically analysed using Kruskal-Wallis non-parametric test, followed by Dunn's post test. *=$p<0.05$, **=$p<0.01$.

5.2.2 Evaluation of valid housekeeping genes.

For further evaluation of macrophage transcriptional phenotypes, Taqman Low Density Arrays (TLDA) were used. For this technique, multiple housekeeping genes could be used, therefore, it was necessary to identify the most suitable housekeeping genes prior to further evaluation. For this, the most consistent housekeeping genes after experimental macrophage polarisation were determined by using a housekeeping TLDA plate.

Monocytes were differentiated in M-CSF for 7 days, followed by polarisation stimuli with IFNγ, IL-4 or GM-CSF. In addition to evaluating the best housekeeping gene to use for macrophage polarisation states, studies were also undertaken to determine a housekeeping gene that stayed at comparable CT values from monocyte separation through to macrophage differentiation for consistent analysis of monocytes and macrophages. Therefore monocytes were assessed (n=1) as well as polarised macrophages (n=3). PBMCs and LPS stimulated M0 and M-GM macrophages were also analysed as a comparison.

The CT values were assessed for each condition and every gene on the array plate. Commonly used housekeeping genes such as GAPDH, ACTB and TBP, did not have consistent CT values across all conditions (Figure 5.2). In several of the genes, PBMCs were different to the other conditions, however, as there was no intention to transcriptionally analyse whole PBMCs, this was not a concern. There was also variability observed between the 3 donors used between the macrophage phenotypes. However, CT values for UBC and GUSB were found to be the most comparable across all conditions. These housekeeping genes were therefore carried on into further experiments using TLDA transcriptional analyses.

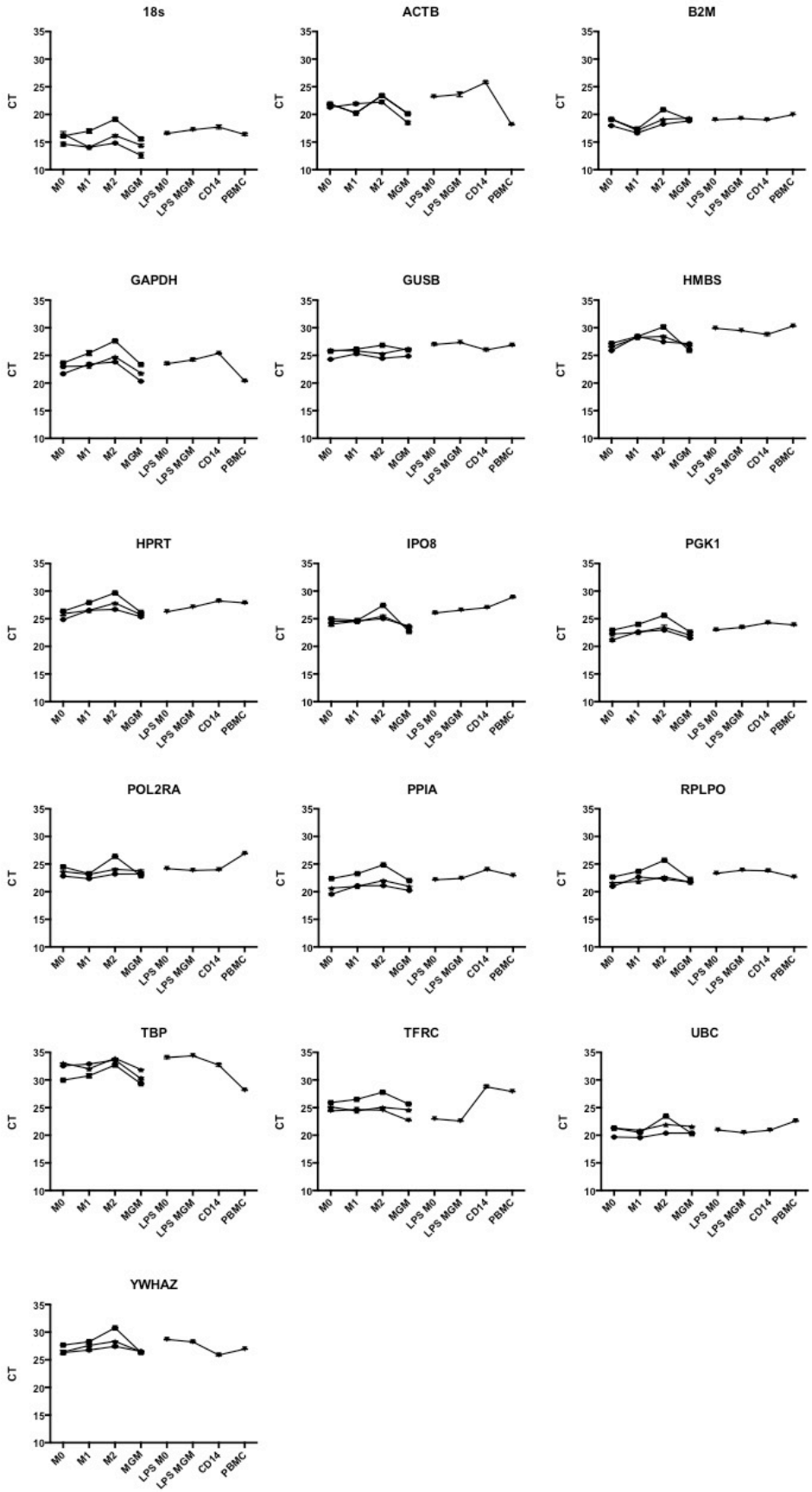


Figure 5.2 Housekeeping Array analysis
cDNA was analysed from 3 buffy coat donors cultured into 4 phenotypes of macrophages with the additional stimuli of LPS in an M0 or MGM macrophage or a CD14 monocyte immediately after separation or a PBMCs. CT values of each labelled gene are shown across all conditions.

5.2.3 The effect of disease stimuli on macrophage polarisation

The initial experiments showed that macrophages can be polarised and expressed different macrophage transcriptional markers depending on the stimuli. To assess the effect of the diseased microenvironment on macrophage polarisation, macrophages were stimulated with synovial fluid from RA patients. Stimulating macrophages with synovial fluid *in vitro*, aimed to artificially recreate the environment in which monocytes infiltrating the tissue would be subjected to. By determining how macrophages differentiate in this environment, we could establish whether these macrophages were similar to particular polarisation states of the experimental macrophages (Figure 5.1). OA synovial fluid had a different effect on monocytes when co-stimulated with GM-CSF in comparison to RA synovial fluid in the previous chapter. Therefore, to determine whether different synovial fluids affected the polarisation of macrophages, RA, and OA synovial fluids were used.

Macrophages stimulated with synovial fluid from RA or OA patients, termed MSF, had some transcript similarities to experimental macrophages, however, despite aspects of specific experimental macrophages, their overall transcriptional profile was different. Individual samples came from buffy coats donated from the Scottish National Blood Transfusion Service (Section 2.2.1). It was observed that there was variation between the buffy coats and their response to synovial fluid. M1 macrophages expressed APOL3 at greater levels, and one buffy coat also expressed this transcript highly with the addition of either RA or OA synovial fluid, however due to the variation observed, it was difficult to confirm a specific MSF transcriptional phenotype (Figure 5.3). CD36 had similar levels of expression across all experimental macrophages, however, there was an up-regulation of this transcript in both OA and RA MSFs. M1 and M-GM macrophages had greater levels of CLEC4E and this was mirrored by both the OA and RA MSFs. M2 and M-GM macrophages had increased expression of CSF2RA in comparison to M0 macrophages, and 1 buffy coat donor MSF had higher expression. The transcript LILRA2 in the OA and RA MSFs was more comparable in lower expression than M0 and M2 macrophages to the M1 and M-GM macrophages. MSR1 and TLR4 transcripts had greater expression in the MSFs in comparison to the experimental macrophages.

Analysis of macrophage transcriptional profiles differentiated with RA and OA synovial fluids showed that MSFs are different from a specific experimental macrophage. However, MSFs had properties of the experimental macrophages, as there was not a transcript that was up-regulated in the MSFs, that was not also up-regulated in another condition. Synovial fluid provides another profile in the spectrum of macrophage activation. There was also no transcriptional profile that was consistent across all donors, therefore it was difficult to conclude a specific MSF phenotype. The RA and OA MSFs had comparable transcriptional profiles suggesting the synovial fluid had a similar impact on macrophage polarisation, however functional studies would be needed to determine whether these macrophages were the same.

As macrophages polarised with OA or RA synovial fluid had a very similar transcriptional phenotypic profile, it was investigated whether synovial fluid from PsA patients also had the same impact on macrophage transcriptional phenotypes. After 7 days differentiation of monocytes with M-CSF, macrophages were further stimulated for 24 hours with RA, OA or PsA synovial fluid.

Transcripts were analysed by qPCR for genes that were identified as markers of specific experimental macrophages (Figure 5.1). The synovial fluid stimulations caused very minor changes to transcription in comparison to M0 macrophages (Figure 5.4) and expression of transcripts in MSFs was much lower than in Figure 5.3. This confirmed the variability between monocyte donors, and the difficulty in making conclusions. However, the RA, OA and PsA MSFs had comparable levels of transcript expression suggesting they were of a similar transcriptional phenotype (Figure 5.4).

The transcriptional observation, suggested a similar transcriptional phenotype between MSFs polarised with RA, OA or PsA synovial fluid. Therefore, to determine whether the similar MSF transcriptional phenotype could be translated into their functional output, chemokine levels were investigated. To ensure the chemokine induction was measured, without interference from the level in synovial fluid, the chemokine content of the synovial fluids that were added to the culture, was deducted from the level of the supernatants. In 2 of the 3 buffy coat donors, LPS up-regulated chemokine expression, acting as a positive control, however, this was not the case for 1 donor, which was

unresponsive to LPS stimulation. None of the chemokines were up-regulated by stimulation with synovial fluids, which would potentially be expected due to the number of pro-inflammatory DAMPs within the fluid. Interestingly, the MSFs had a suppressed level of CCL7 and CCL8 in comparison to M0 macrophages, and the synovial fluid had a similar impact on functional chemokine output, whether it was derived from RA, OA or PsA patients.

Although there is not a robust signature in the transcriptional analysis or the secreted chemokines that have been looked at, it is clear that synovial fluid from any arthropathy has a comparable impact on macrophage polarisation with regards to transcriptional profile or chemokine induction.

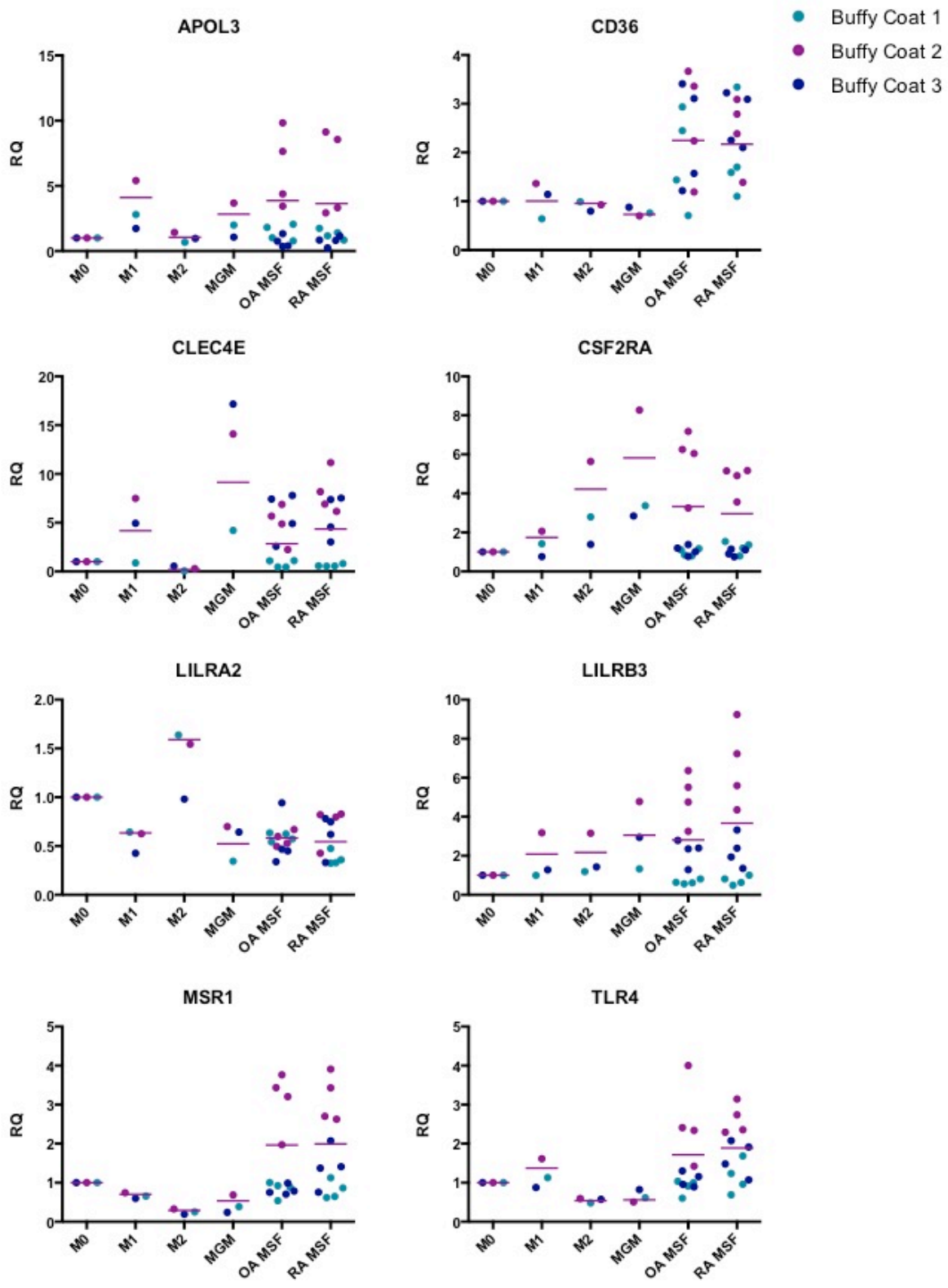


Figure 5.3 Transcriptional analysis by TLDA of macrophages differentiated with RA or OA synovial fluid.
Monocytes isolated from human buffy coats (n=3) were cultured with 100ng/ml M-CSF for 7 days, followed by 24 hour stimulation with: 100ng/ml M-CSF for M0; 20ng/ml IFNγ for M1, 100ng/ml IL-4 for M2; 100ng/ml GM-CSF for M-GM; or 10% synovial fluid (n=4) from RA or OA patients for MSF. Transcripts were analysed by TLDA relative to GUSB housekeeping gene and the RQ calculated over M0 macrophages. Horizontal bar indicates mean.

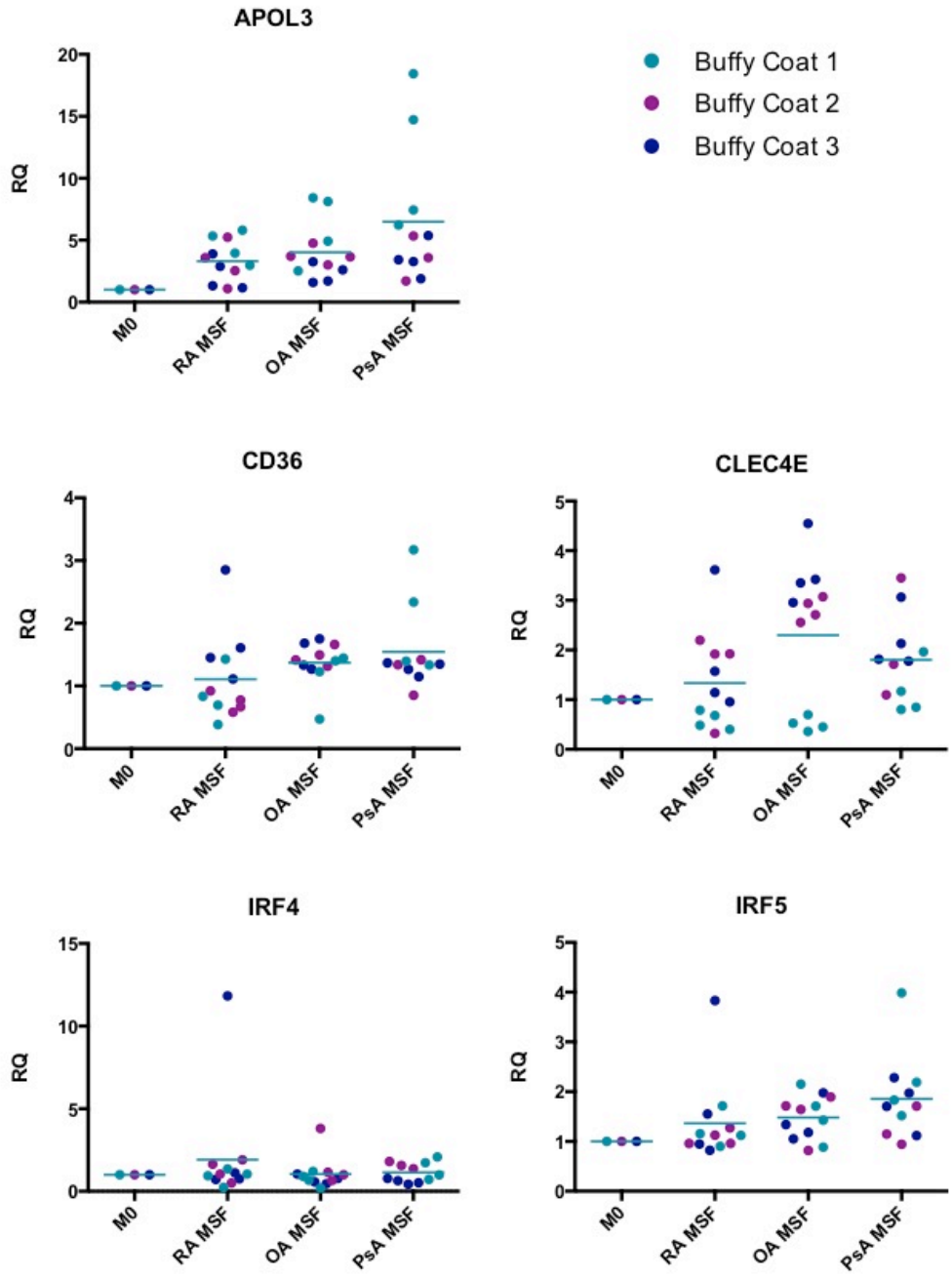
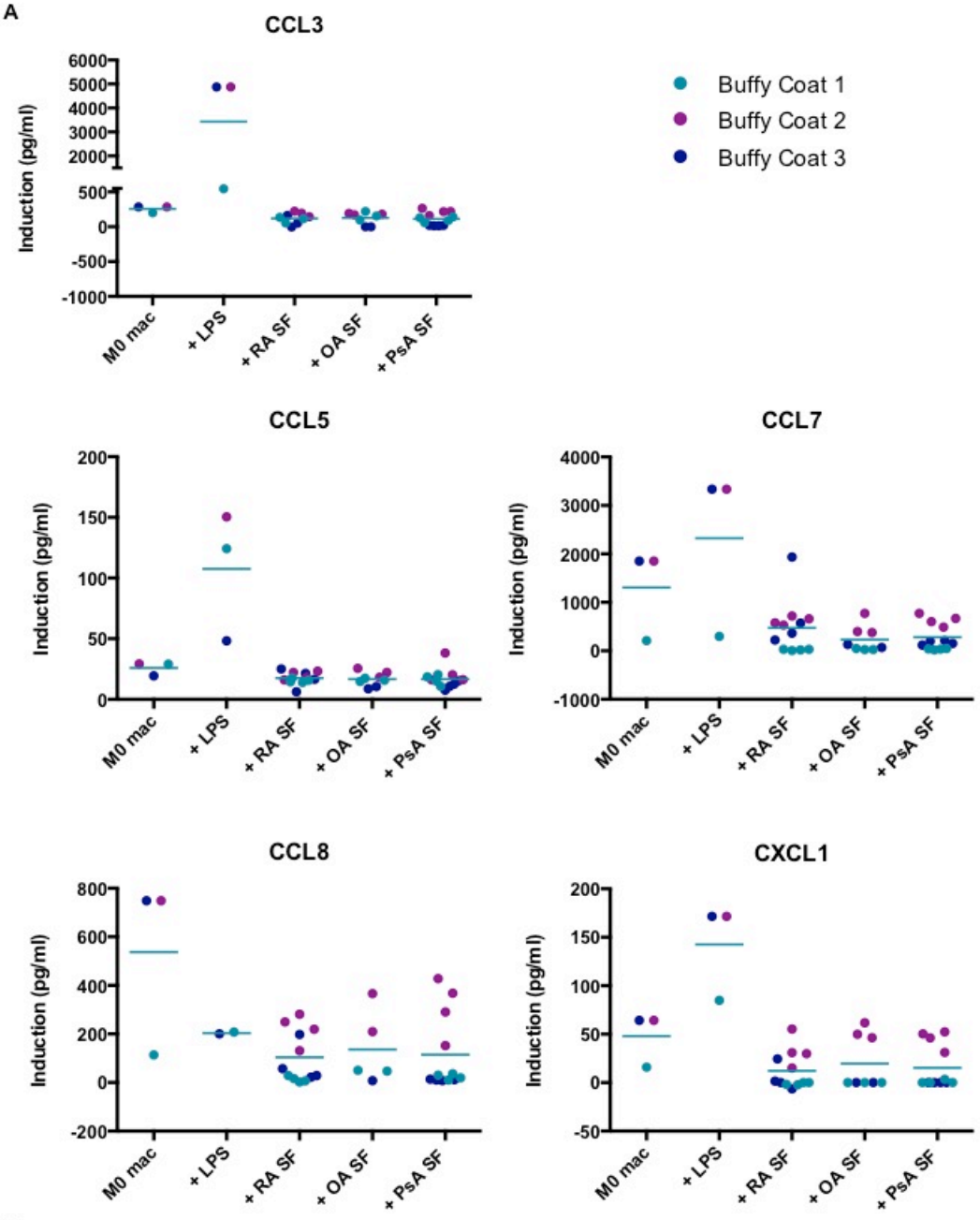


Figure 5.4 Transcriptional analysis by qPCR of macrophages differentiated with diseased synovial fluid.
Monocytes isolated from human buffy coats (n=3) were cultured with 100ng/ml M-CSF for 7 days, followed by stimulation for 24 hours with: 10% synovial fluid (n=4) from RA, OA or PsA patients. Transcripts were analysed by qPCR relative to GUSB housekeeping gene and the RQ calculated in relation to M0 macrophages. Horizontal bar indicates mean.

A



B

	RA				OA			PsA			
	1	2	3	4	1	2	3	1	2	3	4
CCL3		253.56	142.87	92.03	n/d	n/d	n/d	89.57	80.27	53.09	67.83
CCL5	45.08	22.98	157.65	57.05	22.93	9.46	49.12	110.23	65.75	17.3	210.41
CCL7	42.4	332.13	8.93	32.45	n/d	n/d	n/d	21.88	n/d	n/d	n/d
CCL8	307.03	117.09	28.78	19.17	12.3		5.5	17.28	9.74	5.8	23.02
CXCL1	322.21	n/d	91.64	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d

n/d = not detected

Figure 5.5 Chemokine induction after synovial fluid stimulations.
A) Monocytes isolated from buffy coats (n=3) were cultured in 100ng/ml M-CSF for 7 days. M0 macrophages were stimulated with M-CSF for a further 24 hours with or without 15.63ng/ml LPS or with RA, OA or PsA synovial fluid (n=2,3,4). The supernatants were removed and were analysed for chemokine induction by Luminex. B) The synovial fluids themselves were also analysed and the level (pg/ml) that was added to the culture deducted from the MSF to ensure induction was quantified (A). Horizontal bar indicates mean.

5.2.4 Monocyte and macrophage transcriptional profile comparison after synovial fluid stimulation.

The previous data found that the disease origin of the synovial fluid did not impact the macrophage phenotypic markers investigated. To determine whether this was also the case for infiltrating monocytes into the joint, monocytes were also stimulated with synovial fluid and directly compared with the macrophage.

In several of the analysed genes, transcriptional expression of was significantly different between monocytes and macrophages. There were no significant differences in any of the analysed transcripts between unstimulated monocytes and monocytes stimulated with RA, OA or PsA synovial fluid (Figure 5.6). However, despite the lack of significance, transcript expression was more variable across the conditions in comparison to macrophages. Monocytes stimulated with OA synovial fluid had significantly less expression of CSF2RA, CREB1, IRF5 and CCL13 in comparison to PsA synovial fluid stimulated monocytes, suggesting these monocytes have a different transcriptional signature. There was no significant difference between the RA and OA synovial fluid stimulated monocytes, however, there was slightly increased expression of the analysed transcripts in the RA synovial fluid stimulated monocytes. In comparison to macrophage transcript expression, there was significantly less APOL3 transcript in comparison to all MSFs. Monocytes stimulated with OA and RA synovial fluid also had significantly lower levels of CSF2RA in comparison to comparative MSFs. Interestingly this was not the case for the PsA synovial fluid stimulated monocytes. Although there were no significant differences, monocytes stimulated with all synovial fluids had greater levels of CCL2 transcript expression in comparison to the macrophages. CCL13 expression was significantly higher in monocytes compared with their corresponding macrophages when stimulated with RA or PsA synovial fluid.

Looking specifically at the monocytes stimulated with synovial fluid, the PsA stimulated monocytes had significantly greater expression of APOL3, CSF2RA, CREB1, CD36, CCL13 and IRF5 in comparison to the OA synovial fluid (Figure 5.7).

The caveat to this experiment was that it was only tested in one matched monocyte and macrophage donor, however, the use of 4 synovial fluid from each

disease showed the consistent response to these fluids by the monocytes or macrophages. Thus the data generated supports the concept that monocytes are more responsive to disease stimuli in comparison to differentiated macrophages at least, with respect to the transcripts measured in this study.

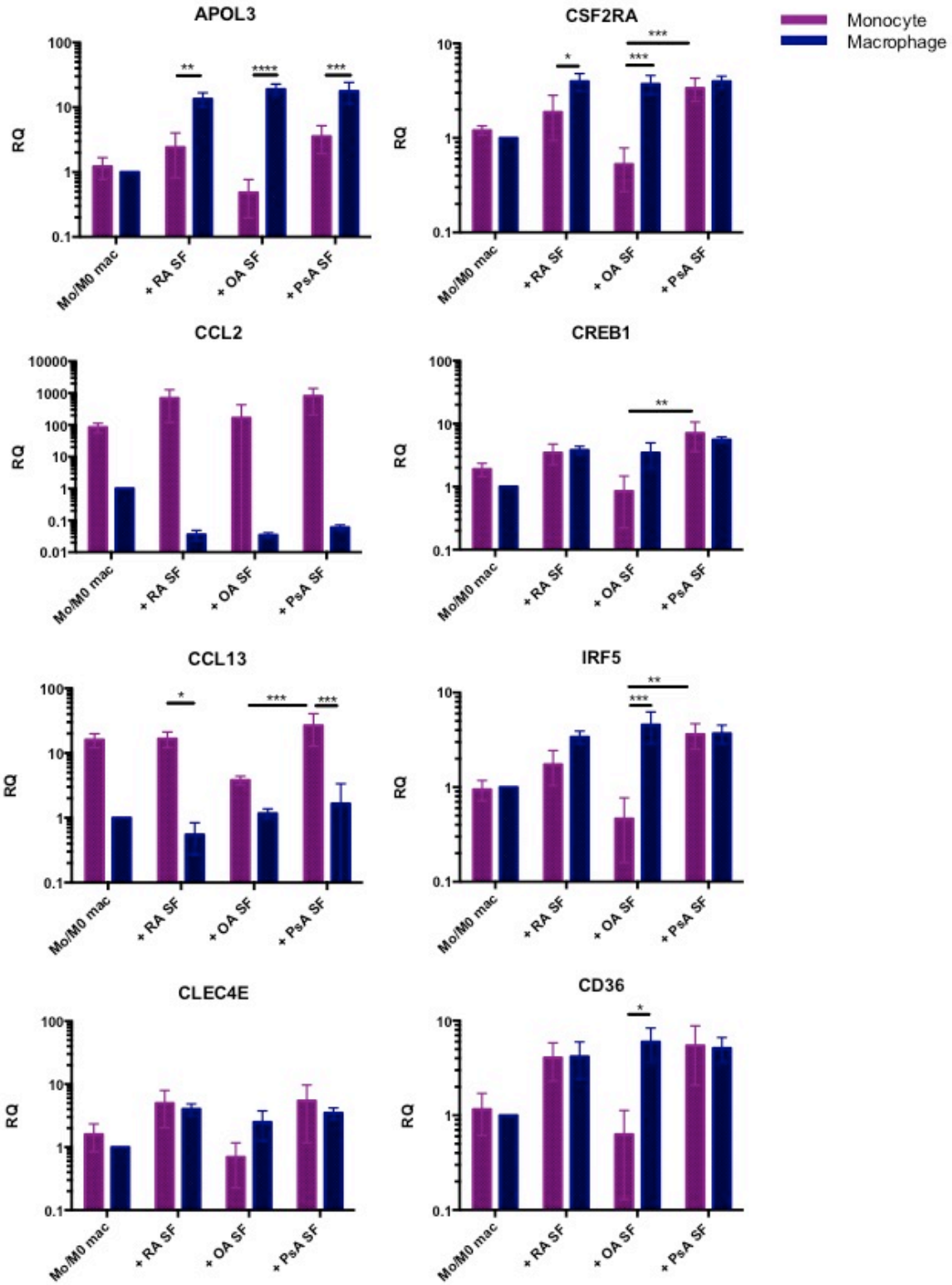


Figure 5.6 Transcriptional comparison between monocyte and macrophages after synovial fluid stimulation.
Monocytes isolated from a buffy coat (n=1) were either cultured in complete media for 24 hours or for 7 days in M-CSF. The stimulations with 10% RA, OA or PsA synovial fluids (n=4) were for 24 hours. Cells were lysed and analysed for transcripts by TLDA in relation to GUSB housekeeping. RQ calculated over the freshly isolated monocyte. MSFs statistically analysed using 2-way ANOVA with Bonferroni's post test. *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

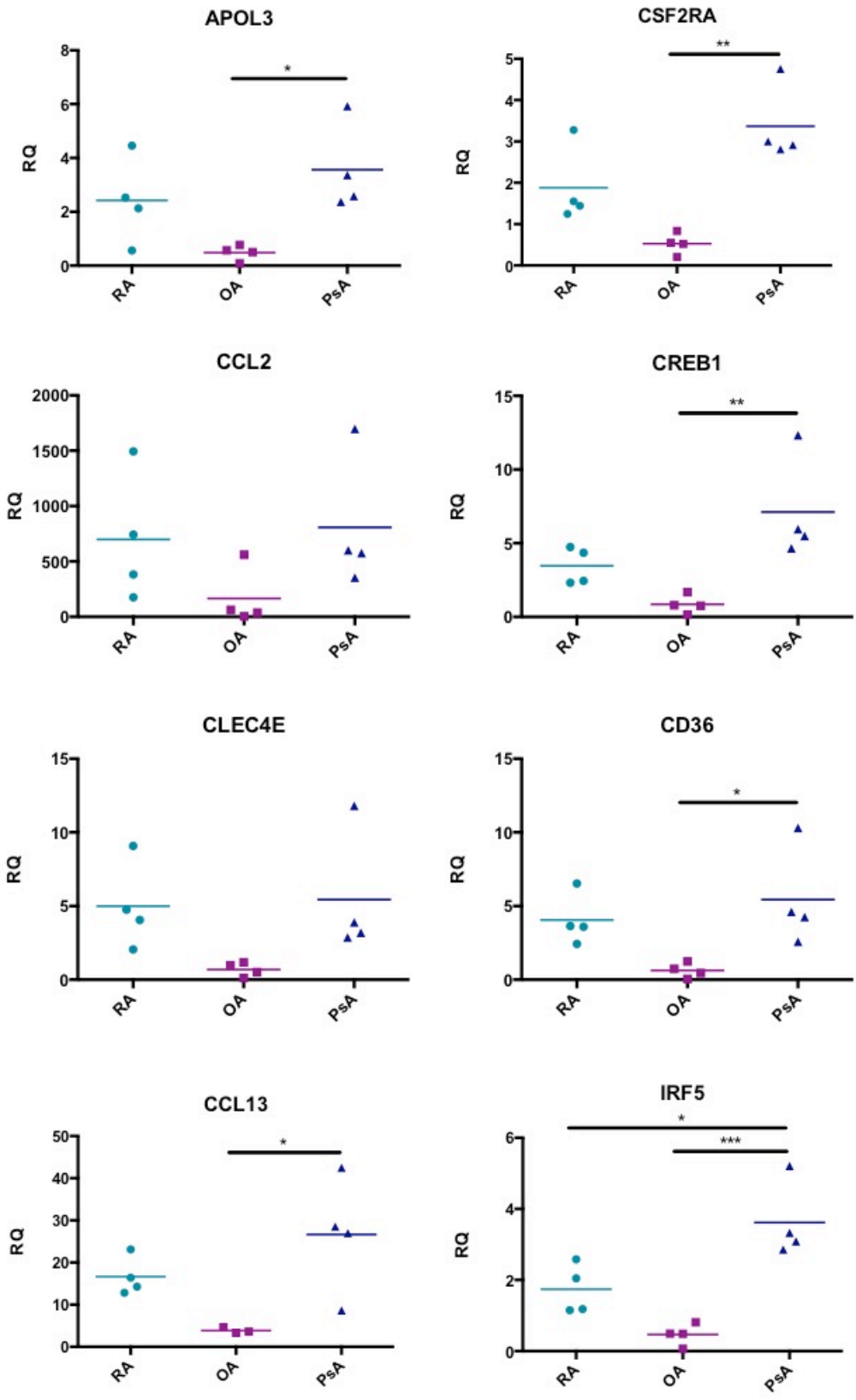


Figure 5.7 TLDA analysis of monocytes stimulated with RA, OA or PsA synovial fluid. Monocytes separated from a human buffy coat (n=1) were stimulated with 10% synovial fluid from RA, OA or PsA patients (n=4) for 24 hours. Cells were lysed and transcripts analysed by TLDA, relative to GUSB housekeeping. RQ calculated in relation to monocytes alone with no synovial treatment. Horizontal bar indicates mean. Statistically analysed using one-way ANOVA and Bonferroni's post test. *=p<0.05, **=p<0.01, ***=p<0.001.

5.2.5 Monocyte phenotype analysis after stimulation with synovial fluid and Mavrilimumab.

Monocytes stimulated with synovial fluid from RA, OA or PsA patients have different transcriptional profiles (Figure 5.7). The previous chapters examined GM-CSF stimulation of monocytes, which causes the induction and the secretion of pro-inflammatory chemokines (Figure 3.2). The interplay between GM-CSF and synovial fluid with regards to monocyte secretion of CCL17 was also analysed. Synovial fluid contains GM-CSF (326), therefore the involvement of GM-CSF on monocyte activation and differentiation was elucidated through the use of Mavrilimumab (CAM3001).

Synovial fluid stimulated monocytes previously showed differential expression of transcripts associated with macrophage polarisation (Figure 5.7). Therefore, to elucidate whether monocyte activation and differentiation were affected after synovial fluid stimulation, an alternative transcriptional profile was analysed. This also assessed whether Mavrilimumab impacted monocyte activation or differentiation. Monocytes were pre-treated with Mavrilimumab (CAM3001) for 30 minutes before the addition of synovial fluid, to allow binding of the antibody to GM-CSFRα prior to the addition of a potential GM-CSF stimulus.

There were no large differences in transcriptional patterns between monocytes that were pre-treated with the isotype, and the monocytes pre-treated with CAM3001 with and without synovial fluid. The pre-treatment with the isotype followed by RA synovial fluid caused an increase in CCL3, CCL4, CD36 and IL-8 transcripts in most buffy coat donors, however this was not a robust finding and there was variability between the buffy coat donors and the synovial fluids used (Figure 5.8). CD1A and TLR4 were up-regulated by pre-treatment with CAM3001 followed by RA synovial fluid. OA synovial fluid stimulation caused a down-regulation after pre-treatment with CAM3001 in CCL22, CHI3L1, CXCL2 and CLEC4E, however these differences were marginal.

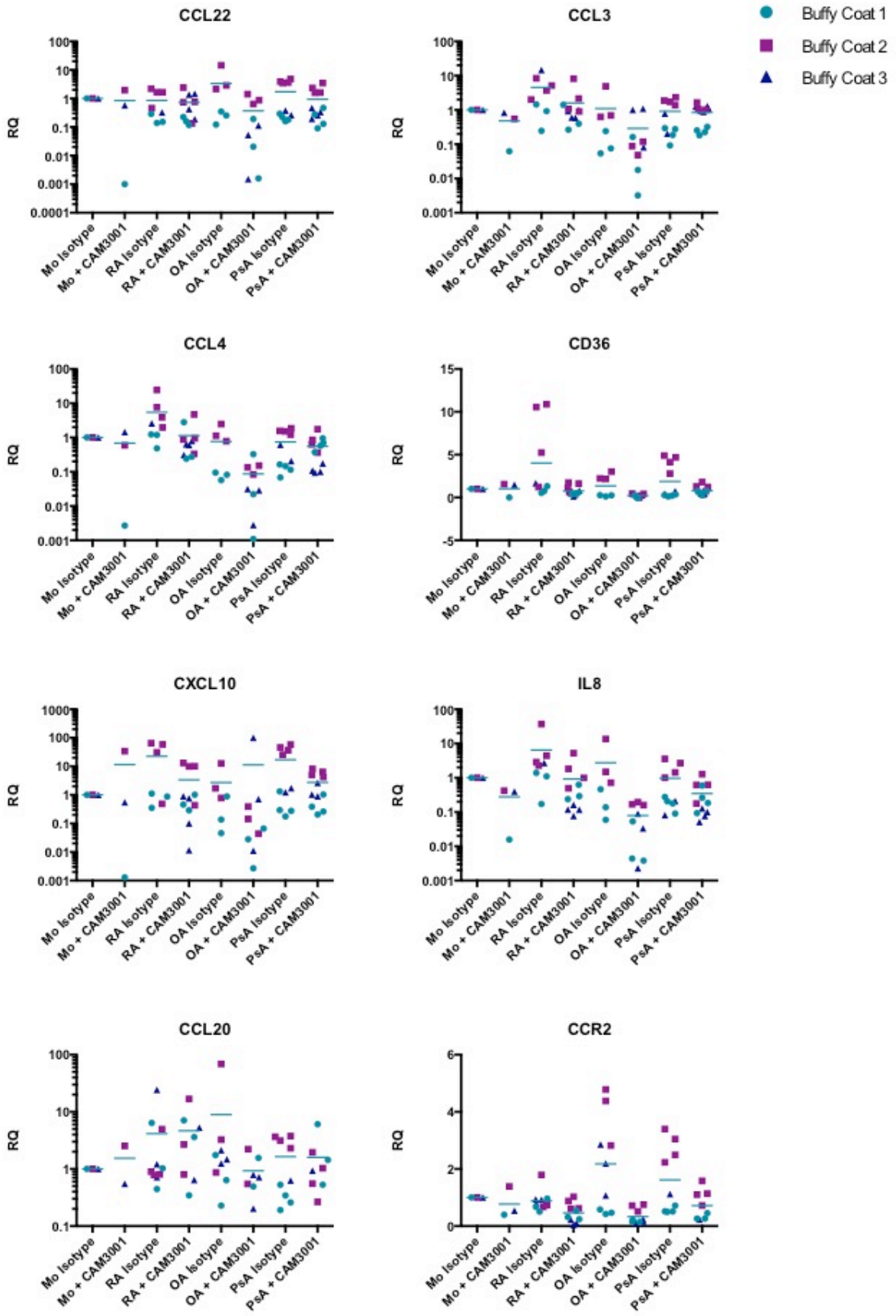
This data shows that there were no significant differences in the transcripts that were analysed. This suggests that Mavrilimumab has no effect on the activation of monocytes, although there was a large amount of variability between the buffy coat donors. Only transcripts were analysed, which prevents accurate

conclusions, therefore further functional analysis or flow cytometric profiles could improve the understanding of the effect of Mavrilimumab on monocytes.

There was substantial variability observed in Figure 5.8 that caused difficulty in interpretation of results. Therefore, to assess whether the observed variability was due to the buffy coat donors, an experiment was performed to limit other variables. In order to ensure consistency, monocytes were all isolated from buffy coats on the same day, and all RNA isolations and qPCR was done at the same time.

Monocytes alone, after 24 hours, up-regulated in CD36 and MRC1 expression, however, CLEC4E was down-regulated (Figure 5.9). RA synovial fluid had no effect on the analysed transcripts after 6 hours, however, after 24 hours, there was a significant increase in MRC1. As monocytes alone after 24 hours also up-regulated MRC1, the RA synovial fluid had no distinct effect on transcript expression. There was no difference with the other analysed transcripts.

There was still variability between the buffy coat donors, despite the measures to keep the experiment consistent. This suggests that the buffy coat donors were causing variability observed in Figure 5.8.


CCL22
CCL3
CCL4
CD36
CXCL10
IL8
CCL20
CCR2
RQ
Buffy Coat 1
Buffy Coat 2
Buffy Coat 3
Mo Isotype
Mo + CAM3001
RA Isotype
RA + CAM3001
OA Isotype
OA + CAM3001
PsA Isotype
PsA + CAM3001

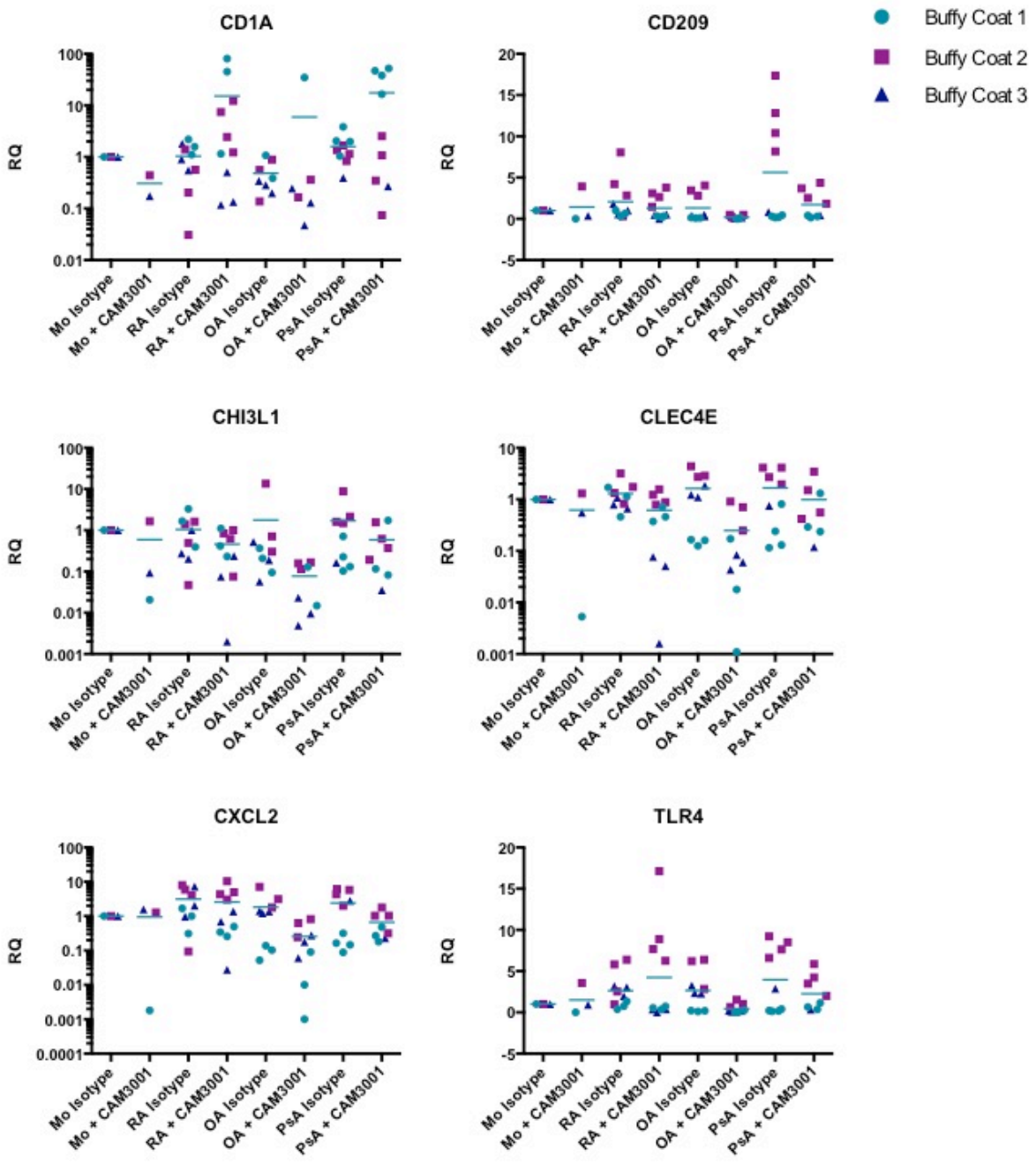


Figure 5.8 Effect of Mavrilimumab on monocyte phenotypes.
Monocytes isolated from human buffy coats (n=3) were pre-stimulated with Mavrilimumab (CAM3001) or the isotype (CAT004) for 30 minutes. After pre-stimulation, synovial fluid from RA (n=4 or n=3 for buffy coat 3), OA (n=3) or PsA (n=4 for 2 buffy coats, n=1 or n=2 for buffy coat 3) patients was added at 10% for 24 hours. Cells were lysed, and transcripts analysed by TLDA in relation to GUSB housekeeping. RQ calculated relative to monocytes with the isotype pre-treatment. Horizontal bar indicates mean.

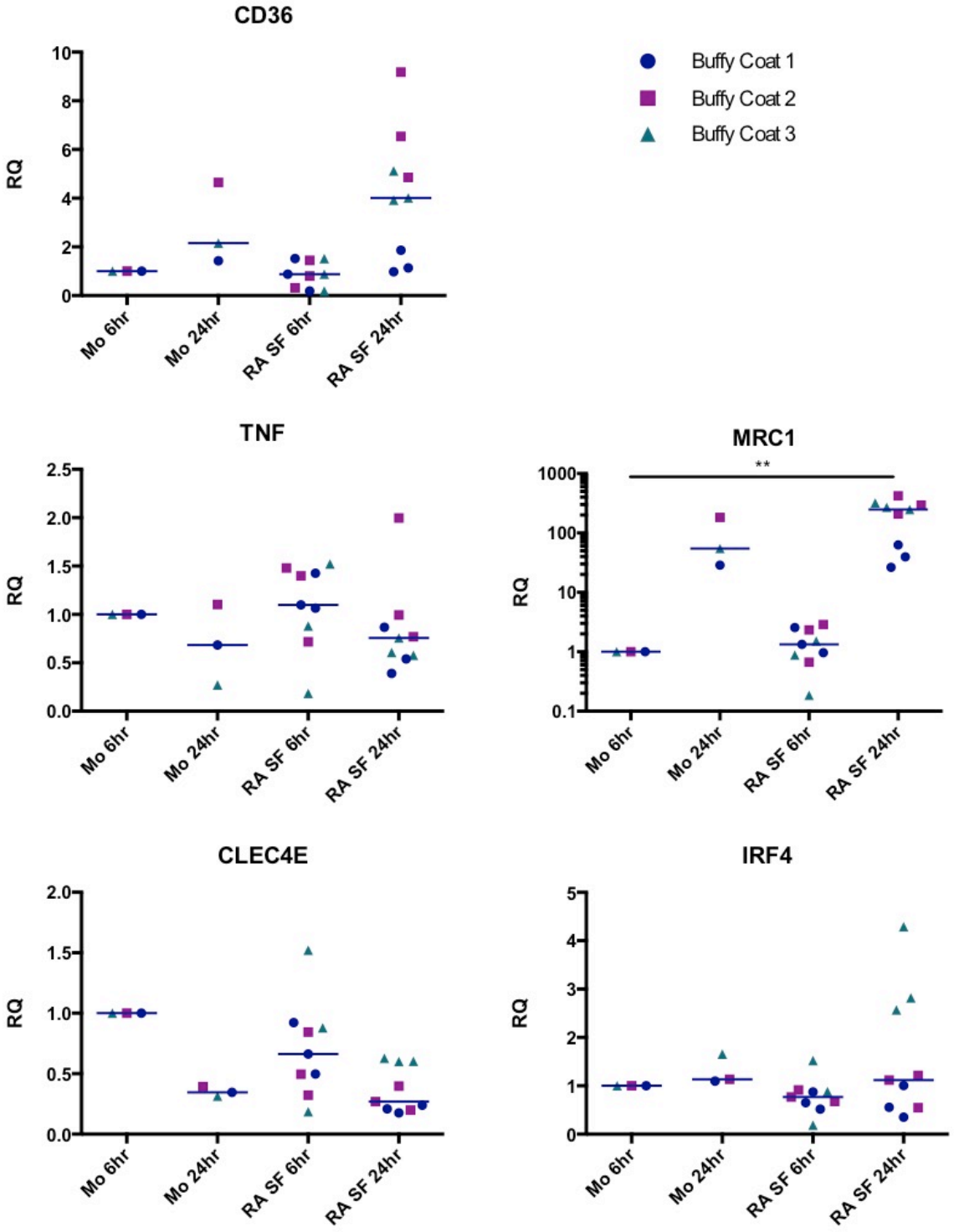


Figure 5.9 Assessment of variability by qPCR.
Monocytes isolated from buffy coats (n=3) on the same day were stimulated with 10% RA synovial fluid (n=3) for 6 or 24 hours. Cells were lysed and RNA extractions were performed at the same time to ensure consistency. Transcripts were analysed by qPCR relative to GUSB housekeeping. RQ calculated in relation to un-stimulated monocytes after 6 hours of culture. Horizontal bar indicates the median. Statistically analysed using Kruskal-Wallis non-parametric test with Dunn's post test. *=p<0.05.

5.2.6 Mavrilimumab caused induction of CCL17 and TNFα in synovial fluid treated monocytes.

Mavrilimumab had little impact on the transcriptional phenotype of synovial fluid stimulated monocytes (Figure 5.8). To determine whether there were any functional differences, cytokine and chemokine secretion were analysed. Isolated monocytes were pre-incubated with Mavrilimumab or the isotype for 30 minutes prior to stimulation with synovial fluid. Chapter 4 analysed how CCL17 was inhibited by synovial fluid after GM-CSF stimulation. Therefore, it was determined whether inhibition of GM-CSF had an impact on CCL17 induction. The pro-inflammatory cytokines: IL-6; IL-8; and TNFα were also analysed.

Secretion of IL-6 was unaffected by pre-treatment with Mavrilimumab (CAM3001), however there was a large amount of variability with the level of cytokine produced. IL-8 was induced by pre-treatment with Mavrilimumab, and one sample was out of range above detection of the assay. The addition of synovial fluid stimulation had no impact on both IL-6 and IL-8 secretion. CCL17 was undetected in the monocytes pre-treated with the isotype, however stimulation of monocytes with RA, OA or PsA synovial fluids caused detectable, but low levels of CCL17 induction (Figure 5.10). Pre-incubation with Mavrilimumab had no effect on the monocytes stimulated with OA or PsA synovial fluid, but caused a significant increase in CCL17 induction in monocytes stimulated with RA synovial fluid. In chapter 4, GM-CSF and RA synovial fluid co-stimulation inhibited CCL17 secretion. Therefore, in this situation, perhaps inhibition of GM-CSF allowed the up-regulation of CCL17. TNFα had a very low level of induction in the monocytes pre-treated with the isotype, which slightly increased in the Mavrilimumab pre-treated monocytes. Surprisingly, in the monocytes pre-incubated with Mavrilimumab, and stimulated with synovial fluids from RA, OA or PsA patients, there was a significant increase in the induction of TNFα in comparison to the monocytes pre-incubated with the isotype.

The significant induction of TNFα after pre-treatment of monocytes with Mavrilimumab was an unexpected and surprising finding, however, this study was only an ‘n’ of 2 buffy coats. Although this suggests that Mavrilimumab causes an up-regulation of TNFα, further studies were required to validate this finding.

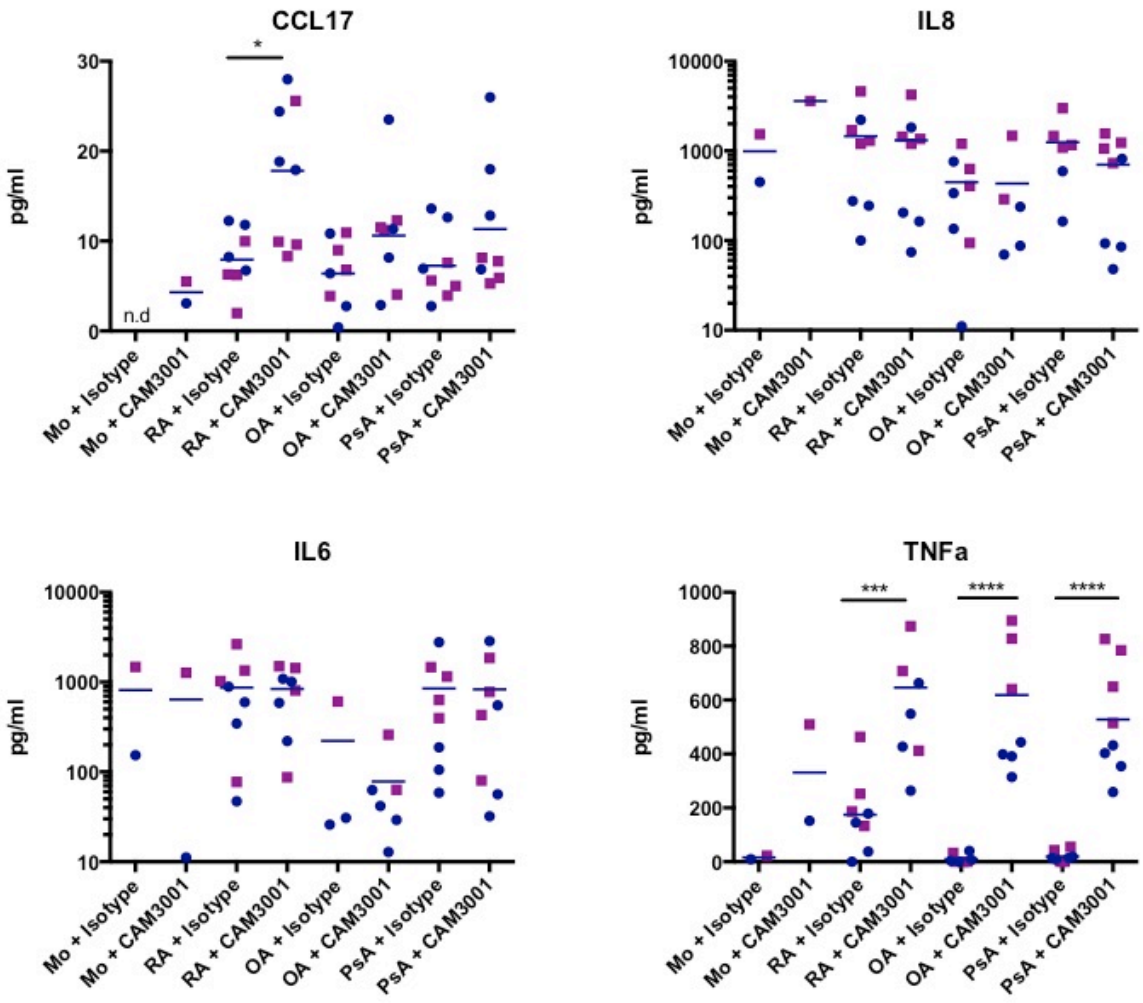


Figure 5.10 Monocytes pre-treated with Mavrilimumab secreted TNFα and CCL17. Monocytes isolated from buffy coats (n=2) were pre-treated with Mavrilimumab (CAM3001) or the isotype (CAT004) for 30 minutes prior to the addition of 10% RA, OA or PsA synovial fluid (n=4). After 24 hours, supernatants were collected and analysed for CCL17, IL-8, IL-6 and TNFα by ELISA. Horizontal bar indicates the mean. Statistically analysed using one-way ANOVA with Bonferroni's post test. *=p<0.05 and ***=p<0.001.

5.2.7 Investigation into the induction of TNFα by Mavrilimumab.

The previous data showed that Mavrilimumab could potentially cause the induction of TNFα in monocytes. Therefore, to analyse this, a dose response of both Mavrilimumab and the isotype was investigated.

Increasing concentrations of the isotype, increasing concentrations of Mavrilimumab and 50ng/ml GM-CSF were used to stimulate monocytes as well as monocytes cultured alone. Stimulation for 4, 16 and 24 hours showed that monocytes alone had a basal level of TNFα secretion (Figure 5.11A). All concentrations of Mavrilimumab resulted in a similar level of TNFα secretion to monocytes alone, whereas the isotype inhibited TNFα production in a dose dependent manner. The GM-CSF positive control caused induction of TNFα, 16 hours after stimulation.

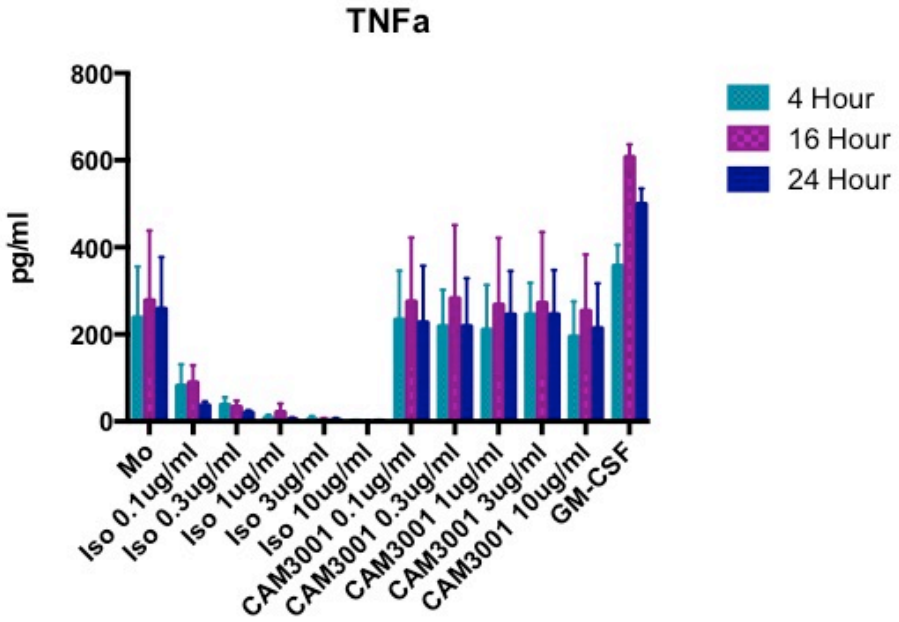
It was an unexpected finding that the isotype for Mavrilimumab inhibited the basal level of TNFα after pre-stimulation. Therefore, it was considered that it could be due to non-specific binding. To examine whether this was the case, monocytes were cultured in media containing human serum to prevent unspecific binding of the antibody. The isotype still inhibited the basal level of TNFα secretion at all analysed concentrations, whereas Mavrilimumab had no change in TNFα production in comparison to the untreated monocyte control (Figure 5.11B).

Therefore, it was necessary to elucidate why this was occurring. One hypothesis was that the isotype was inducing cell death, and therefore preventing the induction of TNFα. To investigate this hypothesis, monocytes were cultured alone, with Mavrilimumab, the isotype or with GM-CSF as a positive control and analysed for apoptosis via FACS (Figure 5.12A-D). Monocytes treated with Mavrilimumab or the isotype had similar levels of apoptosis, comparable with monocytes alone, therefore this was not the reason for inhibition of TNFα. It was possible that the isotype was actually binding to cells, therefore the isotype was fluorescently labelled with vioblue. Using FACS, it was possible to compare binding of the isotype to cells in comparison to unstained cells and FACS beads, which were a positive control. There was a marginal shift in the expression of

isotype labelled-vioblue positive cells in comparison with unstained cells suggesting there was some binding of the isotype to the cells (Figure 5.12E). Controls were used to confirm the isotype was successfully labelled using FACS beads, and so a comparison of vioblue-labelled FACS beads were compared with unstained beads and another pacific blue antibody. The isotype was successfully labelled with the vioblue-fluorescent probe as there was a large shift between unstained and labelled beads (Figure 5.12F) and it was comparable with another pacific blue antibody fluorescence (Figure 5.12G).

These data confirm that Mavrilimumab was not inducing TNFα in monocytes, however, the relevant isotype inhibited TNFα induction. Investigations into the mechanism in which the isotype caused TNFα inhibition were inconclusive, however, the data showed that inhibition was not due to unspecific binding through the use of human serum. Apoptosis was not induced but the isotype had only slight binding to the cells. As I was unable to include an appropriate control antibody there was difficulty in interpretation of the effect of Mavrilimumab on monocytes.

A



B

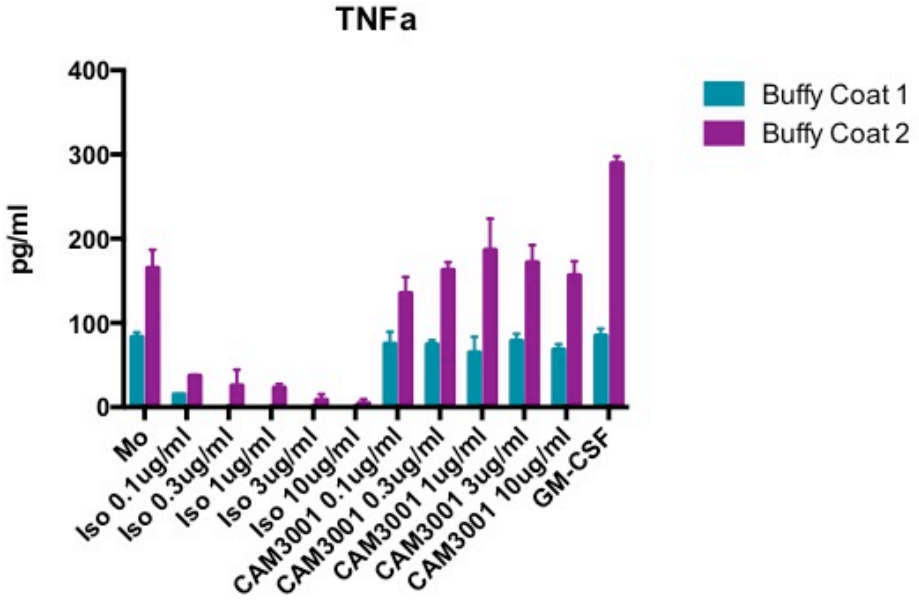


Figure 5.11 Dose response of Mavrilimumab and isotype analysing TNFα induction.
A) Monocytes isolated from buffy coats (n=3) were incubated with increasing concentrations of Mavrilimumab (CAM3001) or the isotype. Supernatants were collected at 4, 16 and 24 hours and analysed by ELISA for TNFα. B) Monocytes separated from buffy coats (n=2) were cultured in media containing human serum and increasing concentrations of Mavrilimumab or the isotype. Supernatants were analysed after 24 hours for TNFα by ELISA. 50ng/ml GM-CSF was used to treat monocytes as a positive control in both A) and B).

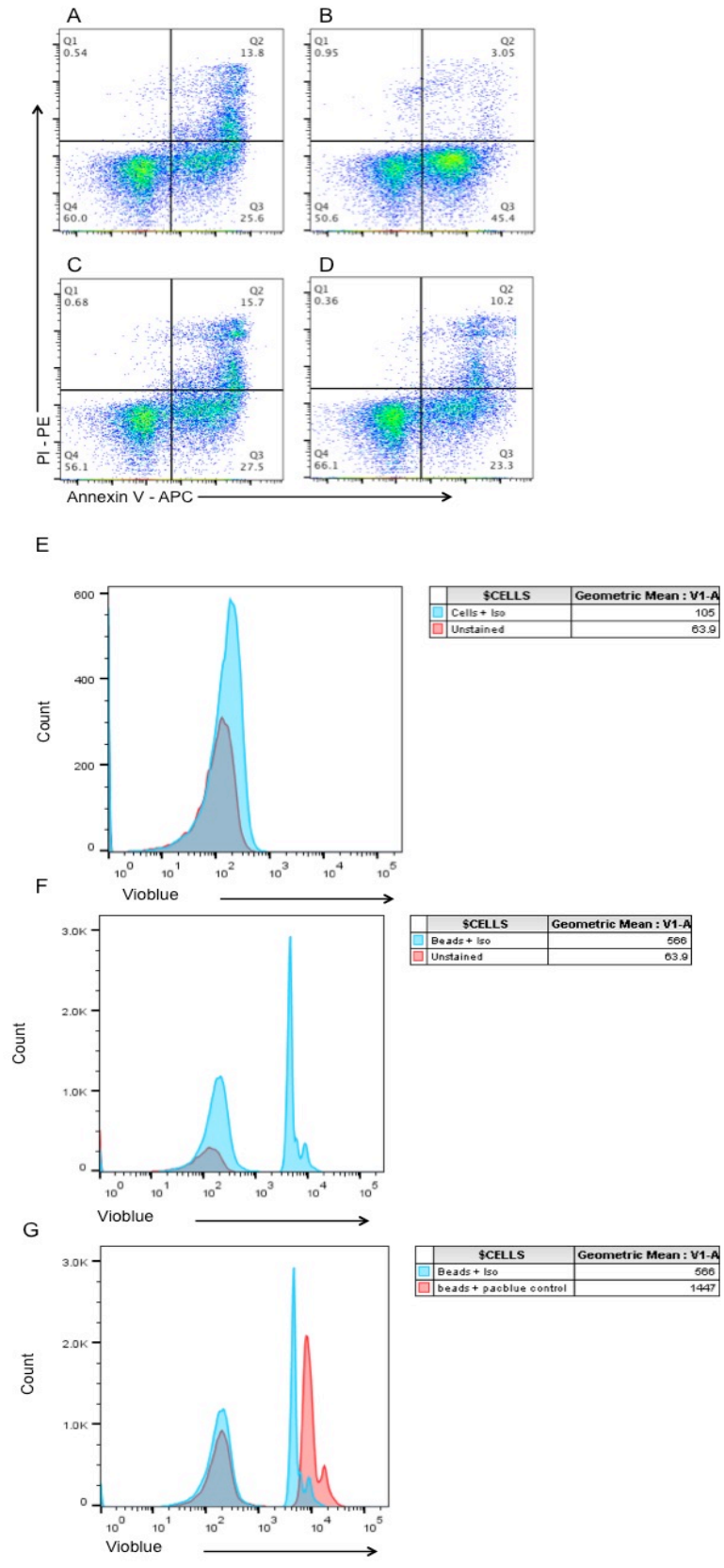


Figure 5.12 Isotype does not induce apoptosis in monocytes.
Monocytes isolated from a buffy coat (n=1) were cultured for 24 hours A) alone or with B) 50ng/ml GM-CSF, C) 10μg/ml Mavrilimumab or D) 10μg/ml Isotype for 24 hours. Monocytes were assessed for apoptosis by Annexin V and PI by FACS. E-G) The isotype was fluorescently labelled with violblue fluorophore and used to analyse binding to monocytes. E) Unstained cells (red) in relation to cells stained with the violblue isotype (blue). F) Beads labelled with the violblue isotype (blue) relative to unstained beads (red). G) Beads labelled with the violblue isotype (blue) in relation to the beads with another pacific blue antibody (red).

5.3 Discussion

Macrophages are highly plastic cells that change their phenotype and activation in response to environmental triggers such as endogenous DAMPs and cytokines (57). In this chapter, investigations were undertaken into macrophage polarisation in response to disease relevant stimuli. Specifically, arthropathy derived synovial fluids were used in order to assess the impact of Mavrilimumab on macrophage phenotypes.

Initial experiments examined macrophage phenotypes under experimental stimuli. The classification system widely used in the macrophage field is the M1 or M2 phenotype. There have been studies suggesting M1 macrophages are *in vitro* derived through stimulation with IFNγ, IFNγ with LPS or GM-CSF, whereas M2 macrophages are derived through stimulation with IL-4, IL-4 and IL-13 or M-CSF (61). In this chapter, macrophages were defined as M0 if they were differentiated with M-CSF alone, M1 for macrophages differentiated with IFNγ, M2 for macrophages differentiated with IL-4 and M-GM for macrophages differentiated with GM-CSF. The nomenclature devised for these experiments was for ease of comparison of macrophages differentiated with synovial fluid with experimental macrophages. We aimed to determine whether there were transcriptional phenotypic similarities between an experimental macrophage and the synovial fluid stimulated macrophages. The M0, M1 and M2 macrophages had distinct transcriptional phenotypes, however the M-GM did not have a specific transcript that was more highly expressed than the other phenotypes. In comparison to the Beyer et al paper (322), in Figure 5.1 there was a significant reduction of CD64 in M2 macrophages in comparison to M1 macrophages. There was also an increase in CD23 expression in M2 macrophages, as well as M1 macrophages highly expressing APOL2, APOL3 and APOL6. Interestingly, Beyer et al., also observed M1 macrophages expressing APOL1, LILRA2 and LILRB3 at significantly higher levels than M2 macrophages, which were not reproduced in this experiment. Krausgruber et al., (327) found M1 macrophages, or macrophages differentiated in GM-CSF to highly express IRF5, whereas in Figure 5.1, M2 macrophages had significantly greater expression of IRF5 than M0 macrophages. M-GM macrophages had a 2.5 fold increase in IRF5 in comparison to M0 macrophages, which could potentially explain why Krausgruber et al., saw an increase in expression. However, M2 macrophages differentiated with IL-4

had greater expression of IRF5. GM-CSF has also been shown to induce IRF4 (328), however this analysis found a greater expression of IRF4 in M2 macrophages. Comparing our findings with the literature shows how there are many different ways of differentiating M1 and M2 macrophages, and variability in assay conditions can lead to differences in expression of transcriptional markers. To improve our findings, we could have used RNAseq or a microarray to fully identify macrophage profiles.

Before examining the effect of synovial fluids on macrophage phenotypic transcripts by TLDA, confirmation of the most consistent housekeeping gene was determined to ensure accurate further analysis. Commonly used housekeeping genes such as GAPDH and ACTB were not consistent across all macrophage phenotypes or the monocyte alone (Figure 5.2). This showed that using a well-defined housekeeping that is popular in the literature is not necessarily the most appropriate for all conditions. In this analysis of 3 buffy coat donors differentiated into macrophages as well as monocytes and PBMCs, the most consistent housekeeping genes were GUSB and UBC, which are less commonly used in the literature. To ensure the housekeeping had a steady CT value, these were used for all further transcriptional analysis.

Analysis of the effect of RA or OA synovial fluid on the polarisation of macrophages was investigated through the analysis of transcriptional profiles. The transcriptional data shown in Figure 5.3 compared monocytes differentiated with experimental stimuli with macrophages stimulated with with RA or OA synovial fluid. Multiple buffy coats and multiple synovial fluids were used to look at the robustness of the observed transcriptional signatures. Both RA and OA synovial fluids had similar effects on the analysed transcripts. This was potentially due to both containing many DAMPs (84). However, this was also surprising considering the previous chapter where OA and RA synovial fluids had different effects on GM-CSF induced secretion of chemokines in monocytes (Figure 4.3). Both OA and RA MSFs had greater expression of CD36, MSR1 and TLR4 in comparison to all experimental macrophages. Despite using multiple buffy coats and multiple synovial fluids to try and gain a robust signature, the data has led us to conclude that there is substantial heterogeneity across healthy donor cells and their response to synovial fluids. This data confirmed that there is a spectrum of activation in macrophages (57) and that the specific

transcriptional analysis of macrophage markers are not necessarily definitive measure of macrophage polarisation. There are other methods of analysis such as flow cytometric analysis of surface receptors and functional assays such as the secretion of cytokines or chemokines (63). RA and OA synovial fluid differentiated macrophages were comparable in the analysed transcript expression. Therefore, we considered whether all arthropathy derived synovial fluids caused a similar phenotype of macrophage. To further investigate, PsA synovial fluids were used and compared with the RA and OA MSF transcript expression. The analysis by qPCR showed the all MSF had a similar transcriptional profile to the M0 macrophages stimulated with LPS suggesting that the origin of the synovial fluid has no impact on the transcriptional phenotypes of macrophages (Figure 5.4). However, the unchanged cellular response between synovial fluids from RA, OA or PsA could be due to: storage as long term storage at -80^{o}C could have caused protein degradation; and the synovial fluid was used at 10% as a greater concentration is cytotoxic, however, this dilutes the cytokines and DAMPs to low concentrations which would have less of an effect than recombinant cytokines. The cytokine content of synovial fluids (Figure 4.1) is very heterogeneous, and in some cases, once diluted, would contain negligible levels of factors, such as cytokines.

Functionally, through chemokine analysis, there were also no differences between macrophages differentiated with RA, OA or PsA synovial fluid. The secretion of chemokines is used to define macrophage phenotypes, with CCL3 and CCL5 secretion associated with M1 macrophage phenotypes (329). CCL7 has also been associated with increased induction in M1 macrophages in comparison to M2 macrophages (330), whereas CCL8 is secreted more in an M2 macrophage (266). CXCL1 secretion is not specifically associated with a phenotype of macrophage, however it is produced upon stimulation with LPS, therefore it is potentially a more pro-inflammatory M1 chemokine (331). These chemokines were analysed using a multiplex assay. Synovial fluids caused no induction of chemokines in macrophages, however as the LPS positive control led to the induction of chemokines, the assay was functional (Figure 5.5). There were also no differences between RA, OA or PsA differentiated macrophages, suggesting that all synovial fluids have a similar impact on macrophage phenotypes. For most of the analysed chemokines, there was no difference in comparison to the

control, however for CCL7 and CCL8, there was a potential suppression of induction with the addition of synovial fluids.

Despite analysing macrophages polarised with synovial fluids from RA, OA and PsA patients via transcriptional markers and chemokine induction, there were no differences. This suggests either that macrophages have less of an inflammatory response to synovial fluid, or that the diluted synovial fluid was not enough of a stimulus for more dynamic activation where differences between the diseases would be observed. RA and OA synovial fluid have widely been compared and there are many differences in the DAMPs and cytokines content (84,133,140). Therefore, as macrophages are plastic and known to alter phenotypes in response to their environment, it would be expected for differences to be observed. There was also a large level of variability between the buffy coat donors used to differentiate into macrophages. This could potentially have been improved with an increase in the number of buffy coats analysed. A large limitation of the phenotypic analysis of macrophages was the factors analysed. FACS analysis of surface receptors would assess the protein expression of multiple markers such as CD206 and CD23, and the analysis of cytokine secretion such as IL-10, IL-6, TNFα or TGFβ could have been used to more thoroughly evaluate macrophage phenotypes.

Macrophages stimulated with synovial fluids from different arthropathies had similar transcriptional profiles and chemokine expression. As macrophages are tissue resident cells, we aimed to determine whether monocytes were more responsive to synovial fluid, as they infiltrate the synovium in response to inflammatory cues. Monocytes had differing levels of most of the transcripts in comparison to macrophages (Figure 5.6), showing that despite originating from monocytes, the differentiation process alters many aspects of the cell's phenotype. Interestingly monocytes transcribe less of CSF2RA (GM-CSFR gene) in comparison to macrophages, which are known to highly express the GM-CSFR on their surface (238). Monocytes had a greater level of CCL2 expression in comparison to macrophages although this was not a significant difference due to the donor variability. Not only were differences observed between monocytes and macrophages, but there were also differences in transcriptional profiles between monocytes stimulated with RA, OA or PsA synovial fluids. PsA synovial

fluid stimulated monocytes had a greater expression of most transcripts in comparison to monocytes stimulated with OA synovial fluid, with monocytes stimulated with RA synovial fluid at a level in between (Figure 5.7). Monocytes appeared to be more responsive to the differences in synovial fluid in comparison to macrophages, which may be due to monocytes infiltrating the synovium in response to inflammatory cues, and therefore the factors within the synovial fluid have more of an impact on the fate of the monocyte (56). The differences observed between diseased synovial fluid stimulations suggest that the components are important with regards to monocyte activation. However, this was only analysed in one buffy coat with 4 different synovial fluids from each disease. This would need to be repeated in a greater number of buffy coat donors to confirm the differences between monocytes and macrophages. Due to the ability of monocytes to respond to synovial fluid from RA, OA or PsA patients in different ways, monocytes were used to take forward and assess for activation markers with the addition of Mavrilimumab.

Pre-treatment of monocytes with Mavrilimumab led to no differences between synovial fluid treated monocytes and monocytes alone. This was hard to interpret due to the large level of variability between the monocyte donors. The variability was confirmed to be due to the monocytes and not a batch effect in repeating the assay. When transcripts were analysed from monocytes that were isolated on the same day, with the whole assay taking place at the same time, there was still variation (Figure 5.9). Mavrilimumab had little effect on monocyte activation transcripts, however due to the variation between monocyte donors, no differences could be observed (Figure 5.8). To improve this experiment, more donors could be used to try to minimise variability as this would allow more conclusions to be drawn. Specific activation markers could be analysed by FACS or functionally by cytokine or chemokine analysis. Analysis of cytokines and chemokines was attempted, but the lack of a suitable unstimulated monocyte control prevented further analysis. The lack of this control also gave the impression that monocytes pre-treated with Mavrilimumab were causing the induction of TNFα and CCL17 (Figure 5.10). Based on the interpretation of the data and the missing control, experiments were repeated to investigate the impact of Mavrilimumab on TNFα secretion. Mavrilimumab caused comparable levels of TNFα induction to the unstimulated monocyte

confirming that Mavrilimumab was not inducing TNFα (Figure 5.11A). During development of Mavrilimumab, studies were conducted into the secretion of TNFα from the monocyte, which showed a down-regulation of TNFα with increasing concentrations of Mavrilimumab (332). The experiments revealed however, that the isotype, used in the studies, caused inhibition of TNFα. The inhibition caused by the isotype was an issue as this prevented comparison with Mavrilimumab, thereby preventing any conclusions being drawn.

We therefore aimed to determine how the isotype was causing such a dramatic dose dependent inhibition of TNFα. Initially human serum was used instead of FBS in the media to block unspecific binding and could prevent the observed inhibition. This was not the case, as even with human serum in the media, there was inhibition of TNFα by the isotype. This suggested that the isotype was either binding to cells, inhibiting TNFα signalling, or that it was causing the cells to apoptose (Figure 5.11B). Apoptosis analysis revealed that monocytes treated with Mavrilimumab or the isotype had similar levels of apoptosis to monocytes alone, suggesting that this was not the cause of TNFα inhibition (Figure 5.12A). Fluorescent labelling the isotype allowed us to assess the ability of the isotype to bind to monocytes. There was a marginal shift in isotype expression in comparison to unstained cells, showing that the labelled isotype was not fully binding to cells. This suggested that by directly binding to the monocyte, the isotype was causing inhibition of TNFα from the basal level. Despite this unusual finding, it was not possible to pursue this any further. However, the hypothesis that the IgG_4 isotype could be binding to Fcγ receptors and causing down-regulation of TNFα is unlikely. It has been shown that binding of immune complexes to Fcγ receptors caused a down-regulation of IL-12 secretion in monocytes (333), however TNFα secretion was induced in macrophages and DCs upon FcγR triggering (68,334). Interestingly, however, binding of mouse FcγRI by small immune complexes have shown to have induce IL-10 responses and down-regulate IL-12 (245). Furthermore FcγRI has been shown to bind IgG_4 (335). The effects of non-complexed IgG4 binding in human monocytes has also been examined in monocyte-derived macrophages, whereby IgG_4 inhibited IFNγ mediated events such as the secretion of CXCL10 via FcγRI (336). Therefore, there could be the potential for IgG_4 to bind FcγRI and inhibit the induction of

TNFα in monocytes, as a hypothesis for the observation in Figure 5.11. However, as the inhibition of TNFα by the isotype was not the main focus of the experiment it was not fully investigated.

This chapter has shown how macrophages stimulated with synovial fluids are a polarised macrophage and a part of the macrophage phenotypic spectrum. The synovial macrophage phenotype, both from the synovial membrane and the synovial fluid also has broad qualities and are incompletely understood (325). These macrophages secrete pro-inflammatory mediators such as TNFα and IL-1, suggesting an M1 phenotype, but also IL-10, IL-1RA and TGFβ, which are more M2-like traits (325,337). Interestingly, the macrophages that were polarised with RA, OA and PsA synovial fluids were comparable both transcriptionally and in chemokine induction. Monocytes were much more responsive to diseased synovial fluids, and differentiating the monocytes in synovial fluids from day 1 rather than in M-CSF first could have created a more accurate *in vitro* derived synovial macrophage. Analysis of Mavrilimumab with respect to monocyte phenotype was inconclusive due to the large level of variability between buffy coat donors and the effect of the isotype on monocytes. In order to move forward with this research, it would be interesting to analyse the phenotype of macrophages directly from synovial membranes and synovial fluid in relation to an *in vitro* stimulated macrophage. Also, the effect of Mavrilimumab on macrophage and monocyte phenotypes stimulated with synovial fluid would be interesting as this would determine whether GM-CSF has in a synovial context has an effect on macrophage activation. However, a more robust assay that would limit donor heterogeneity and an isotype that caused no inhibitory effects on the cells would be required for conclusive analysis of Mavrilimumab. More ways of analysing phenotypes by methods such as FACS could also provide information on the activation of the cells. Overall, this chapter has determined that macrophages that are exposed to synovial fluid are polarised to a unique phenotype. Monocytes, that would potentially infiltrate the synovium, appear more responsive to synovial fluid stimulation and transcriptionally have altered phenotypes dependent on synovial fluid disease origin. Further studies are warranted to understand in greater detail the impact of synovial fluid on macrophage differentiation, as these are key cell types involved in RA pathology.

Chapter 6 General Discussion

This thesis has examined the relationship between the synovial environment and GM-CSF and their impact on monocytes and macrophages. When monocytes infiltrate the synovium, they are exposed to multiple factors that influence their inflammatory response (320). The monocyte response to environmental stimuli, prior to differentiation was unknown. Therefore, we aimed to evaluate the effect of GM-CSF on chemokine responses secreted from monocytes, as well as the influence of the environment on monocytes and macrophage polarisation.

These data have highlighted that monocytes secrete substantial quantities of the CCR4 binding chemokine, CCL17, after 24 hours of stimulation with GM-CSF. GM-CSF is a relevant cytokine to examine as it has been found at high levels within the synovial fluid and $CD4^+$ T cells have been shown to be producers of GM-CSF in the RA synovium (326,338). However, as Chapters 3 and 4 have identified, the capacity of monocytes to secrete CCL17 can be inhibited my multiple factors, including TLR ligands, RA synovial fluid, Small Immune Complexes and IFNα. The ability of RA synovial fluid to inhibit the secretion of a pro-inflammatory chemokine was unique, as RA synovial fluid has previously been found to be stimulatory rather than inhibitory. RA synovial fluid has prevented the apoptosis of neutrophils, thereby leading to the persistence of activated neutrophils, exacerbating the inflammatory pathology (339). Monocytes differentiated with IL-4 or IL-13 for 3 or 6 days had an increase in secretion of CCL18 when co-stimulated with RA synovial fluid, showing that RA synovial fluid can cause an up-regulation in chemokine secretion (340). We found RA synovial fluid inhibited the GM-CSF induced secretion of CCL17, which is a novel inhibitory response opposed to the multiple stimulatory responses that have previously been observed. This suggests that not only are there pro-inflammatory responses involved in RA pathogenesis, but to some level there are more regulatory mechanisms. In this instance, monocytes have the ability, prior to their differentiation, to cause the influx of $CCR4^+$ T cells into the joint, however the synovial milieu prevents this.

Analysis of CCL17, CCR4 and GM-CSFRα in the RA synovium, in Chapter 3, found that CCL17 and CCR4 were prevalent in the tissue. This suggests that despite synovial fluid inhibiting the secretion of CCL17 from monocytes, there must be

other cell types that are secreting CCL17, leading to the infiltration of $CCR4^+$ cells that are observed in the tissue. Dendritic cells are also known to secrete CCL17 upon TSLP stimulation, and TSLP has been found in the RA synovial fluid (180,268). Interestingly, RANKL, which is present within the RA synovium (341) has been found to mediate an increase in the secretion of TSLP induced CCL17 from DCs (342). This suggests that the induction of CCL17 from DCs could be up-regulated by factors within synovial fluid, whereas for monocytes, this induction is inhibited.

Our investigations into the mechanism of inhibition by RA synovial fluid initially analysed pSTAT5 as it was downstream in the signalling cascade from GM-CSFRα. There was no difference observed in unstimulated monocytes compared with monocytes pre-treated with RA synovial fluid. We concluded that pSTAT5 or upstream molecules were not impacted by synovial fluid. Interestingly, STAT6 has been identified as a transcription factor for CCL17 downstream of IL-4 signalling (267). Therefore, we investigated whether this transcription factor was down-regulated after RA synovial fluid signalling. There was a slight down-regulation after RA synovial fluid, but as this was a preliminary study, it was inconclusive, but repeating this experiment would confirm whether STAT6 was involved. Identifying the transcription factor that GM-CSF signalling activates to transcribe CCL17 is important to confirm whether Immune complexes, TLR ligands and IFNα affect activation of this transcription factor.

We have identified that RA synovial fluid differs from OA synovial fluid in the capacity to inhibit GM-CSF induced CCL17 in monocytes. However, the effect of synovial fluid on macrophage polarisation, showed that it was not dependent on the disease origin. Despite synovial fluid polarisation forming distinct macrophages, they had comparable transcriptional profiles despite the origin of the synovial fluid. Whether these macrophages were comparable with macrophages that were directly isolated from the synovium it is unknown, but this would be an interesting comparison. Synovial macrophages are generally associated more with an M1 phenotype due to the pro-inflammatory cytokines, such as TNFα and IL-1, that they secrete. However, they are also known to secrete the more anti-inflammatory cytokine IL-10 (343). This suggests that there could be multiple subsets of macrophages within the synovium. It could

also suggest that the mixed M1 and M2 phenotype observed in our experimentally produced macrophages differentiated with synovial fluid, are similar to those observed in synovial macrophages. Further investigation would be required to examine this observation. Understanding macrophages of the synovium is important to understand RA pathogenesis, as macrophages of the sub-lining layer have been suggested as a potential biomarker to predict therapeutic efficacy (344). For this reason we aimed to establish the effect of Mavrilimumab on macrophage and monocyte phenotypes, as GM-CSFRα was identified at high levels in areas where CD68 was also expressed (Chapter 3). However, due to the large level of variability between human monocyte donors and the isotype phenotype that caused inhibition of TNFα, this could not be achieved.

6.1 Future Work

We found in Chapter 3 that the ability of monocytes to secrete CCL17 was inhibited by TLR ligands. When we observed in Chapter 4 that RA synovial fluid also inhibited GM-CSF induced CCL17 in monocytes, it was assumed that TLR ligands within the fluid were causing this. However, TLR ligands were not the only factor with the capacity to cause inhibition of CCL17. Therefore, future work would include identifying the specific factors within synovial fluid that cause inhibition of CCL17. In order to achieve this, fractionating the synovial fluid based on size would be the initial method to narrow down the investigations. As we know that small immune complexes can mimic the RA synovial fluid in causing inhibition, it would be interesting to isolate the ACPA or RF from the fluid to determine whether they have the same effect. It would also be important to identify the specific mechanism leading to inhibition of CCL17. Our data suggests there could be multiple pathways as there are several factors that cause inhibition of GM-CSF driven CCL17, however, it would be important to confirm this. There is the possibility that there are multiple signalling pathways that lead to the same factor that inhibits transcription of CCL17.

These data have analysed monocytes as a whole, however it would be interesting to determine whether the different subsets of monocytes, categorised by their CD14 and CD16 expression, had varying responses to GM-CSF and RA synovial fluid.

OA synovial fluid had variable effects on the inhibition of CCL17, therefore, it would be important to identify whether the factor within OA synovial fluid that caused inhibition was the same as the RA synovial fluid. OA synovial fluid significantly inhibited GM-CSF induced CCL3 and CCL4, and it would be interesting to understand the mechanism of inhibition, as well as its biological relevance and impact on disease pathology.

It would still be of interest to understand the impact of Mavrilimumab on monocyte and macrophage phenotypes within the synovium, however, a reliable isotype and experimental system would be required for this. Also, analysis via flow cytometry or assessing cytokine and chemokine release as well as transcriptional analysis or RNAseq would give a greater depth to phenotypic profile analysis. Mavrilimumab impacted on CCL17 and CCL22 in serum of patients, highlighting that GM-CSF driven CCL17 and CCL22 could be involved in RA pathogenesis (295).

6.2 Conclusions

This thesis has identified a novel regulatory system within the RA synovium. Monocytes exposed to GM-CSF secrete CCL17, however, in the RA synovial environment, this induction is inhibited. CCL17 chemoattracts $CCR4^+$ cells into the synovium, which upon activation secrete GM-CSF. Therefore, the inhibition of CCL17 could potentially prevent the influx of $CCR4^+$ cells into the synovium, thereby regulating the inflammatory cell infiltration into the joint. However, as there are other cells such as dendritic cells that secrete CCL17, there is still the possibility of $CCR4^+$ T recruitment into the tissue. Inhibition of GM-CSF driven CCL17 by RA synovial fluid was mimicked by TLR ligands, small immune complexes and IFNα. This suggests multiple mechanisms can cause inhibition of this pathway, which need further investigation.

Appendix

Media

Complete RPMI:

500ml Incomplete RPMI (Gibco, Invitrogen)
50ml Heat Inactivated Foetal Bovine Serum (Invitrogen)
5ml Penicillin-Streptomycin (Sigma)

Human Serum RPMI:

500ml Incomplete RPMI (Gibco, Invitrogen)
50ml Human Serum (Biowest)
5ml Penicillin-Streptomycin (Sigma)

T Cell Media:

500ml Incomplete RPMI (Gibco, Invitrogen)
50ml Heat Inactivated Foetal Bovine Serum (Invitrogen)
5ml Penicillin-Streptomycin (Sigma)
5ml L-Glutamine (Sigma)

Complete Dulbecco's Modified Eagle Medium (D-MEM):

500ml Incomplete D-MEM
50ml Heat Inactivated Foetal Bovine Serum (Invitrogen)
5ml Penicillin-Streptomycin (Sigma)
5ml L-Glutamine (Sigma)

Buffers

Cell Separation Buffer:

500ml dPBS (Gibco, Invitrogen)
10ml FBS (Invitrogen)

FACS buffer:

1L 1x PBS
3% BSA
1mM EDTA
0.05% Sodium Azide

ELISA Coating Buffer:

1L 1x PBS

ELISA Assay Buffer:

1x PBS
1% BSA

ELISA Wash Buffer:

1x PBS
0.05% Tween

IHC Citrate Buffer (pH6)

2.1g Citric Acid
1L dH_2O

IHC Wash Buffer (TBST)

1x TBS
0.05% Tween

Protein A/G/L magnetic bead binding buffer

50 mM Tris
150 mM NaCl
In dH_2O, pH 7.5

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