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**NEW PATHWAYS IN THE PATHOGENESIS OF
RHEUMATOID ARTHRITIS**

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MBChB, Med.Sci

**A thesis submitted in fulfilment of the requirement for the Degree of
Doctor of Philosophy**

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Abstract

Rheumatoid Arthritis (RA) is a common chronic autoimmune disease that is characterized by synovial tissue inflammation eventually leading to joint destruction with severe functional deterioration and increased mortality – the underlying pathogenesis of RA remains unsolved. The principle of new therapeutic development is to define and characterize a molecular pathway both in terms of its basic biology and also its context-dependent effects in the synovial compartment. A hallmark pathological feature of RA is a rapid influx and accumulation of immune cells such as monocytes/macrophages into the synovium. Monocytes/ macrophages are major effector cells in RA synovitis, principally acting by releasing TNF- α , IL-6 and other inflammatory cytokines and chemokines. The recruitment of effectors cells is an important step in RA progression and is mediated by chemokines and their receptors. Two pathways will be studied in this project, both with potential relevance to the accumulation and activation of inflammatory leukocytes to the synovium, namely microRNAs and sphingolipid enzymes.

MicroRNAs are a recently discovered class of post-transcriptional regulators that induce mRNA target degradation or translation inhibition. They have been shown to be involved in the regulation of the immune response and the development of autoimmunity. Of particular interest in the context of RA is miR-155, which is upregulated in RA synovial macrophages where it regulates cytokine expression such as TNF- α . Until now little was known about the role of miR-155 in regulating monocyte migration. Therefore, we sought to focus on the functional contribution of miR-155 in monocyte migration by the modulation of the expression of chemokines and their reciprocal chemokine receptors. Firstly the absolute copy numbers of miR-155 transcripts in peripheral blood (PB) and synovial fluid (SF) monocytes of RA and healthy controls were assessed. To examine the role of miR-155 in monocyte migration and retention in the joint space, I overexpressed miR-155 in PB CD14⁺ monocytes of healthy controls and RA patients and examined the expression of chemokines and chemokine receptors mRNA levels by taqman low-density array (TLDA) and quantified the production of these chemokines in culture supernatant by multiplex assay. The role of miR-155 was investigated further using bone marrow monocytes (BMMO) from miR-155^{-/-} and wild type (WT) mice. RA PB and SF CD14⁺ monocytes expressed higher copy numbers of miR-155 compared with healthy controls. RA SF monocytes exhibited the highest expression levels of miR-155. The copy number of miR-155 expression was significantly increased in anti-citrullinated protein antibody (ACPA) positive RA compared with ACPA negative RA. The RA PB monocyte miR-155 copy number correlated positively and significantly with DAS28. Overexpression of miR-155 in PB monocytes led to an increased production of chemokines (CCL3/MIP-1 α , CCL4/MIP-1 β , CCL5/RANTES, and CCL8/MCP2) and a reduction in expression of inflammatory chemokine receptors CCR2 and CCR3 while homeostatic CCR7 was up regulated. Commensurate with this, these receptors were expressed in an opposite direction in

BMMO from miR-155 deficient cells; CCR7 was significantly down regulated and CCR2 and CCR3 expression levels were increased. These observations suggest that CCR2, CCR3 and CCR7 were under the tight control of miR-155 and that this regulation is preserved across the species; together suggesting that miR-155 can act as an important regulator of these receptors. We conclude that deregulation of miR-155 in RA monocytes contributes to monocyte retention at sites of inflammation due to induction of chemokine production and down-regulation of inflammatory chemokine receptors. Furthermore, these data imply that miR-155 levels may reflect RA disease activity and could be a potential diagnostic or clinical disease activity biomarker for RA.

Sphingosine kinases (SPHKs), SPHK1 and SPHK2, are isozymes that phosphorylate sphingosine into sphingosine-1-phosphate (S1P). S1P, a pleiotropic lipid mediator of inflammation, subsequently binds with any of the five G-protein coupled protein S1P receptors (S1PR₁₋₅) and stimulates an array of cellular responses. Defects in S1P/S1PRs signalling have been shown to be associated with various pathologies. Until now however, no comprehensive analysis of expression of its components in RA has been performed. My data show that S1P concentrations were significantly elevated in the serum of RA patients with active disease compared to RA patients in remission and in healthy controls. Moreover, S1P₁, S1P₃, S1P₅ and SPHK1 were differentially regulated in RA immune cell subsets, such as neutrophils, monocytes (CD14⁺), lymphocytes (CD4⁺ and CD8⁺) compared to healthy controls. In addition, compared with osteoarthritis (OA) pathological control, RA synovial tissues were strongly positive for the SPHK1, S1P₁, and S1P₃. Interestingly, RA patients treated with biological DMARDs had attenuated levels of SPHK1, S1P₃ and S1P₅, but not S1P₁, when compared with patients treated with conventional DMARDs. Therefore, my study warrants further investigation of the clinical significance of S1P as a biomarker for disease activity and to explore the utility of novel therapeutic tools available to modulate the SPHK/S1PR/S1P axis in RA with a view to defining new therapeutic possibilities.

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Abbreviations

AD	Airway disease
ACPA	Antibodies against citrullinated peptides antigens
APRIL	Proliferating inducing ligand
BAFF	B cell activating factor
BMMO	Bone marrow monocytes
CCP	Cyclic citrullinated peptides
CD	Crohn's disease
Cer	Ceramide
CIA	Collagen induced arthritis
COX-2	Cyclooxygenase-2
CRP	C-reactive protein
CTLA-4	Cytotoxic T lymphocyte associated antigen-4
CVD	Cardiovascular disease
DCs	Dendritic cells
DMARD	Disease-modifying antirheumatic drug
DNMTs	DNA methyl-transferases
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein–Barr virus
EDG	Endothelial differentiation gene
ER	Endoplasmic reticulum
ESR	Erythrocyte sedimentation rate
FACS	Fluorescent activated cell sorting
FCS	Foetal calf serum
FGF	fibroblast growth factor
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage stimulating factor
GWAS	Genomic wide association study
HAT	Histone acetyltransferases
HDACs	Histone deacetylases
HLA	Human leukocytes antigen
HRCT	High-resolution computed tomography
Hsps	Heat shock proteins
IBD	Inflammatory bowel disease
IHCs	Immuno-histo chemistry staining
IFN	Interferon
IL	Interleukin
ILD	Interstitial lung disease
IL1R2	IL1R2 interleukin 1 receptor
IRAK1	IL-1 receptors-associated kinase-1
LPS	Lipopolysaccharide
MACS	Magnetic-activated cell sorting
MCSF	Macrophage colony-stimulating factor

MHC	Major histocompatibility complex
MiRNAs	MicroRNA
MMP	Matrix metalloproteinase
MS	multiple sclerosis
MTX	Methotrexate
NK	Natural killer cell
NSAIDs	Non-steroidal anti-inflammatory drugs
OA	Osteoarthritis
OASFs	Osteoarthritis synovial fibroblasts
PB	Peripheral blood
PBC	Primary biliary cirrhosis
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PD	Periodontal disease
PDGF	Platelet-derived growth factor
PGE2	Prostaglandin E2
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
QPCR	Quantitative polymerase chain reaction
RA	Rheumatoid arthritis
RASFs	Rheumatoid arthritis synovial fibroblasts
RF	Rheumatoid factors
SAM	S-adenosyl-methionine
SCID	Severe combined immunodeficiency
SE	Shared epitope
SF	Synovial fluid
SHIP-1	SH2 domain containing inositol-5-phosphatase
SLE	Systemic lupus erythematosus
SM	Sphingomyelin
SNP	Single-nucleotide polymorphism
SOCS-1	Suppressor of cytokine signalling-1
S1P	Sphingosine 1 phosphate
S1PRs	Sphingosine 1 phosphate receptors
Sph	Sphingosine
SPHKs	Sphingosine kinases
SSZ	Sulfasalazine
TCR	T cell receptor
TGF β	Transforming growth factor beta
TLR	Toll like receptor
TNF- α	Tumour necrosis factor alpha
TRAF6	TNF- α receptor-associated factor-6
Tregs	Regulatory T cells
UC	Ulcerative colitis
3' UTRs	3' un-translated regions
VEGF	Vascular endothelial growth factor

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Declaration

The work presented in this thesis represents original work carried out by the author within the Institute of Infection, Immunity and Inflammation at Glasgow University. This thesis has not been submitted in any form to any other university.

CHAPTER I
GENERAL INTRODUCTION

1.1 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease of unknown aetiology, mainly affecting the synovial membranes of joints, leading inexorably to damage of the cartilage and bone as well as bursa and tendon sheaths (1, 2). The prevalence of RA in the general population is 0.8-1% and usual age of onset is 40-60 years (1, 3). However, RA may begin at any age and occurs in all races and ethnic groups. There is a female predominance of about 3:1, although the female to male ratio declines with increasing age to 1:1 for onset after sixty years of age. Patients with RA may have insidious onset of symmetric joint pain, swelling that deteriorates over several weeks associated with morning stiffness persisting for over 30 minutes that subsides during the day (4). RA may onset via acute or sub acute presentations in about 25% of patients. On rarer occasions, onset is of a palindromic nature, characterised by recurrent episodes of oligoarthritis with no residual radiological damage (5). Although any joint may be involved in RA, classically synovitis afflicts the proximal inter-phalangeal and metacarpal phalangeal joints as well as wrist, knees, shoulders, ankles, feet, hips and cervical spine (4). These characteristic manifestations are accompanied by symptoms of malaise, weight loss, fever and fatigue.

The inflammatory process of RA can spread to other systems or organs, particularly in patients with severe joint disease and results in extra articular manifestations; these can occur in up to 40% of patients during the course of disease (6). The extra-articular manifestations of RA are many; for example, subcutaneous nodules, pulmonary nodules, pulmonary interstitial fibrosis, pleural effusion, pericarditis, pericardial effusion, vacuities, skin ulceration, Felty's syndrome as well as neurological and haematological complications. The presence of extra articular manifestations are important in determining RA outcomes; RA patients with these manifestations have five-fold increase in mortality as compared to those without extra articular manifestations (6). Thus, RA has a wide clinical spectrum with considerable variability in joint and extra-articular manifestations. However, with early and effective treatment these manifestations are less common. Indeed, there is clear evidence based on recent data suggesting that starting treatment earlier leads to better outcome and even improved rates of disease remission. Therefore, early recognition and aggressive treatment to limit disease sequelae is essential. The

severity of disease may range from mild to very intense; involving multiple organ systems and leading to aggressive damage that causes significant morbidity and mortality. Although RA was once considered to be a relatively benign disorder, it is now known to be a disease with a strong tendency to shorten the lifespan and cause severe disability to a varying degree; accordingly RA is associated with a high social burden and economic cost due to unemployment (7).

Although there is no specific laboratory test to exclude or confirm RA, several lab parameters are associated with diagnosis. These include: erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP), which provides simple indicators about the acute phase response, correlated with clinical assessment of the disease. Moreover, Rheumatoid factors (RF), immunoglobulins directed against the Fc fragment of IgG, are present in the sera of more than 75% of RA patients. Recently, antibodies against citrullinated peptides antigens (ACPA) have been linked to RA, they are highly specific to RA and have been identified in up to 90% of RA patients and they can correlate with disease severity (8). Due to their superior specificity and predictive capabilities for future development of RA, compared to RF, RA patients can be divided serologically into 2 major clinical subtypes, namely ACPA positive and ACPA negative RA. The plain radiography is the most specific and standard investigation in RA diagnosis and pathology. The earliest changes occur in the wrists, feet and consist of soft tissue (s) swelling and juxta-articular demineralization, while later changes include narrowing of joint spaces and erosion. Other imaging diagnostic techniques including magnetic resonance imaging, computed tomography, power Doppler ultrasound and scintigraphy may be used to assess the extent of anatomical changes in RA patients with higher degrees of sensitivity and as such they may in due course replace plain X-rays as the investigation of choice.

Clinical manifestations of RA vary depending on the involved joints and the disease stage. In order to facilitate the consistent identification of patients classification criteria have been developed. Table 1.1 summaries the 1987 American Rheumatism Association revised criteria for RA classification. Until recently, diagnosis of RA should have four or more of these criteria and must be present for at least six weeks to exclude other differential diagnosis of arthritis such as degenerative arthritis (osteoarthritis (OA)) and connective tissue arthritis (4).

Table 1.1 1987 American College of Rheumatology Revised Criteria for rheumatoid arthritis adapted from (4).

Criteria	Definition
Morning stiffness	Morning stiffness \geq 1 hour for at least 6 weeks
Arthritis \geq 3 joints	Synovitis in \geq 3 joint areas simultaneously
Arthritis of hand and wrist	Wrist or hand MCP or PIP joints for at least 6 weeks
Symmetric arthritis	Same joint areas on both sides of the body, for at least 6 weeks
Rheumatoid nodules	Non tender subcutaneous nodules situated over bony prominences
Serum rheumatoid factor	IgM Autoantibody directed against the Fc fragment of IgG
Radiographic changes	Typical of rheumatoid arthritis soft tissue swelling and juxta-articular demineralization, uniform joint space narrowing and erosion

Four of seven criteria are needed for diagnosis of RA and the 14 possible joint areas include hand proximal inter-phalangeal (PIP) and metacarpal phalangeal (MCP), wrist, elbow, knee, ankle, and foot metatarsal phalangeal (MTP) on either side for at least 6 weeks.

Table 1.2 2010 American College of Rheumatology/European League Against Rheumatism classification criteria for rheumatoid arthritis adapted from (9).

Criteria	Score
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
Serology (at least 1 test result is needed for classification)	
Negative RF <i>and</i> negative ACPA	0
Low-positive RF <i>or</i> low-positive ACPA	2
High-positive RF <i>or</i> high-positive ACPA	3
Acute-phase reactants (at least 1 test result is needed for classification)	
Normal CRP <i>and</i> normal ESR	0
Abnormal CRP <i>or</i> abnormal ESR	1
Duration of symptoms	
<6 weeks	0
\geq 6 weeks	1

Score of $\geq 6/10$ is needed for classification of a patient as having definite RA. Joint involvement refers to any swollen or tender joint during examination and is also classified according to location and number of involved joints. Serology, low positive and high positive are equivalent to ≤ 3 and >3 times the upper limit of normal, respectively and are based on the reference range of the laboratory that assesses the biomarker.

Although these criteria provide the gold standard for disease definition they may have significant limitations in allowing earlier RA classification. Recently, therefore, a joint working group have updated these criteria; the 2010 American College of Rheumatology /European League Against Rheumatism (2010 ACR/EULAR) criteria are designed to facilitate the identification of patients who would benefit from early intervention as shown in Table 1.2 (9). A patient with synovitis, not explained by another disease, and who meets these initial criteria with a score of $\geq 6/10$ can be classified as having “definite RA”.

1.2 Rheumatoid Arthritis and Co-Morbidity

RA is a chronic inflammatory disease that mediates many complex effects on patients. Recently, awareness has been increased for the importance of co-morbidities associated with RA. Some co-morbidities of particular concern are cardiovascular disease (CVD), respiratory disease, osteoporosis, risk of infection and some forms of cancer; however, all co-morbidities have a substantial impact on RA disability and mortality.

1.2.1 Cardiovascular Co-Morbidity

Like other autoimmune diseases, RA patients are more likely to develop CVD, and although the exact nature of association is still unclear, the presence of chronic inflammation is considered to be the main link (10). The shared risk may thus reflect a direct impact of RA *per se* and chronic inflammation or could be due to a high prevalence of traditional CVD risk factors such as smoking and diabetes mellitus, both of which are risk factors for development of RA (11). Additionally, management used for RA provide another possible mechanism. Avina-Zubieta *et al.* have reported that patients with RA have increased risk of CVD deaths (12). RA increases the risk of heart failure and the one-year mortality rate after heart failure is higher in patients with RA compared with non-RA controls (13). Moreover, there is strong evidence that patients with RA have higher rates of ischemic heart disease and myocardial infarction (14, 15). Interestingly, patients with RA may have a different clinical course of myocardial disease; they are more prone to recurrent myocardial infarctions with higher rates of mortality than control populations.

Furthermore, associations have been reported between a number of therapies used in RA and CVD. For instance, use of some non-steroidal anti-inflammatory drugs (NSAIDs) has been associated with increasing risk of myocardial infarction (16). Another example is corticosteroids although this dataset is contradictory as the impact reflects a balance of anti-inflammatory and pro metabolic risk overall (17). However, use of methotrexate (MTX) in RA patients reduces the risk of CVD by 21%, including an 18% lower risk for myocardial infarction (18). Of interest, TNF inhibitors appear to reduce the CVD event such as myocardial infarction, strokes and heart failure in patients with RA. However, the impact of TNF inhibitors in cardiac events is not seen as consistently as the effects seen with MTX (19).

1.2.2 Respiratory Co-Morbidity

Similarly, the respiratory system is frequently involved in RA and includes pleural disease, pulmonary nodules, interstitial lung disease (ILD) and airway disease (AD) (20-22). It is now well recognised that RA is associated with reduced life expectancy and although CVD is responsible for majority of RA related deaths, respiratory complications account for 10-20% of overall mortality (23). Recently, Wilsher *et al.* have shown a significant correlation between lung function parameters and high-resolution computed tomography (HRCT) abnormalities with serological markers (RF and ACPA) in newly diagnosed RA (24). Interestingly, antibody against cyclic citrullinated peptides is more highly associated with physiologic parameters or HRCT abnormalities than RF. Furthermore, some studies have suggested that shared epitope (SE) (discuss in genetic factors section) -positive human leukocytes antigen (HLA)-DR β 1 is significantly associated with the presence of obstructive AD (25, 26). Other studies found carriage of HLA-DR4 in RA patients with pulmonary abnormalities, either ILD or AD, was not significantly different from that in patients without this complication (27, 28). This discrepancy in genetic predisposition of pulmonary disease strongly suggests the possibility of contribution by genetic backgrounds other than SE-positive alleles. Most recently, Mori *et al.* clearly showed that carriage of HLA-DR β 1*1501 and *1502 (SE negative alleles) have opposite influences on the incidences of ILD and AD in RA patients; HLA-DR β 1*1502 alleles may confer special predisposition for ILD, while AD is negatively associated with this allele (29).

Furthermore, several studies have demonstrated a significant association between serum levels of anti-CCP and pulmonary disease in RA patients (29-31). Long standing RA; >10 years duration, have positive association with ILD and AD but the association was more stronger with age (29). Finally, ILD in RA is not only related to the disease itself, but may be caused by the treatments used for RA such as MTX, leflunomide and NSAIDs which may directly cause the development of interstitial pneumonitis (32, 33). There are other important issues regarding the potential association of pulmonary disease with RA and lung has been suggested as the site primarily responsible for citrullinated protein production by peptidyl-arginine deiminases, particularly in cigarette smokers (34, 35). In turn these proteins are implicated in triggering the immune system response in genetically susceptible patients.

1.2.3 Osteoporosis

Patients with RA are more likely to develop osteoporosis and osteopenia. Osteoporosis reflects disease activity in early arthritis and most likely results from the release of cytokines that induce bone resorption such as tumour necrosis factor alpha (TNF- α), interleukin (IL)-1 (IL-1) and IL-6 from inflamed synovium (36). In general, bone metabolism in patients with RA is disturbed; while bone formation is reduced in both patients with and without joint destruction only bone resorption is increased in patient with joint destruction (37). Many studies agree that increase in risk of fracture of RA patients is associated with disease activity and severity and part of this is due to reduction of bone mineral density and concomitant steroid therapy as well as disease duration (38, 39). Interestingly, TNF inhibitors appear to decrease the overall fracture rate (40).

1.2.4 Infection

RA is associated with an increased risk of serious infection. There are a number of mechanism that may promote this phenomenon: (i) underlying mechanisms of the disease itself and presence of immune dysregulation (ii) sequelae of co-morbidity conditions, (iii) impact of immune suppressive therapy and induced decreases in immune function as well as (iv) the life style of RA patients such as smoking (41). Infection is one of three leading causes of premature death (42, 43). RA patients

have other serious co-morbid conditions such as lung and kidney disease and diabetes mellitus as well as functional limitation and disability, all of which are also implicated in increased infection risk in RA (41). Most deaths from infectious diseases are of respiratory origin, especially in women. TNF inhibitors and long term use of glucocorticoids have also been implicated in increasing the risk of serious infections up to 2 and 4-fold, respectively in a dose-dependent manner (41, 44).

1.2.5 Malignancy

Although, malignancy remains relatively uncommon, there is some evidence that patients with RA and with high disease activity are estimated to have up to a 25.8-fold increased risk of lymphoma as compared with controls with low inflammatory activity (45). Furthermore, patients with RA have substantial increase risk of lymphoma and lung cancer, with some evidence for a protective effect against breast and colorectal cancer (46). There has been considerable recent interest in the relationship between increased risks of malignancy, skin cancer and lymphoma in particular, in patients receiving immune suppressive therapy (47).

These observations have highlighted the crucial importance of co-morbidity in determining RA outcomes, and also the necessity to consider the impact of RA on the presence and management of co-morbidity. Furthermore, routine screening for co-morbid disease in newly diagnosed RA is important because some of these abnormalities may be present early in the course of the disease despite the patients being asymptomatic or having few symptoms.

1.3 Current Therapies of Rheumatoid Arthritis

Treatment of RA has improved dramatically over the past 1-2 decades. In brief, management of RA has shifted from initial treatment with NSAIDs and corticosteroids to more aggressive and early use of DMARDs (48-50). DMARDs are categorised into two groups - conventional DMARDs (cDMARDs) and biological DMARDs (51). Table 1.3 summarizes the variety of cDMARDs and biological DMARDs that currently used in the treatment of RA. MTX is the drug of choice of initial therapy either as mono-therapy or when used in combination (52) with other

treatment. Other cDMARDs including hydroxychloroquine, sulfasalazine (SSZ) and leflunomide have been shown to be effective in RA either as mono-therapy or in combination with other cDMARDs such as MTX (53). A robust evidence base shows that cDMARDs either in single use or combination improve the symptoms and clinical manifestation as well as control joint destruction in the majority of RA patients. However, although the most commonly used agents and may be clinically effective - their use does not always suffice to prevent joint destruction (54).

A major improvement in outcomes of RA arose with the introduction of biological therapies. Five TNF inhibitors are approved for clinical use; four are monoclonal antibodies (infliximab, adalimumab, golimumab and certolizumab) while one (etanercept) is a soluble receptor fusion protein and (Table 1.3) summarizes the variety of biological DMARDs and related drugs approved for treating RA. Multiple trials have established the efficacy of various TNF inhibitors. They have shown in further improvement in disease activity and provided earlier functional improvement and less progression of radiographic joint damage particularly in combination with methotrexate (55-63). Nevertheless, with TNF inhibitors relatively low numbers of patients achieve sustained remission or are intolerant to this treatment (64, 65). Rituximab is another therapeutic option in the treatment of patients with active and long-standing RA who demonstrate an inadequate response to currently available TNF inhibitors (55, 66). Rituximab is a monoclonal antibody that targets CD20 positive B cells (67). Additionally, abatacept and tocilizumab have been used in the treatment of active RA alone or in conjugation with MTX and they exhibit impressive efficacy in patients with an inadequate response to MTX or after TNF inhibitor (68, 69). Abatacept selectively modulates the co-stimulatory signal required for full T-cell activation, while tocilizumab directly inhibits the IL-6 receptor and thereby inhibits the effector biology of IL-6.

A further accelerated improvement began with the introduction of the 2010 ACR criteria for identifying patients with a relatively short duration of symptoms who may benefit from early institution of DMARD therapy. Several studies have confirmed that early diagnosis and early DMARD treatment increases the chance of achieving remission and reduced joint damage and also improved functional status, which is

strongly associated with radiographic joint damage compared with patients with delayed treatment (54, 70, 71).

So far, it is clear that identification and treatment of early RA is beneficial, but choosing specific treatment regimens for early RA are an equally important issue. In this regard, multiple studies have evaluated this issue, for example the PREMIER study has compared initial combination therapy with initial mono-therapy and shown that the clinical outcomes (ACR20) were higher for MTX than adalimumab, but radiographic progression was less with adalimumab alone than MTX alone. While, the combination group shown better in both measures (72). Crucial data has emerged from the BeSt trial, which was designed with a step-up arm approach, and showed that combination therapy (MTX, SSZ and prednisone, or another option is MTX and infliximab) is, overall, superior to sequential mono-therapy or step-up combination DMARD therapy in early RA (73). This study demonstrated that initial combination therapy resulted in small but significant reductions in the progression of joint destruction and improvements in function at 1 year compared with control group. Similarly, the Finnish Rheumatoid Arthritis Combination Therapy study found that increased remission rates in RA patients who were started on combination DMARD therapy (SSZ, MTX, hydroxychloroquine and prednisolone), as compared with single DMARD therapy at 2 year follow up (50, 74). However, other studies have demonstrated that a proportion of patients respond well to initial therapy with mono-therapy such MTX (57, 75). The ACR's 2012 updates of treatment for RA recommended starting DMARD mono-therapy in any early-RA patient without poor prognostic factors such as functional limitations, extra-articular disease, seropositivity, or erosions; while in early-RA patients with moderate to high disease activity and poor prognostic factors they recommend initial combination DMARDs or TNF inhibitors with or without MTX within the first 6 months of disease.

1.3.1 Unmet Need

Despite the considerable expanded improvement in therapeutic strategies (synthetic and biological DMARDs) of RA, a substantial proportion of patients remain as partial responses and fail to achieve sustained remission. For example, cDMARDs in general and MTX in particular are the hallmark treatment of RA they have a limited efficacy and toxicity problems. Not all patients tolerate these therapies and

many of them fail to achieve an adequate or sustained response to these drugs and consequently fail to improve their outcomes.

Similarly, although TNF inhibitors have undoubtedly and significantly advanced the treatment of RA, a population of patients with refractory RA remains. Of interest, 20-40% of patients treated with TNF inhibitors fail to achieve an adequate response (64, 65). Furthermore, observational studies have shown that TNF inhibitors are associated with a variety of toxicities for example infection. Serious infection, defined as life-threatening or requiring hospitalization or intravenous antibiotics is one of leading cause of morbidity and mortality in RA patients. The proportion of serious infections such as primary tuberculosis (76) and histoplasmosis (77) is significantly greater in patients treated with monoclonal TNF inhibitors (infliximab) than in control groups. Other TNF inhibitors including golimumab and certolizumab, rapidly and significantly ameliorate the manifestations of RA, however, there are increasing incidences of serious infection such as tuberculosis when compared to control population (62, 63). Notably, TNF inhibitor use is also occasionally associated with decreases in the mean white blood cell and platelet counts (78).

Additionally, observational data suggest that malignancies are more common with TNF inhibitors. For example, there is evidence of malignancies occurring in those on infliximab 10mg/kg every 4 weeks; including carcinoma of the breast, squamous cell carcinoma and melanoma and B cell lymphoma (56). Furthermore, patients receiving infliximab or adalimumab also presented with antibodies to double-strand DNA and some patients were diagnosed as having a drug-induced lupus syndrome after two treatments with infliximab at 10mg/kg (56, 78, 79). Multiple sclerosis is now recognized as a complication of TNF inhibitors, particularly etanercept (80). Moreover, anti-TNF therapy is a high cost treatment and may not be appropriate for all patients (81).

Similar to TNF inhibitors, rituximab and abatacept are associated with an increased risk of serious infection. For example, incidence of infections is higher in patients treated with rituximab than in control group with 41% and 38%, respectively and upper respiratory tract infections, nasopharyngitis, urinary tract infections,

bronchitis, and sinusitis are the most common infections in both groups (55). Furthermore, lung cancer risk is higher in patients treated with abatacept than in the general population but it was equivalent to that in the RA population (82).

Thus, even with the advanced management and several therapeutic strategies; either by switching to another TNF inhibitor or by adding rituximab or abatacept or tocilizumab to existing combination therapy, a substantial proportion of patients with refractory RA still exist and fail to respond to therapy (53).

Taken together there remains a significant unmet clinical need in the treatment of RA. There is a need to discover new therapeutics that can maximise the rate of remission and minimize toxicity risk. In addition, tractability of new targets must also come with the advent of biomarkers that can predict those most likely to respond or exhibit toxicity as one moves towards stratified or personalized medicine.

Table 1.3 Conventional, biological DMARDs and related drugs approved for treating rheumatoid arthritis adapted from (51, 83, 84).

Therapy	Max. Dose	Effect	Comments		Main side effects
Conventional DMARDs					
Methotrexate	20mg/week	4-6 weeks	Gold Standard therapy		Gastric irritation, hepatic toxicity and interstitial pneumonitis
Hydroxy-chloroquine	400-600 mg/day	2-6 months	Antimalarial drug		Relatively safe but can lead to eye toxicity
Sulfasalazine	2-3 grams/day	2-6 months	Second most common DMARD		Hypersensitivity, neutropenia and thrombocytopenia
Leflunomide	20 mg/day	4-8 weeks	Efficacy is similar to methotrexate		Diarrhea, reversible alopecia and hepatic toxicity
D-pencillamine		2-6months	Very rarely used now		Bone marrow suppression, rash, renal toxicity
Gold Salt	50mg/ week	4-6 months	Rarely used		Dermatitis, rash, stomatitis and neutropenia
Azathioprin	2.5-3 mg/kg	8-12 weeks	Purine analogy		Immunosuppression and opportunistic infection
Cyclosporine			Potent immune suppressive		Infection and renal insufficiency
Biological DMARDS			Target	Properties	
Etanercept	50mg S/C once/week	2-4 weeks	TNF-α	TNF-α receptors II and Fc fusion protein	TNF inhibitors increase risk of infection and malignancy
Infliximab	IV infusion3-10 mg/kg Day 1, 14,42 then every 8 weeks	2-4 weeks	TNF-α	Chimeric monoclonal antibody to TNF-α	
Adalimumab	40mg S/C once every 2 weeks	2-4 weeks	TNF-α	Recombinant monoclonal antibody TNF-α	
Certolizumab	400mg loading dose then at 2 4 weeks	2-4 weeks	TNF-α	Fab pegylated anti TNF-α	
Golimumab	50mg every 4 weeks	2-4 weeks	TNF-α	Monoclonal antibody to TNF-α	
Tocilizumab	IV infusion 4-8 mg/kg, every 4 weeks	4-8 weeks	IL-6	Humanised anti-IL-6 Receptor monoclonal antibody	Infection, GI perforation also increased lipid parameters and neutropenia
Anakinra	S/C 100mg/ day	2-4 weeks	IL-1	Recombinant IL-1Receptor antagonist	Injection site reaction, increase risk of infection
Rituximab	IV 1000mg followed by two doses within 2 weeks apart	After 3 months	B cells	Chimeric monoclonal antibody anti CD20	Infusion reactions also increase risk of infection and neurological syndromes
Abatacept	IV<60kg/500mg 60-100kg/75mg, >100kg/1gram Or S/C 125mg	Within 3 months	T cells co-stimulation	Human fusion protein (CTLA4-1g)	Infection, malignancy and infusion reaction

1.4 Pathogenesis of Rheumatoid Arthritis

RA is one of a family of autoimmune disorders, where the underlying cause remains incompletely understood. So far, epidemiological as well as genetic and environmental factors have been proposed to have associations with the susceptibility and severity of RA (Figure 1.1). Therefore, identification as well as understanding of these risk factors is extremely important to recognize their contribution to disease development.

1.4.1 Genetic Factors

The crucial role of genetic factors in the etiology of RA development has been highlighted for many years. There are multiple genetic factors that have been associated with disease and estimates of the genetic contribution to RA have ranged between 30% and 60% (85). Some twin studies provided valuable tools to underpin the extent of genetic influence on heritability and concordance of disease development in humans. The heritability of RA among twins is approximately 65%, whereas the estimated concordance, representing the frequency with which a twin sibling with RA were also affected, in monozygotic twins is four times that observed in dizygotic twins (86, 87). Recently, van der Woude *et al.* have estimated the contribution of heritability and concordance between ACPA-positive and ACPA-negative RA twins revealed almost identical heritability rates for both disease subsets. However, the concordance of these 2 subsets was different (88). HLA alleles are known to be associated with RA in many different populations and are thought to account for 50% of the genetic component of RA susceptibility (89). Several studies have showed a substantial genetic association of RA with HLA-DR family alleles (including the DR subtype Dw4 and Dw14) within the class II major histocompatibility complex (MHC) region of chromosome 6 (90, 91). In the human class II region, there are at least 14 different genes, generally found in one of three major sub regions; DR, DQ and DP, and each sub region contains one functional α and β chain to constitute a functional molecule (92). The N-terminal domain of class II β chains is the site of most variability, DR β chains contain 3 major region of variability and the third is the site of most sequence difference between the DR4 subtypes. The specific alleles associated with RA vary according to population; among the most frequently reported alleles are HLA-DR4 and HLA-DR β 1 alleles.

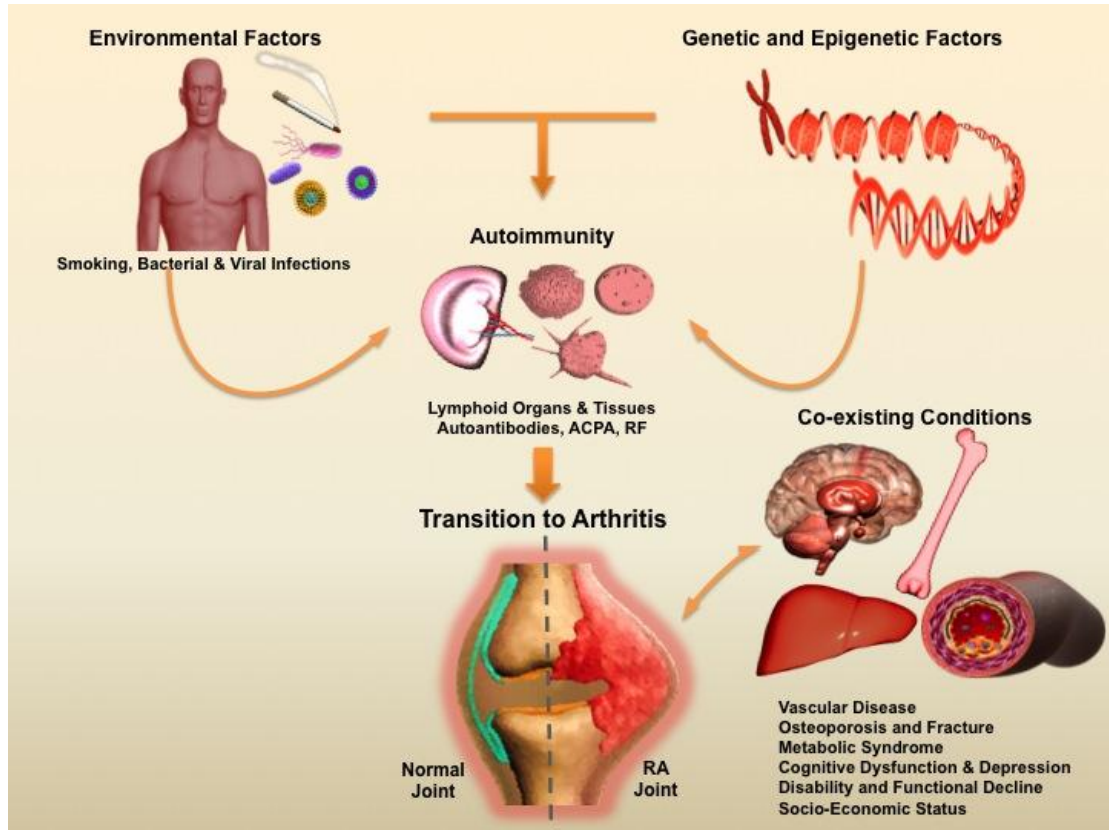


Figure 1.1 Schematic representation of emerging concepts in pathogenesis and onset of rheumatoid arthritis. Environment–gene interactions result in loss of tolerance to self-proteins that may lead to RA-related autoimmunity and disease. For example, smoking triggers the production of anti-citrullinated peptide antibodies (ACPAs), which is generated by post-translational modification in individuals who carry the ‘shared epitope’. At this stage, serum concentrations of rheumatoid factor/ACPA and acute phase proteins, along with cytokines and chemokines start to increase years prior to the onset of clinical disease. The triggers for transition from this period of gradually increasing inflammation “asymptomatic autoimmunity” to development of clinical rheumatoid arthritis with localization of the inflammatory response at joint space “clinically-apparent RA” has not been identified. Patients often report infections, trauma and stress as preceding factors. Adapted from (93).

This complex pattern of association of MHC class II haplotypes with RA led to the proposal of the SE hypothesis, based on the observation that there are shared common amino acid sequences (QRRAA, RRRAA and QKRAA) spanning position 70-74 in the third hyper-variable region of the HLA-DR β molecule (92). Variation in the frequency of SE alleles (DRB1*0101, *0102, *0104, *0401, *0404, *0405, *0408, *0413, *0416, and *1001) in different populations accounts for variation of RA susceptibility and global prevalence of disease (92). Hence, individuals who have the class II HLA-DR β chain; with a shared identical five amino acid sequence (SE hypothesis) are more likely to have RA than those without (90-92). It is unclear how SE contributes to the development of RA but there is a possibility that structural differences between the β chains of the MHC class II molecules may affect the immune response, either during T cell receptor (TCR) repertoire selection, or via antigen/MHC class II interactions during T cell differentiation in the thymus. In addition, single-nucleotide polymorphism (SNP) mapping has shown independent loci within the MHC region, outside the HLA-DR β 1, which are implicated in RA susceptibility.

Recently, striking differences in the pattern of the SE alleles of MHC have been observed to associate only with the risk of ACPA-positive RA but not with ACPA-negative RA (94). Thus, according to ACPA status, there are distinct genetic patterns of MHC associations in the two major clinical subtypes of RA. This observation cannot be entirely accounted for by the SE and clearly indicates that the difference in the serological subtype of disease may be reflected in the difference in genetic predisposition. Indeed, Ding *et al.* have demonstrated that additional loci at HLA-DP β 1, C2-DOM3Z and MICA were associated with MHC, independent of HLA-DR β 1 in ACPA positive RA (94). In contrast, the genetic risk factors of ACPA negative RA are still unclear, although two studies have provided evidence for association of ACPA negative RA with HLA-DR3 and interferon regulatory factor -5 (IRF-5) (95, 96).

On the other hand, genomic wide association studies (GWAS) groups using the Swedish Epidemiological Investigation of Rheumatoid Arthritis and North American RA Consortium, and data from the Wellcome Trust Case-Control Consortium have provided further confirmation that there are significant differences in genetic

association, mainly found in HLA regions, between the two clinical disease subsets. GWAS confirmed that the HLA-DR β 1 contains the most important risk factor alleles accounting for 30-50% of the overall risk and is strongly attributed to the aetiology of ACPA-positive RA. It also provides the clearest demonstration of the presence of a number of genes outside the HLA-DR β 1 gene that are associated with RA susceptibility, such as protein tyrosine phosphatase non-receptor type 22 (PTPN22), cytotoxic T lymphocyte associated antigen-4 (CTLA-4), TRAF1-C5, and STAT4 (97). Although, several genetic susceptibility loci have now been associated with RA, HLA-DR β 1 remains the strongest. Interestingly, most of the well-established genetic variations (PTPN22, CTLA-4, TRAF1-C5 and STAT4) are associated with ACPA positive RA, while IGFBP1 and IRF5 loci are associated with ACPA negative RA (90, 93, 97, 98). These observations suggested a complex hierarchy and multiple molecular pathways that may contribute to the development of RA.

1.4.2 Epigenetics

The preceding literature review clearly indicates that genetic factors in general and HLA-DR β 1 in particular contributes to RA susceptibility. However, identical twins have a concordance rate of 12-15%, which clearly points to other critical elements that may be implicated in RA pathogenesis: epigenetic factors are therefore worthy of consideration (86, 87, 99). In recent years, many complementary lines of evidence suggest that epigenetic changes occurring in RA synoviocytes may play a key role in RA pathogenesis. Epigenetic factors may also operate in immune cell lineages and potentially occur before clinical disease and thereby contribute to susceptibility, *e.g.* induced by smoking, or other toxin exposure. The term epigenetics refers to changes in gene expression caused by mechanisms other than alteration of the nucleotide sequence (100). Although epigenetic modifications may be reversible, they can critically contribute to chromatin structure stability, genome integrity, modulation of gene expression, embryonic development, genomic imprinting and X-chromosome inactivation in females (101). Epigenetic modification of gene expression is mediated by several mechanisms that include DNA methylation, acetylation, phosphorylation, and sumoylation and also via the elaboration and post-transcriptional regulatory function of microRNAs (miRNAs). DNA methylation and acetylation are the most intensively studied pathways therefore I shall discuss them and what is known of their role in RA.

1.4.2.1 Methylation

DNA methylation is generally associated with gene silencing. In mammalian species, DNA methylation is a covalent biochemical modification that occurs by the attachment of a methyl group at the 5th position in the pyrimidine ring of a cytosine within the cytosine-guanine dinucleotide (CpG) resulting in 5-methylcytosine. These may often be grouped in large clusters called CpG islands and are mainly located in the promoter or first exon region of genes. Patterns of DNA methylation reflect activity of DNA methyl-transferases (DNMTs) that catalyse the transfer of a methyl groups from *S*-adenosyl-methionine (SAM) onto cytosine (101, 102). The mammalian DNMTs family consist of five members (DNMT1, DNMT2, DNMT3a, DNMT3b and DNMT3L) that can be divided into *de novo* and maintenance methylation enzymes. DNMT1 is the major enzyme involved in the maintenance of DNA methylation patterns during replication, which recognise established methylation markers and copies them to a newly formed DNA strand. While, DNMT2 is the smallest DNA methyl-transferase and has very weak methylating activity that mainly involved in the recognition of DNA damage. DNMT3 family members DNMT3a, DNMT3b and DNMT3L (the latter lacks an active methyl-transferase site) are responsible for *de novo* DNA methylation during embryogenesis and germ cell development acting by introducing methyl groups into previously unmethylated CpG dinucleotides (101-104). Interestingly, human genome methylation is not uniform and the majority of CpG islands are unmethylated in transcriptionally active genes, with acquired abnormal methylation within the promoter region leading to transcriptional silencing. Indeed, genes silenced by DNA methylation either due to decreased affinity for binding to transcription factors or methyl group recruitment of methyl-CpG binding proteins (MBP) to the promoter region lead to impaired transcriptional processes (105). On the other hand, global hypo-methylation is attributed to chromosomal instability and less imprinting. Thus, inappropriate DNA methylation may lead to various diseases particularly cancers (101).

Recently, there is growing evidence suggesting an important role for DNA methylation in normal biological processes, in the pathogenesis of human diseases in general and RA in particular. In this context, Karouzakis *et al.* showed *in situ* and *in vitro*, that there is significant global genomic hypomethylation in RA synovial

fibroblasts (RASFs), as reflected by the lower nuclear staining of 5-methylcytosine, than in OA synovial fibroblasts (OASFs). This hypomethylation remained significantly reduced even in the presence of TNF- α or IL-1. Furthermore, they point out that in OASFs, stimulation of cell proliferation was accompanied by an increase in DNA methylation but this was not the case in RASFs. The DNMT1:PCNA ratio, proliferating cell nuclear antigen, was significantly lower even in the presence of inflammatory cytokines (106). This observation suggests that there is either a deficiency of production or a decrease in half-life of DNMT1 in RASFs. Indeed, the global DNA methylation was shown to be due to increased polyamine catabolism in RASFs compared with OASFs resulting in increased consumption of SAM, which was 3.3 fold lower in RASFs than that of OASFs (107). This observation of RASFs hypo-methylation is compatible with the study by Nakano *et al.* who demonstrated that genes strongly implicated in RA pathogenesis including inflammation, matrix regulation, leukocyte recruitment and immune response were associated with hypomethylated loci in RASFs (108).

Likewise, global DNA hypomethylation has been observed in peripheral blood mononuclear cells (PBMCs) obtained from RA patients compared with healthy controls (109). Moreover, ACPA-positive RA patients had impaired DNA methylation and lower levels of DNMT1 mRNA than those patients with ACPA-negative disease (109). A further critical observation was reported by Nile *et al.* that contrary to global DNA methylation changes, a single CpG motif at -1099 in the IL-6 promoter region was significantly less methylated in PBMCs from RA (58%) compared to healthy controls (98%) and remained hypomethylated even after IL-6 mRNA induction using bacterial lipopolysaccharide (LPS) (110). Altered patterns of DNA methylation are also observed in proximal CpG motifs of the IL-10 promoter region in RA PBMCs and hypomethylation of this region is inversely correlated with IL-10 mRNA and protein levels in serum (111). Further evidence comes from another study, which employed RA patients as a disease control; they demonstrated that methylation of IL-10 and IL-1 receptor (IL1R2) genes were low in PBMCs of RA patients. These changes were more significant in the latter and are inversely correlated with disease activity of systemic lupus erythematosus (SLE) (112). Recently, it was reported CXCL12/SDF-1 (stromal derived factor-1) expression might be regulated by epigenetic mechanisms. Karouzakis *et al.* have demonstrated

the CXCL12/SDF-1 promoter region is hypomethylated in RASFs compared to OASFs and normal lung fibroblasts (113). Thus, altered DNA methylation in promoter regions of cytokines and chemokines may regulate their expression at transcriptional levels and be responsible for the development or progression of RA in a subpopulation of patients.

DNA methylation also regulates the expression of Death Receptor 3 gene (DR3), a member of the apoptosis inducing tumour necrosis receptor family, in RA synovial cells. Takami *et al.* have demonstrated that there is a striking difference in the methylation pattern of the DR3 promoter region; in contrast to OA synovial cells that were either unmethylated or partially methylated; RA synovial cells were constantly hypermethylated. Furthermore, the expression of DR3 protein was down-regulated in RA synovial cells compared with OA synovial cells. However, genomic analysis studies using PBMCs from healthy controls and RA patients revealed that this region was consistently unmethylated (114).

1.4.2.2 Acetylation

Histones are the main components of chromatin and the DNA strand wraps around histones to generate the nucleosomes; the fundamental organization of chromatin structure. Adding or removing acetyl groups to the N-terminal tail of nuclear histone in nucleosome gene promoters has been implicated in the activation of gene expression (115). Addition of an acetyl group to lysine residues within the histone tail eliminates the positive charge on lysine thus leading to altered interaction with negatively charge DNA. Consequently, the condensed chromatin loosens its structure and this permits a greater level of gene transcription. The extent of gene transcription is dependent on the balance between histone acetylation and histone deacetylation (105, 115, 116). Thus, this post-translational modification of histone occupies an essential part of gene regulation. Histone acetylation and deacetylation are highly regulated by two antagonizing enzymes; histone acetyltransferases (HATs) and histone deacetylases (HDACs) (117). HAT enzymes catalyze the transfer of acetyl groups from acetyl-coenzymes A to lysines and, based on their subcellular localization (either in the nucleus or cytoplasm), they are divided into two subgroups namely A and B, respectively. The former is closely linked to the

transcriptional regulation of gene expression, while the latter is mainly implicated in the acetylation of newly synthesized histones that are free in the cytoplasm (118).

On the other hand, HDACs counterbalance the HAT and are associated with gene silencing. Furthermore, HDACs are classified into four classes depending on their homology to yeast proteins. Class I of HDACs is expressed in all cell types and includes HDAC 1, 2, 3 and 8, sharing homology to yeast transcriptional regulatory reduced potassium dependency gene 3. While, Class II include HDAC 4-7, 9 and 10 and are restricted to tissue specific expression. Class III is closely related to yeast silent information regulatory 2 family of NAD, and include sirtuins 1-7, while HDAC 11 is sub grouped as class IV (117). Altering the distribution pattern of HATs or HDACs on chromatin is associated with either upregulation or the down regulation of gene transcription, respectively.

In recent years, several studies have investigated the relationship of RA and histone acetylation/deacetylation as an important potential contributor to pathogenesis. The majority of these studies have been conducted using HDAC inhibitors (HDACI) in animal models; for instance in autoantibody mediated arthritis beneficial effects arise from HDACI FK228 administration - it inhibits joint swelling, synovial inflammation and joint destruction and causes significant decrease in TNF- α and IL-1 β concentrations in synovium (119). However, Huber *et al.* have clearly demonstrated that there was a significant down regulation of HDAC activity in RA synovial tissues and this decrease was not compensated by a decrease in HAT activity as compared with OA or normal synovial tissue. Thus, the balance of HAT/HDAC is strongly shifted towards histone acetylation in RA synovial tissues. Furthermore, protein expression of HDAC1 and HDAC2 were significantly diminished in RA synovium (118). In this context, the beneficial effect of HDACI in RA is unclear. These inhibitors may target other proteins beyond histone; therefore, illustration of the functional role of individual HDAC in SFs is needed.

However, Horiuchi *et al.* (120) have shown that there was a higher expression of HDAC1 and HDAC2 mRNA level in RASFs than OASFs - targeting of these enzymes enhanced apoptosis and decreased cell counts and proliferation. In addition, silencing the HDAC1 enzyme resulted in decreased matrix

metalloproteinase-1 (MMP) production (120). Likewise, a total increase of nuclear HDAC activity, with no change in HAT activity, is also reported in RASFs compared to OA and normal tissues and is positively correlated with amounts of cytoplasmic TNF- α . Indeed, there was no shift towards histone hyper-acetylation. Moreover, HDAC1 is highly expressed in RASFs at both protein and mRNA level compared to OASFs, while HDAC4 mRNA level is down regulated compared with normal control (121). More recently, increased HDAC has been observed in PBMCs obtained from RA and interestingly both selective and non-selective HDACi regulates the TNF- α and IL-6 production (122). All these studies suggested HDACi seem to have a beneficial effect and could be a possible therapeutic modality for RA; however, these effects might be different between mice and humans and further clinical characterization and evaluation for side effects are required.

1.4.3 Environmental Factors

An accumulation of epidemiological data suggests an important role for environmental risk factors in RA development including smoking, hormones and infection. The most important environmental risk factor is smoking together with other forms of bronchial mucosal stress such as exposure to air pollution and silica-containing dust (123-125). These have been implicated as triggering factors of RA in genetically susceptible individuals (126). Compared with a never smoker, the relative risk of developing RA among smokers is 1.5 fold higher in the general population (127). Smoking contributes to 35% of ACPA positive RA (128). Furthermore, increased risk of developing RA comprises a cumulative dose effect, observed particularly after long duration of smoking >20 years and also affected by the amount of cigarettes smoked per day (129). Interestingly, this risk for RA remains for about 10–19 years even after cessation of smoking (130). Even more intriguingly, among patients with early RA, cigarette smoking is associated with greater disease activity and with higher radiological damage - they develop RA at a younger age than the never smokers (131).

Of recent interest related to pathogenesis of RA, several studies have reported that a gene-environment interaction lead to RA-related autoimmunity and disease. For example, Padyukov *et al.* have proposed smoking as a central driver of gene - environmental interactions in seropositive RA and have shown that relative risk is

2.8 for RF seropositive RA. Indeed, smoking individuals with double SE genes exhibit a substantially higher risk of developing RF seropositive RA than individuals with single SE alleles and those who have never smoked or are smokers without SE associate MHC alleles (127). When considering ACPA positive RA, but not ACPA negative RA, it is believed to occur as a result of the synergistic interaction between the major genetic risk factor; HLA-DR and smoking (132, 133). Indeed, there is a dose response emerging between intensity of smoking determined as pack-years and elevated levels of anticitrulline antibody at the onset of RA. Furthermore, the combination of a history of smoking with the presence of single or double SE genes increases the relative risk of developing RA to 6.5 and 21 fold, respectively compared to those who had never smoked and have a single or double SE ACPA positive RA (132). More recently, Kallberg *et al.* showed that there is a dose dependent association between smoking and SE allele status, and the smoking contributed to 55% in ACPA positive RA that carried a double copy of HLA-DR β 1 SE (128). While, in undifferentiated arthritis, SE allele did not increase the risk of development of RF or ACPA status (133). Interestingly, SE associated genes and smoking are primarily associated with presence of ACPA rather than positivity of RF. Taken together these observations suggest that smoking may represent a significant risk factor of RA development and progression, although the mechanisms underlying this are unclear.

Hormonal and reproductive factors are implicated in the development of RA. The prevalence of RA is greater among woman therefore it is possible that hormonal and reproductive factors may play a role in RA development and expression. Numerous studies have suggest that using the oral contraceptive pill may offer a potentially protective affect either by reducing the risk of developing severe RA or may delay the onset of RA (134). Although the incidence of RA decreases by 70% during pregnancy it can dramatically increase by five fold in the first 3 months post partum (135). Furthermore, breast-feeding is also associated with the onset of RA; breastfeeding woman have a five times higher risk than non-breastfeeding women, however the mechanism is not known but could be due to increased secretion of the proinflammatory hormone prolactin (136). Prolactin, which is known to be proinflammatory, may play a role in the onset of post-partum RA. Another intriguing finding is that people who develop RA have a genetically determined

abnormality of the hypothalamic pituitary adrenal axis, which prevents them from developing an adequate anti-inflammatory response to immune stimulation (137).

Another prominent environmental factor that increases the risk of RA is infection. Several studies have established a possible association between intestinal bacteria and RA. The importance of bacterial flora emerged when rats raised under germ-free conditions developed severe articular inflammation while conventionally raised controls showed only mild disease in an adjuvant-induced arthritis model (138). A variety of studies implicate several infectious agents such as Epstein–Barr virus (EBV), Cytomegalovirus, *Proteus* species, and *Escherichia coli* (*E. coli*) and their products heat shock proteins (Hsps) in the onset and persistence of RA. EBV has long been linked with RA, although the mechanism behind this remains unclear. It is a potent B cell stimulator and antibodies in RA cross-react with EBV nuclear antigens. Furthermore, EBV DNA is expressed 10 times higher in PBMCs of RA than controls (139, 140). EBV is also detected in RA patients synovial fluid (SF) (141).

E. Coli and their product Hsps have been suspected to participate in the autoimmune response during pathogenesis of RA. Hsps are a group of major bacterial antigens; Hsp40, Hsp60 and Hsp70 families. Hsp40 protein represented the one most commonly associated with autoimmune diseases (142). In particular, *E. coli* Hsp40 (DnaJ) and human (DNAJA1 and DNAJA2) are suspected of participating in the pathogenesis of RA. Elevated levels of antibodies against the *E.coli* DnaJ have been shown in serum of the RA patients (143). Furthermore, over expression of human Hsp40s results in increased levels of the anti-DNAJA1 and anti-DNAJA2 antibodies in the synovial tissue and sera of RA patients, respectively (143, 144). Interestingly, DnaJ and DNAJA2 modulate the inflammatory cytokine secretion in PBMCs of RA patients compared to healthy controls (143). Recently, Kotlarz *et al.* demonstrated a significant immunological similarity, not restricted to the conserved J domains but also present in the C-terminal variable regions, between DnaJ and DNAJA1/DNAJA2 proteins. The levels of anti-DnaJ also significantly correlated with anti-DNAJA1 antibodies in the sera of RA patients (145). Although the exact role of bacterial and human Hsp40s in the autoimmune response requires further

elucidation, these observations suggest infection may be involved in arthritis and those proteins have relevance in the induction of RA.

So far, multiple lines of evidence have suggested a link between periodontitis and the risk of RA (146-148). This proposed association between periodontal disease (PD) and RA has been supported by pathological and immunological evidence. Periodontitis is a general term used to describe the chronic destructive inflammation of soft and hard periodontal tissues and ultimately results in tooth loss and is caused by chronic bacterial infection of the gingiva (149). *Porphyromonas gingivalis* (*P. gingivalis*), a highly pathogenic bacterium of the oral flora is currently the only known bacterium with expression of peptidyl arginine deiminase, which is capable of generating citrullinated peptides in periodontium (150). In this context several studies have demonstrated that patients with RA have greater risk of gingival bleeding, calculus formation and tooth and alveolar bone loss than observed in controls (147, 151, 152). Controversially, Mercado *et al.* have reported no association between RA and gingival bleeding and plaque accumulation although they found that patients with RA have greater numbers of missing teeth than controls (153). Furthermore, there is a significant correlation between the severity of PD and RA disease duration (151). Potikuri *et al.* showed that PD is more frequent and severe in non-smokers, disease-modifying anti rheumatic drug (DMARD)-naive RA patients compared with healthy controls (147). Strong evidence suggests that there is a role for smoking as a potential risk factor in the pathogenesis of both RA and also in PD hence raising the possibility of shared aetiology. Moreover, patients with RA have a significant correlation with mean tooth gingival pocket depth that is more pronounced in ACPA positive than ACPA negative RA. Intriguingly, disease activity and autoantibodies (ACPA and RF) titres are higher in RA patients with PD than without PD (147). More recently, data from a case-control study showed a significant association between history of periodontitis and newly diagnosed RA in a Taiwanese population (148). Overall, these data imply that there is a relationship between the presence of PD and the development of RA however there are still uncertainties as to how this operates at a mechanistic level, for example it is intriguing that citrullination in the mouth might create an environment for breach of tolerance. I shall now address this issue in detail.

1.4.4 Autoantibodies and Breach of Tolerance

Antibodies against cyclic citrullinated peptides are highly specific and predictive for RA and widely used in clinical practice as diagnostic markers (154-156). Based on the presence and absence of ACPA, RA disease is categorized into two distinct clinical subtypes. Interestingly, these clinical subtypes are associated with distinct genetic risk factors. Importantly, the HLA-DR β 1 alleles that predispose to RA are predictive of anti-CCP status. Indeed, in positive SE, HLA-DR β 1 alleles are the only risk factors for RA that is positive for anti-CCP antibodies.

The understanding of RA pathogenesis has increased significantly over recent years. It is increasingly clear that in RA as well as other autoimmune diseases asymptomatic autoimmunity is present years prior to the onset of clinical disease. In this context, multiple studies have clearly demonstrated that autoantibodies; either RF or ACPA and serum cytokines or other inflammatory markers are detectable years before the clinically manifested RA (157-161). Of interest, the titre of these autoantibodies, and their specificity profile, as well as the concentration of serum cytokines increases as the onset date of RA approaches (162). For example, del Puente *et al.* and Aho *et al.* observed that RF is present long before overt disease. Similarly, Jorgensen, *et al.* and others have shown that ACPA and RF preceded the onset of clinically apparent RA. Sokolove *et al.* found evidence of autoantibody targeting of several citrullinated proteins including histones, fibrinogen, and biglycan prior to the development of anti-CCP autoantibody (162). Furthermore, Majka *et al.* demonstrated that individuals who were older at the time of diagnosis of RA had longer duration of pre-clinical positivity of autoantibodies (159).

Additionally, several studies have demonstrated elevation of inflammatory markers such as CRP and cytokines in preclinical RA diagnosis samples (163). Rantapaa-Dahlqvist *et al.* showed that the monocyte chemotactic protein-1 (MCP-1) which is implicated in the regulation of the chemotactic activity of leucocytes, was also up regulated in positive autoantibodies patients prior to disease onset compared to control group (164). Jorgensen *et al.* showed that RF/ACPA and TNF- α were elevated in the pre-RA period of 5 years before the onset of clinically apparent disease (165). Furthermore, a later study also showed that many of cytokines that are strongly implicated in RA pathogenesis (TNF- α , IL-6, IL-12p70, interferon (IFN),

IL-2, and IL-15) are highly elevated with increased ACPA epitopes (162). Interestingly, Sokolove *et al.* were also able to demonstrate the utility of ACPA and cytokine profiling to identify individuals at a higher risk for progression to RA (162). However, not all RA patients have detectable circulating pre-RA autoantibodies and indeed, some patient develops positivity for RF 6 years after clinically diagnosis of disease (160).

The earliest event that ultimately leads to development of RA is breach of self-tolerance; this is a phenomenon that means loss of ability to differentiate self-protein from non-self protein. As a consequence of loss of tolerance, patients with RA develop autoantibodies that recognize citrullinated self-proteins including α -enolase, keratin, fibrinogen, fibronectin, collagen, and vimentin. These autoantibodies can present a long period before the symptomatic onset of RA. However, the exact cause of loss of tolerance and development of autoantibodies is not yet clear. Importantly, several studies have examined interactions between genetic and environmental factors that may lead to RA-related autoimmunity and disease. For example, smoking and gingival inflammation have important roles in modifying either the susceptibility to RA or disease severity. Indeed, smoking and infection with tuberculosis was associated with increased RF/ACPA positivity in individuals without RA (166-168). Although, the exact role of the environmental and genetic risk factors in development of RA-specific autoimmunity is not clear there is emerging evidence of an initial immune dysregulation and breach of tolerance at mucosal surfaces. For example, infection with *p. gingival* has been implicated as a potential risk factor in pathogenesis of RA. Taken together these finding suggests that factors such as genetic risk or environmental exposure influences the relationship between the development of RA-related autoantibodies and clinically apparent disease. However, at the earliest stages of disease the break of immunologic tolerance to endogenous citrullinated antigens predisposes to the development of anti-CCP-positive RA.

1.4.5 Synovitis

Normal synovium is a thin membrane that lines the joint capsule and acts as a lubricating tissue between solid tissues so that movement can occur smoothly

without friction. It also participates in normal articular nutrition. This membrane is made up of two layers (subintima and intima) as well as intimal cells, which are generally fibroblasts and macrophages. In RA, joints are characterized by infiltration of inflammatory cells into the synovium and hyperplasia of the synovial lining cells with new blood vessel result in pannus formation (proliferating synovial cells that penetrate the cartilage and bone) (169). Pannus formation leads to the destruction of the adjacent cartilage and sub-chondral bone leading to radiographically detectable erosions as well as ligament and tendon laxity (Figure 1.2). The trigger for synovitis is still unclear but the inflammatory synovitis in RA patients is characterized by a rapid influx and proliferation of inflammatory cells such as monocytes, T and B-lymphocytes as well as neutrophils, leading to increased local concentrations of cytokines, chemokines and proteases. This in turn, results in the acute phase (warmth, redness, swelling, and pain) reaction, effusion and other manifestations of inflammation. Interestingly, multiple studies have demonstrated that the concentration of pro-inflammatory cytokines (*e.g.* $\text{TNF-}\alpha$, $\text{IL-1}\beta$ IL-6) exceed those of anti-inflammatory mediators (*e.g.* IL-1Ra , IL-10) in the RA synovial membrane and this disparity of cytokines most likely contributes to the sustained inflammation of joints that leads to cartilage destruction and bone erosion (170, 171).

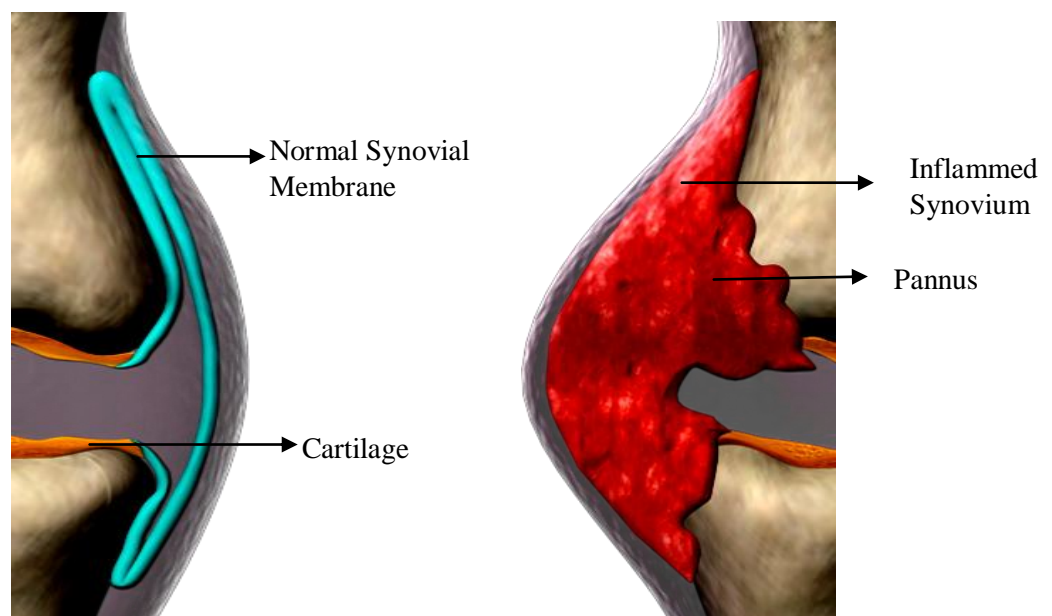


Figure 1.2 Schematic represents synovial joint inflammation

This figure shows the difference of a normal synovial joint compared to an inflamed rheumatoid arthritis synovial joint, which are represented as synovial membrane hyperplasia, cartilage and bone erosion and angiogenesis. Adapted and modified from www.proteinlounge.com.

1.4.5.1 Synovial T Cell Subsets in Synovitis

The involvement of T cells in the pathophysiology of RA is a matter of debate. Although T cells are abundant in synovium their derived cytokines such as IL-2 and INF- γ are rarely detected in either synovium or synovial fluid (SF) (172, 173). More recently the discovery of the novel lineage of T helper 17 cells (Th17) has enabled this view be reconsidered (see below). Several key pieces of evidence implicate the pathogenic role of T cells in RA including mouse data, human genetic associations and targeted therapies. The most convincing evidence for the role of T cells in synovitis is provided by the association of disease severity with HLA-DR alleles within the MHC class II and with lymphoid-specific PTPN22. Moreover, although direct targeting of T cells by cyclosporine or depleting T cells showed limited or no clinical efficiency (174), significant clinical and functional benefit have been observed with the advent of abatacept. Abatacept, a fusion protein containing CTLA-4-immunoglobulin Fc fusion protein, interferes with T cell-antigen presenting cell contact through selective co-stimulation modulation that in turn prevents T cell activation (175, 176). Thus, its clinical efficacy implicates T cells directly in the pathogenesis of RA.

Furthermore, CD4⁺ T helper (Th) cells and their cytokines play a role in initiating and maintaining diverse immune responses within the synovium. Naive CD4⁺ T cells differentiate into diverse phenotypes of effector Th subsets, such as Th1, Th2, Th17, and regulatory T cells (Tregs) under the influence of a network of inflammatory cytokines signals (177, 178). Classically, cytokines regulate the phenotype of effector cells in the synovium; Th1 cells differentiate in the presence of IL-12, and produce IFN- γ and IL-2, while Th2 cells are differentiated by IL-4 and are characterized by the secretion of IL-4, IL-5 and IL-13.

Recently a further effector Th cell lineage has been described and named Th17 that is capable of producing a group of distinctive cytokines IL-17A, IL-17F, IL-22 and IL-21 (179). In addition to Th1, Th2, precursor T cells can differentiate into Th17 cells under the control of transforming growth factor beta (TGF- β) and other cytokines including IL-1 β , IL-6, IL-21 and IL-23 (178). Although, Th1 and Th2 cells have been considered as disease-associated this concept has evolved and now RA is thought to be a primarily a Th17 driven disease. The first evidence for the

importance of Th17 cells and IL-17 in RA was documented by showing elevated levels of IL-17, detected in the serum, SF and synovial tissue of RA patients (180-182). Further evidence for the pathogenic role of Th17 in RA came from several studies that showed acceleration of experimental collagen-induced arthritis (CIA) in mice deficient in the specific p35 subunit of IL-12, which is the main cytokine for the induction of Th1 differentiation. These mice develop severe arthritis and this is associated with increased expression of many inflammatory cytokines in the joint, including TNF- α , IL-1 β , IL-6 and IL-17 (183). Additionally, in the CIA model, serum levels of IL-17 were increased shortly after immunization and IL-17A mRNA was also up regulated in the synovium after the onset of arthritis (184). In contrast, neutralization or deletion of IL-17 caused significantly lessened arthritis with less histological changes such as cellular infiltration and cartilage and bone erosion (185, 186). Of relevance in the current context, TNF- α is mainly involved in the pathogenesis of the early stages of the disease, while IL-17 has been implicated throughout all stages of disease and might contribute to the chronicity of RA (187).

Overall, T cells contribute to inflammation (synovitis) either directly through the production of cytokines (Table 1.4) or interaction with neighboring cells directly or mediated by cytokines and promote their activation. Once activated, T cells are capable of producing cytokines such as INF- γ , lymphotoxin- β , TNF- α and IL-17. IL-17 in turn potentially triggers RA synoviocytes to produce a variety of proinflammatory mediators including IL-1 β , IL-6, TNF- α , CXCL8/IL-8, colony-stimulating factor (CSF) and prostaglandin E2 (PGE₂) (188, 189). Furthermore, IL-17 stimulates fibroblasts to produce neutrophil and T cell attracting chemokines such as CXCL8/IL-8, CCL20/MIP- α , CXCL1/growth related oncogene- α and CXCL2/growth related oncogene- β . Furthermore, IL-17 also induces macrophages to secrete IL-1 β , TNF- α , cyclooxygenase-2 (COX-2), MMP-9 and PGE₂. IL-17 is also capable of mediating monocyte migration via CCL2/MCP-1 (190-193). In addition, T cells can stimulate neighbouring cells (monocytes/macrophages and endothelial cells) in synovium to secrete TNF- α , IL-1 β , IL-6, IL-12, IL-15, IL-18, IL-23, and TGF β through direct cell contact, which in turn further promotes T cell differentiation (194). Moreover, interaction between T cells and fibroblasts could activate the latter and contribute to sustained inflammation of the joints (195). T cells exclusively activated by cytokines such as IL-6, IL-2, IL-15, IL-7 and TNF- α ,

may form an important component of synovial T cell populations and promote the production of IL-6, IL-8 and PGE₂ by synovial fibroblasts (196, 197). Further, TNF- α induces IL-1 β , IL-6, and CXCL8/IL-8, and stimulates fibroblasts to express adhesion molecules that play an important role in recruiting leukocytes to inflammatory sites. Finally, there is evidence that in the synovium, apart from Th1, Th2 and Th17 subsets, there are CD4⁺/CD25⁺ regulatory T cells (Tregs) that express forkhead box p3 (Foxp3) that can suppress effector T cells through production of immuno-suppressive IL-10 and TGF β . Although the precise mechanisms that support Treg differentiation are not clear they appear to include at least IL-10, IL-35 and TGF β . Tregs that have been detected in the synovium and SF exhibit impaired regulatory function (198, 199).

In addition to CD4⁺ T cells there are CD8⁺ T cells in the synovium. The natural function of these cells is related to protection against viral infections and tumours. Activated CD8⁺ T cells can produce very high levels of TNF- α and IFN- γ that may contribute to target cell destruction. Compared with CD4⁺ T cells, CD8⁺ T cells have a limited role in disease progression as described so far, however, synovial CD8⁺ T cell population contains a significant proportion of IFN- γ producing effector cells that might contribute to sustained inflammation by secreting pro-inflammatory cytokines (200). Moreover, CD8⁺ T cells may also contribute to the functional activity of germinal center-like structures in ectopic lymphoid follicles within the synovial membrane (201).

1.4.5.2 Monocytes/Macrophages in Synovitis

Monocytes/macrophages play a central role in the pathogenesis of RA, via the secretion of pro-erosive cytokines and other inflammatory mediators. Monocytes are derived from hematopoietic stem cells, via a granulocyte-macrophage progenitor, which gives rise to monoblasts and subsequently to monocytes that circulate in the blood stream and then move into peripheral tissues for further maturation and differentiation. The process of maturation and differentiation is under the control of a number of cytokines and growth factors for example CSFs, including macrophage colony-stimulating factor (M-CSF) and GM-CSF (202, 203). Monocytes may further differentiate in the tissue depending on the cytokine milieu; for example exposure to IL-4 and GM-CSF drives DC differentiation, irrespective of their subset

(204), and exposure to IL-6 and M-CSF induce macrophage differentiation (205). Macrophages are categorised by their activation status into two distinct subsets; M1 (classical, inflammatory) and M2 (alternative, anti-inflammatory). The former are activated by IFN- γ , GM-CSF, and TNF- α and this M1 subset is also differentiated in response to stimulation with Toll Like Receptor (TLR) ligands *e.g.* LPS. Whereas M2 subset are induced upon exposure to IL-4, IL-13, M-CSF, immune complexes and IL-10 (206).

Macrophage phenotypes are classified based on their function such as host defense, wound healing, and immune regulation (207). In regard to synovitis, classical/inflammatory monocytes are detected in high numbers within inflamed synovial tissues and express a high level of cytokines and chemokines that are strongly implicated in the pathogenesis of RA synovitis, namely TNF- α , IL-1 β , IL-6, IL-23, IL-12, type I IFN, CXCL9-11 as well as reactive nitrogen and oxygen intermediates (207, 208). However, there is accumulating evidence that blood monocytes exist in several functional subsets that exert specific roles in homeostasis and inflammation (209). Interestingly, macrophages serve as a sensitive biomarker of disease because the number of infiltrating macrophages within the inflamed synovial membrane correlates with joint destruction and scores for local disease activity as well as with clinical improvement independent of the therapeutic strategy (210-212). Additionally, macrophages infiltrate the synovial space with other cells; therefore, interaction between the different cell types is likely to be critical. Macrophages also contribute to synovial inflammation via the activation of fibroblasts to induce the secretion of GM-CSF, IL-6 and CXCL8/IL-8. Similarly, they can induce CD4⁺ T cell activation through MHC class II. Monocytes are not only implicated in synovitis but also promote bone resorption by inducing early differentiation of osteoclasts (194). Osteoclasts have been reported to be derived from circulating mature monocytes under the influence of M-CSF and receptor-activator-of-nuclear-factor- κ B ligand (RANKL) (209).

1.4.5.3 Synovial Fibroblast in Synovitis

Synovial fibroblasts (SFs) form the intimal lining of the synovial membrane that in turn lines the joint and contribute directly to the pathogenesis of RA by producing key inflammatory cytokines and proteases (213, 214). The activated phenotype of

RASFs is characterized by a change in morphology, anti-apoptotic behavior and increased invasion properties (215). Available data from severe combined immunodeficiency (SCID) mouse models show RASFs have aggressive behaviour and can migrate between joints, thereby serving as a mechanism that spreads the RA phenotype and consequent cartilage damage to distant sites (216). This unique aggressive phenotype of RASFs appears to be partly responsible for the cytokine rich milieu and ongoing joint inflammation and destruction that are characteristics of the RA synovium. Although several mechanisms have been implicated in generation of the aggressive phenotype, recently, epigenetic changes in RASFs that might alter gene expression and thus function have emerged. RASFs differ from other types of fibroblast such as OASFs and normal-SFs; they highly express proto-oncogenes and specific metallo-proteinases as well as cytokines and adhesion molecules (217-220). Within the joint space SFs reside in close proximity to T cells and macrophages, so interaction between these cells is an important amplification pathway for local inflammation. Activation of fibroblasts is driven by $\text{TNF-}\alpha$ and $\text{IL-1}\beta$, that in turn induce the secretion of a variety of cytokines and chemokines including IL-1 , IL-6 , $\text{TNF-}\alpha$ and MMPs (194, 221). Many of these pathways are further amplified by TLR agonists. Moreover, RASFs are implicated in angiogenesis via elaboration of proangiogenic factors including fibroblast growth factor (FGF), and vascular endothelial growth factor (VEGF). In addition, SFs can contribute to joint destruction directly by invading the cartilage surface or indirectly by producing matrix-degrading enzymes (222).

1.4.5.4 Cytokines in Rheumatoid Arthritis

Although cytokines are a diverse group of proteins, they share a number of properties: they are produced during the effector phase of innate and adaptive immunity, are generally not stored as preformed molecules (some do exist as pro-molecules that can be enzymatically cleaved to active forms), and many of them are produced by multiple diverse cell types. Cytokines act upon many different cells types in turn and often have multiple different effects on the same target. In RA, pro-inflammatory cytokines such as $\text{TNF-}\alpha$, $\text{IL-1}\beta$, and IL-6 , have multiple influences on disease pathogenesis and appear to be responsible for ongoing joint inflammation and destruction. The crucial cytokines that are implicated in RA pathogenesis are presented in Table 1.4.

Table 1.4 Cytokines implicated in the pathogenesis of rheumatoid arthritis adapted from (170).

Cytokine	Cell source	Primary effect in the pathogenesis of RA
IL-1	Monocytes, B cells, synovial fibroblasts	Promotes cytokine and chemokines secretion from fibroblasts and monocytes
IL-6	Monocytes, B cells, T cells, synovial fibroblasts	Induces Th17 cell differentiation and promotes antibody production from B cells
IL-10	Monocytes, T cells, B cells, DCs, epithelial cells	Suppresses the inflammation by down regulating proinflammatory cytokines, also inhibits T cell proliferation and contributes to Treg cell differentiation
IL-12	Macrophages, dendritic cells (DCs)	Th1 cells proliferation and maturation and B cells activation
IL-15	Monocytes, synovial fibroblasts, mast cells, neutrophils, DCs	Promotes activation of T cells, fibroblasts, macrophages and neutrophils and B cell differentiation
IL-17A	Th cells, innate lymphoid cells, synovial fibroblast	Promotes inflammatory cytokines from synovial fibroblasts, monocytes and T cells
IL-18	Monocytes, DCs, platelets, endothelial cells	Promotes differentiation of T cells and cytokines secretion also increase the cytotoxicity
IL-23	Macrophages, DCs	Induce the Th17 proliferation and survival
IL-32	Epithelial cells	Induces TNF α , IL-1 β , IL-6, and chemokines also promote PGE2 release
TNF-α	Monocytes, T cells, B cells, fibroblasts, mast cells	Stimulates monocytes/ macrophages endothelial activation also induces endothelial cell adhesion molecule expression and increases cytokine release, inhibits the regulatory function of regulatory T cell.
INFs	Widespread	Increase MHC expression, enhance macrophages and lymphocytes activation, differentiation, survival also cytoskeletal alternation
APRIL	Monocytes, T cells	Promotes B and T cell proliferation
BAFF	Monocytes, T cells, DCs	B cell proliferation and T cell co stimulation also induce antibody secretion
GM-CSF	Monocytes, T cells, fibroblasts	Regulate myeloid cell, neutrophil and macrophage, production, differentiation and activation
M-CSF	Monocytes, T cells, fibroblasts	Promote monocyte differentiation, proliferation and macrophage survival
TGF-β	Synovial fibroblasts, T cells, monocytes and platelets	Induce Th17 and Treg cell differentiation
FGF family	Synovial fibroblasts, monocytes	Regulate growth and differentiation of mesenchymal epithelial cells
VEGF	Monocytes, platelets, synovial fibroblasts	Angiogenesis
PDGF	Monocytes, platelets, synovial fibroblasts, endothelial cells	Growth factor for various lineages; wound healing

Interleukin (IL), tumour necrosis factor α (TNF α), interferon (IFN), a proliferating inducing ligand (APRIL), B cell activating factor (BAFF), granulocyte/macrophage colony-stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF), transforming growth (TGF β), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF).

1.4.5.5 Chemokine System and Cell Migration

A further critical element of the RA pathogenic sequence is the necessity to recruit and retain leukocytes in the synovial compartment. I shall first discuss the core biology of chemokines and chemokine receptors and then refer to what is known of their role in RA.

1.4.5.5.1 Leukocyte Migration

Leukocyte traffic is a highly coordinated process and a breakdown of the underlying control mechanism might contribute to immune dysregulation and autoimmune disease. Recruitment of circulating leukocytes to the site of inflammation is a key step in the development of an inflammatory process. The control of the traffic of these cells to sites of inflammation involves multiple regulatory steps involving several protein families, including proinflammatory cytokines, adhesion molecules, MMPs and chemokines (223-227). In brief, cells once are activated by inflammatory mediators (*e.g.* damage associated molecular pattern containing molecules), the leukocytes migrate to the site of inflammation. This involves extravasation and chemotaxis. Extravasation and chemotaxis of leukocytes are highly complex processes which begin with rolling of cells along the endothelial surface of the luminal side of blood vessels and during the process of rolling the leukocytes are triggered by endothelial surface bound chemokines, which result in firm but transient leucocyte adhesion, a process termed as activation dependent stable arrest. Subsequently, cells transmigrate through the endothelial layer and underlying basement membrane and are finally released to the tissues in response to chemokine mediated chemo-kinetic signals – whether true gradients exist is currently debated in the field (225, 228, 229). Recruitment or migration of effector inflammatory cells from peripheral blood (PB) to the joint space is therefore a complex process that depends on a cascade of events, mediated by chemokines and their receptors and adhesion molecules.

1.4.5.5.2 Chemokines and Chemokine Receptors

Chemokines play a role in a number of inflammatory diseases through induction of chemotactic activity directing neutrophils, lymphocytes and monocytes and promoting their migration to the inflamed tissues. In addition, chemokines enhance

other cellular responses; they contribute to virus-host interaction, cell survival and angiogenesis. Chemokines are classified in a large subfamily of chemotactic cytokines and are distinguished from other cytokines because they are the only cytokines that act on G-protein-coupled serpentine receptors (230, 231). To date, the human chemokine system comprises about 50 chemokines with a shared homology of between 20 and 80% in amino acid sequence, and 19 chemokine receptors as shown in Table 1.5 (232). Chemokines are small proteins with 60-130 amino acids and based on structural homology they have been classified into four distinct families (CC, CXC, CX3C and C), regarding the number and spacing of conserved cysteine residues in the N-terminal region (228, 233-236). The majority of chemokines belong to CC or CXC subfamilies

1.4.5.5.2.1 CC Chemokines

The CC chemokine subfamily has two adjacent cysteines and have at least 27 members 1 to 28, (CCL10 is the same as CCL9), and bind to at least one of the nine CC Receptors (CCRs). Chemokines of this subfamily usually control the migration of PB cells to sites of tissue inflammation. For instance, CCL2/MCP-1, CCL7/MCP-3, CCL8/MCP-2 and CCL13/MCP-4 are chemo-attractant for monocytes, while CCL3/MIP-1 α and CCL5/RANTES are involved in controlling the migration of eosinophils, macrophages and mast cells (225, 236-239).

1.4.5.5.2.2 CXC Chemokines

The CXC subfamily has one non-conserved amino acid (represented by X) located between two conserved cysteines; this chemokine subfamily consists of 17 members and serves as ligands for the six receptors known as CXCRs (225, 236, 237). These groups of chemokines are classically involved in chemotaxis of neutrophils; additionally CXCL4/PF4 and CXCL10/IP-10 also contribute to monocyte and T cell chemotaxis. Additionally, CXCL12/SDF-1, CXCL13/BCA-1 and CXCL16/GCP-2 bind their respective homeostatic receptors and are involved in lymphoid neogenesis, and are also implicated in the ingress of lymphocytes into the tissues.

CXC chemokines are further subdivided based on the presence and absence of the glutamyl-leucyl-arginyl (ELR) motif at the NH₂-terminal region. Those CXC

chemokines carrying the ELR motif, such as CXCL1/Gro- α , CXCL5/ENA78, CXCL6/GCP-2 and CXCL8/IL-8 are specifically implicated in the migration of neutrophils and promote angiogenesis. While, the subgroup without an ELR motif is chemo-attractant for lymphocytes and inhibits neovascularisation, and these include CXCL4/PF4, CXCL9/MIG, CXCL10/IP-10 and CXCL13/BCA-1. However, CXCL12/SDF-1 lacks the ELR motif but is an angiogenic chemokine (240-243).

1.4.5.5.2.3 CX₃C And C Chemokines

The last two subfamilies are CX₃C and C; in the former family the two conserved cysteines are separated by three amino acids and CXC₃L1 is the only member of this group and is expressed on endothelial cells. While the C subfamily has two cysteine residues and two members XCL1 and XCL2, both are involved in T cell migration and accumulation at the site of inflammation (238, 243-245).

1.4.5.5.2.4 Chemokine Receptors

Chemokine receptors are 7-transmembrane, G-coupled proteins and are expressed in cell membranes. Apart from structural classification according to their sequence similarities that correspond to the chemokine it binds (see below), chemokine receptors are categorised into two classes, according to their expression; constitutive (homeostatic) or inducible upon cell activation (inflammatory). CXCR1, CXCR2, CXCR3, CCR1, CCR2, CCR3, CCR4 and CCR5 CCR6 are considered as inducible (inflammatory) chemokine receptors. In contrast, CXCR4, CXCR5, CXCR6, CCR7 CCR9, CCR10, CCR11, XCR1 and CX3CR1 have a role in basal cell trafficking and homing (246). However, this classification is not absolute and chemokine receptors are extremely complicated. For example, CCR6 is expressed on immature DCs for their response to inducible chemokines, such as CCL20/MIP-3 α and CCL5/RANTES by which they are attracted to sites of inflammation, but this receptor is down regulated as the DCs mature (246, 247). Furthermore, mature DCs lose their responsiveness to most of the inflammatory chemokines through receptor down-regulation, but instead express CCR4 and CCR7 during the antigen recognition process for constitutive chemokines that allow their homing to lymph nodes. Thus, CCR7 is a unique chemokine receptor that governs trafficking of DCs under both inflammatory and homeostatic conditions (248). Another exception, CCR8

expression is primarily restricted to a subset of human CD4 memory T lymphocytes but is upregulated during T cell activation and is a marker of Th2 cells.

Of interest, the chemokine system appears to have redundant specificity. Many chemokines can bind multiple chemokine receptors with high affinity and one receptor can bind many chemokines within a subgroup. However few chemokine receptors bind single ligands such as CXCR4 and CXCR5. In general, the redundancy is mainly associated with inflammatory chemokines since they involve multiple receptors within the same family rather than homeostatic chemokines that are selective for single receptors (223, 235, 236). As shown in Table 1.5 this unusual relationship between ligands and their receptors is mainly observed in receptors and chemokines implicated in inflammatory (inducible) leukocyte migration such as CCR3. In contrast, receptors involved in homeostatic chemokine functions are much more limited and restricted to their specific ligands such as CCR9. However, CCR4, CCR7, and CCR10 are homeostatic chemokine receptors and each has two ligands (249).

1.4.5.5.3 Nomenclature of the Chemokine System

There are two systems for naming chemokines. An older system reflected their assumed function or cell type that produced them, while the latter combines structural motifs with the letter L for ligand and a number, as shown in Table 1.5 (235, 236). However, due to the large number of chemokines discovered and to prevent molecules being given multiple names, a more convenient systematic system of nomenclature was devised. Thus, chemokine receptors have a systematic classification system in which receptors are named according to the subfamily of chemokines they bind, followed by R (for receptor) and a number (235, 236, 238, 243).

In addition to structural classification, chemokines are sub-grouped into two categories depending on their function in inflammation and immunity; homeostatic/constitutive and inflammatory/inducible chemokines (Table 1.5) (223, 231). The homing chemokines are expressed constitutively within lymphoid and non-lymphoid tissues to control physiological leukocyte traffic and development. For example, CCL19/MIP-3 β , CCL21/6CKine and CCL25/TECK are considered to

be constitutive chemokines. Inducible chemokines, in contrast, are those chemokines that have broad target cell selectivity and act on both innate and adaptive immune cells. The main function of these chemokines is to control the recruitment of effector cells including granulocytes, monocytes, natural killer (NK) cells, and effector lymphocytes in infection, inflammation, tissue injury and tumour sites. However, several chemokines cannot be categorised to either of these two functional groups; they are upregulated under inflammatory conditions and also target non-effector leukocytes (precursor and resting cells), therefore they are referred to as dual function chemokines (223).

Finally, chemokines have been grouped into acute and chronic inflammation categories. CXC subfamily members recruit neutrophils to inflammation sites and thereby are considered as acute inflammation chemokines. On the other hand the CC subfamily are responsible for recruiting the cells that mediate chronic inflammation such as monocyte/macrophage, eosinophil and T cells and were considered to be chronic inflammation chemokines (231). However, recent findings that CXCR3 is expressed in effector T cells indicate that this acute and chronic classification is unsatisfactory and should not be adopted for wider use (231).

Table 1.5 Human chemokines and their receptors is adapted from (236, 246).

Systemic name	Traditional name	Expression	Chemokine Receptors
CCL1	I-309	Dual	CCR8
CCL2	MCP-1	Inflammatory	CCR2
CCL3	MIP-1 α	Inflammatory	CCR1, CCR5
CCL4	MIP1- β	Inflammatory	CCR5
CCL5	RANTES	Inflammatory	CCR1, CCR3, CCR5
CCL7	MCP-3	Inflammatory	CCR1, CCR2, CCR3
CCL8	MCP-2	Inflammatory	CCR1, CCR2, CCR3, CCR5
CCL11	Eotaxin-1	Inflammatory	CCR3
CCL13	MCP-4	Inflammatory	CCR1, CCR2, CCR3
CCL14	HCC-1	Inflammatory	CCR1
CCL15	MIP5/HCC-2	Inflammatory	CCR1, CCR3
CCL16	LEC/HHC-4	Inflammatory	CCR1
CCL17	TARC	Dual	CCR4
CCL18	PARC	Homeostatic	Unknown
CCL19	ELC/MIP-3 β	Homeostatic	CCR7
CCL20	LARC/MIP-3 α	Dual	CCR6
CCL21	SLC/6Ckine	Homeostatic	CCR7
CCL22	MDC	Dual	CCR4
CCL23	MPIF-1	Inflammatory	CCR1
CCL24	MPIF-2	Inflammatory	CCR3
CCL25	TECK	Dual	CCR9
CCL26	Eotaxin-3	Inflammatory	CCR3
CCL27	CTACK/ILC	Inflammatory	CCR10
CCL28	MEC	Inflammatory	CCR3, CCR10
CXCL1	Gro- α	Inflammatory	CXCR2
CXCL2	Gro- β	Inflammatory	CXCR2
CXCL3	Gro- γ	Inflammatory	CXCR2
CXCL4	BRAK	Inflammatory	CXCR3B
CXCL5	ENA78	Inflammatory	CXCR2
CXCL6	GCP-2	Inflammatory	CXCR1, CXCR2
CXCL7	NAP-2	Inflammatory	CXCR2
CXCL8	IL-8	Inflammatory	CXCR1, CXCR2
CXCL9	MIG	Dual	CXCR3
CXCL10	IP-10	Dual	CXCR3
CXCL11	I-TAC	Dual	CXCR3
CXCL12	SDF-1	Homeostatic	CXCR4
CXCL13	BCA-1	Homeostatic	CXCR5
CXCL14	BRAK	Homeostatic	Unknown
CXCL16	GCP-2	Dual	CXCR6
CX3CL1	Fractalkine	Homeostatic	CX3CR1
XCL1	Lymphotactin	Homeostatic	XCR1
XCL2		Homeostatic	XCR1

I-TAC, interferon-inducible T-cell α -chemo-attractant; MCP, monocyte chemo-attractant protein 1; RANTES, regulated on activation, normal T-cell expressed and secreted; BCA-1, B-cell-attracting chemokine 1; CTACK cutaneous T-cell-attracting chemokine; ELC, Epstein-Barr-virus-induced gene 1 ligand chemokine; ENA78, epithelial-cell-derived neutrophil-activating peptide 78; GCP-2, granulocyte chemotactic protein 2; Gro, growth-regulated oncogene; IL-8, interleukin-8; IP-10, interferon-inducible protein 10; MDC, macrophage-derived chemokine; MEC, mucosae-associated epithelial chemokine; MIG, monokine induced by interferon; MIP, macrophage inflammatory protein; NAP-2, neutrophil-activating peptide 2; SDF-1, stromal-cell-derived factor 1; SLC, secondary lymphoid-tissue chemokine; TARC, thymus and activation-regulated chemokine; TECK, thymus-expressed chemokine.

1.4.5.5.4 Chemokines in Rheumatoid Arthritis

As mentioned above, the inflamed synovium, synovial fluid and tissues are characterized by infiltration of a mixture of inflammatory cells that include neutrophils, macrophages, T cells, B cells, mast cells and DCs. There is also increased vascularity, including new blood vessel formation and thickening of the intimal layer, which in turn results in destruction of cartilage and the underlying bone (169). Within the synovium of RA patients, the infiltrating inflammatory mononuclear cells and other cells produce various inflammatory mediators such as cytokines and chemokines and matrix degrading enzymes (250). It is not surprising therefore that various chemokines are found in abundance in RA synovium and these have been implicated in inflammatory cell recruitment and angiogenesis (251, 252). In this regard, multiple studies have shown that synovial tissue and fluid as well as serum from RA patients contains high levels of inflammatory and some homeostatic chemokines; a further critical element underlying the pathogenesis of RA. These include CCL2/MCP-1, CCL3/MIP-1 α , CCL5/RANTES, CXCL1/GRO α , CXCL8/IL-8, CXCL5/ENA-78 and CXCL12/SDF-1. Moreover, their functional importance has been confirmed by several studies of animal models of inflammatory arthritis. However, other group of chemokines are proposed to exert anti-angiogenic effects, including CXCL4/PF4, CXCL9/MIG and CXCL10/IP-10 (237, 243, 253-264).

The CC chemokines exert mainly inflammatory functions and are implicated in RA pathogenesis via recruitment and retention of monocytes and T lymphocytes into joint spaces. These include CCL2/MCP-1, CCL3/MIP-1 α , CCL4/MIP-1 β , CCL5/RANTES, CCL7/MCP-3, CCL8/MCP2, CCL13/MCP-4, and CCL20/MIP-3 α (253, 255, 256, 265). These chemokines are strongly expressed in the SF and tissues as well as chondrocytes (MCP-4/CCL13) of arthritis patients. Of interest, RASFs produce these inflammatory mediators in response to TNF- α and IL-1 β . Furthermore, CC chemokines involved in haemostatic trafficking, such as CCL17/TARC, CCL18/PARC, CCL19/ELC and CCL21/SLC, are expressed constitutively and contribute to both the physiological formation of lymphoid structures and lymphoid neogenesis in RA (266, 267). Angiogenesis is an early and further critical event in the pathogenesis of RA by promoting a rapid influx of leucocytes and pannus formation.

The CXC chemokines mainly exert inflammatory rather than haemostatic functions, and synovial macrophages represent a major source of this subfamily. The SF and tissues from RA patients contain elevated levels of CXCL1/GRO α , CXCL8/IL-8 and CXCL5/ENA-78, which are primarily neutrophil chemoattractants and mediators of angiogenesis. CXCL8/IL-8 is also important in regulating the expression of leukocyte adhesion molecules (259-261, 268-272). In addition, CXCL4/PF4, CXCL9/MIG, and CXCL10/IP-10, which are chemoattractants for monocytes and T cells rather than neutrophils, inhibit synovial neovascularization perhaps due to their lack of the ELR amino acid motif (240). Intriguingly, there is a unique group of chemokines that have also been implicated in synovial inflammation although they are homeostatic chemokines. These include CXCL12/SDF-1, CXCL13/BCA-I and CXCL16/GCP-2 (273-275). These chemokines bind their respective homeostatic receptors CXCR4, CXCR5 and CXCR6, and are involved in the synovial lymphoid neogenesis underlying arthritis and are also implicated in the ingress of lymphocytes into the RA synovial tissues.

Fractalkine/CX₃CL1 is up-regulated in macrophages, fibroblasts, endothelial cells and DCs of RA synovial tissues, while the soluble form is present in SF (276, 277). This chemokine plays a dual role as a chemotactic molecule for monocytes and lymphocytes and it also serves as an adhesion molecule for T cells, an angiogenic mediator, and has been associated with rheumatoid vasculitis (276-279). Lymphotactin/XCL1 is mainly implicated in T cell recruitment to RA joints and Blacschke *et al.* demonstrated that in RA synovium it is localised mainly in CD3⁺ sub-lining cells (280). Interestingly, in RASFs lymphotactin/XCL1 is significantly down regulated by MMP-2 production. Thus, in addition to T cell recruitment; it might have an additional function in regulating the mechanisms of disease progression in RA.

1.4.5.5.5 Chemokine Receptors in Rheumatoid Arthritis

The synovium of RA joints express numerous chemokine receptors including homeostatic and inflammatory family members. Among CCRs, the entire group are abundantly expressed in RA synovial tissue and cells and include CCR1, CCR2, CCR3, CCR4, CCR5, CCR6 and CCR7. CCR2 and CCR3 are also expressed on articular chondrocytes (281). CCR4 and CCR6 have been respectively implicated in

leukocyte and Th17 lymphocyte ingress into the RA synovium (282, 283). Among these chemokines CCR5 is strongly expressed in RASFs and may be critical for lymphocyte recruitment into the joint (284). Furthermore, CCR7 localizes the lymphocytic infiltration and DCs in the RA synovium and plays a crucial role in maturation and homing of DCs to lymphocytic aggregates (285). Additionally, CCR7 deficient mice with antigen-induced arthritis are protective against disease due to impaired development and organization of tertiary lymphoid tissues (286). Interestingly, in comparison between peripheral blood and SF monocytes, CCR1 and CCR2 are highly expressed in circulating monocytes and are important for cell recruitment from the circulation to the synovium, while CCR3 and CCR5 are expressed mainly in SF monocytes and implicated in cell retention in the joint spaces (287).

Regarding CXCRs; CXCR1 and CXCR2 are expressed on RA synovial neutrophils and macrophages and these receptors recognize the most relevant inflammatory and angiogenic CXC chemokines (288, 289). CXCR3 is highly expressed in RA synovial tissue and represents the most important receptor in the homing of leukocytes into the RA synovium (290, 291). While other CXC chemokine receptors, CXCR4, CXCR5 and CXCR6 which ligate the homeostatic chemokines CXCL12/SDF-1, CXCL13/BCA-1 and CXCL16/GCP-2 respectively, have been implicated in synovial inflammation and lymphoid neogenesis (228, 262, 289, 292).

Among the XCR1 and CX₃CR1 chemokines receptors, XCR1 expression is present in RA synovial lymphocytes, macrophages and RASFs (280). CX₃CR1 has been implicated in monocyte and lymphocyte recruitment into joint space. Additionally, CX₃CR1 is predominately expressed in synovial macrophages and DCs and also on RA SF lymphocytes and macrophages (245, 277).

1.5 Pathogenesis Leads Discovery

The principle of new therapeutic development in RA is to define and characterize a molecular pathway both in terms of its basic biology and also its context-dependent effects in the synovial compartment. Two pathways will be described forthwith. I shall now introduce molecular pathways that may offer such therapeutic utility in future and upon which I have chosen to investigate in my PhD.

1.5.1 MicroRNA

1.5.1.1 Introduction

MicroRNAs (miRNAs) are a recently discovered class of gene expression regulators that are active in many biological systems (293). They are small, single strand non-coding RNA molecules of 19-24 nucleotides long that negatively regulate gene expression of protein-coding genes at the post-transcription level by affecting the degradation or translation of target mRNA. MiRNAs mediate their regulatory actions by directly binding to 3' untranslated regions (3'UTRs) of specific target mRNA through nucleotides 2-7 in the 5' region of miRNA, known as the miRNA seed regions (294). They have been shown to be involved in the regulation of immune responses and the development of autoimmunity.

1.5.1.2 Biogenesis and Regulation of miRNA

MiRNA biogenesis and target mRNA repression is a complex process starting in the nucleus and ending in the cytoplasm, and involves several different enzymes. Processing and regulation of miRNA occurs at three levels: transcription, processing and subcellular localization throughout many post-transcriptional modifications (295). Recent emerging data show that the processes of biogenesis and regulation are influenced by inflammation and other stress factors. The interesting aspect of miRNA genes is related to their genome position, approximately more than half of miRNA coding genes are found within the introns of protein-coding genes or as independent transcription genes; the exons of untranslated genes (296). Alternatively, while most human miRNA are genomically single, certain miRNAs are grouped in clusters and expressed together such that they may be functionally related or may be involved in the same metabolic pathway (297). A typical example

is provided by the miR17-92 family, which comprises six miRNAs (miR-17, miR-18a, miR-19a, miR-19b-1, miR-20a and miR-92a-1).

MiRNAs are derived from large genomic DNA and transcribed by RNA polymerase II to generate long (several hundred to thousand nucleotides) primary transcripts with a stem-loop structure, referred to as primary miRNA (pri-miRNA) with a 5' cap and a 3 poly A tail (298, 299). Similarly, nuclear RNase III enzyme Drosha along with a double-stranded-binding protein DGCR8 (DiGeorge syndrome critical region gene 8) cleaves the long pri-miRNA to a shorter~ 70 nucleotide stem loop structure known as the precursor miRNA (pre-miRNA) (300). Alternatively, some intronic miRNA precursors, termed mitrons, enter the miRNA biogenesis, bypass the Drosha endonuclease pathway and are processed in the nucleus by RNA splicing machinery (301, 302). In either case, this hairpin structure is recognized by exportin-5, a member of the karyopherin family of protein transport, and is actively transported from nucleus to cytoplasm, where the second processing step is carried out (303). Once in the cytoplasm, pre-miRNA undergo further processing and removal of the hairpin loop by the cytoplasmic endonuclease Dicer (RNAase III enzyme) together with other proteins to generate a short 19-24 nucleotides double stranded miRNA (miRNA: miRNA*) duplex (304, 305). The miRNA duplex is further processed, one "guide strand" is selectively, based on the stability of its 5' end, loaded into the RNA-induced silencing complex (RISC) and functions as the mature miRNA, while the other strand (passenger strand, miRNA*) is degraded (306, 307). Mature miRNA then guides the RISC to cognate target genes, miRNA base pair with their complementary mRNA molecules, and represses the target gene expression by either degrading target mRNA (perfect complementarity) or repressing their translation (imperfect complementarity) and the end result is reduction in the total of amount of target protein (308, 309). A single miRNA is able to target a large number of genes and surprisingly a single miRNA is able to bind ~200 different transcripts (310). Although the miRNAs expression from the transcription of miRNA genes until loading of the RISC complex have been established; understanding the regulation of this process is still incomplete and many questions remain to be answered.

1.5.1.3 MiRNA in Development and Regulation of Immune System Function

1.5.1.3.1 MiRNA in Immune Cell Development

Several studies have highlighted the regulatory role of miRNAs in immune cell development (311-315). Originally, studies were conducted to study the expression profiling of hematopoietic cells during their development; they reported that miRNAs are dynamically regulated during the development of T cells, B cells and granulocytes, and are involved in the regulation of these processes. For instance, miR-181a is one of the miRNAs involved in immune cell development; it is highly expressed in thymus cells and to a lower extent in heart, lymph node and bone marrow and was reported as a positive regulator of B cell differentiation. Moreover, miR-181a has been implicated in thymic T cell differentiation and affects the sensitivity of T cells to antigens by modulating TCR signalling (316, 317). In contrast to miR-181a, miR-105 was linked to B cell differentiation by inhibiting the transition of pro-B cells to the pre-B cell stage (318). MiR-142s and miR-223 are miRNAs reported to have a critical role in T cell and granulocyte differentiation respectively, (316, 319). Taken together these observations provided important insights as to how the miRNA network could impact immune cell development and differentiation.

1.5.1.3.2 MiRNA and Innate Immunity

The innate immune system and their immune cells such as granulocyte, monocyte/macrophage and DCs provide a primary first line of defence against infection and represent the primary initiators of inflammatory responses. Innate immune system and inflammatory signalling is mostly mediated by binding of pathogen associated molecular patterns, found in different pathogens, to TLRs (320, 321). TLRs are expressed at high levels on macrophages and DCs and are implicated in the recognition of a wide range of distinct chemical structures conserved in microbes. For instance, TLR3 and TLR4 recognize double stranded RNA virus and bacterial endotoxin lipopolysaccharide, respectively (322). Emerging studies show the contribution of miRNA networks in the development and function of innate immune system cells (323, 324). For example, miR-17-5p, miR-20a, member of miR17-92 cluster, and related miR-160a have been implicated in macrophages and myeloid

derived DCs differentiation and maturation through the transcription factor acute myeloid leukaemia-1 (AML1; also known as runt-related transcription factor 1, Runx1) (323, 325, 326). The expression levels of these miRNAs are down regulated during the differentiation of hematopoietic progenitor cells into monocytes, while the AML1 is upregulated at protein level. Furthermore, enhanced expression levels of these miRNAs strongly promote blast-cell proliferation and inhibits monocytic differentiation and maturation (327).

Another miRNA shown to promote monocyte and macrophage differentiation is miR-424; enforced expression of this miRNA in NB4 cells induced monocyte maturation by inhibition of nuclear factor I/A (NFIA) expression (328, 329). Moreover, miR-223 has also been shown to profoundly affect granulopoiesis (330). MiR-223 negatively regulates progenitor proliferation and granulocyte differentiation and activation and mice deficient to miR-223 exhibit exaggerated tissue destruction and develop more inflammatory lung lesions as a result of hyper-functional neutrophils (324).

Furthermore, several efforts have also been made to demonstrate the role of miRNAs during the activation of the innate immune system. A number of miRNAs are upregulated during innate immune cell activation. In this regard, functional studies show that some miRNAs are induced by bacterial and viral TLR ligands as well as inflammatory cytokines. For instance, miR-146a, miR-155 and miR-132, were found to be upregulated after exposure of human monocytic THP-1 cells to LPS (331). Furthermore, miR-146a over expression was found to be inducible in cells expressing surface TLRs that sense bacterial components (TLR2, 4 and 5) but not intracellular TLR (TLR3, 7 and 9) which sense viral components (331). Additionally, expression of miR-146a was also found after exposure to pro-inflammatory cytokines such as TNF- α and IL-1 β in an NF-kB dependent manner (331). Further, miR-146 expression upon pro-inflammatory cytokine challenge is not only restricted to immune cells but is also observed in alveolar epithelial cells of the lung as well as in SFs (332, 333).

Like miR-146, miR-155 expression has been identified in response to a wide range of inflammatory mediators; including TLR ligands and pro-inflammatory cytokines

TNF- α and INF- γ (331, 334-337). MiR-155 can positively regulate the inflammatory response via repressing the level of SH2 domain containing inositol-5-phosphatase (SHIP-1), an important negative regulator of phosphoinositide 3-kinase (PI3K) and the downstream AKT pathway, in turn increasing the AKT pathway (338-341). Furthermore, miR-155 targets suppressor of cytokine signalling-1 (SOCS-1), which similar to SHIP-1, is a negative regulator of the TLR pathway and is important in controlling the inflammatory response (339, 341). Notably, miR-155 deficient mice have shown upregulation in the expression of SHIP-1 and SOCS-1, while miR-155 over-expression lead to further down-regulation of their expression. Indeed, mice deficient in miR-155 have suppressed immune response capabilities whereas enforced expression of miR-155 in the bone marrow compartment of mice leads to myelo-proliferative disorders similar to that which occurs after LPS stimulation (340, 342, 343). In addition, targeting either SHIP-1 using small interfering RNA against SHIP-1 or deletion of SOCS-1 in mice resulted in a hematopoietic phenotype that in part reflects miR-155 over-expression (341, 344, 345). Taken together, these observations show that miR-155 can serve as positive regulator of innate immunity.

Although the inflammatory response is important to eradicate microbial pathogens, resolution of inflammation in a timely manner is also important to avoid excessive damage to the host. In addition to the vital role of miRNA network in inflammatory responses, miRNA is also capable of acting as an effector molecule in driving a negative feedback mechanism. For example, miR-146a, miR-9 miR-21 and miR-155 have evolved to limit intense inflammatory states and participate in negative feedback regulation of inflammatory pathways (331, 346-349). Taganov *et al.* showed that miR-146 could function as an effector molecule in driving a negative feedback mechanism to attenuate the TLR response, preventing excess inflammation. MiR-146a/b directly targets TNF- α receptor-associated factor-6 (TRAF6) and IL-1 receptors-associated kinase-1 (IRAK1), which are key molecules downstream of TNF- α and IL-1 β signalling in the TLR4 signalling pathway and all promote inflammation (331, 350, 351). MiR-146a is induced by bacterial pathogens in an NF- κ B dependent manner and elevated miR-146 in turn suppresses the activity of inflammatory pathways by down regulation of TRAF6 and IRAK1, and subsequent results in downstream regulation of TNF- α , IL-6 and IL-1 β (331, 350, 351). MiR-

146a therefore appears to play a role in a negative feedback mechanism and prevents excess inflammation.

Furthermore, miR-9 is upregulated in both monocytes and neutrophils after TLR4 activation via LPS and proinflammatory cytokines such as TNF- α and IL-1 β . MiR-9 induction operates feedback control on the NF- κ B-dependent responses to maintain a constant level of NF- κ B1 protein expression (346). In addition miR-21 was recently identified as a negative regulator of inflammatory responses through down regulation of NF- κ B signalling, driving a switch to anti-inflammatory responses via secretion of IL-10 (349). MiR-21 is upregulated in response to TLR4 activation of macrophages and operates through targeting mRNA encoding PDCD4, a tumour suppressor inflammatory protein, which leads to activation of NF- κ B by unknown mechanisms (349).

MiR-155 is part of the negative feedback loop, however, its role seems to be complex, possibly owing to its controlling expression of both pro and anti-inflammatory mediators or due to a wide range of relevant targets (352). For example, miR-155 may participate in a negative feedback loop in human myeloid derived DCs, miR-155 deletion showed significantly increased expression of IL-1 β and other proinflammatory cytokines in response to LPS stimulation (337). Furthermore, miR-155 represses the expression of the signalling protein TAK1-binding protein 2, which promotes inflammation, which may be key for its anti-inflammatory effects (337). However, miR-155 in certain settings can positively regulate the inflammatory pathway by negatively regulating SHIP-1, which is a potent inhibitor of many inflammatory pathways including the AKT pathway. Interestingly, AKT signalling has recently been shown to negatively regulate miR-155 and miR-125b expression but positively regulates miRNAs let-7e and miR-181c expression in macrophages (353). More recently, miR-155 has been shown to convey atheroprotective effects; hematopoietic deficiency of miR-155 leads to more inflammatory atherosclerotic lesions by increasing leukocyte recruitment to plaque and also decreased plaque stability (354). It clearly suggests the existence of miR-155 in a negative feedback loop and thereby miR-155 may diminish instead of enhancing the inflammatory response.

DCs are important components in the innate immune system and serve a crucial function in initiating and regulating immunity. MiRNAs have been implicated in regulation of distinct aspects of DCs biology. For instance, miR-34 and miR-21 are shown to be important for proper DCs differentiation by targeting *WNT1* and *JAG1*, whereas miR-155 regulates their maturation by targeting the transcription factor PU.1 (355, 356). In addition, another study reported that myeloid DCs from miR-155 deficient mice show impaired ability to support T cell activation, despite expressing normal levels of MHC class II and co-stimulatory molecules, which indicates that miR-155 is not required for maturation (357). However, miR-155 may participate in a negative feedback loop in myeloid derived DCs as mentioned above.

Taken together the aforementioned information strongly suggest that miRNA network are important in controlling innate immune responses, which are induced by bacterial or viral TLR ligands, as well as inflammatory cytokines. In addition, miRNAs have a vital role in coordinating the appropriate behavior of the immune system; influencing both aspects of positive and negative feedback.

1.5.1.3.3 MiRNA and Adaptive Immunity

Similarly, miRNAs also have an important role in regulating the adaptive immunity response. In the immune system, miRNA appear to play a key role in lineage induction pathways of T cells and also have a strong role in the induction, function and maintenance of the regulatory T-cell lineage. T cell development in the thymus and their activation in the periphery is controlled by a complex protein signalling and miRNA network. Several studies shown that the miRNA expression profiling in T cells vary between T cells subsets and stage of development (312, 358, 359). Adding to this complexity, the level of miRNA expression appears to be inversely correlated with the activation status of the cells (312). For example, proliferating T cells express mRNAs with shortened 3' UTRs and fewer miRNA target sites than those seen with resting T cells, suggesting they are at that stage less susceptible to regulation by miRNAs (360). Additional evidence that confirms the importance of miRNA in the development of mature T cells comes from observation that mice with specific deletion of Dicer have hypo-cellularity and the severity of the phenotype is dependent on the stage of development at which Dicer is removed (361, 362).

So far, two specific miRNAs have been implicated in T cell development and differentiation namely, miR-181a and the miR-17-92 cluster (311, 312, 363). The best evidence for miR-181a playing a role in T cell development and differentiation; its level is correlated inversely with the activation status of T cells – such levels are dysregulated across naïve, effector and memory CD8 T cells (311, 312). Furthermore, miR-181a controls thymic development of immature T lymphocytes for differentiation into mature cells by modulating the strength of TCR signalling. MiR-181a increases the sensitivity of TCR signalling by directly targeting multiple protein phosphatases involved in the attenuation of signal transduction (311, 317). While, the miR17-92 cluster targets the mRNA encoding pro-apoptotic proteins, leading to increased T cell survival during development (363). This suggested that the hypocellularity of thymic and peripheral T cells might be mediated in part by loss of miR-17-92.

Once the mature naïve T cell is in the periphery, the regulatory role of miRNA is more clear; to control the differentiation of T helper cells into distinct effector T helper cell subsets (364). For instance, Rodriguez *et al.* have clearly demonstrated that CD4⁺ T cells deficient in miR-155 are biased towards Th2 differentiation and enhanced production of Th2 cytokines (IL4) via targeting the transcription factor c-Maf (a potent trans-activator of the IL-4 promoter) (357). Consistently, Bnaerjee *et al.* showed that over expression of miR-155 in CD4⁺ T cell promotes Th1 differentiation, while its inhibition enhances T cell bias towards the Th2 phenotype (365). Moreover, mice with the T cell deletion in SHIP-1, an important negative regulatory of many inflammatory pathways, exhibit defective Th2 differentiation (365). These findings were confirmed by using miR155^{-/-} mice in CIA and experimental autoimmune encephalomyelitis (EAE) models in which defective Th1 and Th17 cells responses were observed (343, 366, 367). However, other studies show that miR-155 is not involved in Th1 differentiation and as such more studies are required and thus the precise molecular mechanism for the discrepancy between these studies is still not well characterized (357, 366). Further, miR-326 promotes Th17 cell development by targeting ETS1, which is an established repressor of Th17 cell development (368).

MiRNAs are also important in regulating differentiation of Treg, that function to suppress inflammatory responses. For instance, mice with global deletion of either Drosha or Dicer in Treg expressing cells results in the reduction of Foxp3 Treg cells induction and is disproportionate to that of other T-cell subsets both in the thymus and the periphery (369-372). This numerical loss leads to systemic and lethal inflammatory lesions.

Further, miRNAs play a crucial role in controlling Treg function and miR-155 has the best evidence for miRNA playing a role in this regard. MiR-155 is directly regulated by Foxp3 and is critical for maintaining cell homeostasis and overall survival; miR-155 deficient mice have impaired development of Tregs (373-375). This likely operates via SOCS1, a negative regulator of the IL-2 signalling pathway. Additionally, over expression of miR-155 during thymic differentiation is essential to maintain Treg proliferation activity and increase their sensitivity to their principle growth factor IL-2 (374). However, other miRNAs expressed by Treg cells that enhance suppressive function have yet to be identified and further studies are required.

In B cells, miRNAs appear to have a key role in early and effector cell differentiation, i.e. at both levels of antigen-independent development in the bone marrow and antigen-dependent selection in secondary lymphoid organs. For example, absence of Dicer enzyme during early B cell development led to a complete developmental block in early B cell progenitors and have also a survival defect at the progenitor to precursor B cell transition (376). Similarly, absence of miR-17-92 leads to a developmental block at the pro-B to pre-B transition due to an increase in Bim expression (377). While, ectopic over-expression of the miR-17-92 family results in enhanced B-cell proliferation and survival by down regulating the expression of pro-apoptotic gene Bcl-2/Bim (377). Indeed, the miR-17-92 cluster has been associated with lympho-proliferative disease both in human and mice and this is thought to involve the direct targeting of transcription factors; Bcl-2 (378). Unlike, the miR-17-92 family, miR-155 controls important aspects of B-cell biology particularly during the stage of B cells response such as development of germinal centre B cells, the generation of isotype- switched, high-affinity IgG1 antibodies and memory responses (357, 379, 380).

1.5.1.4 MiRNA in Diseases of Immunological Origin

MiRNAs dysregulation has been linked to the pathogenesis of many diseases of immunological origin by their wide regulatory role in basic cell biology, metabolism and in specific elements of innate and adaptive immune function. Therefore, it is becoming increasingly clear that miRNAs regulation may be involved in a broad spectrum of immunological disorders, including the autoimmune diseases. However, the role of miRNAs are only beginning to be explored in the context of autoimmunity but it is accepted that they are involved in variety of cellular processes such as apoptosis, differentiation, immune cells development and immune responses. Although, the exact impact of miRNAs in pathologies of autoimmune disease have yet to be fully understood but several mechanisms have been suggested include loss or down regulation of particular miRNA due to mutation or transcriptional down regulation, as well as upregulation of certain of miRNA either due to mutation or consequent to gene amplification. Moreover, mutation at the 3'UTR of the target mRNA or its gene may influence the binding site or transcription upregulation and this result in suppressed production of its target proteins. Interestingly, there is now compelling evidence that links dysregulation of miRNAs network and pathogenesis of autoimmune disease, include RA, systemic lupus erythematosus (SLE), multiple sclerosis (MS), primary biliary cirrhosis (PBC), ulcerative colitis (UC), and psoriasis.

1.5.1.4.1 Multiple Sclerosis

Multiple Sclerosis (MS) is a chronic autoimmune inflammatory demyelinating disease of the central nervous system (CNS), in which myelin specific CD4⁺ T cells becomes activated in the peripheral immune compartment, cross the blood brain barrier and promote neurological damage (381). Only recently, publications underscoring the role of miRNAs in the molecular mechanism of MS, have investigated miRNAs profiles in the PB of MS patients and some were conducted in the brain lesions of different disease activity of MS patients (382-388). For example, miR-155 is over expressed 10 fold higher in active MS brain white matter lesions than normal brain white matter (387). As I mention before, due to its various functions in the immune system, it is likely that miR-155 also has different functions in MS pathology by controlling the immune cells development and differentiation. MiR-155 promotes the differentiation of inflammatory T cells, including the Th17 cell and Th1 cell subsets via targeting and down regulation of the transcription factor

C-Maf, which has an important role in the development of Th2 cells, where the differentiation is shifted towards the Th1 phenotype (343, 357). Indeed, the miR-155^{-/-} mice were highly resistant to EAE, a model system for studying MS (343).

Furthermore, miR-326 is one of the most upregulated miRNAs in active MS lesions versus normal brain white matter and is also over expressed in PB leukocytes of MS patients with relapses than comparable cells from normal controls (384). Interestingly, miR-326 is also able to promote Th17 differentiation and promoting of T cell- dependent tissue inflammation, therefore, mice lacking miR-326 show a reduction of symptoms in EAE by preventing Th17 cell differentiation through targeting Ets-1, a known negative regulator of Th17 differentiation (384). While both miR-148a and miR-126 are upregulated in the inactive MS brain lesion compared to control brain tissue, the latter is also down regulated in blood samples from MS patients versus healthy controls (382, 383, 387).

Recently, there are several lines of evidence that suggest that a certain number of miRNAs are not only involved in pathophysiology of MS by regulating the innate immune response, but are also critical for relapse of the disease. Otaegui *et al.* examined miRNA expression in the PBMCs of MS patients in relapse and remission status compared to controls and demonstrated that miR-18b and miR-559 are related in some way to the relapse while the miR-96 play a role in remission (386). Thus, miRNAs are of potential importance in the molecular mechanisms of disease and their expression could be useful as a biomarker of the relapse status. By contrast, miR-124 is expressed only in resident macrophages of brain and spinal cord (microglia), but not in other PB monocytes and macrophages, both in the normal CNS and during EAE (389). Interestingly, miR-124 promotes cellular quiescence by directly targeting the mRNA encoding C/EBP- α and reduces expression of this protein under steady state conditions, while in EAE miR-124 is down regulated and thus contributes to CNS inflammation (389). These observations clearly implicated miRNAs in the regulation of neuro-inflammation and can alter the magnitude of the inflammatory response.

1.5.1.4.2 Inflammatory Bowel Disease

Ulcerative colitis (UC) and Crohn's disease (CD), collectively termed Inflammatory Bowel Disease (IBD), are chronic complex disorders characterized by wide variation in clinical presentation with a relapsing and remitting clinical course (390, 391). Although, both UC and CD are involved in gastro-intestinal inflammation, they differ in location and the nature of the inflammation. UC is limited to the mucosal layer of the rectum and colon; in contrast, CD is mainly transmural inflammation and can affect any part of the gastrointestinal tract from the mouth to the anus (390, 391). Recently growing evidence indicates that there is altered expression of the tissue miRNAs profile in UC and CD as well as in PB (392-394). Several of these studies have examined miRNA expression in epithelial cells of patients with crohn's ileitis, crohn's colitis and active ulcerative colitis and suggest that abnormalities in miRNA expression may contribute to the molecular mechanisms of disease as well as distinguishing between diseases states. Comparison of miRNA profiles isolated from colonic biopsies for healthy controls and patients with active UC, inactive UC and chronic active CD revealed that active UC was associated with differential expression of 11 miRNAs; 3 (miR-192, miR-375, and miR-422b) were significantly decreased, whereas the other 8 miRNAs were significantly increased in active UC tissues compared to healthy control tissues (392). Particularly, miR-192 and miR-21 were the most highly expressed in active UC tissue compared to healthy controls. Interestingly, miR-192 correlated inversely with the expression of MIP-2 α , (chemotactic cytokine) and immunohistochemistry confirmed its expression in colonic epithelial cells (392).

Furthermore, differential miRNA expression pattern in patients with active versus inactive UC are distinct, for instance, the miRNAs were increased in active UC, 4 of the 8, miR-23a, miR-16, miR-24, and miR-29a, were increased in colonic tissues of both active and inactive UC, while the expression of the other 4 was mostly consistent with healthy control levels. In contrast, miRNAs decreased in active UC exhibited increased expression of miR-375 and miR-422b in inactive UC compared to healthy control, while miR-192 was unchanged in inactive UC (392). On the other hand, differential miRNAs profiling in the subgrouping crohn's ileitis and crohn's colitis reveals that 3 miRNAs: miR-23b, miR-106, and miR-191 were significantly increased in active Crohn's colitis and are not UC-associated miRNAs (393).

Furthermore, that study similarly reported that miRNAs profiling in Crohn's ileitis versus healthy control and showed that miR-16, miR-21, miR-223, and miR-594 were over-expressed in active terminal ileal CD tissues (393).

Comparison of miRNA expression profiles in PB of active CD and inactive CD with those from healthy controls revealed that five miRNAs, were significantly over expressed while two miRNAs, miR-149* and miRplus-F1065 were significantly down regulated in active CD compared to healthy controls (394). In particular, four of the five miRNAs including miRs-199a-5p, 362-3p, 532-3p and miRplus-E1271 were over expressed in PB of active CD compared with inactive CD, while the miR-340* was significantly expressed in both active and inactive CD. In contrast, miR-149* expression was significantly decreased in PB of both active and inactive CD but miRplus-F1065 was decreased only in active CD (394). Similarly, analysis of the miRNA expression in PB samples of active UC patients and healthy controls demonstrated that twelve miRNAs, includes miR-28-5p, miR-151-5p, miR-199a-5p, miR-340*, miR-3180-3p, -miRplus-E1271, miRplus-F1159 miRplus-E1035, miRplus-1153, miR-523-3p miR-103-2* and miR-362-3p, were significantly upregulated in active UC patients with miR-103-2* and miR-362-3p being most highly expressed compared with controls (394, 395). Notably, the first 9 miRNAs were increased in PB of active UC but not in blood of patients with inactive UC, and miR-505* was decreased in both active and inactive UC compared to healthy controls (394, 395). Interestingly, PB profiling of miRNAs expression can distinguish the active CD and active UC and further analysis indicates that 10 miRNAs were significantly increased in active UC compared to active CD and only miR-505* was significantly decreased in active UC (394). Overall, differentially expression of miRNAs in PB could be useful tools for diagnosis and early biomarkers methods to determine patient's disease course.

1.5.1.4.3 Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a chronic inflammatory systemic autoimmune disease that affects multiple organs or systems, including skin, joints, kidneys, and the CNS. The clinical manifestations of SLE are secondary to autoantibody mediated destruction of host cells or trapping of antigen-antibody complexes in capillaries of organs resulting in inflammation and tissue damage

(396). It is becoming increasingly clear that miRNAs may play an important role in SLE pathogenesis. Dai *et al.* revealed that comparison of the miRNA profile in SLE patients and healthy controls identified a group of 16 miRNAs that are differentially regulated in SLE PBMCs; 7 miRNA (miR-196a, miR-17-5p, miR-409-3p, miR-141, miR-383, miR-112, and miR-184) were down regulated, while 9 miRNA (miR-189, miR-61, miR-78, miR-21, miR-142-3p, miR-342, miR-299-3p, miR-198, and miR-298) were upregulated, compared to healthy controls (397). The same group has analysed the miRNA profiling of kidney biopsies taken from lupus nephritis patients versus controls and revealed 66 miRNA were differentially expressed compared to controls; 36 miRNAs were upregulated and 30 were down regulated (398). Furthermore, Tang *et al.* examined the miRNA expression in the PBMCs of SLE patients during a relapse and remission, versus normal controls, demonstrating that the decrease expression of miR-146a in SLE patients inversely correlated with clinical disease activity and over activation of the type I IFN pathway (399). Interestingly, over-expression of miR-146a inhibits the induction of type I IFNs pathway in PBMCs of SLE patients, this suggested that miR-146a could be the causal factor in abnormal activation of type I IFN in SLE (399). These data highlight the potential important role of miRNA expression both as diagnostic markers and as factors implicated in the pathogenesis of SLE.

1.5.1.4.4 Rheumatoid Arthritis

Abnormalities of miRNA expression may contribute to the molecular mechanisms that underpin RA pathogenesis. After the first report provided by Stanczyk in 2008 describing dysregulation of miRNA networks in RASFs, several investigators examined miRNAs either in circulation, plasma and PBMCs, or within the SF or synovium tissue and suggested there is a link between miRNA function and RA pathogenesis (Figure 1.3). Pauley *et al.* clearly demonstrated the PBMCs of RA patients exhibited between 1.8 fold and 2.6 fold increase in miR-146a, miR-155, miR-132 and miR-16 expression compared to healthy controls and increased miR-146a and miR-16 expression correlated with disease activity (400). These observations supported the notion that miR-146a could be a promising diagnostic biomarker of autoimmune disease. Similarly, Murata *et al.* provide the first description of increased concentrations of miR-16, miR-146a, miR-155 and miR-223 in RA SF, where as miR-132 was not significantly different, as compared to OA SF.

Interestingly, increased plasma concentration of these miRNAs inversely correlated with tender joint counts and plasma miR-16 also inversely correlated with 28-joint disease activity score (401). Additionally, miR-146a expression in RA CD4⁺ cells was positively correlated with the levels of TNF- α in both PB and SF (402).

Of relevance in the current context, several studies have also evaluated miRNA profile expression in RA synovial tissue and RASFs in particular. It is becoming increasingly clear that many miRNAs are either upregulated or down regulated in rheumatoid tissues. Among these; miR-124a is significantly under expressed compared with OA synovial tissue and over expression of miR124a in RASFs results in arrested cell cycle at the G1 phase and suppressed MCP-1 (403). Koch *et al.* reported that MCP-1 was highly expressed in both synovial tissue and SF, which is important for monocyte migration and retention in joint spaces; thus, low levels of miR-124a may promote leukocyte migration (404). More recently, Niederer *et al.* have demonstrated that basal expression of miR-34a*, the passenger strand of miR-34a, was also down regulated in RASFs compared with OASFs and negatively correlated with X-linked inhibitor of apoptosis protein expression in SFs. However, expression levels of miR-34a, miR-34b/b*, and miR-34c/c* did not differ between RASFs and OASFs (405).

In contrast, miR-155 and miR-146a were more highly expressed in SFs of RA patients than those from patients with OA (332, 406). Furthermore, *in situ hybridization* and double immunofluorescence staining revealed RA synovial lining and sub lining layers are positive for miR-146a and miR-155 and primarily in CD68⁺ macrophages (332, 367). In addition, Stanczyk *et al.* reported that RA SF monocytes displayed higher levels of miR-155 compared with RA PB monocytes (406). Induction of miR-155 in RASFs significantly suppress MMP-3 and 1, these data suggested a possible role of miR-155 in modulating downstream tissue damage (406). Recently, our group showed that RA SF CD14⁺ monocytes express higher levels of miR-155 as compared with RA PB CD14⁺ monocytes and inhibition of miR-155 significantly increased the expression of SHIP-1 mRNA, a potential target of miR-155, in RA SF CD14⁺ monocytes (367). Taken together these observations suggest a dysregulation of miRNA expression in RA patients that may contribute to disease pathology. Moreover, these data suggest a possible role of miRNAs to serve

as a diagnostic and/or prognostic marker in an array of inflammatory disorders, including but not limited to RA.

MicroRNA and Rheumatoid arthritis

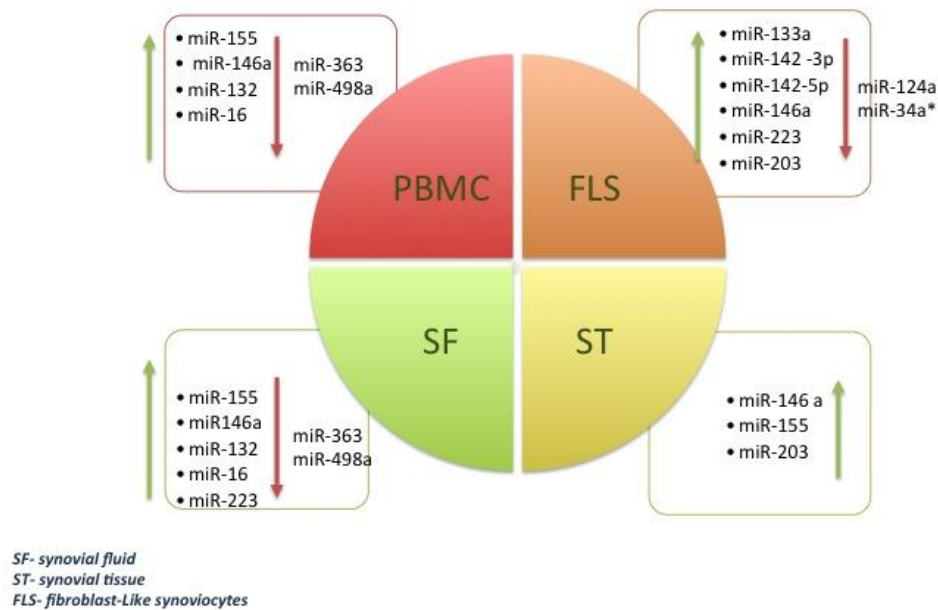


Figure 1.3 Schematic diagrams represents dysregulation of miRNAs levels in rheumatoid arthritis either in circulation (PBMCs) or within the synovial fluid (SF) or synovium tissue and synovial fibroblast (SFs).

1.5.1.5 MiRNAs in Cell Migration

A hallmark pathological feature of RA is infiltration and accumulation of inflammatory cells in the synovium of the joint. The recruitment and retention of effector cells, including monocytes to the joint space is a critical element of the pathogenetic sequence in RA progression and is mediated by chemokines and their receptors. Thus far it has not been known whether miRNAs regulate this element of pathology. Increasing our understanding of RA pathogenesis and miRNAs has become one of the necessary elements to answer many questions as well as clarify the story of RA pathogenesis.

Recently, many members of the miR family have been implicated directly or indirectly in the regulation of cell movement and migration. Of particular interest are emerging studies showing the contribution of miRNA networks to monocyte migration and chemokine/chemokine receptor system. For instance, miR-124a in RASFs significantly suppresses MCP-1 as mentioned in details in section 1.5.1.4.4. (403). Another miRNA implicated in cell movement, miR-34a, is under expressed in CD44⁺ prostate cancer cells, initiating and metastatic cells, and overexpression of miR-34a in these cells leads to suppressed tumour regeneration and metastasis, whereas targeting miR-34a expression in CD44⁻ cells resulted in tumour expansion and metastasis (407).

Additionally, miR-155 has been detected in human pulmonary fibroblasts in response to TNF- α and IL-1 β and could also alter their migration. Ectopic expression of miR-155 in these cells leads to induction of caspase-3 activity and results in increases in fibroblast migration on a type I collagen substrate (408).

Likewise, Dagan *et al.* reported that, miR-155 directly represses native human germinal center associated lymphoma (HGAL) expression by binding to its 3'-UTR mRNA in human lymphoma cell lines (409). Enforced expression of miR-155 decreases expression of HGAL protein and increases SDF-1 stimulated lymphoma cell motility (409). HGAL is a specific gene involved in negative regulation of lymphocyte and lymphoma cell motility by directly interacting with actin and myosin and by activating the RhoA signalling pathway (410, 411).

However, in pre-eclampsia miR-155 suppresses trophoblast movement. Pre-eclampsia is a placental dependent disorder causing maternal and perinatal morbidity and mortality and incomplete invasion of extra villous cytotrophoblasts that invade the uterine arterioles has been recognized as a common pathological feature of pre-eclampsia. Recently miRNA profiling in two studies showed that seven miRNAs were upregulated including miR-155 (412, 413). Compared with placentas from woman with normal pregnancies, the placenta from woman with severe pre-eclampsia displayed higher levels of miR-155. Dai *et al.* showed manipulation of miR-155 levels in trophoblasts by forcing their expression reduced the ability of these cells to migrate, while transfection with miR-155 anti-miR reversed the negative effect on migration. (414). Overall these observations strongly suggested that miR-155 regulates multiple genes involved the cell biological processes including cell migration.

The foregoing literature clearly shows that miRNAs in general and miR-155 in particular regulate several inflammatory pathways including cell movements, of relevance to the synovial immune response. Thus far the ability miRNAs to directly regulate leukocyte recruitment to the synovial lesion have not been extensively investigated beyond the few datasets alluded to above. Therefore in my thesis I sought to explore a potential link between miRNAs in general and miR-155 in particular with chemokine and chemokine receptor expression in RA.

1.5.2 Novel Signal Pathways in Rheumatoid Arthritis

There is increasing interest in identifying signalling pathways that might offer therapeutic utility in RA. This has been pioneered by the advent of Janus kinase (JAK) inhibitors but more studies are required to gain a fuller understanding of the potential of such pathways to offer therapeutic value. I have elected to focus on one such pathway that offers promise as a future therapeutic target.

The Sphingosine kinases/sphingosine 1 phosphate (SPHKs/S1P) axis have been proposed to have a significant role in the induction of various types of inflammatory responses and disease pathologies i.e. cancer, arthritis, asthma and ulcerative colitis (415). Recent investigations in animal experimental arthritis (CIA) have clearly demonstrated that serum S1P levels are elevated and SPHK1 expression increased in the synovial membrane of mice with arthritis (416, 417). Further evidence supports S1P's key role via S1P receptors (S1PRs) in promoting synoviocyte proliferation, induced cyclooxygenase-2 (COX-2) expression and PG2 production (418). Additionally, S1P levels were elevated in RA SF (416). The SPHK2 is highly expressed in RASFs (419) and its targeting in CIA models results in increased disease activity and also increase serum levels of pro-inflammatory mediators such as IL-6, TNF- α , and IFN- γ (417). Thus, SPHK1 and SPHK2 have distinct roles in regulating the development of inflammatory arthritis (417). Therefore, sphingolipid enzymes and their products may have a different role in the pathogenesis of RA. Until now however, no comprehensive analysis of expression of its components in RA has been performed.

1.5.2.1 Synthesis and Metabolism of Sphingolipids

Over the past decade, sphingolipids and their metabolites have emerged as a new class of potent bioactive molecules, regulating diverse pathophysiological processes (420-422). Sphingolipids are important chemical structures in the cell membrane and formation of these molecules is mediated through one of two pathways; the *de novo* synthesis or through a salvage pathway. The *de novo* synthesis of sphingolipids begins with the condensation of serine with palmitoyl CoA at the cytoplasmic face of the endoplasmic reticulum (ER) to create 3-keto-sphinganine catalysed by serine palmitoyl transferase (423). This product (3-keto-sphinganine) then undergoes two rapid enzymatic reactions; firstly it is reduced to sphinganine (dihydro-sphinganine

(DHS)) by 3-keto-sphinganine reductase and subsequently acetylated to dihydro-ceramide by dihydroceramide synthase. The latter is converted to ceramide by desaturase (424, 425). Once the dihydro-ceramide and/or ceramide are formed they are translocated from the ER to the lumen side of the Golgi apparatus, where they can be converted to dihydro-sphingomyelin (DH-SM) and sphingomyelin (SM) respectively, by SM synthase (424-426). Otherwise, the dihydro-ceramide and/or ceramide are converted to dihydroglucosylceramide and glucosylceramide on the cytosolic surface of the Golgi apparatus, after translocation into the luminal side of the Golgi; these molecules are converted into lactosylceramide and more complex sphingolipids (424).

1.5.2.2 Sphingomyelin Cycle

SM is an important membrane sphingolipid molecule formed as a precursor of essential lipid intermediate molecules in the sphingomyelin cycle, particularly ceramide (Cer), sphingosine (Sph) and S1P, which have been implicated in cell differentiation, proliferation and apoptosis (420, 421). The metabolism of SM is regulated by the activity of different sphingomyelinase (SMase) isoforms (421, 427, 428). The function of each isoform is entirely related to its intracellular localisation and mechanisms of activation.

The hydrolysis of SM by SMase leads to the generation of Cer (429). Once the Cer is generated it can be phosphorylated by ceramide kinase to form ceramide-phosphate or it can be a substrate for SM synthase to yield SM or degraded further by ceramidase to generate Sph. In the same manner, Sph can be either phosphorylated by sphingosine kinases (SPHKs) to S1P or can be utilised by ceramide synthase to generate Cer (420-422, 424). While, S1P degradation is achieved via two different pathways, either by the dephosphorylation backs to Sph via S1P phosphatase or irreversible cleavage by S1P lyase to hexadecenal and phosphoethanolamine and removal from the sphingomyelin cycle Figure 1.4 (430, 431). Therefore, formation and degradation of sphingolipids are interconnected and interdependent and the dynamic balance between ceramide and S1P in the sphingomyelin cycle can drive cellular processes either into cell survival or cell death.

1.5.2.3 Sphingosine Kinase

Sphingosine kinases (SPHKs) are recently discovered enzymes which belong to the lipid kinase family that includes ceramide kinase, diacylglycerol kinase (DAGK) and phosphatidylinositol 3- kinase (PI3K). SPHKs modulate diverse cellular responses (415). To date, two isoforms of SPHKs, namely SPHK1 and SPHK2, have been cloned in humans and mice (432-435). These isoforms contain five conserved domains (C1-C5) and the ATP binding site is present within C2, the C1-C3 contains a unique catalytic domain, which is also found in DAG kinase and ceramide kinase; however, C4 appear to be unique to SPHKs (432-437). Though SPHK1 and SPHK2 are highly homologous, the latter has 200 additional amino acids and four predicted trans membrane regions, while the former, has three calcium and calmodulin binding sequences and several protein kinase-binding sites (432-437).

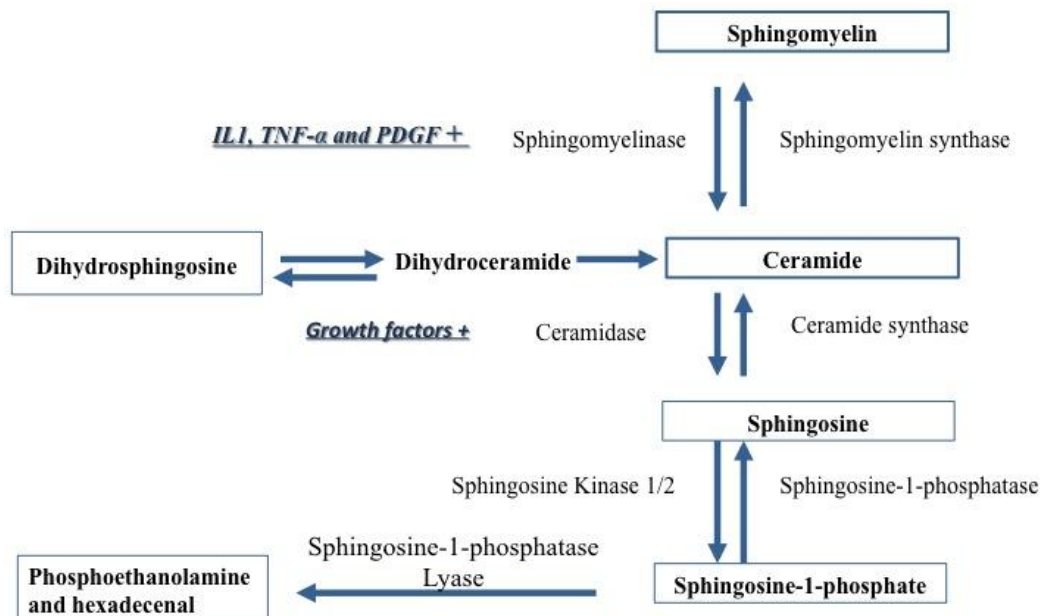


Figure 1.4 Diagrammatic representation of the pathways of Sphingomyelin metabolism. The hydrolysis of sphingomyelin (SM) by sphingomyelinase (SMase) leads to the generation of ceramide (Cer). Cer is then phosphorylated by ceramide kinase to form ceramide-phosphate or it can be a substrate for SMase to yield SM or degraded further by ceramidase to generate sphingosine (Sph). The Sph is then phosphorylated by sphingosine kinases (SPHKs) to S1P or it can be utilised by ceramide synthase to generate Cer. The S1P degradation is caused by two different pathways; either by the dephosphorylation back to Sph via S1P phosphatase or irreversible cleavage by S1P lyase to hexadecenal and phosphoethanolamine and removal from the sphingomyelin cycle (adapted from Wong et al., 2009 (429)).

Northern-blot and quantitative real-time polymerase chain reaction (QPCR) analyses demonstrated that SPHK1 and SPHK2 have different kinetics, tissue expression as well as temporal expression patterns during development, which gives a clue that they regulate distinct cellular and molecular functions, and could be regulated by different mechanisms (424). SPHK1 is predominantly found in lung, spleen, kidney and blood whereas SPHK2 is highly expressed in heart, liver, brain and kidney (438). In humans, SPHK1 and SPHK2 are localised to chromosomes 17q25.2 and 19q13.2, respectively (439). These isoforms are predominately present in the cytoplasm; however, recent studies have shown that the SPHKs have distinct subcellular distribution, which varies according to the tissue types. SPHK1 is a ubiquitous cytosolic protein that rapidly translocates to the plasma membrane after activation, while the SPHK2 is mostly localised to the nucleus.

Moreover, the SPHK isoforms differ in their substrate preferences; SPHK1 prefers D-erthro- sphingosine and D-erthro-dihydro sphingosine, while SPHK2 prefers erthro phyto-sphingosine and threo-dihydrosphingosine. The most well known competitive inhibitors of SPHK1 are threo-dihydrosphingosine (DHS) and N, N dimethylsphingosine (DMS), while SPHK2 is inhibited by DMS (436, 440, 441).

1.5.2.3.1 Mechanism of Activation and Regulation of Sphingosine Kinase

There is a growing list of agonists that have been reported to increase SPHKs activity namely:

- Agonists of G-protein coupled receptors (GPCR) such as acetylcholine, bradykinin, lysophosphatitic acid (LPA) and S1P.
- Agonists of receptor tyrosine kinase-like platelet derived growth factor (PDGF) (442), nerve growth factor (NGF) (443), epidermal growth factor (EGF) (444) and VEGF (445).
- Cross linking of immunoglobulin receptors (446, 447) and other biological agents such as IL-33, TNF- α , C5a and Ca²⁺ increasing agents as well as phorbol esters (415, 448-450).

Many of these stimuli cause a rapid onset and transient activation of SPHKs. Although, modulation of SPHK activity by these agonists is not well understood, the regulation of SPHKs appears to be occurring at both transcriptional and post-

transcriptional levels, including phosphorylation, protein-protein interaction, phosphatidic acid, Ca^{2+} and subcellular localization (448, 451-453). The exact pathway and molecular mechanisms by which SPHKs isoforms are activated is still under intensive investigation and there are some challenging clues postulated by various studies (432, 433, 436, 454). One of the important clues is that both SPHK1 and SPHK2 are found in the cytosol while their active substrate Sph is found in the cell membrane, thus there is translocation of SPHK either by direct association with membrane components or by bringing it close to its substrate. However, translocation to the plasma membrane could be one of the regulatory processes for SPHK1 activation, but not for SPHK2 (432, 433, 436, 454).

Furthermore, regulation of SPHK1 by $\text{TNF-}\alpha$, requires it to bind to TNF receptor-associated factor-2 (TRAF2) resulting in activation of the enzyme, which in turn is required for TRAF2 mediated activation of NF- κB and subsequently prevents apoptosis by $\text{TNF-}\alpha$ (450). In addition, it was shown that deletion of the TRAF2 binding consensus site prevents the interaction of SPHK1 with TRAF2 and the stimulation of SPHK1 by $\text{TNF-}\alpha$ (450). Likewise both phorbol ester, phorbol 12-myristate 13-acetate (PMA) also induce protein kinase C mediated phosphorylation of SPHK1 and its translocation to the plasma membrane (448). Taken together these observations show that both $\text{TNF-}\alpha$ and phorbol ester induce phosphorylation of SPHK1 at serine-225. This is mediated by extracellular signal-regulated kinase (ERK1/2) and this phosphorylation was required for agonist stimulation of SPHK activity and translocation of SPHK1 to the plasma membrane (453).

Similarly, C5a which is produced following activation of the complement system and stimulates C5aR; CD88 in human macrophages is capable of inducing a rapid and transient increase of SPHK1 activity and membrane translocation with elevation of the S1P level (455). However, previous studies have compared a number of physical and enzymatic properties between the SPHK1 from human placenta and from *E. coli* and demonstrated little difference indicating that human SPHK1 is not post-translationally modified and SPHK1 has a substantial basal activity (435).

Whereas, in some cells these stimuli (agonists) induce a more sustained increase of SPHK activity, which lasts for hours or days; such as NGF mediated SPHK activity

in neuronal cells and stimulation of fibroblasts with TNF- α (456, 457). The biphasic course of SPHK activation is most likely consistent with a first rapid increase in enzymatic activity, which is then followed by increased transcription.

Several studies have shown that Ca^{2+} regulates SPHK1 activity by initiating an increase in cytosolic Ca^{2+} that is required for catalytic activity (458-460). Though the Ca^{2+} modulation of SPHK activity is unclear, some of the studies have shown that SPHK1 binds to Ca^{2+} /calmodulin and enhanced the translocation of SPHK1 to the plasma membrane, but not its catalytic activity (432, 433, 461). Furthermore, it was shown that the Ca^{2+} induced activation of SPHK, using Ca^{2+} increasing agents that enhanced basal S1P formation, whereas chelating agents of intracellular Ca^{2+} inhibited the stimulation of S1P production in HL-60 and HEK-293 cells (458, 460). On the other hand, SPHKs are involved in the receptor-induced Ca^{2+} mobilization from intracellular stores; indicating some complexity of Ca^{2+} /SPHKs interaction (462).

Several protein-protein interaction studies suggested that the activity of SPHK1 was also enhanced by proteins that directly interact with SPHK1 such as protein kinase A, platelet endothelial cell adhesion molecule-1 and others (437, 463, 464). However, the physiological significance of this interaction has not been well understood. For example, tyrosine kinases Lyn and Syk were recently identified proteins in mast cells that directly interact with SPHK1 (neither of these kinases phosphorylate the SPHK1), which results in increased activities of both these kinases and the recruitment of SPHK1 to Fc ϵ RI shortly after stimulation (465). Although much less information is available about the regulation of SPHK2 activity, many reports have suggested that it can be stimulated by various agonists and also interacts directly with several intracellular proteins (466).

1.5.2.4 Sphingosine 1-Phosphate (S1P)

S1P is a pleiotropic sphingolipid identified in the early 1990s as a potent second messenger (467, 468). It has emerged as a novel lipid mediator (469) and found to be associated with regulation of biological activities, like cell migration, proliferation, survival, suppression of apoptosis (470, 471) and cytoskeletal organization (424, 472-477). The cellular S1P concentration is tightly regulated by

its formation from sphingosine by the activity of SPHKs and by its degradation through two distinct mechanisms; reversible dephosphorylation of S1P into sphingosine by S1P phosphohydrolases as well as lysophospholipid phosphohydrolases and irreversible degradation by a pyridoxal phosphate dependent S1P lyase to trans-2 hexadecanal and ethanolamine phosphate Figure 1.5 (430, 431).

In the basal state, S1P generation and degradation is well balanced and results in low cellular concentrations of S1P (478, 479). The S1P concentration in human blood ranges from 100nM to 4μM. Furthermore, it depends on the detection methods used as well as the type of species under investigation (480). In the serum, S1P is bound to albumin and other plasma proteins (481, 482). In the blood, S1P is chiefly found in erythrocytes and thrombocytes since these cells lack S1P lyase and erythrocytes lack S1P phosphohydrolases (483). Recent studies have observed that erythrocytes and vascular endothelial cells could be responsible for maintaining S1P concentrations in plasma (480, 484-487). However, thrombocytes contain a much higher concentration of S1P (487, 488). Importantly, a physiological gradient exists between human tissues as well as plasma and interstitial fluids (489), which is required for the homing of immune cells to lymphoid tissue and regulating their egress to lymphatic and blood circulation.

1.5.2.5 S1P/S1PRs

S1P is a pleiotropic cytokine that mediates an array of important biological processes (490-497) by binding specifically with G protein-coupled S1P receptors (S1PRs) (498-500). In addition, S1P acts as a second messenger independent of its receptors (501-503) and also weakly binds to other LPA receptors like P2Y receptor (504-506). These GPCRs also called the endothelial differentiation gene (EDG) family of proteins (507-509) with low nM affinity. The EDG receptors were recently classified as S1PRs (490, 510-512). To date, the S1PR family has five members namely, EDG1/S1P₁, EDG5/S1P₂, EDG3/S1P₃, EDG6/S1P₄ and EDG8/S1P₅. The S1P and di-hydro S1P specifically bind with these receptors to elicit various biological processes (513). In mammals, these receptors display tissue specific expression pattern, S1P₁₋₃ are found in all tissues while S1P₄ is restricted to lymphoid hematopoietic tissues and lung and S1P₅ are mainly localized to brain and skin (447, 501-503).

S1P acts in an autocrine or/paracrine way to induce an array of cellular and molecular functions (Figure 1.5) (470, 514-516). S1P has shown to be transported from the intracellular space to the extracellular space via an ATP binding cassette transporter, ABCC1 in fibroblasts and mast cells (517, 518). All the five types of S1PRs, like other GPCRs, undergo various regulatory processes like the removal from the cell membrane into the cytoplasm and vice versa. The S1PRs are phosphorylated by specific GPCR kinases (GRKs) on their serine and threonine residues by Akt/PKB that occur within minutes of the receptor activation. This leads to the recruitment of β -arrestins that binds with the agonist bound S1PRs (519, 520) to uncouple the S1PRs from the G-proteins and cause the internalization of the S1PRs, which is called desensitization. Internalized S1PRs are either recycled back to the plasma membrane, which is called re-sensitization or transported to lysosomes for degradation and ultimately causes the down-regulation of S1PRs (415, 521).

1.5.2.6 An Overview of S1P/S1PRs in Health and Disease

1.5.2.6.1 S1P₁

(Synonyms: S1PR1, EDG1)

S1P₁ is expressed in most immune cells and tissues (415). It binds to S1P with high specificity and affinity, and regulates an array of cellular and molecular events such as cytoskeletal rearrangements and migration of mast cells toward antigen and Fc ϵ RI-triggered degranulation (511), migration of resident stem cells in the skeletal muscle to improve tissue regeneration (522), differentiation of endothelial cells (498), inhibition of sprouting angiogenesis during vascular development (523), egress of immature B cells from bone marrow into blood (524) and induction of inflammation (488). S1P₁ is essential for vascular maturation (525) and the loss of S1P₁ during embryonic development leads to malformed embryonic hearts and early death of mouse embryos (526). The impaired expression of S1P₁ prolongs the survival of chronic lymphocytic leukaemia B-cells in patients with unfavourable prognosis (527). The upregulation of S1P₁ in peripheral T cells significantly reduces the progress of EAE in mice (528). On the other hand, over expression of S1P₁ was observed in diseases such as gall bladder adenocarcinoma (529), thyroid cancer and neoplasia (530), cell migration and invasion in Wilms tumour (531), lymphatic

metastasis in tumours (532), haemorrhage (533) and synovial hyperplasia, inflammation and RANKL- induced osteoclastogenesis in RA (534, 535).

1.5.2.6.2 S1P₂

(Synonyms: S1PR2, EDG5)

S1P₂ is expressed consistently in many tissues and cells such as mouse bone marrow derived mast cells (511), T lymphocytes (536), primary human bronchial epithelial cells (537), glioma cell lines (538) and stria vascularis of cochlea (539). It plays a role in cellular processes such as survival, growth, proliferation, migration, transformation, activation and mast cell degranulation (511). It plays a vital role in the wound healing process during acute lung injury by inducing the proliferation of hepatic myofibroblasts (540). S1P₂ plays a pivotal role in the function of the vasculature and is an important agent for the proper maintenance of hemodynamic (541) and myogenic differentiation (542), proper functioning of the auditory and vestibular systems (543), and counteracting the effect of IL-1 β in human chondrocytes in OA patients (544). However, it was found to be associated with pathological retinal angiogenesis (545), pathogenesis of endothelial injuries in diabetic nephropathy (546) and vasodilatation associated with anaphylactic shock (547).

1.5.2.6.3 S1P₃

(Synonyms: S1PR3, EDG3)

S1P₃ binds to various molecules including S1P with variable affinity. It was found that S1P₁, S1P₂, and S1P₃ function co-ordinately during embryonic angiogenesis (548). It plays a role in the recruitment of anti-inflammatory monocytes to micro-vessels during implant arteriogenesis (549), Ca²⁺ release from internal stores (550), cell proliferation and survival (551, 552). S1P₃ is chiefly involved in the remodelling, proliferation, and differentiation of cardiac fibroblasts, cardio-protection from ischemia/reperfusion injury *in vivo* (553). However, it was associated with the development of diseases such as oedema, lymphoid cancer, angio-immunoblastic T cell lymphoma, and neoplasia (554). The activation of S1P₃ by S1P and FGF may play a key role in glial proliferation, reactive gliosis and brain tumour formation (555).

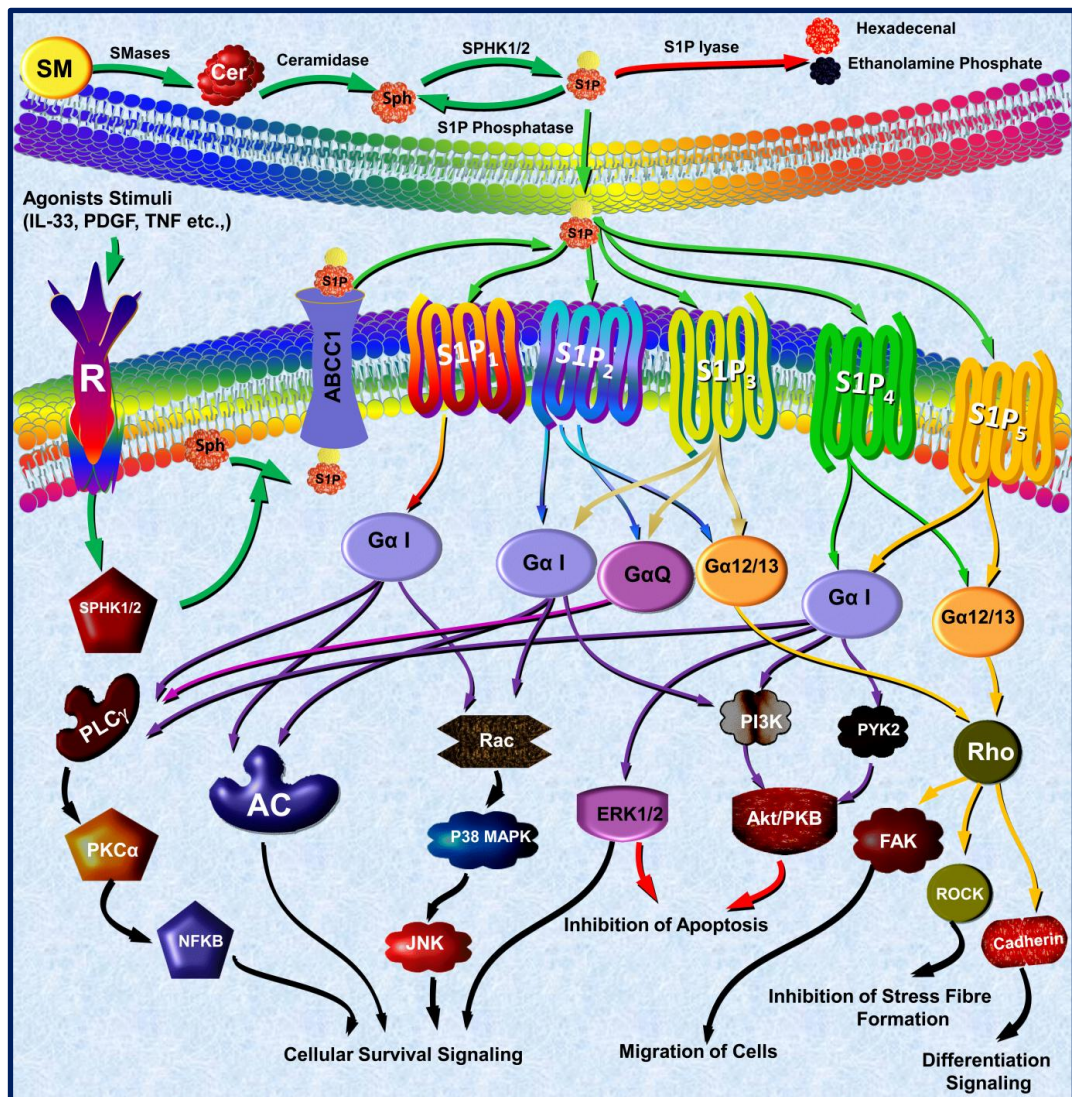


Figure 1.5 S1P/S1PR axis mediated signalling. The agonists (IL-33, PDGF, TNF- α) induce the expression of SPHK1/2 that subsequently converts sphingosine into S1P. Ceramide and sphingosine inhibit proliferation and induce apoptosis, while S1P stimulates growth and suppresses apoptosis, regulates angiogenesis, which is critical for the tumour progression. Furthermore, S1P goes outside the cell through ATP binding cassette (ABC) transporter C1 (ABCC1) and exerts its action through the G-protein coupled S1PR 1-5 in an autocrine and/or paracrine fashion to regulate cell proliferation, growth, differentiation, migration, apoptosis and other cellular processes (adapted and modified from Aarthi *et al.* 2011(415)). (Image is kindly supplied by Dr Peter Pushparaj).

1.5.2.6.4 S1P₄

(Synonyms: S1PR4, EDG6)

S1P₄ binds to S1P and involved in cell signalling in various cell types (552). S1P₄ is highly expressed in blood cells compared to other tissues. It is coded by an intron less gene and is mainly expressed in lymphoid tissues (552). It regulates various intracellular molecules, release of Ca²⁺ from internal stores (556), chemotaxis (557), regulation of immune response, activation of adenylate cyclase and phospholipase C γ (PLC γ) (556, 558) and inhibition of T cell proliferation as well as secretion of effector cytokines such as IL-2, IL-4 and IFN γ and increased production of anti-inflammatory cytokine, IL-10 in S1P₄ only T cells (559). Until now, S1P₄ is not associated with any disease pathologies (415).

1.5.2.6.5 S1P₅

(Synonyms: S1PR5, EDG8)

Like other S1P receptors, S1P₅ interacts with S1P and is involved in cellular signalling processes in a variety of cells (552). S1P₅ is highly expressed in NK cells and oligodendrocytes of the CNS compared to other tissues (560). Unlike other S1PRs, S1P₅ is chiefly restricted to the brain and the skin (552). It plays a role in the egress of NK cells from lymph nodes and bone marrow (561). Like S1P₄, it is also not associated with any disease pathologies (415).

1.5.2.7 SPHK/S1PRs and Axis in Rheumatoid Arthritis

Recently, several efforts have also been made to demonstrate the role of SPHKs/S1P in autoimmune disease in general and RA in particular (416, 417, 562, 563). Multiple studies have clearly demonstrated that the expression of SPHK1/2 was increased in RA synovium. In addition, the levels of S1P in the SF of RA patients were found to be significantly higher than those of OA patients (416). Of relevance in the current context, elevated SPHK1, S1P, and S1P₁ levels have been detected in RA synovium and the S1P/S1P₁ axis played a role in synovial proliferation and induced cyclooxygenase-2 COX-2 expression (418). Also, S1P was required for the ability of the TNF- α to induce COX-2 leading to PGE2 production (418). Also the increased level of SPHK2 in the RASFs was found to be contributing to the proliferation (419).

The role of SPHKs/S1P in RA has been studied using a non-specific SPHKs inhibitor, DMS, and RNA interference (RNAi) approach against SPHKs. Additionally, DMS significantly reduced the levels of TNF- α , IL-6, IL-1 β , CCL2/MCP-1, and MMP-9 in cell-contact assays using both Jurkat-U937 cells and RA PBMCs. Furthermore, intraperitoneal administration of DMS in the CIA model, significantly inhibited disease severity and reduced articular inflammation and joint destruction. Furthermore, similar reduction in incidence and disease activity was observed in mice treated with SPHK1 knock-down using the RNAi method (417). These results provide us with a clue that the S1P/S1PRs axis modulation may provide a novel approach in treating or managing chronic autoimmune conditions like RA.

Since, the pleiotropic modulator S1P and its five receptors may have a role in the pathogenesis of RA, the understanding of sphingolipid signal transduction pathway will be essential for the development of novel therapeutic for RA. Until now however, no comprehensive analysis of expression of its components in RA has been performed.

1.6 Aim

The purposes of the present study are to;

- 1) Explore a role for a novel miR-155 regulatory pathway for monocyte migration in RA via modulation of chemokine and chemokine receptor system. This study investigated the role of miR-155 on chemokine and chemokine receptor expression using human blood monocytes and bone marrow derived monocytes and macrophages from miR-155^{-/-} and WT mice.
- 2) Explore the differential expression of SPHK1/2, S1P and its five receptors in the blood cells and the synovium of RA patients compared to healthy controls and OA patients.

CHAPTER II

MATERIALS AND METHODS

2.1 Reagents

Histo-hypaque gradient-1107 and leukocyte separation-1119 were purchased from Sigma-Aldrich, USA. Human CD8⁺, CD4⁺, and CD14⁺ micro beads, human CD15-FITC, CD14-FITC, CD8-FITC, CD4 PE, Mouse IgG2a-FITC and mouse IgG2a-PE, auto-MACS running buffer, auto-MACS rinsing solution all were purchased from Miltenyi Biotec Inc. SPHK1, S1P₁, S1P₂, S1P₃ and S1P₅ primary antibodies from Abcam plc, Cambridge, UK, while SPHK2, S1P₄ antibodies were purchased from Santa Cruz Biotechnology. BD Cytotfix/Cytoperm and Fixation/Permeabilization Solution Kit were purchased from BD Biosciences. High Capacity cDNA Reverse Transcription Kit and Fast SYBR Green Master Mix were ordered from AB Applied Biosystems, USA. The Sphingosine 1 Phosphate ELISA Kit (S1P) K-1900 was purchased from Echelon Biosciences Inc and QPCR primers for SPHK1/2 and S1PR₁₋₅ were ordered from Integrated DNA Technologies BVBA. The Fc Receptor blocker was purchased from Wako Chemicals GmbH.

miRIDIAN mimic hsa-miR-155, miRIDIAN microRNA mimic negative control, miRIDIAN microRNA mimic transfection control with Dy547 were purchased from ABgene Limited. N-TER[™] nanoparticle siRNA transfection system (Sigma), ammonium chloride solution, Stem cell Technologies, while, RNeasy mini kit, miScript reverse transcription Kit, Hs_miR-155_1 miScript primer assay, Hs_RNU1A_1 miScript primer assay, miScript SYBR Green PCR Kit, all purchased from Qiagen Germany. Mouse CD11b micro beads and mouse CD115 micro beads kit were purchased from Miltenyi Biotec Inc. Further, APC anti-mouse CD115 (CSF-1R) antibody, PE/Cy7 anti-mouse Ly-6C antibody, FITC anti-mouse CD11b antibody, PE anti-mouse Ly-6G antibody, FITC Rat IgG2b, κ Isotype Ctrl antibody were all purchased from Biolegend. Murine M-CSF was purchased from Peprotech.

Human cytokine panel I milliplex catalog ID MPXHCYTO-60K-12 and human cytokine panel II milliplex catalog ID MPXHCYP2- 62K-05 were purchased from Merck Millipore.

2.2 Collection of Peripheral Blood and Synovial Fluid Samples

Peripheral blood (PB) samples were obtained from healthy controls and RA patients, who were older than 18 years of age and capable of providing informed consent. All the RA patients recruited to this study met the diagnostic criteria of 2010 ARC/EULAR. All procedures received Ethics Approval. Groups were age and sex matched. Dr. David McCrery, a rheumatologist at Rheumatology clinic, Glasgow Royal Infirmary (Glasgow, U.K.) and I within the department, collected blood samples from arthritis patients and controls respectively. Blood samples were collected into lithium heparin vacuettes and were transferred to the research unit. Diagnosis of RA was made based on classification criteria described in the introduction. Healthy donors did not have a history of any diseases. Further, SF samples were collected from RA patients at various routine outpatient Rheumatology Clinics at Glasgow Royal Infirmary or Glasgow Stobhill Hospital (Glasgow, U.K.). All samples were collected into lithium heparin vacuettes.

2.3 SPHK1/2 and SIPR₁₋₅ Study

For the SPHK1/2 and SIPRs study the total amount of blood sample taken from both healthy controls and RA patients was thirty-five ml; thirty ml of which were used for cell isolation and sorting while the other five ml of blood was used for serum separation to measure S1P levels.

2.3.1 Isolation of Serum Samples

Five ml of PB was collected in a serum separation tube and allowed to clot and then centrifuged (3000 RPM) at room temperature for 10 minutes. Serum was aliquoted and stored at -80°C until analysis. Serum samples from healthy controls (n=20) and RA patients (n=40) were used to assay the S1P level by using a competitive S1P ELISA KIT (S1P) K-1900 (Echelon Biosciences Inc., USA) as per manufacturer's instructions. Samples were analysed in triplicates.

2.3.2 Isolation and Purification of Human Leukocytes from Blood

For isolation of CD14⁺ monocytes, T lymphocytes (both CD4⁺ and CD8⁺ cells) and neutrophils, density gradient centrifugation of 30 ml heparinized blood was performed. This was done using Histo-Hypaque gradient-1107 and leukocyte

separation-1119 (Sigma-Aldrich, USA) according to manufacturer's instructions with minor modification. In brief, 3ml of Histo-Hypaque gradient-1107 was carefully layered on top of 3ml of leukocyte separation -1119 in 15ml conical centrifuged tubes. Then approximately 6ml of diluted PB (diluted 1:1 with phosphate buffered saline (PBS)) was carefully layered on top and centrifuged at 2500 RPM at room temperature for 25 minutes. After centrifugation two distinct layers were obtained, the interface mononuclear cell-rich buffy coat layer and the opaque layer containing neutrophils were collected using a Pasteur pipette and transferred to clean tubes labelled as PBMCs and neutrophils respectively. After that cells were washed with cold PBS and centrifuged at 1500 RPM for 5 minutes. This step can be repeated two to three times for better cell purification, and numbers of viable cells from both tubes were counted by using haemocytometer and trypan blue staining. Finally, PBMCs and neutrophils cells were resuspended in the appropriate amount of PBS until further serial purification.

2.3.2.1 Serial Purification of CD14⁺ Monocytes, CD4⁺ and CD8⁺ T Lymphocytes from PBMCs

Purification of CD14⁺ monocytes and both subset of T lymphocytes (CD4⁺ and CD8⁺ cells) were carried out in cascades by magnetic-activated cell sorting (MACS) using the Auto-MACS separator according to manufacturer's protocol (Miltenyi Biotec). The PBMCs were isolated as previously described and washed with MACS buffer (PBS containing calcium and magnesium, 2% Foetal Calf serum (FCS) and 100U/mL penicillin and 100µg/ml streptomycin) and incubated at 4°C with anti-CD16/CD32 (Fc Block) to reduce non-specific binding by Fc receptors. Cells were then incubated with 80µl of MACS buffer and 20µl of human CD8⁺ micro beads (Miltenyi Biotec) per 1×10^7 cells for 15 minutes at 4°C. After the incubation cells were washed with approximately 10ml of MACS buffer and centrifuged at 1500 RPM for 5 minutes. Supernatant were discarded and the cells were resuspended in 5ml of MACS buffer and purified by positive selection on an Auto-MACS separator using the "possel" programme. The positive fraction was run through the "possel" program again in order to increase the cell purity, which ranged between 95-98%, as assessed by flow cytometry. The numbers of viable CD8⁺ cells were counted using a haemocytometer and trypan blue staining. Cells was resuspended in the appropriate amount of MACS buffer and incubated on ice until further analysis.

The number of viable cells from the negative fraction of purified CD8⁺ cells were counted and incubated with 80µl of MACS buffer and with 20µl of human CD14⁺ micro beads (Miltenyi Biotec) per 1x10⁷ cells for 15 minutes at 4°C. After the incubation cells were washed and centrifuged as previously described and then purified by positive selection on Auto-MACS separator using the “possel” programme twice. The number of viable CD14⁺ cells were counted using trypan blue staining and resuspended in the appropriate amount of MACS buffer until further analysis. The purity of enriched CD14⁺ monocytes from the second passage was assessed by FACS analysis. The number of viable cells from the negative fraction of purified CD14⁺ cells were counted and incubated with 80µl of MACS buffer and with 20µl of human CD4⁺ micro beads (Miltenyi Biotec) per 1x10⁷ cells for 15 minutes at 4°C. The cells were washed, centrifuged and purified as previously described, and finally the number of viable CD4⁺ cells were counted using trypan blue staining and resuspended in the appropriate amount of MACS buffer until further analysis. The procedure for the cascade purification of CD14⁺ monocytes, CD4⁺ and CD8⁺ T lymphocytes from PBMCs using the MACS and Auto-MACS separator are shown on Figure 2.1.

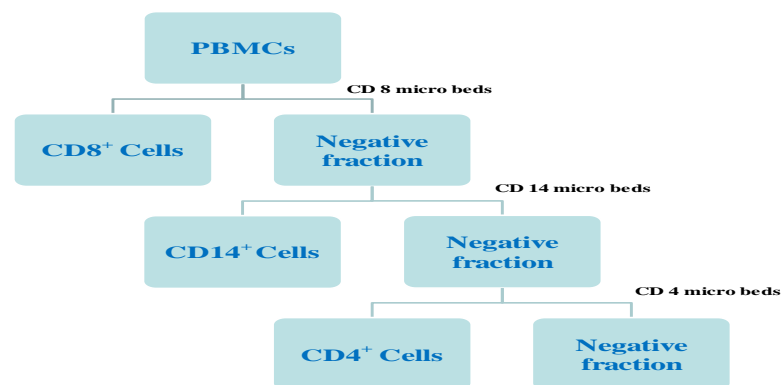


Figure 2.1 Cascades purification of CD14⁺ monocytes and both CD4⁺ and CD8⁺ T lymphocytes from PBMCs using magnetic-activated cell sorting (MACS) Auto-MACS separator.

After isolation and purification of (CD15⁺ neutrophils, CD14⁺ monocytes, CD4⁺ and CD8⁺ T cells) from PB, the cell suspension was split into three tubes. One tube was used to assess the cell purity (on the same day), while the second and third tube were used to examine the intracellular expression and mRNA levels of SPHK1/2 and SIP₁₋₅ using FACS technique and QPCR, respectively.

2.3.2.2 Assessment of Cell Purity

Assessment of cell purity was performed on the same day of cells purification. The 100µl of enriched cell suspension (approximately 1×10^6 cells) was stained with 10µl of specific antibodies or appropriate isotype control. The CD15⁺ neutrophils, CD14⁺ monocytes and CD8⁺ T cells were stained with CD15, CD14 and CD8 FITC antibodies, respectively and isotype control (mouse IgG2a FITC). While CD4⁺ cells were stained with CD4 PE antibody and isotype control mouse IgG2a PE. All were incubated for 15 minutes in dark at 4°C. The cells were washed with 2ml of FACS buffer (PBS containing calcium and magnesium, + 2% FCS + 0.1mM EDTA) and centrifuged 1500 RPM for 5 minutes. The cells were then resuspended in 300-500µl of FACS buffer and acquired use a FACS caliber (BD Biosciences) with Cell Quest Pro Software (BD Bioscience, USA) and data analysed via FlowJo software (Tree Star Inc., USA) (Figure 5.2).

2.3.2.3 Intracellular Protein Staining

For intracellular protein levels of SPHK1/2 and SIPR₁₋₅ 1ml of cell suspension (1×10^7) of each type of purified cells: CD15⁺ neutrophils, CD14⁺ monocytes and both CD4⁺ and CD8⁺ T lymphocytes were washed with PBS and centrifuged at 1500 RPM for 5 minutes and then cells were fixed by addition of 1ml of 4% paraformaldehyde (Sigma) and incubated for 20 minutes at 4°C. After incubation the cells were then washed twice with 2ml FACS buffer and resuspended in 1ml of FACS buffer and stored at 4°C until the analysis.

On the day of analysis, each tube of purified cells were washed with FACS buffer and centrifuged at 1500 RPM for 5 minutes and supernatant discarded completely. To fix and permeabilize cells, the cells were incubated with 300µl/tube with cytofix cytoperm (BD Biosciences, USA) for 30 minutes at 4°C. The cells were then washed twice with 1ml cytoperm wash (BD Biosciences, USA), and then resuspended in 1ml of wash buffer. Aliquot of 100µl of each type of cells were incubated for 30 minutes at 4°C with primary antibodies for SPHK1/2 or SIPR₁₋₅ or appropriate isotype controls; the SPHK1, SIP₁, SIP₃ and SIP₅ share the same isotope control (primary rabbit antibody), while the other share the primary goat antibody as isotype control (Table 2.1). Incubation followed by 1ml wash with cytoperm wash and then cells incubated with appropriate secondary antibody labelled with FITC for

30 minutes at 4°C. Finally cells were washed with 1ml cytoperm followed by 2ml washing with FACS buffer, and then resuspended in 300-500µl of FACS buffer and acquired using a FACS calibre with Cell Quest Pro Software and data analysed using Flowjo software.

Table 2.1 Primary antibodies for intracellular SPHK1/2 and SIPR₁₋₅ staining and appropriate secondary antibodies and isotype controls.

Primary antibody	Supplier	Isotype control	2 nd antibody
Human SPHK1	Abcam plc	Rabbit antibody	Anti Rabbit FITC
Human SPHK2	Santa Cruz inc	Goat antibody	Anti Goat FITC
Human SIP₁	Abcam plc	Rabbit antibody	Anti Rabbit FITC
Human SIP₂	Abcam plc	Rabbit antibody	Anti Rabbit FITC
Human SIP₃	Abcam plc	Rabbit antibody	Anti Rabbit FITC
Human SIP₄	Santa Cruz inc	Goat antibody	Anti Goat FITC
Human SIP₅	Abcam plc	Rabbit antibody	Anti Rabbit FITC

2.3.3 Immunohistochemistry Staining for SPHK1/2 and SIPR₁₋₅

Synovial tissue specimens were obtained from RA and OA patients at the time of arthroscopic biopsy or total joint replacement surgeries. All RA and OA patients fulfilled the diagnostic criteria for RA and OA classification respectively, and written consent form was obtained from all subjects. Synovial tissue specimens were preserved in 10% formalin, embedded in paraffin and then serially sectioned onto microscope slides at 5µm thickness. Immunohistochemistry staining (IHCs) procedure was performed at room temperature over two days by using a primary antibody and a biotinylated secondary antibody with Avidin/Biotin Complex and DAB chromagen (ABC) method.

Briefly, tissue sections from the RA and OA patients were heated in a tissue drying oven (GenLab) for 30-35 minutes at 60°C and deparaffinised twice in xylene for 5 minutes. After that tissues were rehydrated twice through descending series alcohol for 30 seconds. For antigen retrieval, slides were immersed in 0.01M-citrate buffer, pH 6.0 and boiled in a microwave for 20 minutes. Slides were then left to cool for 15 minutes before being washed in distilled water for 5 minutes and washed twice in TBST for 5 minutes. Later, sections were incubated for 30 minutes with 20% horse serum in TBST containing diluent of avidin D (from Avidin blocking kit) at room temperature in a humidified box. To prevent non-specific binding section were

incubated with Fc Receptor blocker (Wako Chemicals GmbH, Germany) for 30 minutes after tapping off the Blocking serum. After that slides were incubated overnight with primary antibodies to SPHK1/2 or SIPR₁₋₅ or appropriate isotypes control (Table 2.2). The next day, sections were washed and incubated with 0.05% hydrogen peroxidase in methanol for 30 minutes to block any endogenous peroxidase activity and sections were further washed and incubated with appropriate biotinylated secondary antibody diluted in 2.5% human serum in TBST (1:200) for 30 minutes. Sections were incubated with Avidin/Biotin Complex for 30 minutes. Complex was made by mixing 2 drops of solution A with 5mls TBST mixed followed by 2 drops of solution B, from the Standard Vectastain ABC kit. Sections were washed twice in TBST for 5 minutes and stained with DAB chromagen following manufacturer's instructions. Finally, sections were washed with water for 5 minutes and counterstained with Harris Heamatoxyline for 15 second and then washed in running water for 2 minutes and then dehydrated through an ascending series of alcohol to xylene. The sections were mounted using DPX mounting solution and were allowed to air dry before covering with coverlids. Pictures of sections were taken using an Olympus BX 41 microscope, DP 25 camera and Axiovision software. The extent and intensity of staining in synovial lining cells, sub-lining layer and vascular endothelial cells were graded on a scale of 0-4+ (0= no cells, 1= < 25%, 2= 25-50%, 3= 50-75%, 4= 75%) by two observers blind to the nature of the samples, on 2 separate occasions.

Table 2.2 Antibodies for immuno-histo chemistry staining show antibody target of SPHK1/2 and SIPRs and their working concentration for each antibody with appropriate isotype controls and secondary antibodies. Dilutions were not mentioned as stock concentration of antibodies can vary.

Primary Antibodies	Working Concentration	Supplier	Isotype Control	Secondary antibodies
Rabbit Polyclonal human SPHK1	2.5µg/ml	Abcam plc	Rabbit IgG	Biotinylated antiRabbit (1:200)
Goat Polyclonal human SPHK2	2.5µg/ml	Santa Cruz	Goat IgG	Biotinylated antiGoat (1:200)
Rabbit Polyclonal human SIP₁	2.5µg/ml	Abcam plc	Rabbit IgG	Biotinylated antiRabbit (1:200)
Rabbit Polyclonal human SIP₂	2.5µg/ml	Abcam plc	Rabbit IgG	Biotinylated antiRabbit (1:200)
Rabbit Polyclonal human SIP₃	1.8µg/ml	Abcam plc	Rabbit IgG	Biotinylated antiRabbit (1:200)
Rabbit Polyclonal human SIP₅	2.5µg/ml	Abcam plc	Rabbit IgG	Biotinylated antiRabbit (1:200)

2.3.4 Gene Expression in Purified Cells

2.3.4.1 RNA Extraction and Reverse Transcription

The third aliquot from freshly isolated CD15⁺ neutrophils, CD14⁺ monocytes and both CD4⁺ and CD8⁺ T lymphocytes were centrifuged at 1500 RPM for 5 minutes and then lysed for total RNA extraction using the 350µl buffer RLT (Qiagen RNeasy mini kit, Qiagen, Germany) according to the manufacturer's instructions. The quality of the RNA was determined spectrophotometrically at 260 nm. RNA samples were stored at -80°C until further analysis. The total RNA was reverse transcribed into cDNA according to the manufacturer's instructions using the High Capacity cDNA reverse transcription kit obtained from Applied Biosystems, UK.

2.3.4.2 Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

Quantitative RT-PCR was performed on triplicate samples using the ABI 7900HT Fast Real Time PCR System (Applied Biosystems, UK) with human SPHK1/2, S1PR₁₋₅ primers using the SYBR green method. Primers were all designed using Primer Express software (Applied Biosystems). The PCR reaction was carried out according to the manufacturer's protocol (Applied Biosystems, UK). Reaction mixtures of 20µl contained 10µl SYBR green master mix with the ROX dye as the passive reference, 5mM MgCl₂, 200µM dATP, dCTP, dGTP, 400µM dUTP, 1.25 U AmpliTaq Gold DNA polymerase, 0.5U AmpErase uracil N-glycosylase (UNG), 300nM forward and reverse primers (each 3.6µl), distilled water (0.8µl) and 2µl cDNA. All reagents were obtained from Applied Biosystems, UK.

To compensate for variation in amount of RNA taken, expression of target genes was normalized to an endogenous control (β-actin). For this analysis the comparative C_t method (ΔC_t) was used (564). The ΔC_t values were generated via the subtraction of C_t value of target gene from C_t value of β-actin control. Next, ΔΔC_t values were obtained by calibrating the ΔC_t of target gene against mean ΔC_t of calibrator (mean ΔC_t healthy control).

$$\Delta\Delta C_t = (C_{t \text{ target}} - C_{t \text{ reference}}) \text{ Calibrator} - (C_{t \text{ target}} - C_{t \text{ reference}}) \text{ Sample}$$

Relative quantitative values were calculated as 2^{-ΔΔC_t} and results are expressed as fold change over healthy control.

2.4 MicroRNA study

For the miRNA study, 35-40ml of PB from RA patients (n=24) and from age and sex matched healthy controls (n=22) were used for in vitro experiments. In addition, SF samples were collected (n=11). The characteristic clinical and laboratory information of RA patients are presented in detail within Chapter III.

2.4.1 Purification of Human CD14⁺ Monocytes from PB and SF

Blood samples were placed into sterile 50ml centrifuge tubes with an equal volume of wash medium (RPMI 1640 medium [Gibco, Invitrogen, Carlsbad, CA, USA] containing 100U/mL penicillin and 100µg/ml streptomycin). Diluted blood was slowly layered on top of 3ml Histo-Hypaque gradient-1107 in a 15ml centrifuge tubes. Samples were spun at 2500 RPM for 25 minutes at room temperature, which separates the blood samples into three layers. The very bottom layer is red blood cells then Histo-Hypaque, followed by a white ring layer, which contains PBMCs. PBMCs layers was carefully taken up and transferred to new tubes and mixed with wash media and spun at 1500 RPM for 5 minutes. The supernatant was carefully taken off, and the cell pellet was resuspended in the appropriate amount of wash media and numbers of viable cell were counted by using haemocytometer and trypan blue staining.

SF samples were centrifuged at 3000 RPM for 10-15 minutes at room temperature. The SF supernatant was carefully taken off using a Pasteur pipette, and the cell pellets resuspended in wash medium. Cell suspension was pipetted up and down several times and passed through Nitex mesh (Cadisch and Sons, London, UK). Next, cells suspension was carefully layered on top of 3ml Histo-Hypaque gradient-1107 and centrifuged 2500 RPM for 20 minutes at room temperature. Cell pellets were washed by addition of 10 ml wash media and the numbers of viable cells were counted using a haemocytometer and trypan blue staining.

Purification of CD14⁺ monocytes was carried out from isolated PBMCs and SF pellets using the CD14 micro beads and Auto-MACS separator according to manufacturer's protocol (Miltenyi Biotec) as we mentioned before. The positive selection of purified CD14⁺ cells was run using the "possel" program in order to

increase the cell purity, which ranged between 95-98% (Figure 3.1). Number of viable CD14⁺ cells was determined via a haemocytometer with trypan blue staining. Cells were cultured at 37°C in 5% CO₂ controlled environment and treated as described below.

2.4.2 Cells Culture and Transfection

Enriched PB CD14⁺ monocytes were seeded in 24-well plates at a concentration of 0.35×10^6 cells per 600µL of complete RPMI 1640 medium (RPMI 1640 medium [Gibco, Invitrogen, Carlsbad, CA, USA] containing 10% FCS, 100U/mL penicillin and 100µg/ml streptomycin L-Glutamine (2mM). PB CD14⁺ monocytes were either transfected with hsa-miR-155 mimic, or mimic negative control/miRNA mimic transfection control labelled with Dy547, at 20nM concentration using the N-TER nanoparticle siRNA transfection protocol or were left un-transfected, as an additional control. It is important to mention that preliminary studies in our lab optimized the transfection efficiency and concluded that the 20nM was the optimal transfection concentration based on unpublished QPCR data (Kurowska-Stolarska *et al.*). After transfection, cells were incubated at 37°C in 5% CO₂ controlled environment for 24 h. 24 h later some wells were stimulated with different doses (2, 10 or 100ng/ml) of LPS. Cells were cultured for 16-18 h further. After that culture supernatants were transferred into 1.5 Eppendorf tubes and centrifuged at 1500 RPM for 5 minutes at 4°C. The culture supernatants were collected for cytokine and chemokine analysis (see below) and stored at -20°C, while the cells were lysed in 700µl of Qiazol for RNA extraction and gene expression analysis (see below) and store at -20°C.

Efficiency of CD14⁺ monocytes transfection with miRNA mimic was monitored with miR-155 mimics and control mimic labelled with Dy547 using FACS analysis. Any experiments where the transfection efficiency was below 60% were excluded from further analysis. In addition, in those experiments with transfection efficiency $\geq 60\%$ TNF- α production from cultured CD14⁺ monocytes transfected with miR-155 mimics was measured using the TNF- α ELISA to serve as an additional control for the efficiency of CD14⁺ monocytes transfection. Furthermore, analysis was carried out to examine whether transfection influenced cells viability, therefore, cell viability was assessed before and after transfection by trypan blue staining.

2.4.3 Animal Experiment

2.4.3.1 Mice

Male miRNA-155 deficient (miR155^{-/-}) mice on a C57BL/6 (B6) background were purchased from Jackson Laboratories. Wild Type (WT) control littermates were produced by back crossing miR-155^{-/-} mice with B6 mice. Experiments were carried out according to the guidelines of the UK Home Office.

2.4.3.2 Optimization of Bone Marrow Monocytes Isolation

2.4.3.2.1 CD11b⁺ Cells Isolation and Culture

Bone marrow cells were isolated from the femurs and tibia of mice (WT and miR155^{-/-} mice). In brief, bones were cleaned of muscle, then cut through the top and the bottom of each bone, and bone marrow was flushed out with sterile PBS into sterile petri dish using a 0.3mm needle and 2.5ml syringe. Bone marrow suspension was pipetted up and down several times and passed through Nitex mesh (Cadisch and Sons, London, UK) and spun down at 1500 RPM for 10 minutes. The supernatant discarded completely while the cell pellet was resuspended in complete media after incubation on ice with ammonium chloride solution (0.8% NH₄Cl/0.1mM EDTA; Stem Cell Technologies, Grenoble, France) for 10 minutes to lyse red blood cells (RBC). Cells were washed twice with MACS buffer and number of viable cells was assessed using a haemocytometer and trypan blue staining.

Cells were then incubated at 4°C with anti-CD16/CD32 (Fc Block) to reduce non-specific binding by Fc receptors, for 10 minutes. Bone marrow cells from both WT and miR155^{-/-} mice were incubated with 80µl of MACS buffer and 20µl of mouse CD11b⁺ micro beads (Miltenyi Biotec) per 1x10⁷ cells for 15 minutes at 4°C. After the incubation, cells were washed, centrifuged and purified by positive selection on an Auto-MACS separator using the “possel” programme. The positive selection process was then repeated to increase cell purity. Purity of cells was greater than 97% as assessed by FACS analysis staining with anti mouse CD11b antibody.

Cells were cultured in 12–well plates (2×10^6 cells in 600 μ l complete media) at 37°C in 5% CO₂ controlled environment. After 24 h some wells were stimulated with 100ng/ml LPS for a further 24 h and other wells stimulated with media (control group). Similar to human experiments, culture solutions were transferred into 1.5ml Eppendorf tubes and spun down 1500 RPM for 5 minutes at 4°C. Culture supernatants were collected (for cytokine and chemokine analysis) and stored at -20°C, while cells were lysed in 700 μ l of Qiazol (for RNA extraction and gene expression analysis) and stored at -20°C.

The enriched CD11b⁺ cells were stained with anti mouse CD11b, Ly6C, Ly6G and CD115 antibodies and analysis was performed by FACS techniques. Thus, CD11b⁺, proved not to be a good marker for bone marrow monocytes as CD11b⁺ positive fractions were contaminated with neutrophils. Therefore, bone marrow monocyte was isolated based on their expression of CD115, another bone marrow monocyte marker.

2.4.3.2.2 CD115⁺ Cells Isolation and Culture

To obtain bone marrow monocyte with appropriate purity, bone marrow cells suspensions were prepared as described above and cells were sorted based on their expression of CD115 using mouse CD115⁺ micro beads Kit (Miltenyi Biotech) and Auto-MACS separator. Likewise, CD115 was not a good marker to sort the bone marrow monocyte population. At this stage I decided to sort the bone marrow enriched CD115⁺ cells using FACS Aria sorter. Although, the purity was acceptable but the number of cells was very low. I therefore decided to sort fresh whole bone marrow on the basis of CD11b, Ly6C, Ly6G and CD115 expression using the FACS Aria sorter only.

2.4.3.2.3 Sorting Whole Bone Marrow using the FACS Aria sorter

Bone marrow cells suspensions were prepared as described above. FACS Aria-based sorting and analysis was used to isolate bone marrow monocytes and verify their quality and the quantity. The cell yield from whole bone marrow isolations reached $40 \pm 4 \times 10^6$ cells/donor mouse (n=4) each experiment. To reduce non-specific binding by Fc receptors bone marrow cell suspensions were incubated with anti-CD16/CD32

at 4°C for 10 minutes. After that, cells were stained with cell lineage specific antibodies; CD11b, Ly6C, Ly6G and CD115 (Table 2.3) and incubated for 15-20 minutes at 4°C. All experiments were controlled with appropriate isotype antibodies and unstained cells.

Firstly, dead cells were excluded from analysis by adding 10µl 7-aminoactinomycin D (7-AAD; BD Biosciences) to each sample before acquisition and cells were then sorted on the basis of CD11b, Ly6C, Ly6G and CD115 expression by live-gated cells under sterile conditions. In general, lymphocytes and granulocytes were identified based on their forward-/side-scatter properties and subsequently analysed for lineage specific markers. CD11b is a marker of myeloid population on mouse including monocytes/macrophage and granulocyte. Lymphocytes were negative for the granulocyte markers CD11b and Ly-6G. Ly-6G is lymphocytes antigen 6 complex loci G (L6G) expressed by the myeloid lineage during their bone marrow development; monocytes only express L6G transiently during their development, while granulocytes expressed this marker in bone marrow and periphery. Post sorting purity of bone marrow monocytes (CD11b, Ly6C and CD115 and a lack of Ly6G) was performed in all experiments and all data generated were analysed using FlowJo software (Tree Star Inc, OR, USA).

In some experiments, total RNA samples were extracted directly after sorting from bone marrow monocytes (2 mice/group in a total of 4 experiments) and samples sorted at -20°C until further analysis. In other experiments, freshly purified cells were seeded in 12-wells plates in complete media; and after 24 h some wells were stimulated with LPS (100ng/ml) or left un-stimulated and cultured for further 24 h (4 mice/group in a total of 2 expts). The analysis was performed on culture supernatant and total RNA samples harvested post-culture.

Table 2.3 Monoclonal primary antibodies were used for sorting bone marrow monocytes using FACS Aria sorter.

Antibody	Isotype	Source/ Cat No.
FITC anti-mouse CD11b Antibody	Rat IgG2b	Biolegend/101206
PE/Cy7 anti-mouse Ly-6C Antibody	Rat IgG2c	Biolegend/128017
APC anti-mouse CD115 (CSF-1R) Antibody	Rat IgG2a	Biolegend/135509
PE anti-mouse Ly-6G Antibody	Rat IgG2a	Biolegend/127607

2.4.3.3 Generation of Bone Marrow Macrophages

To generate macrophages from bone marrow precursors of WT and miR-155^{-/-} mice (n= 4/groups), total bone marrow cells were plated (2×10^6 cells/ml) into Petri dishes (Sterilin, UK) in total volume of 5ml complete medium supplemented with 20ng/ml mouse M-CSF and cultured at 37°C in 5% CO₂ for 6 days. On day 3 of culture, non-adherent cells were removed and replaced with fresh media supplemented with 20ng/ml mouse M-CSF. On day 6 of culture, media and non-adherent cells were removed by washing with RPMI 1640 and the adherent cells were collected with cell scrapers (Costar). After counting, cells were seeded into 6 well plates (1.5×10^6 /ml) in complete media supplemented with 0.5ng/ml mouse M-CSF. 24 h thereafter, (100ng/ml) LPS was added to some wells for a further 24 h. Culture supernatants were collected and total RNA samples were harvested using 700µl of Qiazol and samples sorted at -20°C until further analysis.

2.4.4 Cytokine and Chemokine Analysis

2.4.4.1 ELISA Assay

Enzyme Linked Immunosorbent assay (ELISA) was used to measure production of mouse and human TNF- α concentration in culture supernatants according to manufacturer's protocol. Capture antibody diluted in PBS buffer was coated onto Immunol microtiter (Thermo Labsystems) plate and incubated overnight at 4°C. Plates were then washed with 0.05% Tween/PBS before being blocked with blocking buffer (0.5% bovine serum albumin; BSA in PBS), to block non-specific binding, and incubated for 1 h at room temperature. Following washing, 100µl samples and standards were added in triplicate to the plate and incubated for 2 h at room temperature. Standard was made using recombinant mouse or human cytokine dissolved in complete media at a maximum concentration of 2000pg/ml and serially diluted 1:2 including two wells containing only medium (blank) to obtain an eight point standard curve. Plates were washed again; detection antibody was added and incubated for a further 1 h at room temperature. Plates were then washed and streptavidin-HRP was added to each well and plates were incubated at room temperature for 30 minutes. The plates were washed and substrate solution (TMB) (Biosource) was added for 30 minutes. 100µl stop solution (Biosource) was used to stop the reaction. Plates were read at 450nm in a microplate reader (Dynex

Technology). A list of human and mouse capture and detection antibodies are presented in Table 2.4.

Table 2.4 Cytokine analysis by ELISA and concentration antibodies and appropriate secondary and detection antibodies in assay.

Cytokine	Supplier	Capture antibodies	Detection antibodies	Stre. HRPABs
Hu. TNF-α	Invitrogen	Anti human TNF- α 0.250mg/0.125ml	Anti human TNF- α Biotin 0.250mg/0.125ml	0.25ml
Mu. TNFα	eBiosciene	Anti mouse TNF- α 1/250 for 1 plate	Biotin-conjugated Anti human TNF- α (polyclonal)	1/250 for 1 plate

2.4.4.2 Luminex Assay

The culture supernatants of human CD14⁺ monocytes were examined for a set of cytokines and chemokines using the Multiplex Kit panel I and II (Millipore UK) on a Bio-Plex system (Bio-Rad). The predesigned plates were used to assess the effect of overexpression regulation of miR-155 on the secretion of inflammatory cytokines and chemokines from culture of PB CD14⁺ monocytes of healthy controls (n=15) and RA patients (n=16). All samples were tested in triplicate. The predesigned plates measured TNF- α , IL-1 β , IL-10, CCL2/MCP-1, CCL3/MIP-1 α , CCL4/MIP-1 β , CCL5/RANTES, CCL7/MCP-3, CCL8/MCP-2, CCL13/MCP4, CCL17/TARC, CCL19/MIP-3 β , CCL20/MIP-3- α , CCL21/6CKINE, CCL22/MDC, CXCL1/GRO, CXCL5/ENA-78, CXCL7/NAP-2, CXCL8/IL-8, CXCL9/MIG, CXCL10/IP-10, CXCL11/I-TAC, CXCL12/SDF-1, CX₃CL1/Fractalkine, lymphotactin and VEGFA.

In brief, 96-well filter plates were pre-wet with assay buffer and shaken on a plate shaker for 10 minutes at room temperature. Next, assay buffer was gently removed by vacuum. Samples and standards were added into appropriate wells and beads with immobilized antibodies were added to all wells and incubated overnight at 4°C on a plate shaker. Plates were then washed twice with wash buffer after removing fluid with vacuum. Detection antibody was added to each wells and incubated on a plate shaker for 1 h at room temperature; streptavidin-HRP was added for a further 30 minutes and agitated at room temperature. Plates were washed twice and fluid removed with vacuum. Following washing, 150 μ l of fluid sheath was added to all wells and plates were run on Luminex 100TM. Data were analysed using the Bio-Plex software.

2.4.5 Gene Expression Analysis

2.4.5.1 RNA Extraction from Cell

Total RNA was extracted from PB monocytes after transfection with miR-155 mimic/negative mimic controls using miRNeasy mini Kit (Qiagen) following manufacturer's instructions. In addition, RNA was extracted from PB or SF monocytes of RA patients and PB monocytes of healthy controls to evaluate the absolute copy number of miR-155. In brief, cells were lysed and disrupted with 700µl Qiazol and gently agitated and stored at -80°C until required for RNA extraction. Frozen samples were placed on the bench top at room temperature for 5 minutes until samples completely thawed. After that 140µl chloroform was added to tubes and shaken vigorously then centrifuged for 15 minutes at 1200 rpm at 4°C. This separated the samples into three different layers. The bottom layer (red) corresponded to the organic phase then a white interphase and the upper (colourless) aqueous phase containing RNA. One and half volumes of 100% ethanol was added to upper aqueous phase after transferring to new collection tube and mixed thoroughly before applying to the RNeasy spin column. The columns were centrifuged for 15 seconds at 8000g and the flow through discarded. Wash buffer, RWT, (350µl) was then applied to the column, centrifuged (15 seconds, 8000g) and the flow through discarded followed by 15 minutes incubation with DNase at room temperature. The columns were washed with 350µl of wash buffer RWT, centrifuged and the flow through was discarded. Buffer RPE (500µl) was applied to the column, which was then centrifuged at 8,000g. A second volume of RPE was added, to ensure all contaminants were removed, and the column was centrifuged at 8000g for 2 minutes. RNA was eluted in RNase-free water (30-50µl) and centrifuged at 8000g for 1 minute.

The quantity and quality of RNA was determined using a spectrophotometer. The absorbance of the RNA is measured at 260 and proteins at 280nm. The ratio of these readings give an indication of the quality of the RNA, a ratio of 1.8 to 2.1 indicates that the RNA is good quality and has limited contamination with protein. Also we checked the RNA integrity number (RIN) and I included only samples with RIN above 7 for further analysis.

2.4.5.2 cDNA Synthesis

cDNA was transcribed from RNA using miScript Reverse Transcription Kit (Qiagen) according to manufacturer's instruction. For each sample, 60ng template RNA was placed in an RNase free tube containing reverse-transcription master mix, required for first-strand cDNA synthesis, then mixed and placed on ice. Briefly, RT master mix was made up to contain 4µl miScript RT buffer 5X, 1µl miScript Reverse Transcriptase Mix and made up to a final volume of 20µl with RNAase-free water. Samples were incubated at 37°C for 60 minutes, followed by a 5 minutes incubation at 95°C. Samples were either stored on ice for immediate analysis by qPCR or stored at -20°C.

2.4.5.3 Generating Standards for qPCR

Standard Real time-PCR allows relative differences in gene expression between samples to be evaluated but does not allow for absolute quantification. To assess the absolute levels of miR-155 transcript in RA samples (PB/SF) and healthy controls we generated standard curves for miR-155 and U1.

To prepare the template cDNA: 5µl of 20µM miR-155 mimic (10pmoles) was added to 10µl of RNase free water and denatured for a 5 minutes at 95°C (using standard PCR machine) and then quickly cooled down in ice for 5 minutes. After that a mixture of 4µl miScript RT buffer 5X and 1µl miScript Reverse Transcriptase Mix was added and mixed with the denatured miR-155 mimic and made up to a final volume of 20µl. The mix was incubated at 37°C for 60 minutes, followed by a 5 minute incubation at 95°C. Calculation of copy number of miR-155 was done as described below. The house-keeping standard gene probe, RNU1A was kindly provided by Dr. Derek Gilchrist.

2.4.5.4 Calculating Transcript Copy Numbers

Transcript copy numbers within each standard was calculated using a series of formulae and facts as follows:

5µl of 20µM mimic = 0.1nmoles

Then, to convert moles into copy number, the number of molecules per mole is 6.023×10^{23} copies (Avogadro's constant)

$$\begin{aligned}
 \text{No. of Copies DNA} &= \text{Avogadro's Constant} \times \text{moles} \\
 &= 6 \times 10^{23} \times 1 \times 10^{-10} \\
 &= 6 \times 10^{13}
 \end{aligned}$$

To obtain a standard curve, purified PCR products were diluted in serial 10-fold dilution in nuclease-free water to generate a range of standards from 1×10^3 to 1×10^9 . Each of these standards was used as a template cDNA in each QPCR assay.

2.4.5.5 SYBR Green Quantitative Polymerase Chain Reaction

The qPCR protocol assessing target transcript expression was based on a SYBR Green method. Master mix and miScript primer assay (MS00003605) (all from Qiagen) were used according to manufacturer's protocol. The expression of RNU1A (MS00013986) was used as endogenous control. Briefly, cDNA samples were prepared as previously described and assessed in triplicate in 20 μ l final reaction volumes in a 96 well plate format using the ABI 7900HT Quantitative Real Time PCR System (Applied Biosystems, UK). Reaction mixtures of 20 μ l contained 10 μ l SYBR green master mix with 2 μ l of each Universal primers and Assay primers, 5 μ l distilled water and 1 μ l cDNA or template standard cDNA. In all assays, the samples and standards were tested in triplicate. To compensate for the amount of RNA in reaction mix, normalisation of the target genes with an endogenous control (RNU1A) was performed.

2.4.5.6 Normalising QPCR Data

To permit effective comparison of miR-155 gene levels between samples, the amount of RNU1A was used as a comparator housekeeping gene. In general, the amount of gene transcripts can vary between samples due to a variety of factors including differential rates of RNA degradation, varying efficiency of the RNA extraction procedure and the efficiency of the reverse transcription reaction. Therefore, to control for the amount of miR-155 transcripts, it was normalized to RNU1A. To calculate the absolute transcript levels for miR-155 we used the formula below:

$$\text{Copy number of gene of interest} / \text{copy number of RNU1A gene} \times 10^6$$

This gives the copy number of the gene of interest per 1,000,000 copies of RNU1A

2.4.5.7 TaqMan Low Density Arrays

TaqMan Low-Density Arrays (TLDA) is 384-well microfluidic plate that allows multiplex qPCR. Specific primers for chemokines and chemokine receptors expressed in murine bone marrow monocytes/macrophages or human PB CD14⁺ monocytes and 18S as an internal control were used. Total RNA was extracted from WT and miR-155^{-/-} bone marrow monocytes either directly after sorting or after 24 h culture and LPS stimulation using miRNeasy mini kit (Qiagen) as described before. Similarly, total RNA was extracted from PB CD14⁺ monocytes of healthy controls and RA after transfection with miR-155 mimics. In both case, a total of 550ng RNA was reverse-transcribed with High Capacity RNA-to cDNA Kit (Applied Biosystems) following manufacturer's guidelines. An RT master mix was made up per reaction to contain 10µl RT buffer, 1µl 20X Enzyme Mix and 550ng template RNA the reaction made up to a final volume of 20µl with RNAase-free water. Samples were mixed and run on a PCR machine: 37°C for 60 minutes; 95°C for 5 minutes and finally hold at 4°C. The cDNAs were made up to a final volume of 100µl with RNAase- free water. The cDNA was then loaded onto TLDA plates after 1:1 dilution with TaqMan PCR Master Mix No AmpErase (ABI Ltd), and was run on 7900HT TaqMan reader using SDS software. ΔC_t values were generated using 18S as an endogenous control, and $\Delta\Delta C_t$ values were obtained by normalizing the mean ΔC_t with the untreated control. Results are expressed as fold change over control.

2.5 Statistical analysis

The data were analysed by Graph Pad Prism version 5.0 software and all values were presented as mean \pm standard error mean (SEM). Statistics used in this thesis were Mann-Whitney *U* test, student's t-test and a Krusal Wallis test with Dunn's multiple comparison tests. A P value less than or equal to 0.05 has been used as a cut-off value to assign the statistical significance.

CHAPTER III
THE ROLE OF MIR-155 IN CHEMOKINE AND
CHEMOKINE RECEPTOR EXPRESSION

3.1 Introduction and Aim

Rheumatoid arthritis (RA) is a chronic autoimmune disease that is characterized by synovial tissue inflammation eventually leading to joint destruction. Monocytes/macrophages are believed to be major effector cells in RA synovitis, operating primarily by producing cytokines *e.g.* TNF- α , IL-6, IL-1 β , prostaglandins, *e.g.* PGE2 and variety of matrix metalloproteinases (170). The recruitment of effector cells, including monocytes to the joint space is an important step in RA progression and is mediated by chemokines and their receptors. Chemokines provide an evolutionarily conserved molecular mechanism that allows cells to move from one tissue compartment to another. They are implicated in RA pathogenesis as proinflammatory mediators that regulate leukocyte recruitment and retention within the synovial and articular space. Monocytes/macrophages in RA constitutively express many chemokines, while SFs produce only lower level of chemokines in the normal state. Chemokines are abundantly expressed in synovial tissue, SF and PB of RA patients (237). A number of chemokines, including CCL2/MCP-1, CCL3/MIP-1 α , CCL4/MIP-1 β , and CCL5/RANTES, are implicated in RA pathogenesis and are expressed in SF (254-256). Furthermore, a number of specific chemokine receptors including CCR1, CCR2, CCR3, and CCR5 are highly expressed in RA PB and SF monocytes (287). Moreover, expression of CXCR4 is much higher in RA synovium than in OA (565). Chemokines and their receptors are important for each stage of migration; some play a role in monocytes recruitment from circulation, and other may be involved in monocytes retention in the joints (566). Understanding the regulation of monocytes/macrophage accumulation in RA synovium is important and will provide insight into the inflammatory nature of rheumatoid synovitis.

MiRNAs are a recently discovered class of post-transcriptional regulators (293, 294). They have been shown to be involved in the regulation of immune responses and the development of autoimmunity. Of particular interest are emerging studies showing the contribution of miRNA network to monocyte migration and the chemokine/chemokine receptor system. For instance, induction of miR-124a in RA synovial fibroblasts significantly suppresses CCL2/MCP-1, which is important for monocyte migration and retention in joint space (403).

In a recent study, we have performed an array analysis of the expressed miRNAs in RA SF CD14⁺ cells (367). One of the miRNAs are shown to be highly expressed in SF CD14⁺ cells was miR-155. Our interest was particularly taken by the role of miR-155 in this regard. To characterize the mechanism by which miR-155 might contribute to the pathogenesis of RA, we demonstrated that miR-155 is upregulated in synovial membrane and SF macrophages from patients with RA (367). Further we overexpressed miR-155 in PB CD14⁺ monocytes and found that this led to an increase in the production of pro-inflammatory cytokines such as TNF- α (367). This observation suggested that miR-155 might be involved in regulating the biology of these cells. Until now, little has been known of the role of miR-155 in regulating chemokine production, chemokine receptor expression in monocytes and their consequent migration. In the current study, I tested the hypothesis that miR-155 may regulate monocyte migration in RA patients through modulating the expression of the chemokine and chemokine receptor system.

We have chosen monocytes to characterize the mechanism by which miR-155 might contribute to pathogenesis of RA. Monocytes/macrophages are central effectors of synovitis and considered as key producers of proinflammatory cytokines and chemokines. Further, monocyte/macrophages expressed higher levels of miR-155 than lymphocytes in blood from patients with RA (400). The clinical significance of these cells is that they positively correlate with clinical symptoms and the degree of joint damage (212). They also represent a sensitive biomarker to change after effective treatment; reduced sub-lining CD68 expression in synovial biopsies correlates with efficacy of anti-rheumatic treatment independently of the therapeutic strategy (567). Further, monocytes/macrophages are a major source of cytokines in RA synovium such as TNF- α and IL-6 and targeting these cytokines has yielded a marked clinical impact on RA treatment.

To understand the role of miR-155 in monocyte migration and retention in the joint space, I overexpressed miR-155 in PB CD14⁺ monocytes of healthy controls and RA patients and examined the expression of chemokines and chemokine receptors at mRNA levels by TLDA as well as the production of chemokines in culture supernatants by multiplex assay.

3.2 Results

3.2.1 Patient Characteristics

Demographic data as evaluated at the time of PB collection from RA patients from the routine outpatient Rheumatology Clinic, Glasgow Royal Infirmary (Glasgow, U.K.) are listed in Table 3.1. Available data included age, sex, disease duration, tender joint (TJ) and swollen joint (SJ) counts as well as laboratory parameter such as ESR, CRP, RA factor and ACPA positivity. Disease activity of RA patients recruited to this project was calculated using the Disease Activity Score (DAS28). DAS28 is combined index that assess the number of swollen and tender joints and measure the ESR or CRP and provide a number between 0 and 10, indicating the disease activity and represented as thresholds for high and low disease activity. All the patients recruited to this project were RF positive.

Table 3.1 Demographic, clinical and laboratory information of rheumatoid arthritis patients.

Subject	Age	Sex	DD	ACPA	ESR	CRP	DAS28	Treatment
RA1	59	F	16	+	22	9	3.5	HCQ+MTX+SSZ
RA2	61	M	8		75	46	3.85	MTX
RA3	62	F	13	+	60	11	5.16	LEM
RA4	73	F	33		12	0.5	2.89	MTX+ Adal
RA5	63	M	27		16	8.6	4.38	MTX+ Inflix
RA6	67	M	27		7	3.7	2.2	HCQ+MTX+SSZ
RA7	70	F	15	+	40	12	4.6	MTX+ Etan
RA8	62	F	22	+	26	7	3.8	SSZ
RA9	53	M	10		38	3.3	4.7	SSZ
RA10	67	M	4		14	3	3.9	MTX+ Gold
RA11	59	M	17	+	19	5	3.9	MTX
RA12	67	F	41	+	31	5.2	4.36	LFM+HCQ
RA13	55	F	8	+	10	2.4	2.95	SSZ
RA14	75	F	9		20	5.1	2.4	MTX+ SSZ
RA15	58	M	5	+	41	27	5.3	HCQ+MTX+SSZ
RA16	71	F	10	+	31	1.2	3.36	HCQ+MTX+SSZ
RA17	70	F	13	+	34	10	5.2	MTX+ Adal
RA18	62	F	22	+	10	5	2.5	MTX
RA19	58	M	20	+	60	22	5.8	MTX+ SSZ+ Adal
RA20	36	F	4	+	30	6.6	4.6	MTX+ SSZ
RA21	63	F	30	+	60	11	4.8	SSZ
RA22	74	F	37	+	40	13	4.95	SSZ+HCQ
RA23	60	F	15	+	12	1.6	3.2	MTX+ SSZ+ Inflix
RA24	76	F	21	+	15	5.6	4.4	MTX+ SSZ

DD; disease duration, ACPA; Anti-citrullinated protein antibody, ESR; erythrocytes sedimentation rate and the normal ESR is <20mm/h for female and <10 mm/h for male CRP; C-reactive protein and the normal CRP is < 4.9mg/l, ESR, SSZ; Sulfasalazine, HCQ; Hydroxychloroquine, MTX; Methotrexate, LEM; Leflunomide, Adal; Adalimumab, Inflix; Infliximab, Rifux; Rifuxumab, Etan; Etanercept

3.2.2 Purity of Sorted CD14⁺ PB and SF Monocytes

PB and SF monocytes comprised a homogeneous population that uniformly expressed CD14 –and this was achieved by using the gating strategy against isotype control. Isolation of CD14⁺ monocytes from PB of healthy controls (n=22) or PB and SF of RA patients (n= 24 and 11 respectively), was conducted using CD14⁺ micro beads and purified by positive selection on an Auto-MACS separator using the “possel” programme as described in Materials and Methods. To confirm that sorted PB CD14⁺ monocytes from healthy controls or RA patients were monocytes, cell purity was checked using FACS to identify CD14⁺ positive cells and the number of cells in each quadrant (%) was determined. In an average of all experiments from both healthy controls and RA PB, the purity of CD14⁺ monocyte populations was 95% and 93% as shown in Table 3.2. The dot plot presented in Figure 3.1A, B and C shows a representative plot of positive CD14⁺ monocytes from PB of healthy controls and RA as well as SF, respectively. Figure 3.1D shows a representative histogram of cells that stained positive for CD14⁺ from healthy controls and RA PB as well as SF.

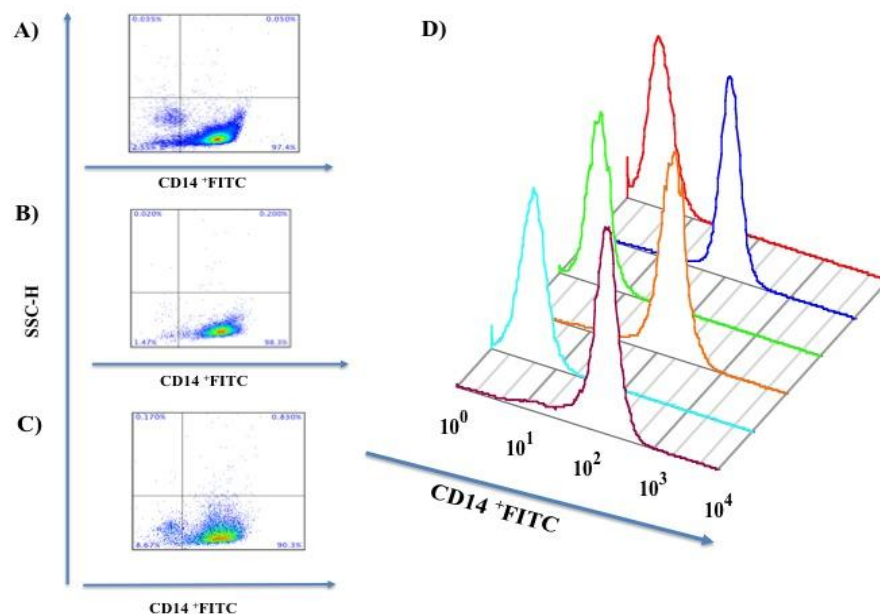


Figure 3.1 Percentages of CD14⁺ monocytes positivity was determined by flow cytometry analysis. Representative dot plots of enriched CD14⁺ monocyte population from PB of A) healthy controls, B) RA and C) SF of RA patients after auto-MACS sorting. With gating strategy the percentage of cells in each quadrant was determined to calculate the purity of the isolated cells. D) Representative histogram analysis of the number of cells that stained positive for CD14⁺ monocytes from healthy controls and RA PB and SF against isotype control (red, green and light blue lines are isotype control, while dark blue, orange and purple lines are HC, RA and SF respectively).

Table 3.2 Cell purity and transfection efficiency of PB CD14⁺ monocytes isolated from both healthy controls and RA patients. Cell purity was determined using flow cytometry analysis to identify CD14⁺ positive cells and presented as %. PB CD14⁺ cells were transfected with miR-155 mimic or control mimic and transfection efficiency of cells were monitored with control mimic labelled with fluorescence dye Dy547 using flow cytometry. Based on previous experiment in the lab we set threshold for transfection efficacy at 60%. While any experiment with transfection efficiency below 60% (Red marked) was withdrawn from further analysis. Data were presented as %. TE; transfection efficiency, HC; healthy control, RA; Rheumatoid arthritis

Healthy controls			Rheumatoid Arthritis		
Subject	CD14 ⁺ Purity	TE	Subject	CD14 ⁺ Purity	TE
HC1	97	86	RA 1	99	41
HC2	97	59	RA 2	94	76
HC3	95	50	RA 3	97	64
HC4	97	64	RA 4	93	69
HC5	97	76	RA 5	92	82
HC6	96	85	RA 6	90	65
HC7	96	43	RA 7	90	51
HC8	98	63	RA 8	92	40
HC9	98	71	RA 9	93	70
HC10	98	87	RA 10	94	86
HC11	97	37	RA 11	97	40
HC12	98	35	RA 12	97	60
HC13	98	85	RA 13	96	65
HC14	95	40	RA 14	97	64
HC15	90	78	RA 15	94	82
HC16	93	74	RA 16	90	37
HC17	93	40	RA 17	92	73
HC18	98	78	RA 18	98	25
HC19	93	80	RA 19	85	87
HC20	98	81	RA 20	90	88
HC21	92	85	RA 21	92	31
HC22	90	81	RA 22	94	48
			RA 23	92	82
			RA 24	90	64

3.2.3 MiR-155 is Upregulated in PB and SF Monocytes of RA Patients

3.2.3.1 MiR-155 Copy Number is Highly Expressed in PB and SF Monocytes of RA Patients

MiR-155 expression has been confirmed recently to be strongly elevated in RA synovial biopsies, and is upregulated in RA SF-derived CD14⁺ monocytes. However prior studies are potentially confounded by the use of relative expression calculations and as such may be subject to errors arising from uncontrolled variables, particularly affecting ‘control’ miR species against which comparison is drawn for relative expression. Absolute quantification is thus preferable to relative expression. I therefore calculated the absolute copy numbers of miR-155 transcripts in PB and SF CD14⁺ monocytes of RA patients and compared this with the copy number of healthy controls. I did this by generating standard curves for miR-155 and RNU1A; the latter was used as a housekeeping gene, and transcript levels were assessed by quantitative PCR using SYBR green. In all assays, samples/standard transcript copy numbers were calculated as described in Materials and Methods. The analysis was performed on total RNA samples harvested 48 h after culture of CD14⁺ PB and SF monocyte of RA patients (n= 24 and 11 respectively), while healthy control CD14⁺ monocyte PB were used for comparison (n=22). It is important to mention that I calculated the miR-155 copy numbers after 48 h culture and do not show the baseline expression - this was because I decided to evaluate absolute copy numbers after the original experimental design was set – I thus was required to work with the cell populations and a sample cohort by then already gathered. Reassuringly, and consistent with recent studies in which fold change was only examined, RA PB and SF monocytes/macrophages showed statistically significantly higher copy number of miR-155 than cells from controls. RA SF macrophages contained the highest copy number compared with healthy controls (P<0.0001) Figure 3.2A. Additionally, the mean miR-155 copy number was also significantly higher in samples from RA SF-derived CD14⁺ cells compared with RA PB CD14⁺ monocytes (P<0.009) (Figure 3.2A).

3.2.3.2 MiR-155 Copy Number after Challenge with Different Doses of LPS

MiR-155 has been previously shown to be upregulated by TLR ligands in PB CD14⁺ monocytes (367). I therefore analysed the copy number of miR-155 in both healthy controls and RA patients' PB CD14⁺ monocytes before and after treatment with different doses (2 and 10ng/ml) of LPS. Purified PB CD14⁺ monocytes were cultured on 24-well plates, after 24 h LPS was added to some wells for a further 16-18 h. Total RNA was extracted to examine the transcript levels of miR-155. Overall, copy numbers of miR-155 was significantly increased in PB CD14⁺ monocyte of healthy controls and RA patients after challenge with different doses of LPS Figure 3.2B compared with transcript levels obtained with no LPS treatment. In healthy controls CD14⁺ monocyte treated with LPS expressed significantly greater levels of miR-155 transcript than those observed in cells without stimulation particularly with 10ng/ml LPS. Importantly, miR-155 copy number in RA PB CD14⁺ monocytes treated with 2 and 10ng/ml were expressed almost two and four times higher than those observed in cells without LPS stimulation respectively. Compared to healthy controls, RA CD14⁺ monocytes treated with LPS or without LPS stimulation displayed a significantly higher copy number of miR-155. The highest difference was observed with LPS stimulation at a concentration of 10ng/ml.

3.2.3.3 Seeking Correlation of PB Monocyte miR-155 with a Variety of Clinical Indices and Lab Biomarkers

Next, PB monocyte miR-155 copy number was correlated against a variety of clinical indices and lab biomarkers using the RA patient's characteristic data (Table 3.1). It was noted that miR-155 copy number were positively and significantly correlated with DAS28 of RA patients according to the two-tailed Pearson Correlation Coefficient ($R=0.728$ and $p=0.0001$) (Figure 3.2C). Further analyses revealed that miR-155 copy number was positively and significantly correlated with TJ and SJ counts (Table 3.3). Because miR-155 is associated with TJ/SJ, a correlation of miR-155 levels with disease activity and lab biomarkers would be expected. Next, miR-155 copy number was plotted against lab biomarkers of inflammation namely CRP and ESR. A significant positive correlation was found between ESR and miR-155 copy number ($R=0.546$, $P=0.006$) whereas this analysis

did not show any significant correlation with CRP (Figure 2D). Finally, as expected, there was a positive and significant correlation between the CRP and ESR ($R=0.672$, $P=0.0001$) in RA patients' samples recruited to this project as shown on Table 3.3 and Figure 3.2E).

Further statistical analysis revealed that the copy number of miR-155 was statistically higher in patients with high ($n=4$) or moderate ($n=15$) disease activity (according to DAS28) ($p<0.007$ as determined by 1 way ANOVA test/Kruskal-Wallis test) than those patients with remission state ($n=5$) or healthy controls ($n=22$) Figure 3.2F. Patients with high disease activity demonstrated the highest copy number compared with healthy controls and RA patients with remission. Furthermore, miR-155 copy number was significantly elevated in patients with high disease activity compared with those with moderate disease activity ($p=0.015$) or remission state ($p=0.04$).

Next I analysed the expression of miR-155 copy number within ACPA expression categories. There was a significantly increased expression of miR-155 copy number in ACPA positive RA compared with ACPA negative RA ($p=0.033$) Figure 3.2G. So far, ACPA have been linked to RA and, indeed are highly specific to RA and have been identified in up to 90% of RA patients and they in turn correlate with disease severity. However, there was no correlation between the observed increase in the miR-155 copy number and the patients' age, disease duration or medication. In our clinical data of patients, all of the RA patients were being treated with cDMARD; 6 patients were also receiving a biological DMARD. Further analysis was conducted to examine whether drug therapy influenced miR-155 copy number. There was no significant difference in miR-155 copy number between patients receiving treatment with biological treatments such as Etanercept, Adalimumab or Infliximab plus cDMARD and those RA patients treated with cDMARD alone Figure 3.2H.

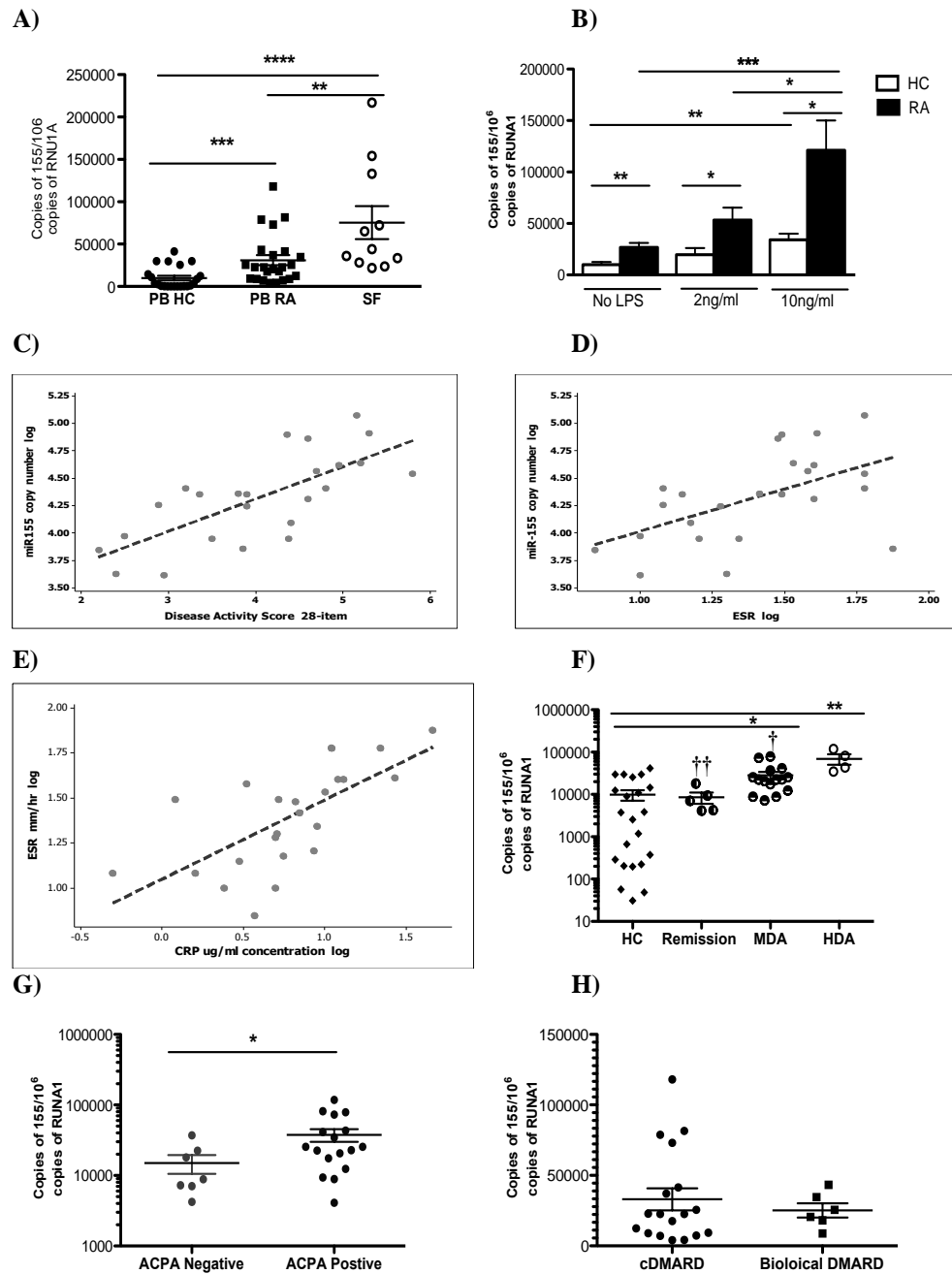


Figure 3.2 A) Absolute copy numbers of miR-155 transcripts in PB and SF CD14⁺ monocytes of RA patients (n= 24 and 11 respectively) and compared this with the copy number of healthy controls (n=22). Total RNA samples of CD14⁺ monocytes were converted to cDNA and the levels of miR-155 transcripts were assessed by quantitative PCR measuring absolute copy numbers using the SYBR green and preparing standard curves for miR-155 and RNU1A as described in Materials and Methods. The copy numbers of miR-155 is normalized to 1×10^6 copies of RNU1A as housekeeping gene. B) MiR-155 copy numbers in PB CD14⁺ monocytes of healthy controls and RA patient in absence (HC=22, RA=24) and presence of different doses of LPS (2ng/ml (HC=18, RA=22) and 10ng/ml (HC=9, RA=16)). The statistical difference from control was determined using Kruskal-Wallis test and Mann-Whitney U test between the groups. C) Correlation between miR-155 copy number and DAS28, each dot represents patient disease activity versus their miR-155 copy number. D) Correlation miR-155 copy number with ESR of RA patients. E) Correlation between the lab biomarkers of inflammation CRP and ESR of RA patients. F) Copy number of miR-155 against disease activity state based on DAS28 (Remission, Low, Moderate or high Disease activity) compared with healthy controls and difference were determined by 1 way ANOVA test/Kruskal-Wallis test. G) MiR-155 transcript levels versus clinical disease subsets (ACAP positive vs. negative) RA. H) MiR-155 copy number between the RA patients receiving treatment with the cDMARD or biological DMARD. The statistical difference was determined using Kruskal-Wallis test and Mann-Whitney U test between the groups, * = $p \geq 0.05$, ** = $p \geq 0.005$ and *** = $p \geq 0.0005$. While, (†) is marked statistically different between patients with high disease activity and those either with moderate disease activity or remission status.

Table 3.3 Correlation analysis of miR-155 copy number against a variety of clinical indices and lab biomarkers. Data were skewed by log or square-root transformation to restore normality. R-value on top with corresponding p-values on the lines below

	MiR155 copy no	Age	DD	ESR	CRP	ACPA	TJ	SJ	DAS28
Age	-0.239								
	0.260								
DD	-0.064	0.464							
	0.766	0.022							
ESR	0.546	-0.136	-0.108						
	0.006	0.526	0.615						
CRP	0.186	-0.169	-0.095	0.672					
	0.384	0.429	0.659	0.000					
TJ	0.631	-0.035	0.127	0.261	0.197	0.480			
	0.001	0.872	0.555	0.218	0.356	0.018			
SJ	0.503	-0.232	-0.301	0.565	0.472	0.067	0.529		
	0.012	0.276	0.152	0.004	0.020	0.756	0.008		
DAS28	0.728	-0.193	-0.055	0.759	0.600	0.367	0.781	0.803	
	0.000	0.366	0.797	0.000	0.002	0.078	0.000	0.000	

DD; disease duration, ESR; erythrocytes sedimentation rate, CRP; C-reactive protein, TJ; tender joint, SJ; swollen joint and DAS28; Disease Activity Score 28. The correlation of miR-155 copy number against a variety of clinical indices and lab biomarkers is kindly done by Dr. Charles McSharry.

3.2.4 Transfection Efficiency of CD14⁺ Monocytes

In order to evaluate the functional implications of the increased miR-155 expression described in the experiments above, I sought to optimize a transfection method that would deliver a mimic to a majority of cells without compromising viability. I monitored transfection efficiency with control mimic (*C.elegans* miR-67) labelled with fluorescence dye Dy547. There was no labelled miR-155 at the time of my experiments. This control miR has similar length and nucleotide composition (different sequence) to miR-155 mimic. Therefore, efficiency of transfection of human CD14⁺ monocytes with miR-155 mimic was monitored by miR-155 mimics and control mimic labelled with Dy547 by flow cytometry. Transfection efficiency of healthy controls' varied between 35- 87% and the mean was 67% (Table 3.2 and Figure 3.3A). For RA patients, transfection efficiency ranged between 25-88% with a mean 62% (Figure 3.3B). Based on previous experiments in the lab, we set the threshold for transfection efficiency at 60%, in both healthy controls (n=15) and RA (n=16) samples. Any cell culture with transfection efficiency below 60% (Red marked) was withdrawn from further analysis (we set this rule a priori). In experiments that showed transfection efficiency above 60%, TNF- α level was measured using ELISA to serve as an additional control for the efficiency of transfection Figure 3. 3C. Consistent with previous studies (367), overexpression of miR-155 triggered the production of TNF- α compared to cells transfected with control mimic.

Next, I assessed if the transfection affected the cells' viability. Therefore, CD14⁺ monocytes were transfected with miR-155 mimic or left un-transfected and cell viability determined by haemocytometer and Trypan Blue staining. Transfected cells were analyzed to determine the percentage of dying and viable cells before and directly after transfection and also over next 48 h compared to the that un-transfected cells. As shown in Figure 3.3D, transfection of CD14⁺ monocytes with miR-155 were not associated with any significant alteration in cell viability, compared to control cells left un-transfected.

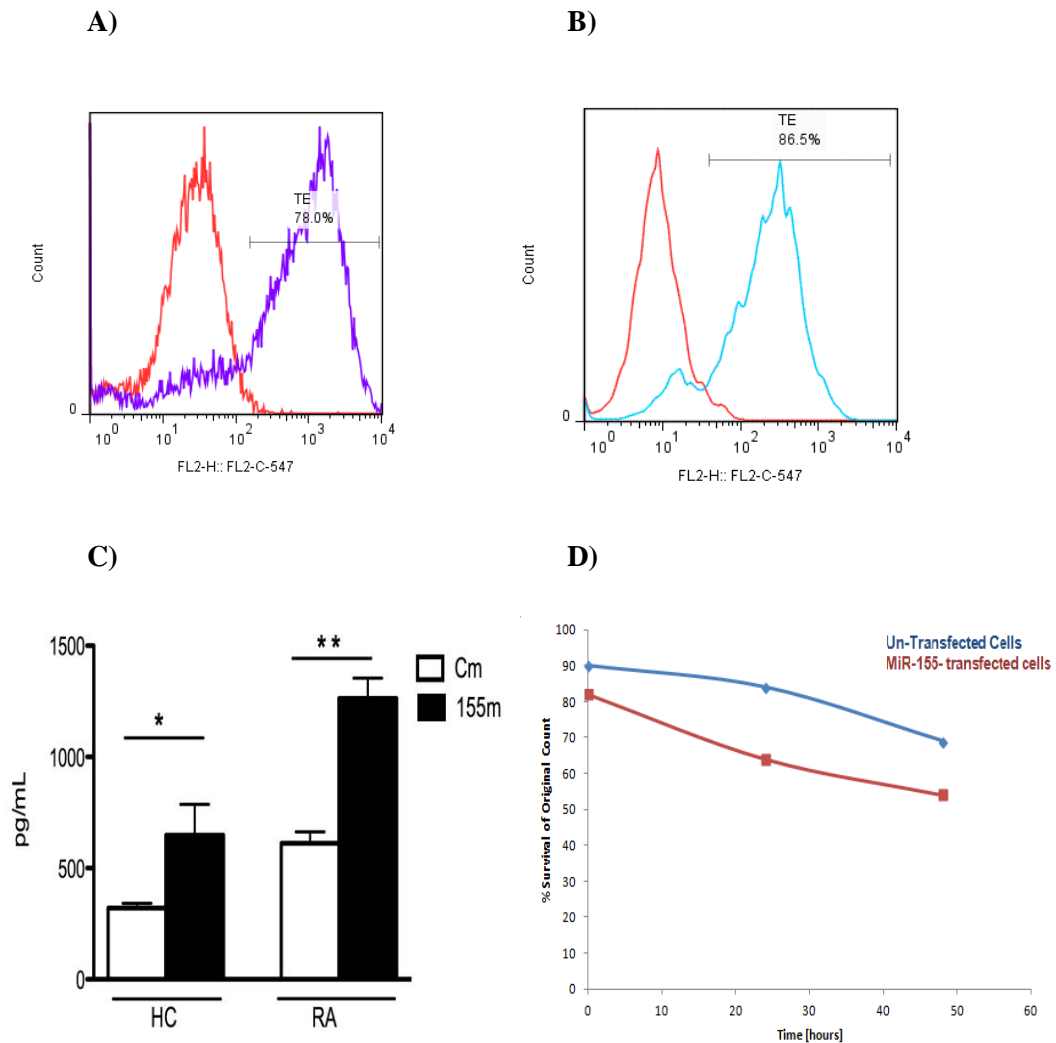


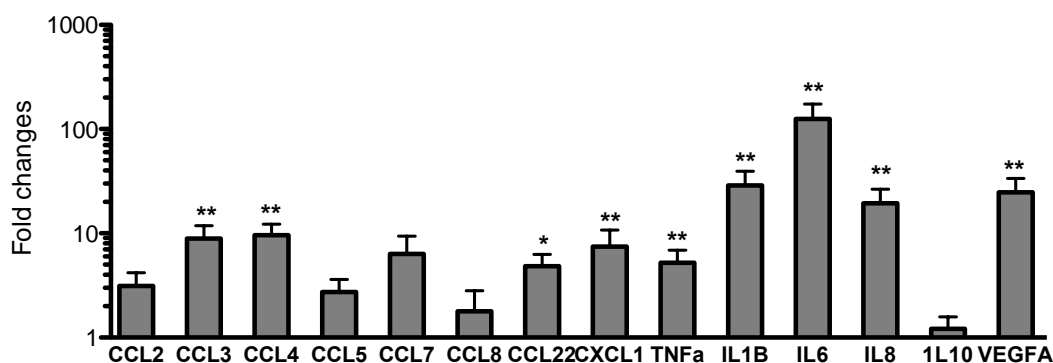
Figure 3.3 Efficiency of transfection of PB CD14⁺ monocytes with miR-155 mimic was monitored in transfected cells with miR-155 mimics or control mimic by control mimic labelled with fluorescence dye Dy547 using flow cytometry as described in Material and Method. A representative histogram analysis of A) healthy controls, B) RA patients of the CD14⁺ monocytes that transfected with miR-155 mimics or control mimic labelled with Dy547. C) TNF- α level within culture supernatants of experiments with transfection efficiency above 60% after transfection with miR-155 mimic or control mimic derived from healthy control (HC) and patients with RA (RA) (n=5) using ELISA. D) A representative experiment (n=1) of viability of CD14⁺ monocytes before and after the transfection with miR-155 was measured with Trypan Blue staining. Transfected cells were counted to determine the percentage of dying and viable cells before and directly after transfection and also over next 48 h compared to that un-transfected cell. Values presented as mean \pm SEM and marked bars are statistically different from control using Kruskal-Wallis test * = $p \leq 0.05$ and ** = $p \leq 0.005$. HC: healthy controls, RA: Rheumatoid arthritis, Cm: control mimic and miR155m: miR-155 mimic.

3.2.5 Cytokine and Chemokine mRNA Expression by PB Monocytes after Overexpression of miR-155

Expression the mRNA of inflammatory cytokines and chemokines after experimental over-expression of miR-155 in PB CD14⁺ monocytes of healthy controls and RA patients (n=8/group) was assessed using predesigned TLDA plates with specific primers to candidate cytokines and chemokines Figure 3.4A and B, respectively. Analysis was performed on total RNA samples harvested 48 h after culture from PB CD14⁺ monocytes transfected either with miR-155 mimic or control mimic and converted to cDNA. Cytokine and chemokine transcription levels were expressed as fold change relative to control mimic transfected cells and after normalization to 18S as housekeeping gene. In healthy controls, miR-155 transfected CD14⁺ monocytes expressed higher levels of CCL3/MIP-1 α , CCL4/MIP-1 β , CCL22/MDC, CXCL1/GRO, CXCL8/IL8, TNF- α , IL-1 β , IL-6 and VEGFA but not CCL2/MCP-1, CCL5/RANTES, CCL7/MCP-3 and CCL8/MCP-2 compared to control mimic transfected cells Figure 3.4A.

Similarly, in RA patients, over-expression of miR-155 in CD14⁺ monocytes induced the expression of mRNAs encoding CCL2/MCP-1, CCL3/MIP-1 α , CCL4/MIP-1 β , CCL5/RANTES, CCL7/MCP-3, CCL8/MCP2 and CXCL8/IL8. As observed, over-expression of miR-155 did not significantly affect the levels of CCL22/MDC, CXCL1/GRO, and VEGFA (Figure 3.4B). The mRNA levels of other pro-inflammatory cytokines, such as TNF- α , IL-1 β and IL-6, were also determined in PB CD14⁺ monocytes after over-expression of miR-155 Figure. 3.4B. Although, TNF- α , IL-1 β and IL-6 mRNA levels were increased this was not statistically significant. Supernatants were collected from PB CD14⁺ monocytes after over-expression of miR-155 or control mimics and analysed by pre-designed luminex assay. Although the exact molecular mechanism is unknown, this observation suggested that miR-155 can contribute to the development of pro-inflammatory response in both healthy controls and RA patients, but could do so in a discrete manner in patients with RA.

A. Cytokine and chemokine expression at the transcript level in PB CD14⁺ monocytes of healthy controls after overexpression of miR-155



B. Cytokine and chemokine expression at the transcript level in PB CD14⁺ monocytes of patients with RA after overexpression of miR-155

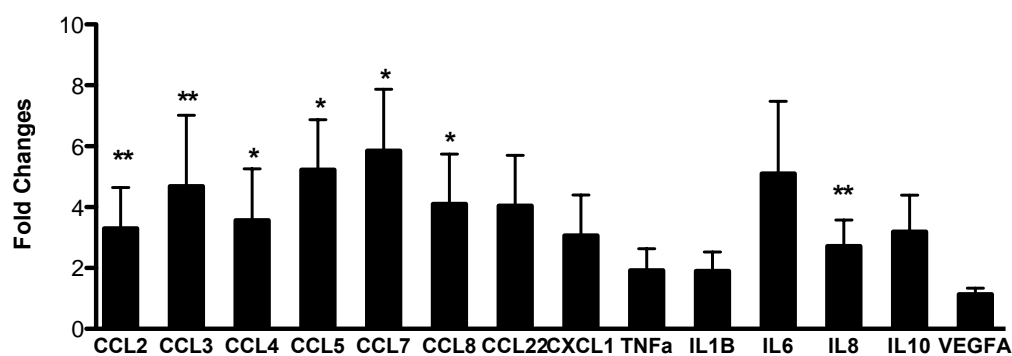


Figure 3.4 Cytokine and chemokine mRNA expression in PB CD14⁺ monocytes from A) healthy controls and B) RA patients (n=8/group) after transfection either with miR-155 mimic or control mimics using the N-TER nanoparticle siRNA transfection system as described in Materials and Methods. Total RNA was extracted 48 h after culture and TLDA plates with specific primers to determined transcript levels of candidate cytokine and chemokine. Transcript levels are expressed as fold change after normalization to 18S as housekeeping gene and calibrated to levels detectable in control mimics transfected cells. These data are representative of 8 separate experiments for each group. Bars show the mean of 3 replicates of total 8 experiments from each group \pm SEM. Marked bars are statistically different from control mimics transfected cells using Mann-Whitney U test; *= $p \leq 0.05$ and **= $p \leq 0.005$.

3.2.6 Overexpression of miR-155 in RA PB Monocytes Significantly Enhanced the Production of Cytokines at the Protein Level

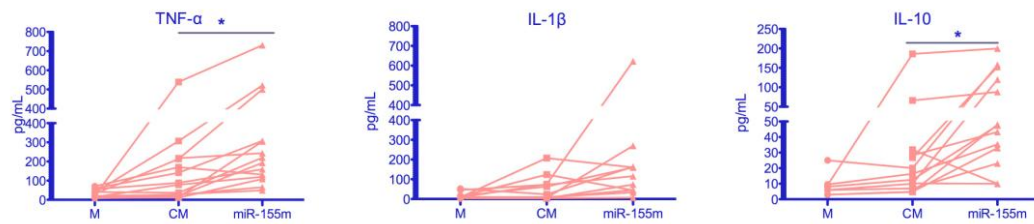
Next, I looked for the impact of over-expression of miR-155 as an important regulator of inflammatory cytokine release. To investigate this, a pre-designed luminex assay was used to assess the effect of over-expression of miR-155 on the production of a set of inflammatory cytokines and chemokines. PB CD14⁺ monocytes from healthy controls (n=15) and RA patients (n=16) were transfected either with a miR-155 mimic or negative control mimic or were left un-transfected as controls. The concentration levels of pro-inflammatory cytokines were measured 48 h after transfection. There was no statistically significant increase in cytokine production in cells transfected with control mimic compared to untouched cells; thus serving as an additional negative control (Figure 3.5A and B), suggesting that the transfection protocol itself has no significant stimulatory effect on cytokine production and justifying the use of a control mimic transfection in all experiments as a control.

In healthy controls, overexpression of miR-155 in CD14⁺ monocytes induced the production of TNF- α , and IL-10, but did not significantly affect the production of IL-1 β Figure 3.5A. While in RA patients, consistent with our previous study, overexpression of miR-155 significantly increased the concentration of multiple pro-inflammatory cytokines, including TNF- α , and IL-1 β . In addition, over-expression of miR-155 triggered production of anti-inflammatory IL-10 in culture supernatants, likely as a part counter-balance mechanism Figure 3.5B.

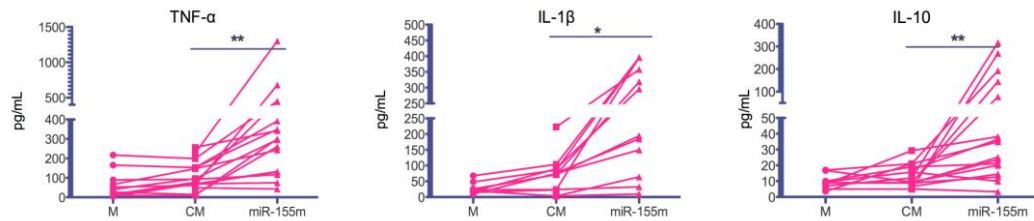
Of interest, RA CD14⁺ monocytes after over-expressing miR-155 displayed higher levels of IL-1 β than that produced by healthy controls cells under the same conditions; 233 \pm 42 pg/ml and 111 \pm 23 pg/ml respectively (P<0.04) Figure 3.5C. Likewise, TNF- α level showed a trend to be higher in RA CD14⁺ monocytes but this increase did not reach the level of significance.

These observations suggest that miR-155 can promote an inflammatory response although how it interacts with such inflammatory pathways to promote cytokine secretion in monocytes is unclear.

A. Cytokine production from PB CD14⁺ monocyte of healthy controls after over expression miR-155



B. Cytokine production from PB CD14⁺ monocyte of RA patients after over expression miR-155



C. Cytokine production from PB CD14⁺ monocyte of RA patients compared with healthy controls after over expression miR-155

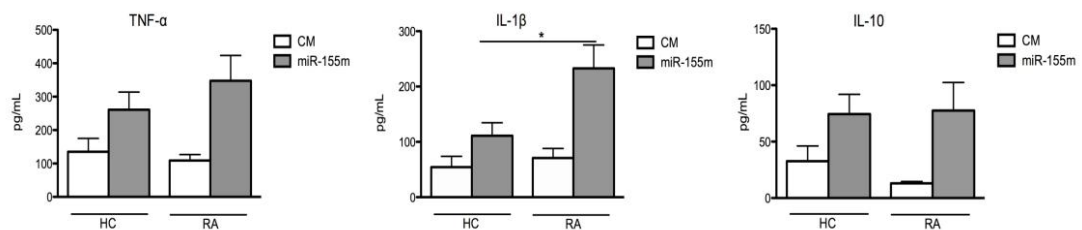


Figure 3.5 Cytokines concentration within culture supernatants of PB CD14⁺ monocyte from A) healthy controls (n=15) and B) RA patients (n=16) after transfection either with miR-155 mimic (miR-155m) or control mimic (CM) using the N-TER nanoparticle siRNA transfection system or left un-transfected (M) as described in Materials and Methods. The concentration levels of proinflammatory cytokines were measured using predesigned multiplex assay and tested in triplicate. The statistical different from the control using Wilcoxon test $*=p \leq 0.05$ and $**=p \leq 0.005$. C) Cytokine concentrations produced by RA CD14⁺ monocyte in compare to healthy controls after over expression miR-155. Values are presented as mean \pm SEM and marked bars are statistical different from the control using Kruskal-Wallis test, $*=p \leq 0.05$ and $**=p \leq 0.005$. HC: healthy controls, RA: Rheumatoid arthritis, Cm: control mimic, miR155m: miR-155 mimic and M; Media.

3.2.7 Overexpression of miR-155 in RA PB Monocytes Induces Production of Chemokines at the Protein Level

I then investigated whether miR-155 over-expression in PB CD14⁺ monocytes could alter their chemokine production. I monitored the effect of miR-155 on chemokine production by transfecting PB CD14⁺ monocytes from healthy controls (n=15) and RA patients (n=16) were transfected with either miR-155 mimic or negative control mimic or were left un-transfected as control. 48 h after transfection the cell culture supernatants were collected, and chemokine production was quantified using multiplex analysis as described in Materials and Methods. Production of some chemokine was significantly increased in cells transfected with miR-155 mimic; however, other chemokine production was not affected by transfection. Therefore, based on chemokine concentrations within culture supernatants of PB CD14⁺ monocytes after over-expression miR-155, the impact of miR-155 can be categorized into 2 groups: (i) chemokines that were significantly up-regulated in response to miR-155 overexpression; CCL3/MIP-1 α , CCL4/MIP-1 β , CCL5/RANTES and CCL8/MCP-2, (ii) chemokines either unchanged (see below) or below the limit of assay detection, namely CCL13/MCP4, CCL17/TARC, CCL19/MIP-3 β , CCL20/MIP-3 α , CXCL9/MIG, CXCL11/I-TAC, CXCL12/SDF-1 and lymphotactin.

3.2.7.1 Chemokines Significantly Upregulated in Response to miR-155 Overexpression

As expected on the basis of mRNA data, the concentration levels of multiple chemokines were significantly increased in culture supernatants of miR-155 transfected cells. First of all, no significant differences were found in chemokine production between non-transfected CD14⁺ monocytes and cells transfected with control mimics, which further confirms the specificity of the effects observed with the miR-155 mimics.

In healthy controls, there were no significant differences observed in the majority of chemokines tested between cells transfected with miR-155 in comparison to cells transfected with control mimic except for CCL3/MIP-1 α . The concentration of CCL3/MIP-1 α was significantly increased in culture supernatants of healthy controls

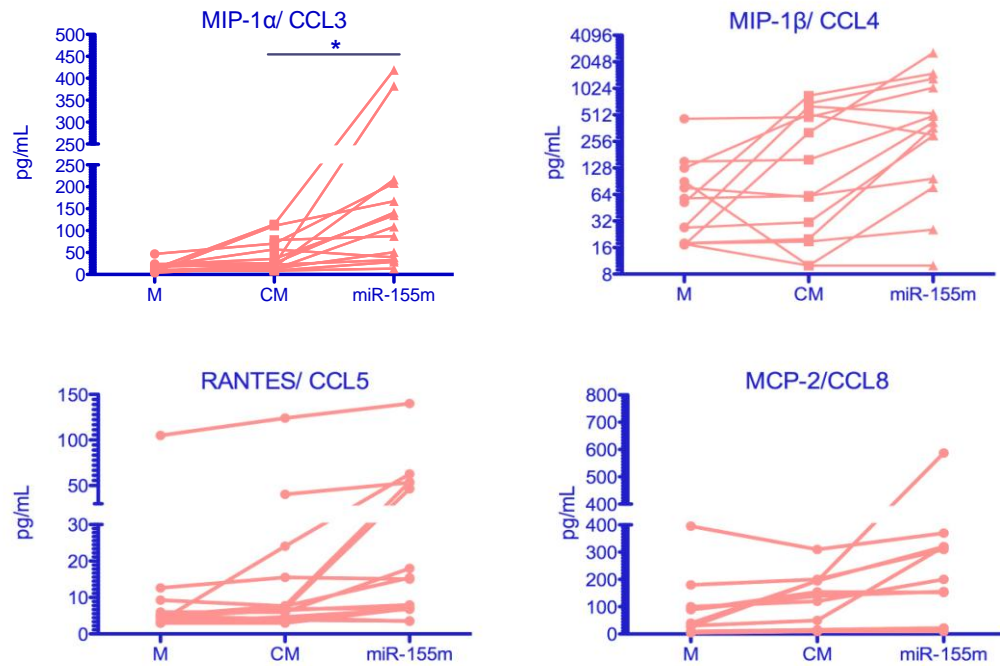
PB CD14⁺ cells transfected with miR-155 compared with supernatants from cells transfected with control mimic Figure 3.6A. In RA patients, over-expression of miR-155 in PB CD14⁺ monocytes triggered the production a variety of chemokines, including CCL3/MIP-1 α , CCL4/MIP-1 β , CCL5/RANTES and CCL8/MCP-2 compared with control mimic transfected cells Figure 3.6B. Notably, CCL8/MCP-2 production (mean \pm SEM) from RA CD14⁺ monocyte in response to miR-155 over-expression was statistically higher than that produced from healthy controls miR-155 transfected cells (801 \pm 164pg/ml vs 245 \pm 55pg/ml) (P<0.03).

These observations suggest that miR-155 in RA CD14⁺ monocytes could be involved in posttranscriptional control of the inflammatory pathways via triggering chemokine production that was implicated in RA synovitis and recruitment and retention of inflammatory cells in the joints space.

3.2.7.2 Chemokines not Affected by miR-155 Overexpression

Some chemokines were unchanged either in healthy controls or in RA PB CD14⁺ culture supernatants of cells transfected with miR-155 compared to control mimic or in control cells left un-transfected. This group of chemokines included CCL2/ MCP-1, CCL7/ MCP-3, CCL21/6CKINE, CCL22/MDC, CXCL1/GRO, CXCL5/ENA-78, CXCL8/IL-8, CXCL7/NAP-2, CXCL10/IP-10 and CX₃CL1/Fractalkine (Figure 3.7 and 3.8). This observation suggested that these chemokines were not regulated by miR-155.

A. Chemokines production from PB CD14⁺ monocytes of healthy controls after overexpression of miR-155



B. Chemokines production from PB CD14⁺ monocytes of RA patients after overexpression of miR-155

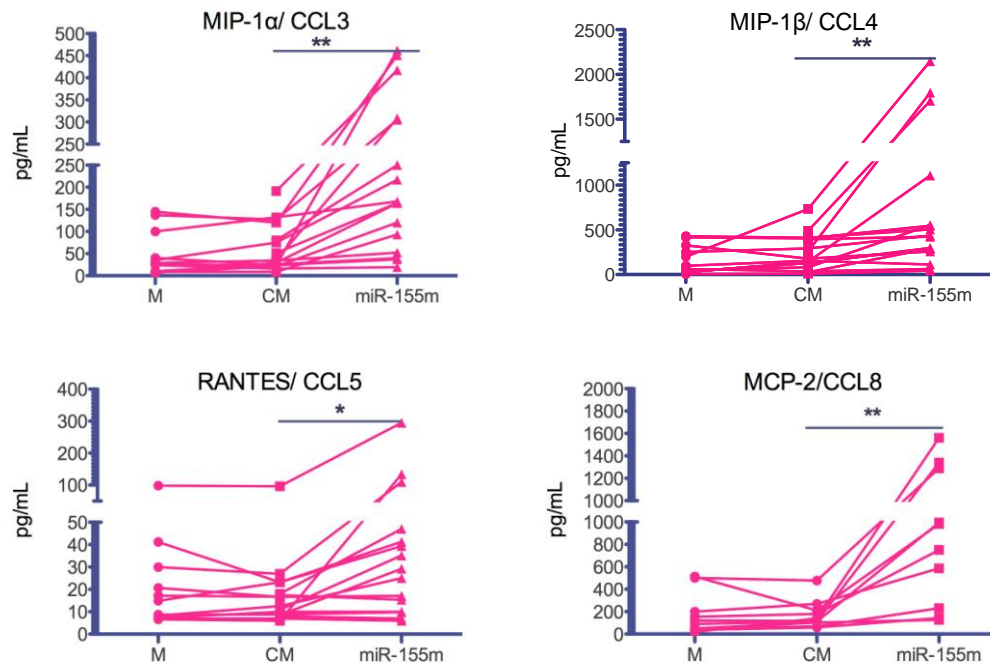


Figure 3.6 Chemokines upregulated in response to miR-155 overexpression. Chemokines concentration within culture supernatants of PB CD14⁺ monocyte from A) healthy controls (n=15) and B) RA patients (n=16) after transfection with miR-155 mimic (miR-155m) or control mimic (CM) using the N-TER nanoparticle siRNA transfection system or left un-transfected (M) as described in materials and methods. The concentration levels of inflammatory chemokines were measured 48 h after culture using pre designed multiplex assay and were tested in triplicate. Statistical significance are determined using Wilcoxon test compare to the control mimic, $*=p \leq 0.05$ and $**=p \leq 0.005$. HC: healthy controls, RA: Rheumatoid arthritis, Cm: control mimic, miR155m: miR-155 mimic and M; Media.

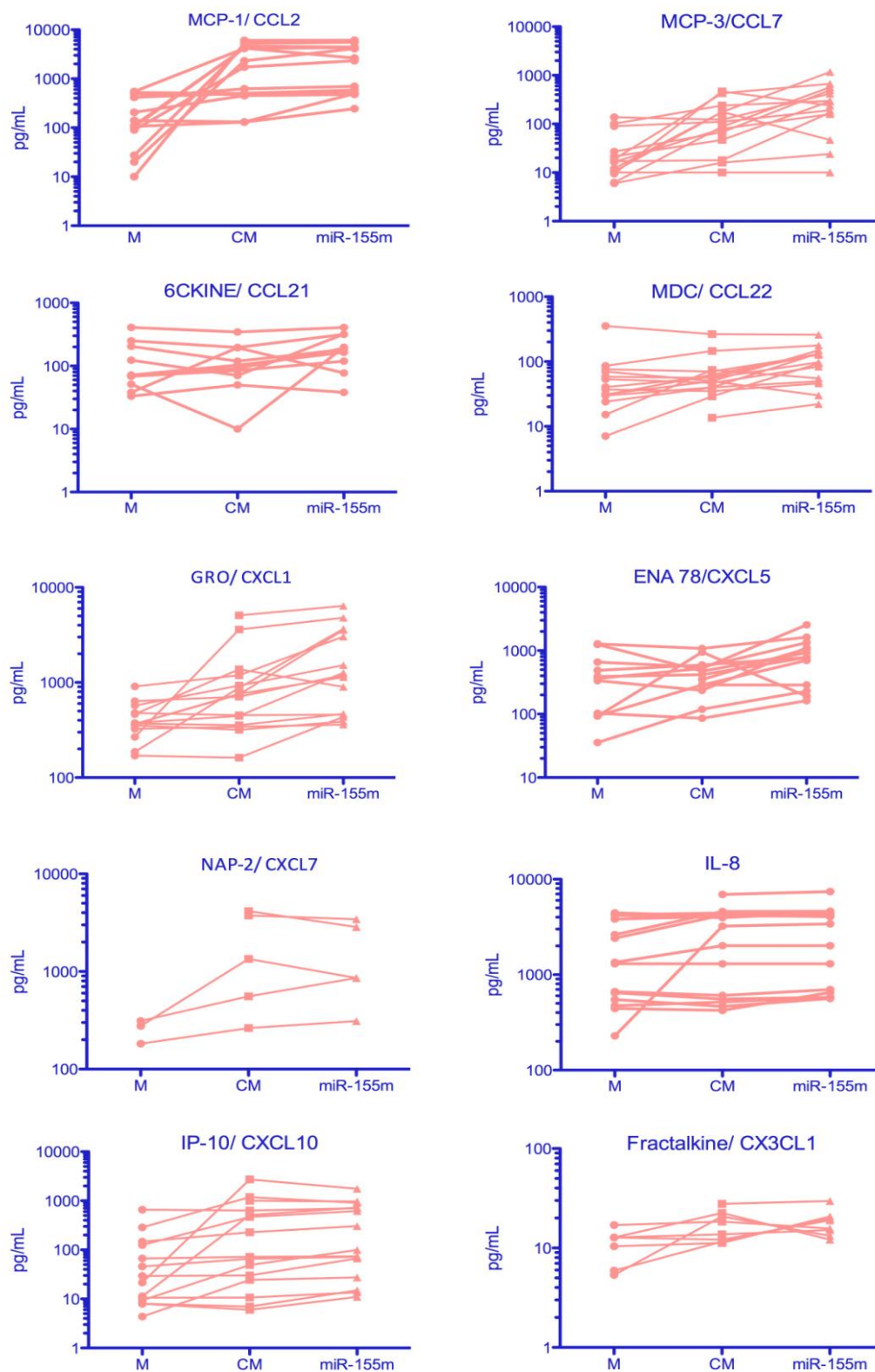


Figure 3.7 Chemokines unaffected by miR-155 overexpression. Chemokine levels within culture supernatants of PB CD14⁺ monocyte from healthy controls (n=15) after transfection with miR-155 mimic (miR-155m) or control mimic (CM) using N-TER nanoparticle siRNA transfection system or left untransfected (M) as described in Material and Method. The concentrations of chemokines were measured using pre designed multiplex assay and were tested in triplicate. HC: healthy controls, RA: Rheumatoid arthritis, Cm: control mimic, miR155m: miR-155 mimic and M; Media.

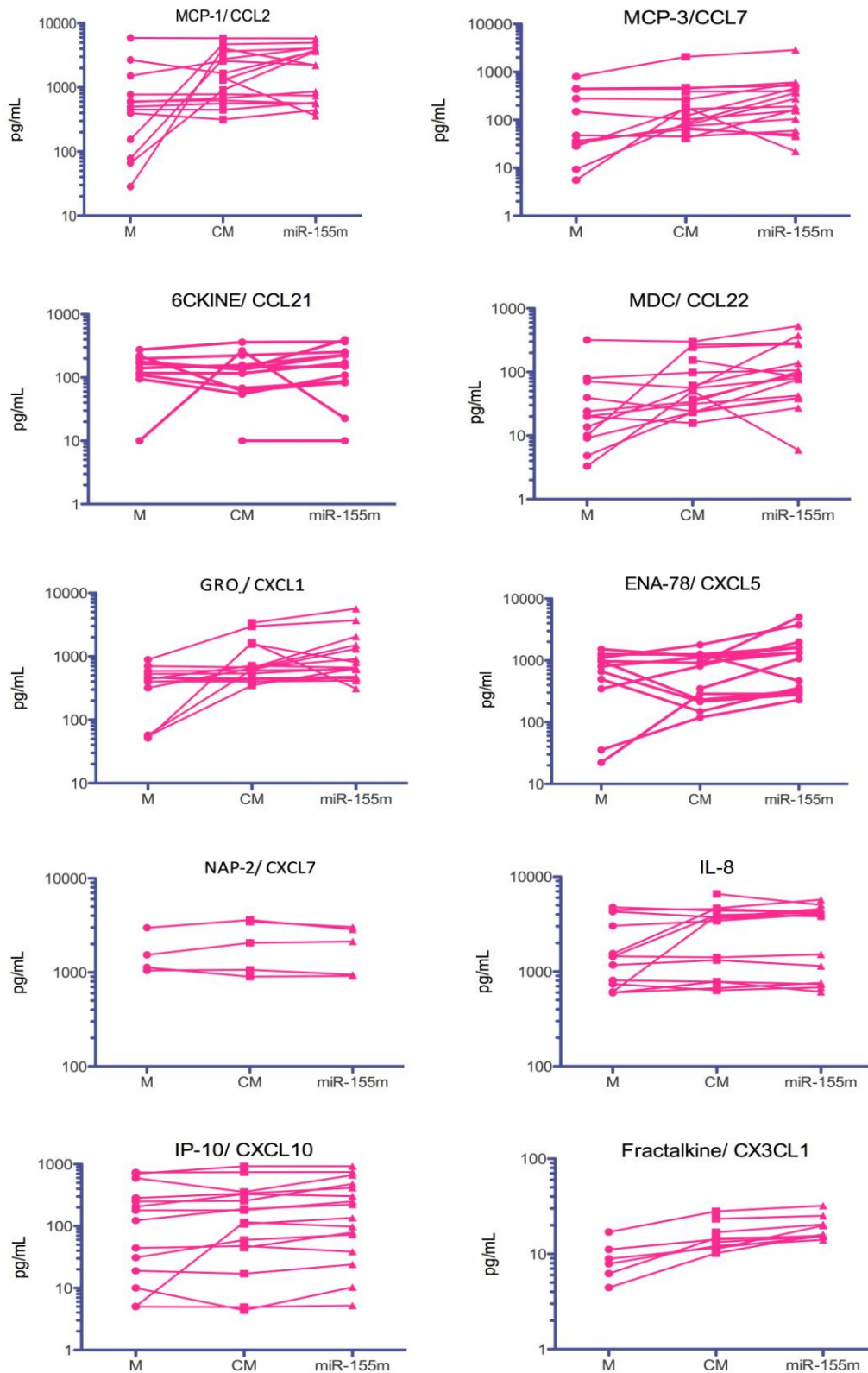


Figure 3.8 Chemokines were unaffected by miR-155 overexpression. Chemokine concentrations within culture supernatants of PB CD14⁺ monocyte from RA patients (n=16) after transfection with miR-155 mimic (miR-155m) or control mimic (CM) using N-TER nanoparticle siRNA transfection system or left un-transfected (M) as described in Material and Method. The concentrations of chemokines were measured 48 h from culture using predesigned multiplex assay and were tested in triplicate. HC: healthy controls, RA: Rheumatoid arthritis, Cm: control mimic, miR155m: miR-155 mimic and M; Media.

3.2.8 Cytokine and Chemokine Production in PB Monocytes after miR-155 overexpression and their Modulation by TLR Agonism (LPS)

Our preliminary study using luminex analysis revealed that the TLR4 agonist (LPS) led to a strong induction of candidate cytokines and chemokines from PB CD14⁺ monocytes, from both healthy controls and RA patients, transfected with miR-155. MiR-155 has been shown to be upregulated by TLR ligands in PB CD14⁺ monocytes (367). I therefore analysed the potential interaction between LPS and miR-155 for cytokine and chemokine production from PB CD14⁺ monocytes after miR-155 overexpression. PB CD14⁺ monocytes derived from healthy controls and RA patients (n=5/group) were transfected either with miR-155 mimics or negative control mimics or were left un-transfected. After 24 h, LPS (100ng/ml) was added to some wells for a further 16-18 h. The concentration of cytokines and chemokines in the cell culture supernatants were measured by predesigned luminex assay.

In healthy controls, LPS (100ng/ml) strongly triggered the CD14⁺ monocytes to produce all the cytokines and chemokines tested, and there were no differences observed between the concentrations produced by cells transfected with miR-155 mimic or control mimic and cells that were left un-transfected (Figure 3.9). Likewise, in RA derived CD14⁺ monocytes there were no significant differences observed in the majority of cytokines and chemokines tested between cells transfected with miR-155 in comparison to cells transfected with control mimic or cells that were left un-transfected (Figure 3.10).

Thus, the presence of LPS abrogated the effect of miR-155 and no distinct difference was observed in cytokine and chemokine production between monocytes transfected with miR-155 mimics or control mimics. However, CD14⁺ monocytes were treated with a particularly high dose; 100ng/ml of LPS, which might have masked any difference in cytokine production. Therefore I suggest that this experiment should be repeated with a lower concentration range of LPS.

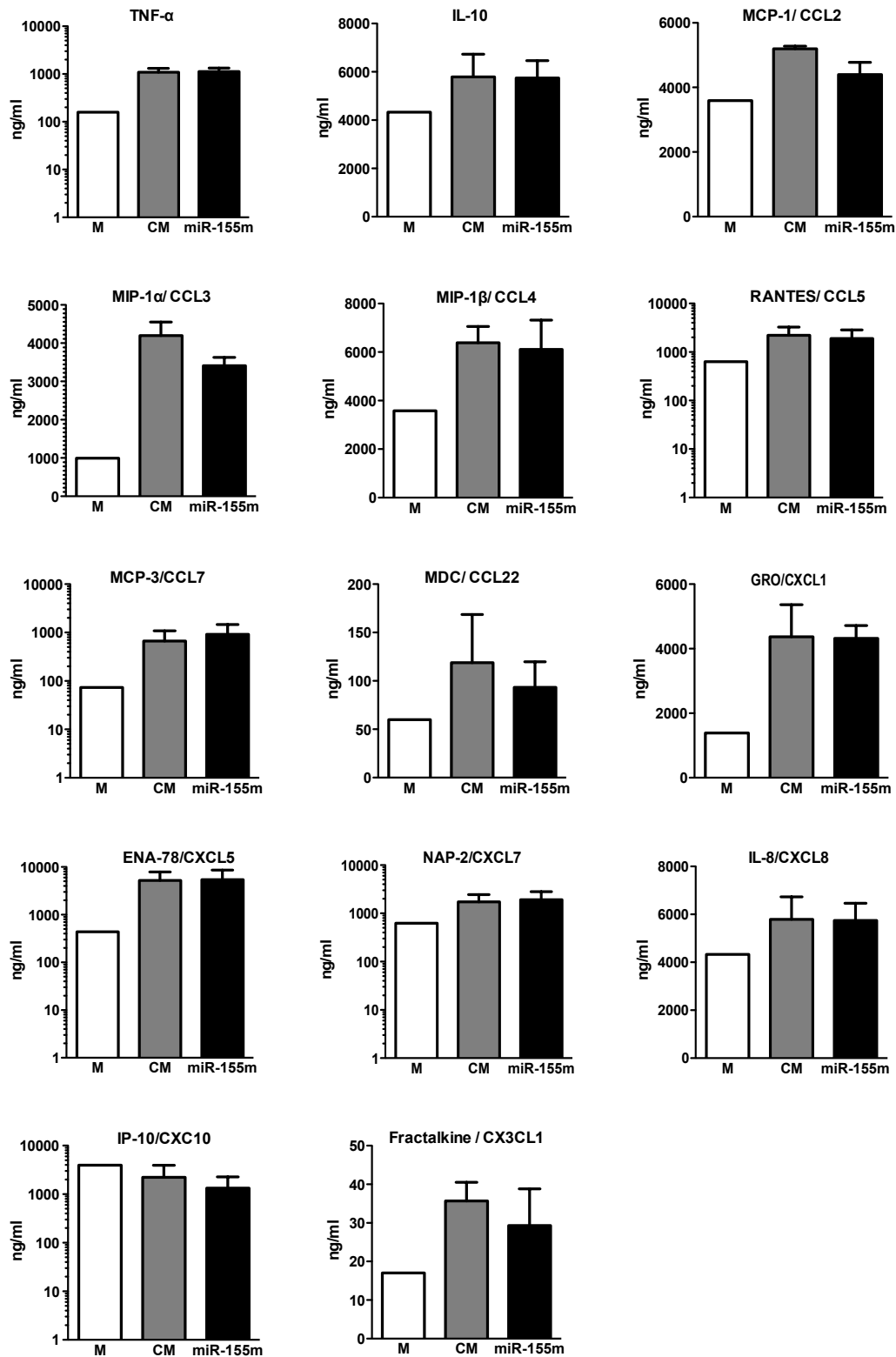


Figure 3.9 Cytokines and chemokines concentration within culture supernatants of PB CD14⁺ monocyte of healthy controls (n=5) after transfection either with miR-155 mimic (miR-155m) or control mimic (CM) using the N-TER nanoparticle siRNA transfection system or left un-transfected (M) as described in Materials and Methods. 24 h thereafter transfected cells were stimulated with (100ng/ml) LPS for a further 16-18 h. Culture supernatants were collected, and cytokines and chemokine production were measured using multiplex assay and tested in triplicate. Values are presented as mean \pm SEM, HC: healthy controls, RA: Rheumatoid arthritis, Cm: control mimic, miR155m: miR-155 mimic and M; Media.

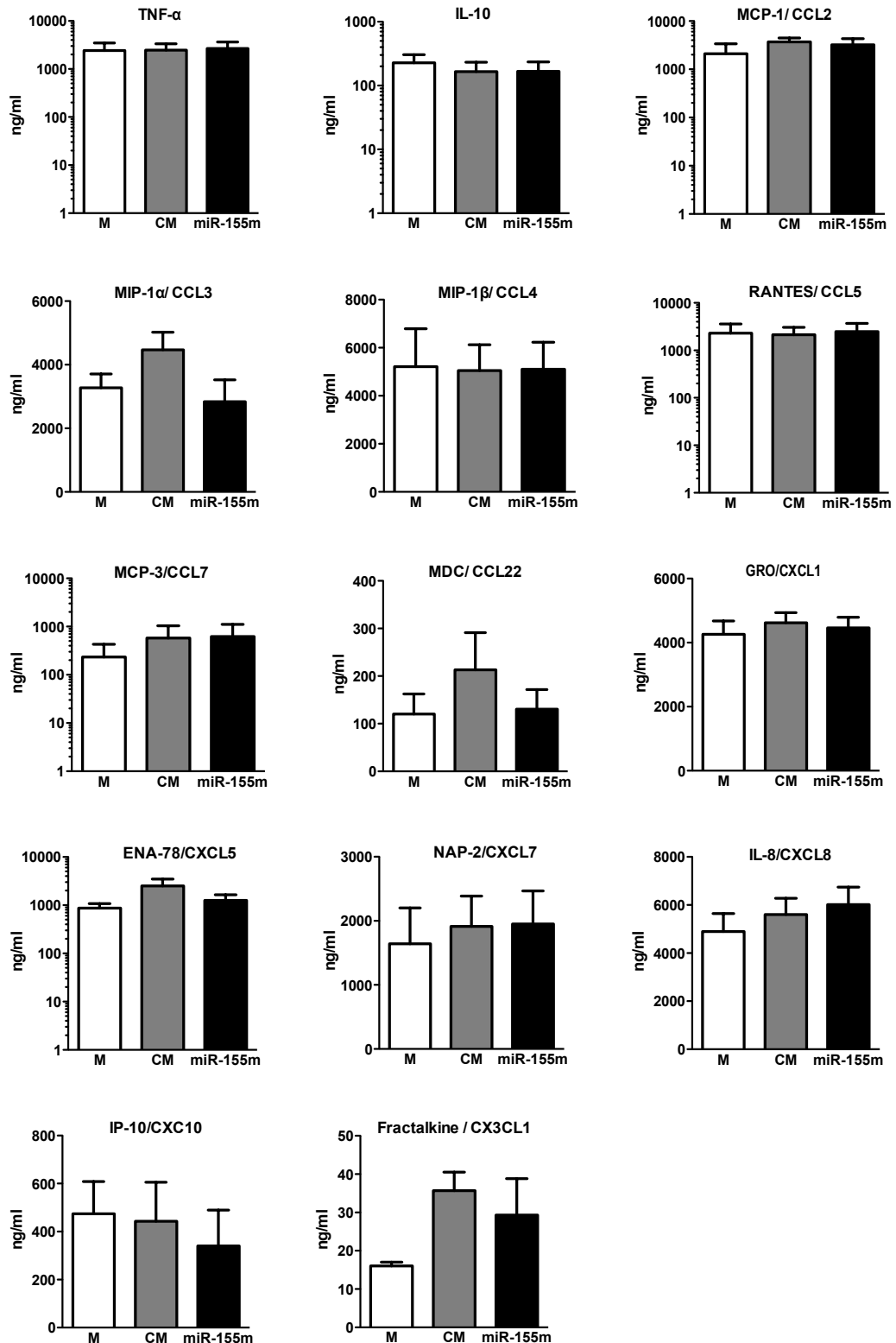


Figure 3.10 Cytokines and chemokines concentration within culture supernatants of PB CD14⁺ monocyte of RA patients (n=5) after transfection either with miR-155 mimic (miR-155m) or control mimic (CM) using the N-TER nanoparticle siRNA transfection system or left un-transfected (M) as described in Materials and Methods. 24 h thereafter transfected cells were stimulated with (100ng/ml) LPS for a further 16-18 h. Culture supernatants were collected, and cytokines and chemokine production were measured using predesigned multiplex assay and tested in triplicate. HC: healthy controls, RA: Rheumatoid arthritis, Cm: control mimic, miR155m: miR-155 mimic and M; Media.

3.2.8.1 TNF- α Production by PB Monocytes overexpressing miR-155 and their modulation by different doses of LPS

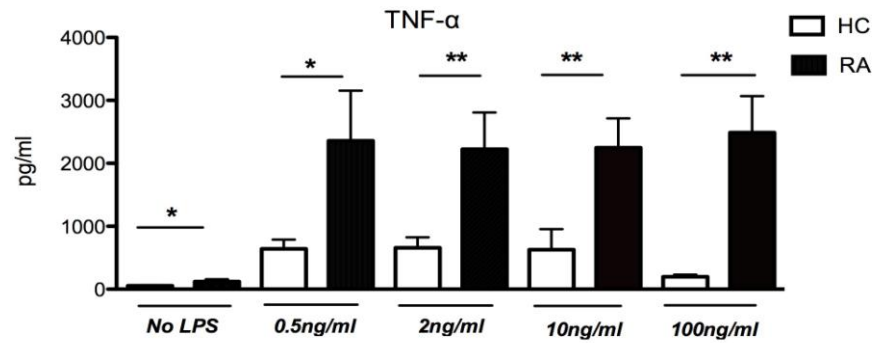
As I mention above, presence of LPS (100ng/ml) led to a strong induction of candidate cytokines and chemokines from PB CD14⁺ monocytes and there was no distinct difference was observed in production between monocytes transfected with miR-155 mimics or control mimics. Therefore I decided to titrate the LPS dose (0.5, 2, 10ng/ml) and measure TNF- α levels using ELISA. To this end, PB CD14⁺ monocytes derived from both healthy controls and RA patients (n=4) were transfected with miR-155 mimic or control mimic or left un-transfected and 24 h thereafter some cells were stimulated with different doses (0.5, 2 and 10ng/ml) of LPS. Culture supernatants were collected 16-18 h after LPS addition.

First of all, I examined the impact of different LPS doses on TNF- α production from CD14⁺ monocytes that were left un-transfected in both healthy controls and RA patients using ELISA. Overall TNF- α production was significantly higher within culture supernatants of cells that derived from RA patients than healthy controls in all conditions (Figure 3.11A). However, levels of TNF- α production were not correlated with increasing the LPS doses. Indeed, even 0.5ng/ml of LPS was sufficient to maximally stimulate cells and trigger TNF- α production.

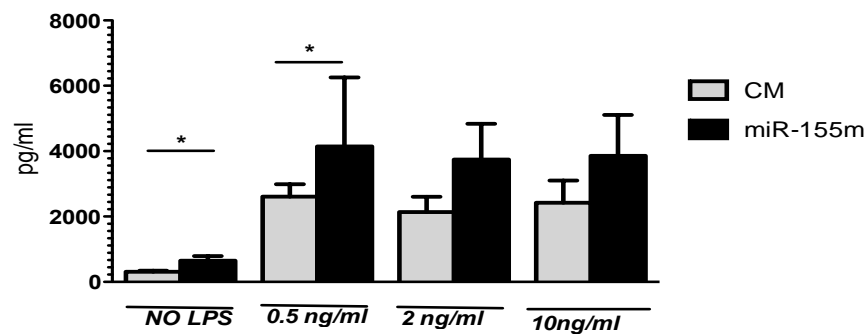
Next, I looked to TNF- α production from CD14⁺ monocytes transfected with miR-155 mimics or control mimics in response to different LPS doses in both healthy controls and RA patients. Reassuringly, and consistent with previous data generated by multiplex assay, TNF- α levels were significantly higher in supernatants from cells transfected with miR-155 mimic than those transfected with control mimic with no LPS stimulation in both healthy controls and RA patients (Figure 3.11B and C), respectively. In healthy controls, TNF- α levels were significantly increased in CD14⁺ monocytes transfected with miR-155 and treated with 0.5ng/ml LPS compared to those cells transfected with control mimic. Consistent with preliminary data, TNF- α concentration in response to 2 and 10ng/ml LPS were also increasing within culture supernatants of miR-155 transfected cells in comparison with control mimic transfected cells but did not reach levels of significance Figure 3.11B. While in RA patients, there was no distinction in TNF- α concentration between cells transfected with miR-155 mimic in comparison with control mimic in response to

different LPS doses Figure 3.11C. Together, this data indicate that LPS leads to a strong induction of TNF- α and the addition of increased expression of miR-155 did not affect its production. Thus, the presence of high concentration of LPS has masked the affect of miR-155 on TNF- α production.

A)



B)



C)

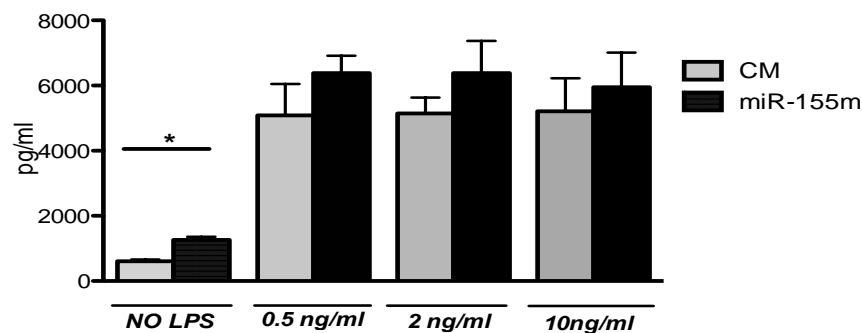


Figure 3.11 A) Concentration of TNF- α within culture supernatants of PB CD14⁺ monocytes of healthy controls and RA patients (n=4) in absence and presence of different doses (0.5, 2 and 10ng/ml) of LPS as were determined by ELISA. While, B) and C) represented the TNF- α levels within the culture supernatants of PB CD14⁺ monocytes that derived from healthy control and RA patients (n=4), respectively and after transfection either with miR-155 mimic or control mimic as described in Materials and Methods. 24 thereafter transfection, cells either un-stimulated or stimulated with a different LPS doses (0.5, 2 and 10ng/ml). Values are presented as mean \pm SEM and statistical significance are determined using Kruskal-Wallis test, *= $p \leq 0.05$ and **= $p \leq 0.005$. HC: healthy controls, RA: Rheumatoid arthritis, Cm: control mimic, miR155m: miR-155 mimic and M; Media.

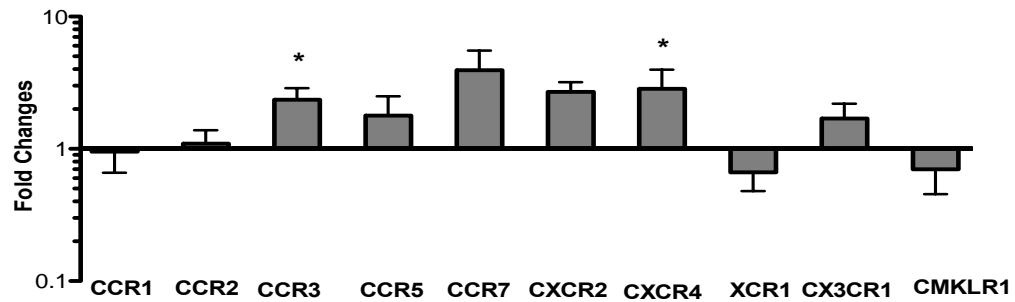
3.2.9 MiR-155 Regulates Chemokine Receptors mRNA Expression

Altered monocyte trafficking and migration in RA patients is under the control of chemokine receptors. Therefore, I investigated the impact of over-expression of miR-155 on chemokine receptor expression in PB CD14⁺ monocytes mRNA of both RA patients and healthy controls.

The role of miR-155 on chemokine receptor expression was investigated using TLDA plates that allow simultaneous measurement of multiple PCR with specific primers for distinct chemokine receptors. In brief, total RNA was extracted from PB CD14⁺ monocytes of healthy controls and RA patients (n=8/group) 24 h after transfection either with miR-155 mimic or control mimics as described in Materials and Methods. After cDNA synthesis and normalization using 18S; a house-keeping gene as a standard, the transcript levels of candidate chemokine receptors were presented as fold-changes after calibration to transcript levels of control mimic transfected cells.

In healthy controls CCR3 and CXCR4 expression was increased in miR-155 transfected CD14⁺ monocytes compared with control mimic transfected cells. While other receptors were slightly upregulated in CD14⁺ monocytes transfected with miR-155 over control mimic transfected cells, none of these differences were statistically significant Figure 3.12A. In RA derived CD14⁺ monocytes, we noted some differences. Interestingly, miR-155 transfected RA PB CD14⁺ monocytes revealed an increase in transcript levels of CCR7; almost three times higher than their control mimic transfected cells Figure 3.12B. In contrast, over-expression of miR-155 induced down-regulation of CCR2 and CCR3, with CCR2 being the most significantly affected. Other chemokine receptors including, CCR4, CCR6, CCR8, CCR9, CCR10 and CX₃CR1 were below the limit of assay detection.

A. Chemokine receptors expression in PB monocytes of healthy control after transfection with miR-155



B. Chemokine receptors expression in PB monocytes of RA patients after transfection with miR-155

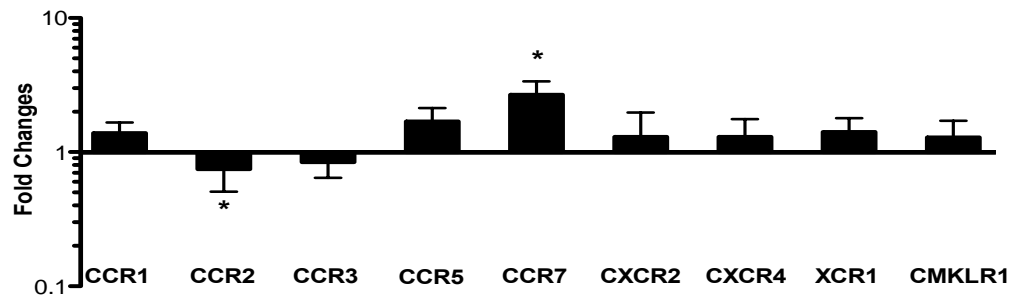


Figure 3.12 Chemokine receptors mRNA expression in PB CD14⁺ monocytes of A) healthy controls and RA patients (n=8/group) after transfection with either miR-155 mimic or control mimics using the N-TER nanoparticle siRNA transfection system. Total RNA was extracted and converted to cDNA as described in Material and Methods and transcripts levels of chemokine receptors were determined using TLDA plates with specific primers for distinct chemokine receptors. The transcript levels of candidate chemokine receptors were presented as fold changes after normalization to 18S as housekeeping gene and calibrated to transcript levels of control mimic transfected cells. Bars show the mean \pm SEM of 3 replicates of total 8 experiments from each group plus or minus SEM. Marked bars are statistically different from control mimics transfected cells using Mann-Whitney U test; $*=p \leq 0.05$.

3.3 Discussion

There is intense interest in unravelling those factors that coordinately regulate cytokine and chemokine production by macrophages in RA synovitis. In addition there is evidence that circulating monocytes in patients with RA are already in a primed or pre-activated state that is somehow implicated in their subsequent recruitment to the synovial space and participation therein to the inflammatory cascade. The factors that regulate such priming in these cells are also poorly understood. Prior studies have implicated miR-155 in the regulation of cytokines in macrophages of synovial origin and strongly suggest that it can play an important role. No studies however have addressed the role of miR-155 at the level of absolute copy number. Moreover no studies have addressed the potential role for miR-155 beyond this to the regulation of chemokine or chemokine receptor expression. Herein I have dissected the role of miR-155 to address each of these issues.

Among various miRNAs, miR-155 has been found to be highly expressed in various samples derived from patients with RA. For example, PBMCs exhibit higher expression of miR-155 in RA patients than OA patients and healthy controls (400). Therefore, this study was initiated to determine if miR-155 could mediate regulatory effects upon chemokine production, chemokine receptor expression in monocytes and their consequent migration. To test this hypothesis, it was important first to focus on exact copy number expression in the PB and SF of RA patients compared to healthy controls. Several studies reported that miR-155 was upregulated in RA PBMCs, RA SF-derived CD14⁺ monocytes and also strongly expressed in RA synovial biopsies (367, 400, 568). However, prior studies only reported levels of miR-155 by calculating the relative expression using quantitative RT-PCR. I now demonstrate that miR-155 copy number is upregulated significantly in both PB and SF CD14⁺ monocytes, particularly in SF, compared with PB monocytes of healthy controls. Further, I provide evidence that miR-155 copy number is also significantly upregulated in both RA patients' and healthy control subjects' PB monocytes following cell activation upon exposure to the TLR4 ligand LPS. Importantly, induction of miR-155 was significantly higher in patients with RA. These data are consistent with previous studies, in which only the fold-change was examined suggesting that they are internally valid (569). In future studies I would like to evaluate whether this early, enhanced copy number is essential upon cell activation

by LPS for the progression of the immune response and the production of inflammatory mediators downstream.

The increased miR-155 observed in cells from RA patients suggests that it could have clinical significance. I therefore sought correlations between miR-155 with parameters of clinical relevance. MiR-155 copy number positively correlated with the total joint (TJ) and swollen joint (SJ) counts, and also correlated with DAS28. In my cross-sectional cohort, all of the RA patients were being treated with cDMARDs, and 6 patients were also being treated with a biological DMARD. Analysis of the influence of drug therapy on miR-155 copy number expression revealed no difference between the two treatment categories. However, due to the small sample size in this study it is recommended that this be re-evaluated in a larger study appropriately powered for biomarker discovery. Thus, whether or how drug therapy influences miR-155 expression must at present remain speculative. Notably, despite the small sample size of RA patients in this study, it was observed that the ACPA positive RA patients have a higher copy number than ACPA negative RA patients. The nature of this interesting relationship between the miR-155 and ACPA has yet to be clearly understood. Since ACPA formation is an early event in the pathogenesis of arthritis observed prior to onset of clinically established RA, and also since miRNAs including miR-155 may be involved in the post-transcriptional modification of proteins and activation of the immune system it will be important in an appropriately powered prospective study to define the exact role of miR-155 in the formation of ACPA. Finally, I demonstrated that the miR-155 copy-number correlated with the ESR; which is a biomarker of acute inflammation. These data collectively imply that miR-155 is involved in the pathogenesis of RA, or its clinical manifestation and could be a promising biomarker for early diagnosis and therapeutic intervention. As proof of principal, several studies have emphasized the clinical significance of miRNA in cancer as highly specific and sensitive non-invasive biomarkers. Recent studies suggest that miRNAs in plasma can be biomarkers for the diagnosis, and predict the drug response of lung, breast, prostate and colorectal cancer. For example, increased concentration of serum miR-155 is a potential clinical biomarker for chronic lymphocytic leukaemia and Waldenstrom's macroglobulinemia (570). Similar studies measuring serum miR-155 in RA patients are therefore indicated.

As a next step, it was essential to address the functional consequence of increased miR-155 in inflammatory arthritis. Few reports so far have addressed this issue. One recent report demonstrated that miR-155 is crucial for the pro-inflammatory activation of human myeloid cells and antigen-driven inflammatory arthritis (367). In the present work, the functional role of miR-155 was explored by creating an over-expression system. There were several important reasons to generate an over-expression miR-155 system:

- (i) SF monocytes/macrophages have particularly high copy-numbers of miR-155 so I aimed to create a system that mimics this.
- (ii) I had access to miR-155^{-/-} mice, which are an appropriate experimental control strain.
- (iii) It is more convenient to get PB than SF from RA patients
- (iv) There are opportunities to get PB from a broader clinical spectrum of RA patients due to major improvement in disease outcomes arising from early diagnosis and introduction of biological therapies.
- (v) This work was an extension of pilot work in our lab which demonstrated that miR-155 over-expressed in RA PB monocytes strongly triggered the mRNA expression and production of cytokines TNF- α and IL-1 β implicated in RA synovitis.

In this study, I confirm the previous observation that enhanced miR-155 expression in RA PB monocytes strongly increased the production of TNF- α and IL-1 β , and their levels were significantly increased at the protein level. I did not see elevation of mRNA levels compared to control suggesting that the level of regulation may be at the post transcriptional stage of cytokine expression, or in cytokine processing.

The next part of my study extended the scope of the effects of increased miR-155 in RA beyond cytokine production to include that of chemokines. Given the regulatory interactions that link the chemokine system (chemokines and chemokine receptors) and cytokines such as TNF- α and IL-1 β , I hypothesized that miR-155 would also exert regulatory effects therein. To our knowledge, this is the first study providing systematic evidence that miR-155 has a role in chemokine and chemokine receptor expression in RA PB monocytes that are implicated in cell recruitment and migration at the joint spaces. I have shown that miR-155 over-expression significantly increased the expression and production of CCL3/MIP-1 α , CCL4/MIP-1 β ,

CCL5/RANTES and CCL8/MCP-2 from RA PB at both mRNA and protein levels. These data are consistent with previous studies showing that these chemokines through their receptors, CCL3/MIP-1 α (ligand for CCR1, CCR3 and CCR5), CCL4/MIP-1 β (CCR5), CCL5/RANTES (CCR1 and CCR5) and CCL8/MCP-2 (CCR2), are considered to be implicated in RA pathogenesis via recruitment and retention of monocytes and T cells in joints (571). Thus, miR-155 might be involved directly or indirectly in monocyte recruitment and retention, which in turn resulted in progression and persistence of synovitis.

In the present study we observed that CCL2/MCP-2, CCL7/MCP-3 and IL-8/CXCL8 mRNA levels were significantly increased following miR-155 over-expression but there was no corresponding increase in protein levels. This was in contrast to the observations in this lab by Kurowska-Stolarska *et al* (367) in which IL-8/CXCL8 protein as well as mRNA was increased in response miR-155 over-expression. The differences between the two studies may reflect minor methodological differences such as processing time prior to purification of cells from PB or the relatively small sample size previously employed in the Kurowska-Stolarska *et al* study. The implication from the different observations suggests that miR-155 is acting as a fine tuner rather than obligate partner in chemokine production such that rather subtle differences in approach may alter its assessed functional significance. This will be important to explore prior to therapeutic interventions going forward.

To summarize, the above data suggest that within the synovium the significant higher expression of miR-155 might mediated chronic synovitis through increase expression and local production of cytokines and chemokines that play a critical role in articular inflammation and recruitment of inflammatory cells into the joint space that are necessary for driving disease pathology. However, to address a potential mechanism by which the cytokines and chemokines are secreted in response to miR-155 over-expression, we hypothesised that this could be due to a direct functional effect of the TNF pathway. Although the intracellular signalling pathway for activation of cytokines and chemokines is complex, the TNF pathway has a fundamental role and could be one candidate pathway for investigation. Preliminary experiments determined the effects of anti-TNF (adalimumab at 10 μ g/mL) on miR-155 induced the production of cytokines and chemokines from PB monocytes in

vitro. These unpublished results demonstrated that anti-TNF induced the down-regulation of production of cytokines and chemokines from PB monocytes that over-expressed miR-155. Adalimumab efficiently neutralized the production of TNF α , IL-1 β , MIP-1 α and MIP-1 β . Although the results suggested that this down-regulation could be due to a direct functional effect of adalimumab, future studies will be required to define the range of pathways that might mediate such effects. We cannot, however, exclude a role of miR-155-regulated targets in the modulation of monocytes/macrophage cytokine responses. Compelling experimental evidence identifies miR-155 target transcripts that are of potential interest in regulation of inflammation *e.g.* SHIP-1 and SOCS-1. Recently our group showed that miR-155 regulated the expression of SHIP-1 and a novel tyrosine kinase receptor (AXL) in RA PB and SF CD14⁺ monocytes. Both represent potent inhibitors of many inflammatory pathways such as PI3K AKT that may lead to increased production of pro-inflammatory cytokines such as TNF- α , IL-1 β and IL-6, which are in turn implicated in the development of chronic arthritis (367, 572).

In contrast, other potential monocyte chemokines did not appear to be regulated by miR-155 pathway in PB RA monocytes. These include CCL2/MCP-1, CCL7/MCP-3, CCL21/6CKINE, CCL22/MDC, CXCL1/GRO, CXCL5/ENA-78, CXCL8/IL-8, CXCL7/NAP-2, CXCL10/IP-10 and CX3CL1/Fractalkine. The functional significance of this should be explored in future studies using selective inhibitors, or gene-silencing, when such reagents become available. I conclude, however, that other miRs will likely co-regulate the range of chemokines expressed in monocytes. This is rational from an evolutionary point of view in terms of host defence in which critical checkpoints could be vulnerable to subjugation by microbial species to the detriment of effective protection.

Chemokine receptors govern the directional movement of cells toward their chemokine ligands. Inflammatory chemokine receptors in RA patients fall into 2 as yet loosely defined categories. Some are involved in cell recruitment (*e.g.* for monocyte; CCR1 and CCR2), and others are important in cell retention (*e.g.* monocytes at joint spaces; CCR3 and CCR5) (287). In the present work, I showed that in RA PB monocytes over-expressing miR-155 there was a reduction in mRNA expression of the inflammatory chemokine receptor CCR2, and in contrast there was

an increased mRNA expression of CCR7. CCR7 is a homeostatic chemokine receptor that is involved in the release of monocytes from the bone marrow to periphery and into draining lymph nodes (209). These observations suggest that not only does TLR-4 stimulation induce a switch of chemokine receptor expression from inflammatory receptors to homeostatic receptors (573), but also that miR-155 might mediate this same effect. Commensurate with this, some of these receptors showed the opposite reciprocal expression patterns in mouse monocytes that were deficient for miR-155 (chapter IV); bone marrow monocytes of miR-155 deficient mice expressed significantly higher mRNA transcript levels of inflammatory chemokine receptors CCR2 and significantly lower mRNA transcript levels of CCR7. This suggests that endogenous miR-155 may act as an important regulator of chemokine receptors in monocytes leading to their retention at sites of inflammation. The mechanism of this regulation is currently unknown. Analysis of 3'UTRs of chemokine and chemokine receptors (using online Target Scan software; <http://www.targetscan.org/>) shows that none of the affected chemokine/chemokine receptors are directly targeted by miR-155. This finding suggests that miR-155 most likely functions by regulating proteins in the signalling pathways implicated in chemokine and chemokine receptor system expression.

Thus, dysregulation of miR-155 in RA monocytes can contribute to the production of pro-inflammatory chemokines by these cells and to their accumulation at the sites of inflammation through the autocrine/paracrine patterns (Figure 3.13). A possible scenario is that miR-155, expressed at high copy number in RA SF monocyte/macrophages, triggers chemokine production that in turn leads to recruitment of monocytes and other cells such as T cell into the joint space and synovium. In parallel, miR-155 suppresses the expression of the inflammatory chemokine receptor CCR2 that may play a role in the retention of cells expressing it in joint spaces. In addition, my data indicate that CCR7 is positively regulated by miR-155. CCR7 and its corresponding ligands have been implicated in lymphoid neogenesis. In RA synovium, CCR7 is mainly expressed in infiltrating lymphocytes and DCs and plays a crucial role in maturation and homing of DCs to lymphocytic aggregates (285). Consistent with a crucial role of CCR7 in the regulation of development and organization of tertiary lymphoid tissues, CCR7 deficient mice are protected against collagen induced arthritis (286). Thus, I can speculate that

inflammatory monocytes expressing high levels of miR-155 and CCR7 can give rise to inflammatory DCs that are directed to ectopic lymphoid structures in the synovium.

Together, these data provide preliminary evidence that miR-155 modulates the mRNA expression of chemokines and chemokine receptors in monocytes; however, to characterize their functional consequences appropriate chemotactic assays are required. To address this, some experiments were conducted in our lab to evaluate monocyte migration using the method of Matrix Gel slide and Two Photon Microscopy. Purified PB CD14⁺ monocytes were transfected with control oligo or miR-155 or anti-miR-155 labelled with different fluorochromes CFSE (Green) or with CMTPX (Red) and incubated overnight to allow their movement into a matrix gel. The next day the distance the cells moved was examined with Two-Photon Microscopy under fluorescent light, but unfortunately, the cells did not attach to collagen matrix and we could not quantify the movement. We tried to identify what caused lack of adhesion and considered and tested the following factors: processing during separation causing cell stress; selection by micro-beads that might affect their adherence, cell stress caused by staining and finally degree of adherence to collagen IV. We identified that positive selection using micro-beads affected the adherence of monocytes to collagen therefore monocytes prepared by a more labour-intensive negative selection method was developed (data not presented). Thus, optimization of a matrix slide migration assay took longer than expected and we did not have enough time to carry out further experiments. We recommend that evaluation of functional role of miR-155 in cell migration could be performed using a trans-well system.

In conclusion, the precise mechanism by which miR-155 might govern articular inflammation is not clear. The data presented in this chapter suggest that miR-155 in part has the ability to exacerbate the secretion of pro-inflammatory cytokines and chemokines as well as to modulate the expression of chemokine receptors and thereby enhance the recruitment of inflammatory cells to the site of inflammation and their local retention and activation.

To gain a better understanding of this process, in chapter IV a similar analysis of chemokines and chemokine receptors was carried out using bone marrow monocytes (BMM) from miR-155^{-/-} and WT mice.

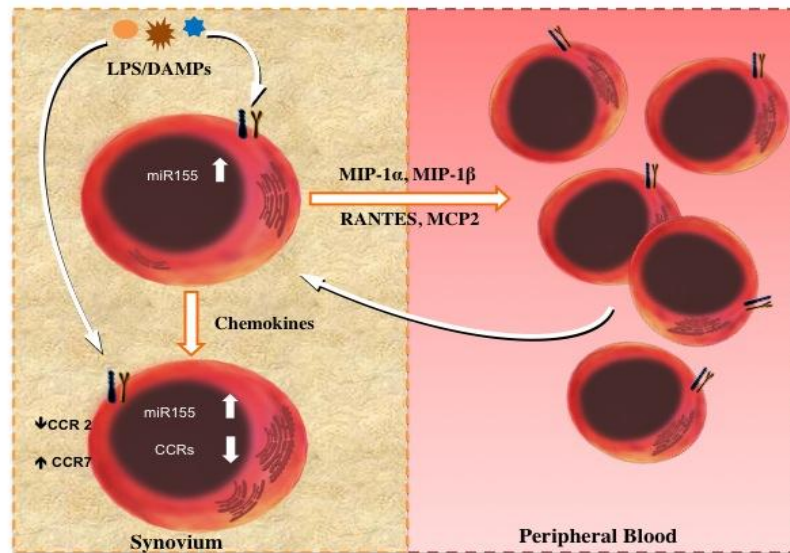


Figure 3.13 Schematic diagrams show a potential role of miR-155 pathway for autocrine and paracrine regulation of monocyte activation and migration and retention in the synovium. In RA synovium, TLR ligands strongly up regulate the miR-155 expression in synovial monocytes/macrophages and this leads to production of pro-inflammatory cytokines and chemokines. Chemokines production subsequently increases monocytes and other cells migration from peripheral circulation to synovium tissues. Simultaneously, high expression of miR-155 leads to down regulation of chemokines receptors in synovium monocytes/macrophages and retention of cells within synovium.

CHAPTER IV

EXPRESSION OF CHEMOKINES AND CHEMOKINE

RECEPTORS IN MIR-155 DEFICIENT MOUSE

MONOCYTES AND MACROPHAGES

4.1 Introduction and Aims

Recently, miR-155 has been implicated in the differentiation and activation of cells of both the innate and the adaptive immune systems (323). Furthermore, its overexpression has been linked to autoimmune disease in general and RA in particular. In humans, upregulated expression of miR-155 in PBMCs and articular tissues of RA patients has been observed (400, 574). Experimental overexpression of miR-155 in monocytes and macrophages triggered the production of TNF- α and IL-6 that are strongly implicated in RA synovitis. Most importantly, inhibition of endogenous levels of miR-155 in synovial macrophages inhibited TNF- α production. Also our group showed that mice deficient for miR-155 developed neither synovial inflammation nor cartilage and bone destruction in CIA model (367). Furthermore, bone marrow-derived macrophages of miR-155 deficient mice exhibit lower expression of some pro-inflammatory mediators, including TNF- α , IL-1 β and chemokines (CXCL1 and CXCL9) compared with WT mice (367). This observation suggested a pro-inflammatory role of miR-155 in articular inflammation.

Recruitment of effectors cells to the joint space is an important step in articular inflammation and is mediated by chemokines and their receptors. Chemokines are characterized by their common ability to direct leukocyte migration via interaction with the chemokine receptors on the leukocytes surface (223). There is an increasing number of evidences suggesting that disruption of this interaction may offer a potential therapeutic approach (289). Thus revealing the mechanism of chemokine and chemokine receptor regulation is an important point in understanding the aetiology and pathogenesis of RA.

In RA synovial tissue, monocytes/macrophages are believed to be major effector cells in synovitis operating by production of cytokines such as TNF- α , IL-6 and IL-1 β and also serving as important producers of chemokines, thereby facilitating entry of immune cells into tissue (170). Furthermore, PB monocytes express many different chemokine receptors (such as CCR1, CCR2, CCR5, CCR8, CXCR1, and CXCR4), facilitating their migration into the synovium to bind their respective ligands that are abundantly expressed in RA synovial tissue (575). I previously demonstrated that in PB monocytes of RA patients, miR-155 modulates the chemokine and chemokine receptor expression system. To further investigate the

role of endogenous miR-155, similar experiments were carried out on bone marrow monocytes cells from miR-155^{-/-} and WT mice.

No previous studies have highlighted chemokine and chemokine receptor expression in miR-155 deficient mice. This part of my work was performed to characterize the expression of chemokine receptors in bone marrow monocytes/macrophages of miR-155 deficient mice compared to WT mice. We hypothesized that monocytes from miR-155^{-/-} mice would show dysregulation of the chemokine or chemokine receptor system and thereby oppose the phenotype of human monocyte overexpressing miR-155.

Several methods and techniques were used to identify and sort bone marrow monocytes. Initially, for the characterization of monocyte populations in bone marrow, markers such as CD11b and CD115 were used as those were previously thought to be specific for monocytes. Later, it is becoming increasingly apparent that CD11b or CD115 alone were inadequate for the identification of bone marrow monocyte populations. It was therefore important to optimize the isolation method of monocytes from the bone marrow of mice to obtain appropriate purity, viability and adequate number of cells allowing to run experiments. Thereafter, I was able to address the primary question of interest namely the impact of miR-155 on chemokine and respective receptor expression.

4.2 Results

4.2.1 Optimization of bone marrow monocytes isolation

To study chemokine receptor expression on bone marrow monocytes (from now onwards mentioned only as BMMO), it was important first to establish a method to isolate pure monocytes from bone marrow with good viability and adequate number. Male miRNA-155 deficient (miR155^{-/-}) mice on a C57BL/6 (B6) background were used for this study. In brief, bone marrow cells were isolated from the femurs and tibia of mice (WT and miR155^{-/-} mice). Cells were washed and incubated with RBC lysis buffer and number of viable cells was assessed by Trypan Blue dye as described on Materials and Methods.

4.2.1.1 Preliminary Identification of BMMO using CD11b⁺ marker

Previous studies in the laboratory enriched mouse BMMO using CD11b⁺ purification Kit (Miltenyi Biotech). It is known that mouse CD11b antigen is strongly expressed on monocytes/macrophages and to a lower extent on granulocytes and NK cells. In my initial experiments, isolation of BMMO from WT and miR155^{-/-} mice (n=4) was conducted using mouse CD11b⁺ micro beads and positive selection on an Auto-MACS separator as shown on Figure 4.1. Purity of isolated CD11b positive cells was greater than 97% as assessed by FACS analysis staining with anti mouse CD11b antibody. In brief, sorted CD11b⁺ cells were seeded in 12-well plates (2x10⁶). 24 h later 100ng/mL LPS was added to some wells for a further 24 h. Total RNA was extracted from CD11b⁺ cells and transcript levels of investigated chemokine receptors were determined by TLDA plates with specific primers as described in Materials and Methods. The transcript levels of expressed chemokine receptors were presented as fold changes after normalization to 18S (housekeeping gene) and then calibrated to WT transcript levels. In the absence of LPS, CD11b⁺ cell of miR-155^{-/-} expressed higher levels of CCR1, CCR2, CCR5 and CXCR4 compared to WT as shown in Figure 4.2. LPS suppressed expression of all chemokine receptors in both WT and miR-155^{-/-} CD11b⁺ cells. Particularly, LPS significantly down regulated inflammatory chemokine receptor expression namely, CCR1, CCR2, CCR5 and CXCR4 (Figure 4.2). However, there was no significance difference in the levels of down-regulation of majority of chemokines receptors between the WT and miR-155^{-/-} mice. Expression of CCR4, CCR6, CCR8, CCR9, CXCR1, CXCR5 and

CXCR6 were below the limit of assay detection. Table 4.1 summarizes expression of chemokine receptors in CD11b⁺ cells of miR155^{-/-} compared to WT mice.

Table 4.1 Summary of chemokine receptors expression in bone marrow CD11b⁺ cells of miR-155^{-/-} compared to WT mice. CD11b⁺ cells were isolated from WT and miR-155^{-/-} mice (3 mice/group) using CD11b⁺ micro beads and Auto-MACS separator. (+), Increased; (-) Un-changed and (*) are statistically different from controls. Data were analysed with student t test * = p \geq 0.05.

	CHR	Increased	≥ 0.05
C-CR	CCR1	+	*
	CCR2	+	*
	CCR3	+	
	CCR4	-	
	CCR5	+	*
	CCR6	-	
	CCR7	+	
	CCR8	-	
	CCR9	-	
	CCR10	+	
CXCR	CXCR1	+	
	CXCR2	+	
	CXCR3	+	*
	CXCR4	-	
	CXCR5	-	
	CXCR6	-	
	CXCR7		
CX₃CR	FKR	+	
XCR1	XCR1	-	

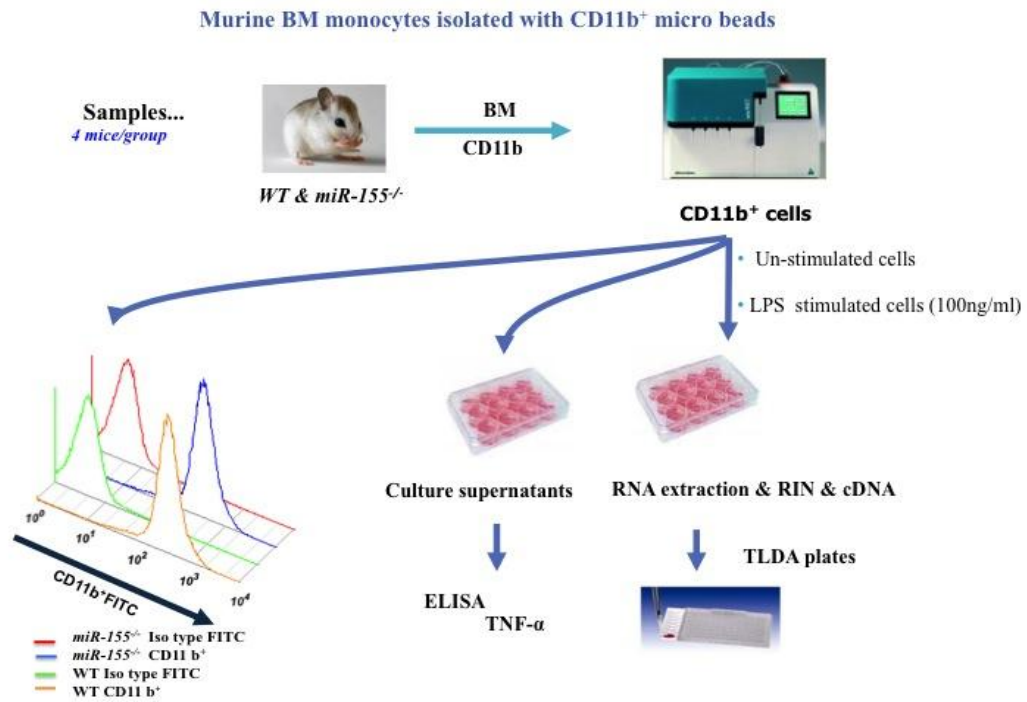


Figure 4.1 Experimental setup for enriched murine bone marrow monocytes using CD11b⁺ micro beads and Auto-MACS separator. Enriched CD11b⁺ cells were seeded in 12-well plates (2x10⁶). 24h later they were either treated or untreated with LPS (100ng/mL) for a further 24 h. Total RNA were extracted from CD11b⁺ cells and transcript levels of candidate chemokine receptors were determined by TLDA plates with specific primers. Representative histograms of CD11b⁺ positive fraction from WT and miR155^{-/-} mice (red and green lines are FITC isotype control, while dark blue and orange lines are CD11b⁺ cells for miR155^{-/-} and WT, respectively) are shown.

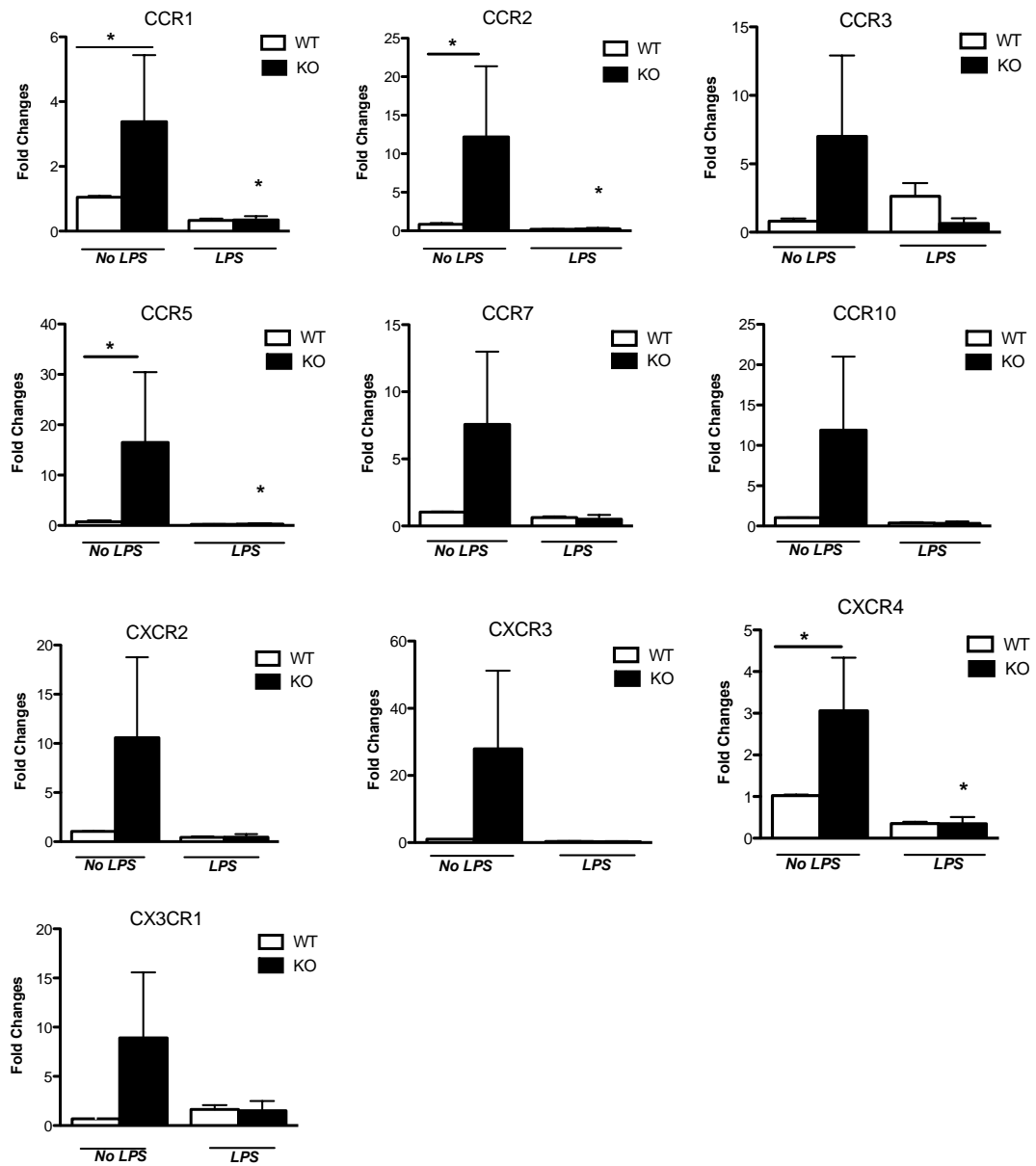


Figure 4.2 Chemokine receptors expression in bone marrow CD11b⁺ positive cells from miR-155^{-/-} and WT mice. CD11b⁺ cells were isolated from WT and miR-155^{-/-} mice (4 mice/group) using CD11b⁺ micro beads and Auto-MACS separator. Enriched CD11b⁺ cells were cultured for 24 h. after that some cells were treated with (100ng/mL) LPS for further 24 h. Total RNA was extracted and TLDA plates with specific primers determined transcript levels of candidate chemokine receptors. Transcript levels are expressed as fold change after normalization to 18S as housekeeping gene and calibrated to one mouse from WT group. All bars for the transcript data show mean fold changes of 3 replicates from each group \pm SEM and marked bars are statistically different from WT untreated LPS group and determined by Kruskal-Wallis test, * = $p \leq 0.05$.

I made a late surprising discovery that many of the $CD11b^+$ cells were not monocytes/macrophages (Figure 4.3). Although the purity of $CD11b$ positive cells was 95% using anti mouse FITC antibody (histogram Figure 4.1), not all of them were monocytes. The majority of the $CD11b^+$ population consisted of neutrophils (dot plot Figure 4.3B and D). The analysis was done with 4 colour flow cytometry techniques. Enriched $CD11b^+$ cells were stained with anti-mouse $CD11b$, $Ly6C$, $Ly6G$ and $CD115$ antibodies as shown in Figure 4.3. It revealed that $CD11b^+$ cells consisted of two populations; $LY6C^+ LY6G^-$ fraction (monocytes) and $LY6C^- LY6G^+$ (neutrophils). A representative experiment demonstrated that WT and $miR-155^{-/-}$ $CD11b^+$ cells consisted of 17% and 12.7% monocytes, respectively (Figure 2B). Thus, $CD11b^+$ was not a good marker for sorting monocyte from bone marrow as $CD11b^+$ positive fractions were contaminated with neutrophils. Therefore, I decided to isolate BMMO based on their expression of $CD115$ (the M-CSF receptor; another monocyte marker) using $CD115^+$ kits (Miltenyi Biotech) and the Auto-MACS separator.

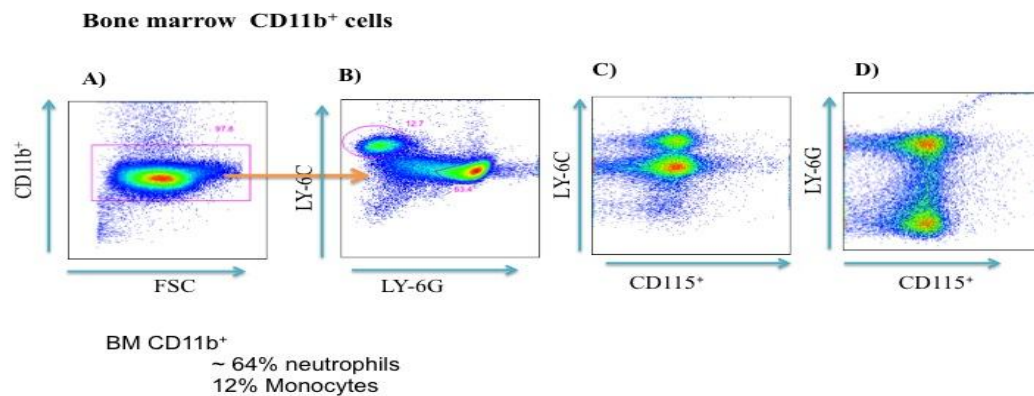


Figure 4.3 A representative purity of $CD11b^+$ cells populations from $miR-155^{-/-}$ mouse experiment that were defined by $Ly6C$, $Ly6G$ and $CD115$ expression. $CD11b^+$ cells were isolated from WT and $miR-155^{-/-}$ mice using $CD11b^+$ micro beads and Auto-MACS separator and analysed for the expression of $Ly6C$, $Ly6G$ and $CD115$ by flow cytometry. **A)** Representative dot plot expression of $CD11b$ staining cells and FSC profile. **B)** The percentages of $LY6C$ and $LY6G$ positive cells in $CD11b^+$ population **C & D)** Representative dot plots demonstrate the expression of $CD115/LY6C$ (monocytes) and $CD115/LY6G$ (neutrophils) on $CD11b$ positive cells. Results are representative of 2 experiments from each group.

4.2.1.2 Identification of BMMO using CD115 marker

CD115 is expressed on monocytes, macrophages, and osteoclasts. CD115 is a receptor for colony stimulating factor-1 (CSF-1) and signalling through CSF-1R regulates monocytes/macrophages proliferation and differentiation. Thus I decided to use CD115 to identify BMMO in mice. Therefore, bone marrow suspensions (as described above) from WT and miR155^{-/-} mice (n=2) were labelled with CD115⁺ micro beads and purified by positive selection on an Auto-MACS separator using the “possel” programme as described in Materials and Methods. To validate that sorted CD115 positive cells from WT and miR155^{-/-} mice were BMMO, cell purity was checked by FACS analysis after staining cells with antibodies against, CD11b, Ly6C, Ly6G and CD115. Data were analyzed using FlowJo software. Interestingly, BM enriched CD115⁺ cells population contained only 51.9% and 55% in WT and miR155^{-/-} mice of LY6C⁺ (monocytes), respectively. So, the purity of monocyte population was still contaminated with LY6G⁺ neutrophils (Figure 4.4B and D). Results are representative of one individual experiment.

Next, I decided to add LY6C marker for my purification strategy. I sorted the bone marrow enriched CD115⁺ cells using the FACS Aria sorter. In brief, CD115⁺ cells suspensions from WT and miR155^{-/-} mice (n=2) were stained with antibodies against CD11b, Ly6C, Ly6G and CD115 expression. Monocyte was sorted based on the expression of CD115⁺CD11b⁺Ly6C⁺ and a lack of the expression Ly6G. From the representative data in Figure 4.5C, almost 79% of enriched CD115⁺ cells population expressed the CD11b⁺; and Ly6C and CD115 (monocytes) constituted 70% from this fraction after sorting based on the expression of CD115⁺CD11b⁺Ly6C⁺ and a lack of the expression Ly6G (Figure 4.5E). Post-sorting purity of BMMO were 95% and 96% for WT and miR-155^{-/-}, respectively (Figure 4.5F and G). Cells viability was also good and determined by using Trypan Blue staining. Although purity and viability were good the total number of BMMO from sorting was very low and was not enough to set up experiments. This was likely due to long and multistep selection process that included micro beads sorting and subsequent processing through FACS Aria that could lead to cell stress and death. I therefore decided to sort fresh whole bone marrow on the basis of CD11b, Ly6C, Ly6G and CD115 expression using FACS Aria sorter only.

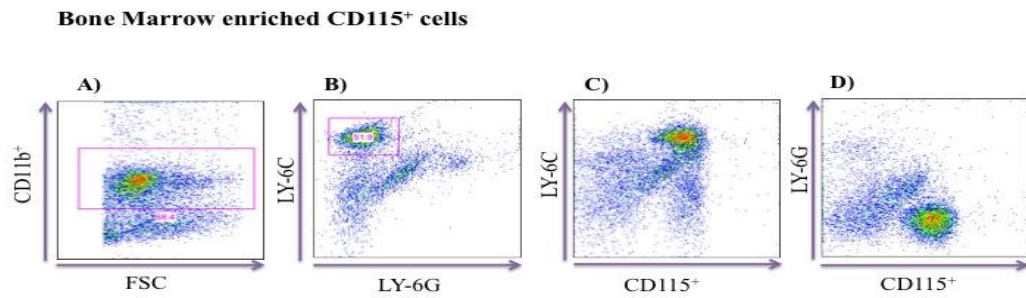


Figure 4.4 A representative WT mouse experiment shows purity of CD115⁺ cells populations that were defined by Ly6C, Ly6G and CD115 expression. CD115⁺ cells were isolated from WT and miR-155^{-/-} mice using CD115⁺ micro beads and Auto-MACS separator and analysed for the expression of Ly6C, Ly6G and CD115 by flow cytometry. A) Representative dot plot expression of CD11b staining cells and FSC profile. B) The frequency of subpopulations of CD11b⁺ cells identified on the basis of LY6C and LY6G expression by flow cytometry. C & D) Representative dot plots demonstrate the expression of CD115/LY6C (monocytes) and CD115/LY6G (neutrophils) on CD11b positive cells. Results are representative of one experiment.

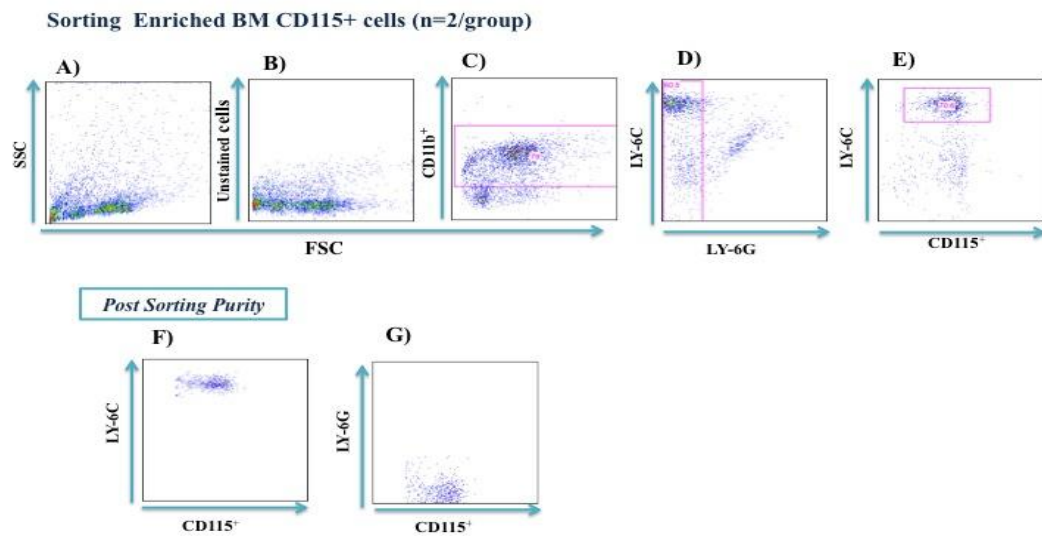


Figure 4.5 A representative miR-155^{-/-} BMMO population defined by CD11b⁺, LY6C, LY6G and CD115 expression on enriched CD115⁺ cells. CD115⁺ cells were enriched from WT and miR-155^{-/-} mice (n=2/group) using CD115⁺ micro beads and Auto-MACS separator. Live cells were sorted on the basis of the expression of CD11b, Ly6C, CD115 and lack of Ly6G using a FACS Aria. A) Representative dot plot expression of enriched CD115 using forward (FSC) and side scatter (SSC) parameters compared to unstained cells (B). C) The frequency of subpopulations of CD11b⁺ cells identified on sorted CD115 positive cells. D & E) Representative dot plots demonstrate the expression of CD115/LY6C (monocytes) and CD115/LY6G (neutrophils) on CD11b⁺ fraction from enriched CD115 cells. F & G post-sorting purity BMMO (based on their expression of Ly6C and CD115) was 95%. Results are representative of one experiment.

4.2.1.3 Sorting Whole Bone Marrow using the FACS Aria Sorter

Whole bone marrow cells suspensions were prepared as described in Materials and Methods from WT and $\text{miR155}^{-/-}$ mice ($n=2$). Cells were incubated with anti-CD16/CD32 at 4°C to reduce non-specific binding via Fc receptors after lysing red blood cells. After that cells were labelled with cell lineage specific antibodies (CD11b, Ly6C, Ly6G and CD115) as shown on Table 2.3 and incubated for 15-20 minutes at 4°C. All experiments were controlled with appropriate isotype antibodies and unstained cells. Furthermore, all acquisition was performed under sterile conditions.

First I used CD11b expression to identify the entire myeloid population on total whole bone marrow cells. This gating strategy resulted in far superior separation of CD11b^+ populations (Figure 4.6C) and revealed that, there were two populations in total BMMO; CD11b negative and CD11b positive that comprised (48% and 45%) and (41% and 53%) for WT and $\text{miR155}^{-/-}$ mice, respectively of the total of BM cells. Next, live CD11b^+ cells were identified by gating on the 7-AAD negative fraction (Figure 4.6D). Monocytes were then sorted based on their expression of CD11b, Ly6C and CD115 and a lack of Ly6G (Figure 4.6E and F). BMMO post sorting purity was performed for both WT and $\text{miR155}^{-/-}$ mice ($n=2$) (Figure 4.6G and H). Post sorting purity and viability of BMMO from WT and $\text{miR155}^{-/-}$ mice were (92% and 98%) and (96% and 98%) respectively. Furthermore, the total number of sorted monocytes was suitable to run my experiments. All data generated were analysed using FlowJo software.

Finally, analysis confirmed my earlier conclusions that most of the CD11b^+ subset was not monocytes, as some of them lacked LY6C expression (Figure 4.6D). Also my experiments revealed that the CD11b and CD115 couldn't be used to identify pure monocytes in bone marrow. Sorting monocytes based on all three markers CD11b, Ly6C and CD115 expression and a lack of Ly6G is the best methods for the identification of BMMO population.

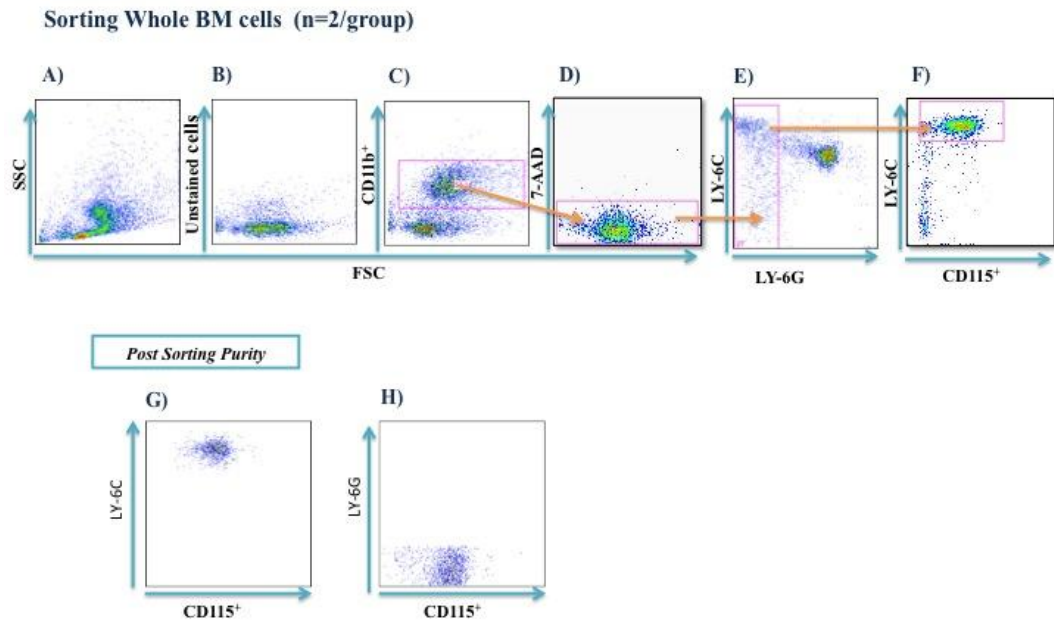


Figure 4.6 A representative of miR-155^{-/-} BMMO population defined by CD11b⁺, LY6C, LY6G and CD115 expression on whole bone marrow cells suspension. BM murine cells suspensions were prepared from WT and miR-155^{-/-} mice (n=2/group) as described in Materials and Methods. Cells were labelled with cell lineage specific antibodies then sorted for the expression of CD11b, Ly6C, Ly6G and CD115 by live-gated cells using a FACS Aria. A) Representative dot plot expression of whole bone marrow cells using forward (FSC) and side scatter (SSC) parameters compared to unstained cells (B). C) CD11b⁺ expression on whole bone marrow cells demonstrate entire myeloid population on 7-AAD negative cells also show CD11b⁺ subpopulations; CD11b negative fraction versus CD11b positive populations. D & E) Representative dot plots demonstrate the expression of CD115/LY6C (monocytes) and CD115/LY6G (neutrophils) on CD11b⁺ fraction from enriched CD115 cells. F & G post-sorting purity BMMO (based on their expression of Ly6C and CD115) were 92% Results are representative of 6 experiments.

4.2.2 Chemokine Receptor mRNA Expression in BMMO from miR155^{-/-} Mice

4.2.2.1 Basal expression of chemokine receptors in miR155^{-/-} mice

The result obtained from the preliminary analysis of chemokine receptors expression on miR155^{-/-} mouse CD11b⁺ cells suggested that endogenous miR-155 could be a negative regulator of the expression of chemokine receptors. MiR-155^{-/-} bone marrow CD11b⁺ expressed significantly higher level of the inflammatory receptors CCR1, CCR2, CCR5 and CXCR4. Similar analysis was performed on pure monocyte populations from WT and miR-155^{-/-} mice. In brief, the basal expression of chemokine receptors was examined in RNA of BMMO that were sorted on the basis of CD11b, Ly6C, CD115 and lack of Ly6G expression using FACS Aria sorter as described above. Total RNA samples were extracted directly after sorting (2mice/group/of total 4 exps). After cDNA synthesis TLDA plates were performed with primers specific for mice chemokines receptors. In miR-155^{-/-} mice transcription levels of chemokine receptors within BMMO were presented as fold change. Their expression was normalization to 18S (housekeeping gene) and then calibrated to transcript levels of WT mice.

Consistent with the preliminary data on CD11b⁺ cells, analysis of the chemokine receptors expression in miR-155^{-/-} BMMO confirmed the upregulation of a number of chemokine receptor transcripts levels compared with WT. As shown in Figure 4.7, miR-155^{-/-} BMMO expressed significantly higher levels of CCR1, CCR2, CCR3, CCR5, CCR9, CXCR3, CXCR4 and CX₃CR1. In contrast CCR7 was statistically down regulated in miR-155^{-/-} BMMO compared to WT mice (Figure 4.7). No alterations in CXCR2, CXCR5 and CXCR6 expression were observed. Other chemokine receptors (CCR6, CCR8, CCR10, CXCR1 and XCR1) were below the limit of detection in both WT and miR-155^{-/-} BMMO. Together these data suggested that endogenous miR-155 is a negative regulator of the expression of chemokine receptors and might be one the factors that contributes to optimal expression of some chemokine receptors in BMMO and could therefore be involved in regulating the biology of these cells.

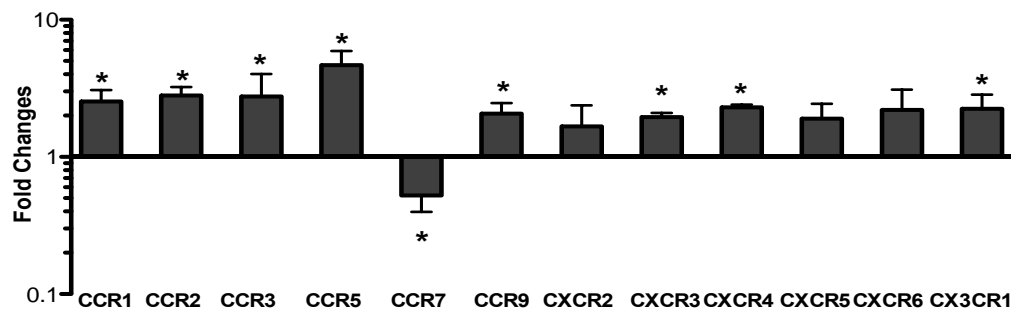


Figure 4.7 Chemokine receptor expression in BMMO that were sorted on the basis of CD11b, Ly6C, CD115 and lack of Ly6G expression. Total RNA samples were extracted directly after sorting (2mice/group/of total 4 *exps.*) and transcripts levels of chemokine receptors expression were determined by QPCR using TLDA plates. Chemokine receptors expressions in miR-155^{-/-} BMMO were presented as fold change after normalization to 18S as house keeping gene and then calibrated to transcript levels of WT mice. All bars for the transcript data show mean fold changes of 3 replicates of total 4 experiments from each group \pm SEM, and statistical significances were evaluated by using Mann-Whitney test * = $p \leq 0.05$.

4.2.2.2 Chemokine Receptor Expression after LPS Stimulation

LPS stimulation is known to down regulate inflammatory chemokine receptor expression in monocytes (576-578). Next I tested whether miR-155 is involved in that process. BMMO were sorted as described earlier from both WT and miR-155^{-/-} mice (4mice/group/of total 2 exps.). Likewise, freshly purified cells were seeded in 12-wells plates (2x10⁶) and after 24 h some wells were stimulated with LPS (100ng/ml) or left un-stimulated and cultured for further 24 h. The analysis was performed on total RNA. Chemokine receptors transcription levels were normalization to 18S. I examined the impact of LPS on chemokine receptor expression in WT and miR-155^{-/-} monocytes.

First, to study the impact of LPS on expression of chemokine receptor expression in either the WT or miR-155 deficient mice, the transcript levels of chemokine receptors expressed in BMMO of WT or miR-155^{-/-} LPS treated samples were calibrated by WT or MiR-155^{-/-} LPS un-stimulated transcript levels (Figure 4.8A and B), respectively. Overall, exposure of BMMO to LPS resulted in down regulation of expression of a majority of inflammatory chemokine receptor transcripts in both WT and miR-155^{-/-} monocytes compared to respective LPS un-treated monocytes. For example, BMMO of WT mice exhibited down regulation of inflammatory chemokine receptors, with CCR1, CCR2, CXCR2 and CXCR4 being most highly down regulated after the treatment with LPS Figure 4.8A. BMMO of miR-155^{-/-} mice showed a trend to down regulation of the same receptors (CCR1, CCR2 and CXCR4) after treatment with LPS compared to non-LPS miR-155 deficient monocytes (Figure 4.8B). However, its important to mention here I conducted two experiments therefore its was impossible to determined levels of significance. In contrast, in neither WT nor MiR-155^{-/-} mice LPS did not affect the expression levels of CCR5, CCR7 and CCR9.

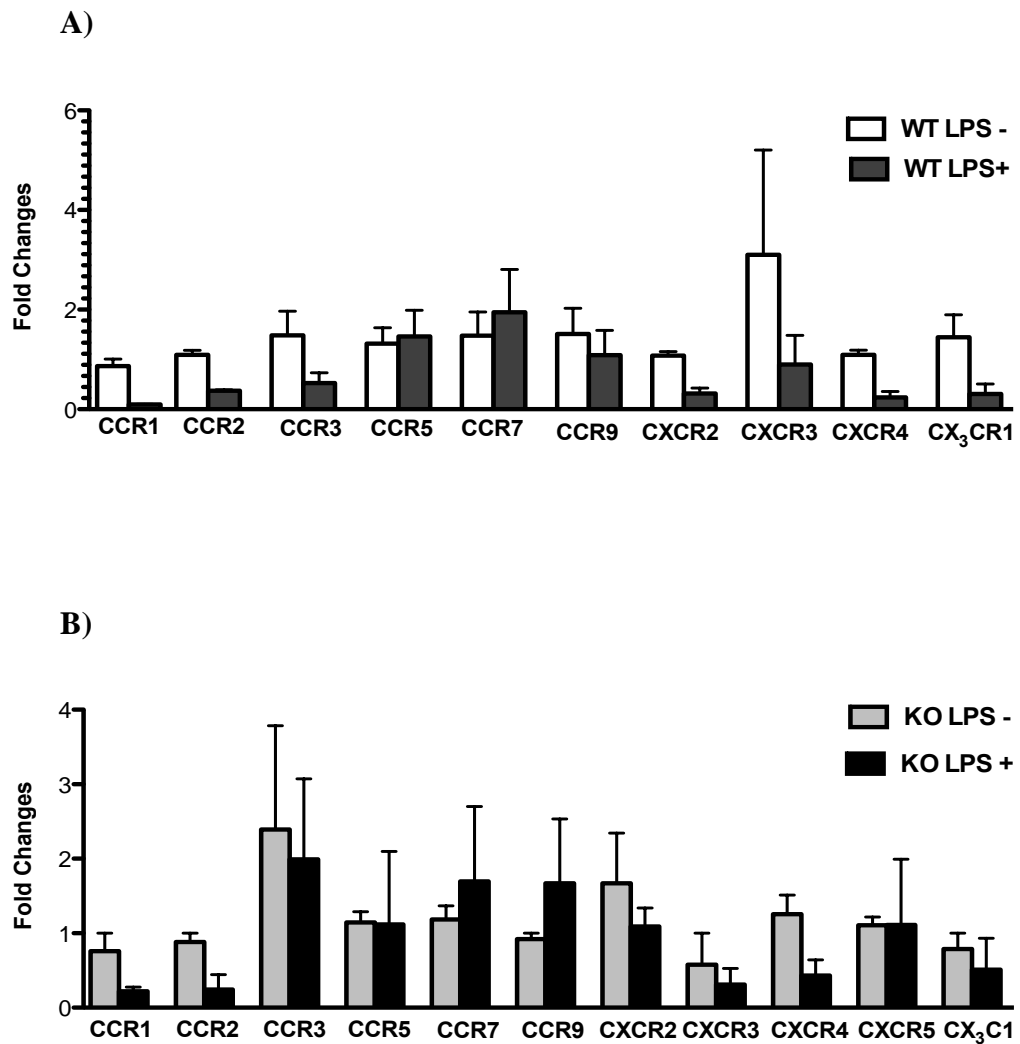


Figure 4.8 Quantitative analysis to study the impact of LPS in chemokine receptor mRNA expression by BMMO of miR-155 deficient mice or WT after LPS treatment. BMMO were sorted on the basis of CD11b, Ly6C, CD115 and lack of Ly6G expression from whole bone marrow cells of WT and miR-155^{-/-} mice (4mice/group/of total 2exps) using FACS Aria. BMMO were seeded in 12-wells plates (2x10⁶) and after 24 h stimulated with LPS at 100ng/mL or left untreated for further 24 h and chemokine receptors expression determined by TLDA plates. Transcripts of chemokine receptors expressed in cells of WT or miR-155^{-/-} after LPS treated were calibrated to (A) WT LPS untreated or B) KO LPS untreated, respectively. All bars show mean fold change of 2 replicates \pm SEM compared with controls. Its important to mention here I conducted two experiments therefore its was impossible to determined levels of significance.

Next I examined whether the expression level of chemokine receptors differed between WT and miR-155^{-/-} mice after LPS stimulation. I used one mouse from the LPS un-treated group of WT as a calibrator control (Figure 4.9). Firstly, in contrast to freshly sorted monocytes, WT and miR-155^{-/-} monocytes cultured for a total time of 48 h (24 h before and 24 h after LPS stimulation) in complete media did not differ in chemokine receptor expression (Figure 4.9). Indeed, both WT and miR-155 deficient monocyte showed similar phenotype in terms of chemokine receptor expression once incubated in culture media, likely due to effect of serum. Therefore, I decided to examine the effect of culture and media on chemokine receptor expression in comparison to basal expression (see below, Figure 4.10). While, analysis of LPS-treated cultures demonstrated that BMMO of WT and miR-155^{-/-} mice showed significantly lower magnitude of down-regulation of CCR1, CCR2, and CXCR4 transcripts compared to WT untreated cells (Figure 4.9). Notably, CXCR2, CXCR3 and CX₃CR1 were also down regulated after LPS stimulation but compared to controls; did not reach the levels of significance. No alterations in CCR5, CCR7 and CCR9 expression were observed. Taken together these observations suggested that miR-155 does not contribute to LPS induced down-regulation of inflammatory receptor in monocytes as the same effect where observed in both WT and miR-155^{-/-} BMMO.

Chemokine receptor expression in BMMO both WT and miR-155^{-/-} mice after 48 h cultures was compared to basal expression. One mouse from WT or miR-155^{-/-} basal expression were used to calibrate the transcript levels of chemokine receptors expression in BMMO of WT or miR-155^{-/-} after 48 h culture (Figure 4.10A and B), respectively. Up regulation of CCR3, CCR7 and CXCR2 in WT monocytes and CCR3, CCR7, CCR9 and CXCR2 in miR-155 deficient cells were observed. Of interest, CCR1 and CCCR2 show either unchanged or a trend of down regulation after 48 h culture compare to basal expression.

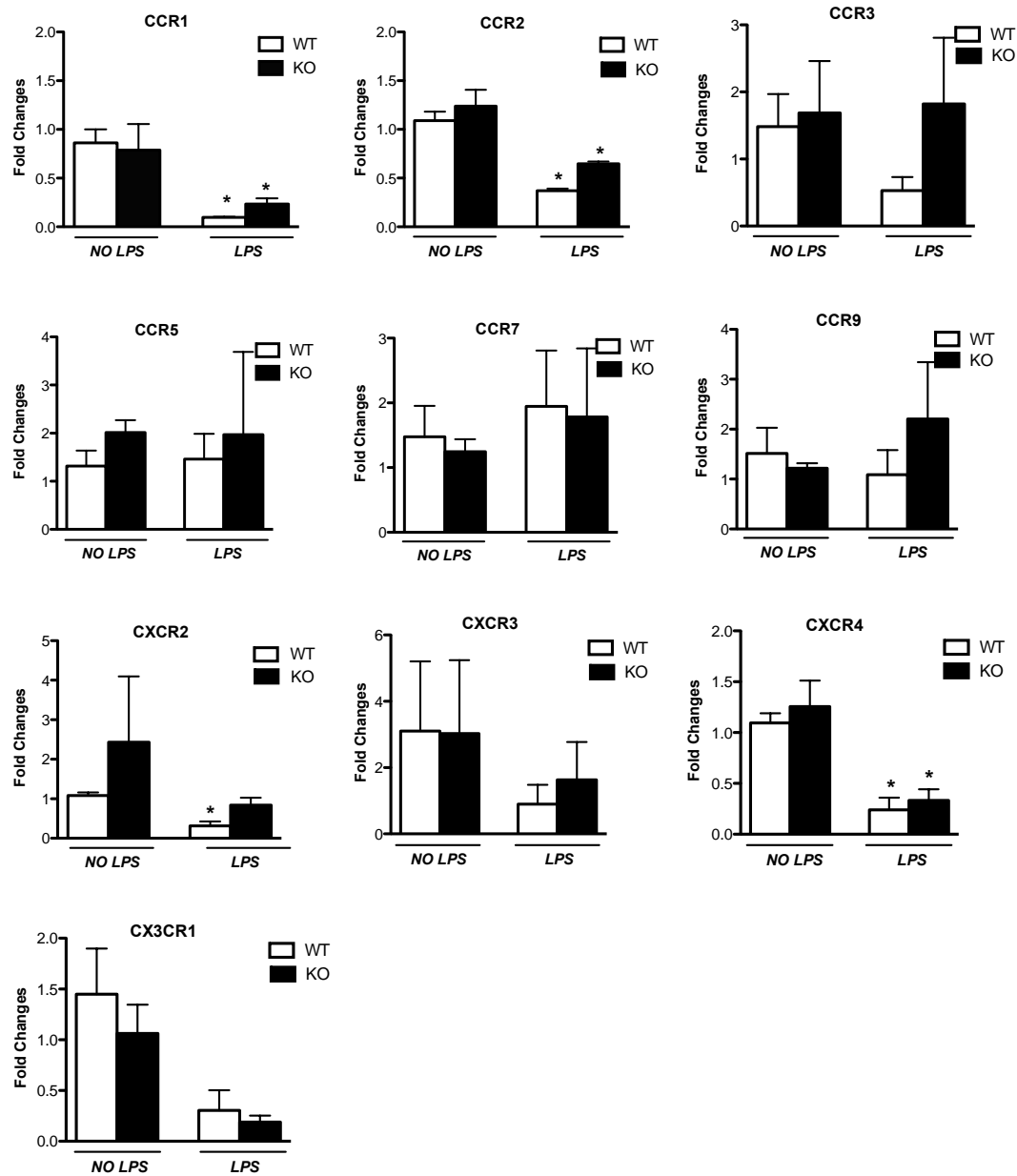


Figure 4.9 Quantitative analysis to study the impact of LPS in chemokine receptor mRNA expression by BMMO from miR-155 deficient mice or WT after LPS treatment. BMMO were sorted on the basis of CD11b, Ly6C, CD115 and lack of Ly6G expression from whole bone marrow cells of WT and miR-155^{-/-} mice (4mice/group/of total 3exps) using FACS Aria. BMMO were seeded in 12-wells plates (2x10⁶) and after 24 h stimulated with 100ng/mL LPS or left untreated for further 24 h and chemokine receptors expression determined by TLDA plates with candidate chemokine receptors. Transcripts levels of chemokine receptors expressed in cells of WT or miR-155^{-/-} before and after LPS treated were calibrated by one mouse from of WT LPS untreated group. All bars show mean fold change of 2 replicates ± SEM compared with controls. Marked bars are statistically different from WT LPS un-stimulated cells and determined by Kruskal-Wallis test, *P ≤ 0.05.

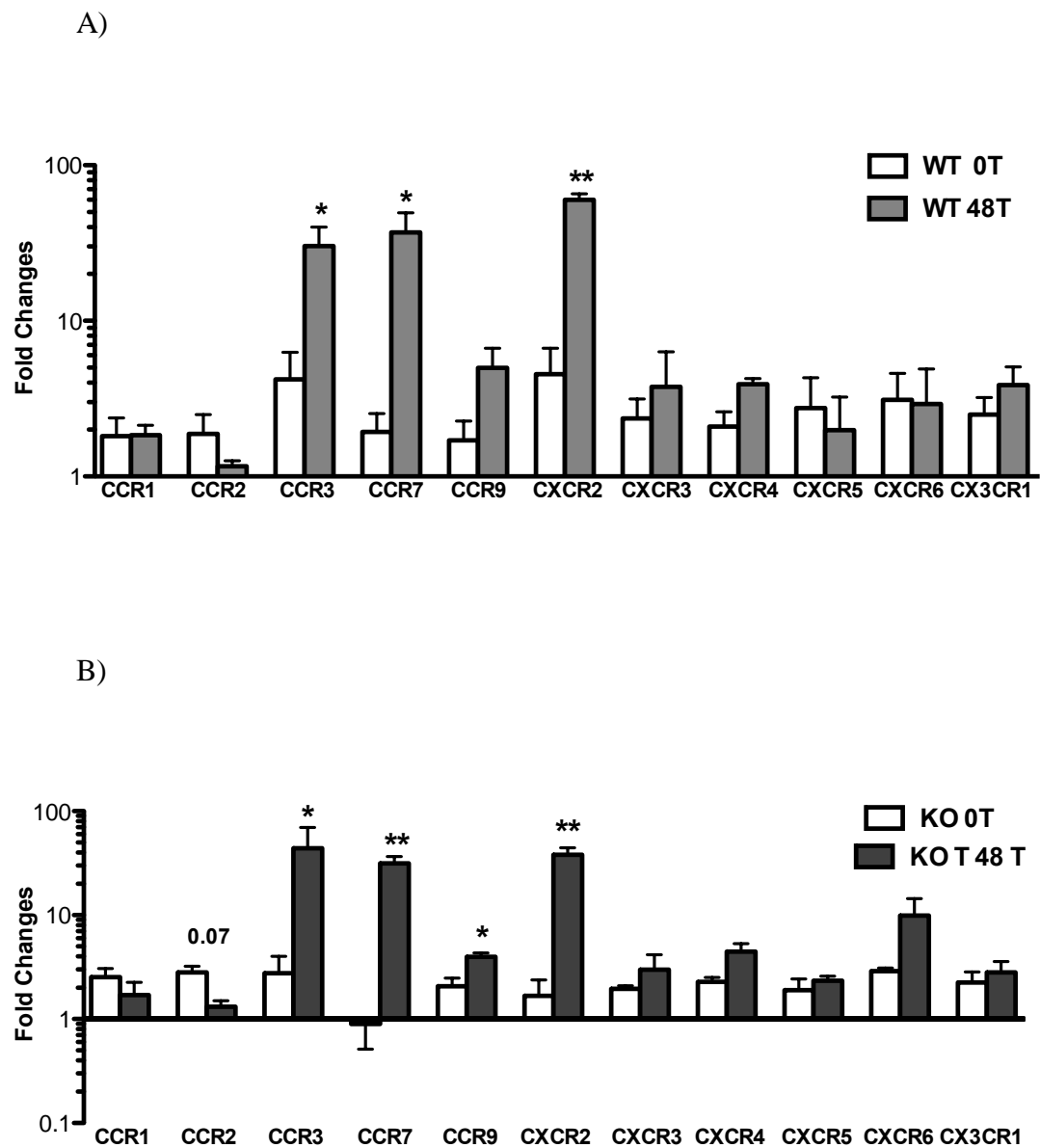


Figure 4.10 Quantitative analysis of chemokine receptors mRNA expression in BMMO of WT and miR-155 deficient mice that directly after sorting or after 48 h of culture. RNA from freshly sorted cells (2mice/group/of total 4exps) and 48 h cultured (4mice/group/of total 3exps) were isolated and TLDA plates were performed with primers specific for mice chemokine receptors. Cells cultured for 48 h were calibrated against the transcript levels of one mouse from basal expression A) WT and B) KO, respectively. All bars show mean fold change of 2 replicates \pm SEM compared with controls. Marked bars are statistically different from control group and determined by Kruskal-Wallis test; * $P \leq 0.05$.

4.2.3 Cytokine and Chemokine Production by BMMO of miR-155^{-/-} Mice

Next, I looked at the production of soluble mediators by miR-155 deficient BMMO. After 24 h from culture, cells were stimulated with 100ng/ml of LPS and supernatant collected 24 h later. Cytokine and chemokine levels were measured by Luminex assay (Figure 4.11). Overall, miR-155 deficient cells showed a tendency towards lower production of pro-inflammatory cytokines and chemokines. However, IL-6 was the only cytokine significantly decreased in miR-155 deficient cells compared to WT. Furthermore, miR-155^{-/-} BMMO produced significantly less IL-6 upon stimulation with LPS. Similar trends were observed with TNF- α , IL-12 and CCL2/MCP-1. IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-10, IL-13, IL-17, IFN- γ , KC, CXCL9/MIG, VEGF, FGF and GM-CSF were below the limit of detection.

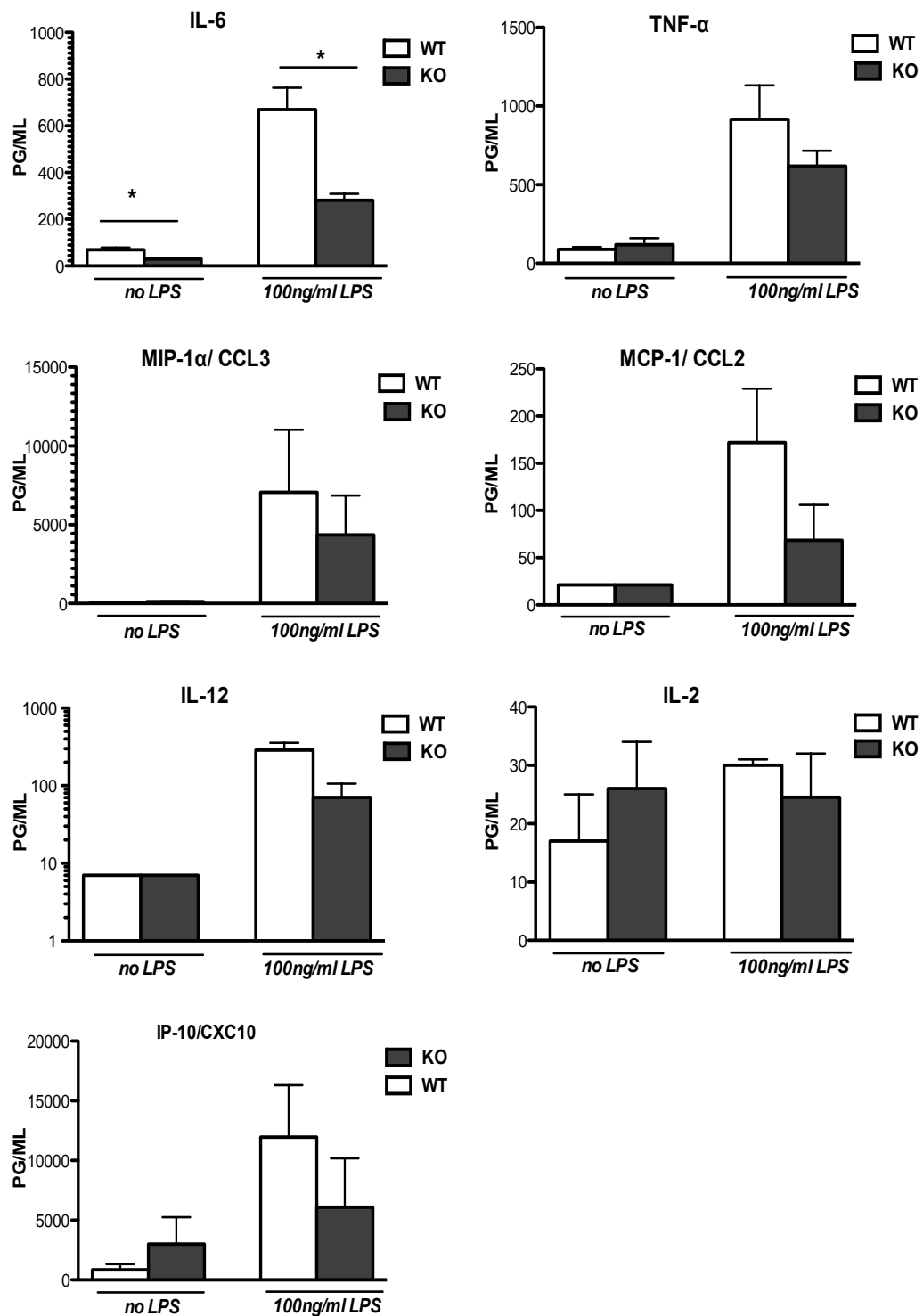


Figure 4.11 Cytokines and chemokines level produced by BMMO of miR-155 deficient mice. BMMO were sorted on the basis of CD11b, Ly6C, CD115 and lack of Ly6G expression from both miR-155^{-/-} and WT mice (4mice/group/of total 2exps). Cells were cultures 24 h and then stimulated with (100ng/mL) LPS or left untreated for 24 hours and cytokines and chemokines level were measured in the supernatant by Luminex assay. All bars show mean of 2 replicates \pm SEM compared with controls. Marked bars are statistically different from control group using Kruskal-Wallis test; *P \leq 0.05.

4.2.4 Chemokine Receptor Expression in miR-155^{-/-} Macrophages

I then investigated the expression of chemokine receptors in bone marrow–derived macrophages of both WT and miR-155^{-/-} mice using TLDA plates. Macrophages were grown from murine bone-marrow precursors in complete medium supplemented with mouse M-CSF for 6 days. On day 3 of culture, media with non-adherent cells were removed and replaced with fresh media. After that cells were seed on 6 wells plates (1.5×10^6 /ml) for 24 h. Some wells were stimulated with LPS (100ng/ml) or left un-stimulated and cultured for further 24 h. One mouse was used to generate WT LPS un-treated macrophages and was used as a calibrator in analysis.

Bone marrow–derived macrophages of miR-155 deficient mice expressed higher levels of CCR1 and CXCR4 compared to WT macrophages (Figure 4.12). In addition, CCR3 expression was increased in miR-155^{-/-} mice macrophages compared to the WT but did not reach the levels of significance ($P=0.06$) (Figure 4.12).

Next, exposure of bone marrow–derived macrophages to LPS resulted in a down regulation of expression of the majority of inflammatory chemokine receptor transcripts in both WT and miR-155^{-/-} macrophages compared to un-treated cells. As shown in Figure 4.12, LPS treated bone marrow–derived macrophages of WT displayed significant down-regulation in the expression of CCR1, CCR2, CCR3, CXCR3 and CXCR4 with CXCR4 being the most highly down regulated. Likewise, LPS treated miR-155^{-/-} macrophages exhibited down-regulation of the CCR1, CCR2, CCR5, CXCR3 and CXCR4 compared to un-treated WT macrophages (Figure 4.12). Interestingly, CCR1 was significantly down regulated in miR-155 deficient macrophages in comparison to WT after LPS stimulation.

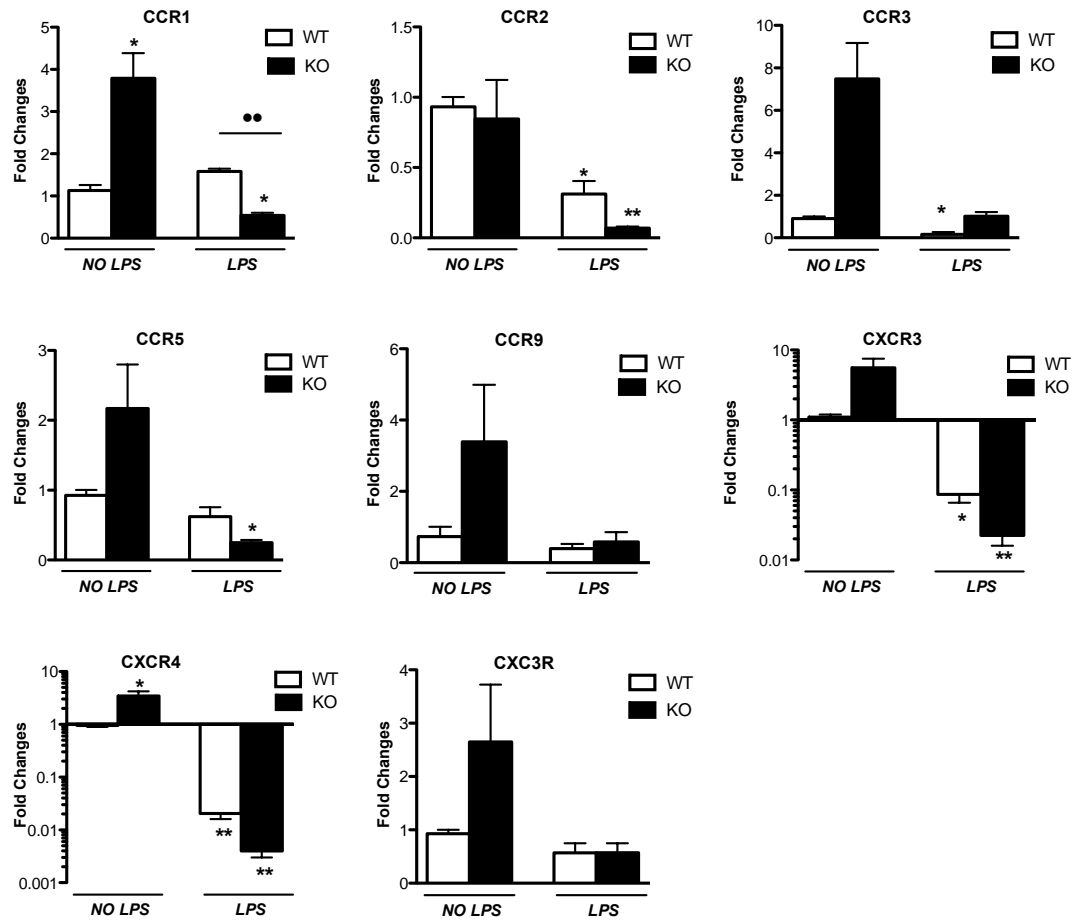


Figure 4.12 Chemokine receptors expression in bone marrow derived macrophages of miR-155^{-/-} and WT mice. Bone marrow-derived macrophages either from WT or miR-155 deficient mice (n=3 mice/groups) were seeded on 6 wells plates (1.5x10⁶/ml) for 24 h. After that cells were either left untreated or were stimulated with 100ng/mL LPS for 24 h. Total RNA was extracted post cultures and QPCR and TLDA plates were performed with primers specific for mice chemokine receptors. Transcript levels of chemokine receptors in miR-155^{-/-} macrophages were presented as fold change after standardization against 18S and calibration to transcript levels of WT -treated cells. All bars for data show mean \pm SEM. Marked bars are statistically different from LPS un-stimulated WT cells determined by Kruskal-Wallis test; *P \leq .05, **P \leq .005, while • sign mean there is a significant difference between the LPS stimulated cells.

4.3 Discussion

Monocyte traffic from blood into tissue occurs constitutively and this is increased during inflammation where they then can play a central role in pathogenesis. In RA infiltration and accumulation of monocytes/macrophages in synovial tissue is a key first step of the complex process that ultimately leads to joint destruction. Therefore identification of molecules involved in cell migration may facilitate the development of new therapeutic strategies. An increasing number of reports demonstrate a crucial role for miR-155 in the pro-inflammatory activation of human myeloid cells and antigen-driven inflammatory arthritis but its effects on inflammatory cells migration and accumulation at sites of inflammation have not been defined yet. In the previous chapter, I showed that overexpression of miR-155 would enhance inflammation via dysregulation of chemokine or chemokine receptors expression in human PB monocytes. Therefore, the main aim of this chapter was to examine chemokine receptors expression in BMMO of miR-155 deficient mice compared to WT mice in steady state condition and upon LPS stimulation.

First of all, it was important to establish a method to isolate pure monocytes from the bone marrow of miR-155 deficient mice with good viability and adequate numbers. Monocytes comprise heterogeneous populations that are defined on the basis of differential expression of various myeloid markers. CD11b is highly expressed on monocytes but is also abundant on neutrophils (579, 580). Similarly, the Gr-1 (Ly6C-Ly6G) family of antigens has been used to identify neutrophils, monocytes and DCs. The Ly-6G is almost exclusively neutrophil specific, while Ly-6C is expressed on monocytes (579, 580). Furthermore, monocyte colony-stimulating factor receptor CD115 is expressed on monocytes, macrophages and DCs (581, 582). In our study we sorted BMMO based on the combinations of expression of CD11b, Ly6C and CD115 and lack of expression of the neutrophil marker Ly6G using the FACS aria sorter, and that approach gave us a high purity monocyte population.

For many years, it has been appreciated that monocytes are a highly dynamic population; both mobilization from bone marrow to bloodstream or recruitment from blood to sites of inflammation is mainly regulated by chemokine-chemokine receptor interactions. Recent reports suggest that blood monocytes consist of several

functionally diverse subsets characterized by distinct chemokine receptor expression (209). In this study, we observed that BMMO of miR-155^{-/-} mice express higher levels of many chemokine receptors, including CCR1, CCR2, CCR3, CCR5, CCR9, CXCR3, CXCR4 and CX₃CR1, while CCR7 is significantly down regulated as compared to WT cells. In summary, this suggests that endogenous miR-155 plays a role in regulation of monocyte trafficking in a homeostatic manner (583-585). This effect is likely to be mediated in steady state since in many of my experiments I did not seek to up regulate miR-155 *a priori* yet noted effects in the miR-155 knockout state.

Although it is well established that CCR2 is necessary for the recruitment monocytes to sites of inflammation, namely “synovial tissue” (287), recent evidence demonstrates that CCR2 is also essential for the mobilization of newly-formed monocytes out the bone marrow to the peripheral circulation. Tsou *et al.* have clearly demonstrated that CCR2^{-/-} mice have a marked reduction in a subset of circulating blood monocytes due to failure of monocytes to exit the bone marrow with a concomitant increase in bone marrow monocytes and monocyte precursors (584). This suggests that CCR2 expressed on monocytes is not only required in response to inflammation but also appears to be important in the mobilization of monocytes from the bone marrow to the blood under normal homeostatic state and for normal monocyte homeostasis. Our data suggest that miR-155 may act as an important negative regulator of CCR2 expression in monocytes affecting the migration of monocytes out of bone marrow to the periphery.

Another two chemokine receptors that appear under control of miR-155 are CCR5 and CX₃CR1. They are thought to contribute to monocyte homeostasis by decreasing monocyte numbers in PB (586, 587). One possibility is that CX₃CR1 directs monocyte trafficking from PB into non-inflammatory tissue (588). Interestingly, Geissmann *et al.* demonstrated existence of at least two major subsets of circulating murine monocytes; firstly, a short-lived inflammatory subset CX₃CR1^{low}CCR2⁺ that selectively home to inflamed tissue. Their phenotype resembles that of classical human CD14⁺CD16⁻ monocytes. The second subset of patrolling monocytes is characterized by longer half-life and high expression of CX₃CR1^{high}CCR2⁻. This subset homes constitutively to non-inflamed tissues where

they can differentiate into resident macrophages (588). This subset is analogous to human CD14⁺CD16⁺CD64⁻ monocytes. Given that miR-155 deficient monocytes express higher levels of both CCR2 and CX₃CR1, miR-155 may orchestrate homeostatic trafficking of both monocyte populations. Consistent with my data Etzrodt *et al.* have demonstrated that miR-155 is highly expressed in Ly-6C^{low} monocytes (CX₃CR1^{high}CCR2⁻) (589). Like miR-146, miR-155 could be one of the factors that may regulate the balance between monocyte subset in the circulation.

In addition, miR-155 deficient monocytes showed upregulation of CXCR4 expression. Although, it is well established that SDF-1/CXCR4 interactions play a role in migration and retention of monocytes in RA synovium (565), CXCR4 also seems to be important in the regulation of monocyte trafficking to and retention in the bone marrow under homeostatic conditions as demonstrated by Wang *et al.* (585). Our data suggest that balance between monocyte migrations in and out of bone marrow is regulated by miR-155. To functionally explore the contribution of miR-155 to the regulation of monocytes migration, monocyte mobilization in and out of bone marrow in response to their ligands should be tested. One attractive experiment would be to perform adoptive transfer of bone marrow from WT and miR-155KO with congenic allele CD45.1 in 1:1 ratio to irradiated WT mice and then evaluate blood and bone marrow monocyte populations 8 weeks after bone marrow reconstitution.

I also demonstrate that miR-155^{-/-} bone marrow derived macrophages express higher CCR1 and CXCR4 compared to WT mice. Thus, miR-155 might have a dominant role in regulation of the CCR1 and CXCR4 in both monocytes and macrophage.

Consistent with higher expression of various chemokine receptors in miR-155 deficient monocytes that I presented in this chapter, Donners *et al.* showed more inflammatory monocytes (LY-6C^{high}) and reduced number of resident subsets in the circulation of miR-155 deficiency mice (354). These findings indicate that endogenous miR-155 may act as an important homeostatic regulator of chemokine receptors in monocytes. Furthermore, this study confirms previous observations that the lack of miR-155 is sufficient to reduce the production of the IL-6, by resting and LPS stimulated monocytes, which is of particular importance in the RA pathogenesis

as IL-6 is validated clinical therapeutic targets (366, 367). However, understanding the biological significance of endogenous miR-155 as a negative regulator of these receptors in homeostatic state needs further evaluation. Most of these queries will be answered in the future by investigating receptors' protein expression using FACS and by performing cell migration assays in which the particular contribution of miR-155 to the regulation of monocytes migration can be dissected. For example, to determine whether this change in receptor transcript levels is associated with an altered chemokine responsiveness, BMMO should be examined for their ability to migrate toward ligands.

We next hypothesized that altered chemokine receptor expression in miR-155 deficient mice may underpin the inflammatory effects of TLR4 and therefore investigated the impact of LPS on chemokine receptor expression in BMMO from these mice compared to WT mice. Several studies have reported regulation of inflammatory chemokine receptor expression by ligands for TLR2 and TLR4 through many mechanisms including inhibition of transcription or receptor internalization induced by TLR induced chemokine(577). The data obtained from these experiments demonstrated that TLR4-ligand (LPS) induced reduction in transcript levels of some chemokine receptors in both miR-155 deficient monocytes and macrophage. Interestingly, LPS suppressed the inflammatory chemokine receptors such CCR1, CCR2, and CXCR4, however, it did not affect the expression of homeostatic e.g. CCR7. Of interest, there was the same degree of down regulation of a majority of chemokine receptor in both WT and miR-155 monocytes suggesting that miR-155 is not obligatory in this process. According to the microRNA prediction algorithms none of chemokine receptors are predicted to be a direct target of miR-155. Thus future work needs to be done to identify a target or set of multiple targets responsible for the miR-155 mediated effect on chemokine receptor system. One of the possibilities to be tested is whether the changes to the composition of chemokine receptors on miR-155KO cell are due to the fact that miR-155 is predicted to target M-CSFR, a survival and maturation factor for monocyte and macrophages.

In summary, our study demonstrated that under steady-state conditions, endogenous miR-155 attenuated inflammatory chemokine receptor expression and drives CCR7

expression. This implicates the positive role of miR-155 in recruitment of monocytes to LN, where they can differentiate towards DCs. Upon LPS stimulation, most inflammatory chemokine receptors (*e.g.* CCR1, CCR2, CXCR4) were down regulated regardless of the expression of miR-155. In contrast inflammatory soluble mediators were reduced (*e.g.* IL-6) in miR-155 deficient BMMO suggesting a positive role of miR-155 in regulation of proinflammatory cytokines and chemokines.

CHAPTER V

ROLE OF SPHK/S1PR AND THE S1P AXIS IN RHEUMATOID ARTHRITIS

5.1 Introduction and Aims

My prior studies focussed on miRNA mediated regulation of intracellular effector function in leukocytes in RA. I wished in parallel studies to also address a candidate signal cascade for expression and potential functional importance. The increasing interest in the development of small molecular inhibitors for the treatment of RA lead me to consider this as an area for further investigation. I now report a series of experiments addressing the expression of a family of lipid kinases in RA tissues.

One particular success of human genome sequencing by Next Generation Sequencing technologies was the revelation that the human “kinome” consists of 518 kinases (590). The kinases or phosphotransferases are enzymes that biocatalyze the transfer of a phosphate group to their specific substrate proteins and regulate an array of cellular processes (590). SPHK, a type of lipid kinase, may be activated by various stimuli (415). SPHK is ubiquitous and has two isozymes, SPHK1 and SPHK2. Both isozymes have five highly conserved domains (415). The sphingosine backbone of the sphingolipids, a key structural part of plasma membrane in mammalian cells, forms ceramide by the coupling of a fatty acid by an amide bond. Ceramide is deacylated by ceramidase to release sphingosine that is subsequently phosphorylated either by SPHK1 or SPHK2 to produce the highly pleiotropic cytokine, S1P. S1P binds with any one of the five S1PRs on the plasma membrane in an autocrine or paracrine manner to promote different types of cellular and molecular responses (415). To maintain homeostasis, the cellular concentration of S1P is critically controlled in low concentrations. S1P phosphohydrolases, lysophospholipid phosphohydrolases or S1P lyase enzymes degrade S1P into ethanolamine and hexadecenal (430, 431, 478, 479).

Studies have indicated that the S1P/SPHK axis plays a significant role in the induction of various types of inflammatory responses and disease pathologies in cancer, arthritis, asthma, and ulcerative colitis (415). Recent investigations in animal experimental arthritis using the CIA model have clearly demonstrated that the serum S1P level are elevated and the SPHK1 expression was increased in the synovial membrane of mice with arthritis (416, 417). Furthermore, SF of RA patients exhibited higher levels of S1P than those with OA (416). It was observed that the S1P/S1P₁ axis played a role in synovial proliferation and induced COX-2 expression

(418). S1P was also required for the ability of the TNF- α to induce COX-2 leading to PGE2 production (418) and the level of SPHK2 are found to be increased in synovial fibroblasts contributing to increased proliferation (591).

Moreover, defects in the S1P/S1PR signalling have been shown to be associated with various pathologies. Hence, in our present study, we have investigated the critical role and clinical significance of S1P/S1PR axis in PB cells and the synovium of RA patients in comparison to healthy controls and OA patients, respectively.

To explore the role of S1P/S1PR axis in the pathogenesis of RA we examined the intracellular expression and mRNA levels of SPHK1/2 and S1PRs in isolated PB CD15⁺ neutrophils, CD14⁺ monocytes and both CD4⁺ and CD8⁺ T lymphocytes of RA patients compared to healthy controls (n=10) using FACS technique and QPCR, respectively. Sorting of PB cells was performed using density gradient with different density of Histo-Hypaque gradient. Followed by serial purification of CD14⁺ monocytes and both CD4⁺ and CD8⁺ T lymphocytes using micro beads and an Auto-MACS separator as described in Materials and Methods. Next I extended this work to study the SPHK1/2 and S1PRs expression in PB cells of RA patients receiving different therapeutic regimens, including SSZ (n=11), MTX (n=10) biological DMARDs (n=9) and compared these to healthy controls (n=10). Furthermore, I examined the expression and localization of SPHK1/2 and S1PRs in the synovial tissues from patients with RA and compared to OA (n=4) by single IHCs analysis and Vector Elite ABC Methods. Competitive ELISA assessed S1P level in serum of RA patients compared with healthy controls.

5.2 Results

5.2.1 Patient characteristics

PB samples were obtained from healthy controls and patients with RA who met the diagnostic criteria of 2010 ARC/EULAR (592). Patients' samples were derived from the routine outpatient Rheumatology Clinic, Glasgow Royal Infirmary (Glasgow, UK). Patient demographics and clinical details such as the disease duration, treatment regimens and biochemical parameters are presented in Table 5.1. Disease activity of RA patients recruited in this project was calculated using the DAS28. All the RA patients involved in this study were sero-positive for RF.

5.2.2 Detection of S1P in RA serum

The levels of S1P in the serum of PB obtained from healthy controls (n=20) and RA patients (n=40) were measured using a competitive ELISA on triplicate samples. S1P was significantly higher in RA patients' serum up to $58.9 \pm 8.1 \mu\text{M}$ (mean \pm SEM) than those observed in serum of healthy controls ($16.5 \pm 2.9 \mu\text{M}$) ($P=0.0001$) Mann-Whitney U test (Figure 5.1A). Further analysis was carried out to examine whether drug therapy influenced S1P levels. Therefore, S1P concentration was examined in the serum of RA patients who were receiving different therapeutic regimens including SSZ (n=11), MTX (n=10), biological DMARD (n=9) compared to healthy controls who were receiving no immune modifying therapeutics (n=10). RA patients treated with conventional or biological DMARDs (Infliximab, Adalimumab, Tocilizumab, Rituximab) showed statistically significantly higher levels of S1P than that observed from healthy controls (Figure 5.1B). Additionally, RA patient treated with biological DMARD expressed the highest levels compared with healthy controls. Although, S1P levels tended to be high in RA treated with biological DMARD than those treated with cDMARD (SSZ and MTX) there were no significant differences in S1P levels between the three treated groups (Figure 1B). This may reflect an underpowered study.

Next, I examined the serum concentrations of S1P and its relationship with RA disease activity. From the data presented in Table 5.1, patients with RA were categorised to remission state (n=9) or low (n=7), moderate (n=18) and high (n=6) disease activity according to the DAS28 score measured cross sectionally in the

clinic. Overall, serum S1P levels were significantly higher in RA patients with (Low, Moderate and High) disease activity ($P < 0.0001$ as determined by 1 way ANOVA test/Kruskal-Wallis test) than those found in RA patients with remission status or healthy controls (Figure 5.1C). Patients with high disease activity demonstrated the highest levels compared with healthy controls and RA patients with remission or low disease activity. Additionally, S1P levels were significantly elevated in patients with high or moderate disease activity ($99 \pm 13.8 \mu\text{M}$ and $69 \pm 2.75 \mu\text{M}$) compared with those with low disease activity or remission state ($35.4 \pm 2.5 \mu\text{M}$ and $29 \pm 2.5 \mu\text{M}$), respectively (Figure 5.1C).

I sought correlation between S1P levels and DAS28 and other lab parameters using the RA patient's characteristic data presented in Table 5.1. A significant positive correlation was found between S1P (μM) levels and the DAS28, according to the two-tailed Pearson Correlation Coefficient (Pearson Correlation 0.523, $P = 0.001$) (Figure 5.1D). The correlation analysis of S1P levels against a variety of clinical indices and lab biomarkers (Table 5.2) showed that S1P positively correlated with TJ and SJ counts. Furthermore, S1P levels were correlated positively with ESR (Pearson Correlation 0.327, $P = 0.018$) (Figure 5.1E). However, there was no correlations between serum S1P and CRP in RA patients.

Table 5.1 Demographic, clinical and laboratory information of rheumatoid arthritis patients recruited in this study.

Subject	Sex	Age	DD	CRP	ESR	DAS 28	Medication
RA1	F	61	12	17	50	5	SSZ
RA2	F	62	8	32	16	4.5	HCQ+MTX
RA3	F	66	23	1.8	20	5.2	HCQ+MTX
RA4	F	75	16	26	55	6	Non
RA5	F	50	23	21	70	5.5	HCQ+MTX+SSZ
RA6	F	41	14	0.6	17	1.77	HCQ+MTX+Adal
RA7	F	53	16	1.8	17	2.3	HCQ+MTX+SSZ
RA8	M	73	13	2.5	14	2.96	HCQ+MTX+SSZ
RA9	F	48	17	1.9	12	3.2	MTX
RA10	F	53	10	5	27	4.9	HCQ+MTX+SSZ
RA11	F	62	13	7.6	35	5.3	HCQ+SSZ
RA12	F	77	47	51	60	6	SSZ
RA13	M	54	26	21	16	3.1	HCQ+SSZ
RA14	M	64	29	20	44	4.9	HCQ
RA15	M	64	36	4.3	12	2.6	SSZ
RA16	F	79	17	7.6	16	1.97	MTX
RA17	F	81	22	9.1	7	2.4	MTX
RA18	F	70	29	5.4	7	2.2	SSZ
RA19	F	58	5	8.3	40	3.3	MTX
RA20	F	79	35	16	54	3.6	MTX
RA21	F	60	13	18	46	5	SSZ
RA22	F	67	19	2	21	2.6	SSZ
RA23	M	67	26	16	20	3.1	MTX
RA24	F	51	34	2.2	15	3.9	MTX+ Tocil
RA25	F	64	12	2.3	9	4	SSZ
RA26	F	75	48	13	37	4.6	SSZ
RA27	F	68	32	4	26	4.6	MTX+ Adal
RA28	F	68	27	1.2	40	2.9	MTX+ Adal
RA29	F	75	27	6	30	4.08	MTX
RA30	F	80	35	8.6	40	5.5	SSZ
RA31	F	52	28	0.6	10	2.8	MTX
RA32	F	73	13	2.9	12	3.9	SSZ
RA33	F	76	22	3.2	10	3	MTX
RA34	M	59	17	2.1	22	3.8	SSZ
RA35	F	74	22	0.5	16	2.5	MTX
RA36	F	49	17	13	14	2.1	MTX+ Inflix
RA37	F	69	29	71	73	4.97	MTX+ Inflix
RA38	F	65	32	5.4	25	2.5	MTX+ Adal
RA39	F	59	28		3512	4.9	MTX+ Rifux
RA40	F	43	12	4	22	3.3	MTX+ SSZ+Adal

DD; disease duration, ESR; erythrocytes sedimentation rate and the normal ESR is <20mm/h for female and <10 mm/h for male CRP; C-reactive protein and the normal CRP is < 4.9mg/l, ESR, SSZ; Sulfasalazine, HCQ; Hydroxychloroquine, MTX; Methotrexate, Adal; Adalimumab, Tocil; Tocilizumab, Inflix; Infliximab, Rifux; Rifuxumab,

Table 5.2 Correlation analysis of S1P levels against a variety of clinical indices and lab biomarkers. Data were skewed by log or square-root transformation to restore normality. Data were presented as R-value and P-value below. R-value on top with corresponding p-values on the lines below

	S1P	Age	DD	CRP	ESR	TJ	SJ
Log age	-0.172						
	0.289						
Log DD	-0.150	0.348					
	0.356	0.028					
Log CRP	0.107	0.251	0.103				
	0.513	0.118	0.526				
Log ESR	0.372	0.089	0.017	0.620			
	0.018	0.584	0.920	0.000			
Sqrt TJ	0.563	0.086	-0.036	0.388	0.371		
	0.000	0.598	0.824	0.013	0.019		
Sqrt SJ	0.566	0.124	-0.113	0.355	0.520	0.824	
	0.000	0.445	0.486	0.025	0.001	0.000	
DAS28	0.523	0.171	0.072	0.544	0.689	0.841	0.892
	0.001	0.292	0.660	0.000	0.000	0.000	0.000

DD; disease duration, ESR; erythrocytes sedimentation rate, CRP; C-reactive protein, TJ; tender joint, SJ; swollen joint and DAS28; Disease Activity Score 28. The correlation of miR-155 copy number against a variety of clinical indices and lab biomarkers is kindly done by Dr. Charles McSharry.

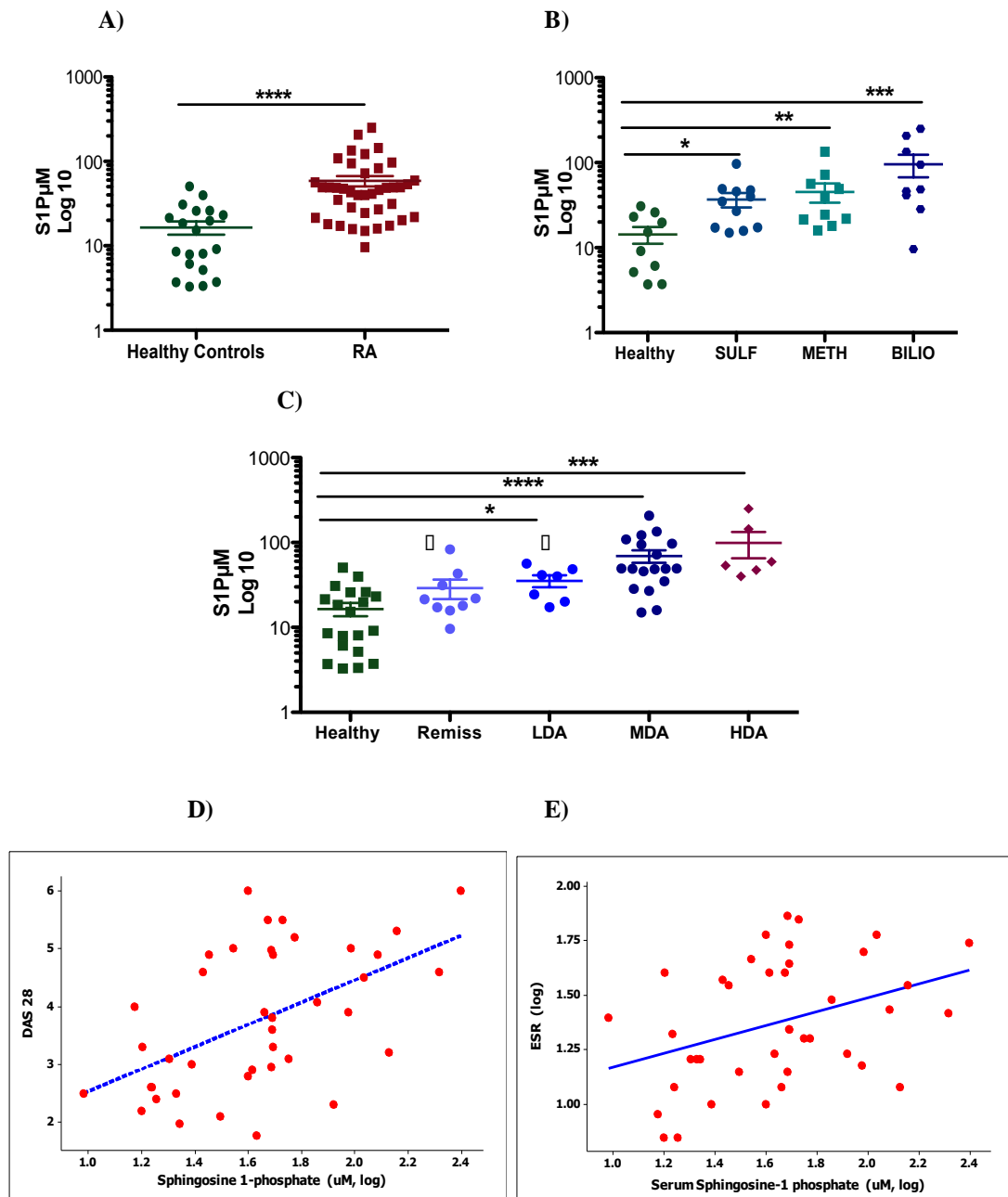


Figure 5.1 A) S1P levels (log S1P) in the serum of both healthy controls (n=20) and RA patients (n=40) using competitive S1P ELISA on triplicate samples as described in Materials and Methods. The values are presented with a mean \pm SEM $p < 0.0001$. Statistical significance was determined using a Kruskal-Wallis test. B) S1P levels (log S1P) in the serum of RA patients receiving different treatment regimens such as Sulfasalazine (n=11), Methotrexate (n=10), Biological agents (n=9), and healthy controls (n=10) using competitive S1P ELISA on triplicate samples. C) S1P levels against disease activity state in RA patients based on DAS28 (Remission, Low, Moderate or High Disease Activity) compared to healthy controls and difference were determined by Kruskal-Wallis test. Values presented with a mean \pm SEM and marked dots are statistically different from healthy controls (* = $p < 0.05$, ** = $p < 0.005$ and *** = $p < 0.0005$), while the marked dot ● indicate the statistical difference between patients with high disease activity and those either with remission or low disease status. Correlation of S1P levels to D) DAS28 and (E) ESR of RA patients.

5.2.3 Optimization and Purity of Sorted Cells from PB of RA patients

To study the differential expression of SPHK1/2 and SIPRs expression in PB of healthy controls and RA patients, it was important first to set out a method to isolated pure cells with good viability and an adequate number of cells. Several methods and techniques were tested for sorting PB cells. In our lab, initially the polymorphonuclear cells (CD15⁺ neutrophils) were isolated by density gradient centrifugation using histopaque gradient-1119. As shown on (Figure 5.2) purification of mononuclear cells (CD14⁺ monocytes and both CD4⁺ and CD⁺ T lymphocytes) were carried out in cascades using MACS and the Auto-MACS separator as described in Materials and Methods. With this protocol, sorted cells (CD15⁺, CD14⁺, CD8⁺, and CD4⁺) were enriched to high purities from the PB of RA patients and health controls. The purity of isolated cells was determined by two-colour flow cytometry as described previously elsewhere and the number of cells in each quadrant (%) were determined; and the protocol yielded purity for all cells between 93-99%.

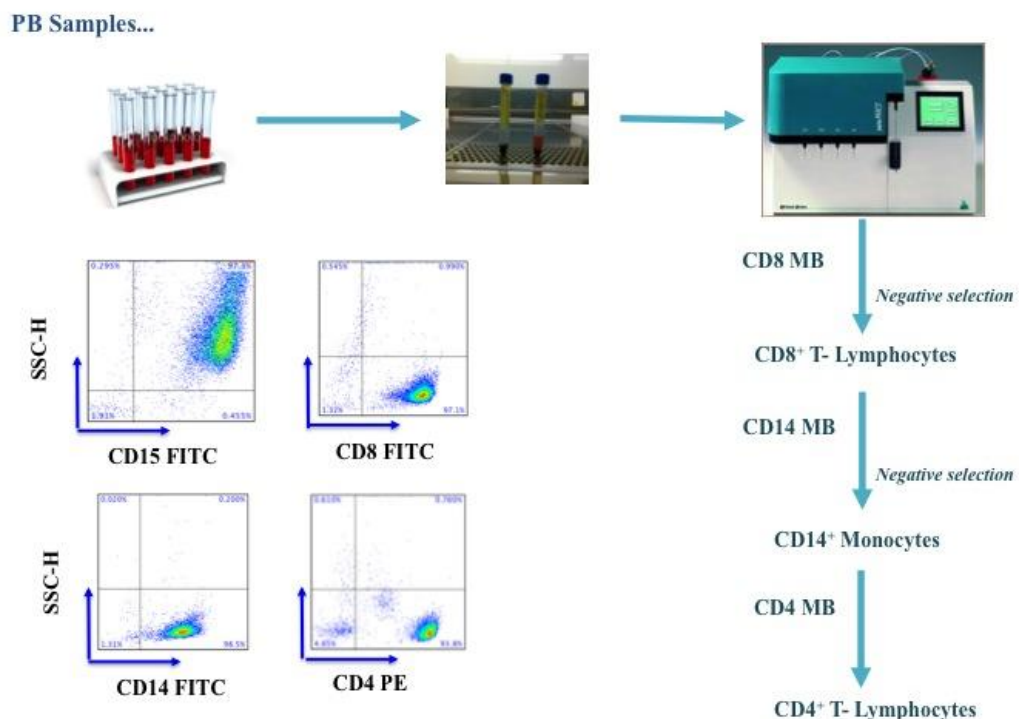


Figure 5.2 Cascade purification of CD14⁺ monocytes and both CD4⁺ and CD8⁺ T lymphocytes from PBMCs of both healthy controls and RA patients (n=10) using magnetic-activated cell sorting (MACS) and Auto-MACS separator. Purity post sorting was determined by two-colour flow cytometry as described in Materials and Methods. Representative dot plots of CD15⁺ neutrophils, CD14⁺ monocyte and both CD4⁺ and CD8⁺ T lymphocytes, and after gating strategy the percentage of positivity cells in each quadrant was determined to calculate the purity of the isolated cells using the Flowjo software.

5.2.4 SPHK1/2 Expression in the PB and Synovium of RA Patients

5.2.4.1 SPHK1 Expression

The differential intracellular expression of SPHK1 in the PB cells (CD15⁺, CD14⁺, CD8⁺, and CD4⁺) of RA patients and healthy controls (n=10) was performed by FACS analysis. Over all, SPHK1 protein was expressed at high levels in all purified cells of healthy controls and RA patients. The percentage change in the intracellular SPHK1 expression was found to be significantly higher only in CD4⁺ T cells of RA patients compared to the healthy controls. SPHK1 expression was increased about 1.4 fold in RA derived CD14⁺ monocytes and CD8⁺ T cells compared to the healthy controls, but these values did not reach my pre-determined levels of significance (Figure 5.3A). I also evaluated the mean fluorescence intensity (MFI) of SPHK1 expression in the purified cells from RA patients, this reflecting target protein density, compared to healthy controls. It was evident that SPHK1 was upregulated in RA CD4⁺ T cells compared to healthy controls. In an average of ten experiments, MFI for SPHK1 in the CD4⁺ T cells was significantly higher in RA patients 21.6±5.1 (mean ± SEM) when compared to healthy controls 11.5±9.2 and (P<0.01 Mann-Whitney U test) (Figure 5.3B and C and Table 5.3). However, there were no significant differences observed in the MFI for SPHK1 in other cells.

In order to assess the expression as well as the precise localization of SPHK1 in RA synovial tissue, I performed IHC analysis. RA and OA synovial tissues (n=4 each disease) were stained with rabbit polyclonal human SPHK1 antibody in order to characterize the expression and localization of the SPHK1 in RA compared to OA tissue. I used respective isotype controls at the same concentration as the specific primary antibodies to differentiate from non-specific staining. I found that SPHK1 protein was clearly over expressed in RA synovium compared to OA tissue (Figure 5.4A). In particular, SPHK1 expression was markedly elevated in the synovial lining layer (SLL), sub-lining layer (SULL) including the infiltrating inflammatory cells and vascular endothelial layer (VEL) (Figure 5.4A) compared to the OA synovial tissues. Although SPHK1 was expressed in synovial tissue of OA patients, it was weak in intensity compare to that expression noted in RA synovium.

To quantitate the extent and intensity of SPHK1 expression in SLL, SULL and VEL of RA and OA synovium tissues, staining scores were calculated using a semi-

quantitative scale. Staining scoring was performed by selection of three representative high power fields per slide and graded on a scale of 0-4+ by 2 'disease-blinded' observers on 2 separate occasions. The numbers of cells with positive staining in each area were then counted manually. Analysis was repeated for 4 patients (RA and OA) synovial tissues respectively. There was a significantly increased expression of SPHK1 in the SLL ($P=0.026$), SULL including the infiltrating inflammatory cells ($P=0.03$) and VEL ($P=0.037$) of the RA synovial tissue compared with finding in OA synovium tissues (Figure 5.4C).

To test whether the intracellular expression of SPHK1 in RA patients was accompanied by differences in mRNA expression, I conducted RT-qPCR. Analysis was performed on total RNA samples obtained from the PB cells ($CD15^+$, $CD14^+$, $CD8^+$ and $CD4^+$) of RA patients ($n=35$) and healthy controls ($n=10$). The transcription levels of SPHK1 is presented as fold change after normalization to β -actin as a housekeeping gene and then calibrated to healthy controls. The mRNA levels of SPHK1 in all the isolated PB cells of RA patients were significantly upregulated compared to healthy controls. In particular, I noted that RA PB $CD4^+$ lymphocytes showed an increase in transcript levels of SPHK1; almost five times higher than their healthy controls (Figure 5.5A).

Next I extended this work to study the gene expression in the same cells of RA patients receiving different treatment regimes, namely SSZ ($n=11$), MTX ($n=10$), biological agent ($n=9$) and compared these individually to healthy controls ($n=10$). Compared to healthy controls, mRNA levels of SPHK1 in all PB cells were significantly upregulated in patients receiving cDMARDs such as SSZ and MTX than those patients receiving the biological treatment (Figure 5.5B). This result may indicate that the biological treatment may have a role in attenuating SPHK1 mRNA towards levels found in the healthy controls.

5.2.4.2 SPHK2 Expression

SPHK2 was not differentially regulated in any of the isolated immune cells from the PB of RA patients compared to the healthy controls (Figure 5.3D). Although, expression and localization of SPHK2 in both RA and OA synovial tissue was weak compared to SPHK1, it still appeared over expressed in synovial lining cells of RA synovium tissues compare with finding in OA synovial tissues (Figure 5.4B).

However, quantitative analysis of SPHK2 showed no significant differences between the RA and OA synovial tissues (Figure 5.4D).

mRNA levels of SPHK2 were clearly and statistically upregulated in all RA PB cells compared to healthy controls (Figure 5.5C). Like SPHK1, in those RA patients treated with biological agent there were no significant differences observed in transcript levels of SPHK2 in PB cells of healthy controls except CD4⁺. CD4⁺ cells show SPHK2 upregulation in both RA patients treated with conventional and biological DMARDs compared to healthy controls (Figure 5.5D). In contrast, mRNA levels were significantly higher in RA patients treated with cDMARD.

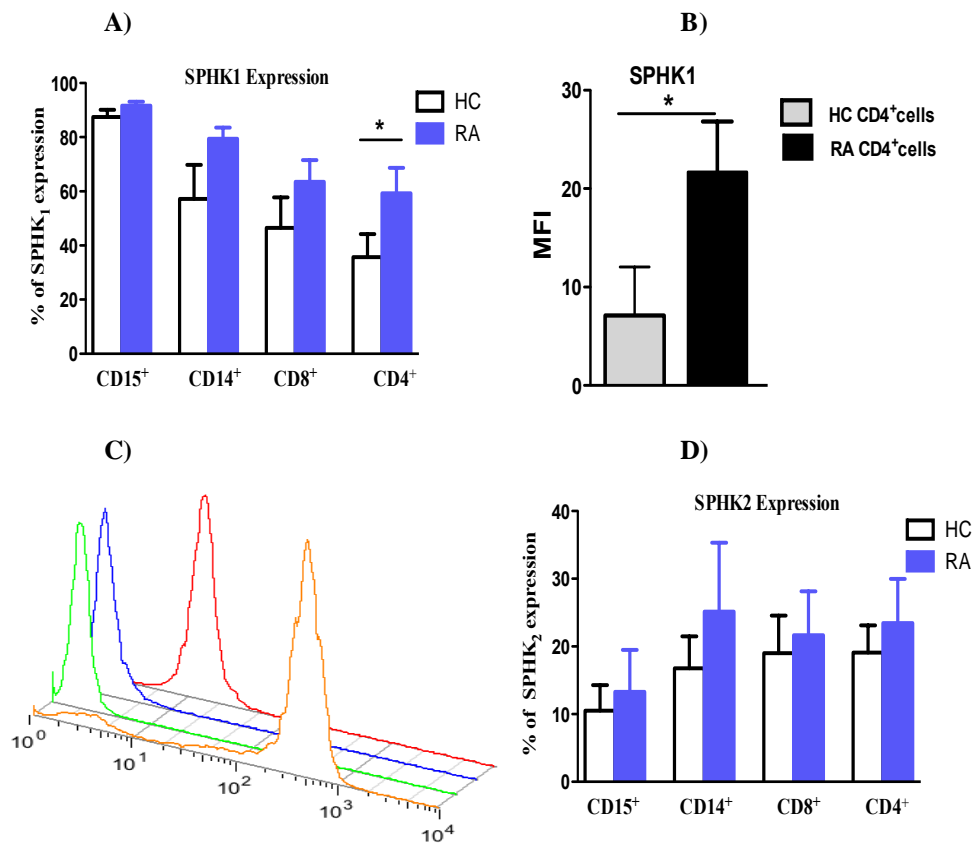


Figure 5.3 Intracellular expression of (A) SPHK1, (D) SPHK2, proteins in neutrophils (CD15⁺), monocytes (CD14⁺) and both (CD8⁺ and CD4⁺) T lymphocytes cells derived from PB of healthy controls and RA patient (n=10) using FACS analysis. Cells were enriched from PB using micro beads and an Auto-MACS separator using the “possel” programme as described in Materials and Methods. Cells were incubated 30 minutes at 4°C with primary antibodies for SPHK1/2 or appropriate isotype and then incubated with appropriate secondary antibody labelled with FITC. After 30 minutes, cells resuspended in FACS buffer and analysed using FACS caliber and Cell Quest Pro Software and Flowjo software. (B) MFI of SPHK1 expression in CD4⁺ T lymphocytes of RA patients, which represent the protein density, compared to healthy controls. (C) A representative histogram analysis of the number of PB CD4⁺ cells that stained positive for SPHK1 from healthy controls and RA patients against isotype controls (Blue and green lines are isotype controls, while red and orange lines are HC and RA respectively). Values presented as mean ± SEM and marked bars are statistically different from control using Kruskal-Wallis test; P ≤ 0.05 has been used as a cut-off value to assign the statistical significance *= p ≤ 0.05.

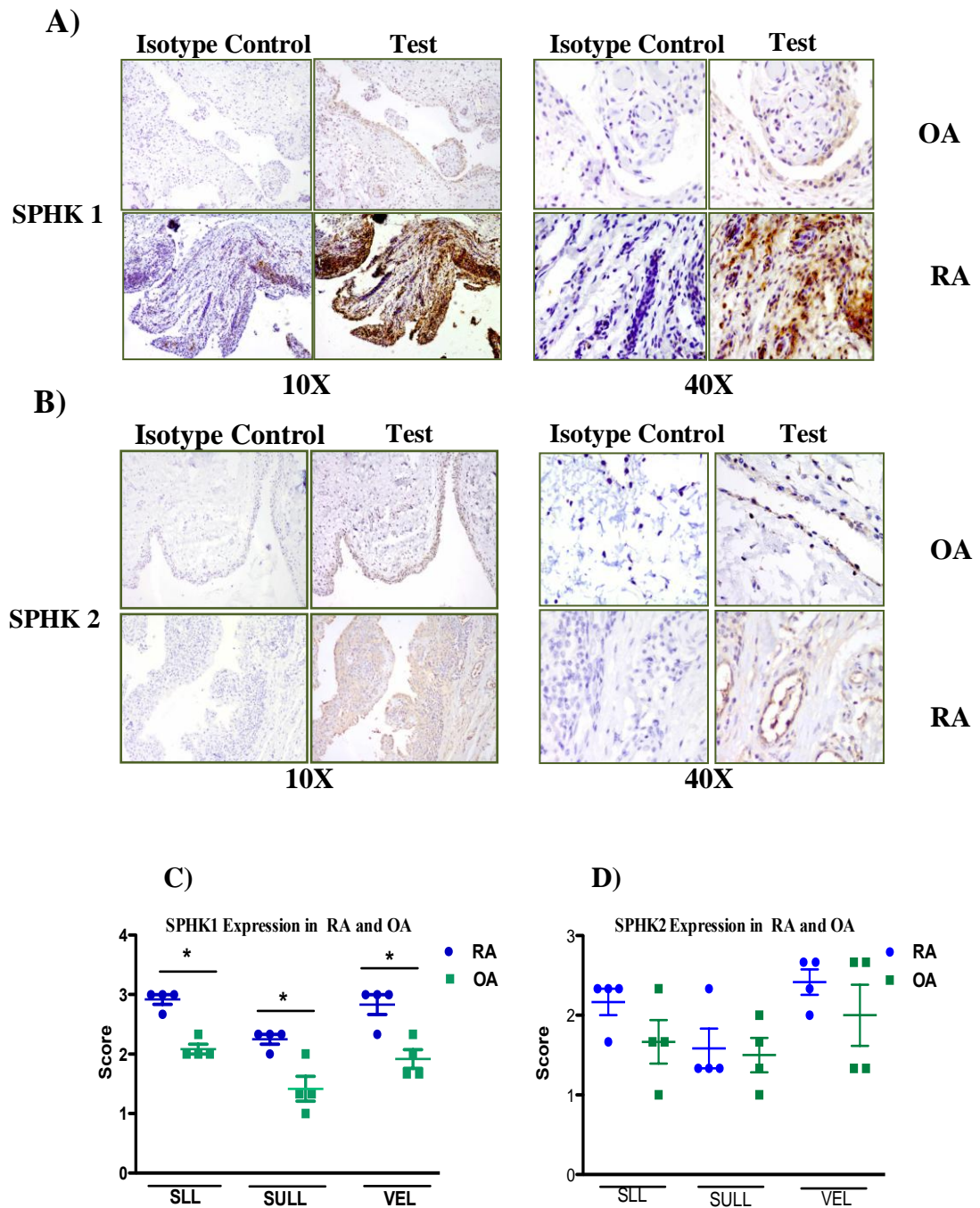


Figure 5.4 A representative of single IHC staining of synovial tissues of RA and OA patients (n=4). Synovium tissues were stained with appropriate primary antibodies for (A) SPHK1, (B) SPHK2 in order to characterize their expression and localization in RA patient compared to OA with respective isotype controls at the same concentration as the specific primary antibodies. The positive staining was indicated by brown deposits in the background appears purple and appropriate isotype controls were negative, (original magnification 10X and 40X). Quantitative analysis of (A) SPHK1 and (B) SPHK2 in the immune-stained RA synovial tissues compared to OA. The extent and intensity of staining with respective antibodies in synovial lining layer (SLL), Sub-lining layer (SULL) and vascular endothelial layer (VEL) from 4 patients with RA and OA were evaluated by 2 blinded observers on 2 separate occasions and graded on a scale of 0-4+ (0= no cells, 2= < 25%, 2= 25-50%, 50-75%, 4=75%). Values are presented as mean \pm SEM. $P < 0.05$ has been used as a cut-off value to assign the statistical significance using Kruskal Wallis test.

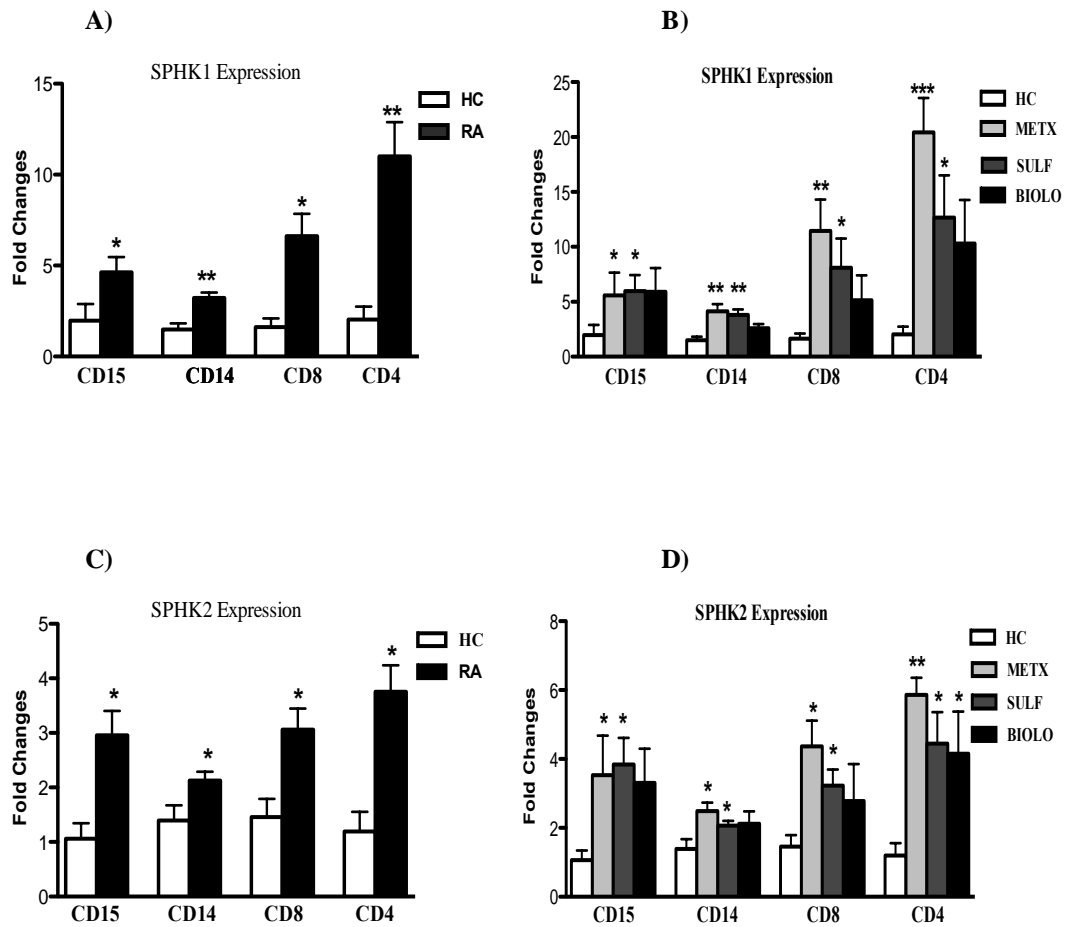


Figure 5.5 The mRNA levels of (A) SPHK1 and (C) SPHK2 in PB cells neutrophils (CD15⁺), monocytes (CD14⁺) and both (CD8⁺ and CD4⁺) T lymphocytes cells derived from healthy controls (n=10) and RA patient (n=35). RNA samples were harvested directly after sorting and converted to cDNA and the levels of target transcripts were assessed by RT-QPCR as described in Materials and Methods. The transcript levels of target gene (SPHK1/2) were presented as fold change after normalization to β -actin as a housekeeping gene and then calibrated to healthy controls. While, B and D represented the mRNA levels of SPHK1/2 expression, respectively in PB cells of RA patients receiving different therapeutic regime, Sulfasalazine (n=11), Methotrexate (n=10), biological agent (n=9) and compare to healthy controls (n=10). Values presented as mean \pm SEM and marked bars are statistically different from control and determined by Kruskal -Wallis test; $P \leq 0.05$ has been used as a cut-off value to assign the statistical significance *= $p \leq 0.05$ and **= $P \leq 0.005$.

5.2.5 S1P Receptor(s) Expression in PB and Synovium of RA Patients

I next decided to systematically evaluate the reciprocal receptors through which S1P may be mediating effect in the RA lesion.

5.2.5.1 S1P₁ Expression

Expression of S1P₁ protein was clearly upregulated in the CD15⁺ neutrophils of RA patients compared with the healthy controls (Figure 5.6A). The MFI of S1P₁ in neutrophils CD15⁺ was significantly higher in RA patients when compared to healthy controls ($P<0.011$) (Table 5.3 and Figure 5.6B and C). Furthermore, S1P₁ expression in CD8⁺ T lymphocytes was elevated, as opposed to healthy controls, but did not reach the level of significance ($P=0.06$).

S1P₁ expression and localization in RA synovium tissues showed a similar cellular distribution as SPHK1 and clearly localized in the SLL, SULL and VEL (Figure 5.7A) in a more extensive pattern than that observed in OA synovial tissues. Quantitative analysis showed that S1P₁ was significantly expressed in SLL ($P<0.04$) and SULL ($P<0.029$) of the RA synovial tissue but there were no significant differences in VEL compared with OA synovial tissues (Figure 5.8A).

Next, I examined S1P₁ mRNA levels in PB cells from both healthy controls and patients with RA. As shown in Figure 5.9A, the RA patients' PB cells exhibited markedly increased expression of S1P₁ compared with healthy controls. When I examined S1P₁ expression in PB cells of RA patients receiving different treatment regimes, I found that although it was highly expressed in RA compared with healthy controls, it was not differentially expressed on any of the groups receiving distinct treatment regimens (Figure 5.9B).

5.2.5.2 S1P₂ Expression

S1P₂ protein was not differentially expressed in any of the isolated PB cells of RA patients when compared to the healthy controls (Figure 5.6D). IHC analysis revealed that S1P₂ was modestly expressed in synovial tissue, mainly in the SLL of RA synovium tissues (Figure 5.7B). However, quantitative localization showed that S1P₂ was significantly overexpressed in SLL ($P<0.047$) on RA synovium but I found

no significant expression difference evident in SULL and VEL comparing RA to OA synovial tissues (Figure 5.8B).

Analysis of mRNA expression in RA PB cells showed no difference in S1P₂ expression in RA compared with healthy controls cells (Figure 5.9C). Similarly, there was no significant difference observed for mRNA levels of S1P₂ in PB cells either in RA patients treated with cDMARDs or biological agents compared to the healthy controls except in the CD4⁺ cells (Figure 5.10D). Levels of mRNA in the CD4⁺ cells exhibited markedly increased expression of S1P₂ in patients treated with MTX compared with healthy controls.

5.2.5.3 S1P₃ Expression

S1P₃ was expressed in very low levels in all sorted PB cells in both healthy controls and RA patients (Figure 5.6E) compared to that observed for other S1PRs. The percentage change in the intracellular S1P₃ staining and FACS analysis revealed that it was significantly upregulated only in the CD15⁺ neutrophils of RA patients when compared to the healthy controls. However, MFI of S1P₃ in CD15⁺ cells was 3.9±2 and 2±9.6 (mean ± SEM) for healthy controls and RA patients, respectively (Table 5.3). I am therefore uncertain of the significance of this at a functional level.

Next I turned to analyses of S1P₃ expression of RA synovial tissues. S1P₃ staining exhibited some similarity to SPHK1 and S1P₁ expression. It was highly expressed in the synovium of RA patients compared to OA patients (Figure 5.7C), and quantitative analysis for S1P₃ staining showed that it was expressed in SLL (P=0.028), SULL (P=0.029) and VEL (P =0.028) of the synovium of RA patients in levels that were higher than those observed in the synovium of OA patients (Figure 5.8C).

The mRNA levels encoding S1P₃ expression were significantly higher in RA PB cells than in those from healthy controls (Figure 5.9E). The mRNA of SPKH1 and S1P₃ expression exhibited some similarity, as both were over expressed in RA patients receiving cDMARDs such as SSZ and MTX than those patients receiving the biological treatment compared to healthy controls. However, with respect to mRNA levels encoding S1P₃ expression in CD14⁺ monocytes the analysis showed no

differences between patients treated with cDMARDs or biological agents compared to the healthy controls (Figure 5.9F).

5.2.5.4 S1P₄ Expression

In contrast to other S1P receptors, S1P₄ was down regulated in CD15⁺, CD14⁺, CD4⁺ and CD8⁺ cells of RA patients compared with healthy controls although this only reached significance for CD4⁺ T cells (Figure 5.6F). I analysed the MFI for S1P₄ in the CD4⁺ T lymphocytes in average of ten experiments. The MFI for S1P₄ in the CD4⁺ T lymphocytes although decreased in RA patients compared to healthy controls (54±20 and 65 ±20) but did not reach the level of significances (P<0.08 Mann-Whitney U test) (Table 5.3). I was not able to detect S1P₄ using IHC in synovial membrane. I did not have a suitable positive control and as such I cannot conclude that this is true down regulation of the receptor. It is equally possible that methodological failure accounts for my observations. A variety of methods were employed (Materials and Methods) but none elicited tissue staining.

In addition, mRNA levels were not differentially expressed in any of the isolated RA PB cell subsets when compared to the healthy controls (Figure 5.9G). Moreover, there was no significant difference for mRNA S1P₄ expression in PB cells in patients receiving conventional or biological DMARDs compared to healthy controls (Figure 5.9H).

5.2.5.5 S1P₅ Expression

The intracellular expression of S1P₅ showed about 2-fold upregulation in PB cells of RA patients compared with healthy controls. As shown in (Figure 5.6G), the percentage changes in S1P₅ expression in RA patients were significantly upregulated in CD14⁺ monocytes and both CD4⁺ and CD8⁺ T lymphocytes (39±310%, 30±8.5% and 28±8.9%) (Mean ± SEM) compared with healthy controls (15.9±9%, 4.4±1.8% and 1.3±0.6%), respectively. Further analysis for MFI was conducted, compared to healthy control, although its still high in RA patients derived cells but there were no significant difference observed (Table 5.3).

S1P₅ was slightly over expressed in the SLL and SULL - the majority of S1P₅ expression was present in infiltrating inflammatory cells of the synovium in both RA and OA biopsies examined (Figure 5.7D). However, quantitative analysis showed no significant differences for S1P₅ in SLL, SULL and VEL of RA synovial tissues as compared to OA synovial tissues (Figure 5.8D).

mRNA levels of S1P₅ were only upregulated in RA PB CD14⁺ monocytes ($P < 0.043$) but not in the other PB subsets compared with healthy controls (Figure 5.9I). Moreover, there was no significant difference in the S1P₅ expression in CD15⁺ and CD8⁺ cells either in patients receiving cDMARDs or biological agents compared to healthy controls (Figure 5.9J). However, S1P₅ expression in CD4⁺ and CD14⁺ cells was significantly upregulated in RA patients receiving cDMARDs than those patients receiving the biological treatment compared to healthy controls (Figure 5.9J).

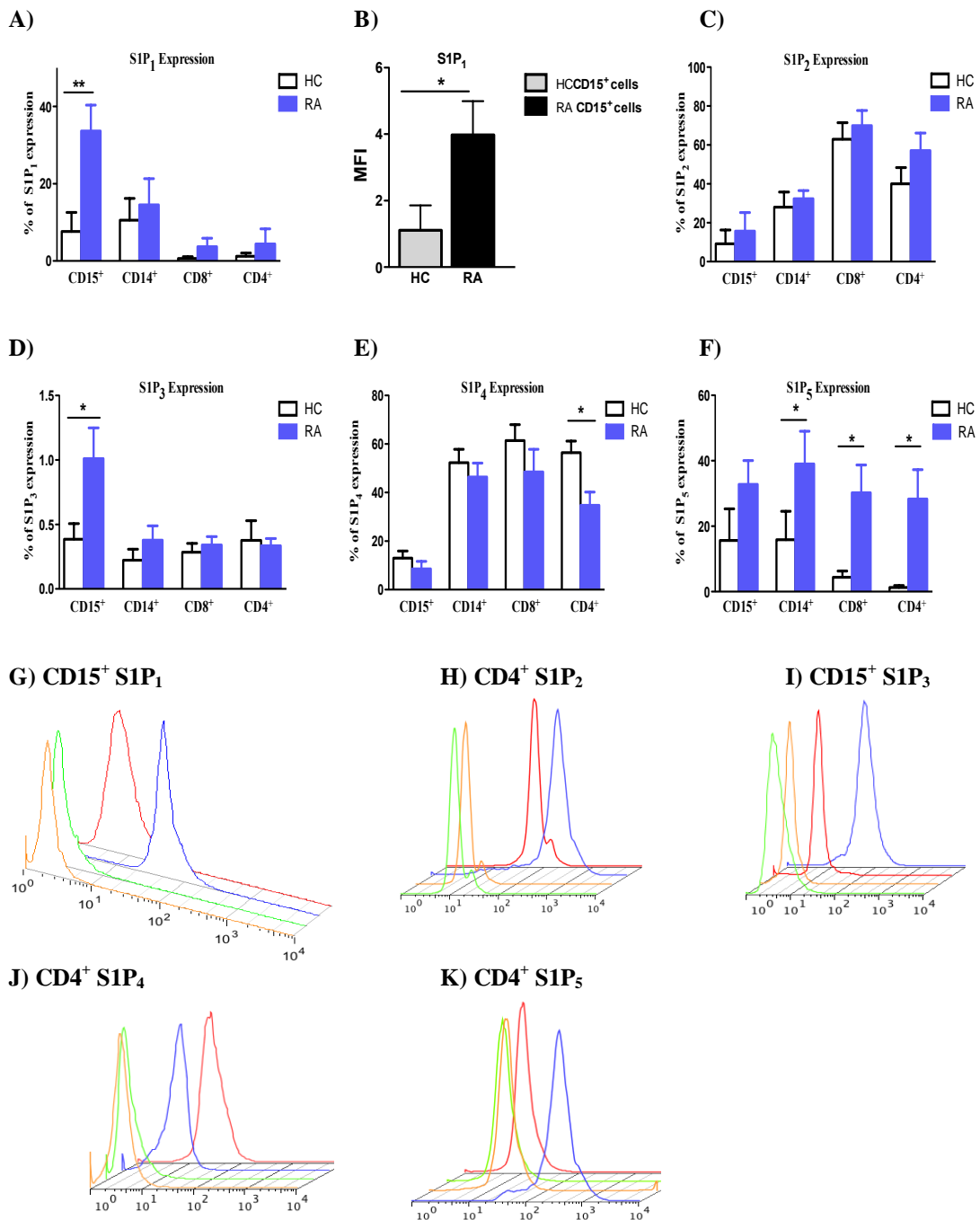


Figure 5.6 Intracellular expression of (A) S1P₁, (C) S1P₂ (D) S1P₃ (E) S1P₄ and (F) S1P₅ in neutrophils (CD15⁺), monocytes (CD14⁺) and T lymphocytes (CD8⁺ and CD4⁺) cells derived from PB of healthy controls and RA patients (n=10) using FACS analysis. Cells were enriched from PB using micro beads and an Auto-MACS separator using the “possel” programme as described in Materials and Methods. Cells were incubated 30 minutes at 4°C with primary antibodies for S1PRs or appropriate isotype and then incubated with appropriate secondary antibody labelled with FITC. After 30 minutes, cells resuspended in FACS buffer and analysed using FACS calibre and Cell Quest Pro Software and Flowjo software. (B) MFI of S1P₁ expression in CD15⁺ neutrophils of RA patients compared to healthy controls. G-K) A representative histogram analysis of the number of cells that stained positive for CD15⁺ S1P₁, CD4⁺ S1P₂, CD15⁺ S1P₃, CD4⁺ S1P₄ and CD4⁺ S1P₅ respectively, from healthy controls and RA PB against isotype control (green and orange lines are isotype control, while red and blue lines are HC and RA respectively). Values presented as mean ± SEM and marked bars are statistically different from control using Kruskal - Wallis test and P ≤ 0.05 has been used as a cut-off value to assign the statistical significance *= p ≤ 0.05.

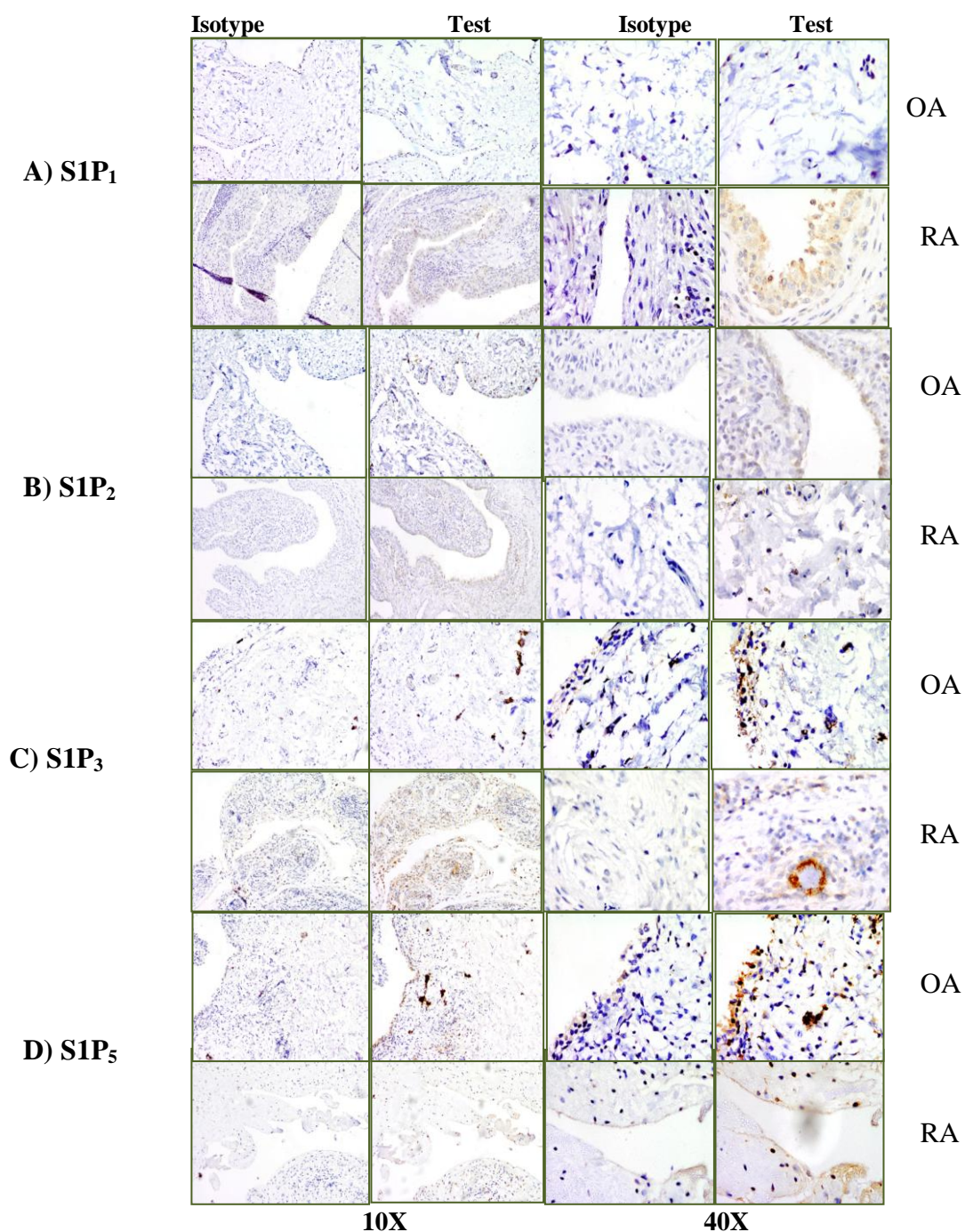


Figure 5.7 A representative of single IHC staining of synovial tissues of RA and OA patients (n=4). Synovium tissues were stained with appropriate primary antibodies for (A) S1P₁, (B) S1P₂, (C) S1P₃ and (D) S1P₅ in order to characterize their expression and localization in RA patient compared to OA with respective isotype controls as same concentration as the specific primary antibodies. The positive staining was indicated by brown deposits in the background appears purple and appropriate isotype controls were negative, (original magnification 10X and 40X).

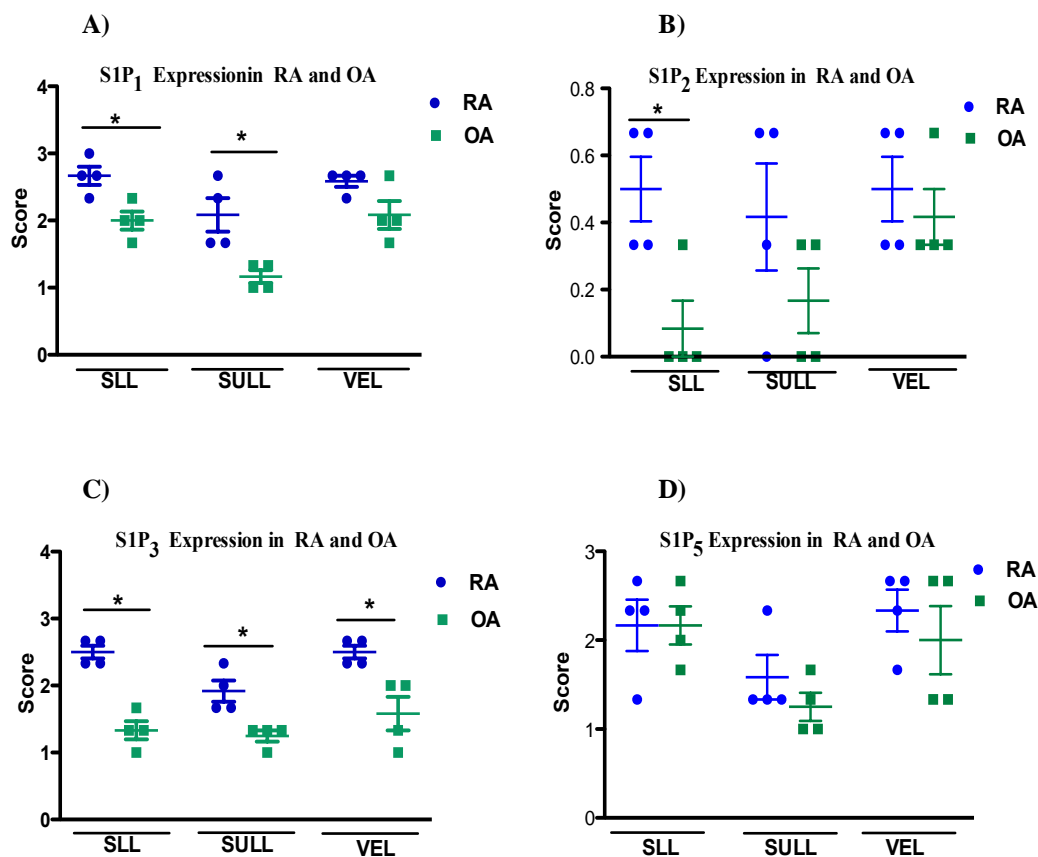


Figure 5.8 Quantitative analysis of (A) S1P₁, (B) S1P₂, (C) S1P₃ and (D) S1P₅ in the immune-stained RA compared with OA synovial tissues. The extent and intensity of staining with respective antibodies in synovial lining layer (SLL), Sub-lining layer (SULL) and vascular endothelial layer (VEL) from 4 patients with RA and OA were evaluated by 2 blinded observers on 2 separate occasions and graded on a scale of 0-4+ (0= no cells, 2= < 25%, 2= 25-50%, 50-75%, 4=75%). Values are presented as mean \pm SEM and P < 0.05 has been used as a cut-off value to assign the statistical significance using Kruskal Wallis test.

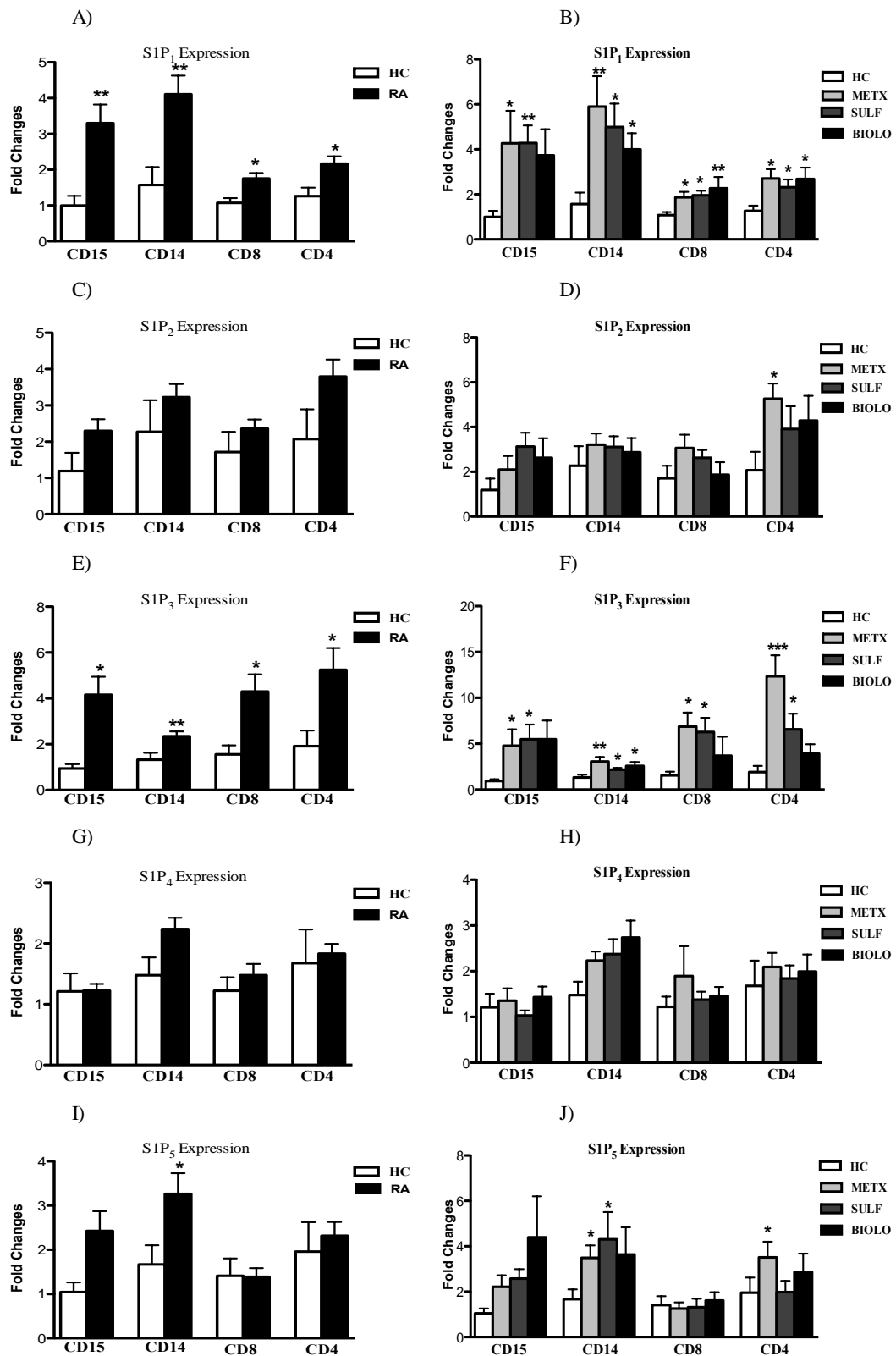


Figure 5.9 mRNA levels of (A) S1P₁, (C) S1P₂, (E) S1P₃, (G) S1P₄ and (I) S1P₅ in PB cells CD15⁺, CD14⁺, CD4⁺ and CD8⁺ from healthy controls (n=10) and RA patient (n=35). RNA samples were harvested directly after sorting and converted to cDNA and the levels of target transcripts were assessed by RT-QPCR as described in Materials and Methods. The transcript levels of target gene (S1P₁₋₅) were presented as fold change after normalization to β -actin and then calibrated to healthy controls. While, B, D, F, H and J represented mRNA levels of S1P₁₋₅ expression, respectively in PB cells of RA patients receiving different therapeutic regime, Sulfasalazine (n=11), Methotrexate (n=10) biological agent (n=9) and compare to healthy controls (n=10). Values presented as mean \pm SEM and marked bars are statistically different from control using Kruskal Wallis test and $P \leq 0.05$ has been used as a cut-off value to assign the statistical significance *= $p \leq 0.05$ and **= $P \leq 0.005$.

Table 5.3. SPHK1/2 and S1PRs intracellular expression in peripheral blood cells of rheumatoid arthritis patients

Cells type		CD15		CD14		CD8		CD4		P value*
Subject		HC	RA	HC	RA	HC	RA	HC	RA	
SPHK1	%	87.4±2	91.6±1	57±12	79.4±4	46±11	63.5±8	35.7±8	59±9	*CD4
	MFI	32.5±7	48.2±7	18.5±4	34.8±9	12.9±5	9.5±3	11.5±9	21.6±5	*CD4
SPHK2	%	10.5±3	13.2±6	16.8±4	25±10	19±5.5	21.6±7	19±4	23.4±7	
	MFI	19±13	11±6	1.9±1	2.1±1	58±20	24±10	26.9±12	11±4.6	
S1P ₁	%	7.6±5	33.6±7	10.5±5	14.5±6	0.6±0.4	3.6±2	1.2±0.8	4.3±4	**CD15
	MFI	1±0.7	4±1	0.7±0.4	0.3±0.2	1.3±0.7	0.8±0.5	1.7±1.4	1±0.9	*CD15
S1P ₂	%	9.2±7	15.6±9	28±8	32±4	63±8	69.9±8	40±8	57±9	
	MFI	0.6±5	1.4±6	1.8±0.7	2.8±0.9	7.8±3	11.8±3	6.9±1.8	7.7±2	
S1P ₃	%	0.4±1	1±0.2	0.2±1	0.4±1	0.3±6	0.34±1	0.4±0.2	0.3±1	*CD15
	MFI	3.9±2	2±9.6	3±2.4	1.9±1	2.7±1	3.4±1.7	2.1±1	5.9±3	
S1P ₄	%	12.9±3	8.5±3	52±6	46.4±6	61±6.5	48±9	56±4.7	34.7±5	*CD4
	MFI	26±13	3.8±1	10.9±5	19±7	56±18	90±36	65±20	54±20	
S1P ₅	%	15.7±9	32.7±7	15.9±9	39±10	4.4±1.8	30±8.5	1.3±0.6	28±8.9	*CD14, 8 and 4
	MFI	18±16	21±9	9.8±3	18±6.8	14±5.6	19±7.6	6±3.5	12±4	

Peripheral blood cells (CD15⁺, CD14⁺, CD8⁺, and CD4⁺) from RA patients (n=10) and healthy controls (n=10) were stained with hSPHK1/2 and hS1PRs monoclonal antibodies or appropriate isotype controls and were analysed by flow cytometry. Positive cells were gated and positive expression of SPHK1/2 and S1PRs cells was analysed. In average of ten experiments, both the number of positive cells (presented as mean per cent) and MFI were determined and were varied between the healthy control and RA patients as shown. The red bars were highlighted to show that S1P₃ was expressed in very low levels in all sorted PB cells in both healthy controls and RA patients. While, S1P₄ was down regulated in CD15⁺, CD14⁺, CD4⁺ and CD8⁺ cells of RA patients compared with healthy controls. Values are presented as mean ± SEM P, <0.05 has been used as a cut-off value to assign the statistical significance.

5.3 Discussion

RA is a chronic systemic inflammatory disease - the aetiology is not clearly defined (590, 592, 593). Although several genetic and environmental factors have specifically been shown to play a role in RA, a critical shift towards the increased production of proinflammatory cytokines compared with anti-inflammatory cytokines in the synovial membranes of joints is closely attributed to the pathogenesis of RA (170). Recent studies have clearly demonstrated that the expression of SPHK1 was increased in RA synovium (416-418). SPHK1 and SPHK2 catalyse biochemical conversion of sphingosine into S1P (415) and in turn can cause the release of proinflammatory mediators from SFs, endothelial cells, and immune cells (416, 594).

In my study, S1P levels were elevated in the serum of RA patients compared with healthy controls. Additionally, patients with active disease exhibited higher levels of serum S1P than patients with low disease activity and were significantly reduced in RA patients in remission. These data collectively imply that S1P levels are implicated at some level in the pathogenesis of RA, or its clinical manifestation and could be a promising biomarker for disease activity. Consistent with previous studies, serum S1P levels were found to be predictor of both the occurrence and severity of coronary disease (595). Analysis of the influence of drug therapy on S1P serum concentration however, revealed no difference between the patients treated with cDMARDs and those receiving biological agents. Moreover, RA patients taking biological agents had highest increased levels of serum S1P compared with healthy controls. Larger studies are therefore required to formally test the utility of this as a clinically useful marker.

S1P is a pleiotropic molecule and has ability to induce an array of intracellular biochemical reactions leading to the activation of transcription factors such as NFkB, c-Fos, c-Jun, AP-1 and induce the production of proinflammatory mediators (415, 416). SPHK1 expression, but not SPHK2, was elevated in circulating CD4⁺ T lymphocytes and in the synovium of RA patients. Moreover, SPHK1 overexpression was specifically located in the SLL, SULL including mainly infiltrating inflammatory cells and VEL. Increased production of S1P, a potent chemokine, by

SPHK1 in the RA synovium, could therefore be one of the contributing factors for production of inflammatory mediators and the influx of immune cells into joints of RA patients with active disease. The SPHK1 transcript was highly overexpressed particularly in CD4⁺ cells in RA patients. However, SPHK1 expression was attenuated in PB cells of RA patients treated with biological agents. These data may indicate that the inflammation in RA was well controlled in patients treated with biological therapies rather than cDMARDs. Though SPHK2 expression was also found to be relatively higher in all the PB cells of RA patients and lower in RA patients treated with biological agents, SPHK2 protein expression was not observed in synovium of RA patients.

Neutrophils play a key role in the pathogenesis of various autoimmune diseases (596). Neutrophil recruitment is one of the main characteristics of RA. A very recent study has shown that C5a receptor (C5aR) and Fcγ receptors (FcγR) pathways independently play a role in the recruitment of neutrophils in an animal model of inflammatory arthritis (597). Moreover, neutrophils have been shown to form neutrophil extracellular trap (NET) termed as NETosis (598) in the serum and SF of RA patients (599). It was also observed that NETosis correlated with the levels of ACPAs and systemic inflammatory markers in RA patients compared with healthy controls and OA patients (599). In our study, S1P₁ and S1P₃ were significantly overexpressed in the neutrophils of RA patients. In addition, S1P₁ and S1P₃ were also elevated in SLL, SULL and VEL of the RA synovium. Overexpression of S1P₁ and S1P₃ in neutrophils in RA patients may drive the recruitment of neutrophils towards S1P gradient in the synovial membranes of joints. However, the role of S1P/S1PR axis in NETosis is yet to be established in autoimmune inflammatory diseases. Similarly, S1P₁ and S1P₃ transcripts were highly overexpressed in the PB cells of RA patients receiving both cDMARDs and biological treatments. It has clearly indicated that gene expression of S1P₁ in PB cells of RA patients are poorly controlled by different treatment regimens and it would be useful to devise a therapy to control levels of S1P₁ to specifically attenuate inflammatory signals or pathways initiated through the S1P/S1PR axis in RA.

S1P₄ is highly expressed in blood cells as well as lymphoid tissue (552, 559). Interestingly, S1P₄ was down regulated in all the immune cells in RA. It was shown

that unlike S1P₁, S1P₄ could not induce the T lymphocytes to produce cytokines and chemokines (559) and S1P/S1P₄ axis mediates immunosuppressive effects by inhibiting proliferation of T lymphocytes. Besides, it hampers secretion of effector cytokines such as IFN- γ , IL-4, and IL-2 and promotes the secretion of anti-inflammatory cytokines such as IL-10 from T lymphocytes (559). Hence, down regulation of S1P₄ receptor on neutrophils, monocytes, and both CD4⁺ and CD8⁺ T cells may lead to the uncontrolled secretion of effector cytokines and the decreased production of anti-inflammatory cytokines and subsequently contributing to the pathogenesis of RA.

The expression of S1P₅, a T-bet regulated gene, is greatly limited to brain, skin and oligodendrocytes of the CNS (560, 561). S1P₅ is highly expressed in NK cells and plays a key role in NK cell egress from lymph nodes and bone marrow to the sites of inflammation (560). In our study, S1P₅ expression was elevated in CD14⁺ monocytes, CD4⁺ and CD8⁺ T cells of RA patients. In addition, S1P₅ transcripts were upregulated in CD14⁺ cells in RA patients. The upregulation of S1P₅ was observed in both CD14⁺ and CD4⁺ cells in RA patients treated with cDMARDs compared with RA patients treated with biological agents. Along with S1P₁, the S1P₅ could play a vital role in trafficking of T lymphocytes to the sites of inflammation and promote RA pathogenesis.

In conclusion, our study demonstrated that the S1P/S1PR axis was dysregulated in RA patients. S1P levels were elevated in RA patients under relapse compared with patients under remission. Furthermore, SPHK1 expression was upregulated in crucial innate immune cells such as neutrophils as well as in the RA synovium. Increased expression of S1P₁, S1P₃, S1P₅ and decreased expression of S1P₄, in PB cells and RA synovium, could potentially recruit innate immune cells such as neutrophils, monocytes, T lymphocytes, NK cells from blood, lymph nodes as well as bone marrow to the synovium of the joints and cause production of proinflammatory cytokines and attenuate synthesis and secretion of anti-inflammatory cytokines to perpetuate the RA pathogenesis. Hence, our results merit further consideration, on the clinical significance of S1P as a possible biomarker for disease severity and to explore all the novel therapeutic avenues currently available to modulate SPHK/S1P/S1PR axis in RA.

CHAPTER VI
GENERAL DISCUSSION

Though progress has been achieved in the understanding of many different aspects of RA treatment, the aetiology of RA still remains elusive. Although there are an increasing number of effective biotherapies, a substantial number of RA patients still do not respond sufficiently to current treatment options, and this warrants the identification of new therapeutic targets and the development of novel therapies for RA. The principle of new therapeutic development heavily relies on the characterization of a tractable molecular pathway both in terms of its basic biology and its clinical implications in RA pathogenesis. In this thesis, I have studied two different molecular pathways initiated by miR-155 and S1P, both in terms of their basic biology and clinical relevance to RA.

Recently, the role of miRNAs has been intensively studied in many different fields including RA. There are several lines of evidence suggesting that miRNAs are a novel class of biomarkers and offer potential targets for the treatment of RA. One miRNA that appears to be implicated in RA is miR-155. Its expression is induced by inflammatory cytokines and TLR ligands. I aimed to understand how miR-155 accounts for chronicity of RA mediated by chemokine and chemokine receptors. MiR-155 has been detected in the joints and SFs of patients with RA and has been found to be involved in the regulation of MMPs (406). It also exerts powerful regulatory functions in promoting the production of pro inflammatory cytokines such as TNF- α , IL-1 β and IL-6, which are strongly implicated in RA synovitis (572). Furthermore, evidence from animal models has suggested that miR-155 is critically involved in the adaptive and innate immune reactions leading to autoimmune arthritis (366). Indeed, miR-155 deficiency in mice completely prevents the development of CIA and ameliorates local cartilage and bone destruction. Additionally, deficiency in miR-155 in mice showed reduced expression of articular TNF- α , VEGF and other chemokines, which play a critical role in articular inflammation, neo-angiogenesis of hyperplastic synovium, and recruitment of inflammatory cells into the joint space (366, 572). Recruitment and accumulation of immune cells is an essential element in both onset and chronicity of RA and is mediated by chemokines and their receptors. Chemokines are key molecules in the development of synovial inflammation and regulate cell traffic; their key role is indicated by the fact that many chemokines are highly expressed in RA synovium. My initial aim was to examine the functional contribution of miR-155 in monocyte

migration through the modulation of chemokines and chemokine receptors both in humans and miR-155 deficient mice.

Previous studies have examined miR-155 expression both in PB and RA synovium tissues. To our knowledge, this is the first study to examine the exact copy number of miR-155 in PB and SF CD14⁺ monocytes of RA patients. In accordance with various publications, I have demonstrated that PB and SF CD14⁺ monocytes of RA patients expressed higher levels of miR-155 compared to PB monocytes of healthy people. RA SF monocytes exhibit the highest levels of miR-155. This strongly supports the concept that miR-155 indeed plays a significant role in inflammatory arthritis pathogenesis. In addition, I confirmed our previous observation that pro-inflammatory cytokines, such as TNF- α and IL-1 β , are increased in response to overexpression of miR-155 in RA PB CD14⁺ monocytes. Moreover, PB CD14⁺ monocytes overexpressing miR-155 exhibit increased production of chemokines (CCL3/MIP-1 α , CCL4/MIP-1 β , CCL5/RANTES, and CCL8/MCP-2), of which several have been found to be expressed in RA synovial tissue (575) likely facilitating entry of immune cells into the tissue. Thus, increased levels of miR-155 in patients with RA particularly in SF monocytes might be responsible in part for excessive proinflammatory cytokine and chemokines production from monocytes leading to persistent joint inflammation and destruction.

Monocytes are known to express many different chemokine receptors such CCR1, CCR2, CCR5, CCR8, CXCR1, and CXCR4. In this study I demonstrated that overexpression miR-155 increases CCR7 and suppress CCR2 and CCR3 expression in PB monocytes. The mechanism of this regulation is currently unknown but these observations suggest that miR-155 may act as an important regulator of chemokine receptors in monocytes leading to retention of cells at the sites of inflammation in “joint space”. This is of particular relevance considering that TNF- α inhibitors are clinically effective in the treatment of approximately 75% of people with RA, but about 25% of patients still do not respond to this treatment, and subsequent proportional failures to distinct modes of action, mean that a significant subset of RA patients are only partially or not treated (58). These data reinforce rather than weaken the hypothesis that miR-155 may represent an important target for RA

therapy; it regulates both cytokine and chemokine production up on its overexpression.

Together with the pro-inflammatory effects of miR-155 and significant higher copy number was present in RA patients compared to healthy controls PB monocytes; miR-155 might have some important clinical significance. This has highlighted the clinical significance of miR-155 by virtue of its association with clinical markers, DAS28, TJ/SJ counts and ESR in RA. Additionally, the present study also demonstrated that RA patients with active disease (on the basis of DAS28) exhibit a higher copy number in monocytes than those with mild disease activity or remission status. These data indicate that miR-155 levels may be a useful marker of RA disease activity, perhaps included as part of a poly factorial algorithm. This observation is consistent with very exciting independent publications, where plasma and SF as well as PBMCs miRNAs expression correlated with RA disease activity. By extension from the oncology field, disease specific miRNAs for RA are expected; therefore, further studies may be required for comprehensive analysis of miRNAs in PBMCs, plasma and SFs in large cohorts of patients.

Additionally, I studied chemokine receptor expression and chemokine production from BMMO of miR-155^{-/-} mice. Some progress had been made in examining the functional role of miR-155 in the pathogenesis of autoimmune arthritis using miR-155^{-/-} mice in the CIA model, which develops neither synovial inflammation nor cartilage and bone destruction. However, no previous report demonstrated the differential expression of chemokine receptors in miR-155 deficient BMMO or macrophages. This study indicates that the BMMO and macrophage of miR155^{-/-} in steady state express several chemokine receptors that play an important role in controlling cells homing and migration. Of interest, ectopic expressions of miR-155 in PB CD14⁺ monocytes blunting of their migration by functionally down regulating CCR2 and increase the CCR7 expression (under section 3.2.9). Moreover these receptors are expressed in an opposite way in BMMO of miR-155 deficient cells; CCR7 is significantly down regulated and CCR2 and CCR3 expression are increased. This observation suggests that CCR2, CCR3 and CCR7 seemed to be of under the tight control of miR-155 that is preserved across the species. In addition, dyregulation of chemokine receptor expression in BMMO of miR-155^{-/-} mice raises

the question of whether miR-155 can be involved in the maintenance of monocyte homeostasis under normal homeostatic conditions. Thus, miR-155 could act as an important regulator of these receptors in homeostatic state.

Interestingly, analysis of the cytokine profile in these experiments demonstrated that deficiency in miR-155 leads to the suppression of majority of cytokines and chemokines, which are strongly implicated in RA pathogenesis. The most profound was inhibition of IL-6 in LPS stimulated miR-155 deficient cells as compared to WT mice. Cytokine production and effector function are now a matter of primary importance and represent a crucial factor for disease pathogenesis. Therefore, this reduction in cytokine/chemokine production also supports the hypothesis that miR-155 is involved in RA pathogenesis.

Since studies have shown that the S1P/SPHK axis plays a significant role in the induction of various types of inflammatory responses and disease pathologies, in my second objective, I investigated the potential role as well as the clinical significance of the S1P/S1PR axis in PB cells and the synovium of RA patients compared to healthy controls and OA patients, respectively. I have observed significant positive correlation with serum S1P (μM) levels, the DAS28, a variety of clinical indices, TJ/SJ counts and ESR. S1P levels were increased in RA patients undergoing relapse compared with patients in remission. This suggests that serum S1P could be a potential clinical biomarker for RA disease activity and at least suggests that S1P dependent pathways are implicated in the elevated levels of RA associated inflammation.

To further explore the role of S1P/S1PR axis in the pathogenesis of RA, I examined the intracellular protein expression and mRNA levels of SPHK1/2 and S1PR₁₋₅ in the isolated PB cells of RA patients. SPHK1, S1P₁ and S1P₃ levels were significantly higher at mRNA levels in PB cells of RA patients. IHC analysis further indicated elevated expression of SPHK1, S1P₁ and S1P₃ in the synovium. SPHK1 was significantly expressed in CD4 lymphocytes at protein level. While, S1P₁ and S1P₃ were specifically elevated in RA neutrophils and S1P₅ was elevated in RA CD14⁺ and both CD4⁺ and CD8⁺ T cells. On the other hand, S1P₂ was unchanged compared to healthy controls, while S1P₄ was found to be down regulated in all the PB cells of

RA patients particularly in CD4⁺ cells. Since S1P/S1P₄ axis is immunosuppressive, down regulation of S1P₄ in PB cells of RA patients could hamper the secretion of immunosuppressive cytokines and chemokines and ‘de-repress’ immune function. Moreover, increased expression of S1P₁, S1P₃, S1P₅ and the decreased expression of S1P₄ in PB cells and the RA synovium, could potentially recruit innate immune cells such as neutrophils, monocytes, T lymphocytes, NK cells from the blood, lymph nodes as well as bone marrow to the synovium of the joints and cause the production of proinflammatory cytokines and attenuate the synthesis and the secretion of anti-inflammatory cytokines to perpetuate the RA pathogenesis.

Furthermore, I have observed that compared to the cDMARDs, RA patients treated with biological agents have reduced expression of SPHK1 and S1P₃ in the PB cells of RA patients. These results may indicate that biological treatments may have an important role in the attenuation of SPHK1 mRNA expression similar to the normal levels observed in the healthy controls. Hence, our study demonstrated that the S1P/S1PR axis was indeed dysregulated in RA patients and our results merit further consideration, on the clinical significance of S1P as a possible biomarker for disease severity and to explore all the novel therapeutic avenues currently available to modulate S1P/S1PR/SPHK axis in RA.

Taken together I have embarked on an ambitious series of experiments designed to explore the potential biology and therapeutic or biomarker tractability of two independent inflammatory moieties in the context of RA pathogenesis. My data offer rich detail to each subject area and as such have enhanced the possibilities for therapeutic targeting and diagnostic developments on the basis of both lines of investigation.

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