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# Investigating the role of microRNA in inflammatory cytokine production of macrophages in Rheumatoid Arthritis

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

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## Abstract

**Background:** Rheumatoid Arthritis (RA) is a chronic systemic inflammatory disease, which targets the synovial membrane and joints of patients, resulting in irreversible bone damage, and disability. It is marked by an influx of inflammatory immune cells into the synovium, disrupting the natural resolution of inflammation and resulting in the establishment of chronic disease. The development of new treatments for RA is limited by our incomplete understanding of disease pathogenesis. Macrophages are critically important in the pathogenesis of RA and have been studied intensively due to their ability to initiate and amplify both systemic inflammation and local tissue damage by production of cytokines such as Tumour necrosis factor (TNF)  $\alpha$  and Interleukin (IL)-6. Our understanding of the molecular mechanisms underlying monocyte and macrophage activation in arthritis remains unclear. MicroRNAs (miRs) are a novel class of post-transcriptional regulators. They are short, non-coding RNA molecules that bind to complementary sequences, typically in the 3'untranslated region (UTR) of target genes, resulting in mRNA degradation or sequestration. miRs have been shown to impact various aspects of mammalian biology including cell proliferation, differentiation and the immune response.

**Aims:** To define a specific miR profile of CD14<sup>+</sup> cells separated from peripheral blood (PB) and synovial fluid (SF) of RA patients. Thereafter, to determine the functional targets of these dysregulated miRs, and determine if they play a role in maintaining the pro-inflammatory nature of macrophages in RA.

**Methods and Results:** We identified a miR profile of SF CD14<sup>+</sup> cells in RA. The majority of this study concentrated on miR-125a which was shown to be upregulated in SF CD14<sup>+</sup> cells and PB CD14<sup>+</sup> cells of patients who respond well to conventional disease modifying anti-rheumatic drug (cDMARD) therapy when compared to healthy controls. In these cells miR-125a negatively correlates with clinical measures of disease activity disease activity score 28 (DAS) erythrocyte sedimentation rate (ESR), simple disease activity index (SDAI), clinical disease activity index (CDAI), tender joint count (TJC) and swollen joint count (SJC) whilst positively correlating with serum levels of anti-inflammatory IL-10. This

miR was also inducible by lipopolysaccharide (LPS) in monocytes and expressed at high levels in macrophage colony stimulating factor (M-CSF) macrophages.

Using miR sponge technology we generated a macrophage like, miR-125a null environment in Phorbol Myristate Acetate (PMA) differentiated THP-1 cells. We demonstrated by Enzyme linked immunosorbent assay (ELISA) that miR-125a null cells display a hyper inflammatory response to LPS and produce significantly higher levels of TNF $\alpha$ , IL-6, CCL4 and CCL5 when compared to a reporter control. This phenotype was confirmed in primary human macrophages in which miR-125a was inhibited through transfection with an antagomir. These cells produced significantly higher levels of TNF $\alpha$ , IL-6, IL-12 and CCL4. Interestingly, macrophages in which miR-125a was inhibited expressed significantly lower levels of anti-inflammatory IL-10.

Prediction algorithms identified members of the Toll Like Receptor 4 (TLR4) signalling pathway and inflammatory cytokines as potential miR-125a targets. Through luciferase reporter assays we identified a potential regulatory relationship between miR-125a and TLR4, TNF receptor-associated factor 6 (TRAF6) and CCL4, which may account for the inflammatory phenotype of miR-125a null macrophages. Interestingly overexpression of miR-125a in primary human macrophages results in increased IL-10 production, but also increased TNF $\alpha$  production, highlighting the complicated nature of miR regulation. We demonstrated a potential relationship between miR-125a and the inhibitor of Nuclear Factor Kappa Beta (NF $\kappa$ B), Tumour necrosis factor alpha-induced protein 3 (TNFAIP3), which may account for increased TNF $\alpha$  production after miR-125a expression

**Conclusions:** This study demonstrates that the miR profile of CD14<sup>+</sup> cells from RA patients is different than those of healthy controls. We have identified miR-125a as a negative regulator of macrophage generated inflammation. Based on our data we postulate that under normal conditions, miR-125a fine tunes the macrophage response to LPS/TLR activation and prevents an overactive inflammatory response by regulating NF $\kappa$ B activation, and the production of pro-inflammatory cytokines. This is accomplished by targeting members of the TLR4 signalling pathway and pro-inflammatory chemokines themselves. However, at sites of chronic inflammation, such as in RA, this regulation is impaired. This

may be due to the masking of the effect of miR-125a by the increased expression of its targets during chronic inflammation, and the lack of response to IL-10 of macrophages within the RA joint. Additionally miR-125a regulation may be impaired due to the dysregulation of other important miRs within the RA macrophage.

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## **Author's Declaration**

I declare that, unless otherwise stated, that this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow, or any other Institution.

## Abbreviations

125am	miR-125a mimic
125bm	miR-125b mimic
ACPA	Anti-Citrullinated Protein Antibody
ACR	American College of Rheumatology
ADAMTS-4	metallopeptidase with thrombospondin type 1 motif 4
ADCC	antibody dependent cytotoxicity
Ago	Argonaute
AID	Activation-induced cytidine deaminase
ALL	acute lymphoblastic leukaemia
AML	acute myeloid leukaemia
APC	antigen presenting cell
APRIL	a proliferation-inducing ligand
B reg	Regulatory B cell
BAFF	B cell activating factor of the TNF family
BAK1	Bcl-2 homologous antagonist/killer
BCL-6	B-cell lymphoma protein 6
BLIMP1	PR domain zinc finger protein 1
CAM	cell adhesion molecule
CDAI	Clinical disease activity index
CDAI	clinical disease activity index
CDK	Cyclin dependent kinase
cDMARD	Conventional disease modifying anti-rheumatic drug
CF	Cystic Fibrosis
CIA	Collagen induced arthritis
Cm	Control mimic
CMV	cytomegalovirus
CPNE3	Copeine III
CRP	c-reactive protein
CRT	cell surface calreticulin
CSR	class switch recombination
Ct	Cycle threshold
CTLA	cytotoxic T-lymphocyte-associated protein
CVD	cardiovascular disease
DAMPs	Damage associated molecular patterns
DAS	Disease activity score
DC	Dendritic cells
DGR8	DiGeorge syndrome critical region gene 8
DKK-1	Dickkopf-related protein
DLBCL	diffuse large B cell lymphoma
DMARD	disease modifying anti-rheumatic drug
EBV	Epstein Barr Virus
ELISA	Enzyme linked immunosorbent assay
ERBB2	Receptor tyrosine-protein kinase erbB-2
ESR	erythrocyte sedimentation rate
ETS-1	V-Ets Avian Erythroblastosis Virus E26 Oncogene Homolog
EULAR	European League Against Rheumatism
FAF1	Fas associated factor 1
FGF	fibroblast growth factor
FOXP3	forkhead box P3

GBRC	Glasgow Biomedical Research Centre
GCs	germinal centres
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GWAS	Genome wide association study
HCC	Hepatocellular carcinoma
HDAC11	Histone deacetylase 11
HDL	high density lipoprotein
HRCT	high resolution CT scanning
HSCs	haematopoietic stem cells
Hsp	Heat shock protein
HSV	herpes simplex virus
iBALT	inducible bronchus associated lymphoid tissue
IDT	Integrated DNA Technologies
IFN	interferon
IGFR-1	Insulin like growth factor 1
IgH	immunoglobulin heavy chain
IKK	IKappa B kinase
IKK $\alpha$	Inhibitor of nuclear factor kappa-B kinase subunit alpha
IKK $\beta$	Inhibitor of nuclear factor kappa-B kinase subunit beta
IKK $\epsilon$	Inhibitor of nuclear factor kappa-B kinase subunit epsilon
IL	interleukin
ILD	Interstitial lung disease
IRAK	Interleukin-1 receptor associated kinase
IRF4	interferon regulatory factor 4
Jak	Janus kinase
Jarid2	jumonji, AT rich interactive domain 2
JIA	Juvenile Idiopathic Arthritis
KLF13	Kruppel like factor 13
KO	Knock-out
LDL	low density lipoprotein
LPS	Lipopolysachride
LYP	lymphoid tyrosine phosphatase
MCP-1	monocyte chemotactic protein 1
M-CSF	macrophage-colony stimulating factor
mDC	Myeloid dendritic cell
MHC	Major Histocompatibility Complex
miR	microRNA
MMP	matrix metalloproteinase
MRE	microRNA response elements
mRNA	Messenger RNA
MS	Multiple Sclerosis
mTOR	Mammalian target of rapamycin
MTX	methotrexate
Mut	mutated
NEMO	Inhibitor of nuclear factor kappa-B kinase subunit gamma
NF $\kappa$ B	Nuclear Factor Kappa Beta
NK	Natural killer
NLRP3	Nod-like receptor protein 3
NSAIDs	non-steroidal anti-inflammatory drugs
OA	osteoarthritis

OPG	Osteoprotegerin
ORF	Open reading frame
ORP-9	oxysterol binding protein 9
Ox LDL	oxidised low density lipoprotein
<i>p. gingivalis</i>	<i>Porphyromonas gingivalis</i>
PAD	peptidylarginine deaminase
PAMP	pathogen associated molecular pattern
PB	Peripheral blood
PBMCs	peripheral blood mononuclear cells
PCDP4	programmed cell death protein 4
pDC	Plasmacytoid dendritic cell
PMA	Phorbol Myristate Acetate
PP1	protein phosphatase 1
PRC2	Polycomb Repressive Complex 2
PRRs	pattern recognition receptors
PTPN22	protein tyrosine phosphatase N22
PU.1	Spi-1 proto-oncogene
PUMA	p53 upregulated modulator of apoptosis
q-PCR	Quantitative polymerase chain reaction
RA	Rheumatoid Arthritis
Rab5a	Ras related protein 5a
RANK	receptor activator of NF- $\kappa$ B
RASF	Rheumatoid arthritis synovial fibroblast
RF	Rheumatoid factor
RISC	RNA induced silencing complex
SCID	Severe combined immunodeficiency
SDAI	Simple disease activity index
SE	Shared epitope
SF	Synovial fluid
SHIP-1	inflammation Phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1
SHM	somatic hypermutation
SJC	swollen joint count
SLE	Systemic lupus erythematosus
SNBTS	Scottish national blood transfusion service
SNPs	single nucleotide polymorphisms
SOCS1	suppressor of cytokine signalling 1
STAT	Signal transducer and activator of transcription
TAB1	TAK1-binding protein 2
TAK1	Mitogen-activated protein kinase kinase kinase 7
TAM	Tumour associated macrophages
TCR	T cell receptor
TCZ	Tocilizimab
Tf	Transcription Factor
TGF	transforming growth factor
TIMP	Tissue Inhibitors of Metalloproteinases
TJC	tender joint count
TLDA	Taqman Low Density Array
TLR	Toll like receptor
TLR4	Toll like receptor 4
TNF	tumour necrosis factor
TNFAIP3	Tumour necrosis factor, alpha-induced protein 3

TNIP2	TNFAIP3-interacting protein 2
tolDC	Tolerogenic dendritic cell
TRAF2	TNF receptor-associated factor 2
TRAF6	TNF receptor-associated factor 6
TRAP	Tartrate-resistant acid phosphatase
Treg	Regulatory T cell
TTP	Trisraprolin
Tyk2	Tyrosine kinase 2
Ub	ubiquitin
UTR	Untranslated region
VEGF	vascular endothelial growth factor
WB	Western Blot
WT	Wild type

# Chapter 1 Introduction

## **1.1 Rheumatoid Arthritis**

### **1.1.1 Introduction**

Rheumatoid Arthritis is a chronic inflammatory disease of unknown aetiology that affects approximately 1% of the population. The aberrant immune response affects the synovial membrane and joints of patients, resulting in irreversible deformity, bone damage, and disability[1]. Typically, noticeable symptoms occur between the ages of 30-50 years, with swelling and pain in multiple joints, and evolution towards a symmetric polyarthritis [2]. RA is characterised by an influx of inflammatory immune cells into the synovium and failure of the natural resolution of inflammation, resulting in the establishment of chronic disease. This in turn drives local mechanical failure of the joint. Due to systemic nature of the immune response, RA is associated with many comorbidities such as cardiovascular diseases, metabolic syndrome, bone pathology, particularly osteoporosis, and cognitive dysfunction, especially fatigue [3]. Together this promotes progressive social, economic and personal decline, deteriorating quality of life and reduced life expectancy. Thus RA comprises a disease of substantial unmet need, which merits detailed dissection, pathogenetic resolution and consequent new medicine development.

### **1.1.2 Classification of RA**

Clinical manifestations of RA vary depending on disease stage and activity. Therefore disease identification is dependent on classification criteria defined by the 1987 American College of Rheumatology (ACR) outlined in Table 1.1. Patients are diagnosed with RA if they present with at least 4 of the 7 criteria outlined.

**Table 1.1 ACR classification criteria for diagnosis of RA adapted from [4]**

<b>Criterion</b>	<b>Definition</b>
<b>Morning Stiffness</b>	In and around the joint lasting >1hr for 6 weeks
<b>Arthritis <math>\geq</math> 3 joint areas</b>	Synovitis in 3 or more joints simultaneously for 6 weeks
<b>Arthritis of hand and wrist</b>	At least 1 swollen area for 6 weeks
<b>Symmetric arthritis</b>	Simultaneous involvement of joints on both sides of body for 6 weeks
<b>Rheumatoid nodules</b>	Nodules observed by a physician
<b>Serum Rheumatoid Factor</b>	Presence of abnormal amounts of RF in serum
<b>Radiographic changes</b>	Presence of erosions or bony decalcifications on radiographs

These criteria have provided the gold standard for RA identification over the past 25 years; however they may have limitations in allowing early diagnosis of disease. Therefore an update to these criteria has been issued, the 2010 ACR/European League Against Rheumatism (EULAR) classification criteria. This is to facilitate the identification of patients who would benefit from early intervention. Patients requiring this early intervention must present with clinical synovitis in at least 1 joint, or have synovitis that cannot be explained by another disease diagnosis.

Classification criteria based on ACR/EULAR 2010 are calculated using a score-based algorithm, outlined in Table 1.2. To be diagnosed with “definite RA”, a patient needs a score of  $\geq 6/10$  by adding the score of categories A - D.

**Table 1.2 2010 ACR/EULAR classification criteria for diagnosis of RA adapted from [5]**

Criteria	Score
<b>A. Joint Involvement</b>	
1 large joint	0
2-10 large joints	1
1-3 small joints	2
4- 10 small joints	3
> 10 joints (at least 1 small)	5
<b>B. Serology (At least 1 result)</b>	
Negative RF and ACPA	0
Low RF or low ACPA	2
High RF or high ACPA	3
<b>C. Acute-phase reactants (At least 1 result)</b>	
Normal CRP and normal ESR	0
Abnormal CRP or ESR	1
<b>D. Duration of symptoms</b>	
< 6 weeks	0
≥ 6 weeks	1

### 1.1.3 Epidemiology of RA

Various incidence and prevalence studies have been carried out over the past few years that suggest a large amount of variation in disease occurrence amongst different countries and populations. Studies suggest that those suffering from RA are more likely to be detected in developed countries with a prevalence of 0.5-1.1% in Northern Europe/North America, compared to a prevalence of 0.1-0.5% in developing countries [6-10]. Whether this reflects a true biologic phenomenon or a reporting bias arising from health care priorities is unknown at this time. In general, rates of RA have been falling in the developed world over the last decade. The reason for this is not known, although altered environmental exposures, for example smoking, may be partly involved.

### 1.2 Risk Factors for RA

Mortality rates in those suffering from RA are higher than in the normal population with survival decreasing 3-10 years depending on age of disease onset and severity [11]. The underlying aetiology of RA has proven difficult to

elucidate, as although defined by a specific set of clinical features, RA is now understood to be a multifactorial disease. The significant variations in disease incidence and prevalence between different populations have led to the investigation of RA specific risk factors that exist within populations.

### **1.2.1 Genetic Risk Factors**

There is overwhelming evidence that development of RA has a genetic component:

#### **1.2.1.1 Twin and Family Studies**

To determine the role of genetic factors in the development of disease many studies have evaluated the concordance of disease between related individuals. This is measured using the parameter  $\lambda_R$ , which is the ratio of prevalence of disease among first-degree relations, compared to the general population. For diseases heavily reliant on genetic abnormalities, such as cystic fibrosis (CF) the  $\lambda_R$  is high at 500, whereas the  $\lambda_R$  for RA is more modest at around 2-4 [12-14]. The most powerful strategy to demonstrate the inheritability of RA is the use of twin studies [15, 16].

#### **1.2.1.2 Major Histocompatibility Complex Associations**

A variety of approaches have been taken to resolve the genes associated with the risk of developing RA. Initial studies were primarily by association. However, of late the application of Genome Wide Association Studies (GWAS) has proven most informative. Even more recently, meta-analyses of these GWAS have yielded further insights. The major genetic risk factor identified to date is the presence of HLA-DRB1 alleles. It has been demonstrated that the majority of RA patients share a short sequence in their MHC Class II molecules which lead to the elaboration of the shared epitope (SE) hypothesis [17]. The RA SE refers to a 5 amino acid motif in the DRB chain of MHC Class II coded by several HLA-DRB1 alleles. The role of MHC Class II in antigen presentation lead to speculation that the SE would lead to the presentation of arthritogenic self and foreign peptides resulting in the selection of pathogenic T cells, however the mechanism of antigen presentation by the expression of the shared epitope remains unclear [18, 19]. More recent studies have also suggested that the SE

itself may act as a signal transduction ligand by activating cell surface calreticulin (CRT), an innate receptor, resulting in the activation of the nitric oxide pathways [20]. Additionally, the shared epitope has been shown to be arthritogenic due to its effects on osteoclasts. Holoshitz et al have demonstrated that *in-vitro* treatment of both murine and human cells results with a synthetic SE peptide induces osteoclastogenesis and the production of TNF $\alpha$  and IL-6. Administration of this peptide to mice with collagen induced arthritis (CIA) resulted in early disease onset, with a higher number of osteoclasts present in the joint, and therefore a higher level of bone erosion [21]. Although, the function of the SE remains unclear, what is clear is that the presence of the SE is associated with severe disease [22].

### 1.2.1.3 PTPN22

Although the MHC locus contributes strongly to the genetic risk in RA it can only explain around 30% of inherited risk, therefore other genetic factors must also play a role [23]. New technology has allowed the identification of many other associated risk loci within RA. GWAS studies have identified single nucleotide polymorphisms (SNPs), which may point to the identity of genes that, in turn play a role in predisposition to disease [24]. One of these, a SNP in lymphoid tyrosine phosphatase (LYP), encoded by the protein tyrosine phosphatase N22 (PTPN22) gene. This phosphatase is involved in T-cell receptor (TCR) signalling. The SNP results in altered amino acid expression by substituting an Arginine to tryptophan resulting in a gain of function for LYP and reducing T cell responses to antigen stimulation [25]. This finding seems paradoxical as it is thought that autoimmunity is more likely to arise from hyper responsive, not hypo responsive TCR signalling. However, as the strength of TCR signalling is one mechanism of selecting autoreactive T cells for deletion in the thymus, reduced receptor activation may impair normal T cell selection, and allow the survival of these potentially pathogenic T cells [26]. Recent studies have also implicated LYP in the activation of integrins and therefore cell migration, demonstrating that expression of the disease associated LYP mutant is associated with a failure to regulate T cell migratory responses. In addition to effector T cells, PTPN22 has been shown to regulate the generation and function of Tregs. PTPN22<sup>-/-</sup> T cells exhibit higher levels of Rap1 phosphorylation and increased IL-10 production, resulting in increased suppressive activity when compared to their wild type

counterparts [27]. Therefore, PTPN22 has been shown to be a critical regulator of both effector and regulatory T cells *in-vivo*.

#### 1.2.1.4 TNFAIP3

TNFAIP3 encodes the ubiquitin editing enzyme A20 that is involved in negative regulation of NF $\kappa$ B signalling. Two RA associated SNPs have been mapped to this locus to date. One of these is a missense mutation that substitutes a phenylalanine residue to a cysteine at position 127, resulting in impaired A20 regulatory function. The other, downstream of the TNFAIP3 promoter, reduces the avidity of NF $\kappa$ B binding and transcription of A20, again impairing its function [28, 29]. The contribution of these SNPs to disease is highlighted using mouse models. Genetic ablation of TNFAIP3 in mice results in the spontaneous development of a severe polyarthritis that resembles RA. These mice have high serum levels of pro-inflammatory cytokines including TNF $\alpha$ , which is consistent with sustained NF $\kappa$ B activity in macrophages [30]. A recent study by Lamkanfi et al, has also suggested that TNFAIP3 is also a regulator of inflammasome activation and IL-1 $\beta$  production in arthritis. They have demonstrated that in TNFAIP3 knockout mice, the arthritis which develops is not dependent on TNF-R1, but on IL-1R and inflammasome activation. This activation usually occurs in 2 steps, with the first being an activation signal, usually through a TLR, resulting in NF $\kappa$ B dependent upregulation of the Nod-like receptor protein 3 (NLRP3) inflammasome protein, pro-IL1 $\beta$  and pro-IL18. As NF $\kappa$ B expression is regulated by TNFAIP3, they have shown that knock-out (KO) macrophages produce higher levels of these proteins, resulting in a higher production of pro-inflammatory TNF $\alpha$ , IL-6 and IL-1 $\beta$ . They therefore hypothesise that TNFAIP3, reduces the pool of NLRP3 available for inflammasome generation and pro-inflammatory cytokine production, and that mutations in this molecule will prove pathogenic [31].

Other genetic loci have also been implicated in disease susceptibility including peptidyl arginine deaminase (PADI4). SNPs within this region are important as PADI4 belongs to a family of enzymes that can post transcriptionally convert arginine residues to citrulline. Antibodies against these citrullinated self-proteins (anti-CCPs) are detected in RA and associated with disease severity [32]. Indeed, a recent study by Suzuki et al has demonstrated 17 SNPs within

this gene, 8 of which are strongly associated with RA. These SNPs associate in distinct haplotypes, and this study has demonstrated a particular 'susceptible haplotype' which is more frequent in RA patients. Expression of this susceptible haplotype is associated with higher levels of anti-CCP antibodies, and therefore may be important in the break of tolerance to citrullinated peptides witnessed in RA [33]. This SNP appears to be influential only in Asian populations [34].

A recent GWAS study, published in Nature, of over 100,000 subjects of European and Asian descent, identified a further 42 SNPs bringing the total identified to 101. From this study, it was shown that the genetic risk for RA is shared, in general, among European and Asian populations, and that around 2/3 of RA risk loci are shared with other inflammation and immune related diseases.

Interestingly, the study also demonstrated that RA risk associated genes, significantly overlap with genes associated with haematological cancers. Many of the genetic risk loci identified contained genes that were already the targets of drug therapy, for example Tyrosine kinase 2 (Tyk2), associated with IL-6R, and therefore already the target of Tocilizumab. This study also demonstrated how genetic data could be integrated with other biological data to drive drug discovery. For example Cyclin dependent kinase (CDK) 6 and CDK4, identified here as RA risk genes, are already approved targets for drugs to treat many forms of cancer. One of these drugs, flavopirodol has been shown to ameliorate disease in animal models of RA [24].

### **1.2.2 Gender**

Incidence of RA is higher in females when compared to males with a ratio of 3:1, suggesting a role for X-linked genes and hormones in disease susceptibility [35]. Studies concerning oestrogen exposure and RA have generated varied results. One study has suggested that oral contraceptive use after diagnosis may protect women, but that there is no effect of prior consumption on RA development [36]. What is clear however is that many women with RA experience remission during pregnancy with an increase in the production of anti-inflammatory cytokines affecting disease activity [37]. After the age of 45 the incidence of RA in men increases and approaches that of age matched women, suggesting decreased androgens are associated with disease onset [38].

### 1.2.3 Environmental Risk Factors

Although genetic risk factors confer susceptibility, a concordance rate between monozygotic twins of only 15%, demonstrates that additional factors must contribute, before a genetically susceptible individual develops RA [39].

#### 1.2.3.1 Smoking

The major environmental risk factor identified to date for disease development is cigarette smoking [40]. Smoking is associated with the production of rheumatoid factor (RF) in both RA sufferers and the general population [41, 42]. In addition smokers are more likely to develop extra-articular features of RA such as lung disease and vasculitis adding to disease severity [43, 44]. It has been hypothesised that cigarette smoking can trigger citrullination of self-proteins, and that these modified proteins are more prone to bind to the HLA-DR SE for presentation. This would result in an immune response to citrullinated proteins in individuals expressing the SE, resulting in the production of Anti-Citrullinated Protein Antibodies (ACPAs) [45, 46]. Citrullination is the post transcriptional modification of arginine residues of proteins by which peptidylarginine is converted to peptidylcitrulline. This reaction is catalysed by PAD enzymes, of which 5 isoforms have been described (PAD1-4 and 6). In healthy physiology, citrullinated proteins are present in a variety of cells and tissues, for example citrullination of keratin within the skin enables the final stage of keratinocyte differentiation, however, recent studies have identified this modification in various pathologies including RA and Multiple Sclerosis (MS) therefore it appears that tolerance to citrullinated proteins is lost in RA [47, 48]. Increase in PAD2 and PAD4 have been demonstrated in the RA synovium [49]. Furthermore RA patients can now be split into two clinically relevant subsets based on ACPA expression [50]. ACPAs are present in only 2% of the normal healthy population, but in over 60% of RA patients, [51]. Citrullinated proteins are not present in the normal joint, but can be generated in response to apoptosis and inflammation [52]. During inflammation, citrullination of various different joint proteins has been demonstrated, including vimentin and fibrinogen. Antibodies from RA patients have been shown to react with the citrullinated, but not the normal forms of these proteins [53].

In addition work by Lundberg et al has identified citrullinated  $\alpha$ -enolase as another potential autoantigen. This protein is highly expressed in the RA joint and antibodies are found within the joint to only the citrullinated form of this protein, and are specific for disease [54]. Lundberg et al identified a dominant B Cell epitope in citrullinated  $\alpha$ -enolase, CEP-1, which reacts with 37-62% of RA sera, but only 2% of healthy controls. This epitope shows high sequence identity with bacterial enolase, and anti-CEP-1 antibodies react with both forms of the citrullinated proteins. This suggests the immune system may be primed by bacterial infection, and tolerance may be broken by a reaction to bacterial enolase, resulting in a chronic response to the human form of this protein, found within the joint [55].

### 1.2.3.2 Infection

Several infectious agents have been implicated as risk factors for RA. Infection with *Porphyromonas gingivalis* (*P. gingivalis*) results in the development of periodontitis in genetically susceptible individuals. Periodontitis is also a chronic inflammatory disease that results in bone resorption within the mouth [56]. Levels of *P. gingivalis* correlate with anti-CCP antibody titres [57]. *P. gingivalis* also express a PAD enzyme which can citrullinate both bacterial and host proteins, with citrullinated  $\alpha$ -enolase being found within the SF of RA patients [54, 58]. This suggests infection with *P. gingivalis* may result in the activation of the host immune system to recognise these modified proteins in genetically predisposed individuals. Additional infections have also been implicated in RA pathogenesis such as infection with the human parvovirus B19 that can cause joint manifestations, in which symmetric arthritis develops in the small joints of the hands and wrist. B19 DNA has been detected in affected joints and presence of DNA is associated with expression of the RA associated shared epitope [59]. However B19 DNA has been found to persist in joint tissue of both individuals who go onto develop RA and healthy individual who do not. Therefore the relationship between RA development and B19 infection remains controversial [60]. Another DNA virus with a proposed role in increasing the susceptibility to RA is Epstein Barr Virus (EBV). This association has been identified for many years, as sera from RA patients contains high levels of antibodies against EBV antigens [61]. The HLA-DRB1\*401 associated motif QKRAA is found on EBV glycoprotein gp110. Healthy individuals have antibodies and

antigen specific T cells against this motif, whereas RA patients have a lower response, this may result in a higher EBV viral load, and an on-going immune response [62]. In addition to viral infection, the presence of mycoplasma DNA is found in the PB of around 53% of RA patients, suggesting a systemic mycobacteria infection [63]. Additional studies have also implicated *Escherichia coli* (*E.coli*) and their production of heat shock proteins (Hsps) in the initiation of RA. Hsps are a family of evolutionary conserved proteins that play an important role in cell physiology under normal and stressed conditions. These proteins are evolutionarily conserved between bacteria and humans and have been implicated in disease pathogenesis, due to their sequence similarity [64]. Hsps are categorised by their size, with Hsp40 being the most intensively studied within the joint. *E. coli* derived Hsp40, or DNAJ, and its human equivalent Hdj1, 2 and 3 have been implicated in disease pathogenesis. Studies have shown the presence of anti-DNAJ antibodies in both RA and Juvenile Idiopathic Arthritis (JIA), and these antibodies have been shown to cross react with both human and bacterial forms of the protein [65]. Additionally, high levels of human anti-DNAJA1 and anti-DNAJA2 antibodies have been demonstrated in the sera and synovial tissue of RA patients [66]. Although the exact role of bacterial and human Hsp40s in the autoimmune response requires further investigation, these observations suggest those proteins have relevance in the induction of RA.

The association of infectious disease and the predisposition to RA development is based on the presence of DNA of, and high antibody titres to infectious agents in RA sufferers compared to healthy controls. However no consistent evidence has emerged to identify a single infection for RA development.

### **1.3 Co-morbidities associated with RA**

Although RA is generally characterised by a destructive immune response within the joint space, RA patients, in common with those suffering from other long-term chronic disease, suffer from various co morbidities. Some of these associated diseases such as cardiovascular disease (CVD); depression and the risk of infection or malignancy contribute to the increased mortality rate of RA sufferers.

### **1.3.1 Cardiovascular mortality and RA**

There is extensive evidence that RA patients with well-established disease have increased risk of ischaemic heart disease [67]. In addition, individuals with RA are more likely to develop heart failure when compared to the general population [68]. Cardiovascular risk may be heightened in RA sufferers due to treatment effects. Of some concern is the use of non-steroidal anti-inflammatory drugs (NSAIDs) which appear to generate the highest risk of a heart attack [69]. It has been demonstrated that chronic inflammatory diseases in general have increased rates of cardiovascular disease. This suggests that it is the presence of on-going inflammation that causes mortality and not specifically the RA disease process.

### **1.3.2 Osteoporosis**

The risk of osteoporosis has been demonstrated in RA patients for many years, due to the presence of pro-inflammatory cytokines and activation of osteoclasts resulting in reduced bone density [70, 71]. Increased disease activity and severity are associated with bone loss, suggesting early treatment may be beneficial in patients to prevent bone erosions [72].

### **1.3.3 Lung Disease**

Pulmonary pathology is common during RA and contributes significantly to its mortality rate. Interstitial lung disease (ILD) is the most common and potentially most dangerous manifestation of RA within the lung [73]. The risk of RA-ILD has been demonstrated to be 10% [74]. The prognosis after being diagnosed with RA-ILD is extremely poor with a median survival rate of approximately 3 years, with the preferred treatment option being immunosuppressive drugs, cyclosporine or cyclophosphamide [75, 76]. The biological mechanisms that drive RA-ILD are relatively unclear, and further research is required due to the high mortality rate from this co-morbidity. Biologic therapy paradoxically is also associated with increased mortality in those with RA and lung disease - the cause of this is also unknown.

Recent studies have also examined the lung as a site of initiation out-with the joint, especially in ACPA positive disease, due to the previously discussed link

between smoking and RA. RA related lung disease has been highlighted by the use of high resolution CT scanning (HRCT) which has identified the presence of ACPAs to be associated with parenchymal lung abnormalities and the citrullination of proteins in early RA [77]. In addition, inducible bronchus associated lymphoid tissue (iBALT) has been identified in the lungs of RA patients in which B cells can produce RF and ACPAs. This has led to a hypothesis that suggests smoking triggers citrullination of proteins within the lung resulting in the production of ACPAs by B Cells. A second injury in the synovium then results in citrullination of joint proteins, which results in the development of chronic disease [78].

#### **1.3.4 Malignancy**

RA is also associated with a risk of lymphoma development, which increases dependent on the level of on-going inflammation present [79]. There has been intense investigation into the risk of malignancy associated with treatment i.e. the use of immunosuppressive agents and new biologic therapies. Small studies have reported EBV related lymphomas associated with methotrexate (MTX) treatment, however this was not replicated in larger case studies [80, 81]. Studies have also reported a risk of lymphoma development with MTX and anti-TNF $\alpha$  therapy, however this risk is relatively low [82].

### **1.4 Clinical Manifestation**

The onset of RA is preceded by an asymptomatic pre-articular phase, marked by a breach of self-tolerance and the presence of circulating antibodies, such as ACPAs and RF. These antibodies can be present years prior to clinical manifestation of disease [83]. A transition event then occurs which results in the disease becoming clinically evident as the synovium is transformed from a functional lining two to three cells deep, to a hyperplastic invasive tissue full of infiltrating immune and resident joint cells [84]. These cells initiate and perpetuate on-going inflammation, tissue damage, and joint destruction.

It is now widely accepted that RA is a multi-factorial disease that develops from a complex interaction of environmental triggers, occurring on a genetically

susceptible background. This results in a breach of tolerance and the recruitment of pro-inflammatory cells to the joint [85].

## **1.5 Immunopathology of RA**

The normal joint is composed of two adjacent bony ends that are covered with a layer of cartilage, separated by a joint space and surrounded by the synovial membrane. The normal synovial membrane is <100 µm thick and the synovial lining consists of a thin layer of synoviocytes. RA is characterised by an influx of pro-inflammatory cells into the synovium resulting in the thickening of the joint lining and pannus formation. The pannus contains proliferating synovial cells that establish chronic inflammation resulting in destruction of cartilage and bone. It is important to understand which cells are present in this tissue in order to development novel treatments which can target either cells or the mediators they which produce.

### **1.5.1 Synovial Fibroblasts in RA synovitis**

The intimal lining of the RA synovium demonstrates an increase in both macrophage and RA synovial fibroblasts (RASFs), both of which are critically important in the disease process [86]

RASFs are active drivers of joint destruction through the production of pro-inflammatory cytokines and matrix degrading enzymes [87]. Activation of RASFs occurs in both an autocrine fashion, through their production of fibroblast growth factor (FGF) and transforming growth factor B (TGFβ) and also through their interaction with pro-inflammatory cytokines such as TNFα, IL-1 and IL-17A, produced by other cells within the joint [87]. Activation of these cells promotes many aspects of disease pathology such as cartilage invasion and degradation. RASFs are described as “tumour like” cells, which can adhere to and invade surrounding cartilage [88]. RASFs are primarily responsible for the destruction of cartilage observed in RA, through the production of matrix metalloproteinase’s (MMPs). These destructive enzymes are constitutively expressed at high levels by early passage RASFs and within the synovium, especially at the cartilage/pannus junction [84]. MMP production by RASFs can be up-regulated by pro-inflammatory cytokines TNFα, IL-1β and growth factors

present at high levels in the joint [89]. In healthy individuals MMP production is regulated by the production of Tissue Inhibitors of Metalloproteinases (TIMPs) produced by chondrocytes, fibroblasts and endothelial cells [90]. In RA this balance is disturbed favouring production of high levels of destructive MMPs resulting in cartilage invasion and destruction. Overexpression of TIMP 1 and 3 in the joint by gene transfer has been shown to reduce RASF invasiveness; with TIMP 3 sensitising RASFs to Fas ligand induced apoptosis [91].

In addition to driving joint destruction, RASFs interact with other cells within the synovium to promote the production of pro-inflammatory cytokines. RASFs contribute to T cell recruitment and retention within the joint through the production CXCL12 that recruits T cells into the synovium [92]. In addition, RASFs produce IL-6, TGF $\beta$  and IL-23, which are essential for the differentiation of Th17 cells [93]. These cells also produce molecules required for the formation of germinal centres (GCs), CXCL12 and CXCL13, and increase B cell survival through the production of B cell activating factor of the TNF family (BAFF) [94]. RASFs also act on resident cells within the joint to promote angiogenesis through the production of growth factors such as vascular endothelial growth factor (VEGF), and promote bone degradation through the production of receptor activator of NF- $\kappa$ B ligand (RANKL) and Dickkopf-related protein 1 (DKK)-1 [95]. It also has been shown that microparticles within the synovium containing inflammatory mediators can be released from RASFs promoting the production of pro-inflammatory cytokines and MMPs and contribute to the activation of adjacent cells [96] An intriguing recent study has also attributed the spread of RA in part to RASFs. Initially only a small number of joints are affected by RA, but this usually spreads. A study in mice has revealed a role for RASFs in this dissemination [97].

### **1.5.2 Dendritic Cells in RA synovitis**

Dendritic cells (DCs) are important immune effector cells due to their ability to present antigen to cells and mediate the adaptive immune response [98]. DCs are immature within the periphery, and are activated in response to pro-inflammatory mediators, such as pathogen associated molecular patterns (PAMPs) during infection. After activation, DCs migrate to the lymph nodes, mature, present antigen to T cells, and secrete cytokines that result in T cell

polarisation. In this way they generate and maintain self-tolerance by presenting endogenous self-antigen both in the thymus and periphery resulting in the deletion of autoreactive cells [99]. Depletion of DCs in animal models results in the induction of a fatal autoimmune disease, emphasising their role in maintaining tolerance [100]. Breakdown of self-tolerance is considered to be an important initiating factor in RA. RA SF contains high numbers of both myeloid and plasmacytoid (mDC, pDC) and these cells have been shown to both migrate into and differentiate within the synovium, dependent on the cytokine and chemokine milieu [101, 102]. RA DCs cells *in-vivo* also appear activated as they express high levels of MHC Class II, RANK and RANKL [103].

Presentation of autoantigen to lymphocytes by DCs can in theory result in the evasion of central tolerance and the induction of autoimmunity. Several auto-antigens have been described in the context of RA, such as citrullinated type II collagen that can be taken up, processed and presented to induce auto-reactive T cells by DCs [104]. Additionally, *in-vitro* synovial DCs have been shown to present human glycoprotein 39 to antigen specific T cells [105]. Auto-antigen recognising T cells can then produce pro-inflammatory and cytotoxic mediators and provide help for autoantibody production by B cells. Additionally, DCs isolated from SF produce high levels of pro-inflammatory IL-1, IL-6 and TNF $\alpha$  when stimulated [106].

In addition to activating the adaptive immune response, DCs have also been shown to induce tolerance. These tolerogenic DCs (tolDCs), mature in an anti-inflammatory cytokine milieu with high levels of IL-10 and TGF- $\beta$ , or immunosuppressive substances such as corticosteroids. tolDCs can induce peripheral tolerance in many ways such as blocking T cell clonal expansion, inducing T cell anergy and inducing the differentiation of Tregs [107]. Studies have therefore investigated the clinical use of these cells as therapy for immune driven diseases. To do this, simply, DC precursors are removed from patients, isolated and differentiated to a tolDC phenotype, at which point they are injected back into the patients. Human clinical trials are continuing with tolDCs in immune related diseases such as Type I diabetes showing positive results [108]. This suggests that on-going trials in RA may prove beneficial in the future, opening up further treatment options for patients.

### 1.5.3 T cells in Rheumatoid Arthritis

T cell involvement in RA has been investigated since the identification of the SE. In addition, recent advances in the understanding of the genetics of RA have demonstrated T cell associated SNPs as risk factors for disease development. For example the SNP at PTPN22 locus and the programmed death 1 (PD-1) gene that protects auto-reactive T cells from apoptosis, allowing a breakdown in tolerance [109].

Based on studies in rodent models, RA was originally believed to be a CD4<sup>+</sup> Th1 driven disease, however only low levels of Th1 cytokines were detected within the joint [1, 110]. However with the description of a new subtype of T cells, Th17 cells, which produce the highly inflammatory cytokine IL-17 the rationale around T cell and disease progression was changed. Th17 cells produce a distinct set of cytokines including IL-17A, IL-17F, IL-21 and IL-22, resulting in a specific immune response [111]. Th17 cells were first described in mice and differentiate from precursor cells in the presence of TGF $\beta$ , and a combination of other pro-inflammatory cytokines, such as IL-1, IL-6, IL-21 and IL-23 [112]. IL-17A is found at high levels in the T cell rich areas of the synovium, and higher concentrations of this cytokine have been identified in both the serum and SF of RA patients, when compared to both osteoarthritis (OA) patients and healthy controls, where it correlates with disease activity [113]. Levels of IL-17A are also higher in ACPA positive disease which has been shown to be more erosive [114]. The location of Th17 cell differentiation is unclear, however the presence of IL-6, IL-23 and TGF $\beta$  in the joint creates conditions where differentiation could occur [1]. IL-17 activates both RASFs and macrophages which can then promote inflammation and destruction, through the release of further pro-inflammatory cytokines and destructive enzymes whilst also acting on chondrocytes and osteoclasts to promote activation and differentiation, allowing cartilage and bone degradation to occur [115]. Experiments demonstrate that the severity and incidence of disease is dramatically reduced in mice deficient in IL-17 or its receptor in models of CIA [110]. However, Th1 cells, and IFN $\gamma$  may still play a role in disease. It has been hypothesised that IFN $\gamma$ , may play a dual role in the synovium, initially supporting inflammation, but inhibiting the differentiation of Th17 cells [116]. In addition to the production of cytokines T cell contact with macrophages within the synovium results in the production of

pro-inflammatory TNF $\alpha$ , chemokines and MMPs [117-119]. Among other things, this cell contact mediated production of pro-inflammatory cytokines is prevented by Abatacept (CTLA-4) therapy discussed in 1.5.5.

Regulatory T cells (Treg) have also been found in the peripheral blood and synovium of RA patients [120]. However it has been hypothesised that the regulatory ability of these cells in disease is impaired. Ehrenstein et al have shown that high levels of TNF $\alpha$  in the synovium can affect the ability of Tregs to suppress pro-inflammatory cytokine expression of effector T Cells [121]. Further studies have shown that indeed, TNF $\alpha$  within the joint has the ability to subvert Treg function. A study by Nie et al has shown that TNF $\alpha$  in the synovium induces the expression of the protein phosphatase 1 (PP1) enzyme, which prevents the phosphorylation of forkhead box P3 (FOXP3) and its activity. They also demonstrated that treatment with anti-TNF $\alpha$  decreased PP1 expression and restored FOXP3 phosphorylation and Treg suppressive function [122].

Additionally, others have hypothesised that under inflammatory conditions in the joint, T cell plasticity, allows Tregs to be polarised towards a pathogenic Th17 phenotype. Wang et al have identified IL-17 producing Tregs in the PB and SF of RA patients, suggesting that these cells may lose their suppressive function at sites of inflammation, under high levels of polarising inflammatory cytokines [123]. Other studies have also suggested that effector T cells within the joint are resistant to Treg suppression [124].

#### **1.5.4 B Cells in Rheumatoid Arthritis**

The involvement of B Cells in the pathogenesis of RA has been highlighted due to the success of depletion therapy, such as with Rituximab, and this population plays several roles in the context of autoimmunity. One of the hallmarks of disease is the presence of circulating autoantibodies such as RF and ACPAs, with the presence of ACPAs being associated with a highly erosive disease [125]. Patients can be split into subsets depending of the presence of these autoantibodies, with 50-80% of patients expressing either RF or ACPAs within their sera [126]. Antibody responses take place in B cell areas of secondary lymphoid organs, such as germinal centres, where somatic hypermutation (SHM) and class switch recombination (CSR) takes place. Ectopic GCs have been identified in around 25% of patients in the RA synovium, where B cells can

accumulate [127]. These well maintained structures allow affinity maturation and receptor editing of autoantibody producing B cells and Humby et al have shown that these structures are functional within the synovium resulting in the continued production of pathogenic autoantibodies [127]. The importance of autoantibody producing B cells in RA has been demonstrated by Holmdhal et al who conducted experiments on  $\mu$ MT mice that lack B cells. Immunisation of these mice with Type II collagen does not induce arthritis, and the T cell response to antigen is also disrupted [128]. The most studied of these autoantibodies, RF, was discovered in the PB of patients in 1957 and can appear years before clinical symptoms are witnessed [129]. Patients seropositive for RF have a worse prognosis, correlated with a more severe aggressive disease [130]. It is believed that RF causes damage through the formation of immune complexes and the Fc mediated activation of the complement system.

In addition to the production of autoantibodies B cells can produce pro-inflammatory cytokines and are efficient antigen presenting cells that can activate T cells through co-stimulation. The importance of this role has been demonstrated using a human synovium/ Severe combined immunodeficiency (SCID) model in which it was shown that T cell activation is B cell dependent [131, 132]

### **1.5.5 Osteoclasts and Osteoimmunology in RA**

Bone destruction by osteoclasts is a hallmark of RA [95]. Osteoclasts are highly specialised cells with the ability to remove calcium from, and degrade bone. In a healthy setting osteoclasts, along with osteoblasts maintain bone homeostasis, [133]. During inflammation the balance between these 2 cells is disturbed, and an accumulation of osteoclasts in the joint results in high levels of bone resorption, leading to damage and erosions [134]. Mice deficient in osteoclasts do not exhibit bone erosion despite the presence of inflammation [135].

Enhanced osteoclastogenesis occurs in the joint due to a combination of factors reliant on the influx of monocytic precursors, and their subsequent differentiation into osteoclasts within the inflammatory environment. Osteoclast differentiation requires the presence of M-CSF and RANKL, both of which are present at high levels in the inflamed joint. These molecules bind to

their receptors, RANK and CD115 respectively inducing differentiation [95]. Th17 cells can also influence osteoclastogenesis through the production of RANKL when stimulated with IL-23 and act directly on precursor cells, which has been shown to be a co-factor in osteoclast activation [136, 137]. Clinical trials using a humanized anti-RANKL antibody (Denosumab) has been shown to be protective against joint destruction in RA as osteoclastogenesis is prevented [138]

Inflammation itself can actively suppress bone formation through its effect on Wnt signalling within the joint [139]. Wnt signalling enhances osteoblast differentiation and downregulates osteoclastogenesis through the expression of Osteoprotegerin (OPG) [140]. The high level of inflammatory cytokines in the joint interferes with the ability of the osteoblast to differentiate and therefore repair bone that has been damaged. One important pro-inflammatory cytokine, TNF $\alpha$  can induce the expression of the proteins DKK-1 and Sclerostin which inhibit the Wnt pathway and are found in the synovium of RA patients [136]. These proteins inhibit osteoblast formation and thus inhibit bone formation and repair [139]. This not only blunts bone formation, but also increases bone degradation as lower amounts of OPG are produced within the joint. It is important to address the balance in RA that is clearly disturbed, as prevention of bone degradation would be of great benefit to the patient in terms of pain and functionality of their joints.

### **1.5.6 Monocytes/macrophages in RA Synovitis**

The role of monocytes and macrophages has been studied intensively within RA due to their ability to produce a large range of inflammatory mediators leading to chronic inflammation, tissue damage and remodelling within the joint [141].

Cells of the mononuclear phagocyte system are 'plastic' cells that can differentiate into different cell types depending on growth factors and cytokines present in their environment. Monocytes differentiate from a CD34<sup>+</sup> stem cell via an intermediate monoblast stage in the bone marrow and when they leave they are thought to circulate for up to three days. Monocytes are recruited rapidly to the site of injury or infection and it is now clear that more than one subtype of these cells exists in humans, and these can be phenotypically

separated based on CD14/CD16 expression upon each subtype which in turn mediate discrete roles in the immune response (Table 1-3) [142]. CD14 is a cell surface protein which acts as a co-receptor for LPS, and CD16 is a receptor which has a low affinity for IgG is also known as FcγRIII.

**Table 1.3 Subsets of human monocytes**

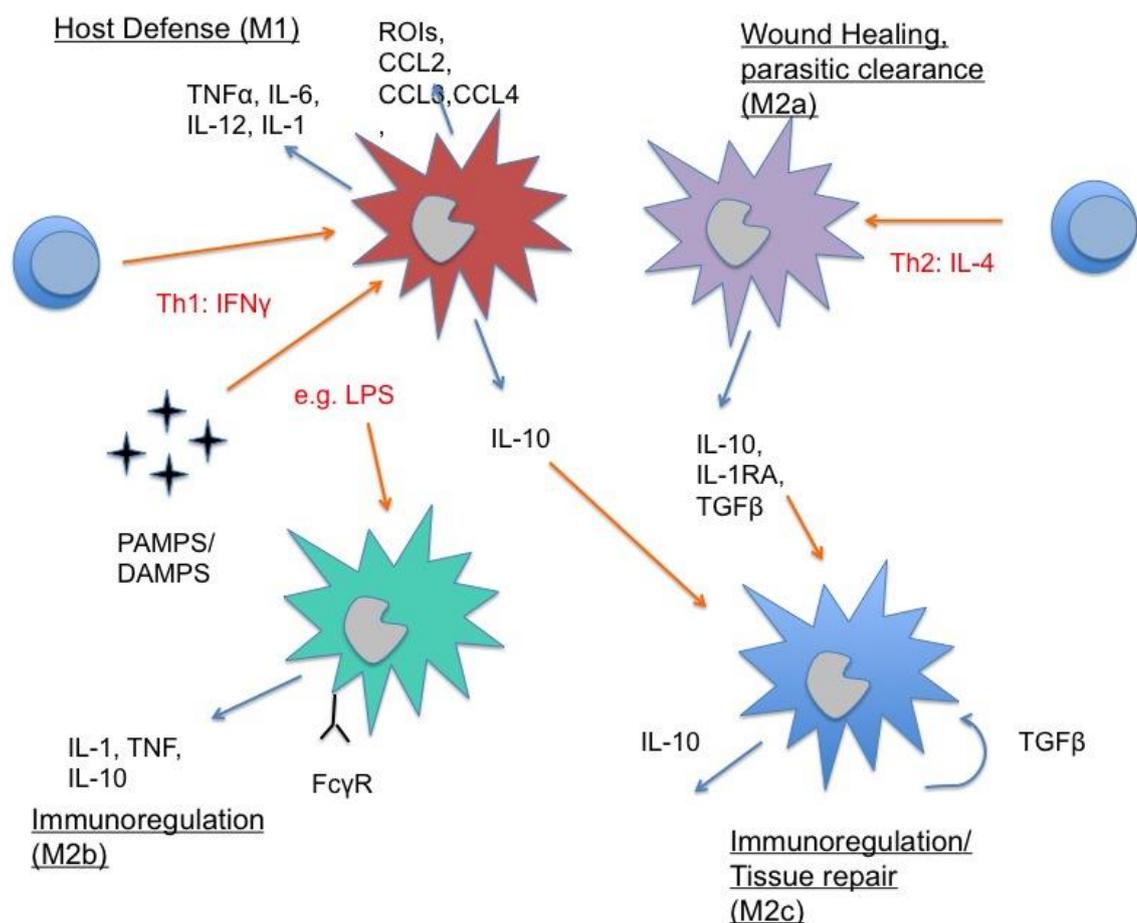
Subset	Markers	Function
Classical	CD14 <sup>++</sup> CD16 <sup>-</sup>	Phagocytosis, anti-microbial, wound healing [143]
Intermediate	CD14 <sup>++</sup> CD16 <sup>+</sup>	Pro-inflammatory- upregulated in various disease states [144]
Non-Classical	CD14 <sup>+</sup> CD16 <sup>++</sup>	Patrolling and anti-viral immunity [145]

CD14<sup>+</sup>CD16<sup>+</sup> monocytes have been shown to express many features of inflammatory tissue macrophages such as high expression of MHC Class II molecules (allowing antigen presentation), high production of pro-inflammatory cytokines, and low production of anti-inflammatory IL-10 [146, 147]. In healthy individuals this population forms 10% of circulating monocytes [146]. It is thought that the differentiation of monocytes is altered in RA patients as PB monocytes express higher levels of adhesion molecules, phagocytic activity and production of pro-inflammatory cytokines [148, 149]. Patients with active RA show higher frequencies of CD14<sup>+</sup>CD16<sup>+</sup> blood monocytes, with the frequency of these cells decreasing in patients that respond well to therapy [144]. The increase in this population can be attributed in some part to the expression of cytokines within the joint that have been shown to drive CD16 upregulation such as TGFβ, M-CSF and IL-10 [150, 151]. These monocytes also express high levels of CCR5 that allow the cells to be recruited rapidly to the joint where they can differentiate under inflammatory conditions to macrophages. It has been proposed that these CD14<sup>++</sup>CD16<sup>+</sup> are the precursors to pro-inflammatory CD16<sup>+</sup> macrophages within the joint [146]. In addition, these ‘intermediate’ monocytes have been implicated in the generation of the pathogenic Th17 response within the joint [152].

In a non-infectious state of chronic inflammation, as witnessed in RA, it is likely that cell to cell contact with T cells contributes to monocyte activation. Rossol et al demonstrated that contact of CD14<sup>++</sup>CD16<sup>+</sup> cells with preactivated T cells causes the cells to produce high levels of TNFα, IL-1β and IL-23. IL-23 is

required to stabilise the pathogenic Th17 phenotype and the production of this cytokine may account for the expansion of the Th17 compartment [153]. Indeed the frequency of Th17 cells correlates with the numbers of intermediate monocytes in RA patients suggesting a direct role for T cell- monocyte contact in the generation of the pathogenic Th17 response in RA [152].

Monocytes migrate from the circulation, through the endothelium and differentiate into macrophages within tissues under the influence of various growth factors such as M-CSF and Granulocyte-macrophage colony-stimulating factor (GM-CSF) [154]. Macrophages, like monocytes exist in different subsets, with differing functions within the immune response depending on the environmental cytokine milieu. To date at least four subsets of macrophages, classified by their immune function and cytokine production have been described (Figure 1.1).



**Figure 1.1 Human macrophage subsets**

To date, 4 subsets of macrophages have been described, characterised by their cytokine production and polarisation environments

Each subset has a different function, with M2 macrophages being more plastic than the classical M1 cells (Table1-4).

**Table 1.4 Subsets of human macrophages**

Subset	Name	Stimuli	Function
M1	Classically activated	LPS/IFN $\gamma$	Defense/pro-inflammatory/microbicidal [155]
M2a	Alternatively activated	IL-4	Anti-inflammatory/wound healing [156]
M2b	Type 2	Fc $\gamma$ R+LPS	Antigen presentation/generation of Th2 response[157]
M2c	Regulatory	IL-10/TGF $\beta$	Inhibit immune response [158]

The number of macrophages in the RA synovium directly correlates with the progression of joint destruction, and response status to a variety of therapeutics, demonstrating the importance of these cells in the clinical context of RA [159, 160]. Activation of macrophages within the joint may occur through the ligation of TLRs PAMPs, or endogenous joint derived particles (DAMPs), released as a result of joint damage, such as fibronectin and Tenascin-C [161, 162]. Similar to monocytes, macrophages can be activated by cell contact within the joint. Macrophage interaction with RASFs results in the production of pro-inflammatory cytokines, with significant cartilage degradation occurring in *in vitro* co-cultures of these cells [163]. Interaction with activated T cells also results in the production of pro-inflammatory cytokines IL-1 $\alpha$ , IL-1 $\beta$  and TNF $\alpha$ , and destructive MMPs [164]. As well as influencing immune cells, cell contact between macrophages and endothelial cells results in the upregulation of specific cell adhesion molecules (CAMs) which are critical for the continued recruitment and retention of activated monocytes within the joint [141]. Macrophages can also be activated in both an autocrine and paracrine way by pro-inflammatory cytokines within the joint.

Once activated macrophages are critical in promoting inflammation and have many functions in RA.

Macrophages are a major phagocytic cell population within the immune system. Within the RA synovium damage to the joint and high levels of pro-inflammatory

cytokines can result in cell death. Dead cells are phagocytosed by macrophages resulting in their activation and the production of pro-inflammatory cytokines such as TNF $\alpha$ , IL-1 $\beta$  and IL-12. In addition macrophages can process and present antigen to T and B cells resulting in the initiation of the adaptive response and perpetuation of inflammation [165]. In addition to the production of cytokines, macrophages also produce high levels of chemokines such as IL-8, CCL3, CCL4 and CCL-2 resulting in the recruitment of further inflammatory cells to the joint [166].

### **1.5.7 Macrophage effector cytokines in RA**

The primary function of macrophages within the RA synovium is the production of cytokines and other mediators of inflammation. Cytokines are a varied group of proteins that can be secreted or membrane bound, and regulate immune function. They are released by almost all cells during the effector phase of the immune response and alter the environment to regulate growth, differentiation and inflammation. Cytokines appear to be crucial at each stage of the pathogenesis of RA and are influential in the establishment of chronic disease. Novel treatments for RA have focused on blocking many of these molecules such as TNF $\alpha$ , IL-6 and IL-1, which will be discussed below.

#### **1.5.7.1 TNF $\alpha$**

TNF $\alpha$ , which is mostly produced by macrophages, is critical in the inflammatory cascade within the joint [141]. It is found at high levels in the synovial membrane and at the cartilage pannus/junction of RA patients, and in general expression correlates with severe disease [167]. Blocking of TNF $\alpha$  has proven extremely important in the clinical setting - its blockade using specific molecular therapeutics confers a clinical response in 70% of patients, reflecting not only its importance in primary pathogenesis, but how disease differs between individuals and at different stages of clinical manifestation [1]. TNF $\alpha$  is important in RA due to its pro-inflammatory effects. It induces the release of additional pro-inflammatory cytokines from cells, such as IL-1, IL-6, IL-23 and G-M-CSF which enhance the inflammatory environment and drive destruction [141]. It also induces the up-regulation of various chemokines such as CCL5, CCL2 and IL-8 resulting in the recruitment and retention of immune cells [141]. TNF $\alpha$  does

not only contribute to retention of, but also to the activation of cells such as osteoclasts, leading to resorption of bone and joint destruction. Additionally, TNF $\alpha$  contributes to angiogenesis and endothelial activation, further aiding the infiltration of destructive immune cells [141].

In addition to TNF $\alpha$ , macrophages also produce a large number of various other pro-inflammatory cytokines that are emerging as critical in the establishment of chronic inflammation.

### 1.5.7.2 IL-1

IL-1, like TNF $\alpha$  is predominantly produced within the joint by inflammatory macrophages and correlates with severe disease progression [168]. Unlike TNF $\alpha$ , IL-1 appears to primarily mediate tissue destruction, by regulating proteoglycan synthesis and therefore bone and cartilage destruction [169]. IL-1 production by macrophages induces the production of MMP-1 and MMP-3, enhancing matrix destruction and providing a critical role for macrophages in bone resorption [170]. IL-1 can bind 2 surface receptors, 1 activatory and 1 inhibitory. The activatory type I IL-1 receptor (IL-1R1) is found on a variety of cells within the RA synovium allowing many cells to respond to the pro-inflammatory cytokine. However expression of its counterpart, the decoy IL-1 receptor (IL-1RII), is low in the synovium [171]. This suggests that the IL-1R balance in RA is disturbed, promoting expression of the pro-inflammatory type I receptor, resulting in chronic inflammation and destruction [172]. However therapeutic interference in this pathway has elicited only modest responses in patients, suggesting problems due to redundancy in IL-1R signalling [173]. In addition to IL-1, macrophages also produce an IL-1R antagonist (IL-1RA) that binds to the receptor but does not induce an intracellular response.

As TNF $\alpha$  inhibition does not aid in recovery of all patients, it is important to continue developing therapies that focus on other inflammatory mediators within the joint. Inhibition of IL-1R by molecules based on IL-1RA (Anakinra) have proven modestly effective in the treatment of RA but has shown some efficacy with regards to slowing joint destruction [174]. However the magnitude of improvement is insufficient to represent a viable therapeutic intervention.

### 1.5.7.3 IL-6

IL-6, along with TNF $\alpha$  and IL-1 $\beta$ , drives production of C-reactive protein (CRP) from the liver and is expressed at high levels in SF where it correlates with DAS [175, 176]. This cytokine plays a central role in the initiation and propagation of inflammation observed in RA. IL-6 exerts its functions through 3 molecules; a membrane bound receptor mIL-6R, the signal transducer gp130 and also a soluble form of receptor, sIL-6R. Binding of IL-6 to mIL-6R results in homodimerisation with gp130 resulting in the formation of a signalling complex. Uniquely IL-6 can also bind to sIL-6R and binding of this complex to membrane bound gp130 also results in activation in a process named trans-signalling [177]. mIL-6R is expressed only on specific cells within the body, whereas gp130 is ubiquitously expressed, widening the populations of cells which can be activated by IL-6 [178]. IL-6 is a multi target cytokine that has wide ranging effects on both the innate and adaptive immune system. It can act on endothelial cells and monocytes to induce the up-regulation of adhesion molecules and the production of CCL2 and IL-8, increasing cellular invasion into the synovium [179]. It can also propagate joint destruction as it induces RANKL production from RASFs, and also the production of MMP1, 3 and 13, resulting in increased osteoclastogenesis and joint destruction [180, 181]. IL-6 also influences the adaptive immune response by driving the induction of pathogenic Th17 cells and the development of autoantibody producing plasma cells [182, 183]. The importance of IL-6 in joint inflammation is highlighted by the effectiveness of the humanised anti-IL-6R antibody Tocilizumab (TCZ) which has been shown to improve clinical symptoms and prevent damage progression in RA [184]. Recent studies using this therapy have implicated IL-6 signalling in lipid metabolism in RA. TCZ treatment across 8 independent clinical trials has shown an increase in serum cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL). The reason for these changes requires more detailed mechanistic studies but likely reflects regulation of scavenger receptor expression and thus altered reverse cholesterol transport [185].

### 1.5.7.4 IL-23

IL-23, another member of the IL-12 family promotes and maintains Th17 differentiation by inducing the production of IL-6, TNF $\alpha$  and IL-1 $\beta$  from synovial

macrophages and RASFs [186]. IL-23 is formed of a p19 and p40 subunit (which is shared with IL-12). The p19 subunit, along with IL-17 is significantly increased in both SF and synovial tissue of RA patients when compared OA [187]. IL-23 is inducible by TLR activation in RASFs and has been shown to play a role in joint destruction through the induction of TNF $\alpha$  and IL-1 $\beta$ , which results in the production of RANKL from these cells [188-190]. Mouse models have demonstrated the importance of IL-23 expression in RA, where transgenic mice that overexpress the p19 subunit demonstrate increased expression of TNF $\alpha$  and IL-1 $\beta$  resulting in systemic inflammation and premature death [191]. However, although the p19 subunit is upregulated within the synovium, levels of the p40 subunit and active IL-23 are low in both SF and PB of RA patients [187]. Previous attempts to block IL-23 in RA have so far failed to prove effective.

#### **1.5.7.5 IL-18**

A profound dysregulation of the IL-18 system exists within RA. IL-18 is produced by CD68<sup>+</sup> macrophages in lymphoid aggregates, and CD14<sup>+</sup> monocytes and neutrophils from the synovial fluid of patients [141, 192]. It has been a focus of interest in RA as levels correlate with the inflammatory marker CRP released by the liver [193]. It is thought that IL-18 exerts its disease causing effects through the induction of TNF $\alpha$ , GM-CSF and Interferon (IFN)- $\gamma$ , the induction of the acute phase response and upregulation of TLR-2 and TLR-4 in synovial cells [194]. IL-18 is also involved in joint destruction through the upregulation of RANKL production by T cells, resulting in higher levels of osteoclast differentiation [87]. Prior studies have shown that IL-18 treatment markedly aggravates arthritis in mice and there appears to be a profound dysregulation of the IL-18 system in patients with RA [87].

#### **1.5.7.6 IL-15**

Macrophage produced IL-15 is detectable in RA synovial fluid by ELISA at comparable levels to TNF $\alpha$  and has chemoattractant properties for memory T cells [195]. Lining layer macrophages are the predominant source of IL-15 within the synovium [196]. IL-15 stimulated T cells have been shown to induce TNF $\alpha$ , IL-1 $\beta$  and IL-8 production from macrophages in a co-culture system [197]. This data suggests a feedback loop in which IL-15 produced by activated macrophages

within the synovium can maintain T cells to further perpetuate inflammation. Baslund et al have trialled a humanised monoclonal anti-IL-15 antibody (HuMax-IL15) that demonstrated a substantial improvement in disease activity according to ACR criteria [198]. These data suggest targeting of IL-15 may produce therapeutic benefit in RA of which phase IIa human studies concurred with.

#### **1.5.7.7 IL-10**

In addition to pro-inflammatory cytokines, synovial macrophages also produce anti-inflammatory cytokines such as IL-1RA (discussed in 1.1.7.2) and IL-10. IL-10 produced by macrophages has autocrine effects and causes down-regulation of antigen presentation and pro-inflammatory cytokines such as TNF $\alpha$ , IL-1 and IL-6 [199]. Despite its anti-inflammatory functions IL-10 is present in the RA synovium [200]. Studies have suggested that co-expression of IL-10 with TNF $\alpha$ , as witnessed in RA may indicate a failure of IL-10 to sufficiently suppress the immune response [201]. Additionally reports have suggested that continual expression of TNF $\alpha$  also causes macrophages and synovial fibroblasts to become unresponsive to IL-10 and this may explain the lack of clinical effect witnessed by IL-10 treatment in RA [202, 203].

#### **1.5.8 Macrophage effector chemokines in RA**

In addition to cytokines, macrophages also produce high levels of inflammatory chemokines within the joint. Chemokines are chemoattractant cytokines that are important in the recruitment and retention of leukocytes at sites of inflammation. Chemokines are classified based on their structures and the position of cysteine residues. Four families of chemokines exist, CXC, CC, C and CX3C, each with differing functions in both health and disease. The previous years have seen an increase in studies aimed at targeting chemokines and their receptors within RA. Important chemokines produced by macrophages will be discussed below.

##### **1.5.8.1 IL-8/CXCL8**

CXCL8 is detected at high levels in SF, sera and involved joints of RA patients, suggesting it plays a direct role in inflammation within the synovium [204, 205]. CXCL8 is produced by macrophages, synovial fibroblasts and endothelial cells

within the joint and is a potent angiogenic factor [206]. Angiogenesis is a hallmark of inflammation and is required for the recruitment of inflammatory cells to the joint space, therefore CXCL8 drives joint inflammation both indirectly, by creating new blood vessels for leukocytes trafficking and also through its direct role as a chemoattractant for neutrophils [207]

#### **1.5.8.2 IP-10/CXCL10**

Synovial fluid levels of CXCL10 are higher in RA compared to OA [208]. CXCL10 expression is induced by IFN $\gamma$  and is a chemoattractant for Th1 cells and is present at high levels in T cell rich areas of the synovium. Immunostaining has shown that macrophages in the lining layer and infiltrating synovial fibroblasts are the main source of this chemokine, which causes the perpetuation of inflammation through its recruitment of pathogenic T cells [209]. Studies have shown that treatment of CIA mice with anti-CXCL10 antibodies suppressed clinical progression and joint erosion of RA by slowing the recruitment of Th1 cells [210]. In addition use of a humanised monoclonal antibody against CXCL10 increased response to methotrexate in patients who previously responded inadequately to the drug, suggesting there may be a clinical effect in targeting CXCL10 [211].

#### **1.5.8.3 CCL2/MCP-1**

CCL2 is expressed at high levels in RA SF when compared to OA, with expression induced by TNF $\alpha$ , IFN $\gamma$ , IL-1 $\beta$  and hypoxia, all of which are found in the RA synovium [212, 213]. Expression of high levels of CCL2 exacerbates inflammation through the recruitment of pro-inflammatory leukocytes, mainly monocytes. Current therapies for RA such as anti-TNF $\alpha$  have been shown to decrease the levels of CCL2 present in the arthritic joint potentially offering a mechanism to explain diminished inflammatory monocyte migration [214]. .

#### **1.5.8.4 CCL5/Rantes**

CCL5 is a chemoattractant for lymphocytes and monocytes among other cells, with messenger RNA (mRNA) being detected in both synovial macrophages and T cells [215]. CCL5 is present in RA SF and serum at higher levels than OA, where expression correlates with number of swollen joint, erythrocyte sedimentation

rate (ESR) and CRP [216]. Expression of CCL5 has been shown to induce IL-6 production from RASFs, and expression of this chemokine is downregulated by treatment with disease modifying anti-rheumatic drug (DMARD) therapy [217].

#### **1.5.8.5 CCL3/MIP-1 $\alpha$ and CCL4/MIP-1 $\beta$**

Both CCL3 and CCL4 are major chemokines produced by macrophages after stimulation with bacterial endotoxins, which have been shown to be present within the joint. Levels of CCL3, a chemoattractant for various cells, including T cells, monocytes and neutrophils, are higher in RA SF compared to OA [218-220]. CCL3 has been shown to directly stimulate osteoclast formation by increasing RANKL and IL-6 production from stromal cells in multiple myeloma, suggesting it could have a similar effect on these cells in RA [221]. CCL4 is also readily detectable in RA SF where its expression correlates with disease activity, and is thought to be involved in the further recruitment of monocytes to the joint [222, 223].

## **1.6 Current Therapies for RA**

Recent advances in understanding the pathogenesis of RA have resulted in the advancement of treatment, from treating pain to treating the aberrant immune response associated with joint inflammation. The advent of biologic therapies, targeting disease propagating molecules has revolutionised prognosis and progression of disease.

### **1.6.1 cDMARDs**

cDMARDs can be used alone or in combination with each other, or a biologic therapy. The best studied cDMARD, MTX remains the foundation of initial therapy, and is used alone and in combination with other treatments. Other cDMARDs which have been shown to be effective in RA treatment both alone and in combination are sulfasalazine, hydroxychloroquine and leflunomide. The exact action of these DMARDs has yet to be clearly understood though increasingly they are recognised to modulate a variety of inflammation associated signalling pathways e.g. NF $\kappa$ B and Adenosine.

### 1.6.2 Anti-TNF $\alpha$ therapy

TNF $\alpha$ , as discussed is a potent pro-inflammatory cytokine with a dominant role in chronic inflammatory and destructive pathways within the joint. It causes macrophage production of pro-inflammatory cytokines IL-1, IL-6 and IL-8, activation of T cells, differentiation of osteoclasts and activation of RASFs to produce MMPs [224]. TNF $\alpha$  was the first cytokine to be fully validated as a therapeutic target within the joint and molecules that knock down its expression have revolutionised the treatment of RA. To date 5 TNF $\alpha$  targeting agents have dominated biological treatment of RA, Etanercept, Infliximab, Adalimumab, Golimumab and Certolizumab. Etanercept is a dimeric fusion protein of human extracellular p75 TNF $\alpha$  receptor linked to Fc region of human IgG1. The others are monoclonal antibodies against TNF $\alpha$  [225]. Efficacies of each of the agents in RA clinical trials are similar and each has been shown to slow radiographic progression. Each of these biologics is more effective when combined with another DMARD, particularly MTX [83, 226-228]

However not all patients respond to anti-TNF $\alpha$  treatment and therefore other treatment options require investigation.

### 1.6.3 Rituximab and B Cell depleting therapy

Rituximab, a mouse/human chimeric IgG1 monoclonal antibody was first developed for use in treating B cell malignancies, but was later licensed for treatment of RA [229]. Rituximab targets B cell specific cell surface antigen CD20 and leads to rapid B cell depletion via antibody dependent cytotoxicity (ADCC) [230]. In the phase III REFLEX trial in which Rituximab treatment was given to 311 patients, compared to 209 placebo, they found that a single course, concomitantly with MTX provided significant and clinically meaningful improvement in patients who had already failed anti-TNF $\alpha$  therapy [231]. Although CD20 is expressed on the majority of circulating B cells it is not expressed on lymphoid progenitor cells within the bone marrow that allows repopulation of autoreactive B cells after treatment, and can lead to relapse. The success of B cell depleting therapy appears dependent on levels of CD27+ memory B cells in the periphery, as a high percentage of memory cells as baseline is associated with early relapse [232].

The success of Rituximab treatment has led to a variety of therapies which target CD20 on the cell surface such as the monoclonal antibodies Ofatumab and Ocrelizumab [233]. Phase I clinical trials in RA are also on-going to investigate the blocking of the cytokine IL-21 [234]. IL-21 is responsible for the differentiation of plasma cells and therefore has a role in autoantibody production [235].

However, not all B cell directed therapies have proven effective and their failure for example, Atacicept within RA is still not fully understood. Atacicept is a fusion protein containing the extracellular domain of transmembrane activator and calcium modulating ligand interactor, fused to Fc portion of IgG which binds to and blocks both BAFF and a proliferation-inducing ligand (APRIL), to inhibit B-cell maturation [236]. However, studies have shown that APRIL may be required for maintenance and generation of IL-10 producing regulatory B cells (Bregs) [237], and therefore, it has been hypothesised that atacicept depletes these protective Breg cells without sufficiently targeting pathogenic subsets.

#### **1.6.4 IL-6R blockade**

The central role of IL-6 in joint inflammation highlighted IL-6 as a potential target in RA. There have now been many trials showing the use of TCZ, a humanised anti-IL-6R monoclonal antibody, on patients with RA. One of these trials, on patients with established RA, who had previously failed one DMARD, found improvements in disease activity within 1 week in patients receiving high doses of TCZ [238]. Further trials of TCZ alone, and combined with MTX, demonstrated that combination therapy was effective in those patients who were previously resistant to anti-TNF $\alpha$  therapy and that TCZ treatment reduced radiographic joint damage [239, 240]

#### **1.6.5 Abatacept and T cell blockade**

Abatacept therapy focuses on targeting the T cell response in RA. Abatacept is a human, soluble fusion protein made up of the extracellular domain of human cytotoxic T-lymphocyte-associated protein (CTLA)-4 linked to modified Fc portion of human IgG. It binds CD80/86 on the surface of the antigen presenting cell (APC) preventing co-stimulation and activation of T cells, whilst reducing

their ability to activate macrophages by cell contact [241]. Clinical trials have demonstrated effectiveness of abatacept therapy in patients with inadequate responses to both MTX and anti-TNF $\alpha$  therapy, offering an alternative treatment option for those patients who are currently failing “gold standard” treatments [242, 243].

### **1.6.6 Chemokine inhibition in RA**

Chemokines are important in recruiting inflammatory cells to the RA joint to perpetuate inflammation. Although indirect effects on chemokine expression appear beneficial in disease, direct targeting of chemokines and their receptors in RA has so far, proved disappointing. Despite promising pre-clinical studies in animal models of disease, chemokine inhibition has largely proved a failure [244]. For example, use of a CCR1 antagonist (MLN3897) provided similar ACR20 response criteria as methotrexate alone in a Phase IIa trial [245]. Additionally, an anti-CCR2 monoclonal antibody also failed to show efficacy in Phase II trials [246]. Chemokine inhibition in RA has proven difficult due to redundancy in the system, and levels required to saturate receptors. However, combination therapy may help to improve the efficacy of these drugs in the future.

### **1.6.7 Other Therapies**

Tofacitinib, a Jak 1/3 inhibitor has been trialled in the context of RA. JAK1 and 3 are Janus kinases (Jak) that mediate signal transduction of cell surface receptors after interaction with cytokines in RA. Tofacitinib, which can be taken orally, has been shown to improve disease in patients with active RA, in combination with MTX [247].

### **1.6.8 Unmet Need**

TNF $\alpha$  blockade shows a response in around 60-70% of patients regardless of DMARD status [248]. It is therefore important to identify patients who will respond to these expensive and potentially toxic therapies, prior to administration. Bacterial infections including sepsis and pneumonia have been reported with the use of anti-TNF $\alpha$  treatment. There is also a risk of the reactivation of latent TB associated with the use of these drugs [249]. It is not only anti-TNF $\alpha$  therapy which comes with a risk of adverse side effects, but

reports of psoriasis and psoriatic arthritis have been witnessed after Rituximab treatment and infection, decreased neutrophil and increased lipids in TCZ treated patients [240, 250].

The success of biologic interventions has revolutionized RA therapy - however considerable unmet needs remain including the need for remission inducing therapeutics, identification of new therapeutic targets and biomarker based prediction of response and toxicity. In this context I elected to study the biology of epigenetic mechanisms, specifically the relevant biology of microRNAs in the context of RA monocyte biology.

## **1.7 MicroRNA**

miRs are conserved short, non-coding RNA molecules approximately 21-23 nucleotides in length which act to post transcriptionally regulate gene expression. Regulation occurs through binding of the miR to complementary sequences, typically in the 3' UTR of target mRNA resulting in repression of translation [251] Almost 2,500 human miRs are now predicted to exist, although possibly not all are functional, that regulate ~60% of human protein coding genes [252]. Due to their key role in regulation of gene expression miRs have been shown to impact various aspects of mammalian biology including cell proliferation, differentiation and the development of the immune response. Aberrant expression of miRs has been implicated in many human diseases including cancer, cardiovascular disease and autoimmunity.

### **1.7.1 Biogenesis of microRNA**

miRs can be located within exons and introns of both coding and non-coding transcripts with studies demonstrating more than 50% are expressed intronically[253]. Intronic miRs can be dually regulated both by their own intronic and host gene promoters, and can also have their own gene [254]

Current models of miR biogenesis suggest a compartmentalised process beginning in the nucleus and ending in the cytoplasm. However as more information emerges concerning this process it is becoming clear that one

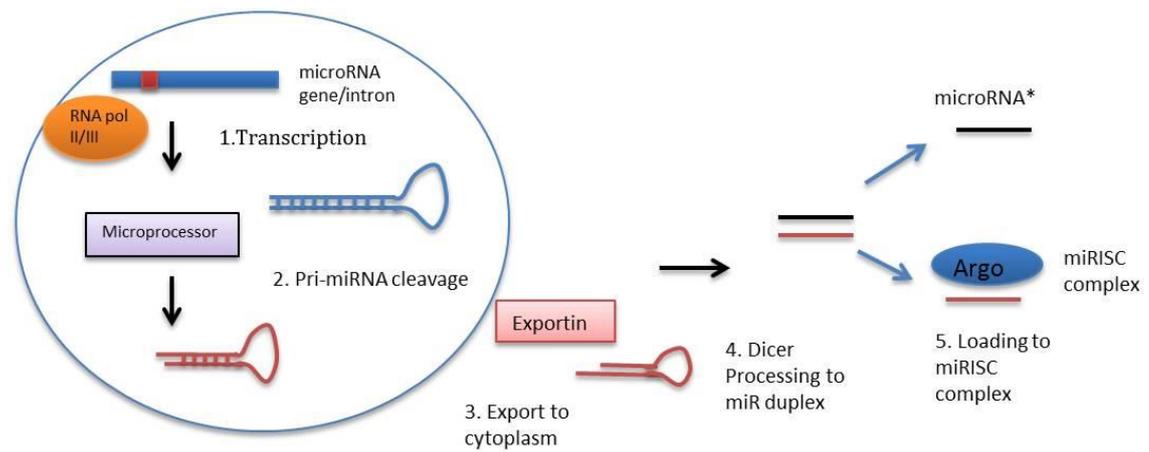
pathway of processing does not apply to all miRs. A canonical pathway of consecutive cleavage of precursor miR has been proposed.

Typically, miRs are transcribed by either RNA polymerase II or III into long primary transcripts that contain either 1 or more hairpin structures, forming the pri-miRNA. Processing of the pri-miR into the final mature miR occurs in several steps [255, 256].

Firstly pri-miRs are processed in the nucleus by a multiprotein complex composed of the RNase III enzyme Drosha and the RNA binding protein DGR8 (DiGeorge syndrome critical region gene 8), dubbed the Microprocessor [257]. Drosha recognises specific sequences at both 5' and 3' ends of the hairpin structure, whilst DRG8 recognises structural features of the pri-miRNA ensuring Drosha cleavage occurs at the precise correct location [258]. Cleavage releases the hairpin structure of the pre-miRNA. This pre-miRNA contains a 5' phosphate and a 3' overhang which is recognized by the transportin protein Exportin 5. Exportin 5 coupled to Ran-GTP enables active transport of the pre-miR from the nucleus to the cytoplasm, where processing continues [259].

It is at this step of processing an alternative pathway can also occur. In addition to processing by Drosha, intron derived transcripts can be released by splicing. These hairpins or mitrons are already the correct size of pre-miRs and can therefore bypass Drosha processing and be transported immediately to the cytoplasm by Exportin 5 [260]

Once in the cytoplasm the hairpin pre-miRNA is further processed by the RNase III enzyme Dicer. Dicer cleaves the hairpin loop of the pre-miR resulting in an unstable ~22 nucleotide miRNA-miRNA\* duplex. The importance of Dicer processing is further illustrated by the lethal phenotype of dicer knock out mice [261]. One strand of the duplex, dependent on base pair stability at the 5' end, is then incorporated into the RNA induced silencing complex (RISC) which is guided to the seed sequence in the 3' UTR of its target mRNA for silencing [262]. The function of the passenger strand (miRNA\*), is so far unknown, however there is some suggestion that this is not simply discarded and degraded, but also plays a biological role [263].



**Figure 1.2 Canonical processing of microRNA**

miRs are transcribed within the nucleus, cleaved and exported to the cytoplasm where further processing takes place. The functional miR stand is loaded onto the RISC complex and is then able to regulate its targets

### 1.7.2 Function of microRNA

MicroRNAs recognize their target mRNA through the principle of Watson-Crick base pairing. Typically miRs recognize complementary base pairs located in the 3' UTR of the target mRNA [264].

Requirements at this 'seed region' (miR nucleotides 2-8) are stringent, as this is the location where the miR recognizes its target mRNA. Nucleotides 2-8 of the seed region must bind complementarily to the 5' tail of the microRNA for target recognition to occur. The requirements at the 3' end of the microRNA are more relaxed and the miR: mRNA duplexes typically contain imperfect mismatches and bulge out with the seed regions [265]. In addition, miRs have now been shown to also bind to sequences in the 5' UTR and coding sequence of target genes opening up alternative mechanisms of regulation [266]

Initial evidence suggested that miRNA mediated regulation of gene expression occurred through repression of mRNA resulting in reduced transcription without altering mRNA levels. However it is now clear that miRNAs function in at least two basic ways, through repression or degradation of mRNA [267]. Which of these methods dominates in mediating microRNA function is still under debate. mRNA degradation has been clearly demonstrated. Many studies have shown that upon transfection with a microRNA mimic or antagomir, levels of mRNA targets containing the miRNA seed region are altered [268]. It is thought that

miRNA regulated degradation occurs under perfect binding conditions and that miRNAs target mRNA to the 5'-3' mRNA decay pathway where they are deadenylated and decapped resulting in endonucleolytic digestion [269]

However as the majority of binding is not perfect, translational repression is also important. Evidence suggests that miRs can repress translation at both pre and post initiation stages. Argonaute (Ago) proteins of the miRISC complex have been shown to compete with the translation initiation factor eIF to inhibit translation initiation [270] In addition it has been demonstrated that the miRISC complex can inhibit translation by interfering with ribosome elongation and increasing proteolysis [271].

### **1.7.3 Prediction of microRNA targets**

Prediction of reliable microRNA targets is essential. The most used approach currently for identifying miR targets is to use computer-based algorithms that scan for complementary microRNA response elements (MREs) within messenger RNA. Target prediction occurs based on a 3-step protocol. Firstly the miR seed region is identified, next 3' UTRs are searched for matching aligning regions. Lastly the site is examined for conservation throughout species. The hypothesis being that evolutionary conserved targets will have been under selective pressure to remain in the genome[272]

Not all algorithms return identical predicted targets for miR, but there is a large amount of overlap and so it is important that many are used simultaneously to determine predicted miR targets.

**Table 1.5 Tools for microRNA target prediction**

<b>Tool</b>	<b>Mechanism of Prediction</b>	<b>URL</b>	<b>Targeting confined to 3'UTR</b>
<b>TargetScan</b>	Stringent seed pairing, site conservation, context score	<a href="http://targetscan.org">http://targetscan.org</a>	Yes
<b>MiRanda</b>	Moderate seed pairing, conservation, number of sites	<a href="http://www.microrna.org">http://www.microrna.org</a>	Yes
<b>PicTar</b>	Stringent seed pairing, site conservation, pairing stability	<a href="http://pictar.mdc-berlin.de">http://pictar.mdc-berlin.de</a>	Yes
<b>RNA.22</b>	Moderate seed pairing, pairing stability, no site conservation	<a href="http://cbcsrv.watson.ibm.com/rna22">http://cbcsrv.watson.ibm.com/rna22</a>	No

The majority of algorithms are built around seed pairing of miR to mRNA. The seed region, nucleotides 2-8 at the 5' end of the miR, has particular importance in miR targeting, and is the most evolutionary conserved region of the miR [273]. Frequently, this region is most complementary to the mRNA, and studies have shown complementarity in this region alone can confer target recognition [265]. However, new data are emerging that suggest that other areas of the miR are also important, and that miR recognition can also occur in both the DNA coding sequence and open reading frame (ORF) [274-276]. As accepted targeting methods are expanded it is important to recognise that computer algorithms, which confine targeting to the 3' UTR may miss genuine targets (Table 1.5). However these algorithms are still widely used, and are an essential part of the beginning of many studies.

Due to the potential of algorithms to generate false positive data, and also miss certain targets depending on their location, it is essential that experimental validation of target interactions is confirmed. This validation is usually carried out using luciferase reporter assays, which can demonstrate the potential of a miR to target its MRE sequence within a reporter plasmid.

In addition, degradation of target mRNAs can be visible by transcriptomic analysis, such as microarrays following ectopic miR expression. The first of these transcriptomic profiles was demonstrated in 2005, where a downregulation of over 100 genes was witnessed after transfection of HeLa cells with either a miR-1 or miR-124 mimic. These downregulated genes were found to contain a consensus sequence, to either miR-1 or miR-124 seed regions, suggesting these mRNAs were targets of these miRs [277]. Since this study, many other groups have employed this method to identify miR targets [278]. Limitations of this method remain problematic, as some miRs exhibit only modest effects on degradation of targets, while others function only at a level of translational repression [277].

As technology advances, new methods of identifying miR targets are continually being introduced, such as immunoprecipitation of RISC components. This is a method by which target mRNAs undergoing direct miR regulation are immunoprecipitated along with Argonaut proteins, which makes up part of the RISC complex. A basic outline of the process involves expression of an epitope tagged Argonaute protein, along with a miR mimic. Lysate is immunoprecipitated with an antibody against the epitope tag, and mRNAs in the sample can then be sequenced [279]. Further modifications have been added to this method, whereby crosslinking of RNA to RNA binding proteins prior to IP, allows deep sequencing of transcripts bound in real time. This method is known as crosslinking IP-Seq or CLIP-seq [280].

As a single miR can target multiple mRNAs, research is progressing to the examination of miR:mRNA networks, and studies will continually evolve to make use of multiple strategies for target identification simultaneously -this comprises the RNA-RNA interactome.

## **1.8 MicroRNA in immune cell development**

Haematopoiesis is a lifelong, highly regulated process wherein pluripotent haematopoietic stem cells (HSCs) give rise to all blood cell lineages. Expression of lineage specific genes is required for differentiation and miR silencing of these cell specific genes is key for correct differentiation. Loss of Dicer in mice leads to lethality in early development as the mice are depleted of stem cells

[261]. Similarly conditional Dicer deletion in HSCs renders these cells unable to reconstitute the haematopoietic system [281]. In lymphoid cells Dicer deletion results in fewer T cells in the thymus and periphery and fewer surviving B cells with a diminished antibody repertoire [282, 283]. Studies have shown a miR expression profile in haematopoietic tissues. For example miR-181 is strongly expressed in the thymus, miR-223 in the bone marrow and miR-142 in all haematopoietic tissues [284]. This expression pattern is consistent with the cellular expression of these miRs after lineage commitment. miR-181 is highly involved in lymphopoiesis, enforced expression of miR-181 both *in-vitro* and *in-vivo* results in a doubling of B cell numbers present in the periphery [284]. In addition to B cell development, miR-181 plays a role in positive and negative selection of thymocytes in the thymus [285], and modulates TCR signalling by altering TCR sensitivity in immature T cells [286]. In contrast, miR-223 appears to be involved in granulopoiesis, as it is not detected in the spleen or thymus [284]. In addition, miR-223 knockout mice exhibit increased numbers of activated neutrophils, resulting in spontaneous development of inflammatory lung pathology [287]. This evidence demonstrates expression of lineage specific miR can regulate and alter haematopoietic differentiation and therefore impact on immune cell generation.

## **1.9 microRNA in immune responses**

### **1.9.1 Innate Immunity**

Innate immunity is the critical first line of defence against infection. The innate immune system is constituted by cells such as macrophages, neutrophils and dendritic cells that sense infection, or damage through highly conserved pattern recognition receptors (PRRs) triggering an inflammatory response [288]. TLRs are an important family of PRRs that are restricted to certain cell lineages that tailor particular responses from specific cells depending on the inflammatory stimuli [289]. It is important that TLR activation and signalling pathways are tightly regulated to ensure an effective immune response, but also that the inflammatory response is resolved when the threat has been dealt with. It has been demonstrated that miRs can regulate the strength and resolution of TLR signalling and therefore a major contributor to the innate immune response. In addition to TLR signalling, miRs can also regulate formation of the innate

inflammasome. The inflammasome is a multi-protein complex that contains a PRR domain, and can therefore be activated by intracellular infection and damage. The function of the inflammasome is to cleave pro forms of IL-1 family cytokines, resulting in the release of their mature forms [290].

miRs can be both induced and downregulated by TLR activation. Three of the most highly studied miRs, miR-146a, miR-21 and miR-155 are induced at high levels after TLR stimulation and act to negatively regulate inflammation in monocytes and THP1 cells.

miR-146a is mainly expressed in immune tissues and is induced in response to LPS, TNF $\alpha$  and IL-1 $\beta$  stimulation in an NF $\kappa$ B dependent manner [291]. This miR is part of a sophisticated negative feedback loop by which TLR ligation activates NF $\kappa$ B that in turn induces the expression of miR-146a. miR-146a has been shown to directly target pro-inflammatory members of the MyD88 signalling pathway TRAF6 and IL-1R-associated kinase 1 (IRAK1) thereby dampening inflammation by regulating pro-inflammatory cytokines and aiding in resolution. Knockout of miR-146a in mice results in an exaggerated immune response and hyper responsiveness to LPS leading to an excessive production of TNF $\alpha$  and IL-6 and increased sensitivity to septic shock. Aging mice also develop spontaneous autoimmunity and tumours in secondary lymphoid organs [292]. These data demonstrate that miR-146a is an important negative regulator of inflammation.

Like miR-146a, miR-21 is also induced by LPS stimulation in monocytes and macrophages in an NF $\kappa$ B dependent manner and similarly negatively regulates TLR activation [293]. miR-21 directly targets programmed cell death protein 4 (PDCD4) and downregulates its expression. This protein is upregulated 8 hours post LPS stimulation and can bind to and inhibit the translation of anti-inflammatory IL-10, thereby promoting an inflammatory state [294]. Mice deficient for PDCD4 are resistant to LPS induced death. PDCD4 expression is downregulated 24 hours post LPS stimulation, due to binding by miR-21, allowing production of IL-10 and resolution of inflammation [295]. PDCD4 thus acts as a switch between pro-inflammatory NF $\kappa$ B activation and anti-inflammatory IL-10 activation through the action of miR-21.

miR-155 has been shown to mediate a central role in regulation of responses to infection by exerting both positive and negative effects on the immune system. Tili et al demonstrated an increase in miR-155 expression following LPS stimulation and increased production of TNF $\alpha$  after overexpression of miR-155. This pro-inflammatory phenotype is mirrored in miR-155 transgenic mice that show increased serum TNF $\alpha$  and increased susceptibility to endotoxin shock [296]. This inflammatory phenotype is likely a consequence of miR-155 targeting of negative regulators of inflammation Phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1 (SHIP-1) and suppressor of cytokine signalling 1 (SOCS1) [297, 298]. miR-155 directly targets SHIP1 resulting in increased activation of Akt kinase after LPS stimulation terminating in increased pro-inflammatory cytokine production. Knock down of SHIP1 in mice results in a myeloproliferative disorder similar to that observed in miR-155 transgenic mice, suggesting miR-155 targeting of SHIP1 is important for the differentiation of immune cells [297]. miR-155 also acts as a positive feedback regulator of antiviral responses by promoting type I interferon signalling through the targeting of SOCS1 in macrophages [298].

Conversely, miR-155 has been shown to negatively regulate the immune response in innate cells by applying a “brake” in the response to infection. Ceppi et al have demonstrated an increase of miR-155 levels in response to LPS in human dendritic cells. This study suggests that miR-155 acts to control the intensity and duration of the immune response after activation through targeting of TAK1-binding protein 2 (TAB2), a signalling molecule downstream of TRAF6 that activates NF $\kappa$ B [299]. miR-155 targeting of TAB2 inhibits the activation of the MAPK signalling pathway, preventing the production of IL-1 $\beta$  and other pro-inflammatory cytokines. However, this only been reported once, and therefore requires more investigation.

In addition to these well studied miR interactions, novel miR that negatively regulate the innate immune response are being described. miR-132 is induced in the monocytic cell line THP1 cells by LPS and also in primary human cells by infection with two separate viruses, the herpes simplex virus (HSV) and the human cytomegalovirus (CMV). This miR negatively regulates the response to these infections by directly targeting the p300 transcriptional co-activator. P300

has also been shown to regulate miR-132 expression itself, forming a feedback loop by which p300 silencing results in a downregulation of pro-inflammatory signalling whilst downregulating its own expression [300]. Furthermore miR-9 is upregulated in both monocytes and neutrophils after activation with LPS and the pro-inflammatory cytokines TNF $\alpha$  and IL-1 $\beta$ . This miR controls NF $\kappa$ B activation by targeting its p50 subunit preventing dimerization with the p65 subunit and the activation of pro-inflammatory cytokines [301].

### 1.9.2 Adaptive Immunity

T Cell activation is a tightly regulated process and recent studies have demonstrated the involvement of miR in the regulation of the T cell response. The regulation of IL-2 production appears to be critical to the function of miRs in regulating T cell activation. Various miRs have been identified which function in this way. miR-155 is upregulated in response to T cell activation where it enhances proliferation through targeting of SOCS1 and allows costimulation through targeting CTLA-4 [302]. In addition miR-181c directly targets IL-2, and miR-9, upregulated after TCR ligation targets PR domain zinc finger protein 1 (BLIMP) and B-cell lymphoma protein (BCL) 6 resulting in increased IL-6 and IFN $\gamma$  production [303, 304].

miR-155 appears to be important in generation of Th17 cells, both *in-vitro* and *in-vivo*. Escobar et al have demonstrated that there is defective Th17 cytokine expression in miR-155 deficient CD4 $^+$  T cells. Their study showed that these T cells express elevated levels of a DNA binding protein, jumonji, AT rich interactive domain 2 (Jarid2). Jarid2 recruits Polycomb Repressive Complex 2 (PRC2) to chromatin, which binds and results in higher levels of H3K27 methylation. H3K27 methylation is involved in the repression of translation, resulting in low IL-22, IL-10, and IL-9 and thereby generates an indirect effect on the production of IL-17A and IL-17F. This study demonstrates miR-155 regulation of chromatin structure, and global changes in gene expression [305]. Additionally, miR-155 seems to play an important role in regulating the interferon response to viral infection in both CD8 $^+$  and natural killer (NK) cells [306, 307]. Recent studies have shown miR mediated gene regulation as critical also for B cell responses. miR-155 is required for normal B cell function and GC formation. This miR is expressed at high levels in mature B cells [308]. miR-155

plays a role in production of antibodies and class switching through targeting of Spi-1 proto-oncogene( PU.1) and Activation-induced cytidine deaminase (AID) [309]. In addition miR-181b also contributes to regulation of antibody class switching, again through targeting of AID [310].

## **1.10 microRNA in Rheumatoid arthritis**

The majority of miR studied in the context of inflammation have been investigated in the context of inflammatory diseases, including RA. Dysregulated expression of miRs has been well documented in RA (Table 1-6). Investigation of these miRs has highlighted novel pathways involved in inflammation and identified new potential biomarkers or targets for treatment. However, although many dysregulated miRs have been identified, in various cell types, the pathogenic function of several within RA still remains to be elucidated. The majority of miR observations are made in primary human cells, with target interactions validated in cell lines, and primary human cells.

**Table 1.6 Dysregulated miR in RA or animal models of RA**

miR	Tissue	Target	Function	Species	Reference
miR-16	↑ PBMCs			Human	[311]
miR-18a	↑ RASFs	TNFAIP3	Allows constitutive NFκB signalling	Human	[312]
miR-19	↓ LPS RASFs	TLR2	Regulation of IL-6 and MMP production	Human	[313]
miR-23b	↓ RASFs	TAB2/3 IKKα	Drives inflammation	Human	[314]
miR-34a*	↓ RASFs	XIAP	Resistance to apoptosis	Human	[263]
miR-124a	↑ RASFs	CDK-2 MCP-1		Human	[315]
miR-132	↑ PBMCs			Human	[311]
miR-146a	↑ PBMCs RASFs CD4+ T cells	TRAF6/ IRAK1 FAF1	Failed regulation Suppresses T cell apoptosis	Human	[311, 316, 317]
miR-155	↑ RASFs PBMCs CD14+	MMP 1+3 SOCS1 SHIP1	Drive inflammation and joint destruction	Human	[318-320]
miR-203	↑ RASFs		Increased IL-6 and MMP-1	Human	[321]
miR-221/222	↑ RASFs			Human/TNFα Transgenic mouse	[322]
miR-223	↑ CD4+ T Cells	IGF1-R	Impairs IL-10 production	Human	[323]
miR-346	LPS RASFs	TTP	TNFα release	Human	[324]
miR-363	↓ CD4+ T cells			Human	[317]
miR-451	↓ Neutrophils	Rab5a CPNE3	Allows neutrophil recruitment	Human	[325]
miR-498	↓ CD4+ T cells			Human	[317]

TNFAIP3, Tumour necrosis alpha inducible protein 3; TLR2, Toll like receptor 2; TAB2/3, Transforming growth factor beta activated protein 2/3; IKKα, IKappa B kinase; CDK-2, Cyclin dependent kinase 2; MCP-1, monocyte chemotactic protein 1; TRAF6, TNFα receptor associated kinase factor 6; IRAK1, Interleukin-1 receptor associated kinase 1; FAF1, Fas associated factor 1; MMP-1, metalloproteinase 1; SOCS1, suppressor of cytokine signalling 1; SHIP1 phosphatidylinositol 3,4,5 triphosphate 1; IGF1-R, Insulin like growth factor 1; TTP, Tristraproline; Rab5a, Ras related protein 5a; CPNE3, Copeine III

Three of the most well studied miR; both in the context of inflammation and RA are discussed below

### 1.10.1 miR-155

miR-155 is up regulated in PBMCs and SF CD14+ cells of RA patients. Exposure of healthy CD14+ cells to RA SF triggers the up-regulation of miR-155, resulting in the production of pro-inflammatory TNFα, IL-6, IL-1β and IL-8, whilst inhibition

results in a downregulation of TNF $\alpha$  production [320]. Although many targets have been identified for miR-155, currently only 1 inflammatory pathway has been validated in the context of RA CD14 $^{+}$  cells and inflammation. miR-155 directly targets SHIP-1 in these cells, allowing constitutive expression of pro-inflammatory cytokines. The contribution of miR-155 to the pathogenesis of arthritis has been investigated by 2 groups using miR-155 knockout mice. These mice were found to be protected against CIA development compared to wild types, with no evidence of bone erosions or inflammatory infiltrate. Additionally miR-155 knockout mice exhibited lower anti-collagen autoantibodies and defective IL-17 polarisation. Interestingly these mice exhibited higher levels of SHIP-1 and lower levels of pro-inflammatory mediators [320, 326].

However, in a K/BxN serum transfer model of arthritis both WT and KO mice develop comparable arthritis, suggesting miR-155 is not involved in antibody mediated effector responses in this disease state. However in this arthritis model KO demonstrated fewer bone erosions, whilst *in-vitro* KO CD14 $^{+}$  cells demonstrated impaired osteoclastogenesis. In addition, S Bluml et al (Abstract presented at “Be the Cure” conference 2014) demonstrated that TNF $\alpha$  transgenic mice on miR-155 KO background do not differ in arthritis severity from WT littermates, suggesting that miR-155, although crucial for TNF $\alpha$  production, is dispensable for its action on bone.

### 1.10.2 miR-146a

miR-146a expression has been found to be increased in various cell and tissue types within the RA joint, including peripheral blood mononuclear cells (PBMCs), RASFs, CD4 $^{+}$  T cells and SF itself (extracellular), where its expression positively correlates with DAS scores [311, 317, 318, 327]. It has been hypothesised that the inflammatory environment within RA causes the up-regulation of this miR, as *in-vitro* miR-146a expression is induced in both RASFs and CD4 $^{+}$  T cells by stimulation with LPS [316, 318]. It has been demonstrated that miR-146a can directly target members of the TLR signalling pathway TRAF6 and IRAK1. However, although miR-146a expression is upregulated in many compartments in RA, levels of TRAF6 and IRAK1 are similar between RA patients and healthy controls, suggesting miR-146a is defective in its regulation of these molecules in RA [311]. Alternatively as one miR can target many different mRNAs it may

highlight that these genes are not targeted by miR-146a in these cells under these specific conditions. Interestingly, along with this role in regulation of inflammation overexpression of miR-146a suppresses osteoclastogenesis. Thus, the miR-146a targets TRAF6 and IRAK1 are also regulators of osteoclastogenesis. This observation was supported *in-vivo* since the injection of double stranded miR-146a in a murine model of arthritis resulted in a downregulation of joint destruction and TRAP positive cells, whilst marginally affecting immune cell infiltration and inflammation [328]. Additionally miR-146a has been implicated in contributing to the “pro-inflammatory phenotype” of T regs in RA. Zhou et al demonstrated diminished upregulation of miR-146a in response to stimulation in RA Treg cells, with lower miR146a in patients with active disease. They hypothesise that this miR-146a defect results in enhanced Signal transducer and activator of transcription (STAT) 1 expression and increased production of pro-inflammatory cytokines by Tregs, contributing to RA pathogenesis [329].

It is therefore interesting to note, that although miR expression may be increased in certain cellular compartments within RA, that their regulation of validated targets can appear defective, underlying the fact that one miR may exert a major effect on different targets in different cells, under different environmental conditions. It also hints at the possibility that although expression of a miR is altered in disease states, its upregulation may not be enough to counteract high levels of chronic inflammation.

### **1.10.3 miR-223**

miR-223 is dysregulated in many cell types within the RA joint. It is one of the most abundantly detectable miRs in RA blood and plasma, where its expression negatively correlates with tender joint count [327]. It is up regulated in RASFs and CD4+ T cells within the synovium, and can have a pro or anti-inflammatory role, depending on the cell type. In T cells, miR-223 has both pro and anti-inflammatory functions, increasing LPS induced IFN $\gamma$ , but modulating IL-17A production through the targeting of the Roquin ubiquitin ligase that reduces IL-17 synthesis [330, 331]. Conversely, miR-223 functions in an alternative manner in monocytes and macrophages, where it enhances anti-inflammatory responses and regulates macrophage polarisation to an M2 phenotype [332]. Unpublished data from this lab group has shown that miR-223 is downregulated in SF CD14+

cells. In these cells it is hypothesised that miR-223 would target the NLRP3 inflammasome, therefore regulating IL-1 $\beta$  production [290]. Thus, its down-regulation in myeloid cells may lead to high production of IL-1 family members in the joints.

On the other hand miR-223 seems to support osteoclast differentiation [333]. Commensurate with this, knockdown of miR-223 in a murine CIA model using a miR-223 lentiviral sponge vector results in reduction of arthritis score and incidence of disease, in addition to a downregulation of erosions and Tartrate-resistant acid phosphatase (TRAP) positive cells within the joint space, suggesting a dominant pathogenic role for miR-223 in animal model of arthritis [334]. However, it is difficult at this moment to say how this will translate to human disease, given the anti-inflammatory role of miR-223 in human macrophages.

## **1.11 microRNA as therapy**

The role of miRs as powerful regulators of inflammation and disease has led to the possibility of using them as therapeutics being explored. As with any novel therapy safety is of paramount importance, therefore currently tissue specific miRs are preferred. Using miRs as therapy will involve either miR replacement, or knockdown of overexpressed miRs in a disease state. Use of synthetic duplexes to mimic miR activity is difficult as there is a risk of off target effects if the mimic is not delivered to the correct cell/tissue. Investigators are trying to overcome this issue to a certain extent by the use of adenoviral vectors for delivery, which can allow for potential tissue specificity. This method is currently in clinical trials for gene therapy. The most widely used approach currently is the use of modified anti-miRs.

The first miR to be considered to have therapeutic potential was miR-122 in Hepatitis C viral infection. miR-122 is the most abundant miR in the liver and is tissue specific. It is also required for replication of the Hep C virus, and inhibition of this miR reduces viral replication [335]. Inhibition of miR-122 using an anti-miR, Miravirsen, is currently in clinical trials. Data from Phase IIa trials suggests that Miravirsen provides continuous anti-viral activity and is well tolerated by patients, demonstrating the clinical utility of target miRs [336]. In

addition to Miravirsen, a clinical trial of MRX34, as a mimic of miR-34a is ongoing in primary and metastatic liver cancer. The rationale behind this being that miR-34a is decreased in many cancers allowing the p53 pathway to be activated and facilitate tumour growth [337]. The phase I trial for this treatment began in May 2013 carried out by Mirna Therapeutics.

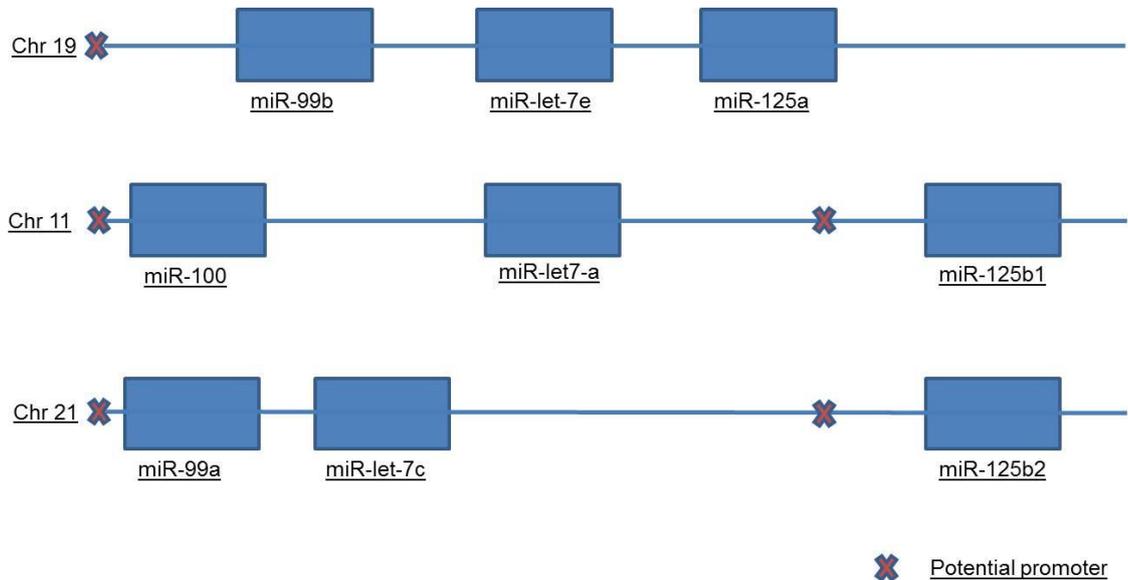
Due to the success of Miravirsen research into the role of novel miRs in health and disease has increased.

## **1.12 The microRNA 125 family**

MicroRNA profiling in the lab revealed that miR-125a is up regulated in RA synovial CD14+ cells compared to PB CD14+ cells

### **1.12.1 Introduction**

The miR-125 family is highly conserved across species, and is composed of three homologues, hsa-miR-125a, hsa-miR-125b1 and hsa-miR-125b2 encoded on 3 different chromosomes. These miRs are expressed in clusters along with members of the miR-99 and Let 7 families (Figure 1.2). Members of the miR-125a cluster are separated by only 1kb, suggesting a single promoter for the whole cluster. However, this has not yet been proven, and the cellular expression pattern of these 3 miRs would suggest that this is not the case. The distance between miR-125b and the other members of the cluster suggest that miR-125b may have a second promoter which allows its expression regardless of other members of the cluster [338].



**Figure 1.3 Genomic organisation of the miR-125 clusters**

The different miR-125 homologues are encoded on different chromosomes. The distance between each family member suggests additional promoters which allow the transcription of miR-125 alone

miR-125a shares the same seed region as miR-125b and it is therefore possible that these miRs may share a similar function. It has been demonstrated however, that these 2 miRs have different cellular expression profiles. In both monocytes and macrophages miR-125a has been shown to be upregulated in response to the TLR4 ligand LPS [301, 339], whereas, in these same cells, LPS has been shown to downregulate miR-125b expression [340, 341]. It is not only in myeloid cells that the difference in expression is demonstrated, miR-125a has been shown to play a role in activated T cells, whereas miR-125b plays a role in naïve T cells [342, 343] This suggests that although homologues, these miRs, and their clustered miRs may play different roles in fine tuning of gene expression, at different stages of the immune response.

## 1.12.2 miR-125 in cancer

Much of the literature surrounding the functional role of miR-125, both a and b, has concentrated on their role in the initiation and maintenance of many different cancer types, acting both as an oncogene, and tumour suppressors.

### 1.12.2.1 miR-125a

miR-125a is downregulated in a number of different cancers, where it would by inference play a tumour suppressor role, such as in oral squamous cell carcinoma

[344], medulloblastoma [345] and chronic lymphocytic leukaemia, where it's underexpression is linked to an inferior disease outcome [346].

The most intensely studied role for miR-125a is as a tumour suppressor through the targeting of the growth receptor, Receptor tyrosine-protein kinase erbB-2 (ERBB2). It has been demonstrated that miR-125a is downregulated in breast cancer tumours overexpressing the Her2 receptor, encoded by the ERBB2 gene. Overexpression of miR-125a in these cells can reduce protein and transcript levels of ERBB2, and in addition, diminish the invasive and migratory potential of these cells [347]. A single nucleotide polymorphism discovered in the miR-125a gene which prevents processing of the pri-miR-125a into a mature miR-125a has also been demonstrated [348]. This variant of miR-125a correlates with low expression of miR-125a, and high Her2 expression in breast cancer tumours, suggesting miR-125a may be used as a prognostic marker of Her2 expression in breast cancer [349]. The role of miR-125a in this growth factor pathway has also been implicated in the aggressive phenotype of acute myeloid leukaemia [350], lung carcinoma [351] and gastric cancer [352]. In addition miR-125a has been shown to inhibit the malignant phenotype of hepatocarcinoma cells through the targeting of MMP11 and VEGFA [353]. Additionally, miR-125a has also been described as an oncogene due to its ability to enhance the invasiveness of urothelial carcinoma[354].

#### **1.12.2.2 miR-125b**

Compared with miR-125a, many more studies have been conducted to determine the role played by miR-125b in cancer. One might speculate that this is due to the clear role it plays as a result of many translocation abnormalities. Increased miR-125b expression can be seen in many myeloid malignancies, where studies have shown it plays a major pro-proliferative role. A direct role for miR-125b in acute myeloid leukaemia (AML) and myelodysplastic syndrome has been suggested, due to the rare chromosomal translocation t (2:11)(p21q23), which results in up to 90 fold increased expression of miR-125b. Upregulation resulted in inhibited terminal monocytic and granulocytic differentiation in HL60 and NB4 leukemic cell lines [355]. In addition, miR-125b has also been implicated in the initiation of acute lymphoblastic leukaemia (ALL), as upregulation of miR-125b occurs through the insertion of this gene into the immunoglobulin heavy chain

locus (IgH,) in all ALL harbouring the t (11:14) q (24:32) translocation. Bousquet and colleagues also demonstrated the causative potential of miR-125b in ALL through the transplant of foetal liver cells that ectopically expressed miR-125b into nude mice. In this study, they found that higher expression of miR-125b increased white blood cell counts in mice, and half of these mice died of diverse leukaemias, such as B-cell and T-cell ALL [356]. Another recent study, carried out by So et al has also demonstrated that miR-125b plays a causative role in leukaemia initiation. They demonstrated that miR-125b overexpression results in enhanced myeloid progenitor output from stem cells, as well as inducing immortality, self-renewal, and tumourigenesis in myeloid progenitors. This occurs through the miR-125b mediated repression of interferon regulatory factor 4 (IRF4), deletion of which results in the development of B cell leukaemia in mice [357, 358].

The role of miR-125b in solid tumours is less clear, like miR-125a, miR-125b can play the role of either a tumour suppressor or oncomir depending on different cell context. Dysregulation of miR-125b has been reported in multiple cancer types, including, but not limited to stomach, colonic, pancreatic, bladder, breast and prostate cancer [347, 359-363]. Upregulation in prostate cancer cells promotes tumour growth by targeting the p53 apoptosis pathway member's p53 upregulated modulator of apoptosis (PUMA) and BAK1 (Bcl-2 homologous antagonist/killer), preventing death of cancer cells by apoptosis. Conversely miR-125b is downregulated in breast cancer, where its expression could inhibit growth of these cells through targeting of Muc1, ERBB2 and ERBB3 [347, 364].

Tumourigenesis has often affected myeloid and lymphoid lineages, and as previously discussed, GWAS studies have shown that RA risk associated genes, significantly overlap with genes associated with haematological cancers [24]. Therefore we were interested to investigate the role of miR-125, in myeloid cells, in inflammatory disease.

### 1.12.3 miR-125 and the immune response

#### 1.12.3.1 miR-125a

miR-125a appears to play a broad role in activating and modulating the immune response, and plays a crucial role in the inflammatory response of macrophages to a variety of stimuli. miR-125a is upregulated in myeloid cells by the recognition of PAMPs by PRRs. Studies have demonstrated that the cluster of miR-125a/99b/Let-7e is upregulated in response to heat killed *Candida albicans*, and the TLR4 ligand LPS in human monocytes [301, 365]. In addition, miR-125a is upregulated in response to TLR2 stimulation by *Listeria monocytogenes* [366]. In each of these conditions, upregulation of the miR is dependent on NFκB activation, suggesting miR-125a is regulated by NFκB expression. Furthermore, miR-125a is upregulated in macrophages that phagocytose oxidised low-density lipoprotein (OxLDL). A direct interaction between oxysterol binding protein 9 (ORP-9) and miR-125a has been demonstrated by luciferase reporter assay. This protein binds both cholesterol and phospholipids, and is downregulated by miR-125a. By inhibiting miR-125a using antagomirs in the presence of OxLDL, Chen et al were able to demonstrate increased level of both ORP-9 mRNA and protein. Additionally, they showed an increased uptake of lipids, increased pro-inflammatory cytokine production, TNFα, IL-6 and IL-2, and also increased expression of the scavenger receptor CD68 from OxLDL stimulated, PMA differentiated THP-1 cells, suggesting a protective role for this miR in atherosclerosis [367].

In addition, miR-125a has been implicated in the control of macrophage polarisation although with conflicting results. In the first study, using murine macrophages, miR-125a has been shown to promote and maintain an M2 phenotype whilst suppressing an M1 phenotype. This study used GM-CSF cultured bone marrow derived macrophages to represent an M1 phenotype and MCSF derived macrophages as an M2 phenotype. Transfection of M1 cells with a miR-125a mimic resulted in lower TNF, IL-12 and iNOS production, whilst increasing IL-4 induced Arg1 expression, a marker of M2 macrophages. This is mirrored in M1 macrophages, where transfection with a miR-125a inhibitor results in higher TNF, IL-12, iNOS, MHC Class II, CD40 and a decrease in IL-4 induced Arg1 expression. This suggests an inhibitory role for miR-125a in M1

macrophage polarisation, commensurate with anti-inflammatory role seen in OxLDL stimulated macrophages. This may be explained in part through the direct targeting of the transcription factor Kruppel-like factor 13 (KLF13) by miR-125a [339].

These studies are contradicted by an additional study investigating the role of miR 125a in the polarisation of human macrophages. Graff et al demonstrated that miR-125a is upregulated in M1 macrophages, stimulated with LPS and IFN $\gamma$  and also in M2b polarised macrophages, stimulated with IgG and LPS, again as a result of LPS stimulation. In addition, they demonstrated that transfection of these cells with a miR-125a mimic, results in a slight increase in TNF $\alpha$  and IL-6 production, in addition to the chemokine CXCL9 that is a marker of the M1 macrophage phenotype. The authors attributed the pro-inflammatory effect of miR-125a to its ability to target the deubiquitin ligase TNFAIP3 demonstrated by Kim et al [368, 369]. Targeting of TNFAIP3 has been shown in this study to allow the constitutive activation of NF $\kappa$ B contributing to the aggressiveness of diffuse large B cell lymphoma (DLBCL), where both of these miRs are overexpressed [369]. It is intriguing that TNFAIP3 is an RA risk associated allele in GWAS studies.

Studies have shown that although macrophages express high levels of miR-125a, the downregulation of this miR is required for osteoclastogenesis.

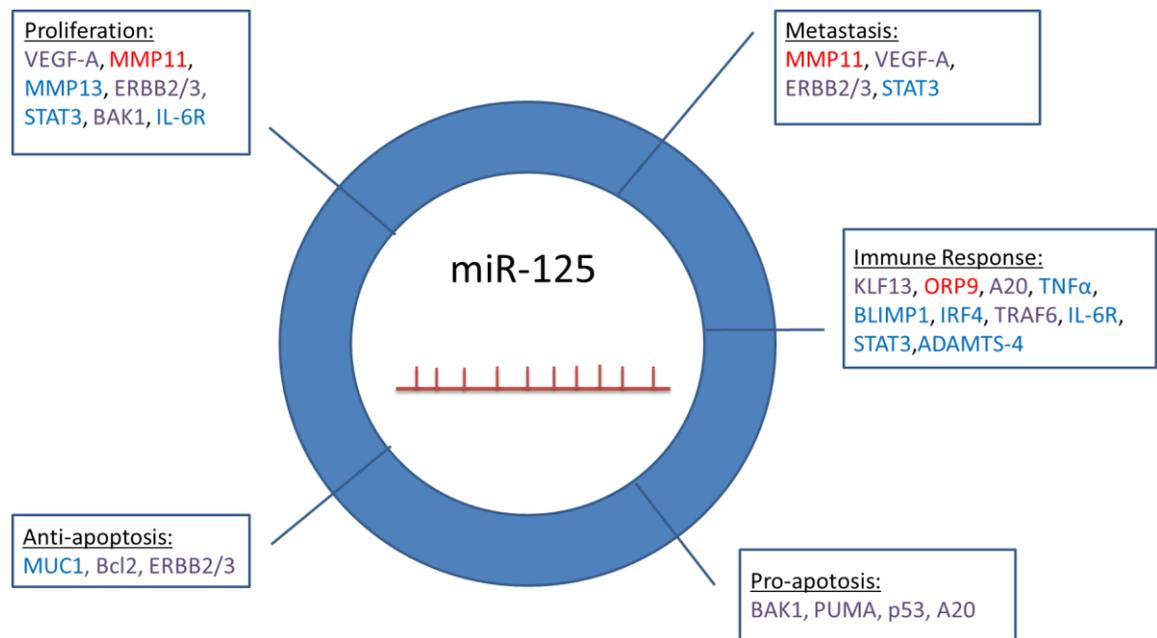
Overexpression of miR-125a prevents osteoclastogenesis through the silencing of TRAF6, a molecule essential for their formation [370]. TRAF6 is also involved in the response to TLR activation, and therefore it is possible that miR-125a silencing of TRAF6 may also aid in resolving the inflammatory response.

In addition to macrophages and osteoclasts, miR-125a plays a role in regulating the activation and effector function of T cells. This miR is downregulated in T cells from Systemic lupus erythematosus (SLE) patients resulting in an overexpression of the transcription factor KLF13 and the chemokine CCL5 [343]. This downregulation results in a missing regulatory feedback loop, which allows the overproduction of CCL5 from terminally differentiated T cells in SLE.

### 1.12.3.2 miR-125b

Due to the intense focus of investigating miR-125b in cancer, fewer studies have concentrated on the role of miR-125b in regulating the immune response. However it has been demonstrated that miR-125b is expressed in macrophages, where it is downregulated in response to LPS in human, mouse and RAW .267 cells [296, 371]. Chaudhuri et al have hypothesised that the role of miR-125b in macrophages is to amplify the innate and adaptive immune response to infection. Overexpression of miR-125b in macrophages results in an increase in MHC Class II, CD40, CD80 and CD86. Additionally, overexpression of miR-125b results in increased apoptosis in these cells. This study hypothesises that miR-125b regulates the activation of macrophages through targeting of IRF4, which is also an inhibitor of the macrophage response [372]. In addition to IRF4, miR-125b can target TNF $\alpha$  itself, and represses expression under homeostatic conditions [296]. Additionally, miR-125b has a role in chondrocyte destruction in OA. This miR is downregulated in OA cartilage when compared with healthy control. It has been demonstrated that miR-125b targets metalloproteinase with thrombospondin type 1 motif (ADAMTS-4), which possesses aggrecanase activity, which can result in degradation of cartilage [373]. Recent studies have also suggested that miR-125b may be used as a biomarker for response to Rituximab therapy in RA. High serum levels of miR-125b associated with good clinical response to Rituximab 3 months after disease flare [374].

The study of miR-125 in various different cancers and immune disorders, has demonstrated a critical regulatory role of this family in various cellular processes, including apoptosis, proliferation, metastasis and the immune response. It has also identified targets of this family that can be viewed in more than 1 of these categories and may aid in the understanding of various disease processes (Figure 1.4).



**Figure 1.4 Validated targets of miR-125 adapted from [375]**

Validated miR125 targets in different cellular processes. Red are validated only for miR-125a, blue are validated only for miR-125b and purple are validated for both miR-125 members

These studies demonstrate the potential multifunctional roles played by the miR-125 family in regulating activation and maintenance of the immune response. Studies in inflammatory disease have so far focused on the expression pattern of these miRs whilst the targets and inflammatory pathways that they regulate still require identification. Thus, I was interested in clarifying the role of miR-125a in human macrophage biology and dissecting the role of increased levels of miR-125a in the activation of RA synovial macrophages.

### 1.13 Aims

We hypothesised that miR expression can differentially influence differentiation and effector functions of macrophages in RA, resulting in pathogenic disease progression. Therefore, the aims of this study were

1. To identify miR species which are dysregulated in RA macrophages
2. Once identified, to determine the regulation of novel miR, and identify their expression levels in different subgroups of RA patients

3. Identify functional targets of these novel miR expressed in macrophages, and determine how they regulate macrophage activation.
4. Identify if dysregulation of these miR plays a role in maintaining the pro-inflammatory nature of macrophages in RA

## Chapter 2 Materials and Methods

## 2.1 General Buffers and Reagents

**Complete RPMI** - RPMI 1640 media supplemented with 10% foetal calf serum (FCS), 2mM L-Glutamine, 100IU/ml penicillin, 100µg/ml streptomycin (all Invitrogen).

**Complete DMEM**- DMEM media supplemented with 10% FCS 2mM L-Glutamine, 100IU/ml penicillin, 100µg/ml streptomycin (All Invitrogen).

**Wash media**- RPMI 1640 medium supplemented with 100IU/ml penicillin, 100µg/ml streptomycin (All Invitrogen)

**MACs/FACs Buffer**- Phosphate Buffered Saline (PBS) supplemented with 2% FCS and 100IU/ml penicillin, 100µg/ml streptomycin (All Invitrogen)

**ELISA wash buffer**- PBS (Invitrogen) with 0.05% Tween (Sigma)

**ELISA assay buffer**- PBS (Invitrogen) with 0.5% Bovine Serum Albumin (BSA) (Sigma)

**Tris-acetate EDTA (TAE) buffer**- To make a concentrated 50x stock solution of TAE dissolve 242 g Tris base 750 mL deionized water. Add 57.1 ml glacial acetic acid and 100 mL of 0.5 M EDTA (pH 8.0). Adjust the solution to a final volume of 1 L.

## 2.2 Cell Culture

All tissue culture experiments were carried out in laminar flow hoods. Cells were cultured under normal culture conditions, in 5% CO<sub>2</sub> at 37°C unless otherwise stated.

### 2.2.1 Culture of Cell Lines

Cell lines were obtained from ATCC ([www.atcc.org](http://www.atcc.org)), a repository for cell lines.

### **2.2.1.1 THP1 Cells**

Cells from the THP-1 cell line were cultured in 25mls of complete RPMI in T75 tissue culture flasks (Corning). Cells were passaged twice a week. Briefly, cells were centrifuged at 200g for 5 minutes to wash and then counted. Cells were seeded at a density of  $2 \times 10^6$  in 25mls of complete RPMI and cultured under normal culture conditions.

### **2.2.1.2 miR-125a Sponge THP-1 cells**

Cells from the THP-1 cell line, which were stably transfected with either the miR-125a sponge or reporter control vectors were maintained by the same method as normal THP-1 cells (Described in Chapter 4). However these cells were also cultured along with 250mg/ml of the antibiotic G418 to allow for selection (Life Technologies).

### **2.2.1.3 Culture of Adherent Human HEK293 Cells**

HEK293 cells were cultured in complete DMEM in T75 tissue culture flasks. Cells were passaged twice a week. Briefly, media was removed from the cells which were then incubated for 3 minutes at 37°C with 5ml 0.05% Trypsin EDTA (Invitrogen) to dissociate cells from the flask. After the cells had detached 7mls of complete DMEM was added to the flask to inactivate trypsin and cells were centrifuged at 200g for 5 minutes at room temperature. Cells were then seeded in a fresh T75 Flask in 15mls complete DMEM and cultured under normal culture conditions.

### **2.2.1.4 PMA differentiation of THP-1 Cells**

The optimum concentration of PMA (Sigma) was experimentally validated and cells were differentiated as follows: THP-1 cells were washed with wash media and counted, after which cells were seeded at a density of  $0.25 \times 10^6$  cells per well in a 24 well plate along with 5ng/ml of PMA. Cells were cultured overnight in PMA, after which media was removed, with fresh RPMI added. Culture media was changed on day 3 and cells were cultured until day 7.

### **2.2.1.5 Freezing Down Cell Lines**

Cell lines, when not in use were stored in liquid nitrogen. Cells were frozen in cryovials at a concentration of  $1 \times 10^6$  in 10% DMSO in complete RPMI. Cells were stored at  $-80^\circ\text{C}$  overnight and then transferred to liquid nitrogen.

## **2.2.2 Primary Human Cell Culture**

### **2.2.2.1 Patient Samples**

All samples were obtained from buffy coats, healthy volunteers and RA patients who fulfilled the 2010 ACR/EULAR criteria for RA. Samples were obtained after obtaining written consent, with the appropriate ethical approvals in place (Research Ethics Committee 11/S0704/7, Internal Ethics Committee 2012073).

All human cells were cultured in complete RPMI, unless otherwise stated

### **2.2.2.2 Separation of peripheral blood mononuclear cells**

Peripheral blood mononuclear cells (PBMCs) were separated using density centrifugation. Blood samples were obtained from buffy coat packs provided by the Scottish National Blood Transfusion Service (SNBTS) or from healthy donors. Briefly, blood was diluted 1:1 in wash media, layered over room temperature Histopaque®-1077 (Sigma) and centrifuged at 350g for 20 minutes at room temperature. The mononuclear layer of cells was transferred to a new 50ml falcon tube and washed in wash media by centrifugation at 200g for 10 minutes at  $4^\circ\text{C}$ . The cell pellet was resuspended in 20mls of ice-cold MACs buffer and cells were counted using trypan blue (Sigma).

### **2.2.2.3 Separation of mononuclear cells from synovial fluid**

Prior to separation cells were pelleted from synovial fluid by centrifugation at 350g for 20 minutes at room temperature. Aliquots of cell free synovial fluid were then stored at  $-80^\circ\text{C}$  for subsequent experiments. Pelleted cells were resuspended in wash media and layered over room temperature Histopaque®-1077. Cells were centrifuged at 350g for 20 minutes at room temperature. Mononuclear cells were then treated as described in 2.2.2.3

#### **2.2.2.4 Purification of CD14+ monocytes from mononuclear cells**

CD14+ monocytes were purified from mononuclear cells using positive selection by AutoMacs according to manufacturer's instructions. Briefly, cells were counted and resuspended in 80µl MACs buffer per  $1 \times 10^7$  cells. A chosen volume of cells, depending on experiment size, was then filtered into 15ml tubes using 70µm filters (BD Falcon). Samples were placed on the AutoMacs rack along with anti-CD14+ conjugated magnetic microbeads (Miltenyi Biotec 130-050-201). Monocytes were isolated from samples using the PosselD programme; this programme ensures maximum cell purity by running the sample over two columns. Purity of the monocyte population was assessed by flow cytometry and was typically >90%

#### **2.2.2.5 Culture of human macrophages**

Human macrophages were differentiated from separated CD14+ cells. These cells were cultured under normal conditions at a density of  $0.25 \times 10^6$  cells per well in a 24 well plate. Cells were cultured in complete RPMI with the addition of 50ng/ml M-CSF or GM-CSF (Peprotech). Cells were cultured for 7 days with the media being replaced on day 3.

#### **2.2.2.6 Culture in Synovial Fluid**

To mimic the synovial microenvironment monocytes were also cultured in 10% synovial fluid. Previous experiments within this lab group, by Dr Mariola Kurowska-Stolarska have shown that 10% SF is the lowest amount of SF which could be used to alter the expression of miR-155, another miR altered in the synovial microenvironment, in CD14+ cells. Monocytes were separated and plated at a density of  $0.25 \times 10^6$  per well in complete RPMI. After plating, 25µl of synovial fluid was pipetted into each well and the plate was gently swirled. The cells were then cultured under normal conditions for 24 hours, after which they were stored for analysis of miR expression.

### **2.2.3 Cell Stimulation**

THP-1, PMA differentiated THP-1, primary human monocytes and M-CSF differentiated macrophages were stimulated with various TLR ligands to

determine their effect on microRNA expression and cytokine production. The concentration of these stimuli is detailed in Table 2.1. These concentrations were chosen based on previous work concerning the regulation of miR-155 within this lab group [320]. Macrophage cells were stimulated with 10ng/ml of LPS due to the extremely high levels of TNF $\alpha$  production if stimulated with 100ng/ml.

**Table 2.1 TLR Cell Stimulations**

Stimuli	Concentration
LPS -TLR4 (Sigma: L2630)	100ng/ml (monocyte) or 10ng/ml (macrophage)
Pam3CSK4 -TLR 1/2 (Invivogen: 112208-00-1)	300ng/ml
Poly I:C -TLR3 (Sigma: P9582)	50 $\mu$ g/ml
CL097 -TLR 7/8 (Invivogen: tlrl-c97)	5 $\mu$ g/ml

Cells and supernatants were stored for further experiments.

## 2.3 Flow Cytometry

### 2.3.1 Assessment of cell purity

Purity of samples collected by AutoMACS isolation was verified by flow cytometry. Cells were washed twice in 500 $\mu$ l of MACs buffer, and resuspended at  $1 \times 10^6$  cells per ml. 100 $\mu$ l of cells was then added to two clean FACS tubes. One tube was used for isotype control and the other for antibody staining. Both tubes were incubated for 20 minutes at room temperature with 10 $\mu$ l of Human Fc block (Miltenyi Biotech: 130-059-901) to prevent non-specific binding. For the purity check antibodies detailed in Table 2.2 were used (all Miltenyi Biotech):

**Table 2.2 Antibodies used to determine purity of CD14 cells**

Antibody	Isotype	Amount (in 100 $\mu$ l)	Source/Cat No.
PE anti-human CD14	IgG2A	10 $\mu$ l	130-091-835
Mouse IgG2A isotype control	IgG2A	10 $\mu$ l	130-091-242

10µl of each antibody was added to 100 µl of cells in each tube and incubated in the dark for 30 minutes at 4°C. Cells were washed in 500µl of MACs buffer with the supernatant discarded. Cells to be analysed immediately were resuspended in 400µl of MACs buffer. Cells to be analysed the next day were resuspended in 200µl of MACs buffer and 200µl FACS FIX (4% paraformaldehyde in PBS). Analysis was performed on a FACS Calibur (BD bioscience) and using Cell Quest software. Cells were compared to isotype control to determine purity.

### **2.3.2 Assessment of Transfection Efficiency**

One method of measuring transfection efficiency was by flow cytometry. In addition to transfection with a targeting miR mimic/inhibitor, cells were transfected with Dy547-labeled mimic based on *C. elegans* miRNA #1: cel-miR-67 (Dharmacon: CP-004500-01). This mimic was labelled with Dy547 that has an absorbance/emission of 557/570 allowing transfection to be monitored and visualised. Briefly, cells were transfected with both a control mimic (Dharmacon: CN-002000-01) and labelled control. Cells were then removed from the culture plate and washed twice in 500µl of MACs buffer. Cells were then resuspended in 400µl of MACs buffer or 200µl of MACs buffer and 200µl FACS FIX and analysed using a FACS Calibur with emission on the FL2 channel. Samples transfected with the labelled mimic were compared to those transfected with control mimic to determine transfection efficiency.

### **2.3.3 TLR4 expression**

TLR4 surface expression was measured on human macrophages and THP-1 cells. Adherent cells were removed from plates using a non-enzymatic dissociation solution (Sigma- C5914-100ML), to minimise cell death. Briefly, reagents were warmed to room temperature and media was removed from cells which were then washed in PBS. After this, 200µl of dissociation reagent was added to each well and the plate was incubated at 37°C for 3 minutes. Cells were then washed in PBS, re-suspended and incubated with Fc block as described previously.

Cells were split into 100µl in 2 tubes to stain:

**Table 2.3 Antibodies used to determine TLR4 expression**

Antibody	Isotype	Amount (in 100 µl)	Source/Cat No.
PE anti-human TLR4	Mouse IgG2A	5 µl	Biolegend 312805
Mouse IgG2A isotype control	Mouse IgG2A	5 µl	Biolegend 400211

Non-adherent THP-1 cells were washed in PBS and incubated with Fc block as previously described.

5µl of each antibody (Table 2.3) was added to the tubes and incubated in the dark for 30 minutes at 4°C. Cells were washed in 500µl of MACs buffer with the supernatant discarded. Cells were resuspended in 400µl of MACs buffer or 200µl of MACs buffer and 200µl FACS FIX, and analysed on the FACSCalibur.

## 2.4 Real Time Polymerase Chain Reaction (q-PCR)

### 2.4.1 RNA Isolation from Cells

Total RNA was isolated from cells using miRnEasy kit (Qiagen). Cultured cells were lysed using 700µl Qiazol (Qiagen) per  $3 \times 10^6$  cells, samples were either stored at -20°C or RNA extraction was continued according to manufacturer's protocol.

Briefly, 140µl of chloroform was added to each sample and shaken vigorously. Samples were centrifuged at 12,000g for 15 minutes at 4°C. All subsequent centrifugation steps were carried out at room temperature. The upper aqueous phase of the solution was transferred to a clean 1.5ml Eppendorf tube and mixed with 525µl 100% alcohol. The solution was transferred to an RNeasy mini spin column provided with the kit and centrifuged for 15 seconds at 8,000g, with the flow through discarded. Next, samples were washed with 350µl of RWT buffer with the flow through discarded. In some cases samples were subject to a DNase digestion step. For this reaction an RNase free DNase kit was used (Qiagen). The reaction consisted of 10µl of DNase I stock solution added to 70µl of RDD buffer, this 80µl solution was added to the column and incubated at room temperature for 15minutes. Subsequently, columns were washed with 350µl of

RWT buffer followed by 500µl RPE buffer (x2) by centrifugation at 8000g for 1 minute with the flow through discarded. Columns were centrifuged one final time to remove excess buffer and the spin column was transferred to a clean, labelled 1.5ml Eppendorf tube. RNA was eluted in 50µl RNase free water (Qiagen) by centrifugation at 8000g for 1 minute.

RNA was stored at -20°C before being measured and at -80°C for longer term storage.

## 2.4.2 Measuring Nucleic Acid Concentration

Nucleic acid concentrations were determined using a Nanodrop ND-1000 spectrophotometer (ThermoScientific). Before each set of samples the equipment was blanked using 1µl of water and then initialised with the same volume. Ratios of absorbance at 260 nm and 280 nm were used to indicate purity of samples. The value of 2.0 for each of these measurements was recommended by the manufacturer as standard to indicate samples free of contaminants. Ratios obtained in these experiments were close to 2, indicating good quality RNA.

## 2.4.3 SYBR green protocol

To measure miR expression miScript primer assays, detailed in Table 2.4 were used (all Qiagen).

**Table 2.4 Qiagen miScript primers used to measure microRNA**

Assay Name	Cat No.
U6 snRNA	MS00033740
Hsa-miR-99a	MS00032158
Hsa-miR-99b	MS00032165
Hsa-miR-125a	MS00033423
Hsa-miR-125b	MS00036629
Hsa-miR-193b	MS00031549
Hsa-miR-511	MS00006993
Hsa-miR-642a	MS00023835
Hsa-miR-642b	MS00023828

To measure mRNA levels primers detailed in Table 2.5 were used

Additionally primers were designed to measure TLR4 variants, and purchased from IDT. The sequences are detailed below:

TLR4-Total

Forward: ACACTCTGTCACTTTGTCACCTC

Reverse: AAGTGGGATGACCTCAGGA

TLR4-001 Long

Forward: AAGGCAGGGAGTATACATTGC

Reverse: CAATCAGGATGTCATCAGGGAA

**Table 2.5 Primers used to measure mRNA using SYBR green qPCR method**

Assay Name	Supplier	Cat No./Assay Name
GAPDH	IDT	Hs.PT.39a.22214836
TNF $\alpha$	IDT	Hs.PT.58.45380900
TRAF6	Qiagen	QT00062888
CCL4	Qiagen	QT01008070
TNFAIP3	Qiagen	QT00041853

**2.4.3.1 cDNA preparation**

cDNA was prepared from RNA samples according to protocol using two separate kits. The first was a miScript II reverse transcription kit (Qiagen). The kit supplies two buffers which can be used depending on species being amplified. HiFlex buffer can be used for interrogation of both mRNA and microRNA. HiSpec buffer can be used to study only microRNA expression. All steps were performed on ice. Briefly, 100-500ng of RNA was added in 10 $\mu$ l to reaction detailed below in Table 2.6

**Table 2.6 miScriptSYBR green cDNA reaction**

Kit Component	Per Reaction ( $\mu$ l)
5x HiFlex/HiSpec buffer	4
10x Nucleics mix	2
RNAse free water	2
Reverse transcriptase	2
Template RNA	10

The reaction was placed in a thermocycler for cDNA synthesis. The cycling conditions were as follows: 60 min at 37 $^{\circ}$ C and then 95 $^{\circ}$ C for 5 min.

Subsequently cDNA was diluted 1 in 10 by adding 180 $\mu$ l of RNAse free water to the 20 $\mu$ l cDNA sample. cDNA not used immediately was stored at -20 $^{\circ}$ C.

cDNA was also prepared using a High Capacity cDNA kit (Life Technologies). Briefly, RNA was standardised to a concentration of 2ng/ $\mu$ l before beginning. Kit components were thawed on ice and a master mix was made up as detailed in Table 2.7

**Table 2.7 High Capacity cDNA reaction**

Kit Component	Per Reaction ( $\mu$ l)
10xRT Buffer	2
25 x dNTP Mix (100 mM)	0.8
RT Random Primers	2
MultiScribe Reverse Transcriptase	1
RNase Inhibitor	1
RNase free water	4.2

10 $\mu$ l of reaction was mixed with 10 $\mu$ l of RNA sample and placed in the thermocycler. The cycle conditions for the cDNA reaction are as follows: 25°C for 10 minutes, 37°C for 120 minutes and then 85°C for 5 minutes. cDNA was then used for further analysis, or stored at -20°C

#### 2.4.3.2 SYBR green PCR for mRNA detection

Reactions were ran in triplicate in 10 $\mu$ l volume using a 384 well PCR plate and Power SYBR® green master mix (Life Technologies), detailed in Table 2.8

**Table 2.8 SYBR green mRNA PCR reaction**

Kit Component	Per Reaction ( $\mu$ l)
Power SYBR green	5
Primer mix	1
cDNA	1
RNase free water	3

A no template control (1  $\mu$ l of water) was also added to ensure no contamination of master mix.

The plate was covered with a MicroAmp optical adhesive cover (Life Technologies) centrifuged at 250g for 1 minute, and placed in an ABI 7900HT real-time PCR machine (Life Technologies) where it was read on cycling conditions detailed in Table 2.9

**Table 2.9 SYBR green mRNA PCR cycling conditions**

	UNG incubation	Polymerase activation	PCR - (40 cycles)	
Temperature (°C)	50	95	95	60
Time (mm:ss)	2:00	10:00	00:15	1:00

A dissociation/melt curve was measured after the run to ensure specificity of primers as follows: Incubate for 15 seconds at 95°C, Incubate for 15 seconds at 60°C and Incubate for 15 seconds at 95°C

#### 2.4.3.3 SYBR green PCR for microRNA detection

cDNA was prepared as described in 2.4.3.1

The miScript SYBR green PCR kit (Qiagen) was used to measure miR expression using SYBR green technology. Reactions were in 10µl volumes as detailed in Table 2.10

**Table 2.10 SYBR green miRNA PCR reaction**

Kit Component	Per Reaction (µl)
QuantiTect SYBR green master mix	5
10x miScript universal primer	1
10x miScript primer assay	1
RNase free water	2
cDNA template	1

The plate was run under the cycling conditions detailed in Table 2.11

**Table 2.11 Cycling conditions SYBR green miRNA PCR**

	Polymerase activation	PCR - (40 cycles)		
Temperature (°C)	95	94	55	70
Time (mm:ss)	15:00	00:15	00:30	:30

## 2.4.4 Taqman Protocol

cDNA was prepared for microRNA analysis using the microRNA reverse transcription kit (Life Technologies). RNA was standardized to 2ng/ $\mu$ l by diluting in RNase free water and specific cDNA reactions were made for each miR. Assays used are detailed in Table 2.12 (Life Technologies)

**Table 2.12 Taqman primers used to measure microRNA**

Assay Name	Cat No.	Assay ID
snRNU6	4440887	001973
Hsa-Let7-a	4440887	000377
Hsa-miR-99b	4440887	000436
Hsa-miR-125a-5p	4440887	002198
Hsa-miR-125a-3p	4427975	002199
Hsa-miR-125b	4427975	000449
Hsa-Let-7e	4440887	002406

The cDNA reaction was made up as detailed in Table 2.13

**Table 2.13 Taqman miRNA cDNA reaction**

Kit Component	Per Reaction ( $\mu$ l)
10xRT Buffer	1.5
25 x dNTP Mix (100 mM)	0.15
MultiScribe Reverse Transcriptase	1
RNase Inhibitor	0.19
RNase free water	4.16
Specific RT primer	3

10 $\mu$ l of RNA was added to 10 $\mu$ l of master mix and cycled under the following conditions 16 $^{\circ}$ C for 30 minutes, 30 $^{\circ}$ C for 30 minutes and 85 $^{\circ}$ C for 5 minutes, cDNA not used immediately was stored at -20 $^{\circ}$ C.

### 2.4.4.1 Taqman PCR for microRNA detection

The PCR protocol was altered for use of universal master mix, no UNG (Life Technologies). Briefly a master mix was made as detailed in Table 2.14

**Table 2.14 Taqman miRNA RT-PCR reaction**

Kit Component	Per Triplicate (µl)
Universal master mix	16.5
Taqman miR assay	1.65
RNAse free water	12.655
cDNA	2.2

Samples were analysed in 10 µl volumes.

The plate was run in an ABI 7900HT real-time PCR machine where it was read under the cycling conditions detailed in Table 2.15:

**Table 2.15 Taqman miRNA RT-PCR cycling conditions**

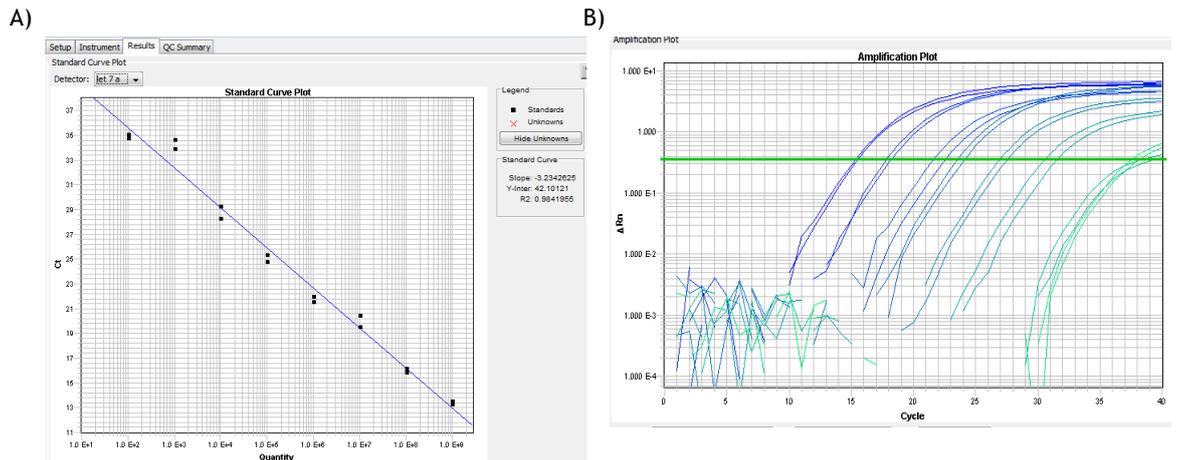
	Stage 1	Stage 2- 40 Cycles	
Temperature (°C)	95	95	60
Time (mm:ss)	15:00	00:15	1:00

## 2.4.5 Analysis of PCR

Results were then analysed using SDS 2.4 software provided by Life Technologies.

### 2.4.5.1 Generation of microRNA standards for RT-PCR

In order to measure absolute copy number of miR, we generated specific miR standards. To do this, we took the miR mimic and denatured at 95°C for 5 minutes. From this reaction we generated cDNA from a known amount of miR and created a standard curve through serial dilutions. This allowed us to relate a Ct value to a copy number of miR through the absolute quantification method. For example shown in Figure 2.1A is a standard curve for Let-7a and the Ct values in Figure 2.1B

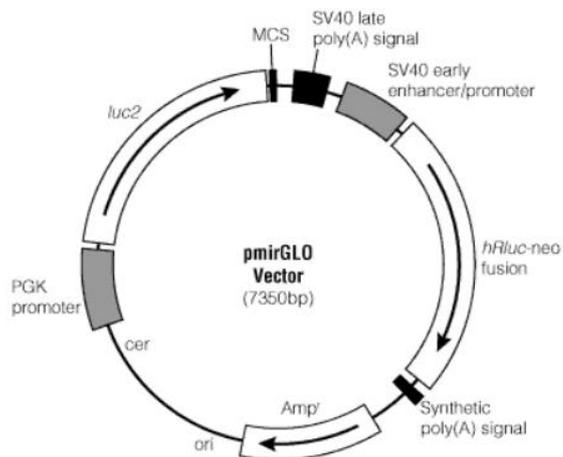


**Figure 2.1 Generation of miR q-PCR Standard for copy number calculation**

PCR standards were generated for each miR studied. A) Standard curve of Let-7a showing quantity of miR present and cycle number detected. B) Ct curves showing amplification of known amounts of Let-7a miR, allowing calculation of copies of miR expressed in a sample

## 2.5 Cloning Techniques: Preparation of reporter plasmids

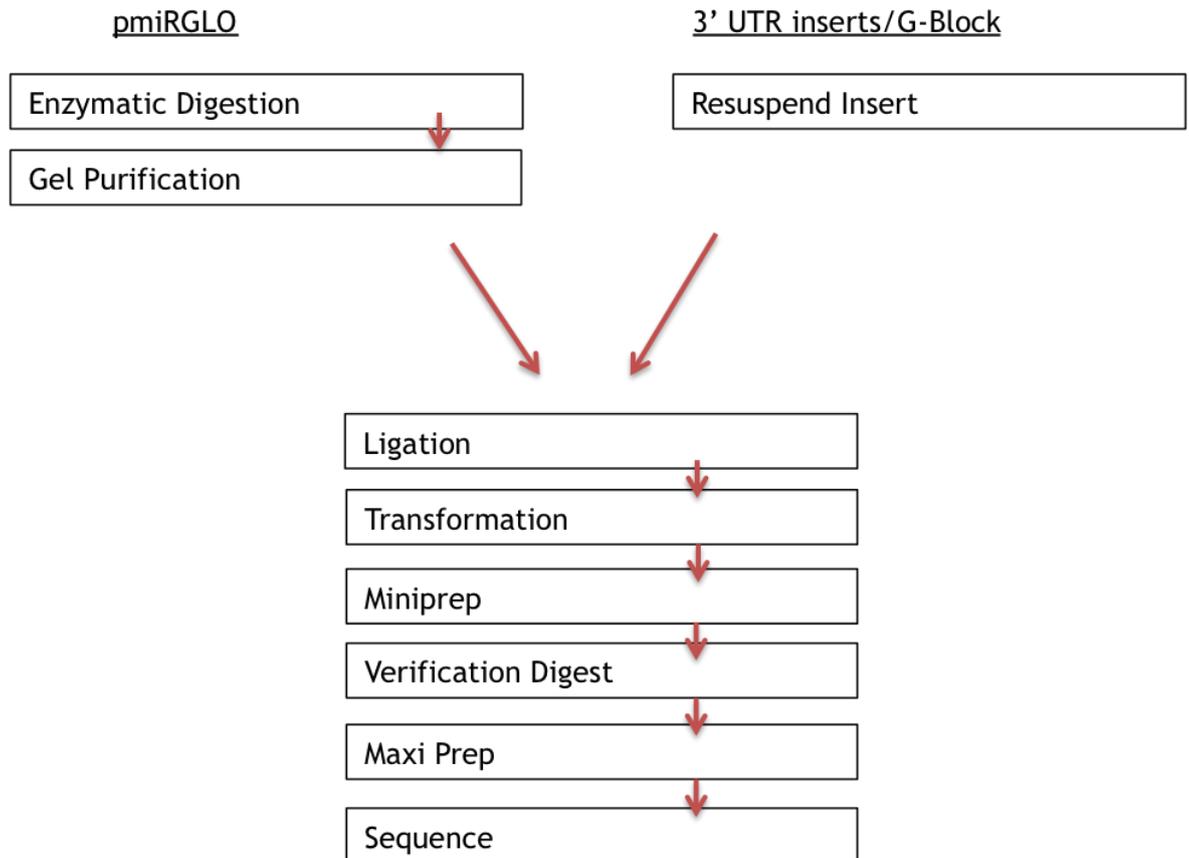
To determine direct interaction between miR-125a and its predicted target gene, reporter plasmids were generated. Fragments of 3' UTR of predicted target were inserted downstream of a luciferase reporter gene in the cloning sites of modified pmirGLO Dual-Luciferase miRNA Target Expression Vector (Figure 2.2) (Promega)



**Figure 2.2 pmirGLO vector**

Schematic of pmirGLO vector purchased from Promega which was used in luciferase reporter assays.

The cloning strategy described in Figure 2.3 was followed to generate reporter plasmids for validating of miR-125a targets



**Figure 2.3 Overview of cloning strategy used to generate reporter plasmids for luciferase assays.**

Inserts were resuspended and ligated into the pmiRGLO vector. This was then transformed into *E.coli* and DNA was extracted. This DNA was verified and sequenced.

### 2.5.1 Designing target G-Block Fragments®

To functionally validate miR-125a targets we used G-Block gene fragments®. G-block fragments are DNA molecules which are synthesised, and are compatible with the Gibson Assembly® Method of cloning. Firstly, 3' UTR sequences containing the potential target site were identified using the ensembl genome browser, and the resulting miR binding site of the target gene was determined using the TargetScan website. This sequence was sent to IDT for synthesis. Ends compatible with the Gibson Assembly® Method were also added to these sequences to allow cloning to take place (GTCTGCAGAGATCTGTTT at beginning of sequence and GTCTAGAGTCGACCTGCAG at the end). Additionally the miR-

125a target site was also mutated to validate miR-125a targeting. The insert was ligated into pmiRGLO vector as follows: 10 µl Gibson Assembly Master Mix, 5 µl pmiRGLO, 5µl G-Block insert. This reaction was then placed in the thermocycler under the following programme: Incubate at 50°C for 60 minutes. The reaction was then transformed into competent *E.Coli*, or frozen for future use. Table 2.16 shows the sequences of G-Blocks generated, with the miR-125a binding site according to either the TargetScan or RNA22 prediction algorithms underlined.

**Table 2.16 Sequences of potential miR-125a targets used for reporter assay**

Name	Sequence
TLR4	<p>GAGAACTACGTGTGAAGGTATTCAAGGCAGGGAGTATACATTGC  TGTTTCCTGTTGGGCAATGCTCCTTGACCACATTTTGGGAAGAG  TGGATGTTATCATTGAGAAAACAATGTGTCTGGAATTAATGGGGT  TCTTATAAAGAAGGTTCCCAGAAAAGAATGTTTCATCCAGCCTCCT  CAGAAACAGAACATTCAAGAAAAGGACAATCAGGATGTCATCAG  <u>GGAATGAAAATAAAAACCACAATGAGATATCACCTTATACCAGG</u>  TAGAATGGCTACTATAAAAAAATGAAGTGTCATCAAGGATATAGA  GAAATTGGAACCCTTCTTCACTGCTGGAGGGAATGGAAAATGGT  GTAGCCGTTATGAAAACAGTACGGAGGTTTCTCAAAAATTA  ATAGAACTGCTATATGATCCAGCAATCTCACTTCTGTATATATAC  CCAAAATAATTGAAATCAGAATTTCAAGAAAATATTTACACTCCCA  TGTTTCATTGTGGCACTCTTCAATCACTGTTTCCAAAGTTATGG  AAACAACCCAAATTTCCATTGAAAATAAATGGACAAAGAAAATG  TGCATATACGTACAATGGGATATTATTCAGCCTAAAAAAGGGG  GAATCCTGTTATTTATGACAACATGAATAAACCCGGAGGCCATTA  TGCTATGTAAAATGAGCAAGTAACAGAAAGACAAATACTGCCTG  ATTTCAATTTATATGAGGTTCTAAAATAGTCAAACCTCATAGAAGCA  GAGAATAGAACAGTGGTTCCTAGGGAAAAGGAGGAAGGGGAGAA  <u>ATGAGGAAATAGGGAGTTGTCTA</u></p>
mTLR4	<p>GAGAACTACGTGGATCCGTATGGATCCAAGGATCCAGTATACAT  TGCTGTTTCCTGTGGGCAATGCTCCTTGACCACATTTTGGGAAG  AGTGGATGTTATCATTGAGAAAACAATGTGTCTGGAATTAATGGG  GTTCTTATAAAGAAGGTTCCCAGAAAAGAATGTTTCATCCAGCCTC  CTCAGAAACAGAACATTCAAGAAAAGGATCCTCAGGATCCATCA  <u>GGAATCCGAAAATAAAAACCACAATGAGATATCACCTTATACCAG</u>  GTGAATGGCTACTATAAAAAAATGAAGTGTCATCAAGGATATAGA  GAAATTGGAACCCTTCTTCACTGCTGGAGGGAATGGAAAATGGT  GTAGCCGTTATGAAAACAGACGGAGGTTTCTCAAAAATTA  TAGAACTGCTATATGATCCAGCAATCTCACTTCTGTATATATACC  CAAAAATAATTGAAATCAGAATTTCAAGAAAATATTTACACTCCCAT  GTTTCATTGTGGCACTCTTCAATCACTGTTTCCAAAGTTATGG  AAACAACCCAAATTTCCATTGAAAATAAATGGACAAAGAAAATGT  GCATATACGTACAATGGGATATTATTCAGCCTAAAAAAGGGGG  AATCCTGTTATTTATGACAACATGAATAAACCCGGAGGCCATTAT  GCTATGTAAAATGAGCAAGTAACAGAAAGACAAATACTGCCTGA  TTTCATTTATATGAGGTTCTAAAATAGTCAAACCTCATAGAAGCAG  AGAATAGAACAGTGGTTCCTAGGGAAAAGGAGGATCCGAGGAT</p>

	<u>CGAGGATCCAGGGAGTTGTCTA</u>
TNFAIP3	GTA <u>ACTGGCAAGGGATGATGTCAGATTCAGCCCAAGGTT</u> CCTCCTCTCCT ACCAAGCAGGAGGCCAGGA <u>ACTTCTTTGGACTT</u> GGAAGGTGTGCGGGGA CTGGCCGAGGCCCTGCACCCTGCGCATCAGGACTGCTTCATCGTCTTGG CTGAGAAAGGGAAAAGACACACAAGTCGCGTGGGTTGGAGAAGCCAGAG CCATTCCACCTCCCTCC <u>CCCAGCATCTCTCAGAGATGTGAAGCCAGATCC</u> TCATGGCAGCGAGGCCCTCTGCAAGAAGCTC <u>AAGGAAGCTCAGGGAAAAT</u> GGACGTATTCAGAGAGTGTGGTAGTTCATGGTTTTTCCCTACCTGCCCCG GTTCTTTCTGAGGACCCGGCA
mTNFAIP3	GTA <u>ACTGGCAAGGGATGATGTCAGATTCAGCCCAAGGTT</u> CCTCCTCTCCT ACCAAGCAGGAGGCCAGGA <u>ACTTCTTTGGACTT</u> GGAAGGTGTGCGGGGA CTGGCCGAGGCCCTGCACCCTGCGCATCAGGACTGCTTCATCGTCTTGG CTGAGAAAGGGAAAAGACACACAAGTCGCGTGGGTTGGAGAAGCCAGAG CCATTCCACCTCCCTCC <u>CCCAGCATCTCTCAGAGATGTGAAGCCAGATCC</u> TCATGGCAGCGAGGCCCTCTGCAAGAAGCTC <u>AAGATCCGAAAATGGACGT</u> ATTCAGAGAGTGTGGTAGTTCATGGTTTTTCCCTACCTGCCCCGTTCT TTCTGAGGACCCGGCA
CCL4	AAAAAGCAGATGTA <u>AATATATGATATCAACAAAAAAGCAGATGTA</u> AATATATGCATTCAAACAGCTGCTCAGAGACAGGAAGTCTTCAG GGAAGGTCACCTGAGCCCGGATGCTTCTCCATGAGACACATCTC CTCCATACTCAGGACTCCTCTCCGCAGTTCCTGTCCCTTCTCTTA ATTTAATCTTTTTTATGTGCCGTGTTATTGTATTAGGTGTCATTTT CATTATTTATATTAGTTTAGCCAAAGGATAAGTGTCCCCTATGGG GATGGTCCACTGTCACTGTTTCTCTGCTGTTGCAAATACATGGAT AACACATTTGATTCTGTGTGTTTTTCATAATAAACTTTAAAATAAA ATGCAGACAGTT
mCCL4	AAAAAGCAGATGTA <u>AATATATGATCCAACAAAAAAGCAGATGTA</u> ATATATGCATTCAAACAGCTGCTCAGAGACAGGAAGTCTTCAGG GAAGGTCACCTGAGCCCGGATGCTTCTCCATGAGACACATCTCC TCCATACTCAGGACTCCTCTCCGCAGTTCCTGTCCCTTCTCTTAA TTTTAATCTTTTTTATGTGCCGTGTTATTGTATTAGGTGTCATTTCC ATTATTTATATTAGTTTAGCCAAAGGATAAGTGTCCCCTATGGGG ATGGTCCACTGTCACTGTTTCTCTGCTGTTGCAAATACATGGATA ACACATTTGATTCTGTGTGTTTTTCATAATAAACTTTAAAATAAAA TGCAGACAGTT
TRAF6	GAAGGATCCTGTAGAATCCTGGAAGTAACCAGATTTTCCTAATAGGGAGA TGATTTTTTTGTGTGCCATCATGTATTTGTTAAAGGCCTATATATAGATAT AAAATATCGTGGAATCTAGTTCTCAGGGAGACCCGCAACTAGTATAAGCT TATAAAGGATCTAAAGATCCATCCACCATTTAAAGTTGTCTGGTAATGAGA GATGACATTGTATCCCCAGAGAGGCCAAATCAGAGTCGCCAGCCAGCGT
mTRAF6	GAAGGATCCTGTAGAATCCTGGAAGTAACCAGATTTTCCTAATAGGGAGA TGATTTTTTTGTGTGCCATCATGTATTTGTTAAAGGCCTATATATAGATAT AAAATATCGTGGAATCTAGTT <u>CAGATCCGACCCGCAACTAGTATAAGCTTA</u> TAAAGGATCTAAAGATCCATCCACCATTTAAAGTTGTCTGGTAATGAGAG ATGACATTGTATCCCCAGAGAGGCCAAATCAGAGTCGCCAGCCAGCGT

TNF $\alpha$	CTTCGAAACCTGGGATTCAGGAATGTGTGGCCTGCACAGTGAAG TGCTGGCAACCACTAAGAATTCAAACCTGGGGCCTCCAGAACTCA CTGGGGCCTACAGCTTTGATCCCTGACATCTGGAATCTGGAGAC <u>CAGGGAGCCTTTGGTTCTGGCCAGAATGCTGCAGGACTTGAGA</u> AGACCTCACCTAGAAATTGACACAAGTGGACCTTAGGCCTTCCT CTCTCCAGATGTTTCCAGACTTCCTTGAGACACGGAGCCCAGCC CTCCCCATGGAGCCAGCTCCCTCTATTTATGTTTGCA
mTNF $\alpha$	CTTCGAAACCTGGGATTCAGGAATGTGTGGCCTGCACAGTGAAG TGCTGGCAACCACTAAGAATTCAAACCTGGGGCCTCCAGAACTCA CTGGGGCCTACAGCTTTGATCCCTGACAGATATCATCTGGATAT <u>CAGGGAGCCTTTGGTTCTGGCCAGAATGCTGCAGGACTTGAGA</u> AGACCTCACCTAGAAATTGACACAAGTGGACCTTAGGCCTTCCT CTCTCCAGATGTTTCCAGACTTCCTTGAGACACGGAGCCCAGCC CTCCCCATGGAGCCAGCTCCCTCTATTTATGTTTGCA

m; mutated, underlined sequences are areas of predicted miR-125a binding, which were then mutated

## 2.5.2 Transformation

3 $\mu$ l of the reaction was pipetted into aliquots of One Shot<sup>®</sup> TOP10 Chemically Competent *E. coli*, incubated on ice for 30 minutes and heat shocked at 42 $^{\circ}$ C for 30 seconds. The cells were then spread on agar plates containing 1 $\mu$ g/ml ampicillin and cultured overnight in an incubator at 37 $^{\circ}$ C. Ampicillin resistance was used to select for those colonies which contained the PCR 2.1<sup>®</sup> plasmid. The next day colonies were picked from plates and cultured overnight in 5mls of LB broth with 1 $\mu$ g/ml at 37 $^{\circ}$ C on a shaker at 225rpm.

## 2.5.3 DNA Extraction

Depending on amount of DNA required, DNA was purified from cultures using a number of different commercially available kits.

For up to 20 $\mu$ g of DNA a QIAprep Spin Miniprep Kit (Qiagen) was used according to manufacturer's instruction. Briefly, 1.5ml of culture was centrifuged at 17,900g for 3 minutes at room temperature. The bacterial pellet was resuspended in 250 $\mu$ l of buffer P1. Next 250 $\mu$ l of buffer P2 was added to lyse cells (solution will turn blue) with the tube inverted 4-6 times. The reaction was neutralized by addition of 350 $\mu$  buffer N3 with the tube inverted 4-6 times. The

solution was centrifuged at 17,900g for 10 minutes with the supernatant transferred to a spin column. The column was centrifuged at 8000g for 1 minute and then washed with 750µl of PE buffer. The column was dried by centrifugation at 8000g for 1 minute with DNA then eluted in 50µl of water.

For up to 500µg DNA a QIAprep Spin Maxiprep Kit (Qiagen) was used according to manufacturer's instructions. Overnight cultures (50-100mls) were centrifuged at 2000g for 30 minutes with the pellets resuspended in 10mls of buffer P1. Next 10mls of buffer P2 was added and the tube was inverted 4-6 times and incubated for 5 minutes at room temperature. To stop the lysis 10mls of buffer N3 was added to the tube which was inverted 4-6 times and incubated on ice for 20 minutes. The solution was then centrifuged at 2000g for 30 minutes. In the meantime a Qiagen-tip was equilibrated with 10mls buffer QBT. The supernatant was then applied to the Qiagen-tip column and allowed to flow through. The column was washed twice with 30mls of buffer QC and DNA was eluted into a clean 50ml tube by adding 15mls of buffer QF. DNA was precipitated by adding 10.5mls of isopropanol to the eluted DNA and centrifuged at 2000g for 30 minutes at 4°C. The supernatant was carefully decanted and the pellet was washed in 5mls of 70% ethanol and centrifuged at 2000g for 10 minutes at room temperature. The supernatant was then decanted and the pellet was allowed to air dry. DNA was then dissolved in 500µl sterile water.

#### **2.5.4 Verification Digest**

To confirm presence of the insert, plasmid DNA was digested with restriction enzymes, sites of which were present in the vector. 10µl of plasmid was digested in 2µl appropriate buffer (NES), 7µl sterile water and 0.5µl of enzyme. The reaction was incubated at 37°C for 1 hour and then ran on a 1% agarose gel. The insert and vector were then cut out and gel purified.

#### **2.5.5 Agarose Gel Electrophoresis**

UltraPure agarose (Invitrogen) was used for agarose gel electrophoresis at concentrations of 1% (w/v) in 1xTAE buffer with addition of ethidium bromide to allow visualisation of DNA under UV light (Sigma). Prior to gel loading, samples were mixed with 1X BlueJuice (Qiagen). Samples were ran alongside 5µl of 1kb

plus DNA ladder (Invitrogen). Gels were placed into an electrophoresis tank filled with running buffer (1x TAE) and ran at 150V for 20-30 minutes, depending on size of expected fragment.

### **2.5.6 Sequencing of Reporter plasmids**

After purification, samples were sent to Source Bioscience for sequencing.

### **2.5.7 Co -Transfection of cells with luciferase reporter plasmid and microRNA**

Transient transfection of HEK 293 cells was accomplished using Attractene transfection reagent (Qiagen) according to manufacturer's instructions.

Shortly before transfection  $12 \times 10^4$  cells were seeded per well of a 24-well plate in 500 $\mu$ l of complete DMEM and incubated for the short time until transfection.

DNA concentration of the reporter plasmid were measured and adjusted to a working concentration of 200ng/ $\mu$ l by diluting in sterile water. microRNA mimics were adjusted to a working concentration of 5 $\mu$ M. To prepare the transfection master mix 0.4  $\mu$ g DNA was diluted in 60 $\mu$ l OptiMem media (Invitrogen).

Subsequently 6pmol of miRNA was added to the DNA and the solution was mixed vortexing. This gives a final concentration of 10nM added to cells. Finally 1.5 $\mu$ l Attractene Transfection Reagent was added to the diluted DNA and the solution was again mixed by vortexing. The transfection master mix was incubated for 10–15 min at room temperature (15–25°C) to allow the formation of transfection complexes. The transfection complexes were added drop-wise onto the cells with the plate gently swirled to ensure uniform distribution of transfection complexes. The cells were then incubated along with the transfection complexes for 24 hours under normal growth conditions.

### **2.5.8 Dual Luciferase reporter assay**

Validation of the interaction of microRNA with 3'UTR target sequence is achieved using a dual luciferase reporter assay (Promega). This assay allows the measurement of 2 different "glow type" luciferase signals sequentially in the

same sample, one from Firefly Luciferase and one from Sea Pansy luciferase, referred to as Renilla.

After 24 hours OptiMem media was removed and cells were washed twice with PBS and lysed by the addition of 200µl of 1X passive lysis buffer to each well. The reaction was incubated at room temperature for 15 minutes on a shaker. Next, 50µl of cell lysate was added in triplicate to a microtitre plate along with 50µl of luciferase substrate and incubated for 10 minutes on a shaker at room temperature, after which the values for luciferase were measured. The renilla reaction was initiated by the addition of 50µl Stop & Glo® Reagent provided with the kit, and incubated for 10 minutes on a shaker at room temperature after which renilla values were measured.

## 2.6 Generation of miR sponge

Generation of the miR-125a sponge, which was designed, created and stably inserted into THP-1 cells and will be discussed in Chapter 4.

## 2.7 Transfection Experiments

Alteration of expression is the basis to determine miR function. All transfection experiments were carried out using miRIDIAN miR mimics and hairpin inhibitors, detailed in Table 2.17, purchased from Dharmacon. Both controls are based on *c.elegans* miR-67, which is confirmed to have minimal sequence identity with miRNAs in human, mouse, and rat. In addition, to validate transfection efficiency, cells were also transfected with a control labelled with Dye 547 described in 2.3.2

**Table 2.17 miR mimics and inhibitors used for transfection**

Name	Cat No.
Control mimic	CN-002000-01
miR-125a mimic	C-300624-05-0005
Control inhibitor	IN-002005-01
miR-125a inhibitor	IH-300624-06-0005
Control mimic Dy547	CP-004500-01

### **2.7.1 Transfection of macrophages**

In order to validate transfection of human macrophages, a number of different transfection reagents were tested.

The best transfection of adherent cells was achieved using TransIT-TKO® Transfection Reagent according to manufacturer's instructions (MirusBio). In all cases the final concentration of miR was 25nM. The TransIT-TKO® reagent was warmed to room temperature and vortexed before use. A transfection master mix was made for each miR/control miR by adding - 7.5 µl 1µM miR, 50µl OptiMem media, and 2.5µl TransIT-TKO® Reagent per well to a tube. This reaction was incubated at room temperature for 30 minutes to allow transfection complexes to form. Before addition to cells, half the media (250µl) was removed from each well and 60µl of transfection complexes was added. Cells were then cultured for 24 hours under normal culture conditions.

## **2.8 Protein measurement**

### **2.8.1 Enzyme-Linked-immunosorbent assay (ELISA)**

A human ELISA kit (Invitrogen) was used to determine the concentration of this cytokine in cell supernatants. 96 well microtitre plates were coated with capture antibody in PBS, covered and incubated overnight at 4°C. The plates were washed with ELISA wash buffer and blocked by the addition of 200µl of assay buffer, to prevent non-specific binding and incubated at room temperature for 1 hour. Next, a standard curve was generated using recombinant human cytokine standards dissolved in blocking buffer. A top standard of 2000pg/ml was serially diluted 1:2 across eight samples. 100µl of each standard was added in duplicate to the plate along with two wells containing only blocking buffer. Samples were run neat or diluted with blocking buffer, depending on experimental set up, with 100µl being added to each well in duplicate. The plates were covered and incubated for 2 hours at room temperature. Next, the plates were washed x2 with ELISA wash buffer and the secondary antibody was added. This step was then incubated for 1 hour at room temperature. The plates were washed as previously and 100µl of streptavidin was added to each well. This reaction was incubated for 20 minutes at room temperature, after

which the streptavidin was washed off and 100µl of TMB chromagen (Biosource) was added to each well. The reaction was then stopped by addition of 100µl stop solution (Biosource) to each well the signal was read at 450 nm on a microplate reader.

**Table 2.18 ELISA antibody concentrations**

Cytokine	Cat No.	Concentration of primary antibody	Concentration of secondary antibody	Streptavidin dilution
TNF-α	CHC1753	0.250mg/0.125mL	0.025mg/0.125mL	1:625
IL-6	CHC1263	0.125mg/0.125ml	0.0025mg/0.125ml	1:2500
IL-12	CHC1563	0.125mg/0.125ml	0.0025mg/0.125ml	1:5000
IL-10	CHC1323	25µg/0.125ml	125µg/0.125ml	1:1250
CCL4	CHC2293	0.125mg/0.125ml	25µg/0.125ml	1:2500

### 2.8.2 Luminex Assay

A cytokine human 25-plex assay (Invitrogen - LHC0009) was used to determine the concentration of 25 cytokines and chemokines simultaneously within differentiated miR-125a sponge cell supernatants. The assay was carried out in accordance with the manufacturer's instructions. Briefly, the plate was pre-wet by washing with wash buffer provided. Next, beads were vortexed and sonicated, then pipetted into each well and washed. Next standards and samples were added with incubation buffer, covered with foil and incubated on a shaker at 500-600 rpm for 2 hours, at room temperature. After 2 hours the plate was washed and the biotinylated antibody added, and incubated on the shaker for 1 hour at room temperature. Finally, the plate was washed, streptavidin added, incubated for 30 minutes and read on the luminex reader.

### 2.8.3 Western Blot

Human CD14+ cells were separated as described in 2.2.2, and seeded at a density of  $3 \times 10^6$  per well of a 6 well plate. Cells were cultured in M-CSF and transfected on day 6. Cells were cultured overnight and then left unstimulated or stimulated with LPS (10ng/ml) for 6 hours.

### **2.8.3.1 Protein extraction**

Protein was extracted from cells using M-PER reagent (Thermo Scientific) according to protocol. Briefly, media was removed from cells which were washed with PBS. Next, 400µl of M-PER was added to each well and shaken gently for 5 minutes. The sample was then pelleted by centrifugation at 14,000g for 10 minutes, with HALT protease inhibitors (Thermo Scientific) added at a concentration of 10µl/ml. Supernatants were then frozen for further analysis.

### **2.8.3.2 Measurement of protein concentration**

Protein concentration was measured using the BCA protein assay kit (Thermo Scientific) according to protocol. Briefly, albumin standards were prepared along with BCA working reagent. Next, 25µl of standard, or sample was pipetted into well of a 96 well plate, along with 200µl of working reagent. The plate was then incubated at 37°C for 30 minutes, before the colour change was measured around 562nm on a plate reader.

Samples were then standardised to 20mg for loading onto gel and denatured in llameli buffer (Biorad) at 95°C for 10 minutes.

### **2.8.3.3 Gel Electrophoresis**

Samples were loaded onto a 10 well 10-12% gel (Invitrogen) which was then placed in a gel tank along with 1xMOPS running buffer (Invitrogen). To allow visualisation of size, a ladder was also added at the same time (Invitrogen). The gel was then ran at 150V for around 1 hour, after which proteins were transferred to a PVDF membrane.

### **2.8.3.4 Transfer and staining**

Proteins were transferred from the gel using the iBLOT dry transfer system according to protocol (Invitrogen). TNFAIP3 expression was then visualised by staining with a TNFAIP3 antibody (Invitrogen -A14028), compared to β-actin (Santa Cruz-SC47778), using the Azure c Series western blot machine. Briefly, the membrane was blocked for 30 minutes and incubated overnight with primary antibody (TNFAIP3 1 in 200, β-actin 1 in 2000) at 4°C. The next day, blots were washed (x3) in TBS-t and incubated for 30 minutes at room temperature with

secondary antibodies (TNFAIP3- Donkey anti rabbit antibody labelled with IRDye 680 RD, and  $\beta$ -actin, donkey anti mouse antibody labelled with IRDye 800CW). Protein expression was quantified using Digital Odyssey Imaging software (Li-Cor).

## **2.9 Statistical Analysis**

Values in this thesis were presented as mean  $\pm$  SEM. Statistical analysis used were indicated in the figure legend and depended on the distribution of results. Parametric tests were used in experiments in which the sample size allowed the identification of a normally distributed population (determined using a Pearson/D'Agostino normalcy test). These tests were used in experiments where the sample size (n) was greater than 6. Additionally parametric tests were used in experiments in which data was normalised (For example in luciferase experiments were control data was normalised to 100%). For small sample sizes, or data in which Gaussian distribution could not be determined, non-parametric tests were used. All statistical analysis was carried out using Graphpad version 6 software. A p value of  $<0.05$  was deemed significant.

## Chapter 3 – Dysregulated expression of microRNA in Rheumatoid Arthritis

### 3.1 Introduction and aims

Rheumatoid Arthritis (RA) is an inflammatory, autoimmune disease, characterised by immune cell infiltration into the synovium. This results in chronic inflammation and destruction of the synovial membrane, cartilage and bone. The cause of RA is currently unknown, with both genetic and environmental factors thought to contribute to disease susceptibility and progression [40, 376]. Monocytes and macrophages play a pivotal role in initiating and perpetuating the inflammation evident in RA. They produce pathogenic pro-inflammatory cytokines and chemokines such as TNF $\alpha$ , IL-6, CCL3, CCL4, and activate other cells such as T cells through cell contact [167, 377, 378]. Although treatment of RA has improved since the introduction of biologic therapies, which target these pro-inflammatory mediators, there are still many patients who do not respond to these interventions. One of the underlying mechanisms mediating this refractory state may be the fact that only a single end product of macrophage activation is targeted, leaving other mediators still bioactive. Therefore looking at the level of integration of activation signals in macrophages may reveal better candidates for therapy. Also to date, there is no clear biomarker for response to therapy. Therefore, many studies are now investigating the existence and characterisation of novel inflammatory mediators, in an attempt to identify new biomarkers, which may aid in identifying responders or non- responders to medication. In addition, studies are on-going to identify novel inflammatory pathways that may lead to new therapeutic interventions.

MicroRNAs (miRs) are a class of post transcriptional regulators that have been the subject of intense interest due to their ability to integrate the regulation of many pathways in response to environment. Recently, these molecules have also they emerged as useful biomarkers in cancer and cardiovascular field. As miR expression varies between cell types, prior studies have determined the miR profile of specific cell subsets, such as regulatory T cells and polarised

macrophages [368, 379]. miR expression can be altered by the cellular microenvironment. Thus, the expression of specific miRs can be changed by various stimuli of relevance to macrophage activation, such as infection, hypoxia, TLR activation and also through maturation signals [301, 380, 381]. Therefore, it has been hypothesised that miRs play a major role in fine-tuning the inflammatory response of a cell by regulating its activation, and the resultant production of cytokines and chemokines.

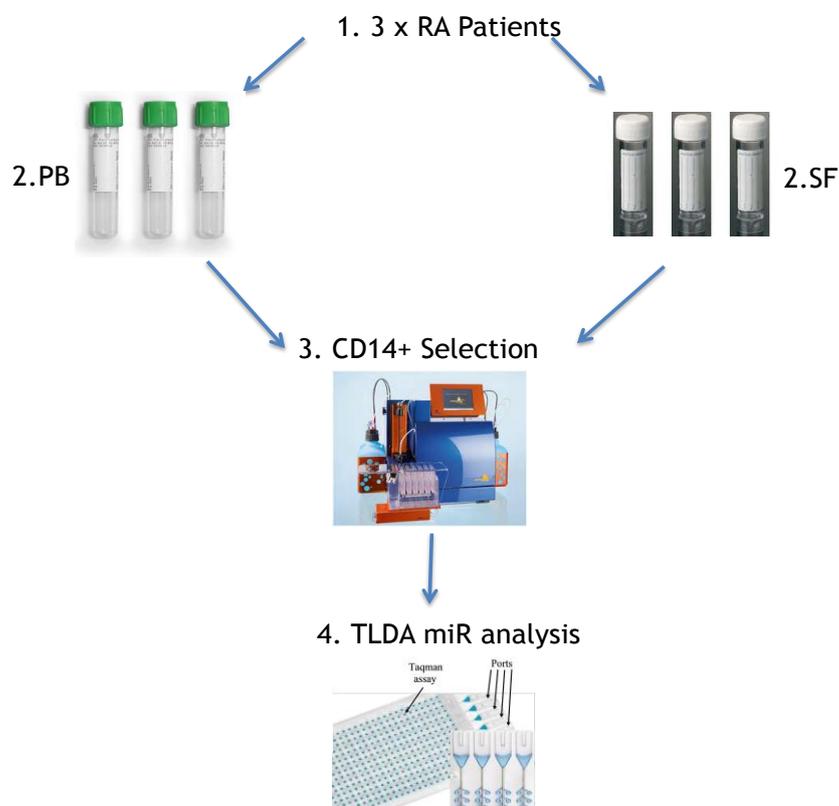
Determining the “normal” miR profile of a cell allows the identification of an aberrant miR profile under disease conditions. Altered miR expression has been demonstrated in various cell types in RA, contributing to the molecular mechanism of disease. For instance, miR-155 is up-regulated in RA PBMCs, RASFs and CD14<sup>+</sup> cells, where in the latter, its targeting of SHIP1, results in over production of the pro-inflammatory cytokines, TNF $\alpha$  and IL-6 [318, 320]. These data suggest that altered expression of miRs may play a major role in the pathogenesis of RA. Further studies have also identified the potential of miRs to be used as biomarkers of response to therapy, such as miR-125b indicating a positive response to Rituximab therapy [374].

To our knowledge there have been no miR profiling studies of monocytes or macrophages performed within the RA synovium. As these cells are a critical driving force behind inflammation, and their activation can be regulated by miR, a study within this lab generated a distinguished miR profile of RA synovial fluid CD14<sup>+</sup>. Therefore, the aim of this chapter was to verify miR dysregulation in RA SF CD14<sup>+</sup> cells, and to identify the environmental stimuli that may be responsible for the unique miR signature of RA monocyte/macrophages within the synovium.

## 3.2 Results

### 3.2.1 microRNA dysregulation in the synovial CD14+ compartment of RA patients

To understand the mechanisms regulating activation of a monocyte upon entering the synovial compartment from circulation, a recent study within this lab group created a differential miR expression profile of RA SF CD14+ cells compared with circulating monocyte populations. This was achieved by comparing paired SF samples to PB samples from the same patient. The experimental set up is summarised in Figure 3.1. PB and SF samples were taken from 3 RA patients and CD14+ cells were separated from each donor using magnetic bead selection. Total RNA was extracted from these cells, reverse transcribed and the microRNA expression profile was generated using a Taqman Low Density Array (TLDA) assay. This technique allow analysis on a PCR plate preloaded with 365 human miR species, allowing the quantification of these miRs simultaneously within the same sample, creating a miR expression profile.



**Figure 3.1 Experimental setup of RA CD14+ microRNA expression analysis**

CD14+ cells were separated from 3 RA PB and SF samples. A miR expression profile of these cells was then created by TLDA analysis.

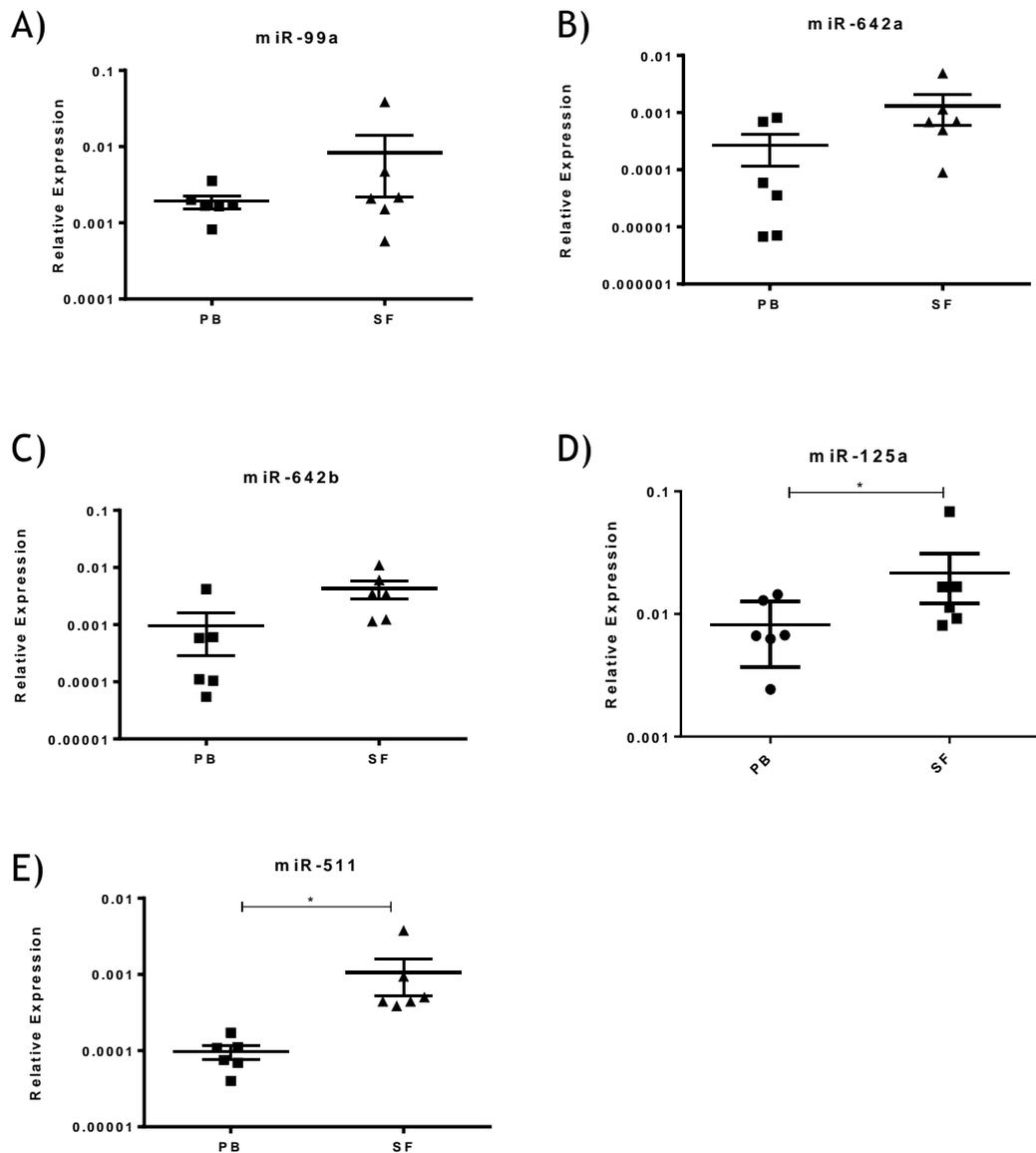
This study identified a number of miRs that are differentially expressed by CD14<sup>+</sup> cells that have entered the synovial microenvironment. One of these miRs, miR-155 has been studied in depth by my lab group, and its contribution to the pathogenesis of RA has been well demonstrated [320]. However, miR-155 is not the only miR to show dysregulated expression within RA SF CD14<sup>+</sup> cells; Table 3.1 shows the fold change of twenty-two miRs that were upregulated by more than 1.5 fold in the SF CD14<sup>+</sup> compartment of three RA patients, when compared with PB CD14<sup>+</sup> cells. Based on the results of this array I focused on validating the expression of candidate miRNAs that had not been previously investigated within the context of RA, shown in red in Table 3.1.

**Table 3.1 The dysregulation of microRNA expression in SF CD14<sup>+</sup> cells from RA patients compared to matched PB CD14<sup>+</sup> cells based on mean fold change between both compartments**

miR	Patient 1	Patient 2	Patient 3	Mean	SD
hsa-miR-511	10.71084	145.8154	97.34261	84.62293	68.4445
hsa-miR-489	1.63467	69.07059	8.956903	26.55405	37.00197
hsa-miR-132	29.36447	2.543824	7.770451	13.22625	14.21833
hsa-miR-642	18.8566	15.07411	5.528925	13.15321	6.868341
hsa-miR-10a	1.629016	23.37671	7.873468	10.95973	11.19751
hsa-miR-146b-3p	9.720295	3.067365	14.7741	9.187253	5.871542
hsa-miR-125a-5p	5.528925	8.562302	10.81528	8.30217	2.652762
hsa-miR-99b	3.670558	11.43988	9.633097	8.247844	4.065683
hsa-miR-125b	2.775985	16.07781	4.534677	7.796157	7.225827
hsa-miR-193b	4.089713	14.31049	1.651754	6.683987	6.716295
hsa-miR-146b-5p	3.863747	3.116655	9.389179	5.456527	3.426201
hsa-miR-99a	1.387029	11.7208	2.018101	5.041978	5.792633
hsa-miR-100	1.144724	8.044477	1.791291	3.660164	3.810664
hsa-let-7c	2.30697	5.433947	2.057654	3.26619	1.881466
hsa-miR-22	1.056286	3.708922	3.035637	2.600282	1.378865
hsa-miR-886-5p	2.380066	3.904125	1.120387	2.468193	1.39396
hsa-miR-708	3.896017	1.085229	4.284121	2.36497	2.994254
hsa-miR-29a	1.971099	1.357544	3.03143	2.120024	0.846822
hsa-miR-29b	2.287862	1.18263	2.14801	1.872833	0.60181
hsa-miR-146a	1.168778	2.977287	1.154286	1.766784	1.048352
has-miR-155	2.599079	1.086735	1.585567	1.757127	0.77063
hsa-miR-629	2.079159	1.259629	1.591071	1.643286	0.412252

Data provided from a previous study by Dr Mariola Kurowska-Stolarska at the GBRC

For my study, I wanted to investigate miRs which were differentially expressed in SF cells, when compared to PB, as this suggests an effect of the synovial microenvironment on miR expression. The previous TLDA analysis (Table 3.1) identified miR, which were indeed increased in the synovial miR environment. Analysis by Dr Mariola Kurowska-Stolarska at the GBRC compared the fold change of expression in SF compared to PB and showed that differences between these compartments exist. I selected miR-125a-5p and miR-99b, because they come from the same miR cluster thereby suggesting coordinated expression. Additionally, searching the literature surrounding this cluster, it appeared that at least miR-125a plays a role in regulating the activation and inflammatory response of macrophages. In addition, I selected miR-511 and miR-642 because they were consistently and strongly up-regulated (more than 5 fold) in SF CD14+ cells of all 3 donors, and had yet to be described within the context of RA, highlighting the novel nature of these observations. Their expression was validated in PB and SF CD14+ cells from six additional RA patients by quantitative PCR analysis. Difference of expression between PB and SF was calculated using a relative expression method. This was calculated by subtracting the housekeeping Ct value, from the Ct value of the miR target and presented as  $2^{-\Delta Ct}$  as amount of product doubled with every cycle. Compared with CD14+ cells from PB, miR-125a and miR-511 expression were significantly increased in SF CD14+ cells from RA patients ( $p < 0.05$ ) (Figure 3.2 D and E), while expression of the other miRs investigated were not significantly altered in the validation cohort (Fig 3.2 A-C).



### Figure 3.2 Expression of dysregulated miR in RA SF CD14+ cells

CD14+ cells were separated from the PB and SF of RA patients. Relative miR expression was measured using Taqman qPCR reagents A-C) Expression of miR-99a, miR-642a and miR-642b are not significantly altered in SF CD14+ cells D and E) miR-125a and miR-511 expression is significantly up-regulated in SF CD14+ cells when compared to PB CD14+ cells. Values presented as mean  $\pm$  SEM of 6 patient samples and marked bars are statistically different between groups using Wilcoxon signed rank test  $*= p < 0.05$ .

### 3.2.2 miR-511 is up-regulated in RA CD14+ cells

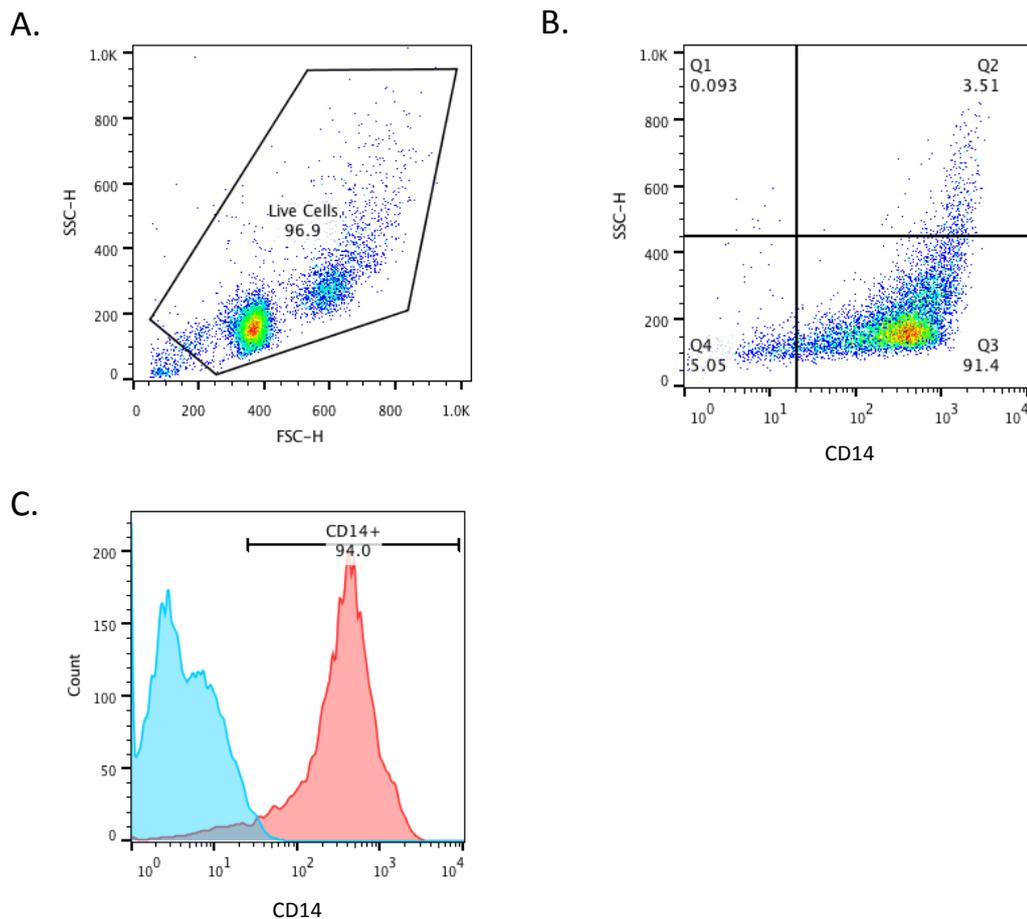
miR-511 was found to be the miR with the highest increase in expression in the original array analysis and was verified to be upregulated in additional RA patient samples. Based on these data, it was decided to further investigate the function of miR-511 in the biology of monocytes and macrophages. To examine the role of miR-511 in these cells, CD14+ cells were isolated as described in

section 2.3.1 of materials and methods, and the purity of this population was verified.

### **3.2.2.1 Purity of CD14+ monocytes**

Both PB and SF monocytes express the CD14 molecule on their surface. This molecule is required for signalling through TLR4 and can also be used as a marker for human monocytes. Therefore these cells can be separated on the basis of CD14 expression using magnetic beads. These beads bind to CD14 on the cell surface, and through a process of positive selection allow these cells to be specifically sorted. Purity of the population is confirmed by flow cytometry, comparing the percentage of CD14+ cells with an isotype control. The purity of cells was >90% in all experiments tested. The dot plot in figure 3.3A shows gating of live cells based on size and granularity (forward scatter, FSC and side scatter, SSC). Figure 3.3B is representative of CD14+ cells from healthy blood samples. Figure 3.3C shows a representative histogram demonstrating positive CD14 expression when compared to an isotype control.

Thus CD14+ cells purified as defined above were then used for all future experiments involving human monocytes and macrophages.



### Figure 3.3 Purity of CD14+ monocytes

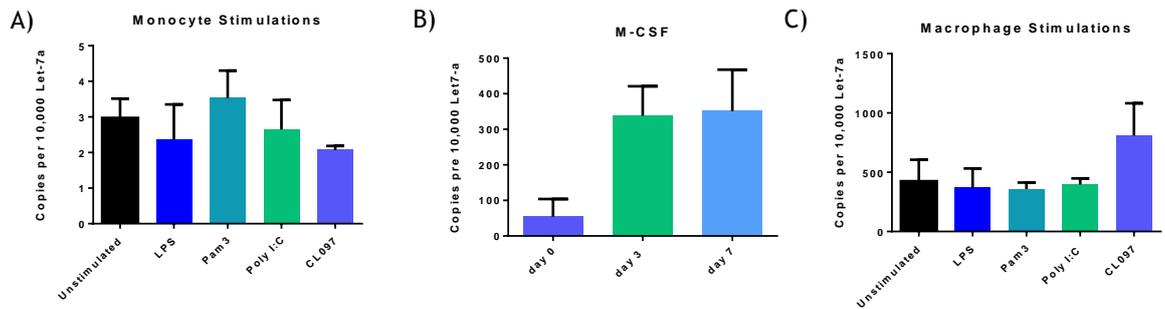
Purity of CD14+ cells was checked by FACs analysis. A) Live cells were gated on based on FSC and SSC. B) Staining of CD14 was measured on live cells, this is a representative dot plot of CD14 expression. C) Representative histogram showing the percentage of CD14+ cells (red) separated when compared to and isotype control (blue).

### 3.2.2.2 miR-511 is expressed at low copy number in healthy monocytes and macrophages and is not induced by TLR stimulation

Previous studies have demonstrated the induction of various miR in response to cellular activation, either by infection or environmental signals. These miR can then function, to both enhance the inflammatory response, or prevent overactive inflammation, by regulating signalling molecules and their inhibitors. Pro-inflammatory mediators such as endogenous TLR ligands, including tenascin-C and fibronectin, that can bind to TLR4, and free nucleic acid that can activate TLR3 are also present within the RA joint, where miR-511 is upregulated in CD14+ cells [162, 382, 383]. I therefore wanted to investigate the effect of TLR stimulation on the expression of miR-511 in healthy monocytes and macrophages. To investigate expression in monocytes, CD14+ cells were

separated from healthy blood, and cultured in 24 well plates, after which cells were stimulated with a broad spectrum of TLR ligands for a further 24 hours. Total RNA was extracted from these cells to allow transcript levels of miR-511 to be determined. For the initial q-PCR experiments absolute copy number of miR was calculated. This was achieved by engineering miR standards as described in the materials and methods section for both miR-511 and Let-7a as a housekeeping control. To be able to perform relative quantification of miR-511, we normalized copy number of miR-511 to 10,000 copies of the abundant housekeeping control, Let-7a. The baseline copy number of miR-511 is low in healthy PB CD14<sup>+</sup> cells (3 per 10,000 let7a) and expression does not appear to be increased following challenge of these cells with a wide range of TLR ligands (Figure 3.4A).

Along with response to inflammatory mediators, miR expression has also been demonstrated to broadly increase during differentiation from monocyte to macrophage [368]. Again, growth factors such as M-CSF are present in the synovial microenvironment where miR-511 expression is significantly increased [384]. Therefore, I wanted to determine if the low copy number of miR-511 seen in monocytes is increased by culture in M-CSF and differentiation towards a macrophage phenotype. To investigate this, CD14<sup>+</sup> cells were separated from 'buffy coat' packs and cultured for 7 days in M-CSF (50 ng/ml), after which RNA was extracted and transcript levels of miR-511 were determined by RT-PCR. Results demonstrated that copy number of miR-511 is increased during macrophage differentiation (Figure 3.4 B). However this increase did not reach statistical significance, and miR-511 continued to be expressed at relatively low copy number; there are less than 500 copies per 10,000 Let-7a in these cells. Additionally, activation of M-CSF driven macrophages through stimulation with TLR ligands (as with monocytes), also does not increase miR-511 expression (Figure 3.4 C).



**Figure 3.4 miR-511 expression in M-CSF differentiated macrophages and TLR stimulated monocytes and macrophages**

Copy number of miR-511 was measured in cultured and CD14<sup>+</sup> cells stimulated with 100ng/ml LPS, 300ng/ml Pam3, 50 $\mu$ g poly I:C and 5 $\mu$ g/ml CL097 using Taqman q-PCR A) Baseline copy number of miR-511 in monocytes is low and is not induced by TLR stimulation. B) Copy number of miR-511 increases during M-CSF differentiation, however there are still low numbers present by day 7. C) Copy number of miR-511 in macrophages is not affected by stimulation with TLR ligands. Values presented as mean  $\pm$  SEM of 3 healthy donors for stimulation experiments (A & C) and mean  $\pm$  SEM of 4 healthy donors for differentiation experiment. No significance was detected using a Friedman Test.

### 3.2.2.3 miR-511 is encoded within the mannose receptor gene

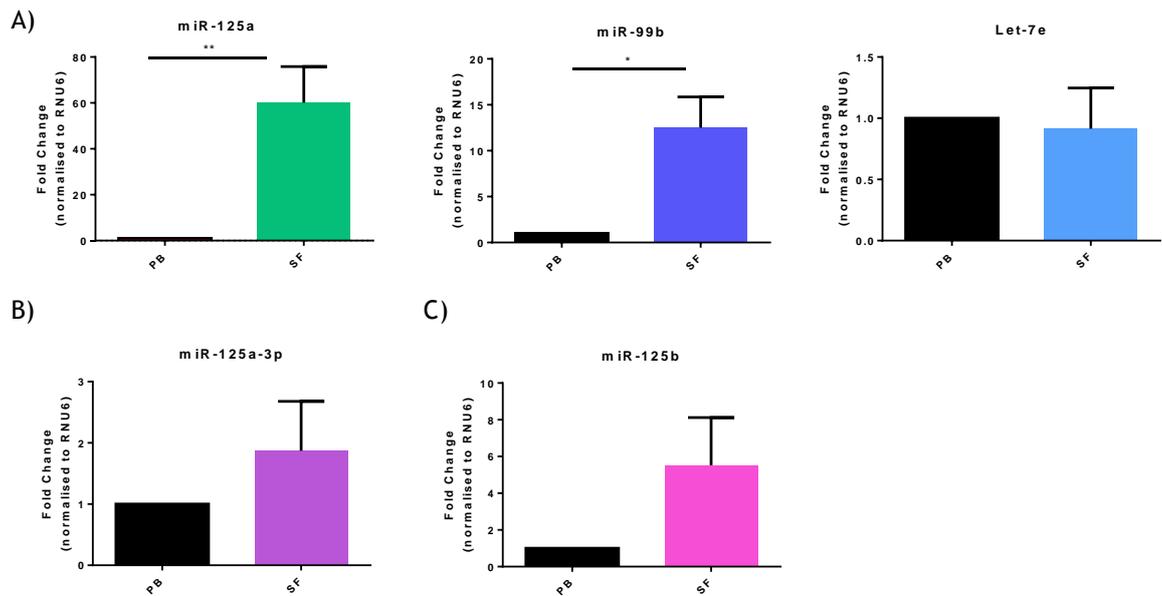
Due to non-response of miR-511 to pro-inflammatory stimulation, we used sequence analysis to examine precisely where the miR-511 is encoded to aid identification of potential transcription activators. We found that miR-511 is encoded within the mannose receptor gene (CD206). It is well described that miRs encoded within the introns of protein coding genes can be transcribed together with the host gene from the same promoter [254]. Expression of CD206 identifies the M2, or alternatively (anti-inflammatory) activated subset of macrophages, and also tumour associated macrophages (TAMs) [385]. This suggested that this miR is potentially induced upon alternative activation of the cell. Therefore, due to the interest of this study in pro-inflammatory monocytes and macrophages, and the apparent low level of expression of miR-511 in these cells, it was decided to concentrate further investigation on another family of miRs that were upregulated in RA SF CD14<sup>+</sup> cells. Nevertheless future studies will be required to follow up the observation and enumerate the copy number in RA SF CD14<sup>+</sup> cells and RA synovial macrophages.

### 3.2.3 miR-125a is up-regulated in SF CD14<sup>+</sup> Cells

In addition to miR-511, two members of the miR-125a cluster were also found to be upregulated in the original array analysis (Table 3.1). Additionally the original analysis also identified miR-125b, a homologue of miR-125a, to be

upregulated in SF CD14<sup>+</sup> cells. Both miR-125a and miR-125b have been shown previously to be important in regulating the activation of monocytes and macrophages [301, 339]. Accordingly, we decided to concentrate our investigation on this family of miRs to elaborate their potential role in pathogenesis.

As we previously demonstrated, not all miRs identified in the initial TLDA analysis were verified to be dysregulated in further RA samples. Therefore, we firstly confirmed the original array data with regards to the miR-125 family. Peripheral blood and SF CD14<sup>+</sup> cells were separated from ten further RA patients, and miR expression was analysed by quantitative RT-PCR. Compared with CD14<sup>+</sup> cells from PB, miR-125a ( $p < 0.005$ ) and miR-99b ( $p < 0.05$ ), but not Let-7e, were significantly upregulated in the SF derived cells (Figure 3.5 A) confirming the TLDA expression data (Table 3.1). The opposite strand of miR-125a, miR-125a-3p is listed as a conserved miR in its own right, with its own set of specific predicted targets. miR-125a-3p is expressed at low levels in RA SF CD14<sup>+</sup> cells (Table 3.2), suggesting that miR-125a-5p is the functional strand within these cells. I therefore decided to make my primary investigations into miR-125a-5p. (Figure 3.5 B). Additionally, although upregulated expression of miR-125b was detected in SF CD14<sup>+</sup> cells, this difference was not significant when compared to PB CD14<sup>+</sup> cells (Figure 3.5 C)



### Figure 3.5 Expression of the miR-125 family in RA CD14+ Cells

Expression of the miR-125a family in CD14+ cells from paired RA PB and SF samples were measured by q-PCR A) miR-125a and miR-99b are significantly up-regulated in synovial fluid CD14+ cells. However Let-7e expression is not altered by the synovial microenvironment. B) miR-125a-3p is expressed at low levels in CD14+ cells, and is marginally increased in SF cells C) Expression of miR-125b is not significantly increased in SF CD14+ cells. PB values have been normalised to 1, with SF represented as fold change compared to PB. Values presented as mean  $\pm$  SEM of 10 patient samples and marked bars are statistically different from PB using a Paired T Test \*\*=  $p < 0.005$  \*=  $p < 0.05$ .

Next, we thought it was important to examine the relative abundance of the expression of the members of this cluster in monocytes. Alternative to the copy number test, a simple inspection of q-PCR Ct values can inform the relative abundance of the expression of gene of interest. Abundance of the transcript decreases with an increasing value of Ct (cycle threshold). The housekeeping RNU6 in these patients' samples remained relatively stable, allowing a comparison of Ct values within these samples. It is generally accepted that a Ct value of 35 represents a single molecule template detection, therefore Ct values above 35 are considered 'noise' from the system [386]. From this we could see that Let-7e is the most abundantly expressed miR in CD14+ cells, and its expression is not increased in SF CD14+ cells. The Ct values also show that although both miR-125a and miR-99b are significantly increased in SF CD14+, miR-125a appears to be more abundant in these samples when compared to miR-99b. Additionally, based on Ct values, we can also speculate that miR-125a is expressed at higher levels in both PB and SF CD14+ when compared to its homologue miR-125b (Table 3.2). RNU6 levels are included in this table in brackets to demonstrate that the housekeeping gene remained relatively stable across conditions and allow a comparison between Ct Values.

**Table 3.2 Average Ct values of miR-125 family members in RA CD14+ cells between PB and SF compartments.**

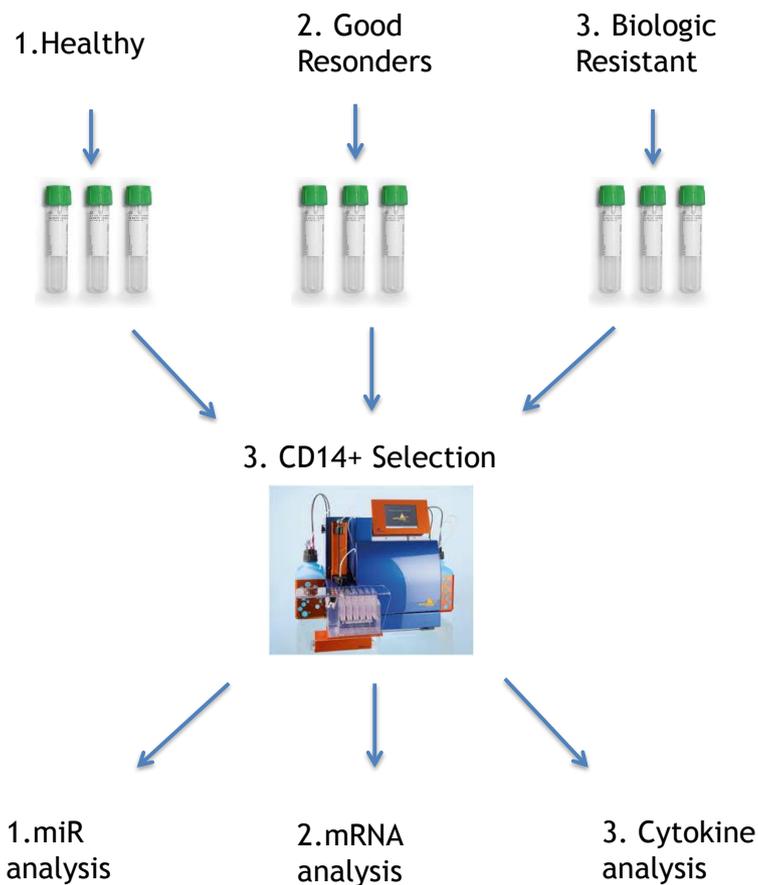
	Average Ct Values	
	PB (RNU6)	SF (RNU6)
miR-125a	29.37 (23.44)	27.24 (23.12)
miR-99b	32.61 (23.25)	29.84 (23.55)
Let-7e	24.71 (22.22)	24.35 (22.12)
miR-125a-3p	34.84 (23.44)	34.23 (23.12)
miR-125b	33.25 (22.22)	31.90 (22.12)

Average Ct values of RNU6 are shown in brackets to demonstrate relative stability of the housekeeping gene

Because of this expression pattern we decided to concentrate our investigation on both miR-125a and miR-99b as they are expressed in PB CD14+ cells and their expression is significantly increased in RA SF CD14+ cells.

### **3.2.4 miR-125a is up-regulated in peripheral blood CD14+ cells of RA patients who respond well to conventional DMARD therapy**

Previous studies have suggested that PB monocytes of RA patients are in an abnormal, 'primed', pro-inflammatory state before they enter the synovium. Therefore, a study was undertaken to determine if the miR profile of PB CD14+ was altered as a result of treatment experience and response characteristics in patients with RA. The experimental setup of this study is summarised in Figure 3.6.



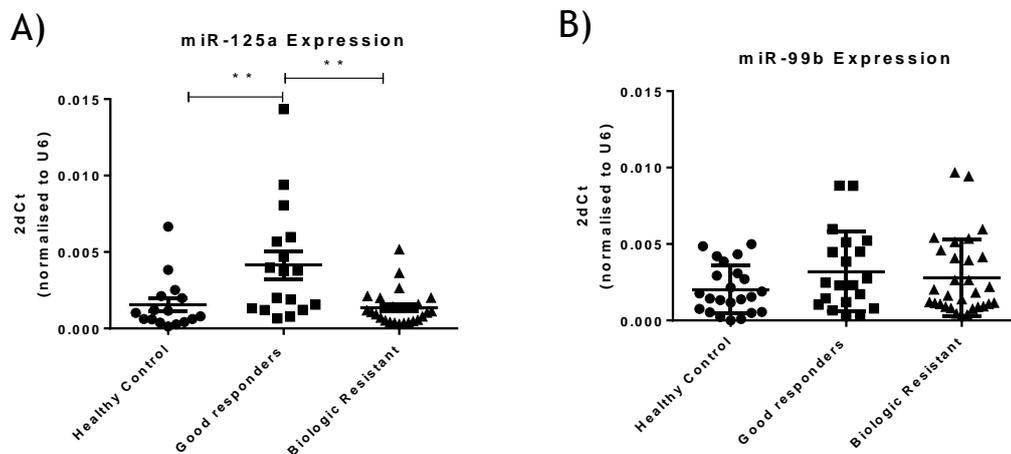
**Figure 3.6 Experimental setup of PB CD14+ analysis from healthy volunteers, patients responding well to cDMARD treatment (Good Responders), and those resistant to biologic therapies (Biologic Resistant).**

Patients and healthy control subjects were recruited to this study and classified into groups based on their response to different therapies. From these samples miR and mRNA studies were performed on separated CD14+ cells from each group. Additionally, cytokine analysis was performed on the serum of these blood samples

Patients and healthy control subjects were recruited to the study by Dr Derek Baxter, Glasgow Royal Infirmary. This cross-sectional cohort was recruited comprising two groups, namely those who responded well to conventional DMARD therapy from the outset and who were therefore judged to be ‘good responders’, and those who had failed DMARD therapy but were also proving to be resistant to biologic treatment - i.e. those who apparently present a therapy resistant phenotype. These groups were gathered to determine if there was a difference in the state of patients, who had distinct clinical response histories over time. Blood samples were taken and CD14+ cells were separated by Miss Lynn Stewart of the Glasgow Biomedical Research Centre (GBRC). RNA was extracted from these cells and miR expression was measured by q-PCR. This allowed the generation of a miR profile of RA PB CD14+ cells, from patients

under different treatment regimes and with this distinct therapy response phenotype.

As miR-125a and miR-99b were significantly up-regulated in RA SF CD14+ cells, I wished to determine if a change in these miRs could also be demonstrated in PB CD14+ of RA patients, by carrying out q-PCR on the RNA which had been isolated from these RA samples. Interestingly, the results demonstrated that miR-125a expression did not differ between healthy and biologic resistant patients. However, its expression was significantly upregulated in the RA good responders group, when compared to both healthy controls and biologic resistant patients with a p value of  $p < 0.005$  (Figure 3.7A). This could suggest a specific activation of miR-125a in those RA patients in whom treatments have been successful - this could either reflect direct effects of the drugs (methotrexate or sulphasalazine) that patients were receiving, or could be an indicator of a disease activity state that is low, or even 'held' in a state of low activation status. Expression of miR-99b did not appear affected by treatment status (Figure 3.7B).



**Figure 3.7 miR-125a and miR-99b expression in PB CD14+ cells of RA patients**  
miR expression was measured in PB CD14+ cells of 3 different groups of subjects. A) miR-125a is upregulated in good responders compared to healthy control and biologic resistant patients B) Expression of miR-99b is not altered between groups. Values presented as mean  $\pm$  SEM and marked bars are statistically different from other groups using Kruskal-Wallis H test \*\*=  $p < 0.005$ .

### 3.2.4.1 miR-125a expression correlates with clinical measures of disease

As miR-125a is increased in good responders (low disease score, DAS28-CRP below 3.0), suggesting a protective role for this miR in response to therapy, we wished to investigate if miR-125a expression correlates with clinical variables of disease. Demographic data, as evaluated at the baseline time-point of PB collection, from subjects are listed in Table 3.3. Available data included age, disease duration, sex, smoking status, swollen joint count (SJC), tender joint count (TJC), simple disease activity index (SDAI), clinical disease activity index (CDAI) erythrocyte sedimentation rate (ESR), C-Reactive protein (CRP), disease activity score 28-ESR (DAS28) and DAS28-CRP

**Table 3.3 Demographics and disease characteristics of study patients**

	Healthy Controls	Good Responders	Biologic Resistant
	n=15	n=15	n=24
Age (years)	32-60 (48)	54-81 (63)	36-78 (59)
Disease duration (months)	N/A	78-367 (242)	72-346 (183)
Female	80%	66%	88%
Smoking status	N/A	Current: 21% Ex: 21% Never: 58%	Current: 25% Ex: 13% Never: 62%
SJC	n/a	1.88	11.13
TJC	n/a	1.59	11.20
SDAI	n/a	9.16	35.92
CDAI	n/a	7.92	33.12
ESR mm/hr.	9.76	22.82	28.96
CRP mg/dl	0.34	1.24	2.48
DAS28-ESR	n/a	3.23	5.86
DAS28-CRP	n/a	2.98	5.44

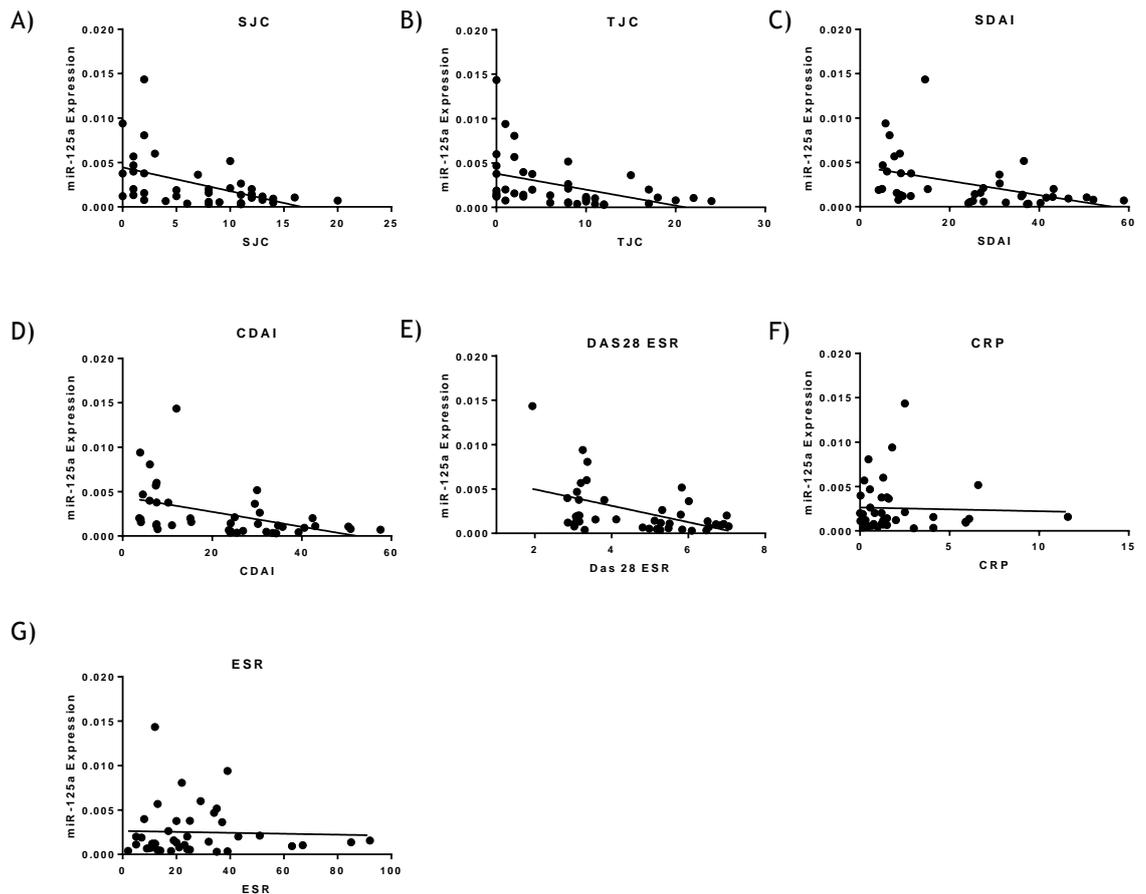
SJC: swollen joint count, TJC: tender joint count, ESR: erythrocyte sedimentation rate, CRP: C-reactive protein, DAS28: disease activity score 28

SDAI, CDAI and DAS 28 values generate a disease score which as a continuous variable indicates a level of on-going disease activity (Table 3.4). We can therefore see that based on these scores, good responders appear to be experiencing low to moderate disease activity, whereas biologic resistant patients are experiencing higher disease activity.

**Table 3.4 Scoring of disease activity in RA patients**

	<b>SDAI</b>	<b>CDAI</b>	<b>DAS28</b>
<b>Score based on</b>	SJC, TJC, patient questionnaire, evaluator questionnaire, CRP value	SJC, TJC, patient questionnaire, evaluator questionnaire	SJC, TJC, patient questionnaire, either CRP or ESR value
<b>Remission</b>	0-3.3	≤ 2.8	≤ 2.6
<b>Low</b>	3.4-11	>2.8 - ≤10	>2.6-<3.6
<b>Moderate</b>	11.1 - 26	>10 - ≤ 22	>3.6-<5.1
<b>High</b>	>26	≥ 22	>5.1

My data demonstrates that miR-125a expression negatively and significantly correlates with TJC and SJC of RA patients (Figure 3.8 A and B). Additionally, miR-125a expression negatively and significantly correlates with SDAI, CDAI and DAS28 ESR of patients (Figure 3.8 C, D and E). Although this association would suggest a relationship between miR-125a expression and disease activity, a correlation was not demonstrated between miR-125a and the indicators of inflammation, CRP and ESR (Figure 3.8 F and G).



**Figure 3.8 miR-125a expression negatively correlates with clinical variables of disease**

A-E) miR-125a expression significantly and negatively correlates with patient SJC, TJC, SDAI, CDAI and DAS28 ESR levels F-G) miR-125a does not correlate with either clinical measures of disease activity CRP or ESR.

The R-value and correlation coefficient for miR-125a and clinical variables of disease are shown in Table 3.5

**Table 3.5 miR-125a correlates with clinical variables of disease**

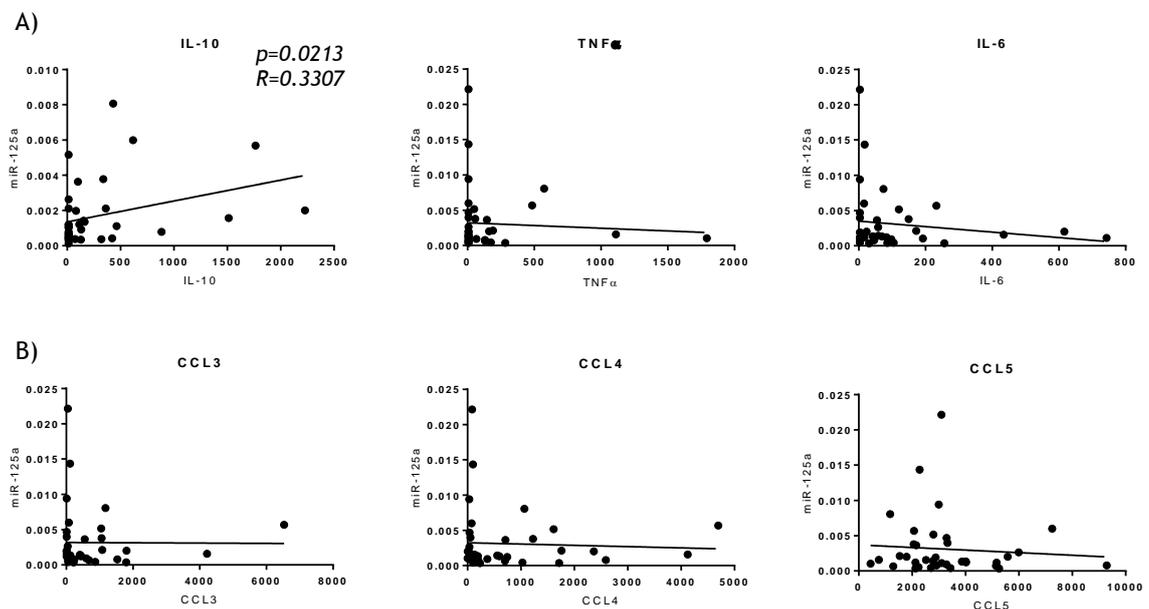
Clinical Variable	MicroRNA-125a relative expression (Correlation coefficient (p value))
SJC	-0.420 (0.0052)
TJC	-0.425 (0.0056)
SDAI	-0.442 (0.0043)
CDAI	-0.448 (0.003)
DAS28-ESR	-0.409 (0.0079)
ESR mm/hr	-0.038 (0.8162)
CRP mg/dl	-0.034 (0.833)

Pearson Coefficient

### 3.2.4.2 miR-125a expression positively correlates with serum IL-10 expression

During the study serum samples were also taken from patients, and a Luminex assay for cytokine expression was carried out by Miss Lynn Stewart and Dr Donna McIntyre, both part of my lab group at the GBRC. As these foregoing data suggested a protective role for miR-125a in CD14<sup>+</sup> cells, we wanted to determine if cellular miR-125a expression correlates with serum cytokine and chemokine levels in RA patients. I therefore took my own dataset and co-analysed it with the cytokine/chemokine dataset provided by my colleagues.

The results suggest that miR-125a expression positively correlates (weakly but nevertheless significantly) with serum levels of the anti-inflammatory cytokine IL-10 ( $R=0.3307$ ,  $P=0.0213$ ) but did not correlate with other pro-inflammatory cytokines such as TNF $\alpha$  and IL-6 (Figure 3.9A). In addition cellular expression of miR-125a did not correlate with soluble pro-inflammatory chemokines such as CCL3, CCL4 and CCL5 (Figure 3.9B).

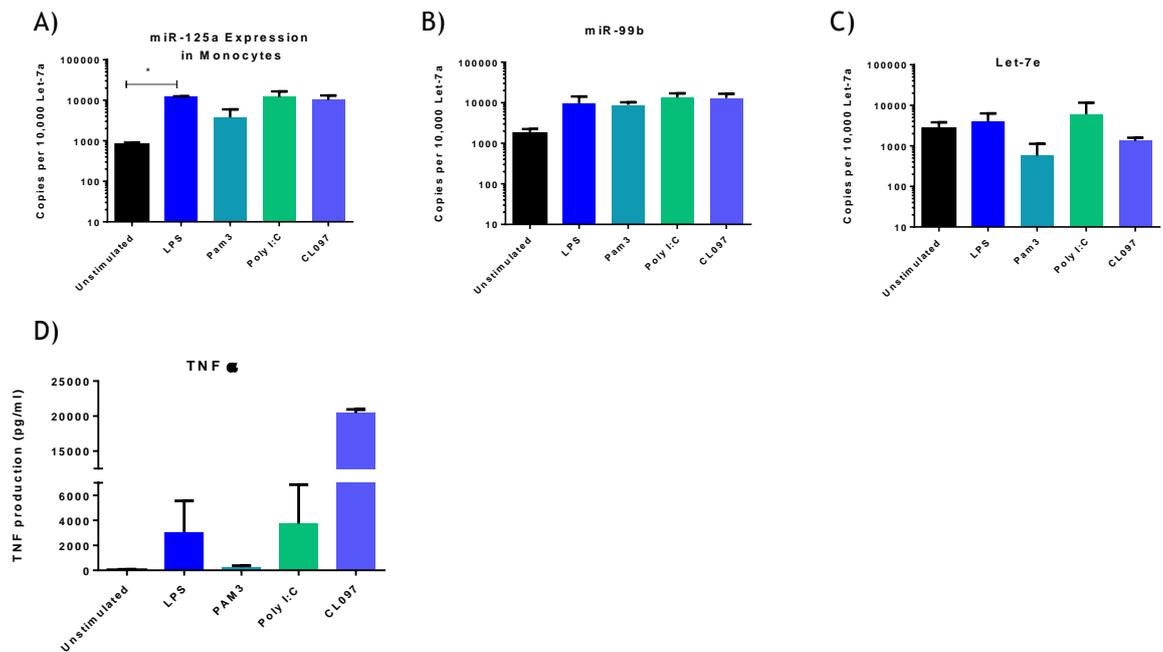


**Figure 3.9 miR-125a expression and cytokine and chemokine production from RA PB CD14<sup>+</sup> cells**

Serum cytokine and chemokine expression was measured using a Luminex assay. A) miR-125a positively correlates with IL-10 serum levels. However miR-125a expression does not correlate with other important disease cytokines such as TNF $\alpha$  and IL-6. Each dot represents miR-125a expression versus cytokine expression. B) miR-125a expression does not correlate with serum chemokine expression from RA patients. Each dot represents miR-125a expression versus chemokine expression.

### 3.2.5 miR-125a is up-regulated in response to LPS stimulation in healthy monocytes

As miR-125a and miR-99b are upregulated in SF CD14<sup>+</sup> cells, I next set out to examine the regulation of these miRs, and the cluster member, Let-7e, in healthy CD14<sup>+</sup> monocytes in order to try to reconstruct the pathway that leads to the synovial 'miR125a phenotype'. Firstly, I decided to examine the effect of TLR stimulation. SF contains many endogenous TLR ligands such as fibronectin and Tenascin-C. In addition, it has been shown that expression of various TLR2 and TLR4 ligands are upregulated in RA synovium along with their receptors on macrophages and other cell lineages [387, 388]. To investigate this, CD14<sup>+</sup> cells were separated from healthy buffy coat samples and cultured for 24 hours in varying concentrations of different TLR ligands. The expression of the miR-125a cluster was then measured by Taqman RT-PCR. The results demonstrated that baseline copy number of miR-125a and miR-99b was similar in monocytes. However, copy number of miR-125a alone was significantly upregulated ( $p < 0.005$ ) by culture for 24 hours with LPS (100ng/ml) (Figure 3.10A). The expression of miR-99b mirrored that of miR-125a, but did not demonstrate statistical significance between experimental conditions (Figure 3.10 B). Let-7e appears to be regulated in an alternative manner in activated monocytes. It is expressed at a higher basal copy number and its expression does not appear to be altered by stimulation with TLR ligands (Figure 3.10C). Activation of these cells was verified by measurement of the pro-inflammatory cytokine TNF $\alpha$ , which is produced by monocytes in response to TLR stimulation. This was measured using an ELISA assay (Figure 3.10D).



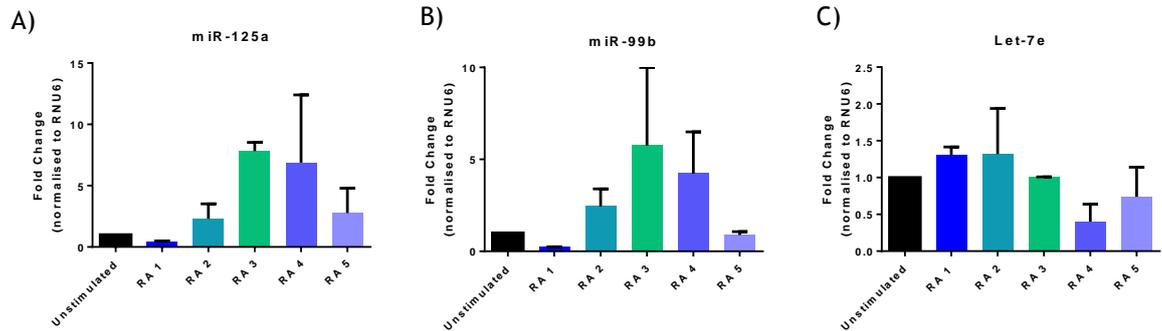
### Figure 3.10 miR-125a cluster expression in TLR stimulated monocytes

Expression of miRs in human CD14<sup>+</sup> monocytes were measured using Taqman q-PCR. A) miR-125a is significantly up-regulated in monocytes by stimulation with LPS. B) miR-99b expression also increases after stimulation, but this is not significant. C) Let-7e expression does not appear to be affected by TLR stimulation in monocytes. D) TNF $\alpha$  ELISA demonstrates that cells are activated and producing TNF $\alpha$  after TLR stimulation. Values are represented as copies per 10,000 Let-7a as mean  $\pm$  SEM of 3 healthy donors and marked bars are statistically different from other groups using a Friedman Test with Dunn's multiple comparison test. \* $p < 0.05$

#### 3.2.5.1 miR-125a can be up-regulated by culture in synovial fluid

miR-125a and miR-99b are expressed at low levels in healthy PB CD14<sup>+</sup> monocytes (Ct values of around 30 for miR-125a and 32 for miR-99b), however the miR-125a cluster is upregulated in SF CD14<sup>+</sup> cells and also by TLR4 activation. I therefore set out to investigate if culture in RA patient derived SF itself can cause upregulation of the miR-125a cluster. Healthy CD14<sup>+</sup> cells were separated as before, but were cultured overnight in 10% synovial fluid from 5 different RA patients. The results demonstrated that culture in SF has differing effects on miR expression across different donors. As previously observed, miR-125a and miR-99b appear to follow the same expression pattern, with miR-99b being expressed at a lower level, when compared to miR-125a. Their expression was increased by RA3 and RA4 SF and not by RA 1 and RA 5 SF, however this change in expression did not reach statistical significance and appeared donor variable (Figure 3.11 A and B). This suggests that synovial environment is capable of inducing miR-125a expression in monocytes. Further studies are

required with increase number of donors and SFs. It would also be important to look at a wider time course and range of SFs.



**Figure 3.11 miR-125a family expression in CD14+ cells cultured in RA SF**

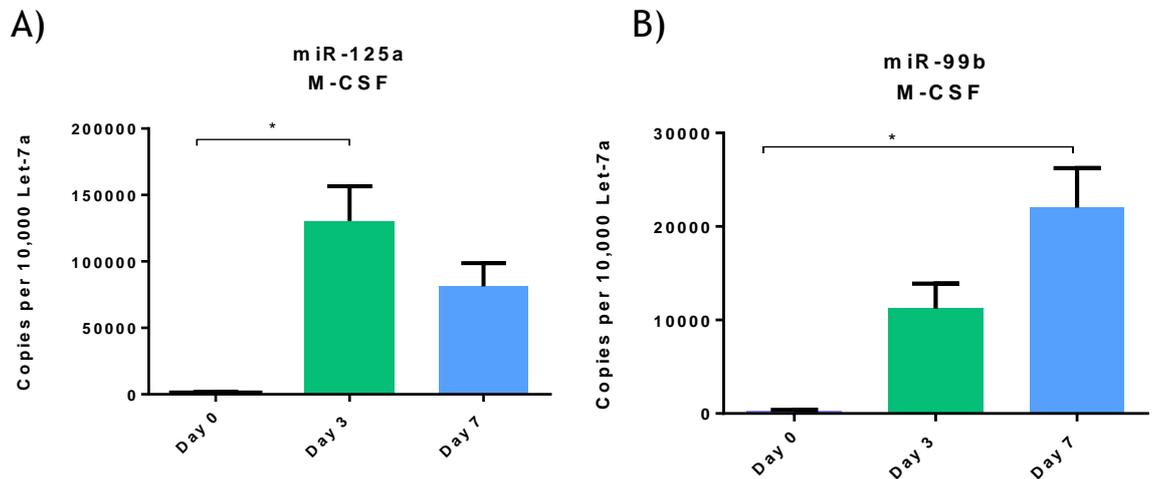
Culture of cells in 10% RA synovial fluid has the potential to upregulate both A) miR-125a and B) miR-99b C) Let-7e expression appears variable in culture with 10% synovial fluid. Values are represented as mean  $\pm$  SEM of 3 healthy donors cultured in 5 different RA synovial fluids

Although part of the same cluster, Let-7e does not appear to be regulated in the same way as miR-125a and miR-99b. Its expression is not altered between PB and SF CD14+ cells, it does not appear inducible by activation of TLRs in monocytes, by culture in SF or differentiation into macrophages. For these reasons I decided to focus on the other members of the miR-125a cluster which are inducible by inflammation in the monocyte/macrophage lineage, and are overexpressed in RA macrophages. These miR may prove more relevant in regulating of these cells in the pathogenesis of RA.

### 3.2.6 miR-125a and miR-99b are up-regulated in response to M-CSF

It has been reported that the miR profile of monocytes changes broadly upon differentiation to macrophages, reflecting the different biological functions of these two cell types. [368]. Macrophage differentiation factor M-CSF is abundant in RA SF thus I decided to test whether M-CSF maturation signals affect expression of miR-125a and miR-99b [384, 389]. Healthy CD14+ cells were separated, as previously described, and differentiated into macrophages by culture for 7 days in M-CSF with the media replaced on day 3. RNA was extracted from cells and miR expression was measured by Taqman RT-PCR. Copies of miR-125a significantly increased by day 3 in culture with M-CSF and although the copy number was lower on day 7 it was still higher than in resting monocytes (Figure 3.12 A). Copies of miR-99b also increase by day 3 in culture with M-CSF, with this increase becoming significant by day 7, but expression of

miR-99b appears up to 10 times lower than that of miR-125a on day 3 (Figure 3.12 B). These data suggest that both miR-99b and miR-125a may play a role in macrophage biology. Particularly high copy number of miR-125a points to potential dominant role of this miR in the regulation of the activation of macrophages.



**Figure 3.12 miR-125a and miR-99b expression in M-CSF macrophages**

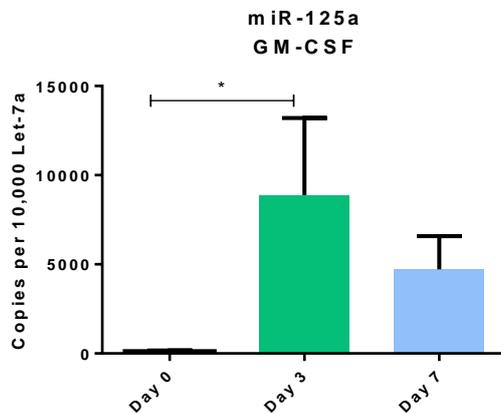
CD14<sup>+</sup> cells were differentiated towards a macrophage phenotype over 7 days with M-CSF and miR expression was measured using Taqman PCR. A) miR-125a expression significantly increases by day 3 in culture and remains highly expressed at day 7 B) miR-99b expression significantly increases by day 3 and continue to rise by day 7. Values are represented as copies per 10,000 Let-7a of 5 healthy donors as mean  $\pm$  SEM and marked bars are statistically different from other groups using a Friedman multiple comparison test. \* $p < 0.05$ .

miR-99b is expressed at low levels in resting and activated monocytes but increases upon macrophage differentiation indicating that it may be involved in activation of these cells. Previous studies carried out in HSPCs which examine this cluster of miR have also demonstrated higher expression of miR-125a compared to miR-99b expression, suggesting miR-125a plays a dominant function within this cluster of miR. Published literature on the function of miR-99b alone is scarce, and studies appear to concentrate on descriptive, expression level investigation. Concurrent with our data set, which shows significantly increased levels of miR-99b in macrophages, validated targets of miR-99b such as mTOR and TRAF2 are important in macrophage biology [390, 391]. However, due to the higher expression levels of miR-125a, and it's interesting expression pattern in RA PB CD14<sup>+</sup> cells, we decided to concentrate the rest of our investigations on

this miR, given the time scale of the project. However further investigations will need to be undertaken to examine the role of miR-99b within these cells.

As cells can be exposed to both M-CSF and GM-CSF within the synovium, with potentially important effects on subset generation, or at least some degree of functional polarisation, next we wanted to examine the expression levels of miR-125a after differentiation with GM-CSF [389]. As described previously, CD14+ cells were separated from human buffy coat packs and differentiated towards a macrophage phenotype using the growth factor GM-CSF, with media changed on day 3. miR-125a expression was measured by q-PCR on day 0, day 3 and day 7.

### 3.2.6.1 miR-125a is up-regulated in response to GM-CSF



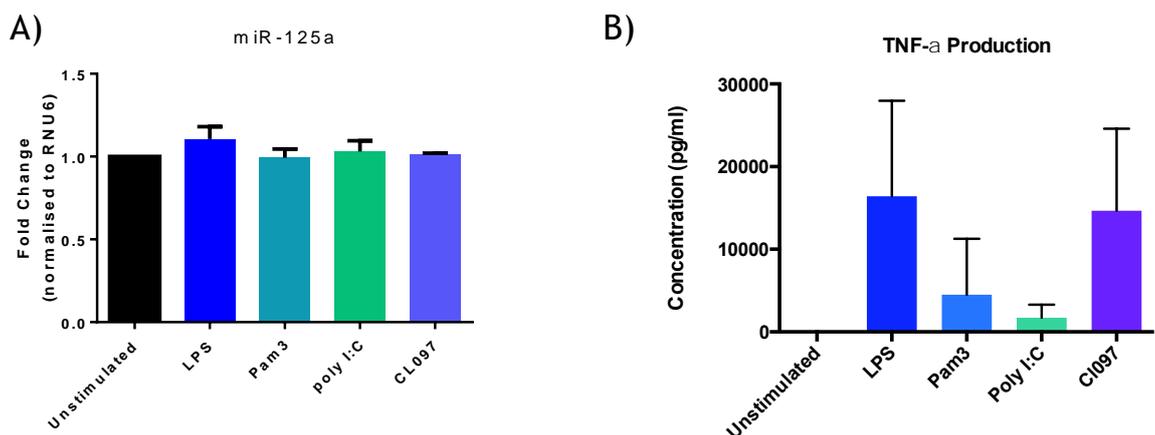
#### Figure 3.13 miR-125a expression in GM-CSF derived macrophages

CD14+ cells were cultured for the indicated time-points with the growth factor GM-CSF. Expression of miR-125a was significantly increased by day 3 in culture. Values are represented as copies per 10,000 Let-7a of 4 healthy donors as mean  $\pm$  SEM and marked bars are statistically different from other groups using a Friedman multiple comparison test. \* $p < 0.05$ .

Results demonstrated that as with M-CSF, miR-125a expression is significantly increased by day 3 in culture with GM-CSF. Again, the level of miR-125a decreases by day 7 but is still over 5 times higher than levels in monocytes. Also it is clear from miR-125a copy number that expression of miR-125a is up to 10 times higher in M-CSF derived macrophages than GM-CSF derived macrophages (Figure 3.13) suggesting that the biology of this miR may have discrete impact in different macrophage lineages.

### 3.2.7 miR-125a expression is not altered in response to TLR activation in macrophages

As previously shown, miR-125a is induced by LPS stimulation in monocytes (Figure 3.10 A) and differentiation towards a macrophage phenotype with both M-CSF and GM-CSF (Figures 3.12 A and 3.13). As miR-125a is expressed at its highest levels in M-CSF driven macrophages, indicating an important functional role in these cells, I wanted to determine if expression can be further regulated in response to TLR stimulation. CD14<sup>+</sup> cells were separated, as described previously from healthy buffy coat packs, and cultured for 6 days in M-CSF. On day 6 various TLR ligands were added and cells were stimulated for an additional 24 hours. For this analysis I decided to determine the fold change in miR-125a expression compared to cells alone. I considered relative expression sufficient as I had already demonstrated the high basal copy number of miR-125a in these cells (Figure 3.12 A). Results showed that TLR stimulation did not affect miR-125a expression in these cells (Figure 3.14 A), suggesting a different functional role in macrophages, compared to monocytes. Activation of cells was verified by measuring the amount of TNF $\alpha$  produced by the cells by ELISA. It appears the stimulation was effective as all stimulated samples produced TNF $\alpha$  (Figure 3.14 B).



**Figure 3.14 miR-125a expression in TLR stimulated macrophages**

CD14<sup>+</sup> cells were separated from healthy blood and differentiated for 7 days in M-CSF. After which cells were stimulated for 24 hours with varying TLR ligands, and miR-125a expression was measured using RT-PCR. A) miR-125a expression in macrophages is not affected by TLR stimulation. B) Cells were activated as demonstrated by TNF $\alpha$  production. Unstimulated is normalised to 1 with other conditions being expressed as fold change compared to unstimulated. Values are presented as mean  $\pm$  SEM of 3 donors. Values are not significantly different using a Repeated measures ANOVA.

### 3.2.8 miR-125b is regulated in an alternative manner to miR-125a in monocytes and macrophages

miR-125b is a homologue of miR-125a, and although it is expressed on a different chromosome, it contains an identical seed region (Figure 3.15). This identical seed region suggests that these two miRs have the potential to regulate the same genes. Reports in the literature have demonstrated that their co-expression differs depending on cell type and environmental stimuli. However, as miR-125b is present in RA SF CD14+ cells, it was important that we validate its expression pattern in monocytes and macrophages to ensure we attribute functional effects to the correct miR.



**Figure 3.15 The mature miR-125a and miR-125b sequences**

miR-125a and miR-125b have identical seed regions shown in blue, suggesting they may have identical targets. The only nucleotide difference between these miRs are indicated in red.

#### 3.2.8.1 miR-125a RT-PCR assay is specific for miR-125a and does not detect miR-125b

As both miR-125a and miR-125b have an identical seed region, and the whole sequence differs in only one nucleotide, it is important that reagents employed in my studies were specific for miR-125a. To analyse miR-125a expression I performed q-PCR using two different systems, Taqman, and SYBR green technology. Taqman q-PCR was useful for samples from which the RNA yield was low as it required minimal RNA amounts (1-10 ng per sample). According to the manufacturers, this technology is also highly specific and can distinguish between highly homologous sequences, with only a single nucleotide mismatch. However as this technology is expensive, I also used SYBR green technology. Therefore, I wanted to ensure that miR-125a PCR primers would not detect miR-125b within samples and vice versa.

I designed an experiment to test the specificity of the miR-125a SYBR green primers. miR-125a and miR-125b mimics were denatured and a standard curve

of known amounts of microRNA was generated, and replicated to a cDNA template (as with PCR standards described in materials and methods). A q-PCR reaction was then performed using this standard curve with miR-125a SYBR green primers. This was to determine if miR-125a primers could amplify miR-125b cDNA within a sample. Table 3.6 shows that miR-125a primers can amplify cDNA from a miR-125a standard from around  $10^5$  copies and above. However, these primers cannot detect miR-125b expression at the same level, for example, instead of detecting  $10^9$  copies at cycle 13, it is detected at cycle 30. The miR-125a assay cannot detect miR-125b at less than  $10^7$  copies. This demonstrates that miR-125a SYBR primers can be used to confidently detect miR-125a expression within macrophages without detecting miR-125b.

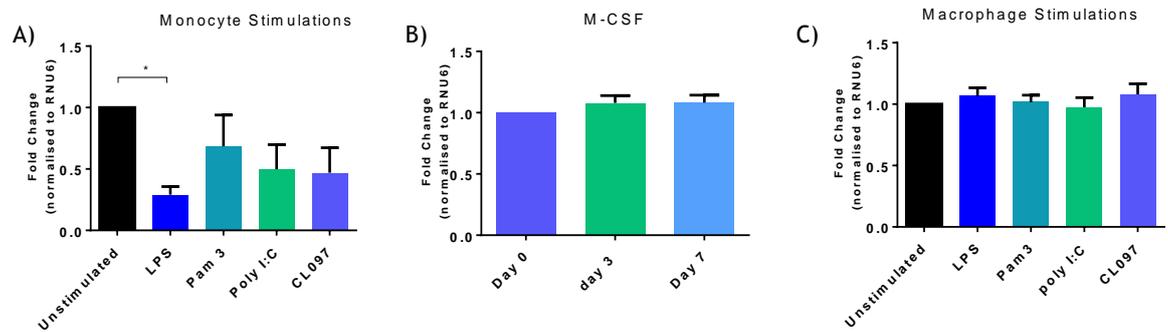
**Table 3.6 miR-125a SYBR green assay specificity**

miR-125a RT-PCR Primers		
Copy Number of miR standard	miR-125a cDNA (Avg Ct Value)	miR-125b cDNA (Avg Ct Value)
$10^9$	13	30
$10^8$	19	34
$10^7$	25	36
$10^6$	29	Undetermined
$10^5$	32	Undetermined

Standards of miR-125a and miR-125b were generated. A q-PCR assay was then performed with miR-125a SYBR green primers to determine if these primers would amplify miR-125b cDNA from the generated standard. This assay amplifies miR-125a cDNA from  $10^5$  copies and above, whereas it does not detect miR-125b expression at less than  $10^7$  copies, demonstrating the specificity of the primers for only miR-125a.

### **3.2.8.2 miR-125b is regulated in an alternative manner to miR-125a in monocytes and macrophages**

Next, I wanted to investigate whether miR-125b is regulated in the same way as miR-125a in human monocytes. As before, healthy CD14+ cells were separated and cultured for 24 hours in various TLR ligands, after which miR-125b expression was measured by RT-PCR. Results showed that miR-125b is downregulated in monocytes after stimulation with LPS, Poly I:C and CLO97, although this difference reached significance only after LPS stimulation (Figure 3.17 A). Additionally, unlike miR-125a, miR-125b expression also does not appear to be inducible by differentiation from monocyte to macrophage with M-CSF, or by stimulation with TLR ligands in these differentiated cells.



**Figure 3.16 miR-125b expression in monocytes and macrophages**

A) miR-125b expression is significantly downregulated by LPS stimulation in human monocytes. B) miR-125a expression is not increased during M-CSF driven differentiation C) miR-125b increases across GM-CSF differentiation D) miR-125b expression is not altered by TLR activation in human macrophages. Values are represented as mean  $\pm$  SEM of A) 3 and B and C) 4 healthy donors and marked bars are statistically different from other groups using repeated measures one-way ANOVA and Tukeys multiple comparison test. \* $p < 0.05$ .

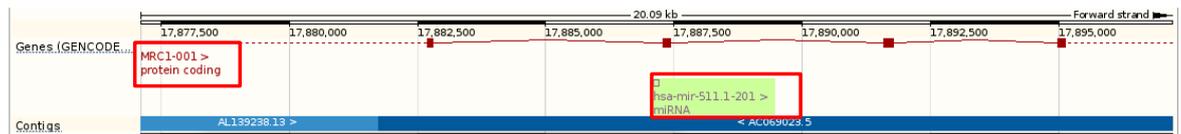
These data demonstrate that miR-125a and miR-125b are regulated differently in monocyte and macrophages suggesting a different function for these miR within these cell types. Levels of miR-125b were not measured using copy number and instead a fold change calculation was demonstrated. Therefore we cannot directly compare expression levels of miR-125a and miR-125b within these cells.

### 3.3 Discussion

I am interested in the understanding of the regulation of RA macrophage activation. My laboratory showed that RA synovial monocytes potentially possess a unique microRNA profile that could in turn shed light on the pathogenic mechanisms operating therein. The aim of this chapter was to identify and validate miRs that were dysregulated in synovial CD14<sup>+</sup> cells when compared to PB CD14<sup>+</sup> cells. As these samples were matched, the expression profile identified alterations in miR expression likely caused by exposure to the synovial microenvironment.

Firstly, we identified miR-511 as being highly upregulated in SF CD14<sup>+</sup> cells. However, miR-511 was expressed at low copy number in both healthy monocytes and M-CSF differentiated macrophages, and did not appear inducible by inflammatory stimuli. These data contradict a previous study by Tserel et al, which suggests that miR-511 is highly upregulated in response to TLR4 stimulation in macrophages [392]. However, in this study, CD14<sup>+</sup> cells were differentiated towards a macrophage phenotype using the GM-CSF growth factor as opposed to M-CSF. Additionally, the concentration of LPS used for stimulation, 1µg/ml, was 10 times higher than the 100ng/ml used in present experiments. These differences in experimental set-up, may account for the differences witnessed between results.

We identified miR-511 as an intronic miR, encoded within the mannose receptor gene (Figure 3.18). Intronic miRs can be transcribed either from their host gene, or by an independent promoter. A study by Squadrito et al, demonstrates that miR-511 is transcriptionally co-regulated along with the CD206 gene. Up-regulation of CD206 will therefore be accompanied by an increase in the expression of miR-511 and vice versa. This study also suggested that overexpression of miR-511, does not alter the pro-inflammatory response of macrophages, and suggests that the activity of miR-511 is specific to TAMs [385].



**Figure 3.17 miR-511 location within the genome**

A screenshot from the ensembl database demonstrates that miR-511 is an intronic miR encoded within the CD206 gene

This tightly controlled expression pattern may explain the low copy number of miR-511 witnessed in both resting and activated macrophages in the present study. As TLR 4 activation is also a method of inducing M1 macrophages, which do not express CD206, this could also account for low levels of miR-511. Thus, although we observed a substantial increase in fold change expression of miR-511 in RA CD14+ cells, this was associated with low absolute copy number in both monocytes and macrophages when I explored expression *ex vivo*. Therefore, we decided to no longer investigate miR-511 in the context of pro-inflammatory RA macrophage activation. However, it may prove important in the future to determine the consequence of the high level of upregulation of this miR. It is possible that this miR is highly upregulated in CD206+ macrophages within the synovium, which function to repair damage. A novel diagnosis technique for RA has recently been proposed by the company Navidea, in which a labelled CD206 compound (manocept) can identify a population of CD206 macrophages in early RA affected joints, which are not present in unaffected joints (source: Navidea website). Therefore this population of macrophages may prove important in future studies and expression of miR-511 should be explored, particularly in early RA biopsies.

Next, I turned my attention to the miR-125a cluster, of which 2 members, miR-125a and miR-99b are significantly increased within SF CD14+ cells. miR-125a, miR-99b and Let-7e form a miR cluster located on human chromosome 19. The close proximity of these miRs suggests that there is a single promoter for this cluster, and that all members should be expressed under the same conditions. However, from my own, and other studies, it does not appear that these miRs are regulated in the same manner [393, 394]. Only miR-125a and miR-99b are significantly increased in RA SF CD14+ cells and expression of miR-99b appears to mirror that of miR-125a, albeit at lower levels. Both miR-125a and 99b are

inducible by LPS, culture in RA derived SF, and expressed at high levels in macrophages, although miR-125a is up to 10 times higher when the two are compared. Considerably higher levels of miR-125a in SF CD14<sup>+</sup> cells, and human macrophages suggest that during its biogenesis, it undergoes alternative methods of regulation that allow it to accumulate at higher levels than other members of the cluster, such as higher transcript stability and reduced decay. We can therefore hypothesise that miR-125a can be independently, or preferentially expressed under certain conditions and in certain cell types, including macrophages. Moreover, we can hypothesise from expression values that miR-125a is the dominant miR in this cluster, and previous studies have also suggested that miR-125a is solely responsible for the function of this cluster, at least with respect to stem cell differentiation. Overexpression of miR-125a alone in these cells is enough to cause haematopoietic expansion [395].

We therefore decided to concentrate continued investigation on miR-125a. My first key observation is that miR-125a is expressed in, and upregulated in PB CD14<sup>+</sup> cells of RA patients who respond well to cDMARD therapy. Expression of miR-125a in these cells negatively correlates with RA disease activity scores, DAS28 ESR, SDAI and CDAI, along with the number of tender and swollen joints, hinting at protective role for this miR in these cells. Additionally miR-125a cellular expression positively correlates with serum levels of anti-inflammatory IL-10, again suggesting a positive role for this miR in the circulation, in terms of primed circulating monocytes, and potentially thereafter at sites of inflammation. These data suggest that detection of miR-125a in PB CD14<sup>+</sup> cells could be used to identify response to DMARD therapy, in line with a recent study by Murata et al that has also suggested that serum levels of miR-125a may be used as a biomarker for diagnosis of RA [396]. Therefore it may be important to examine miR-125a expression in PB cells of other diseases with an inflammatory component, such as OA and SLE, where in the latter, downregulation of miR-125a has already been detected in CD4<sup>+</sup> T cells [343]

An increase in miR-125a expression in LPS stimulated monocytes, and healthy cells cultured in SF suggests that exposure to inflammatory stimuli may induce miR-125a expression. It has been previously shown, in a study by Bazzoni et al, that miR-125a is LPS inducible in monocytes [301]. Additionally, a study by Monk

et al demonstrated up-regulation of miR-125a-3p, after exposure to LPS in murine BMDMs, demonstrating the difference between human and murine myeloid cells [365]. Personal communication from the Loctati group suggests that miR-125a-3p is indeed the active strand in murine macrophages, whilst 5p is the active strand in human cells. To further elucidate the role played by SF in increasing miR-125a expression, it would be important in the future to increase the amount of donors in this experiment. Additionally the components of the SF should be phenotyped for example through Luminex, to identify factors that may cause upregulation. We have shown that miR-125a is upregulated in response to both LPS, and the growth factors M-CSF and GM-CSF, which are present in SF. However we cannot say for certain that these factors are present in our cultures without phenotyping the SF. In addition, miR-125a expression could also be investigated after culture in other SF, such as from OA patients to determine if this is a RA specific association.

Extremely high copy number of miR-125a in M-CSF macrophages suggests that this miR may play an important role within these cells. Expression of miR-125a is lower in GM-CSF derived macrophages, in line with a study by Banerjee et al which also showed higher levels of miR-125a in M-CSF v GM-CSF derived macrophages [339]. Unlike monocytes, miR-125a is not further inducible in macrophages stimulated with LPS, suggesting an alternative role for this miR between these cells, reflecting the differing functions of monocytes and macrophages.

miR-125b, a homologue of miR-125a is expressed as 2 identical forms on chromosome 11 and chromosome 21, in clusters with Let-7 and miR99 family members. This miR is also expressed in RA PB and SF CD14+ cells, where its expression is increased, but not significantly. Both miR-125a and miR-125b have identical seed regions, and therefore can potentially target the same mRNAs. However, it has been previously suggested that miR-125b is regulated in an alternative way to miR-125a, at least in monocytes. Our results showed that miR-125b, is downregulated by LPS stimulation, and not induced during M-CSF differentiation. Examining the Ct values would suggest that miR-125b is expressed at lower levels than miR-125a in macrophages, and that although miR-125b has been more deeply studied, that miR-125a may play a more important

role within these cells. To say for certain, it would be important in the future to determine copy number of the miR-125 family with SF CD14<sup>+</sup> cells. This would allow comparison between family members, and therefore allow us to speculate of the physiological importance, or dominance of each of these miRs. In addition, it would be important to calculate copy number of miR-125b in both monocytes and macrophages, again to allow a comparison with miR-125a and to demonstrate which miR is more abundant, and potentially more physiologically relevant in these cells.

In summary, we found that miR-125a is strongly upregulated in RA SF monocytes, thus at sites of inflammation and in PB monocytes of patients responding well to therapy (low disease score). Investigating the mechanisms responsible for its up-regulation we identified TLR4 stimulation and M-CSF as potential factors increasing miR-125a expression. The next part of this study will focus on the functional consequence of manipulation of miR-125a expression in macrophages and the pathways which are under the regulation of this miR. Based on the data obtained in this chapter showing that miR-125a in PB CD14<sup>+</sup> cells negatively correlates with SJC, TJC and DAS28, whilst positively with IL-10 gives a hint towards protective function of miR-125a and suggests that its high-expression at site of inflammation could be a part of a counterbalance mechanism. This I will test formally in the following chapters.

## Chapter 4- Investigating miR-125a function

### 4.1 Introduction and aims

The previous chapter has demonstrated the dysregulation of miR-125a in both PB and SF CD14<sup>+</sup> cells of RA patients, along with its upregulation by inflammatory mediators and growth factors. In this chapter, I will focus on the functional consequences of miR-125a dysregulation on *in-vitro* cytokine production by monocytes and macrophages. This will be investigated using a stably expressed miR-125a sponge transgene, and gain and loss of function experiments in human macrophages.

The role of miRs in 'fine tuning' gene expression has led to extensive investigation of dysregulated expression patterns in disease states, including the majority of cancers, cardiovascular and rheumatic diseases [397-399]. Sequence analysis suggests that there are around 2,500 miRs in the human genome, and as each miR has the potential to regulate many mRNAs, specific functions in different cellular and environmental contexts are only beginning to be discovered [400]. Compared to the number of computationally predicted miR targets, relatively few interactions have been experimentally validated. The majority of *in-vitro* studies to date have relied on gain and loss of function experiments, through the transfection of double stranded miR mimics and antisense inhibitors to investigate the role played by miRs in specific cell types. Problems associated with gain of function experiments have been acknowledged, such as the unwanted activation of the interferon response, saturation of the RISC complex, due to supra-physiological concentrations of mimic, and the inhibition of non-physiological targets [401-403]. Under loss of function conditions, effect on target mRNAs is witnessed by inhibition of a physiological concentration of miR. Therefore the endogenous function within a cell at a given time can be investigated. There are 3 generally accepted methods to study loss of miR function; through the generation of gene knockouts, transfection of antisense inhibitors and the use of transgene encoded miRNA sponges. Generating gene knockouts is a difficult, time consuming and costly procedure, which may not result in an obvious phenotype, due to a redundancy in function of closely related miRs, the fact that gene knock outs remove both 5p and 3p miR strands, and a lack of conservation between the species. The

latter seems to be a case for miR-125a as human cells predominantly use miR-125a-5p strain while in mouse antisense miR-125-3p is used. Therefore, due to cost and difficulty, other options of inhibition are usually undertaken first. Transfection of cells with miR inhibitors, although transient, can be informative. However, transfection reagents are expensive and their use is complicated by toxicity unless concentration and time-course conditions are optimised. In addition, certain cells can be difficult to transfect, limiting cell types which can be investigated. Currently, creation of miR sponges, expressed from stably integrated transgenes appears to be the most effective method to study loss of specific miRs within cells. miR sponges are transcripts expressed under the control of a strong promoter which contain tandem binding sites complementary to the target miRNA sequence. These transcripts effectively sequester miRs, preventing miR binding of target genes, and creating an environment effectively null for the sequestered miRNAs [404]. miR sponges can be stably inserted into the genome of cell lines to create ongoing miR inhibition, allowing long term experiments to be performed.

One of the most common applications of stably expressed sponge transgenes is to use these cells to mimic the down regulation, or reverse the up-regulation of miRs observed in various disease states. An example of this is a study by Valastyan et al, which identified miR-31 as an important regulator of breast cancer metastasis. The authors identified the downregulation of miR-31 in aggressive metastasising tumours, and so generated a stably expressing miR-31 sponge breast cancer cell line, to mimic this environment. These miR-31 null cells were injected into mice and formed macroscopic metastases in the lungs, indicating that miR-31 plays a role in controlling breast cancer metastasis [405].

Macrophages are key players in the pathogenesis of RA through the production of pro-inflammatory cytokines such as TNF $\alpha$  and IL-6, and chemokines, such as CCL2, CCL3 and CCL4 within the inflamed synovium. It is thought that they can be activated by pro-inflammatory mediators released by damaged joint tissue, and dead cells within the joint, resulting in the activation of TLRs and other receptors. Therefore, pathways which control the activation of these cells are tightly regulated during normal homeostasis, to prevent an overwhelming inflammatory response. During RA this normal immune homeostasis is disturbed,

and the reason for this altered immune response are the subject of many investigations. It is well documented that miRs play a role in the response to infection and can regulate the production of pro-inflammatory cytokines and chemokines by macrophages. For example, LPS induced upregulation of miR-132 can cause the downregulation of TNF $\alpha$  production through targeting the signalling molecule TRAF4 [320, 406].

We therefore hypothesised that the dysregulation of miR-125a seen in RA CD14+ cells would alter their function, resulting in a difference in cytokine and chemokine production. We therefore wanted to determine the role of miR-125a in monocytes and macrophages in resting state, and in response to activation by a TLR4 ligand.

### Chapter Objectives

- Design, generate, and validate a miR-125a sponge vector which can be stably inserted into the genome of THP-1 cells
- Determine the effect of a miR-125a null environment on cytokine and chemokine production in response to LPS by THP-1 derived macrophages
- Determine the effect of gain and loss of miR-125a on cytokine and chemokine production in response to LPS by human primary macrophages

## **4.2 Results**

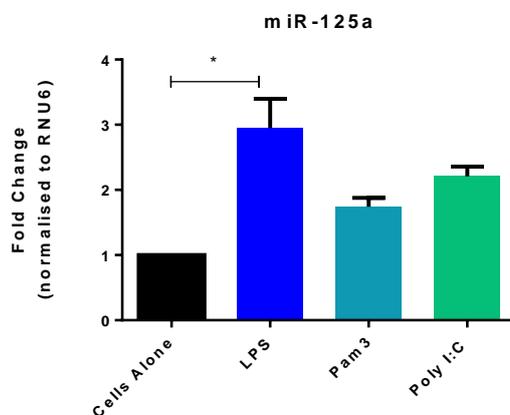
### **4.2.1 Investigating miR-125a function using a miR Sponge**

miR sponges are important tools that can be designed and generated to investigate the long term inhibition of miRs, within specific cell types. We designed a miR-125a sponge to investigate the role played by this miR in response to TLR4 activation, in both monocytes and macrophages. The sponge vector was designed, generated and stably transfected into THP-1 cells, that could then be used to investigate the response of macrophages to inflammation.

#### 4.2.1.1 miR-125a regulation in THP-1 cells

We decided to use the monocytic THP-1 cell line in which to stably express the miR-125a sponge vector. THP-1 cells are monocytic in origin, and importantly retain the ability to differentiate into cells with a robust macrophage-like phenotype, and as such are a well-characterised cell line commonly used as a surrogate to investigate the function of human monocyte and macrophages in-vitro.

Firstly, to ensure THP-1 cells were an appropriate choice for miR-125a sponge expression, we determined the expression and regulation of miR-125a within these cells. We wanted to examine the expression of miR-125a in response to TLR activation, and determine if regulation in THP-1 cells mirrored that of primary human monocytes. Cells were plated and stimulated with LPS (100ng/ml), Pam3 (300ng/ml) and Poly I:C (50µg/ml) for 24 hours. Afterwards expression of miR-125a was measured by q-PCR (Figure 4.1).



**Figure 4.1 miR-125a expression in stimulated THP-1 Cells**

THP-1 cells were stimulated with 100 ng/ml LPS, 300 ng/ml Pam3 and 50 µg/ml Poly I:C for 24 hours and miR-125a expression was measured by SYBR green q-PCR. miR-125a expression is significantly increased by stimulation with LPS, but not Pam3 or Poly I:C. Cells alone (Unstimulated) was normalised to 1, and all other conditions are expressed as a fold change compared to it. Values presented as mean  $\pm$  SEM of 3 separate experiments and marked bars are statistically different from cells alone using a paired T-test  $*=p < 0.05$ .

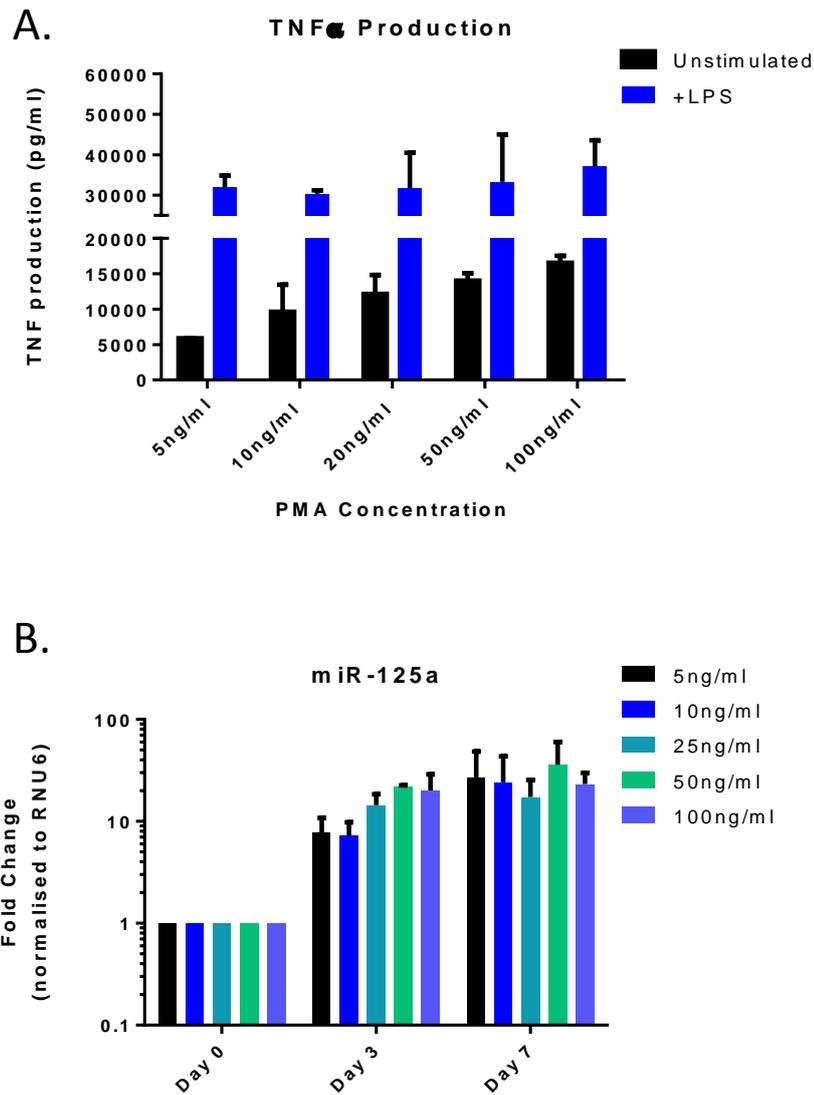
We observed that miR-125a expression in THP-1 cells mirrors that of primary human monocytes (Figure 3.10). Basal expression is low, as determined by the

PCR Ct value. Expression is significantly induced by LPS, but not Pam3 or Poly I:C stimulation (Figure 4.1).

Next, we wanted to determine if miR-125a expression was induced in THP-1 cells by differentiation towards a 'macrophage' phenotype with phorbol myristate acetate (PMA). PMA is a well-established differentiation agent, however the optimum concentration of PMA required to differentiate THP-1 cells has been the source of debate. High concentrations of PMA induce cell activation, resulting in the production of TNF $\alpha$  and other inflammatory cytokines. Therefore, we sought to optimise the concentration of PMA required to induce differentiation, and expression of miR-125a, while keeping TNF $\alpha$  production low. We were guided by a study by Park et al, which suggested 5 ng/ml of PMA was sufficient to induce differentiation of THP-1 cells towards an adherent phenotype, without causing undesirable gene up-regulation [407]. To test this experimentally, THP-1 cells were differentiated for 7 days with various concentrations of PMA ranging from 5 ng/ml-100 ng/ml. Cells were then left unstimulated, or stimulated with 10 ng/ml of LPS. TNF $\alpha$  expression was measured in these cells using an ELISA assay, and miR-125a expression was measured in resting cells by q-PCR. In resting cells, differentiation with 5 ng/ml PMA was enough to induce adherence and differentiation, while keeping low amounts of TNF $\alpha$  production. Cells differentiated under all concentrations of PMA are capable of responding to LPS by producing an increase amount of TNF $\alpha$  (Figure 4.2A).

Levels of TNF $\alpha$  were not measured in a control which had not been treated with PMA as I wanted to compare TNF $\alpha$  production of cells which had been cultured for 7 days in PMA.

To evaluate whether PMA induced THP-1 to macrophage differentiation mirrors M-CSF driven primary monocyte to macrophage differentiation in terms of miR-125a expression, we looked at miR-125a expression at day 0 (monocyte), day 3 and 7 upon culture with PMA. Expression of miR-125a increases around 10 fold by day 3 and is further increased by day 7, with levels of miR-125a being similar across different PMA concentration (Figure 4.2B).



**Figure 4.2 TNF $\alpha$  and miR-125a expression in PMA differentiated THP-1 cells.**

THP-1 cells were differentiated using differing concentrations of PMA. TNF $\alpha$  expression in these cells was measured by ELISA and miR-125a expression was measured by q-PCR. A) Differentiation with 5 ng/ml induces the lowest amount of TNF $\alpha$  production from resting cells. Despite this, these cells produce similar amounts of TNF $\alpha$  when stimulated with 10 ng/ml of LPS. B) miR-125a expression is increased by culture with PMA across a 7 day time period. The up-regulation of miR-125a is similar under all concentrations of PMA. Values presented as mean  $\pm$  SEM of 2 separate experiments.

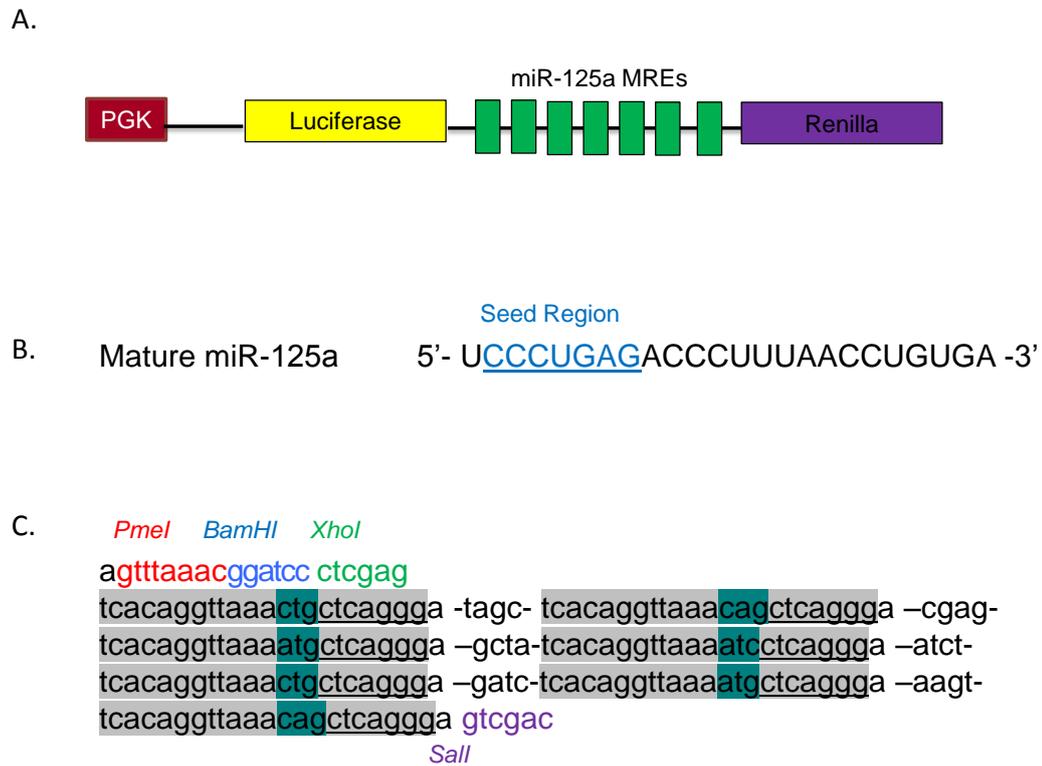
We concluded from these data that THP-1 cells are suitable to study the function of miR-125a in macrophages, as its expression in these cells appears to mirror that of primary human monocytes and macrophages. In addition, we have concluded that 5 ng/ml PMA is sufficient to differentiate THP-1 towards macrophage-like phenotype cells without activating them. Therefore cells will be cultured at this concentration in future experiments.

#### 4.2.1.2 Generation of miR-125 sponge

The miR-125a sponge was constructed by inserting tandem miR-125a binding sites downstream of a luciferase reporter gene driven by a phosphoglycerate kinase (PGK) promoter (Figure 4.3A). The vector used for these experiments was a modified version of the pmirGLO vector used for luciferase reporter assays as described in section 2.5 of Materials and Methods.

I was aided in the sponge design by Dr Derek Gilchrist of the GBRC. The miR-125a sponge is a reverse complementary nucleotide sequence to the full length mature miR-125a sequence. To design the sponge, firstly, the sense strand of the mature miR was taken and the seed region identified (Figure 4.3B). A nucleotide sequence perfectly complementary to this region was then designed and inserted 7 times to introduce 7 tandem miR-125a binding sites. This sequence is underlined in Figure 4.3C. Three random nucleotides were inserted immediately downstream of the seed region. This “bulged” binding site has been proven to increase the efficacy of miR sponge sequences. It has been demonstrated that perfectly complementary miR target sequences can trigger mRNA degradation, mediated by the RISC complex. Gentner et al have shown that “bulged” binding sites within a miR sponge design are more effective and can mimic natural miR regulation of targets via the sequestration rather than degradation of sponge transcripts, preventing miR recycling [408]. These regions are highlighted in blue in Figure 4.3C. Next, random spacer regions were designed to link the tandem repeats. These spacers allowed the binding of the maximum amount of RISC complexes to the miR sponge.

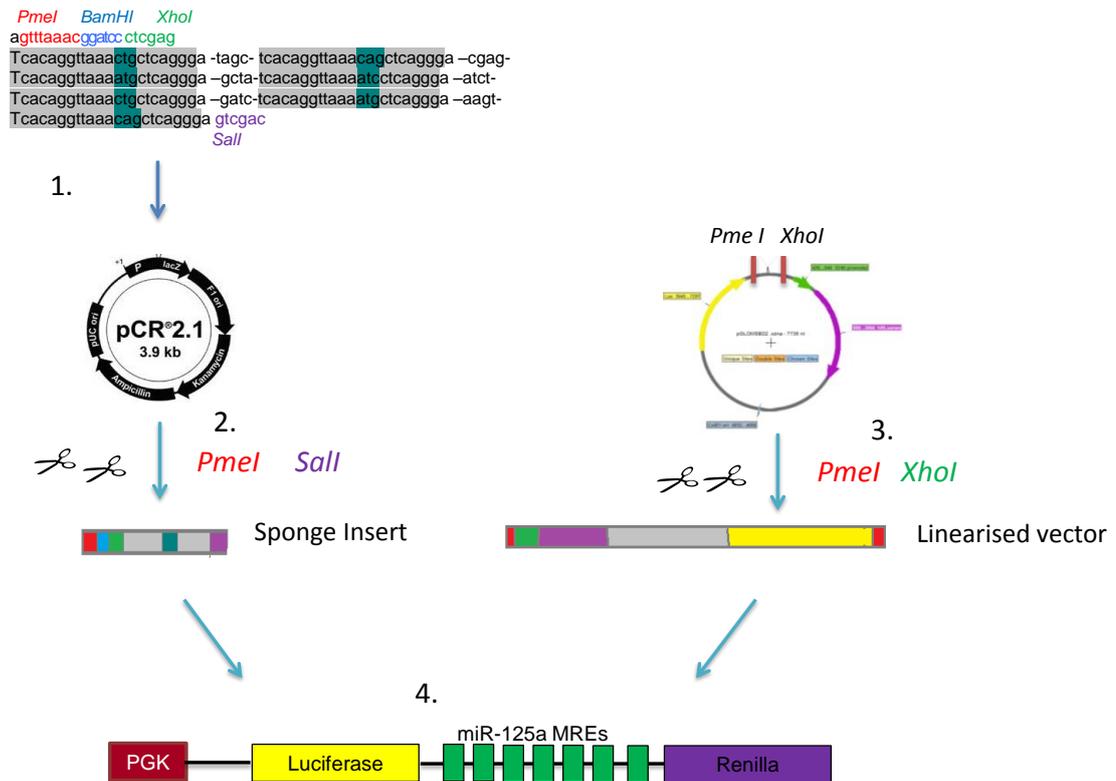
To allow directional cloning, restriction sites were added at the 5' and 3' ends of the sequence (Figure 4.3C).



#### Figure 4.3 Schematic and sequence of miR-125a Sponge

A) Basic schematic showing the location of the 7 miR-125a binding sites downstream of a luciferase gene driven by a PGK promoter. B) Mature miR-125a sequence with the seed region underlined and highlighted. C) Sequence of miR-125a sponge insert showing regions complementary to the mature miR-125a sequence highlighted in grey. The complementary seed region, important for binding miR-125a is underlined. Each seed region is separated from the rest of the sequence by a bulge region, highlighted in blue. This is inserted to ensure binding of maximum RISC complexes. Each section of 7 complementary sequences is separated by linker sequences. The *PmeI*, *BamHI*, *XhoI* and *Sall* restriction sites used for cloning are also indicated.

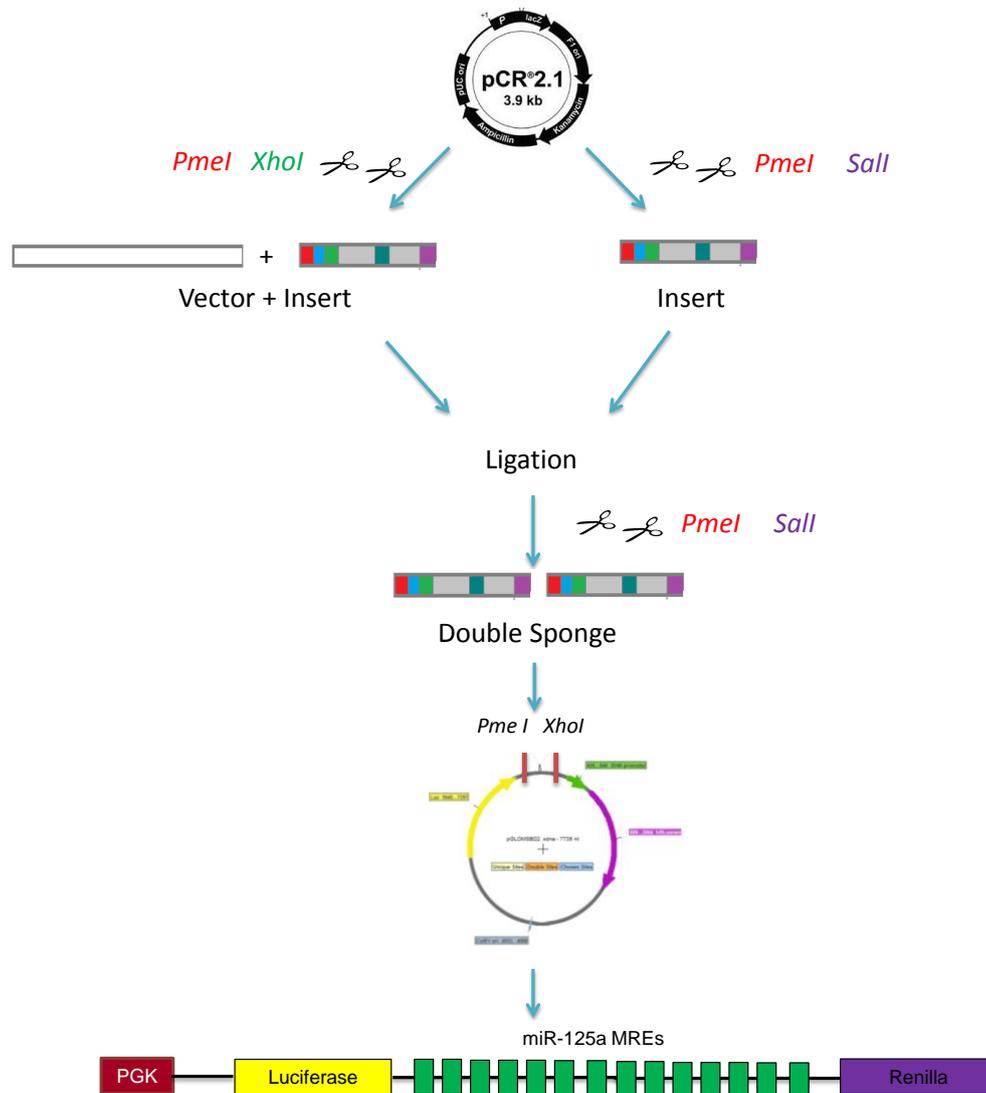
To generate the miR-125a sponge, the DNA sequence described above was ordered and resuspended in water. This sequence was then cloned into the vector pCR2.1-TOPO, creating the vector pCR2.1-miR125a-SpX7. The sponge sequence was then excised from this vector as a *PmeI/Sall* fragment and cloned into the *PmeI/XhoI* sites of pmiRGLO, creating the final vector: pmiRGLO-miR125a a functional miR-125a sponge vector containing 7 miR-125a MREs (miR-125a-SpX7) (Figure 4.4).



**Figure 4.4 Generation of single miR-125a Sponge**

1. The miR-125a sponge insert was resuspended in water, ligated into the subcloning vector pCR 2.1 and transformed into competent *E. coli*. 2. The miR-125a insert was excised from pCR 2.1-miR125a-SpX7 using *PmeI* and *Sall* restriction enzymes, as these sites had been inserted at either end of the construct. 3. pmirGLO vector was digested with the enzymes *PmeI* and *XhoI*, creating complementary sites for ligation of the miR-125a sponge insert. 4. The miR-125a sponge insert was ligated downstream of the luciferase gene of the pmirGLO vector creating a functional miR-125a sponge vector.

We hypothesised that a sponge transgene containing more miR-125a binding sites would increase the efficiency of inhibition; therefore we generated a sponge vector with 14 miR-125a binding sites. To do this we went back to pCR 2.1-miR125a-SpX7 vector and inserted an additional copy of the miR125a sponge sequence doubling the number of potential miR-125a binding sites. The resulting miR-125a sponge sequences contained 14 miR-125a sites, this was subsequently inserted as a *PmeI/Sall* fragment into the *PmeI/XhoI* sites of pmirGLO, creating pmirGLO-miR-125a-SpX14. (Figure 4.5).



**Figure 4.5 Generation of miR-125a double sponge**

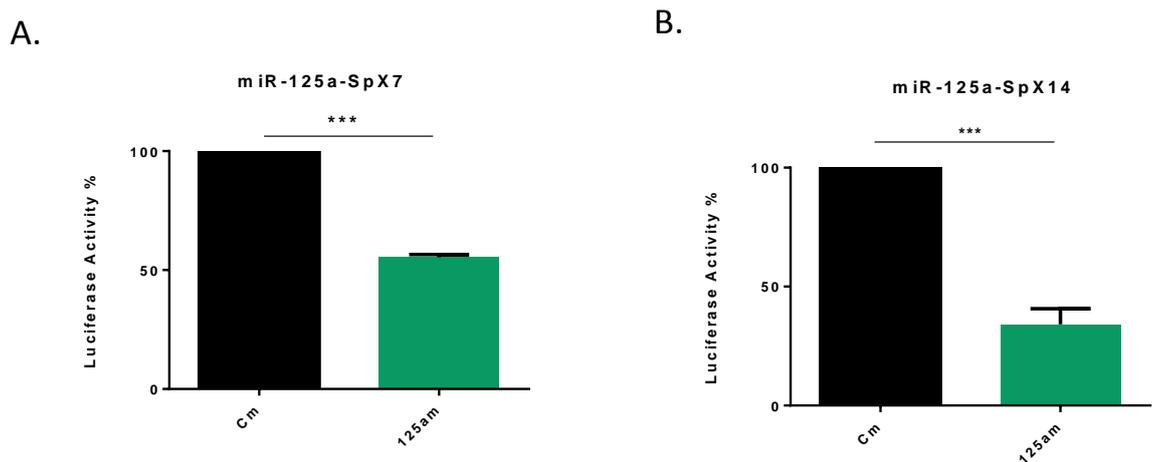
The double miR-125a sponge was generated by linearising the single sponge in PCR 2.1 with *PmeI* and *XhoI* enzymes, while the insert was also cut out using the *PmeI* and *Sall* enzymes. The insert was then re-ligated in the PCR 2.1 vector and grown in competent cells. PCR 2.1 DNA was then cut with *PmeI* and *Sall* enzymes to cut out the double insert which can then be ligated into complementary sites in the pmirGLO vector.

#### 4.2.1.3 Efficacy of miR-125 sponge

After the generation of both sponge vectors we tested their activity to ensure they could bind miR-125a successfully, and also to determine which construct had the most significant effect. We used a luciferase reporter assay. HEK293 cells were co-transfected with either miR-125a-SpX7 or miR-125a-SpX14 vectors, and either a control mimic (Cm) or miR-125a mimic (125am), and cultured for 24 hours. Binding of miR-125a to the complementary repeats within the sponge vector will cause a knockdown of luciferase activity, which can be measured by

a luminometre. Knockdown by the miR-125a mimic is compared to luciferase expression after transfection with the Cm, which will be treated as 100% luciferase activity.

The results showed a significant knockdown of luciferase activity in conditions. However, the miR-125a-SpX714 expressed the highest knockdown of luciferase activity (Figure 4.6).



**Figure 4.6 Test of miR-125a sponge construct in pmirGLO vector**

HEK293 cells were co-transfected with either a Cm or 125am and a miR-125a sponge plasmid (X7 or X14 construct), then cultured for 24 hours. Luciferase activity after transfection was measured and normalised to Renilla expression. A) Transfection of a 125am results in a significant decrease in luciferase activity when compared to a Cm using the single sponge construct B) Transfection of 125am results in a significant decrease in luciferase activity when compared to a Cm using the double sponge construct. Values presented as mean  $\pm$  SEM of 5 separate experiments and marked bars are statistically different from Cm using a paired T-test \*\*\*= $p < 0.0005$

As knockdown of luciferase activity was higher with the miR-125a-SpX14 construct, stable transfection and subsequent assays will be performed with this vector.

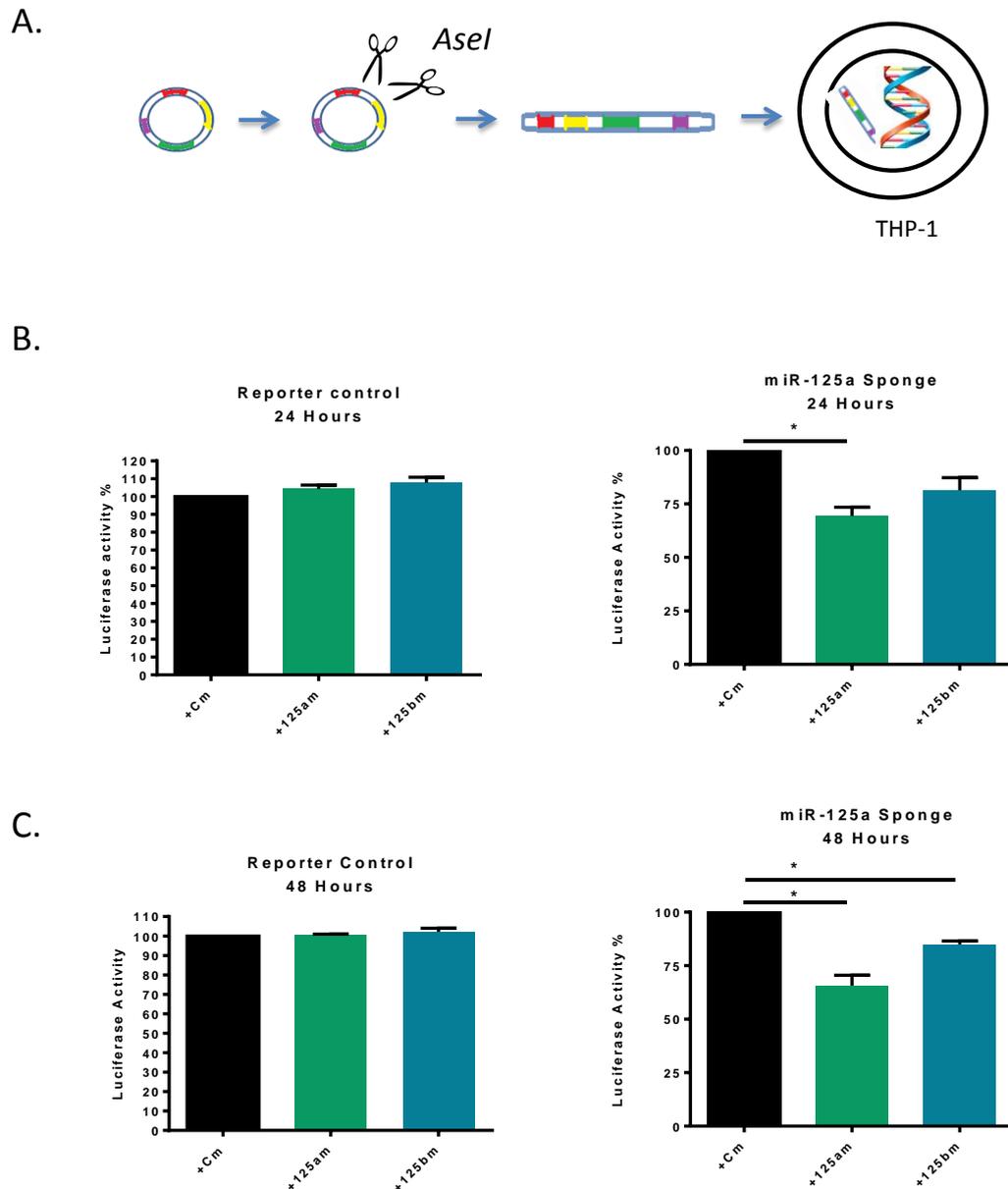
#### 4.2.1.4 Generation of Stable THP-1 Cell line

To enable the investigation of miR-125a in THP-1 derived macrophages, we generated a THP-1 cell line which stably expressed the miR-125a sponge. Plasmid DNA was linearised using *AseI* enzyme; DNA was then precipitated and introduced into THP-1 cells by electroporation (Figure 4.7A). Alongside this another flask of THP-1 cells was transfected with the parental pmirGLO vector the resulting cells were named Reporter Control cells. Cells were selected for

resistance to the antibiotic G418, as expression of the vector confers resistance to this antibiotic.

Stably transfected cells were tested for the transgene expression. Both reporter control and miR-125a sponge expressing cells were plated and transfected, with either a Cm, 125am or a miR-125b mimic (125bm). Cells were then cultured for either 24 or 48 hours, after which luciferase activity was measured. The sponge vector should also bind miR-125b as it has an identical seed region to miR-125a, and so luciferase activity should also be decreased in these samples.

There was luciferase expression in all conditions, which demonstrates the presence of both the reporter control vector and miR-125a sponge vector within these cells. After 24 hours culture, there was no change in luciferase activity in transfected reporter control cells, but there was a significant decrease in luciferase activity in miR-125a sponge cells which had been transfected with a 125am, compared to a Cm (Figure 4.7B). After 48 hours culture, again, there was no change in luciferase activity in transfected reporter control cells, but there was a significant knock down of luciferase activity in miR-125a sponge cells which had been transfected with both a 125am and 125bm, compared to a Cm (Figure 4.7C). The knockdown of luciferase activity in cells expressing the miR-125a sponge, but not the reporter control vector, demonstrates that the miR-125a sponge is expressed and functional in response to miR-125 binding in these cells. As is shown in Figure 4.7C, miR-125a sponge is able to bind miR-125b although to a lesser extent compared to miR-125a. This demonstrates that although both miRs share the same seed region, additional factors also influence miR binding to their target genes.

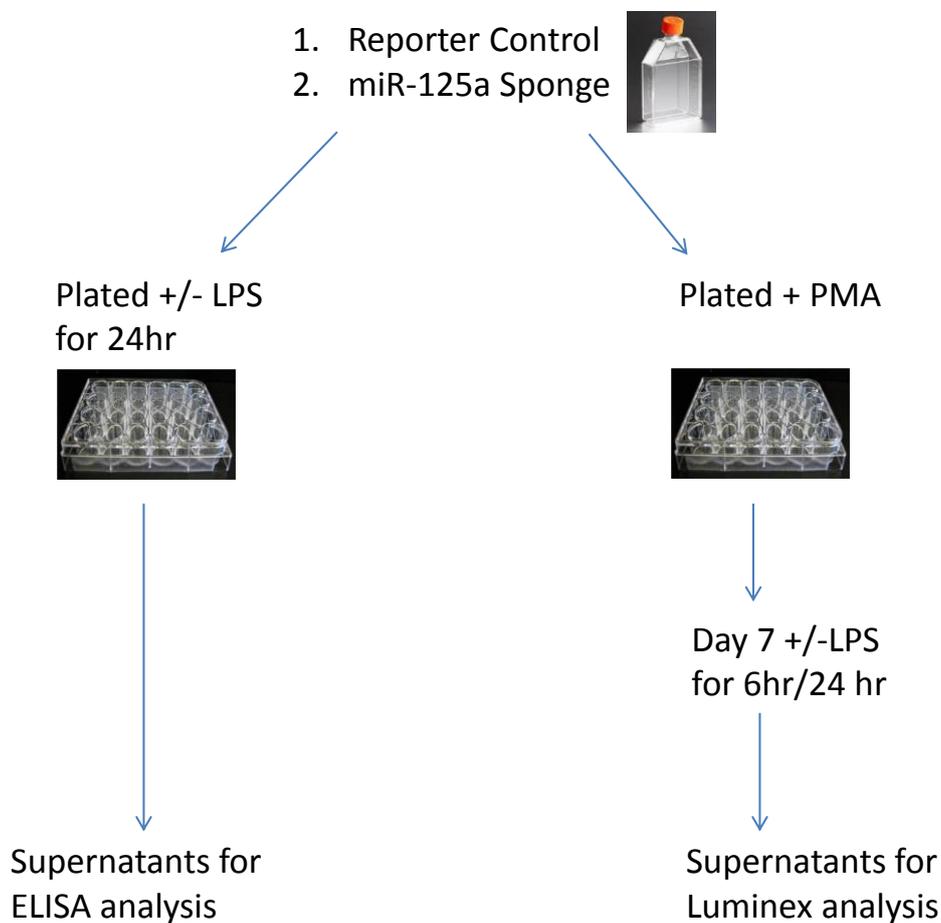


#### Figure 4.7 Generation of functional THP-1 miR-125a stable cells

THP-1 cells which stably expressed functional miR-125a sponge transgenes were generated. A) Circular plasmid DNA was linearised using the restriction enzyme *AseI* and DNA was transfected into THP-1 cells by electroporation. B) Stable THP-1 cells were transfected with either a Cm, miR-125am or a miR-125bm and luciferase activity was measured. After 24 hour transfection a significant decrease in luciferase activity was detected in cell expressing miR-125a sponge transfected with miR-125am compared to Cm. C) . After 48 hour transfection there was a significant decrease in luciferase activity in cells expressing miR-125a sponge transfected with both miR-125a and miR-125b mimics compared to control. Values presented as mean  $\pm$  SEM of 3 separate experiments and marked bars are statistically different from Cm using a paired T-test  $*=p < 0.05$ .

## 4.2.2 Cytokine profile of Stable THP-1 Cells

After generating THP-1 cells which stably expressed the miR-125a sponge vector, we examined the cytokine and chemokine profile of these cells. THP-1 stable cells were plated and either left unstimulated or stimulated with LPS (100ng/ml) for a period of 24 hours and supernatants were stored for cytokine analysis. Next, THP-1 stable cells were differentiated towards macrophages with PMA and either left unstimulated or stimulated after 3 or 7 days with LPS (10ng/ml). This stimulation was for a period of 6 or 24 hours. Supernatants were taken for cytokine and chemokine analysis (Figure 4.8).



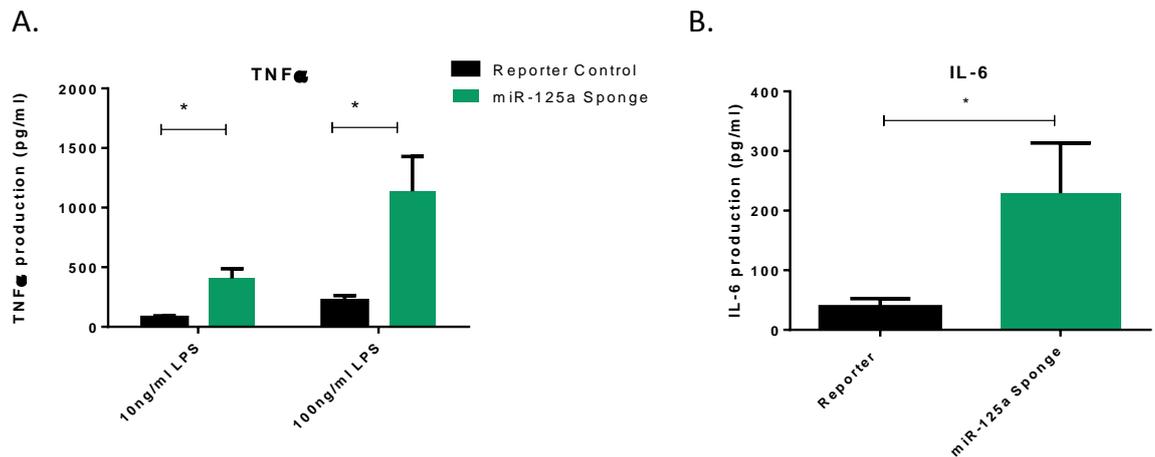
**Figure 4.8 Experimental setup of cytokine and chemokine expression profile of miR-125a sponge expressing THP-1 cells**

First THP-1 cells were left resting or stimulated with LPS for a period of 24 hours, after which supernatants were taken and cytokine expression was measured by ELISA. Next, THP-1 cells were differentiated towards macrophages for 3 and 7 days with PMA, and left resting or stimulated with LPS for a period of 6 or 24 hours, after which cytokine and chemokine expression was measured by Luminex assay.

#### **4.2.2.1 Cytokine profile of THP-1 cells which stably express miR-125a sponge**

We have demonstrated that whilst miR-125a expression is low in resting THP-1 cells, it can be induced by LPS stimulation. LPS induces the production of cytokines from monocytes and we therefore wanted to investigate the effect that loss of miR-125a would have on cytokine production in these cells. We also examined the resting profile of these cells to determine if loss of miR-125a induced the spontaneous production of specific cytokines. Both reporter control and miR-125a sponge cells were stimulated with 10 ng/ml and 100 ng/ml of LPS. After 24 hours, supernatants were taken from these cells, and levels of cytokines classically induced by LPS stimulation, TNF $\alpha$ , IL-6, IL-12 and IL-10, were measured by ELISA.

Results demonstrated that unstimulated cells did not produce detectable levels of cytokines. In addition, LPS stimulated cells did not produce detectable levels of IL-10 or IL-12 at either LPS concentration. However, miR-125a sponge expressing cells produced significantly higher amounts of TNF $\alpha$  after stimulation with both 10 and 100 ng/ml of LPS compared to cells which expressed the reporter control (Figure 4.9A). In addition, miR-125a sponge cells also produced significantly higher amounts of IL-6 after stimulation with 100 ng/ml of LPS (Figure 4.9B). These cells did not produce detectable amounts of IL-6 when stimulated with 10 ng/ml of LPS.



#### Figure 4.9 Cytokine expression of miR-125a sponge THP-1 cells

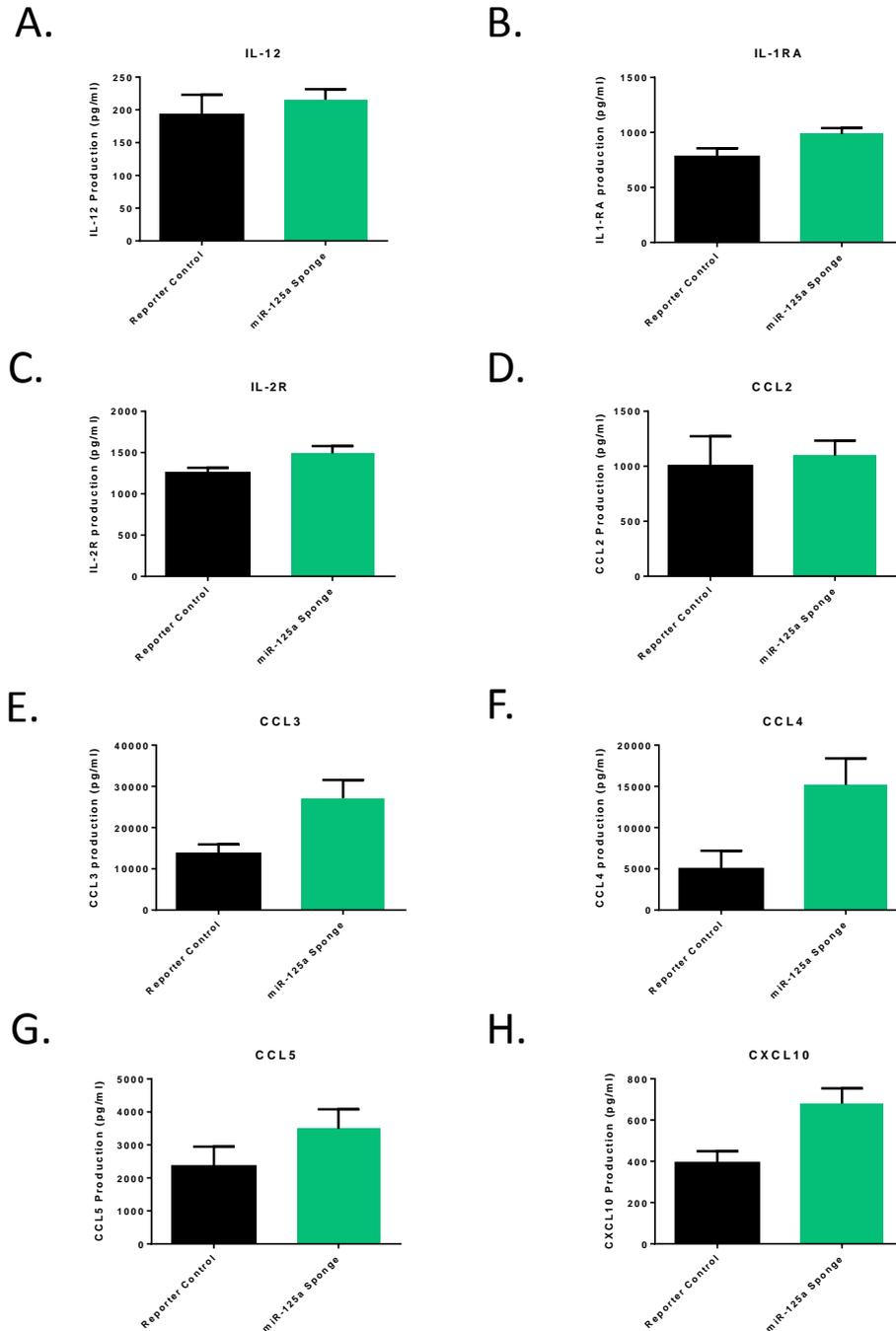
THP-1 cells expressing both the reporter control and miR-125a sponge vectors were stimulated with LPS and cytokines were measured. A) Cells were stimulated with both 10 ng/ml and 100 ng/ml LPS. TNF $\alpha$  production was significantly higher in cells that expressed the miR-125a sponge vector when compared to those expressing the reporter control vector. B) IL-6 production was not detectable when cells were stimulated with 10 ng/ml of LPS. IL-6 production after stimulation with 100 ng/ml LPS is significantly increased in cells expressing miR-125a sponge when compared to the reporter control. Values presented as mean  $\pm$  SEM of 4 separate experiments and marked bars are statistically different from reporter control using a Mann Whitney test  $*=p < 0.05$ .

#### 4.2.2.2 Cytokine and chemokine profile of day 3 THP-1 differentiated miR-125a sponge macrophages

As miR-125a expression is increased in PMA differentiated cells by day 3 and further increased by day 7, we decided to examine the effect loss of miR-125a activity had on the cytokine profile of PMA differentiated cells, along with the response to LPS in these cells at both time-points.

Differentiated cells were either left unstimulated or stimulated with 10 ng/ml of LPS for a period of 6 or 24 hours. Supernatants were taken from these cells and analysed by a pre-designed Luminex Assay. Out of the 25 cytokines and chemokines on the panel, 13 were produced at measurable levels after LPS stimulation. These cytokines and chemokines will be discussed further.

It should be noted that differentiation with PMA activates THP-1 cells. Therefore, although TNF $\alpha$  was not detectable in unstimulated cells, 8 of the 25 cytokines and chemokines were produced at measurable levels in these differentiated cells.

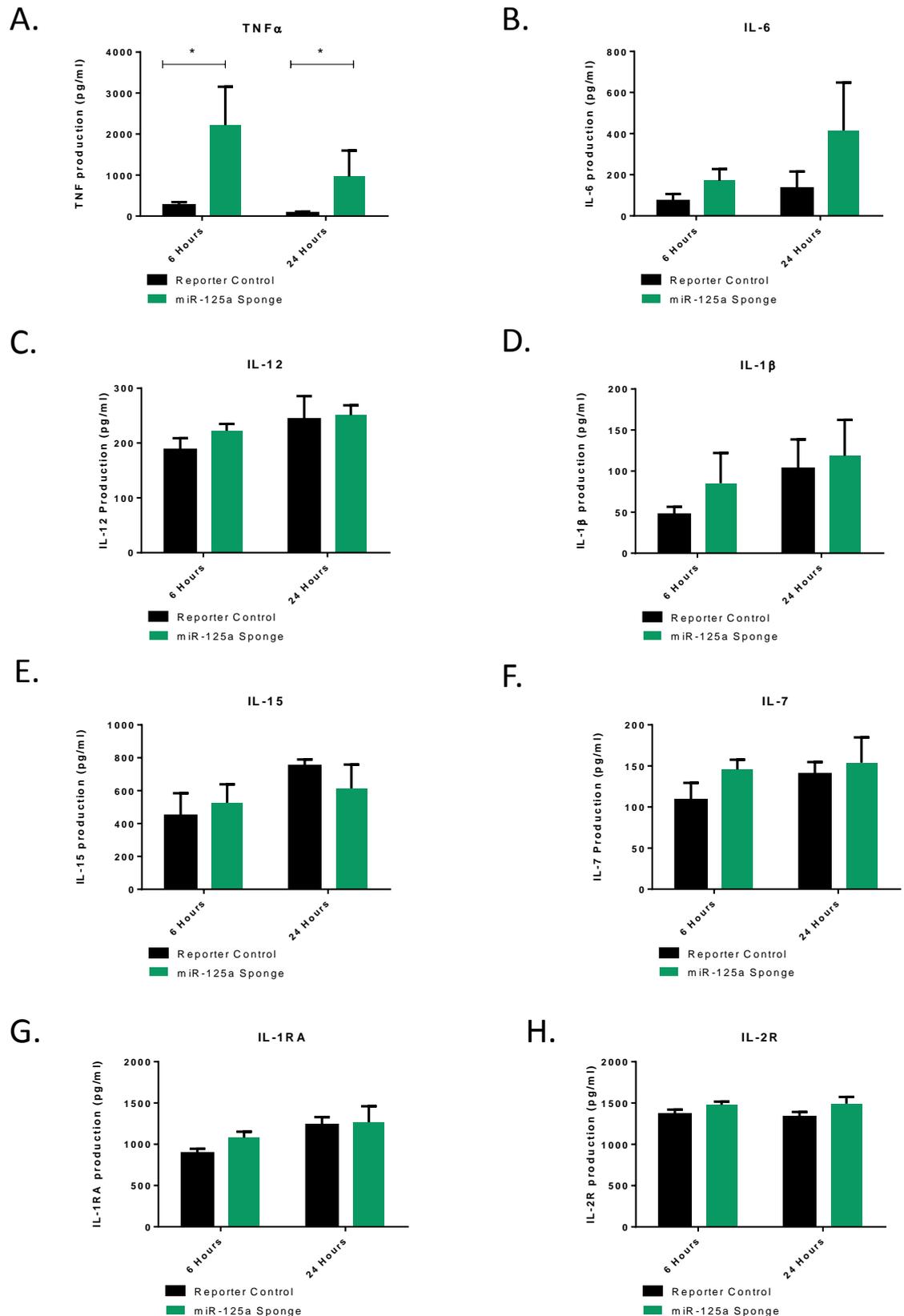


**Figure 4.10 Cytokine and chemokine expression of resting day 3 PMA miR-125a expressing sponge cells**

THP-1 sponge cells were differentiated with PMA for 3 days and cytokine expression was measured by Luminex assay. There is no difference in cytokine and chemokine production in resting cells on day 3 using a Mann Whitney Test. Values presented as mean  $\pm$  SEM of 4 separate experiments.

Although detectable, there was no significant difference in cytokine and chemokine production between miR-125a sponge and reporter control cells (Figure 4.10).

After LPS stimulation, miR-125a sponge expressing THP-1 derived macrophages produced significantly higher levels of TNF $\alpha$  at both the 6 hour and 24 hour time points when compared to reporter control cells. Similarly, production of IL-6 showed a tendency to be up-regulated in miR-125a sponge expressing cells (Figure 4.11A and B). There was no significant difference in production of IL-12, IL-1 $\beta$ , IL-7, IL-1RA or IL-2R between miR-125a sponge and reporter control cells at this time-point (Figure 4.11 C-H).



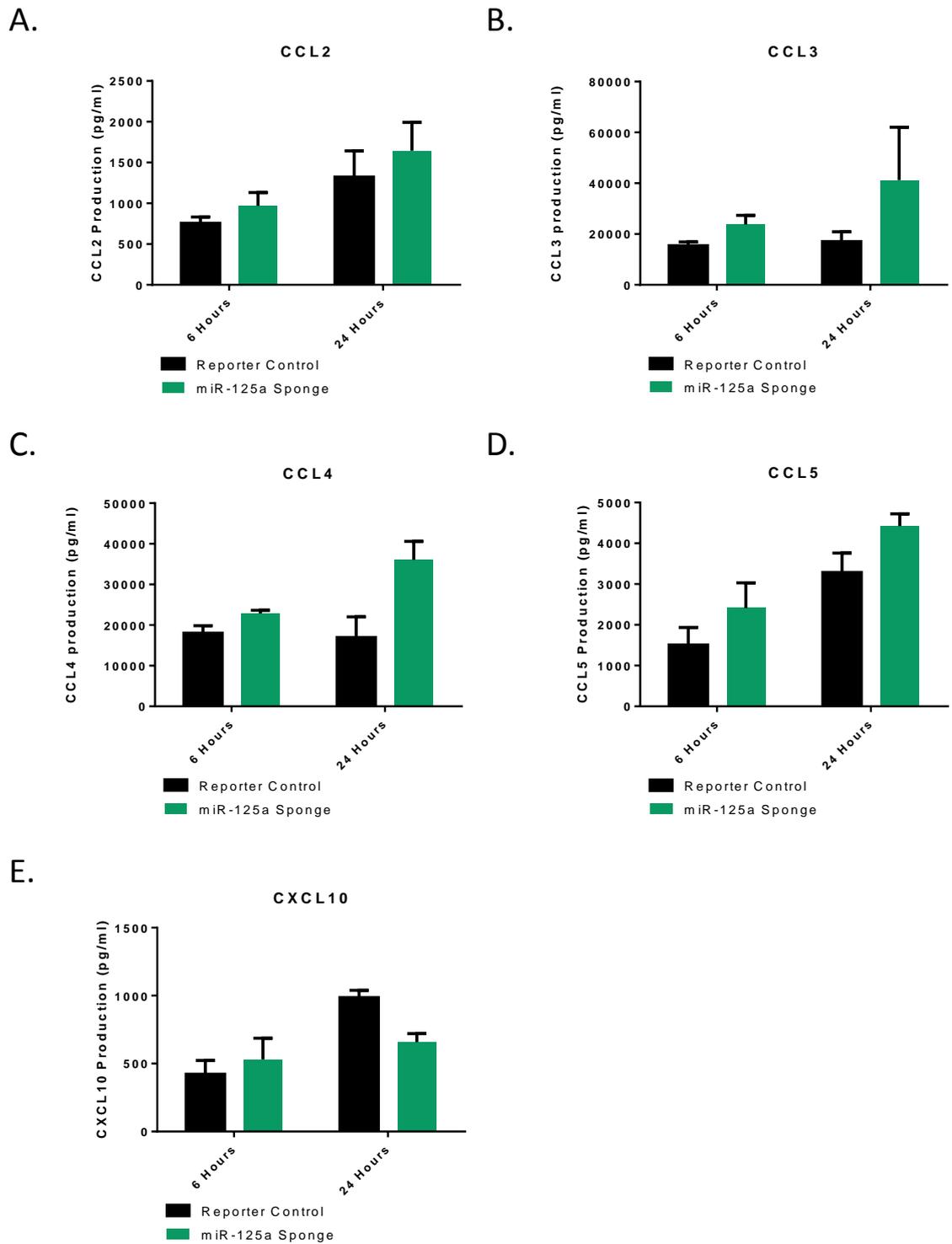
**Figure 4.11 Cytokine expression of LPS stimulated day 3 PMA miR-125a expressing sponge cells**

THP-1 sponge cells were differentiated with PMA for 3 days, stimulated with LPS and cytokine expression was measured by Luminex assay. A) There is a significant increase in TNF $\alpha$  production in cells which express the miR-125a sponge compared to the reporter control B-H) There is no significant difference in the production of other cytokines measured between miR-125a

sponge cells and reporter control cells. Values presented as mean  $\pm$  SEM of 4 separate experiments and marked bars are statistically different from reporter control using a Mann-Whitney test  $*=p < 0.05$

Chemokine expression was also measured in the supernatants of these day 3 differentiated cells. Results showed that there was no significant difference in production of CCL2, CCL3, CCL4, CCL5 and CXCL10 between reporter and miR-125a sponge cells (Figure 4.12 A-E). However, after 24 hour stimulation with LPS, miR-125a sponge expressing cells appear to produce more CCL4 than those expressing the reporter control with a trend towards significance with  $p=0.059$ .

In summary, neutralisation of miR-125a in THP-1, and THP-1 derived macrophages leads to an increase in production of TNF, IL-6 and CCL4.



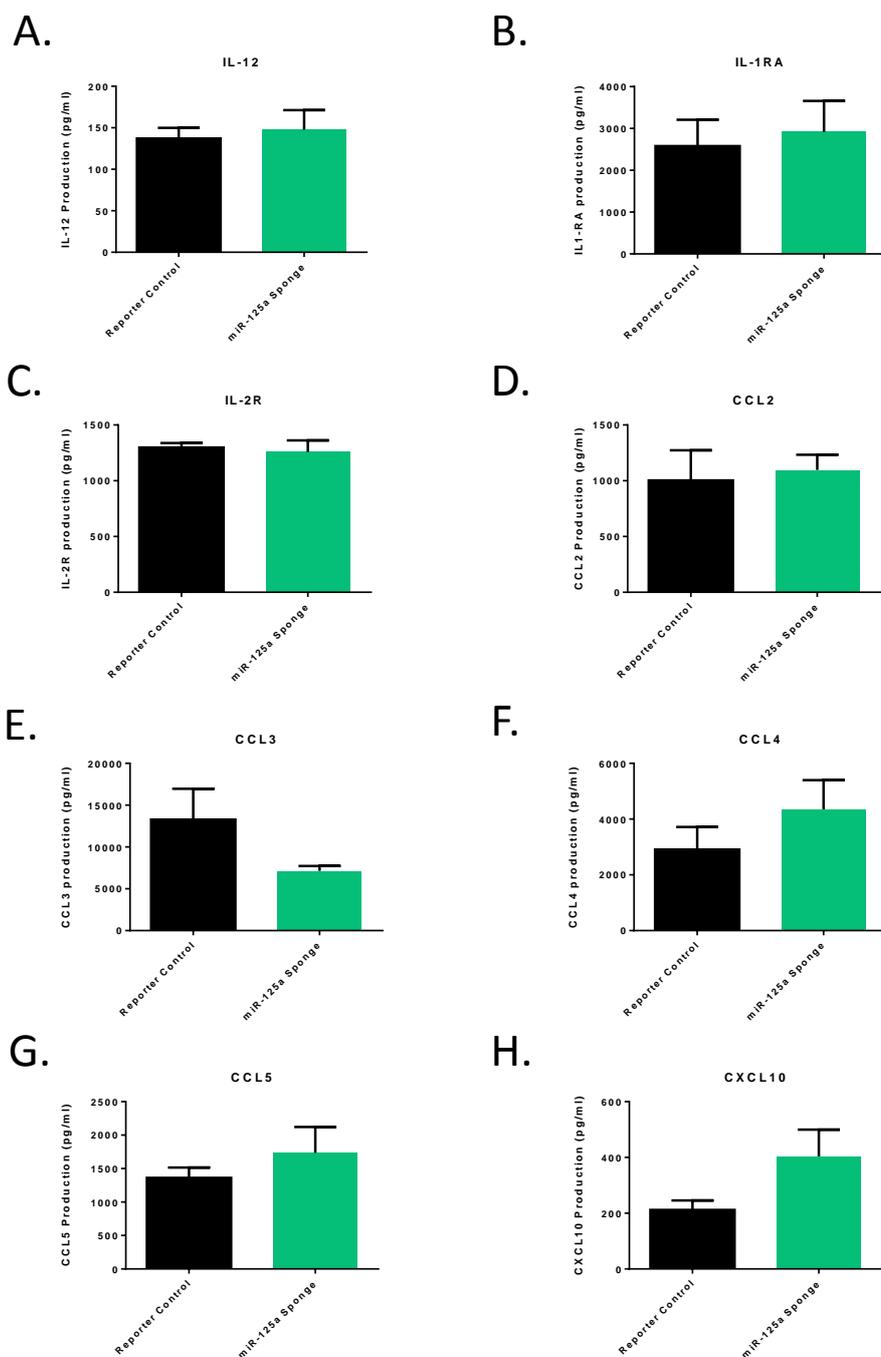
**Figure 4.12 Chemokine expression of LPS stimulated day 3 PMA miR-125a expressing sponge cells**

THP-1 sponge cells were differentiated with PMA for 3 days, stimulated with LPS and chemokine expression was measured by Luminex assay. There was no significant difference using a Mann-Whitney test in any of the chemokines measured. Values presented as mean  $\pm$  SEM of 4 separate experiments.

#### **4.2.2.3 Cytokine and chemokine profile of day 7 PMA differentiated miR-125a sponge expressing THP-1s**

As miR-125a expression in these cells is at its highest at day 7 in culture, we next wanted to determine the cytokine and chemokine expression of day 7 PMA differentiated THP-1 cells. As previously described, this was achieved by using a pre-designed Luminex assay. Cells were again stimulated with 10 ng/ml LPS for a period of 6 and 24 hours, after which supernatants were analysed.

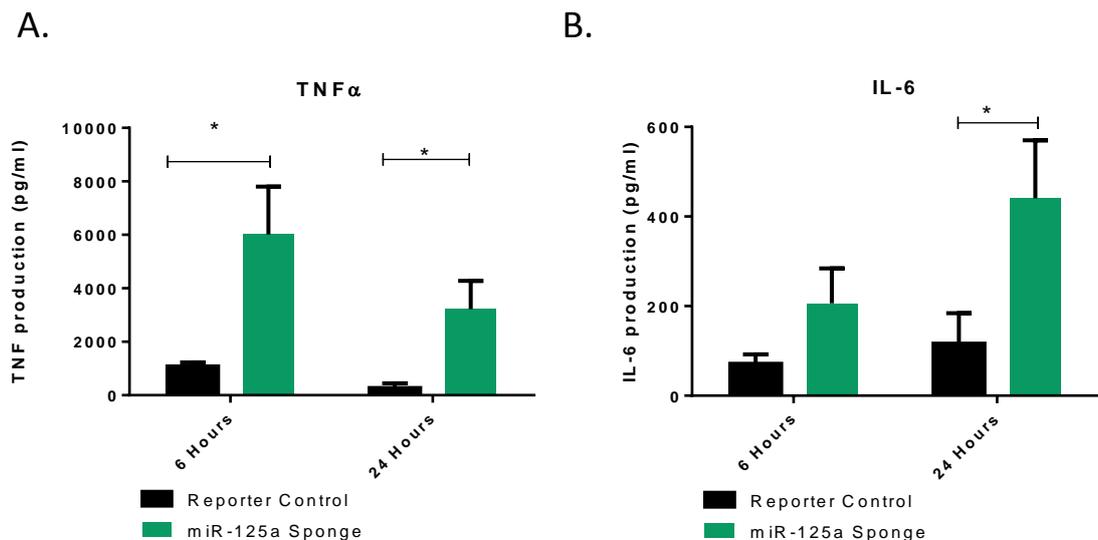
Again, results demonstrate that differentiation of cells with PMA activates cells to produce cytokines and chemokines. However, in day 7 cells there was no significant difference in production of any of the mediators, between cells expressing the miR-125a sponge, or the reporter control (Figure4.13).



**Figure 4.13 Cytokine and chemokine expression of resting day 7 PMA miR-125a expressing sponge cells**

THP-1 sponge cells were differentiated with PMA for 7 days and cytokine expression was measured by Luminex assay. Although detectable, there was no difference in production of cytokines and chemokines measured. Values presented as mean  $\pm$  SEM of 4 separate experiments.

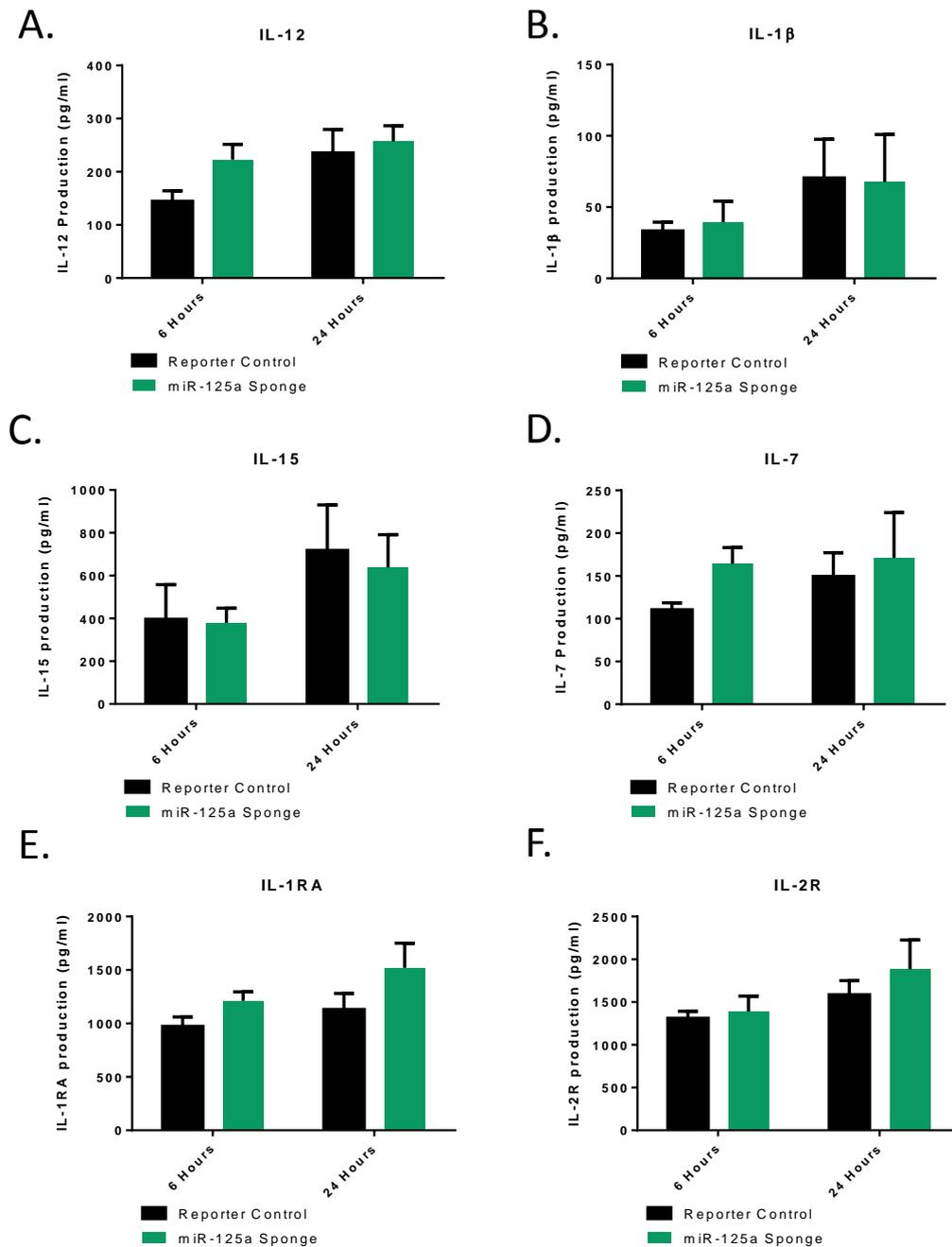
However, after LPS stimulation, cells expressing the miR-125a sponge vector produced significantly higher TNF $\alpha$  than those expressing the reporter control vector at 6 hours and 24 hours after stimulation (Figure 4.14A). By day 7, miR-125a sponge cells also produce significantly higher levels of IL-6, after 24 hour LPS stimulation (Figure 4.14B). These results demonstrate a consistent increase in the production of TNF $\alpha$  and IL-6 in cells which do not express functional miR-125a.



**Figure 4.14 TNF $\alpha$  and IL-6 expression of LPS stimulated Day 7 PMA differentiated miR-125a sponge cells**

THP-1 cells were differentiated using PMA for 7 days, stimulated with LPS and cytokine expression was measured using a Luminex assay. A) TNF $\alpha$  production is significantly increased after stimulation with LPS in miR-125a sponge expressing cells when compared to reporter control cells at both 6 and 24 hour time-points. B) IL-6 production is significantly increased after stimulation with LPS in miR-125a sponge expressing cells when compared to reporter control cells after 24 hours. Values presented as mean  $\pm$  SEM of 4 separate experiments and marked bars are statistically different from reporter control using an Mann-Whitney \* $p < 0.05$ .

In addition to TNF $\alpha$  and IL-6 other cytokines were also evaluated. Results revealed no significant difference in the production of IL-12, IL-1 $\beta$ , IL-15, IL-7, IL-1RA and IL-2 receptor (Figure 4.15 A-F). The anti-inflammatory cytokine IL-10 was not detectable in the supernatants of stimulated PMA differentiated cells. This is supported by Morris et al, who found that PMA differentiated THP-1 cells require stimulation with high doses of LPS (between 100ng-1 $\mu$ g) to produce IL-10 [409].

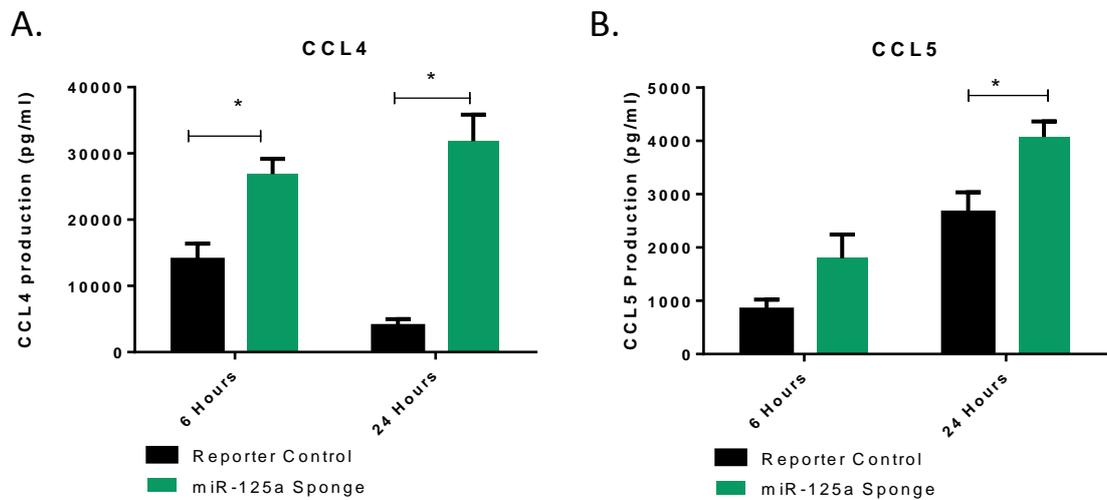


**Figure 4.15 Cytokine expression of LPS stimulated day 7 PMA differentiated miR-125a sponge cells**

THP-1 cells were differentiated using PMA for 7 days, stimulated with LPS and cytokine expression was measured using a Luminex assay. A-F) Cytokines unaltered by the expression of a miR-125a sponge. Values presented as mean  $\pm$  SEM of 4 separate experiments and there is no significant difference using a Mann-Whitney test.

In addition to cytokines, cells expressing the miR-125a sponge produced significantly higher amounts of chemokines. CCL4 was upregulated at both 6 hour and 24 hour time points after LPS stimulation (Figure 4.16A). In addition,

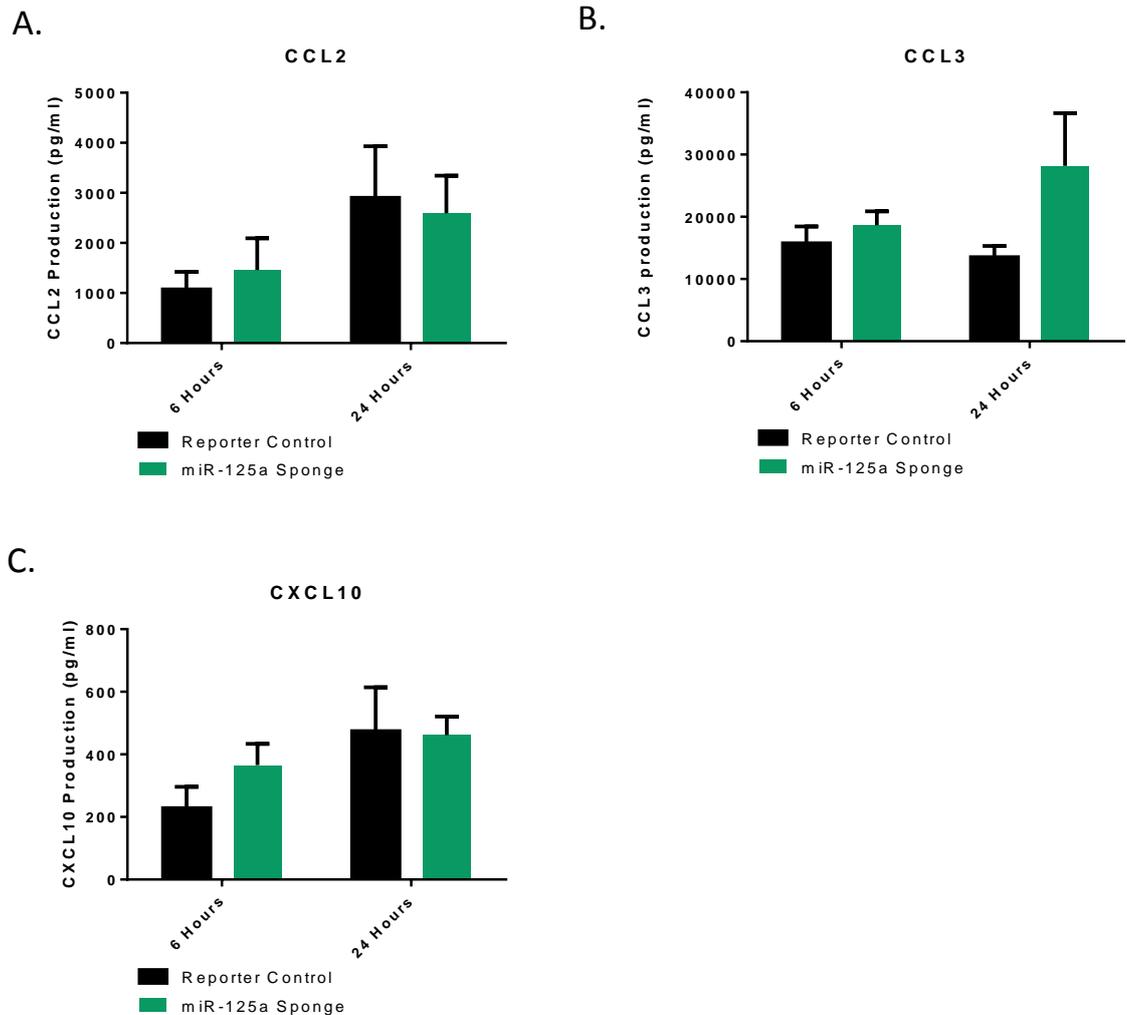
miR-125a sponge cells also produced higher levels of CCL5, this difference reaching significance after 24 hours stimulation (Figure 4.16B).



**Figure 4.16 CCL4 and CCL5 expression in day 7 PMA miR-125a sponge cells**

THP-1 cells were differentiated using PMA for 7 days, stimulated with 10 ng/ml LPS and chemokine expression was measured using a Luminex assay. A) CCL4 production was significantly increased in cells that expressed the miR-125a sponge when compared to reporter control after stimulation with LPS at both 6 hour and 24 hour time-points. B) CCL5 production is increased in cells that express the miR-125a sponge when compared to the reporter control, this difference reaches significance after 24 hours. Values presented as mean  $\pm$  SEM of 4 separate experiments and marked bars are statistically different from reporter control using a Mann-Whitney test. \* $p < 0.05$ .

We observed no difference in expression of CCL2, CCL3 and CXCL10 (Figure 4.17 A-C).



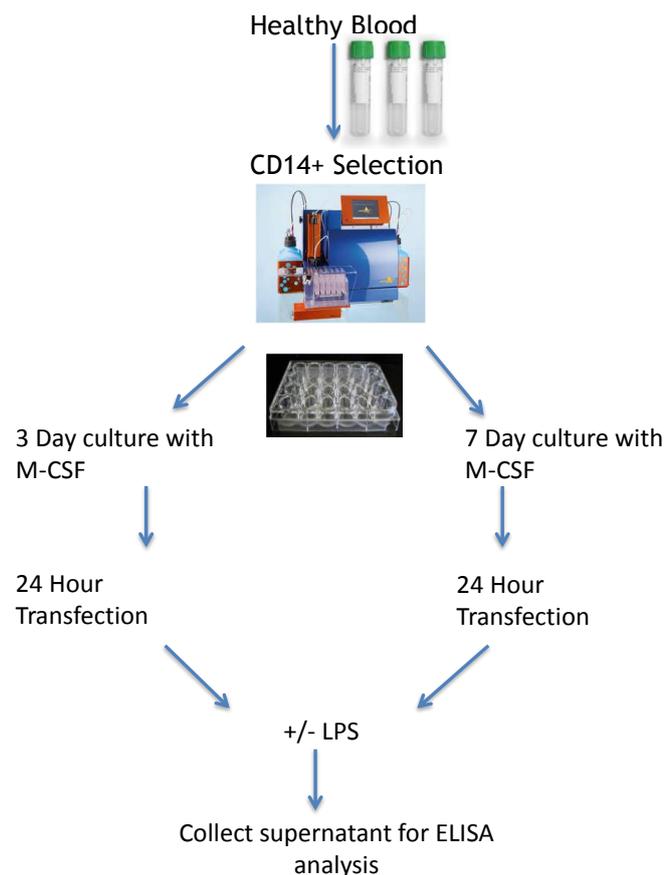
**Figure 4.17 Chemokine expression in LPS stimulated day 7 PMA differentiated miR-125a sponge cells**

THP-1 cells were differentiated using PMA for 7 days, stimulated with LPS and chemokine expression was measured using a Luminex assay A-C) Production of CCL2, CCL3 and CXCL10 was not altered by expression of the miR-125a sponge. Values presented as mean  $\pm$  SEM of 4 separate experiments and no statistical difference was detected using a Mann-Whitney Test

These data demonstrate a specific phenotype in monocytes and macrophages which have reduced miR-125a activity. It is clear that in both cell types, loss of miR-125a results in significantly higher TNF $\alpha$  production and to a lesser extent IL-6 production. By day 7 of differentiation miR-125a sponge cells also produce significantly higher amounts of the chemokines CCL4 and CCL5 suggesting miR-125a is involved in regulating the production of these chemokines. This pattern of change has been shown to be quite robust as the experiment was set up on 4 separate occasions, with a similar result generated.

### 4.2.3 Cytokine profile of primary human macrophages after inhibition of miR-125a

Although we have generated a cytokine and chemokine profile of THP-1 derived miR-125a null macrophages, it is important to validate this expression profile in primary human macrophages. To do this human CD14<sup>+</sup> cells were separated from healthy buffy coat packs and differentiated towards a macrophage phenotype using the growth factor M-CSF (50 ng/ml) for 3 and 7 days. Since primary cells are hard to transfect with DNA vectors, we used 22 nt long miR-125a sequence specific inhibitor or control inhibitor. Macrophages were transfected on either day 3 or day 7. After 24 hours transfection, macrophages were left for an additional 24 hours either unstimulated or LPS stimulated (10 ng/ml) for 24 hours. Supernatants were then harvested for analysis via ELISA (Figure 4.18).



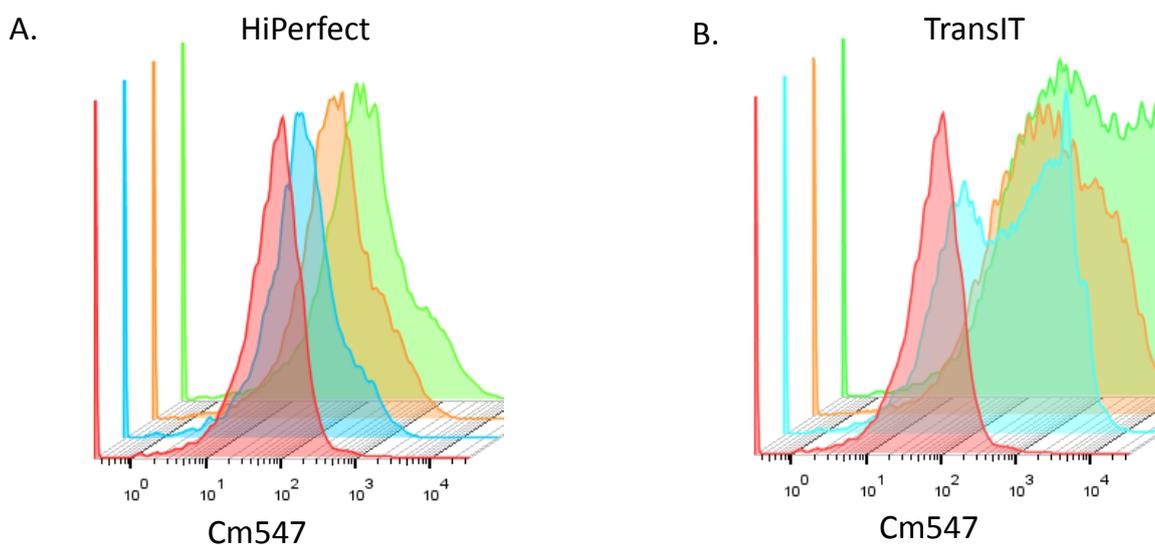
**Figure 4.18 Experimental set-up of human macrophage transfection**

CD14<sup>+</sup> cells were separated from buffy coat packs and differentiated for 3 and 7 days with the growth factor M-CSF (50 ng/ml). Cells were transfected on day 3 or day 7 for 24 hours and then cultured for a further 24 hours with or without LPS (10ng/ml). Supernatants were taken from these cells for analysis by ELISA.

#### 4.2.3.1 Transfection optimisation of human macrophages

Previous transfection experiments carried out within the lab group using primary human macrophages had been carried out using a transfection reagent from Thermo Scientific - Dharmafect. However, when this reagent was tested in these experiments, under conditions previously validated, we observed high levels of cellular toxicity. Therefore we decided to validate transfection reagents. The transfection reagents we chose were HiPerfect reagent (Qiagen) recommended for transfection of macrophages and TransIT TKO reagent (Mirus Pharmaceuticals), which was also recommended for the non-toxic transfection of adherent cells.

Transfection efficiency can be determined by flow cytometry through transfection with a Cm labelled with a dye (547) which can be visualised by FACS. Therefore, I decided to transfect cells with differing concentrations of this mimic (10-50 nM) with the recommended amount of transfection reagent (2  $\mu$ l HiPerfect and 2.5  $\mu$ l of TransIT). Cells were visually inspected before FACS analysis to determine their adherence and removed by dissociation reagent to minimise cell death. Results (untransfected (red), 10 nM Cm547 (blue), 25 nM Cm547 (orange) and 50 nM Cm547 (green)) demonstrated that irrespective of mimic concentration, transfection efficiency of cells was low when transfected with HiPerfect reagent, reaching only 11% at the highest concentration (Figure 4.19A). In contrast, TransIT reagent achieved transfection efficiency of 68% with 25 nM Cm547 concentration (Figure 4.19B). This was marginally higher than with 50 nM Cm547 (64%), and had been previously shown by our lab group to be effective at knocking-down specific miR targets (Kurowska-Stolarska, unpublished results). In addition, previous experiments within our lab demonstrated that a transfection efficiency of over 60% is enough to demonstrate target knockdown, therefore I decided to use this concentration of mimic in further experiments. Percentage of positively transfected cells are shown in



**Figure 4.19 Transfection optimisation of human macrophages**

Human macrophages were transfected with a labelled control mimic and differing concentrations of transfection reagent. Untransfected (red), 10 nM Cm547 (blue), 25 nM Cm547 (orange) and 50 nM Cm547 (green). A) Transfection efficiency with HiPerfect reagent was low, reaching only 11% with the 50 nM concentration. B) Transfection efficiency with TransIT reagent was satisfactory, reaching 67% with 25 nM Cm547.

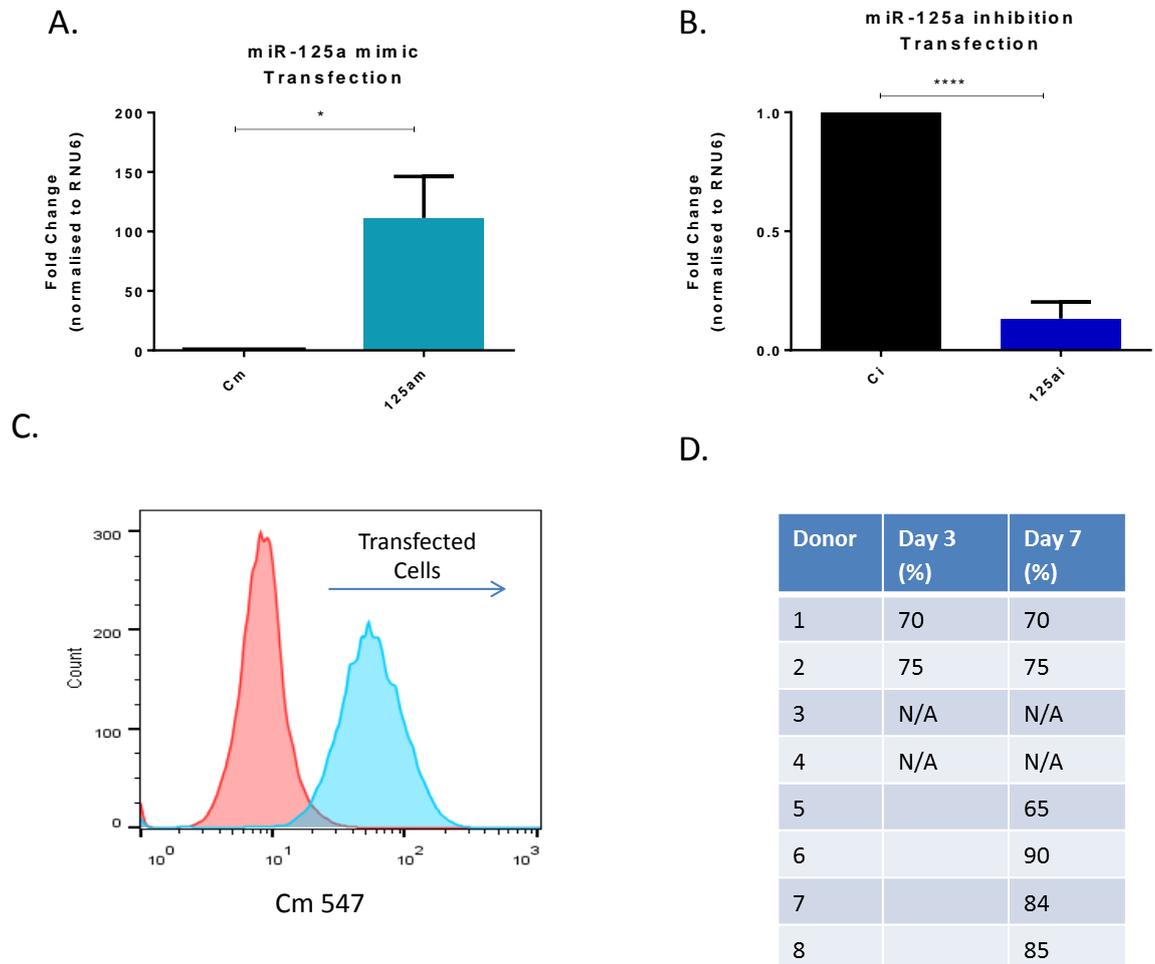
**Table 4.1 Percentage of cells transfected using HiPerfect or TransIT reagent**

Transfection optimisation was performed using human macrophages and two transfection reagents. Human macrophages were transfected with differing concentrations of Cm547, and the suggested amount of transfection reagent. Percentage of transfected cells was measured using Flow Cytometry

Concentration of cm547 (nM)	Percentage Transfected Cells	
	HiPerfect Reagent	TransIT Reagent
10	6	56
25	8	68
50	11	64

For each macrophage donor, transfection efficiency was confirmed using either this FACS based method, or by validating miR-125a expression by q-PCR, or both. The FACS based method gives a good indication of how effective transfection is, however it is important to verify miR-125a knockdown by q-PCR. Q-PCR analysis demonstrated that macrophages transfected with a miR-125a mimic expressed significantly higher miR-125a, and cells transfected with a miR-125a inhibitor expressed significantly lower miR-125a as expected, demonstrating that transfection was effective (Figure 4.20 A and B). Additionally, for all

experiments verified by FACs, transfection efficiency was above 65% and in some cases reached 90% (Figure 4.20 D). An example of a FACS plot showing transfection efficiency is shown in Figure 4.20 C, the red histogram represents cells transfected with Cm, whilst the blue histogram represents cells transfected with the labelled Cm (547).



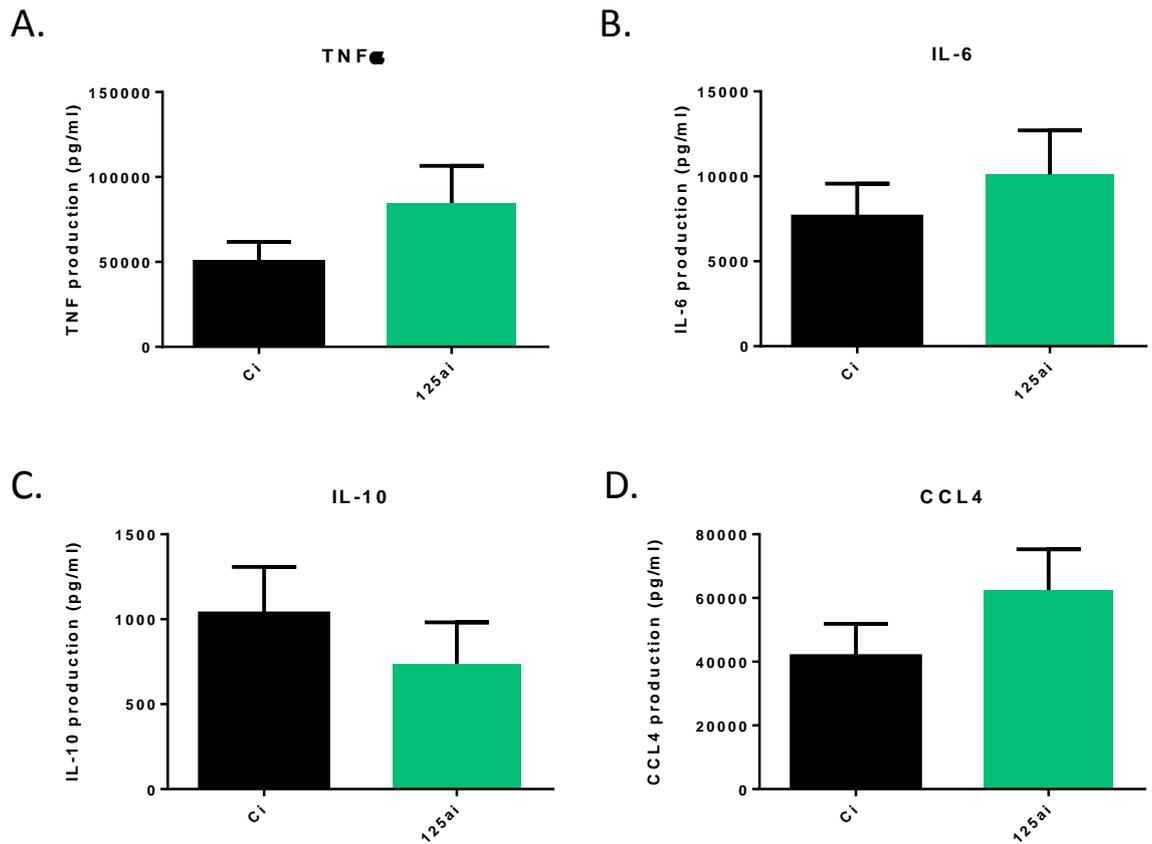
#### Figure 4.20 Transfection efficiency of human macrophages

Human macrophages were transfected with miR-125a mimic and inhibitors and transfection efficiency was measured by q-PCR and FACS. A) macrophages transfected with a miR-125a mimic express significantly higher levels of miR-125a as measured by q-PCR B) macrophages transfected with a miR-125a inhibitor express significantly lower miR-125a as detected by q-PCR C) Example of measurement of transfection efficiency by FACS D) Table showing the transfection efficiency of human macrophages as determined by FACS analysis. Values represent mean  $\pm$  SEM of 8 experiments, with marked bars being significantly different when using a Paired T test. \*= $p < 0.05$ , \*\*\*\*= $p < 0.0005$ .

#### **4.2.3.2 Cytokine and chemokine expression of day 3 macrophages transfected with a miR-125a inhibitor**

Once we had determined the correct reagents for transfection we wanted to verify if the expression profile observed in the miR-125a sponge cells was also present in human macrophages transfected with a miR-125a inhibitor. We determined cytokine expression in day 3 differentiated cells, as we have already shown that miR-125a expression is at its highest in these cells (Figure 3.12A). Guided by data obtained using miR-125a sponges, we tested production of TNF $\alpha$ , IL-6, IL-12, CCL4 and IL-10.

Unstimulated cells did not produce detectable levels of TNF $\alpha$ , IL-6, IL-10 or CCL4 (data not shown). Upon LPS stimulation, Day 3 macrophages transfected with a 125ai did not produce significantly different amounts of TNF $\alpha$ , IL-6, IL-10 or CCL4 when compared to those cells transfected with a Ci (Figure 4.21 A-D). However, as with miR-125a sponge cells, there is a trend towards higher production of TNF $\alpha$ , IL-6 and especially CCL4 ( $p=0.08$ ). Interestingly it appeared that, conversely to the production of pro-inflammatory cytokines, production of IL-10 was lower in cells transfected with 125ai ( $p=0.08$ ).



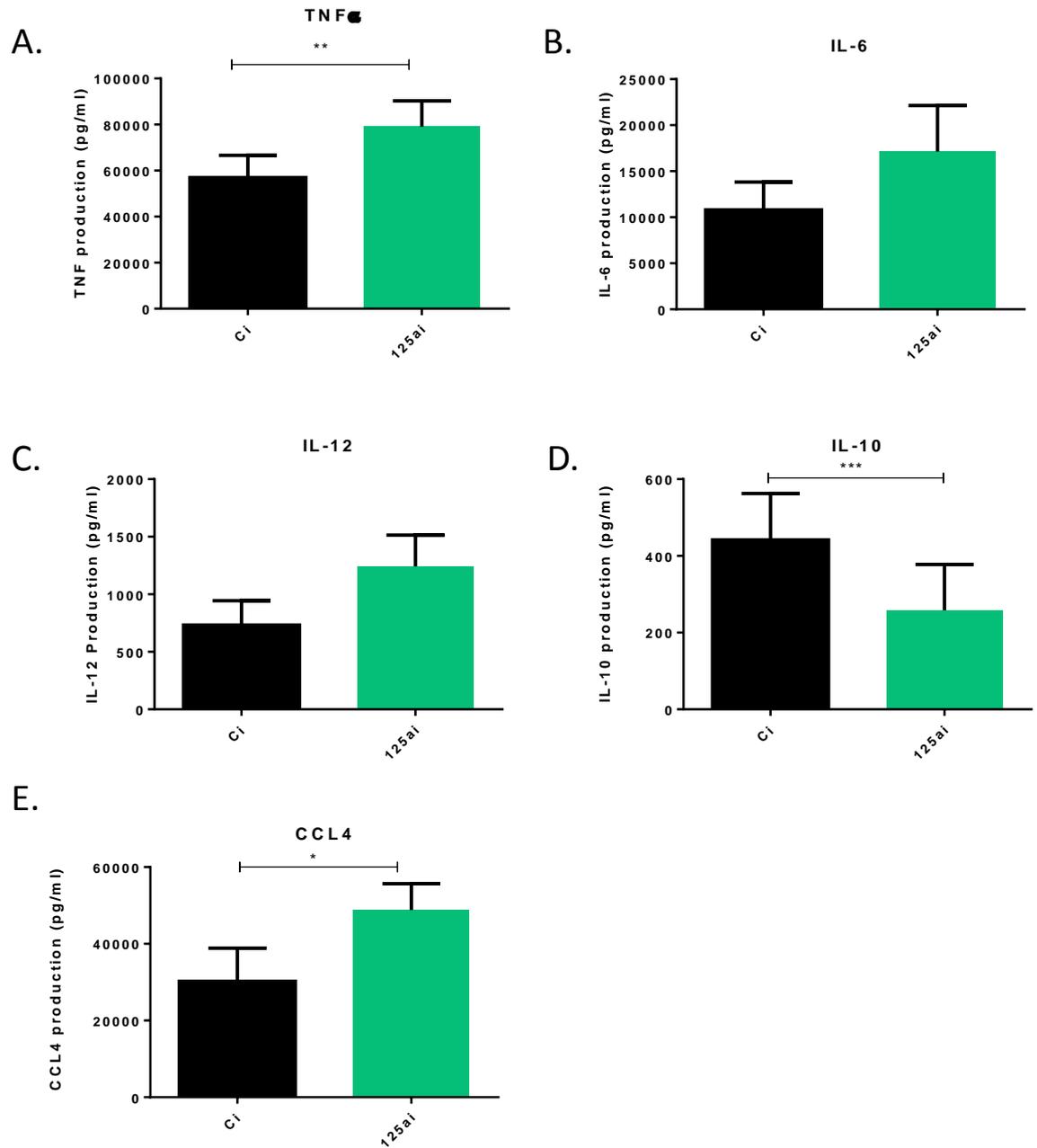
**Figure 4.21 Cytokine and chemokine profile of day 3 macrophages transfected with a miR-125a inhibitor**

Human macrophages were differentiated with 50 ng/ml M-CSF and transfected with either Ci or miR-125ai, after which cells were stimulated with 10 ng/ml LPS. Cytokines were measured by ELISA after 24 hours in culture (A-D). Transfection with a miR-125ai does not significantly alter cytokine or chemokine expression in day 3 macrophage. Values presented as mean  $\pm$  SEM of 4 separate experiments and no statistical difference was detected from Ci using a Wilcoxon matched-pairs signed rank test.

#### 4.2.3.3 Cytokine and chemokine expression of day 7 macrophages transfected with a miR-125a inhibitor

Next we investigated the cytokine and chemokine expression profile of day 7 fully differentiated human macrophages transfected with a 125ai as we have shown that the copy number of miR-125a remains high in these cells.

As with day 3 macrophages, unstimulated cells did not produce detectable levels of TNF $\alpha$ , IL-6, IL-12, IL-10 and CCL4. However, upon LPS stimulation human macrophages transfected with a 125ai produce significantly higher amounts of TNF $\alpha$  and CCL4 (Figure 4.22 A, C and E), and significantly lower amounts of IL-10 (Figure 4.22D). There was no significant difference in production of IL-6 or IL-12 (Figure 4.22 B and C).



**Figure 4.22 Cytokine and chemokine expression of day 7 human macrophages transfected with a miR-125a inhibitor**

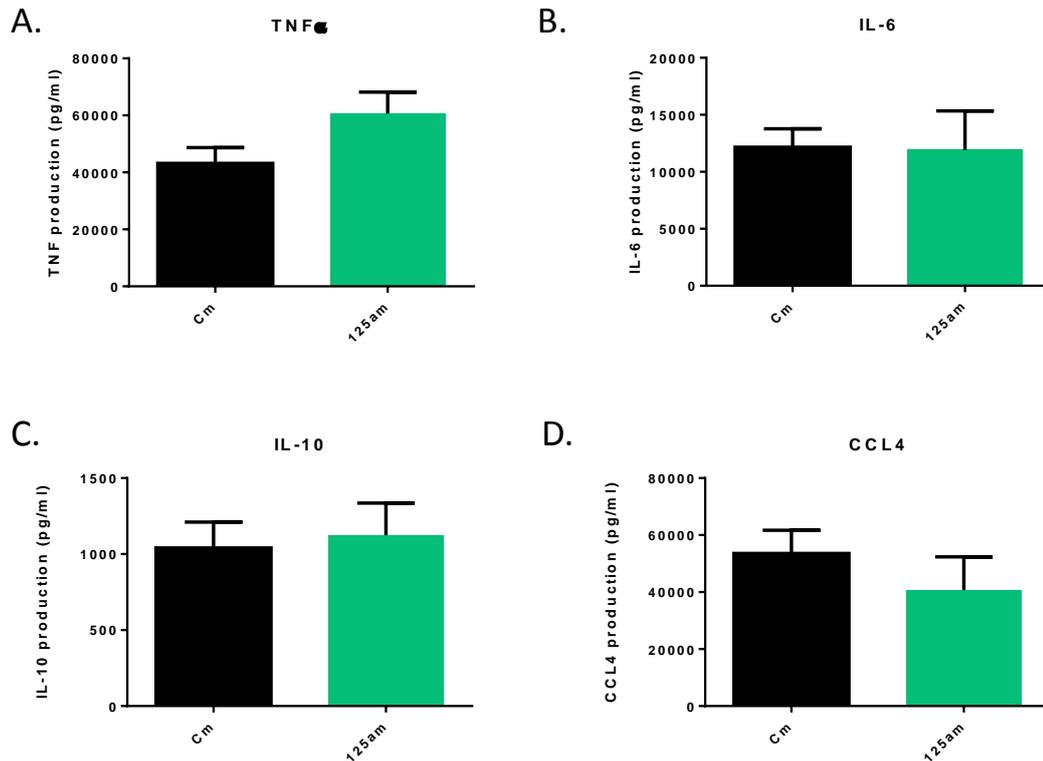
Human macrophages were differentiated with 50 ng/ml M-CSF and transfected with either a Ci or miR-125ai, after which cells were stimulated with 10 ng/ml LPS. Cytokines were measured by ELISA after 24 hours in culture A) TNF $\alpha$  production was significantly higher in cells transfected with a miR-125ai B and C) There was no significant difference in the production of IL-6 and IL-12 between Ci and miR-125ai transfected cells D) IL-10 production was significantly decreased in cells transfected with a miR-125ai E) CCL4 production was significantly increased in cells transfected with a miR-125ai when compared to Ci. Values presented as mean  $\pm$  SEM of 8 separate experiments and marked bars are statistically different from Ci using a paired T test, \* $p < 0.05$ , \*\* $p < 0.005$  \*\*\* $p < 0.005$ .

In summary, primary human macrophage produce significantly higher amounts of pro-inflammatory TNF $\alpha$ , CCL4 and IL-6 (PMA cells) upon miR-125a inhibition, whilst producing significantly lower levels of IL-10.

#### **4.2.3.4 Cytokine and chemokine expression of day 3 human macrophages transfected with a miR-125a mimic**

Finally, I wanted to determine whether overexpression of miR-125a would have the opposite effect on the expression of TNF $\alpha$ , IL-6, IL-10, IL-12 and CCL4. Previously I have demonstrated that transfection with a 125am increased the amount of miR-125a within a sample by up to 200 fold. Taking this approach permits the identification of potential miR-125a targets, but also may highlight off target effects. As before, cells were differentiated using 50 ng/ml M-CSF for 3 and 7 days, transfected for 24 hours with either a Cm or 125am and then left unstimulated, or stimulated for 24 hours with 10 ng/ml LPS, with supernatants taken for ELISA.

As in previous experiments, unstimulated cells did not produce detectable levels of cytokines or chemokines. Results demonstrated that as with 125ai transfection, expression of TNF $\alpha$ , IL-6, IL-10 and CCL4 were not significantly altered in day 3 human macrophages by transfection with a miR-125a mimic (Figure 4.23).



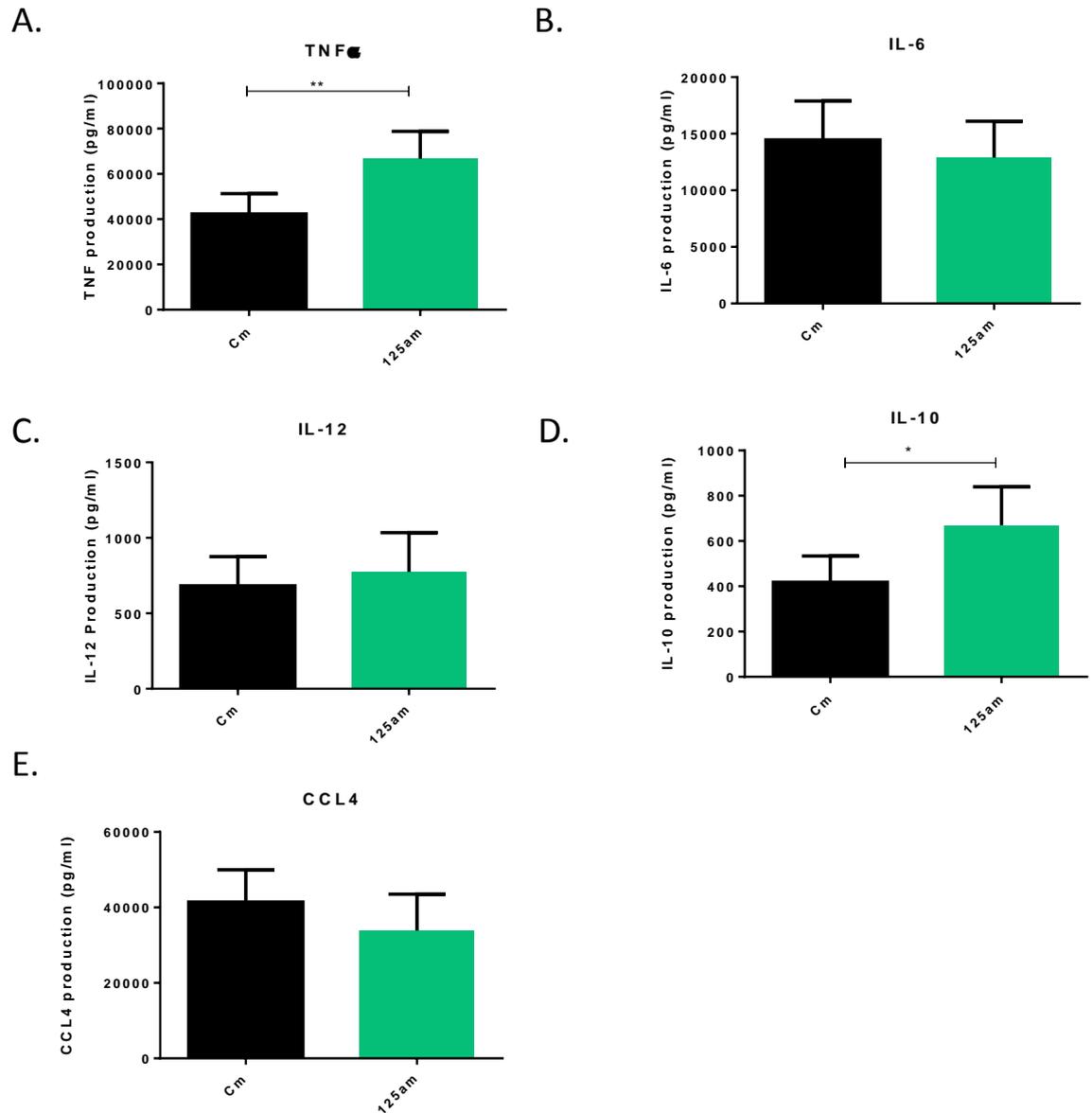
**Figure 4.23 Cytokine and chemokine profile of day 3 human macrophages transfected with a miR-125a mimic**

Human macrophages were differentiated with 50 ng/ml M-CSF and transfected with either a Cm or miR-125am, after which cells were stimulated with 10 ng/ml LPS. A-D) Expression of TNF $\alpha$ , IL-6, IL-10 and CCL4 were not significantly altered by transfection with a miR-125am. Values presented as mean  $\pm$  SEM of 4 separate experiments and no statistical difference was detected from Cm using a Wilcoxon matched-pairs signed rank test

#### 4.2.4 Cytokine and chemokine expression of day 7 human macrophages transfected with a miR-125a mimic

The most remarkable differences induced by the inhibition of miR-125a were observed at day 7 in both PMA differentiated THP-1 cells and M-CSF differentiated primary macrophages. Therefore, we decided to investigate these cytokines in the context of day 7 differentiated macrophages that had been transfected with a miR-125a mimic. I hypothesised that the significant increase in production of TNF $\alpha$ , IL-12 and CCL4 seen in these cells would be reversed in mimic transfected cells. This was the case for CCL4 as overexpression of miR-125a in day 7 human macrophages resulted in lower production of CCL4, this difference trended towards significance with a  $p=0.07$  (Figure 4.24E). Interestingly TNF $\alpha$  production was not reduced, but was significantly increased in these cells (Figure 4.24A).

Based on the miR-125a inhibitor data which demonstrated a decrease in IL-10 production, I hypothesised that miR-125a overexpressing macrophages will produce more IL-10. This was also proven correct, as day 7 human macrophages transfected with a miR-125a mimic produce increased levels of IL-10 (Figure 4.24D).



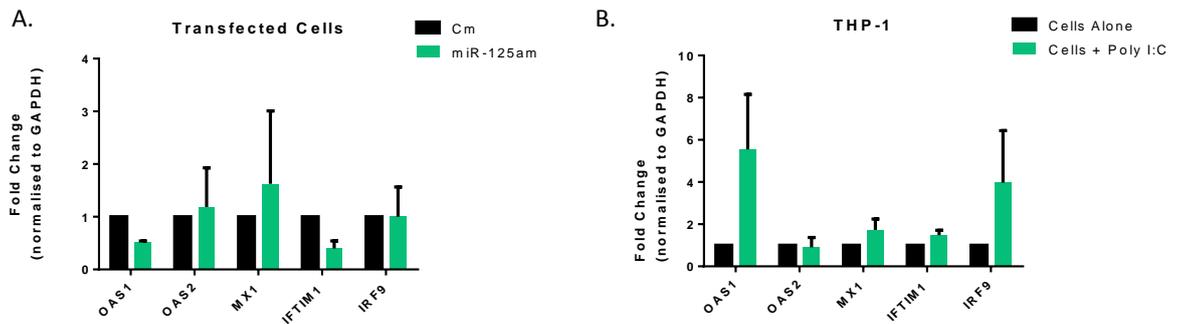
**Figure 4.24 Cytokine and chemokine profile of day 7 human macrophages transfected with a miR-125a mimic**

Human macrophages were differentiated with 50 ng/ml M-CSF and transfected with either a Cm or miR-125a, after which cells were stimulated with 10 ng/ml LPS. A) Overexpression of miR-125a resulted in significantly higher production of TNF $\alpha$  B) Overexpression of miR-125a did not alter IL-6 production C) Overexpression of miR-125a did not alter IL-12 production D) Overexpression of miR-125a resulted in a significant increase in IL-10 production E) Overexpression of miR-125a did not significantly down-regulate CCL4 production. Values presented as mean  $\pm$  SEM of 8 separate experiments and marked bars are statistically different from Cm using a Paired T Test, \*p,0.05,\*\*p<0.005.

One of the many problems associated with the use of miR mimics is the possible undesirable immunogenicity of the double stranded RNA mimic itself. Due to the increased TNF $\alpha$  witnessed in cells transfected with both miR-125a mimics and inhibitors we wanted to investigate the immunogenicity of this mimic.

To do this we wanted to measure a variety of interferon response genes by q-PCR. Interferons are the host's first line of defence against viral infection, and can be activated by dsRNA, a common viral replicative intermediate, through TLR 3, 7 and 8. TLR 7 and 8 also recognise UG-rich motifs which exist in mature miR-125a.

To investigate immunogenicity, I measured the interferon response genes *OAS1*, *OAS2*, *MX1*, *IRF9* and *IFITM1* in transfected macrophages (125am v Cm), and THP-1 cells stimulated with Poly I:C, an activator of TLR3 as a positive control. This immunogenicity assay was used in a study by Haraguchi et al to ensure that their novel synthetic miR did not induce these IFN response genes. THP-1 cells stimulated with Poly I:C demonstrate an induction of *OAS1*, *MX1* and *IRF9*. Haraguchi et al also demonstrated an induction of *IFITM1*, which was not observed in this study (Figure 4.25B). Both *OAS1* and *IFITM1* are downregulated in miR-125a transfected cells and although *MX1* is induced to similar levels as in stimulated THP-1s, this is only in one out of 2 donors (Figure 4.25A). Due to the difference between the positive control of Poly I:C stimulation and 125am transfected cells, I can confidently say that the 125am does not induce an interferon response.



**Figure 4.25 Immunogenicity assay**

RNA from human macrophages transfected with a Cm and miR-125am was analysed by q-PCR to measure levels of IFN response genes, *OAS1*, *OAS2*, *MX1*, *IRF9* and *IFITM1*. Additionally these genes were measured in THP-1 cells, stimulated with Poly I:C as a positive control. A) *OAS2*, *MX1* and *IRF9* expression was induced in 1 of 2 donors, however both *OAS1* and *IFITM1* were down-regulated in these cells. B) In THP-1 cells stimulated with Poly I:C all genes except *OAS2* were induced in both donors. Values presented as mean  $\pm$  SEM of 2 separate experiments.

### 4.3 Discussion

In this chapter, we demonstrated that miR-125a plays a role in regulating the cytokine and chemokine production by macrophages in response to LPS. It is clear from the results, that inhibition of miR-125a results in the establishment of a pro-inflammatory phenotype in these cells. Stable miR-125a inhibition, through a miR-125a sponge, in THP-1 cells resulted in increased TNF $\alpha$  and IL-6 production. This profile was also present in THP-1 derived macrophages in which miR-125a inhibition resulted in a significant increase in TNF $\alpha$ , IL-6, CCL4 and CCL5 production. This pro-inflammatory phenotype was also present in primary human macrophages in which miR-125a was inhibited. These macrophages produced significantly higher amounts of TNF $\alpha$ , IL-12 and CCL4 and also lower amounts of anti-inflammatory IL-10. Interestingly, overexpression of miR-125a in human macrophages demonstrated a significant upregulation of IL-10 and TNF $\alpha$ , suggesting a dual role for miR-125a in TNF $\alpha$  regulation.

The alteration of miR expression is crucial in determining its function within a cell. The majority of previous studies have relied solely on gain and loss of function experiments *in-vitro*, through transfection of double stranded miR mimics and single stranded antisense inhibitors. In this study we used a novel method for inhibiting miR expression, first introduced by Ebert et al in 2007, and

generated a functional miR-125a sponge. This miR sponge vector was stably inserted into the genome of the THP-1 cell line to allow the study of miR-125a function in THP-1 derived macrophages where expression is high. The miR-125a sponge vector contained a luciferase reporter gene which allowed the activity of the construct to be measured. Vectors can also be created which express a fluorescent marker such as GFP. This allows the construct to be visualised by other techniques such as FACS and under a light microscope and would be useful for further experiments.

Due to metabolic and morphological similarities, the human monocytic cell line, THP-1, can be differentiated to macrophages by PMA. High levels of PMA have been shown to upregulate the production of pro-inflammatory cytokines and chemokines, whilst masking the response to other activatory factors such as LPS. Park et al reported that 5 ng/ml of PMA was enough to stably differentiate THP-1 cells towards macrophages without overexpression of TNF $\alpha$ , CCL5, IL-1, CXCL10 or CCL4. As we wanted to examine the response of miR-125a sponge cells to LPS, we set out to optimise the differentiation of THP-1 cells. We found that 5 ng/ml PMA was sufficient to induce the cells to become adherent, while producing lower amounts of TNF $\alpha$ , detectable by ELISA but not Luminex. We examined the response of both day 3 and day 7 differentiated cells with or without stimulation, at both 6 and 24 hours. These two time-points were chosen to investigate both early and late response to LPS. Results demonstrated that PMA itself induced cells to produce specific cytokines and chemokines. This was not surprising as PMA is routinely used along with ionomycin to activate T cells. However, reassuringly, there was no difference in cytokine and chemokine production between miR-125a sponge and reporter expressing cells. Additionally, the level of soluble mediators within the supernatant had decreased by day 7, consistent with 5 days in media without PMA.

At day 3 only TNF $\alpha$  production was significantly higher in miR-125a sponge expressing cells. By day 7, there was also a significant increase in IL-6, CCL4 and CCL5 in these cells. Both IL-6 and CCL5 were increased only after 24 hours stimulation, suggesting that miR-125a may indirectly regulate these mediators.

PMA differentiated macrophages did not express IL-10 at detectable levels. Reports of IL-10 production from PMA differentiated THP-1s are contradictory

depending on the stimulus used, concentration of PMA and culture duration [409, 410]. We can therefore speculate that LPS stimulus at 10ng/ml was not high enough to activate this pathway. In fact, Morris et al have hypothesised that high concentrations of LPS are required to induce production of IL-10 from these cells [409]. Future studies could therefore investigate IL-10 production from miR-125a sponge expressing cells stimulated with higher concentrations of LPS.

The pro-inflammatory phenotype of miR-125a sponge expressing PMA cells was mirrored by primary human macrophages transfected with a miR-125a inhibitor. By day 7, miR-125a inhibited macrophages produced significantly higher levels of TNF $\alpha$ , IL-12 and CCL4. Interestingly, production of IL-10 in these cells was significantly downregulated. Transfection of these cells with a miR-125a mimic did not produce the results expected with regards to TNF $\alpha$ , which was also significantly increased in these cells, suggesting a dual role for miR-125a in TNF $\alpha$  regulation. Transfection of macrophages with a miR-125a mimic did however significantly increase production of IL-10, suggesting regulation of this cytokine.

miRs alter cytokine and chemokine production by either directly targeting these molecules themselves, or regulators within the signalling cascades of these responses. Based on the profile of these cells in response to LPS, we can hypothesise molecules which would account for this pro-inflammatory phenotype and propose models by which miR-125a may regulate these important mediators.

The dual role of miR-125a in regulating cytokine production has been hinted at previously, through the regulation of NF $\kappa$ B in CD34+ cells of myelodysplastic syndrome. Gomez et al demonstrated that overexpression of miR-125a in normal cells resulted in an increase in NF $\kappa$ B activity, whereas after TLR activation, it was inhibition of miR-125a that had this effect [393]. Additionally, there have been previous conflicting studies detailing the role played by miR-125a in polarisation of macrophages. Banerjee et al have demonstrated a decrease in TNF $\alpha$  and IL-12 production after overexpression of miR-125a in LPS stimulated, GM-CSF, differentiated murine macrophages. On the other hand, Graff et al demonstrated a slight increase in TNF $\alpha$  in M1 polarised macrophages after LPS stimulation [339, 368]. The differences in results from these experiments may be due to their choice and characterisation of cells. The Graff study of

macrophage polarisation also used human M-CSF differentiated macrophages which would explain the similar results to this study.

The list of predicted targets for miR-125a also suggests this miR may play differing roles in regulation of inflammatory cytokine production. According to prediction algorithms (TargetScan and RNA22), miR-125a can potentially target pro-inflammatory mediators TNF $\alpha$ , TLR4, TRAF6 and NF $\kappa$ B essential modulator kinase (NEMO). On the other hand miR-125a also potentially targets negative regulators of inflammatory signalling TNFAIP3, interferon regulatory factor 4 (IRF4), Inhibitor of nuclear factor kappa-B kinase subunit epsilon (IKK $\epsilon$ ) and TNFAIP3-interacting protein 2 (TNIP2). We can therefore speculate that miR-125a, depending on cell type and cellular environment may play differing roles in regulating the inflammatory response. Interestingly TRAF6 also mediates IL-12 production in response to infection, which may explain the upregulation of this cytokine after miR-125a inhibition [411].

The increased expression of TNF $\alpha$  in culture may also have effects on the production of other inflammatory mediators such as IL-6 and CCL4. However the list of potential miR-125a targets again allows us to construct hypotheses which can explain alterations in these molecules.

We have demonstrated that miR-125a is a negative regulator of CCL5 expression in response to LPS. CCL5, a chemokine with chemoattractant properties for T cells has been found to be upregulated in a number of autoimmune diseases, including RA and Systemic Lupus Erythematosus (SLE) [216, 343]. miR-125a regulation of CCL5 has also been observed by another group. Zhao et al have shown a downregulation of miR-125a in SLE CD4<sup>+</sup> T cells, which correlates with an upregulation of CCL5. They demonstrate that miR-125a directly targets the transcription factor (tf) kruppel like factor 13 (KLF13) which binds the CCL5 promoter and induces its expression in T cells. Overexpression of miR-125a results in a decrease in both this tf and CCL5 protein [343]. KLF13 is also expressed in both PMA differentiated THP-1 cells and primary human macrophages [339, 412]. We can therefore speculate that miR-125a regulates CCL5 production, through the targeting of KLF13 in these cells in a similar manner to T cells. In addition to CCL5 it appears CCL4 expression is also negatively regulated by miR-125a. CCL4, a chemoattractant for a variety of

immune cells, is a potential target of miR-125a. Direct targeting of this chemokine would explain the early upregulation (after 6 hours) witnessed post LPS stimulation, in miR-125a expressing sponge cells.

Another important pro-inflammatory cytokine upregulated after the inhibition of miR-125a is IL-6. This may be the result of miR-125a targeting of molecules involved in the IL-6 signalling pathway. miR-125a is predicted to bind both membrane bound IL-6R and also gp130. In addition to the receptor, miR-125a also potentially targets the downstream signalling molecule STAT3. It is known that IL-6 can have autocrine effects on macrophages, therefore we can hypothesise that under miR-125a inhibition, levels of STAT3 increase, allowing production of higher amounts of IL-6, which can then be responded to in macrophages, as levels of IL-6R and gp130 also increase.

This data suggests that loss of miR-125a results in an increased inflammatory response to LPS through the increased production of pro-inflammatory cytokines. What is interesting however, is the production of IL-10 in these cells. IL-10 is a potent anti-inflammatory cytokine which regulates inflammation. It is important that inflammation in response to injury or infection is regulated, to prevent an unreasonable immune response, which can result in autoimmunity. Only 3 families of miR contain conserved binding sites within the IL-10 3'UTR, miR-27, miR-194, and interestingly the Let-7 family. The relationship between miR-125a and IL-10 production suggests miR-125a directly targets a negative regulator of IL-10 production. From the list of miR-125a targets we have hypothesised a number of negative regulators, which may account for this IL-10 expression pattern. miR-125a potentially targets IL-10R itself and negative regulators of IL-10 production- V-Ets Avian Erythroblastosis Virus E26 Oncogene Homolog (ETS-1) and histone deacetylase 11 (HDAC11).

The phenotype observed in miR-125a mimic transfected macrophages highlights the limitations of these types of studies. Firstly, the mimic used for transfection is a double stranded RNA molecule and previous studies have demonstrated the ability of these mimics to activate the interferon response through TLR3 and therefore disrupt any functional analysis. To attempt to address this possibility we measured levels of INF response genes, OAS1, OAS2, MX1, IFTIM1 and IRF9 in transfected cells and also in Poly I:C stimulated THP-1 cells as a positive control.

The results from the transfected cells were different than the positive control, in which there was an induction of OAS1, MX1, IFTIM1 and IRF9. In transfected cells there was a downregulation of OAS1 and IFTIM1. However on closer inspection, these 2 genes are potential miR-125a targets. The induction of IRF9 was not to the same level as witnessed in the positive control, therefore we cannot conclude that miR-125a itself is immunogenic. Another limitation of mimic studies is that the introduction of high levels of mimic into a cell results in non-physiological levels of miR which can then have off target effects. We can therefore also speculate that the effect of miR-125a on TNF $\alpha$  production is one of these off-target effects.

To my knowledge this study is the first to generate and use a functional miR-125a sponge to investigate the role of this miR in the response to LPS in macrophages. miR-125a regulation of NF $\kappa$ B, TNF $\alpha$ , IL-12 and CCL5 have been identified previously. However no concrete pathway of regulation of TNF $\alpha$ , or IL-12, have been identified to date. This is the first study to identify a link between miR-125a expression and regulation of IL-6, IL-10 and CCL4. From this data, so far, we can speculate that upregulation of miR-125a in SF CD14 $^{+}$  cells forms part of an attempt at regulating the high levels of inflammation within the joint. As previously demonstrated miR-125a expression is increased in PB CD14 $^{+}$  cells of those responding well to DMARD therapy. This functional study suggests that in these patients miR-125a may contribute to increased levels of IL-10 production, whilst applying a break to the production of pro-inflammatory IL-12, IL-6 and CCL4 by macrophages, however its role in TNF $\alpha$  production remains to be elucidated.

The following chapter will go onto test the hypotheses identified here with regards to miR-125a regulation of its potential targets.

## Chapter 5 – miR-125a is a Regulator of TLR4 Signalling

### 5.1 Introduction and aims

The previous chapter has demonstrated that dysregulation of miR-125a in macrophages alters the cytokine and chemokine response to LPS in these cells. Inhibition of endogenous miR-125a results in the development of a hyper inflammatory phenotype manifested by increased production of TNF $\alpha$ , IL-6, IL-12 and decreased production of anti-inflammatory IL-10. Interestingly, superfluous overexpression of miR-125a results in increased production of both IL-10 and TNF $\alpha$ . This chapter will therefore investigate potential mechanisms underlying miR-125a mediated phenotypes, through the use of online prediction algorithms and then validation experiments.

MiRs function to regulate gene expression through targeting complementary sequences, usually within the 3'UTR of target genes, resulting in their down-regulation. A major goal of miR research is therefore to attribute a functional role to a miR within a given cell type, as by understanding its function, it may prove therapeutically useful in disease. To understand miR functions, their predicted target genes must be identified and validated. As one miR can target many different mRNAs, it is therefore possible that miRs can regulate different pathways concurrently, making them attractive molecules for therapy. An example of this is miR-34a in cancer development. This miR has been shown in various studies to function as a tumour suppressor in many forms of cancer, including breast, brain and liver malignancies, and multiple targets of this miR have been identified. Diage et al have recently demonstrated that delivering a miR-34a mimic to mice suffering from hepatocellular carcinoma (HCC) causes regression of tumours. A single delivery has been shown to inhibit genes in 5 oncogenic pathways: Wnt/Catenin, Hedgehog, VEGF, c-Met and MAPK, whilst stimulating the activation of the suppressed p53 pathway [413]. Concurrently, miR-34a replacement therapy for liver cancer has now entered phase I human clinical trials, demonstrating the importance of these molecules in the regulation of almost all cellular processes [337].

As we have identified an up-regulation of miR-125a in RA CD14<sup>+</sup> cells, and demonstrated a pro-inflammatory phenotype in macrophages after miR-125a inhibition, it is important to identify and validate direct targets of this miR, to determine its functional role within these cells in both health and disease.

miR target identification is accomplished by using computer-based algorithms that identify MREs within mRNAs (reviewed in 1.6.3). Additionally, many studies also begin by using transcriptomic analysis such as microarrays to investigate mRNA expression after transfection of a miR of interest. This method narrows down the number of potential targets, as it is based on experimental changes in mRNA. However, microarrays remains relatively expensive and, as some miRs show only modest effects on degradation of target transcripts, it is feasible to use only online prediction algorithms to identify targets responsible for the miR driven cell phenotypes.

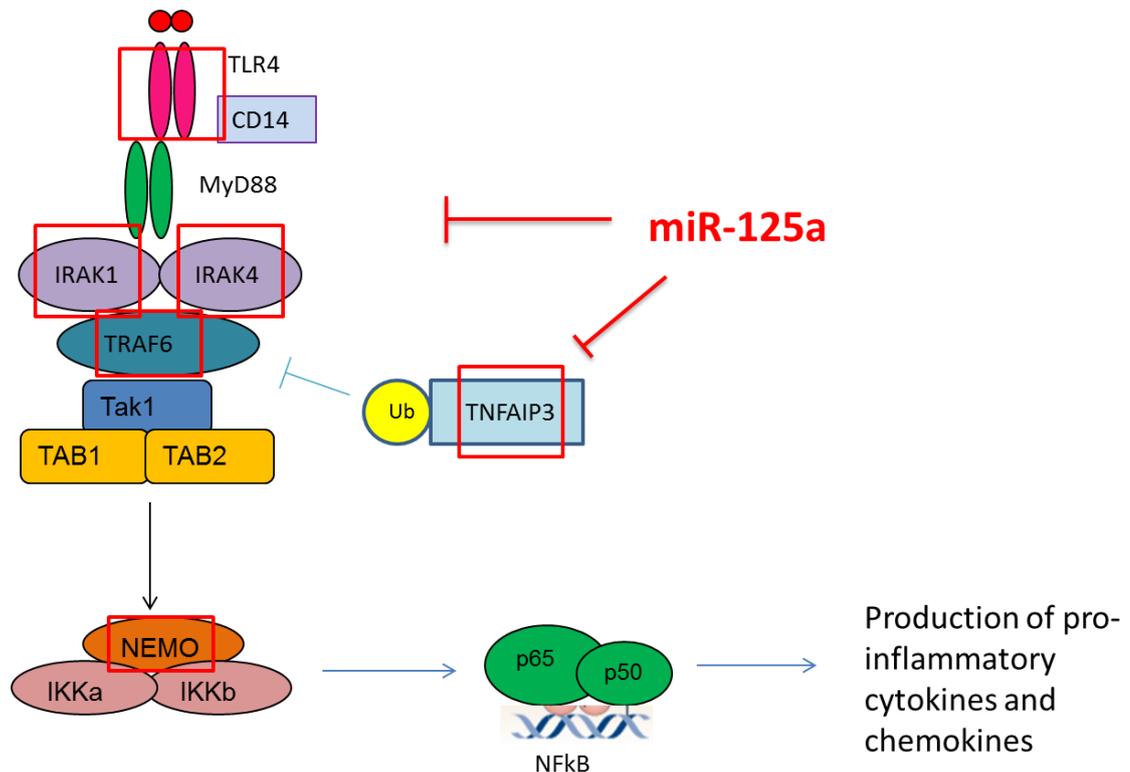
Chapter objectives -

- Identify and validate miR-125a targets in macrophages.
- Functionally demonstrate miR-125a regulation of candidate targets.

## 5.2 Results

### 5.2.1 miR-125a potentially targets members of the TLR4 signalling pathway

As the pro-inflammatory macrophage phenotype was witnessed after activation with the TLR4 ligand LPS, we searched for potential miR-125a targets related to the activation and function of this signalling pathway. Using *in-silico* analysis by using the TargetScan and RNA22 online algorithms, we identified 6 molecules which were potential miR-125a targets, but were also fundamental in the activation and regulation of the TLR4 signalling pathway. These targets were TLR4 itself, and the downstream signalling molecules IRAK1, IRAK4, TRAF6, NEMO (Inhibitor of nuclear factor kappa-B kinase subunit  $\gamma$ ) kinase and the inhibitor of NF $\kappa$ B, TNFAIP3 (Figure 5.1).



**Figure 5.1 Simplified schematic of potential miR-125a targets involved in TLR4 signalling** miR-125a potentially targets at least 7 members of the TLR4 signalling pathway (highlighted within red boxes). NEMO, Inhibitor of nuclear factor kappa-B kinase subunit  $\gamma$ ; Ub, ubiquitin.

For each of the potential targets we examined their characteristics using the TargetScan and RNA22 algorithms (Table 5.1). Firstly we used the TargetScan algorithm to determine if miR-125a potentially targeted the molecule and examined its context score. The context score is the sum of the contribution of six features: site-type, 3' pairing contribution, local AU contribution, position, TA (target site abundance) and SPS (seed-pairing stability). The higher the context score, the more likely that it will be a positive miR-125a target. Additionally, we examined the seed match that was predicted, 8mer displays perfectly complementary binding to the seed region, and 7mer and exact match to 7 of the nucleotides within the seed region.

Next, we identified if the binding site was conserved across species, and finally if the predicted target was also predicted by the RNA22 algorithm.

**Table 5.1 In-Silico Analysis of TLR4 signalling pathway**

Potential Target	Targetscan	Context Score/Percentage	Seed Match	Conserved	RNA22
TLR4	Yes	-0.05/33	7mer-1A	Poorly	Yes
IRAK1	Yes	-0.17/78	7mer-1A	Poorly	Yes
IRAK4	No				Yes
TRAF6	Yes	-0.14/65	8mer	Conserved	No
NEMO	Yes	-0.33/94	8mer	Poorly	Yes
TNFAIP3	Yes	-0.33/94	8mer	Conserved	Yes

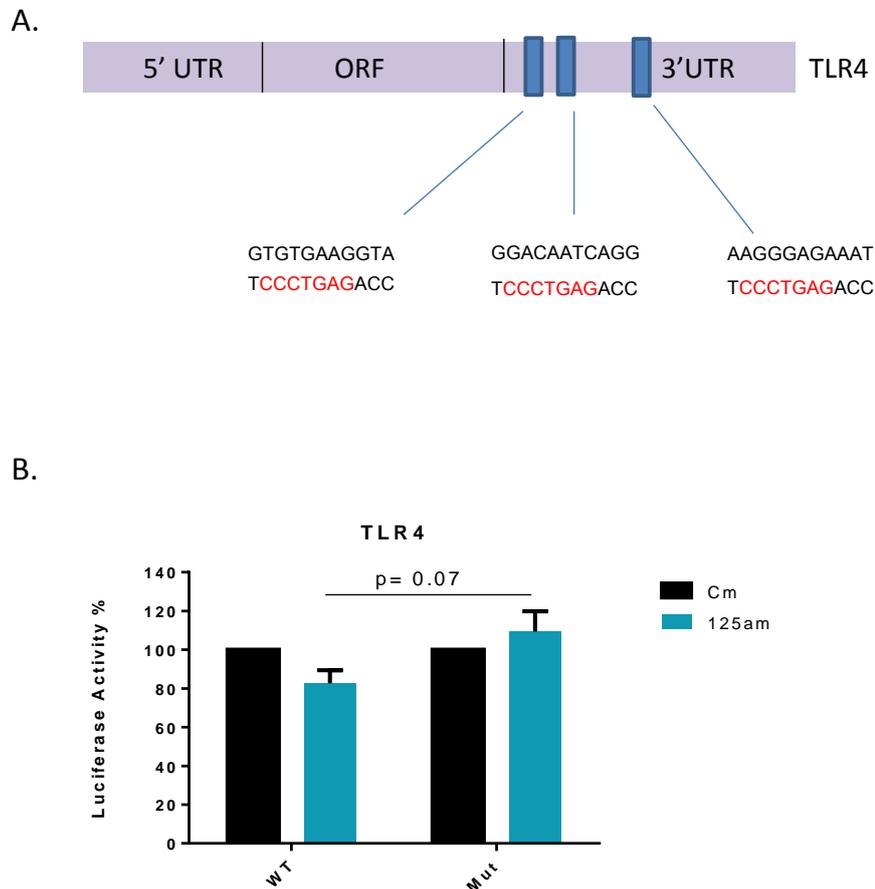
Context Score-indicates how likely it is that the molecule will be a positive target based on the contribution of different features including seed pairing, AU content, stability and abundance. Seed Match-identifies the complementarity of binding between the miR seed region and the site in the mRNA 3'UTR (7mer-7 nucleotide match, 8mer-8nucleotide match).

Based on this information we decided to investigate miR-125a targeting of TLR4, TRAF6 and TNFAIP3. The 3'UTRs of TRAF6 and TNFAIP3 both contain conserved miR-125a binding sites, with context score percentage above 60%. Although the context score of miR-125a binding of TLR4 is low, the presence of 3 potential binding sites identified by the RNA22 algorithm, and its importance in the activation of inflammatory pathways suggested it would be important to investigate the potential regulation of this molecule by miR-125a.

### 5.2.1.1 miR-125a potentially regulates TLR4 expression

As it was beyond the scope of this project to investigate each of these molecules individually, we decided to begin at the receptor level and validate TLR4 as a miR-125a target gene. Using the RNA22 algorithm, we identified 3 potential miR-125a binding sites in the 3' UTR of TLR4 (Figure 5.2A), designed a G-Block® construct (as described in 2.5.1) which would include these binding sites, and inserted this sequence downstream of a luciferase reporter gene (as described in 2.5.1). This construct was termed wild-type (WT) as it contained the miR-125a MREs. Concurrently, we designed a sequence in which these binding sites were altered, termed mutated (Mut) (sequences of G-Block fragments detailed in 2.5.1). If miR-125a directly targets the TLR4 gene, luciferase activity should be decreased with the WT construct. Alterations in the binding sites, as in the Mut construct, would prevent miR-125a binding, and therefore rescue downregulation of luciferase, confirming direct targeting of TLR4 by miR-125a.

To analyse luciferase expression, HEK 293 cells were co-transfected with either the WT or Mut TLR4 construct, and either a Cm or 125am, and cultured overnight. After this, cells were lysed, and luciferase expression was measured (Figure 5.2B).



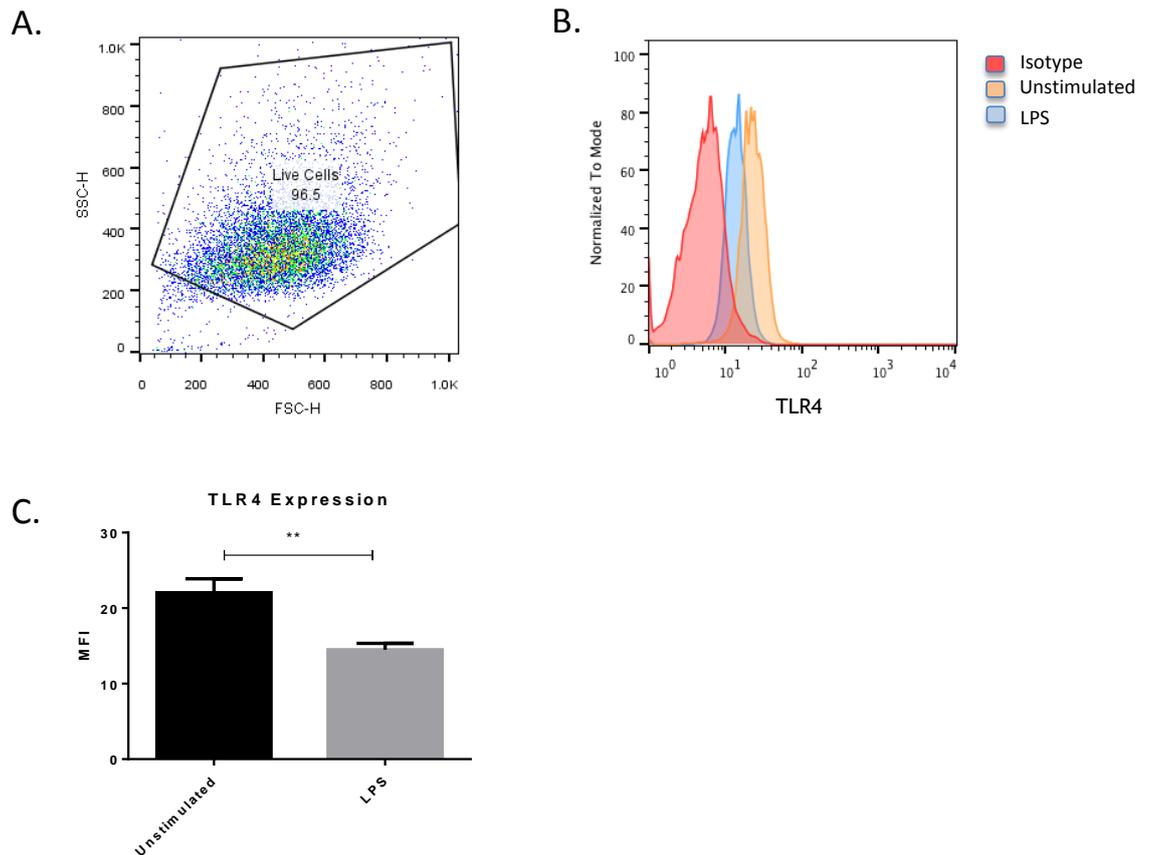
### Figure 5.2 Targeting of TLR4 by miR-125a

A) Using the RNA22 algorithm we identified 3 potential miR-125a binding sites (shown in red) in the 3'UTR of the TLR4 gene. B) HEK293 cells were transfected with either WT or Mut constructs along with a Cm or miR-125am and luciferase expression was measured after 24 hours in culture. Luciferase expression was downregulated by 125am in only cells transfected with WT construct, this downregulation was rescued in cells which were transfected with Mut construct. Values presented as mean  $\pm$  SEM of 4 experiments.  $p=0.07$  unpaired t-test WT vs. Mut. Cm: Control mimic, 125am: miR-125a mimic.

Results demonstrated that cells transfected with the WT TLR4 construct and a 125am displayed slightly lower luciferase expression than those transfected with a Cm, suggesting miR-125a may directly target TLR4. In this experiment we compared the luciferase expression between WT and Mut constructs which have been co-transfected with a miR-125a mimic, and found that knockdown of luciferase expression in the WT was rescued by mutation of the miR-125a binding

sites within the 3'UTR in the Mut construct. This suggests that knock down of luciferase is dependent on the presence of miR-125a MREs. This difference did not reach significance ( $p=0.07$ ) when analysed by an unpaired t-test, and therefore we cannot confidently conclude that miR-125a directly targets TLR4. However, this knockdown in luciferase expression suggests a potential relationship, which may be elucidated through further experiments. Therefore, we decided to investigate this relationship further at the protein level by flow cytometry.

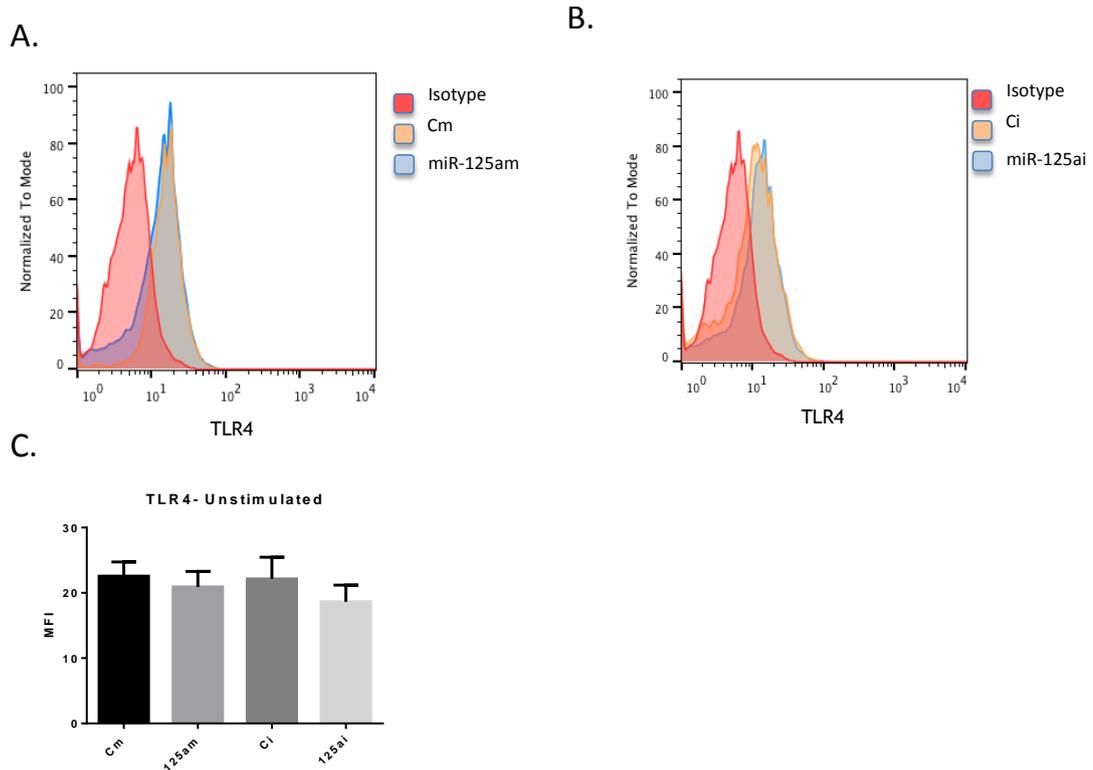
Before the manipulation of miR-125a levels, we firstly wished to determine the expression of surface TLR4 on resting healthy macrophages, and its expression after activation with LPS (10ng/ml). To do this we differentiated CD14<sup>+</sup> cells towards a macrophage phenotype with M-CSF (50 ng/ml), as described previously (in 2.2.2.4). On day 7, we removed the adherent macrophages using a dissociation solution, stained for TLR4, and analysed by flow cytometry. We gated on live cells based on size (FSC) and granularity (SSC) (Figure 5.3A), and results showed that almost all macrophages expressed TLR4 on their surface (Figure 5.3B blue histogram). The results also demonstrated that macrophage surface TLR4 expression is significantly decreased by 24 hour LPS stimulation, in keeping with the role of TLR4 expression in the induction of LPS tolerance (Figure 5.3 B orange histogram and C).



### Figure 5.3 Macrophage surface TLR4 expression

Macrophages were differentiated using M-CSF (50ng/ml) and stimulated with LPS (10ng/ml) for 24hr or left unstimulated. Cells were then stained for surface TLR4 expression. A) Live cells were gated on based on size and granularity B) Representative histogram showing TLR4 staining of macrophages. Almost 100% of human macrophages express surface TLR4 (blue histogram). This expression is decreased by stimulation with LPS (orange histogram). C) Mean Fluorescence Intensity (MFI) values show that TLR4 expression is significantly downregulated by LPS stimulation. Values presented as mean  $\pm$  SEM of 4 experiments, \*\* =  $p < 0.005$ , paired t-test.

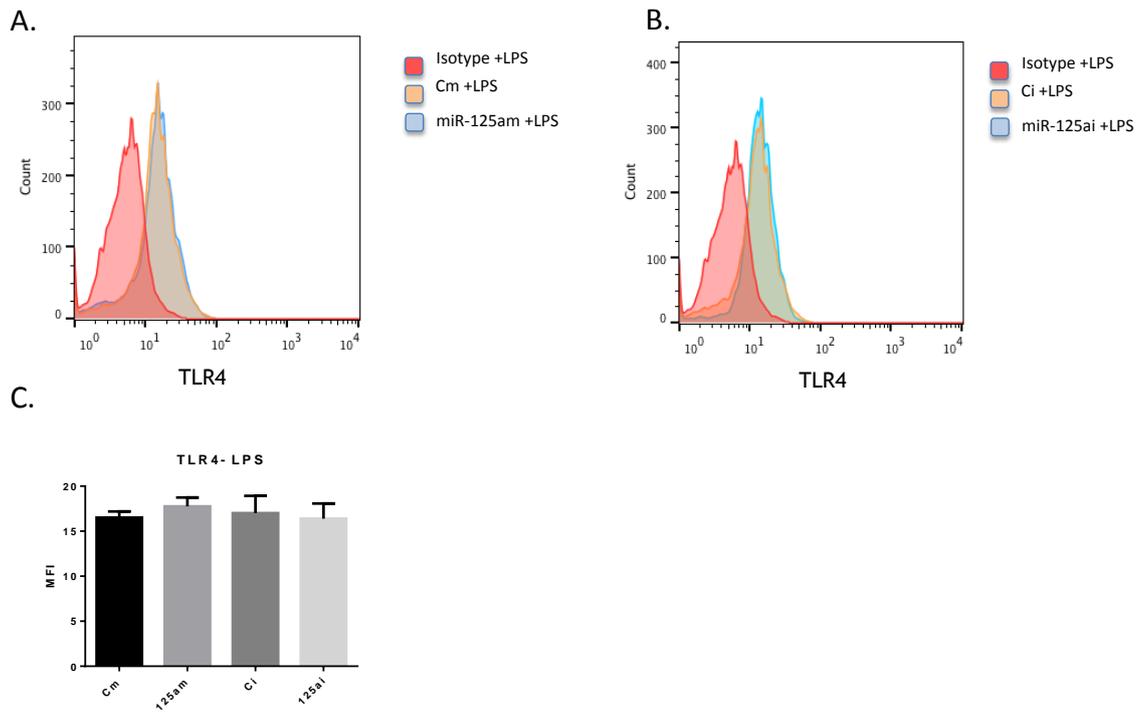
As we could measure TLR4 on the surface of human macrophages, we next set out to determine if the manipulation of miR-125a levels within these cells would change expression of surface TLR4. To examine the effect of overexpression of miR-125a, healthy day 7 macrophage cells were transfected with a Cm or miR-125am, and to examine the effects of inhibition, cells were transfected with either a Ci or miR-125ai. After transfection, cells were cultured overnight. Results demonstrated that although macrophages express high levels of surface TLR4, manipulation of miR-125a in these cells did not alter TLR4 expression. There was no significant difference in expression between cells overexpressing miR-125a (Figure 5.4A and C), or in cells in which miR-125a was inhibited (Figure 5.4B and C), shown by comparison of histograms, and comparison of MFI values.



**Figure 5.4 Surface TLR4 expression of resting transfected macrophages**

Levels of miR-125a were manipulated in macrophages through transfection, after which TLR4 expression was measured by flow cytometry. A) Overexpression of miR-125a does not alter surface TLR4 expression. B) Inhibition of miR-125a does not significantly alter surface TLR4 expression. C) MFI values confirm that manipulation of miR-125a does not alter surface TLR4 expression. Values presented as mean  $\pm$  SEM of 4 experiments, and no significant difference was observed using a paired t test.

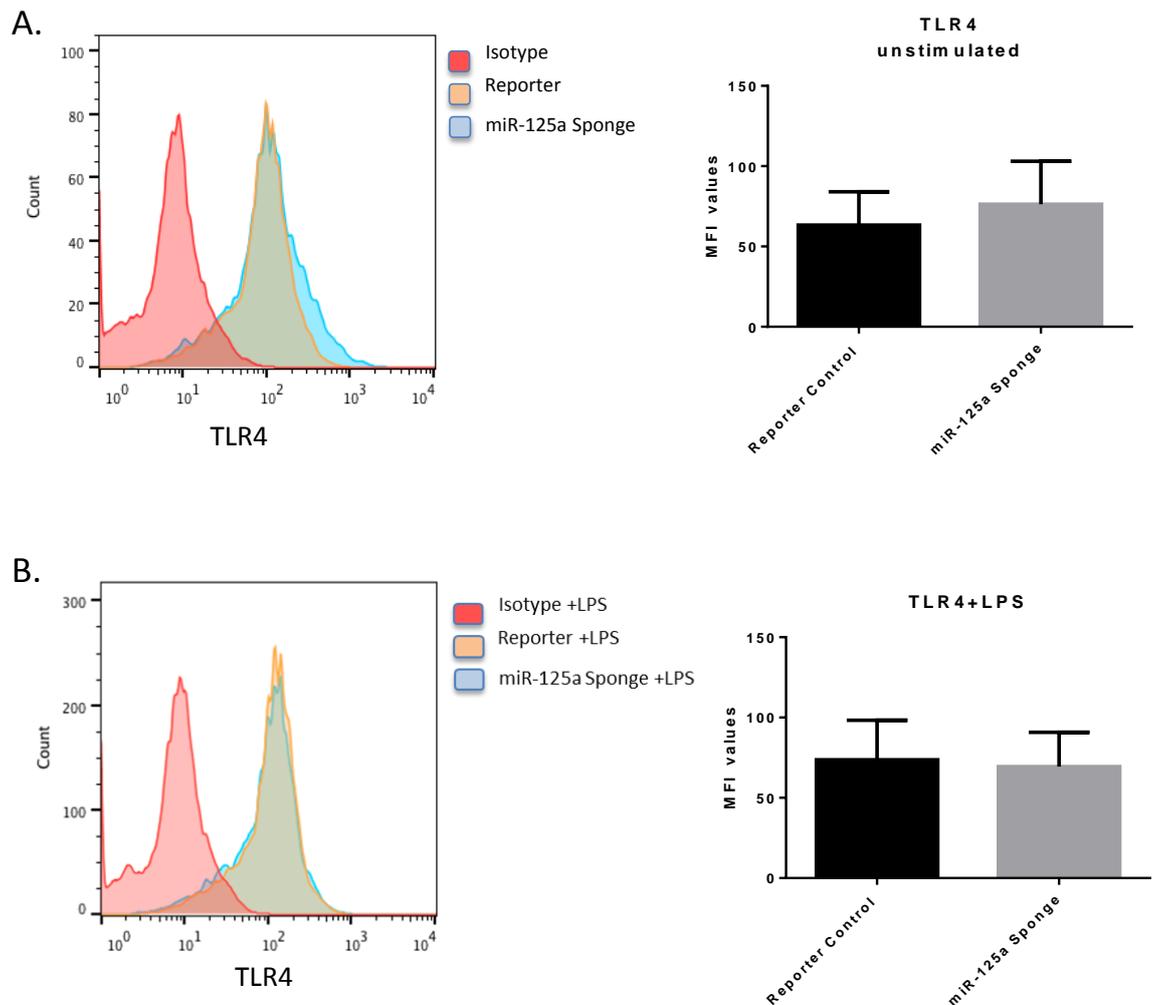
As the pro-inflammatory phenotype was witnessed in activated macrophages, in subsequent experiments we examined the expression of surface TLR4 in LPS stimulated macrophages (10ng/ml). Again, results demonstrated that overexpression and inhibition of miR-125a by transfection had no effect on surface TLR4 on activated macrophages (Figure 5.5 A-C), comparing both cell percentage and MFI values.



**Figure 5.5 Surface TLR4 expression of LPS stimulated transfected macrophages**

Levels of miR-125a were manipulated in macrophages through transfection, after which cells were stimulated with LPS (10ng/ml) and TLR4 expression was measured by flow cytometry. A) Overexpression of miR-125a does not alter surface TLR4 expression. B) Inhibition of miR-125ai does not alter surface TLR4 expression. C) MFI values confirm that manipulation of miR-125a does not alter surface TLR4 expression. Values presented as mean  $\pm$  SEM of 4 experiments, with no significance detected after analysis with a paired t-test.

In the final testing of our hypothesis we examined TLR4 expression on miR-125a sponge containing THP-1 cells, as sponge technology is more efficient in inhibiting miRs than anti-miRs. Firstly, we examined undifferentiated cells and found that although these cells express high levels of TLR4, there is no difference between reporter control, or miR-125a sponge expressing cells, regardless of LPS stimulation (Figure 5.6 A and B).



### Figure 5.6 TLR4 expression of miR-125a THP-1 sponge cells

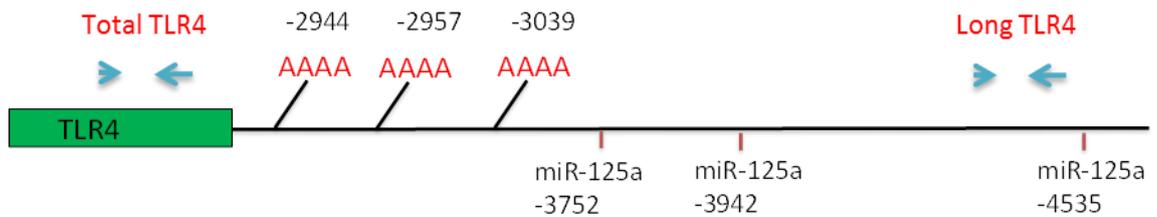
Reporter control and miR-125a sponge expressing THP-1 cells were stained for surface TLR4 expression, and analysed by flow cytometry. A) Representative histogram of TLR4 staining in unstimulated cells. Although cells expressed high levels of TLR4 compared to isotype (blue histogram), there was no difference in TLR4 expression between reporter control (red histogram) or miR-125a sponge (orange histogram) expressing THP-1 cells, as also shown by MFI values. B) There was also no difference in TLR4 expression between reporter control (red histogram) or miR-125a sponge (orange histogram) expressing LPS stimulated THP-1 cells, as also shown by MFI values. Values presented as mean  $\pm$  SEM of 2 experiments.

As we could not identify a direct relationship between miR-125a and TLR4 we next examined the mRNA transcript of TLR4. We wanted to investigate polyadenylation of TLR4 in macrophages, to determine if different transcripts of this molecule were expressed by the cell under different conditions.

A number of different polyadenylation sites have been identified within TLR4 (Figure 5.7), which would cause the expression of a shorter transcript, that would not contain miR-125a binding sites.

We therefore wanted to determine if indeed these shorter transcripts were expressed by macrophages after LPS stimulation, removing the ability of miR-125a to regulate TLR4.

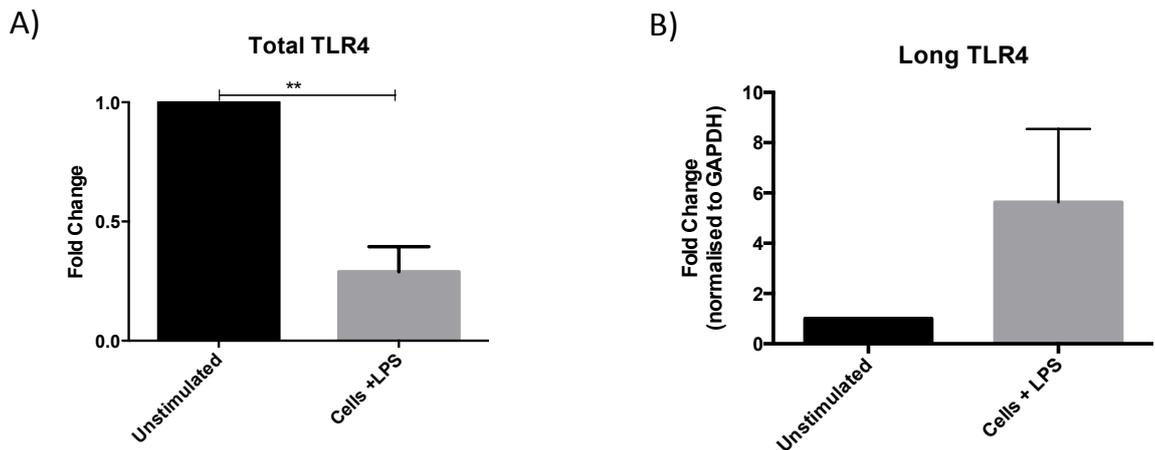
We designed PCR primers which would amplify total TLR4 (a region of the mRNA expressed by all transcripts) and long TLR4 ( a longer version of the transcript) which would contain miR-125a binding sites (Figure 5.7).



**Figure 5.7 Validated TLR4 polyadenylation sites**

NCBI details polyadenylation sites within the 3'UTR of TLR4. These sites are indicated in red in the figure above, and show that at least 3 of these variants would cause the loss of sites required for miR-125a regulation. Additionally PCR primers for different transcript variant sites are indicated with blue arrows.

Macrophages were cultured for 7 days as previously described, and on day 7 they were either left unstimulated, or LPS stimulated for 24 hours, after which TLR4 variant expression was measured by q-PCR.



**Figure 5.8 TLR4 variants expressed by resting and LPS stimulated macrophages**

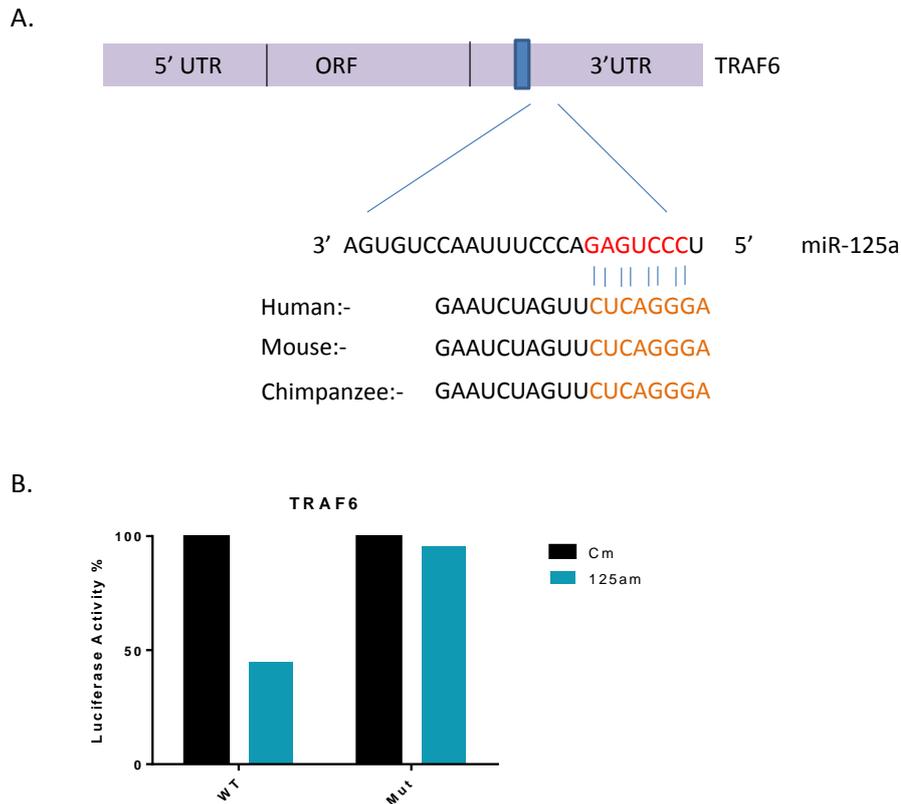
q-PCR primers were designed that would amplify total TLR4 expression and the expression of a long variant of TLR4. These variants were measured in both unstimulated and LPS stimulated macrophages. A) Expression of total TLR4 is significantly decreased after stimulation with LPS. B) Expression of the longer variant of TLR4 appears to increase after LPS stimulation. Values presented as mean  $\pm$  SEM of n=4 experiments (total TLR4) and n=3 experiments (Long TLR4) and marked bars are statistically different from each other using a paired T-Test \*\*=p<0.005

Results showed that LPS stimulation of macrophages significantly decreases the expression of total TLR4 in macrophages (Figure 5.8A). Additionally, it appears that although total TLR4 is downregulated after LPS expression, levels of the longer variant appear to increase when compared to unstimulated cells (Figure 5.8 B).

As we could not identify a direct relationship between miR-125a and TLR4 we next wanted to investigate miR-125a targeting of additional molecules within the TLR4 pathway, which may be responsible for the phenotype, we have identified.

**5.2.1.2 miR-125a regulates TRAF6 expression**

Using the TargetScan algorithm, we identified a highly conserved miR-125a MRE in the 3'UTR of the TRAF6 gene. TRAF6 is a key transduction molecule in the TLR signalling pathway. This site was conserved between human, mouse and chimpanzee, suggesting an important regulatory function of this site within mammals (Figure 5.9 A). Again, we designed a G-Block® construct which would include this binding site, and a separate construct in which this MRE was mutated. This sequence was then inserted downstream of a luciferase reporter gene. As previously, to analyse miR-125a binding potential, a luciferase reporter assay was carried out.



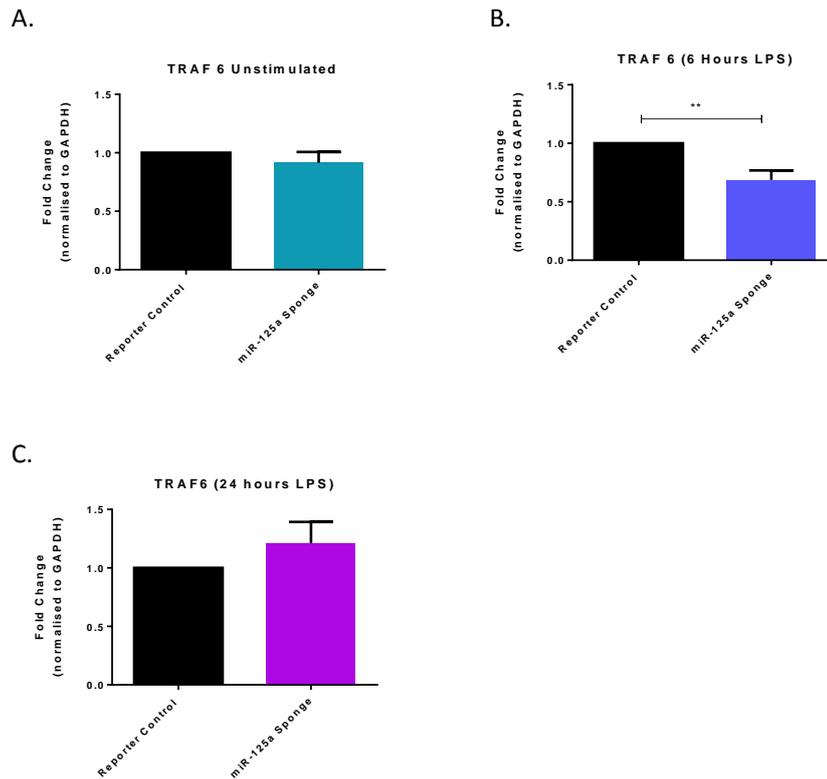
### Figure 5.9 Targeting of TRAF6 by miR-125a

A) Using the TargetScan algorithm we identified a potential miR-125a binding site in the 3'UTR of TRAF6 (shown in red) which was highly conserved across vertebrate species (shown in orange)  
 B) HEK293 cells were transfected with either WT or Mut constructs along with a Cm or miR-125am and luciferase expression was measured after 24 hours in culture. Luciferase expression was downregulated by 125am in only cells transfected with WT construct. However results shown represents only 1 preliminary experiment.

Results demonstrated that transfection of the WT construct along with a miR-125am resulted in a decrease in luciferase expression when compared to a Cm. This decrease in luciferase expression was then rescued by mutation of the miR-125a MRE (Figure 5.9 B). Due to time constraints and HEK293 cell culture problems, this experiment was only performed once. We are confident however that miR-125a does in fact target TRAF6 in macrophages, as a recent study led by Guo et al confirmed that miR-125a directly targets TRAF6 in monocyte derived-osteoclasts, and its overexpression can prevent osteoclastogenesis [370].

Therefore, we next wanted to determine if PMA differentiated miR-125a sponge expressing cells expressed higher levels of TRAF6. As miRs have been shown to repress translation and cause a measurable downregulation of mRNA, we differentiated cells for 7 days as previously, and analysed the expression of

TRAF6 mRNA by q-PCR. We investigated TRAF6 expression in unstimulated cells, and cells stimulated with LPS for both 6 and 24 hours, as we had previously witnessed differences in pro-inflammatory cytokine production at these time points.



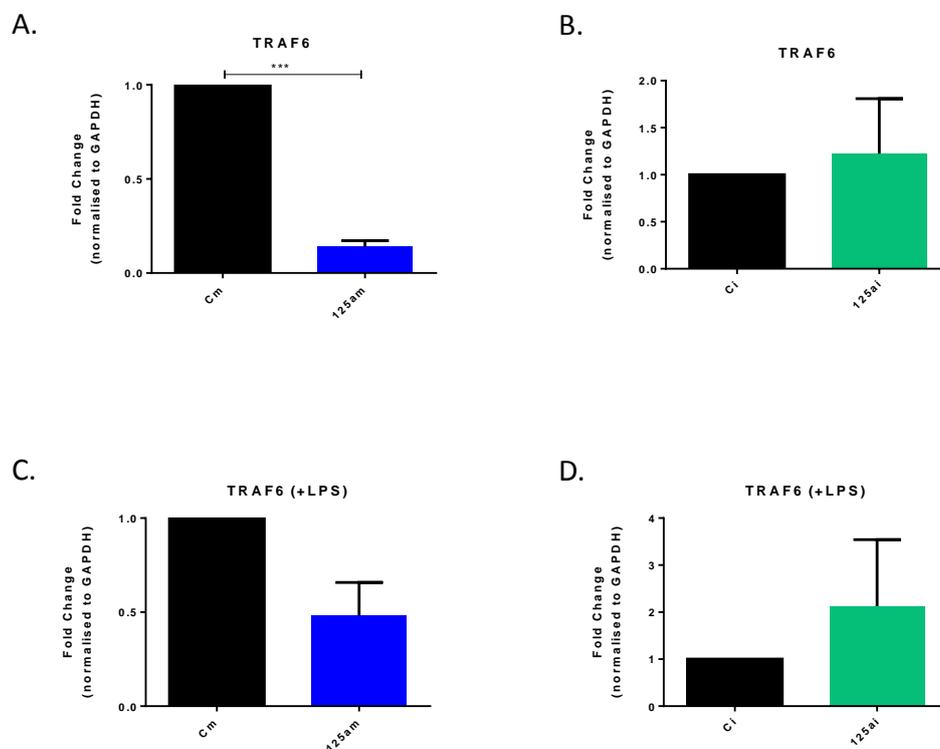
**Figure 5.10 TRAF6 mRNA expression of miR-125a sponge expressing cells**

Expression of TRAF6 mRNA was measured by q-PCR in 7 day PMA differentiated THP-1 cells expressing miR-125a sponge. A) The expression of TRAF6 mRNA is not altered between reporter control and miR-125a sponge expressing unstimulated cells. B-C) Cells were stimulated with LPS (10ng/ml) for 6 and 24h. The expression of TRAF6 mRNA appears significantly lower in miR-125a sponge expressing cells compared to reporter control cells when stimulated with LPS for 6 hours. There is no significant difference in the expression of TRAF6 mRNA between reporter control and miR-125a sponge expressing cells after 24 hour LPS stimulation. Values presented as mean  $\pm$  SEM of 4 experiments and marked bars are statistically different from each other using an unpaired t-Test \*\*= $p < 0.005$

Results demonstrated that expression of TRAF6 was not significantly altered between differentiated reporter control and miR-125a sponge expressing cells when in a resting state, or stimulated with LPS for 24 hours (Figure 5.10 A and C). However, results showed that TRAF6 mRNA is significantly downregulated in differentiated miR-125a sponge expressing cells 6 hours after LPS stimulation

when compared to reporter control (Figure 5.10 B). This suggests that miR-125a may indeed interact with TRAF6 in the early response to LPS.

Next, we wanted to determine whether similar miR-125a/TRAF6 mRNA relationship exists in primary human macrophages in which miR-125a levels had been manipulated. We prepared cDNA of cells from earlier phenotyping experiments showing an inhibitory effect of miR-125a on cytokine production (Chapter 4). Briefly, healthy CD14<sup>+</sup> cells were separated and differentiated, as previously described, and miR-125a levels were manipulated by transfection on day 7. Cells were left unstimulated, or stimulated with LPS for 24 hours, after which levels of TRAF6 mRNA was measured by q-PCR.



### Figure 5.11 TRAF6 mRNA in miR-125a transfected human macrophages

Human macrophages were differentiated for 7 days, after which miR-125a expression was manipulated by transfection followed by stimulation with LPS (10 ng/ml) for 24hrs and levels of TRAF6 mRNA were measured by q-PCR. A) Expression of TRAF6 mRNA is significantly downregulated in non-simulated macrophages overexpressing miR-125a. B) TRAF6 expression is not altered in unstimulated macrophages in which miR-125a is inhibited. C) TRAF6 mRNA expression is lower in LPS stimulated macrophages which overexpress miR-125a. D) There is no difference in TRAF6 mRNA expression in LPS stimulated macrophages in which miR-125a is inhibited. Values presented as mean  $\pm$  SEM of 4 experiments (apart from D, n=2 experiments) and marked bars are statistically different from each other using a paired T-Test \*\*= $p < 0.005$ .

Results demonstrated that overexpression of miR-125a significantly decreased the expression of TRAF6 mRNA in resting cells, with this downregulation trending towards significance in cells stimulated with LPS ( $p=0.06$ ) (Figure 5.11 A and C) supporting the hypothesis that miR-125a has potential to regulate TRAF6. However, there appeared to be no difference in TRAF6 mRNA expression between cells that were transfected with a Ci or miR-125ai, regardless of LPS stimulation (Figure 5.11 B and D). Levels of TRAF6 mRNA in LPS stimulated macrophages was only measured in 2 samples, due to RNA quality from cells, and time constraints. Moreover, the lack of TRAF6 inhibition upon endogenous miR-125a neutralisation could be due to the selection of a time-point in which conditions have not been optimised (24hr). We showed that in LPS stimulated miR-125a sponge THP-1 cells interaction occurred at 6 but not 24hr time point. Therefore, this requires additional time course experiments, that will include more donors and early time points.

We have shown that miR-125a has the potential to bind TRAF6, demonstrated by the preliminary luciferase assay and miR-125 overexpression experiments in macrophages. Further studies are required to fully dissect the role of TRAF6 in the pro-inflammatory phenotype seen when endogenous miR-125a is inhibited in our system.

### **5.2.2 miR-125a potentially targets pro-inflammatory TNF $\alpha$ and CCL4**

In addition to signalling molecules, we also investigated whether miR-125a can directly target pro-inflammatory cytokines themselves, which would explain an increase in these cytokines when miR-125a is inhibited.

Again, we used the target algorithms TargetScan and RNA22 to determine if miR-125a could potentially regulate any of the cytokines we have identified as significantly altered in macrophages in which miR-125a is inhibited, TNF $\alpha$ , IL-12, IL-6 and CCL4.

From this search we determined that there are no potential binding sites in the 3' UTRs of IL-12 or IL-6, and therefore increases in these cytokines must be due

to indirect interactions. However, predication algorithms suggested that miR-125a may directly regulate both TNF $\alpha$  and CCL4 (Table 5.2).

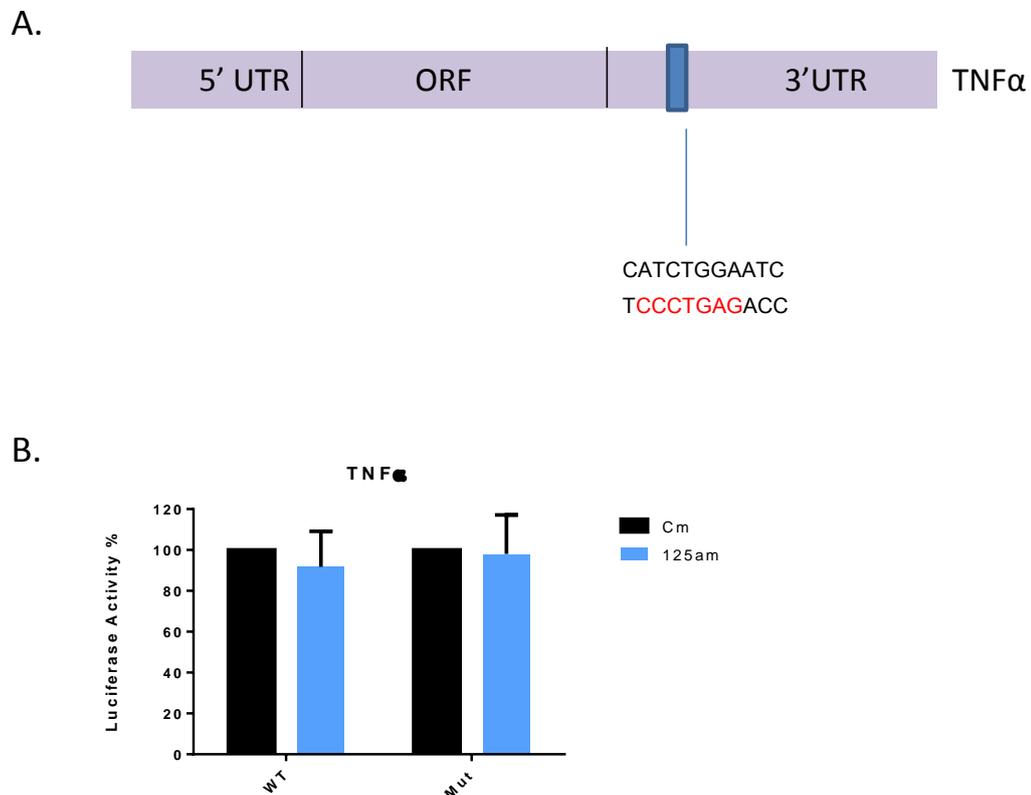
**Table 5.2 In-Silico analysis of cytokines potentially targeted by miR-125a**

Potential Target	Targetscan	Context Score/Percentage	Seed Match	Conserved	RNA22
TNF	No	N/A	N/A	N/A	Yes
CCL4	Yes	-0.15/68	7mer-1A	Poorly	Yes

Context Score-indicates how likely it is that the molecule will be a positive target based on the contribution of different features including seed pairing, AU content, stability and abundance. Seed Match-identifies the complementarity of binding between the miR seed region and the site in the mRNA 3'UTR (7mer-7 nucleotide match, 8mer-8nucleotide match).

### 5.2.2.1 miR-125a does not directly target TNF $\alpha$ mRNA

The RNA22 algorithm shows a potential miR-125a binding site in the 3' UTR of the TNF $\alpha$  gene (5.12 A). Again, to confirm this functionally we used a luciferase reporter assay. As previously, we generated WT and Mut constructs which contained either a sequence with the WT binding site, or a sequence in which this site was altered.



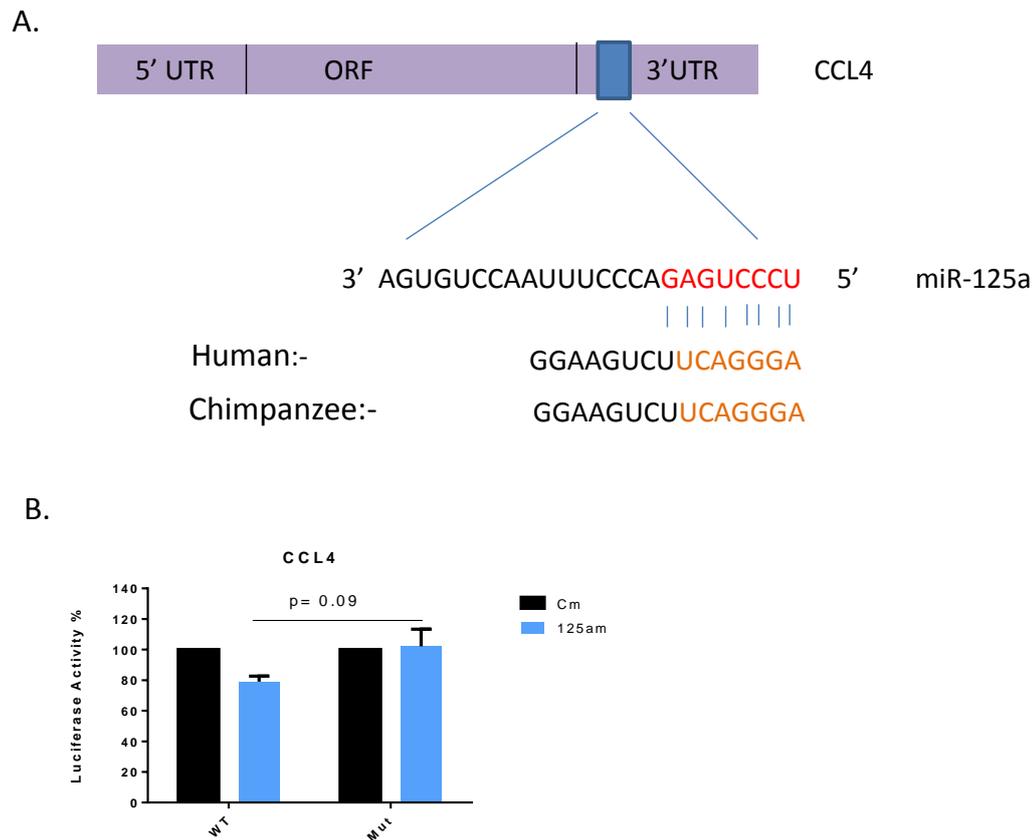
**Figure 5.12 Luciferase reporter assay of TNF $\alpha$**

A) Using the RNA22 algorithm we identified a potential miR-125a binding site in the 3'UTR of TNF $\alpha$  (shown in red) B) HEK293 cells were transfected with WT or Mut construct and Cm or miR-125a mimic. 24hrs later, luciferase activity was measured by a luminometre. Luciferase reporter assay of both WT and Mut constructs suggests TNF $\alpha$  is not a direct target of miR-125a. Values presented as mean  $\pm$  SEM of 4 experiments; with no significant difference observed between WT and Mut using an unpaired t-test

Results demonstrated no difference in luciferase activity in samples which were transfected with either the WT construct and a miR-125a mimic or the Mut vector and miR-125a mimic (Figure 5.12 B). Therefore, we concluded that miR-125a does not directly target and regulate TNF $\alpha$ , and this interaction cannot be responsible for the increase of TNF $\alpha$  witnessed in macrophages in which miR-125a is inhibited.

#### **5.2.2.2 miR-125a potentially regulates CCL4 expression**

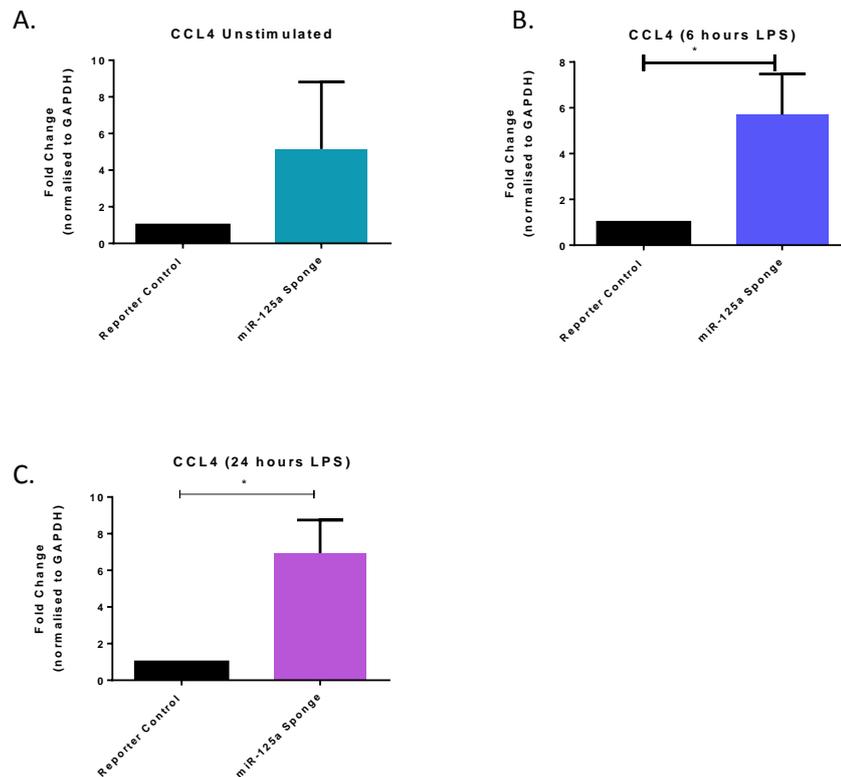
Next, using the TargetScan algorithm we identified a poorly conserved site in the 3' UTR of the CCL4 gene, conserved between humans and chimpanzees, but not present in mouse (Figure 5.13A). To determine if miR-125a can directly target the 3' UTR of CCL4, again we used a luciferase reporter assay.



### Figure 5.13 Luciferase reporter assay of CCL4

A) Using the TargetScan algorithm we identified a potential miR-125a binding site in the 3'UTR of CCL4 (shown in red) which is partially conserved across vertebrates (shown in orange). B) HEK293 cells were transfected with either WT or Mut constructs along with a Cm or miR-125am and luciferase expression was measured after 24 hours in culture. Luciferase expression was downregulated by 125am in only cells transfected with WT construct. Values presented as mean  $\pm$  SEM of 4 experiments.  $p=0.09$  unpaired t-test. WT vs. Mut. Cm: Control mimic, 125am: miR-125a mimic

Results showed that cells transfected with the WT construct and a miR-125a mimic had a lower luciferase expression when compared to a Cm. Additionally this downregulation was rescued when the miR-125a binding site was mutated, suggesting that CCL4 is a potential target of miR-125a, however this difference was not significant ( $p=0.09$ ) and therefore further experiments are required to fully validate this interaction (figure 5.13 B). We next examined the mRNA level of this chemokine in differentiated miR-125a sponge expressing cells. As previously, cells were differentiated with PMA for 7 days, and CCL4 mRNA was measured by q-PCR.

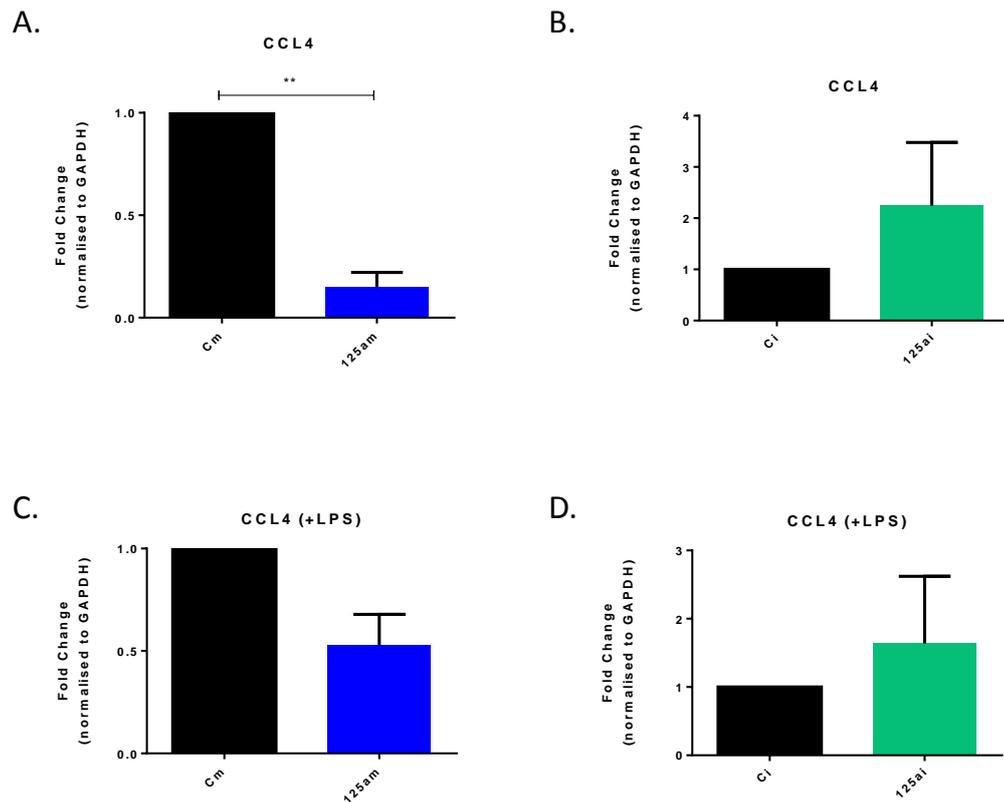


#### Figure 5.14 CCL4 mRNA expression of miR-125a sponge expressing cells

Expression of CCL4 mRNA was measured by q-PCR in PMA differentiated THP-1 cells expressing miR-125a sponge and stimulated with LPS (10ng/ml) for 6 or 24h or left unstimulated. A) The expression of CCL4 mRNA is not significantly altered between reporter control and miR-125a sponge expressing unstimulated cells. B) The expression of CCL4 is significantly increased in miR-125a sponge expressing cells when compared to reporter control after 6 hour LPS stimulation. C) The expression of CCL4 is significantly increased in miR-125a sponge expressing cells when compared to reporter control after 24 hour LPS stimulation. Values presented as mean  $\pm$  SEM of 4 experiments and marked bars are statistically different from each other using an un-paired T-Test  $*=p<0.05$

Results demonstrated that miR-125a sponge expressing cells show higher levels of CCL4 mRNA when compared to reporter control cells (Figure 5.14 A-C). This difference becomes significant after LPS stimulation (Figure 5.14 B and C).

Next, we examined CCL4 mRNA levels in healthy human macrophages in which miR-125a levels were manipulated by transfection. Cells were differentiated as previously described, and transfected on day 7.



**Figure 5.15 CCL4 mRNA expression of miR-125a transfected human macrophages**

Human macrophages were differentiated for 7 days after which miR-125a expression was manipulated by transfection and stimulated with LPS (10 ng/ml) for 24h or left un-stimulated. The levels of CCL4 mRNA were measured by q-PCR. A) Expression of CCL4 mRNA is significantly downregulated in macrophages overexpressing miR-125a. B) CCL4 expression is not altered in unstimulated macrophages in which miR-125a is inhibited. C) CCL4 mRNA expression is not significantly altered in LPS stimulated macrophages which overexpress miR-125a. D) There is no difference in CCL4 mRNA expression in LPS stimulated macrophages in which miR-125a is inhibited. Values presented as mean  $\pm$  SEM of 4 experiments and marked bars are statistically different from each other using a paired T-Test \*\*= $p < 0.005$ .

Results showed that overexpression of miR-125a in human macrophages results in decreased CCL4 mRNA expression. This mRNA downregulation is significant in resting cells (Figure 5.15 A and C). However, CCL4 mRNA does not appear to be significantly higher in cells in which miR-125a is inhibited, regardless of LPS stimulation (Figure 5.15 B and D).

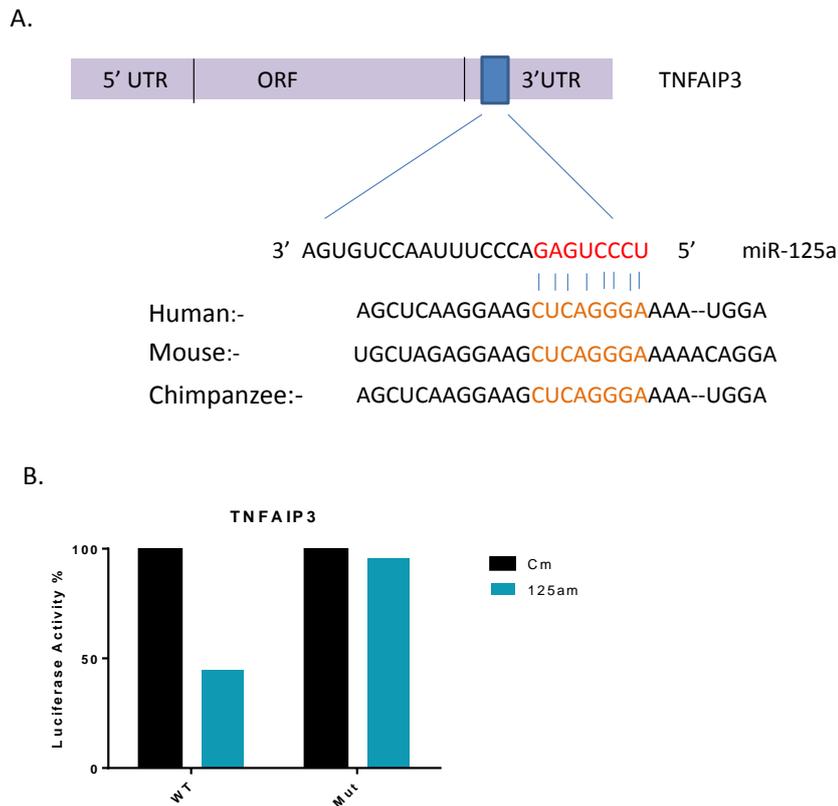
We have shown that upon strong neutralisation of endogenous miR-125a as seen in miR-125a sponge transfected cells, CCL4 mRNA levels are increased and upon overexpression of miR-125a in human macrophages, CCL4 mRNA levels are down-regulated supporting the hypothesis of miR-125a and CCL4 interaction. However, determining whether this is due to a direct targeting of CCL4 or a result of miR-

125a fine tuning LPS signalling pathway, requires additional luciferase assay and functional experiments that include more donors, time points and broader concentration of inhibitor.

### **5.2.3 miR-125a overexpression downregulates TNFAIP3, an important negative regulator of NFκB signalling**

In addition to investigating the pro-inflammatory phenotype caused by inhibition of endogenous miR-125a, we wanted to further investigate the mechanism behind the unexpected phenotype of macrophages overexpressing miR-125a, in terms of TNFα production. We have clearly demonstrated that macrophages which overexpress miR-125a produce significantly higher levels of not only IL-10 but also TNFα after LPS stimulation. We felt that this requires further investigation as this may preclude use of miR-125a mimic as a potential therapy in chronic inflammation.

As we outlined in point 5.2, TargetScan algorithm identified an important regulator of NFκB signalling, the E3 ubiquitin ligase TNFAIP3 as a potential miR-125a target. The 3'UTR of TNFAIP3 contains a highly conserved potential miR-125a binding site, which is conserved between human, mouse and chimpanzee, again, indicating its potential importance in the regulation of this gene in mammals (Figure 5.16 A).



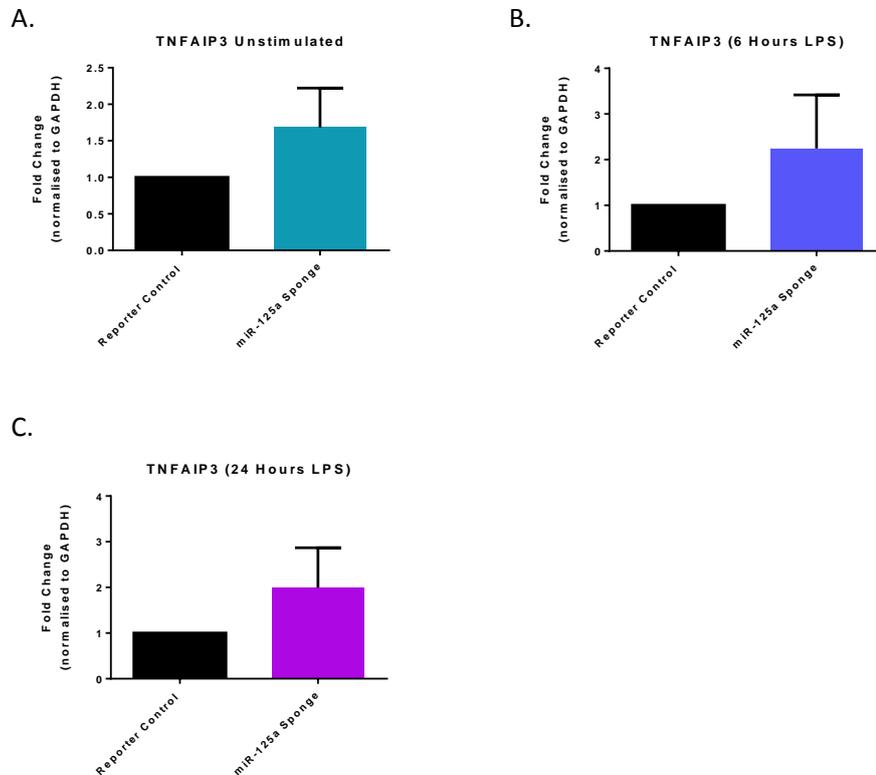
**Figure 5.16 Luciferase reporter assay of TNFAIP3**

A) Using the TargetScan algorithm we identified a potential miR-125a binding site in the 3'UTR of TNFAIP3 (shown in red) which was highly conserved across vertebrate species (shown in orange)  
 B) HEK293 cells were transfected with either WT or Mut constructs along with a Cm or miR-125am and luciferase expression was measured after 24 hours in culture. Luciferase expression was downregulated by 125am in only cells transfected with WT construct. However results shown represents only 1 preliminary experiment.

As previously, to analyse miR-125a binding potential, a luciferase reporter assay was carried out. Results demonstrated that transfection of the WT construct along with a miR-125am resulted in a decrease in luciferase expression, which was rescued by mutation of the miR-125a MRE (Figure 5.16 B). However, again, due to time constraints this experiment was only performed once, and requires repetition to conclusively validate miR-125a targeting of TNFAIP3.

We are confident however that miR-125a directly targets TNFAIP3 in macrophages as our data was confirmed recently by Kim et al, who have shown that miR-125a directly targets TNFAIP3 in DLBCL cells, resulting in constitutively active NF $\kappa$ B signalling [369].

To investigate whether this is a case in our experimental system, we first investigated levels of TNFAIP3 mRNA in differentiated miR-125a sponge expressing cells by q-PCR.

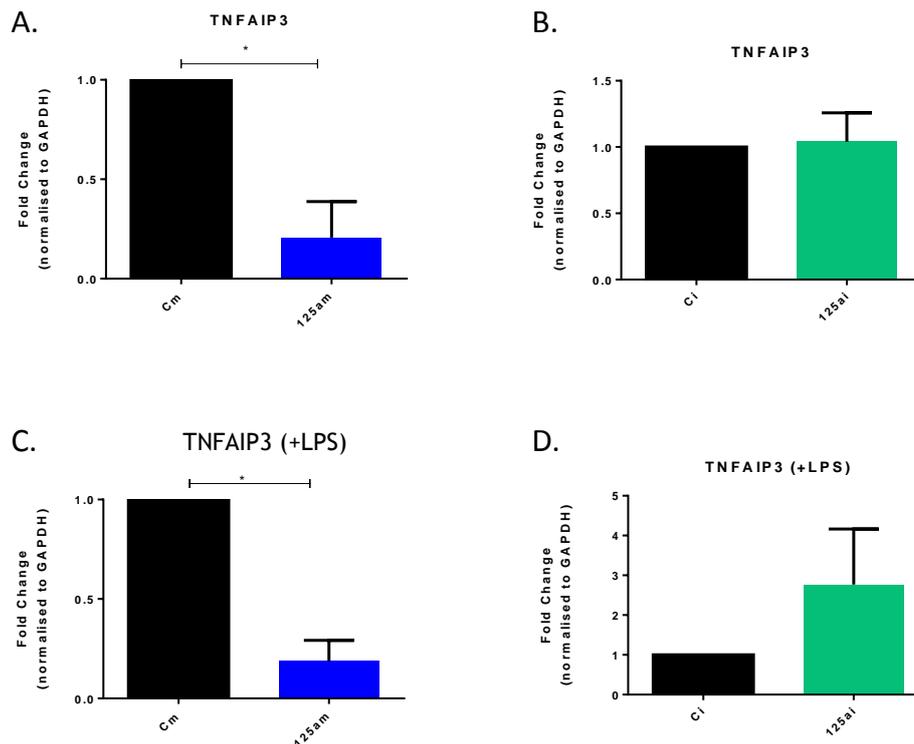


**Figure 5.17 TNFAIP3 mRNA expression of miR-125a sponge expressing cells**

Expression of TNFAIP3 mRNA was measured by q-PCR in PMA differentiated THP-1 cells expressing miR-125a sponge. A) The expression of TNFAIP3 mRNA is not altered between reporter control and miR-125a sponge expressing unstimulated cells. B) The expression of TNFAIP3 is not significantly altered between reporter and miR-125a sponge expressing sponge cells after 6 hour LPS stimulation C) There is no significant difference in the expression of TRAF6 mRNA between reporter control and miR-125a sponge expressing cells after 24 hour LPS stimulation. Values presented as mean  $\pm$  SEM of 4 experiments;

Results demonstrated a trend towards higher levels of TNFAIP3 in miR-125a sponge expressing cells, when compared to the reporter control (Figure 5.17A). This difference also appeared to increase after exposure to LPS (Figure 5.17 B and C). However, these differences were not significant at any of the time points analysed.

We next wanted to measure the mRNA levels and protein in healthy macrophages in which miR-125a expression was manipulated. As before, macrophages were differentiated for 7 days, and miR-125a levels were manipulated by transfection.



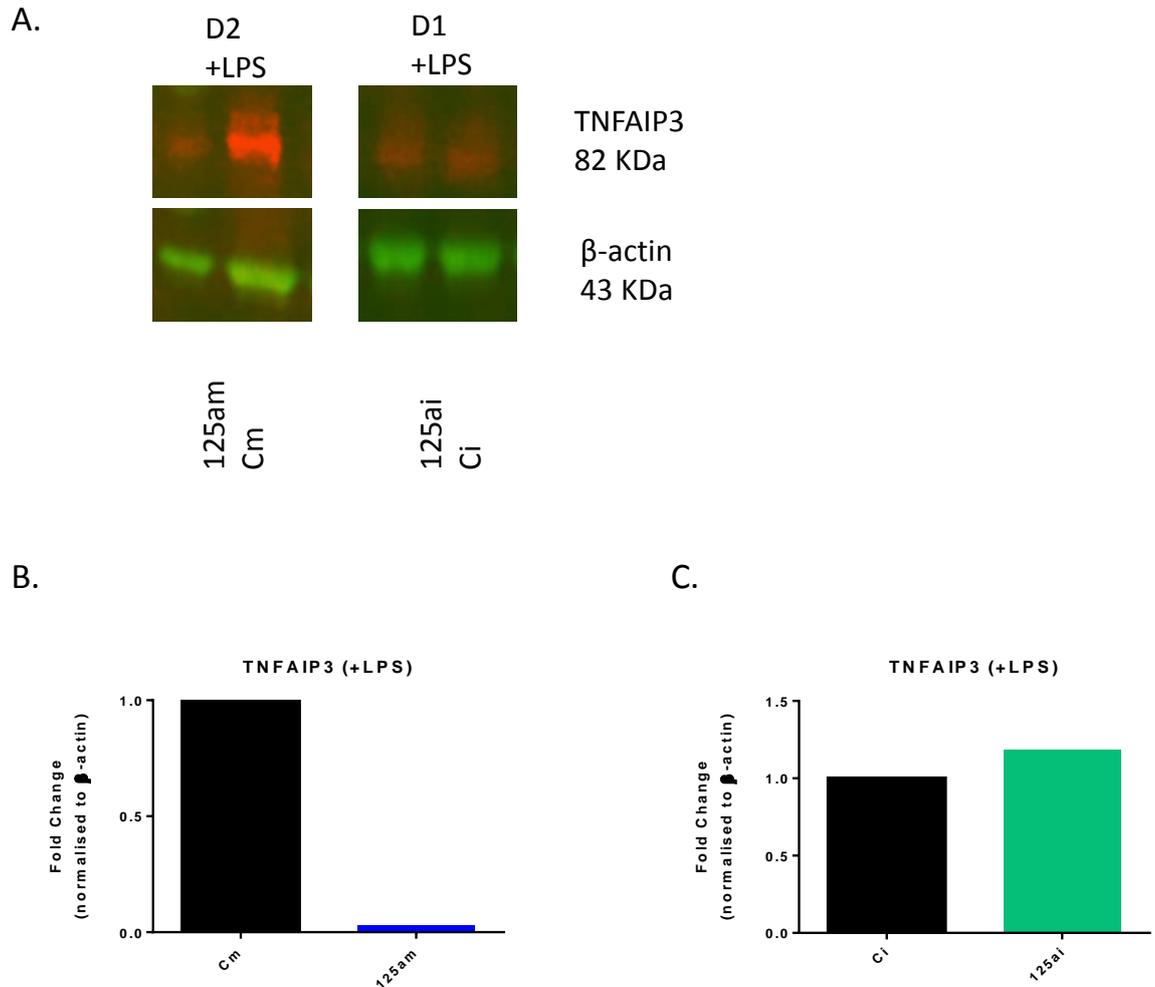
### Figure 5.18 TNFAIP3 mRNA expression of miR-125a transfected human macrophages

Human macrophages were differentiated for 7 days, after which miR-125a expression was manipulated by transfection and levels of TNFAIP3 mRNA were measured by q-PCR. A) Expression of TNFAIP3 mRNA is significantly downregulated in macrophages overexpressing miR-125a. B) TNFAIP3 expression is not altered in unstimulated macrophages in which miR-125a is inhibited. C) TNFAIP3 mRNA expression is significantly decreased in LPS stimulated macrophages which overexpress miR-125a. D) There is no difference in TNFAIP3 mRNA expression in LPS stimulated macrophages in which miR-125a is inhibited. Values presented as mean  $\pm$  SEM of 4 (D n=2) experiments and marked bars are statistically different from each other using a paired T-Test  $*=p<0.05$ .

Results demonstrated that TNFAIP3 mRNA is significantly decreased in macrophages which overexpress miR-125a, in both resting and LPS stimulated cells (Figure 5.18 A and C). However, similarly to miR-125 sponge THP-1 cells, there appears to be no difference in the expression of TNFAIP3 mRNA between cells when endogenous miR-125a is inhibited, regardless of LPS stimulation (Figure 5.18 B and D). These results suggest that miR-125a targeting of TNFAIP3 in macrophages, which overexpress miR-125a, may account for the increase in TNF $\alpha$  production witnessed in these cells after LPS stimulation. Again, levels of TNFAIP3 mRNA in LPS stimulated macrophages was only measured in 2 samples,

due to RNA quality from cells, and time constraints, and so this experiment requires repetition.

As we had demonstrated a significant decrease in mRNA expression, we next wanted to confirm this targeting at the protein level in macrophages by western blot (WB). Healthy CD14<sup>+</sup> cells were differentiated as previously described, and miR-125a expression was manipulated by transfection on day 7. Cells were cultured overnight, after which they were left under resting conditions, or stimulated with LPS for 6 hours. After this, cells were lysed, proteins extracted, and TNFAIP3 expression was measured by WB.



### Figure 5.19 TNFAIP3 protein expression in macrophages

Macrophages were differentiated for 7 days with M-CSF (50 ng/ml). On day 7 miR-125a expression was manipulated by transfection. Cells were cultured overnight, and then stimulated for 6 hours with LPS (10ng/ml) after which TNFAIP3 expression was measured using a Western Blot using fluorescence infrared detection. A) WB analysis showed that cells transfected with a miR-125am expressed lower TNFAIP3 protein when compared to a Cm. Additionally there was no detectable difference in TNFAIP3 expression between cells transfected with a Ci and miR-125ai. B) Analysis of WB protein expression, through 'integrated intensity' values, when compared to  $\beta$ -actin housekeeping shows a decrease in TNFAIP3 protein expression in those cells in which miR-125a was overexpressed. C) Analysis of WB protein expression, when compared to  $\beta$ -actin housekeeping shows no difference in TNFAIP3 protein expression in those cells in which miR-125a was inhibited. Values presented from 1 preliminary experiment.

Results from the WB experiment demonstrated that TNFAIP3 protein expression is low in resting macrophages, and we were not able to detect its expression. However, along with q-PCR data, we can demonstrate that TNFAIP3 expression is increased in response to LPS. In LPS stimulated macrophages overexpression of miR-125a leads to lower levels of TNFAIP3, whereas there was no significant increase in TNFAIP3 expression in macrophages where miR-125a was inhibited (Figure 5.19 A-C). These data further suggests that superficial high levels of

miR-125a can directly target TNFAIP3 in human macrophages after LPS stimulation, resulting in a downregulation of transcription and protein expression, and thus leading to high TNF $\alpha$  production. However, the endogenous miR-125a at the levels induced by M-CSF and TLR stimulation is not involved in TNFAIP3 regulation.

### 5.3 Discussion

In this chapter we aimed to identify direct mechanisms responsible for the anti-inflammatory role of miR-125a in macrophages. We selected potential targets through the use of the online algorithms, TargetScan and RNA22. These targets were tested in luciferase reporter assays, and then functionally validated through the analysis of mRNA and protein levels. We were able to show potential targeting of CCL4, by miR-125a, by luciferase assay and also provided some preliminary data on miR-125a targeting of members of the TLR4 signalling pathway such as TRAF6. We can therefore postulate that under inflammatory conditions miR-125a functions in an anti-inflammatory manner, by regulating important pro-inflammatory pathways.

Firstly, we identified TLR4 itself as a potential miR-125a target, and this target interaction was suggested further by the luciferase reporter assay. However, this interaction was weak, and when surface protein expression was measured by flow cytometry, both in human macrophages and miR-125a sponge expressing cells, no significance difference in expression could be detected. This was interesting as the slight decrease in luciferase activity observed during the reporter assay suggested a potential level of targeting of TLR4 by miR-125a. We therefore interrogated the TLR4 gene for alternative polyadenylation sites, to ascertain if this may affect miR binding. Many protein-coding genes contain a number of polyadenylation sites which allow one gene to code for several different mRNA isoforms, which differ at the 3' end. As polyadenylation changes the length of the 3' UTR, it can therefore remove miR binding sites within the mRNA. This phenomenon has been identified in cancer cells, where a transformed cell lines have been shown to broadly express mRNA isoforms with shorter 3' UTRs. This allows oncogenic activation through the removal of miR regulation, increased mRNA stability, and increased protein expression [414].

We therefore wished to investigate which variants of TLR4 were expressed on human macrophages, as if the 3'UTR was shortened, this may account for the lack of miR-125a regulation of TLR4. The National centre for biotechnology (NCBI) website contains information which identifies 11 validated polyadenylation sites for TLR4, 3 of which would cause the expression of a truncated TLR4 mRNA, resulting in a loss of miR-125a binding sites (Figure 5.7). Due to this, it is important to validate which variant of TLR4 is expressed by macrophages, both under resting and stimulated conditions, to determine the regulative capabilities of any miR on this gene.

We tried to address this question close to the end of this project. We designed two sets of primers which would amplify total TLR4 (part of the mRNA which all variants expressed) and a longer variant of TLR4 (which would contain miR-125a binding sites). Firstly, we wanted to investigate the effect LPS stimulation would have on these transcripts. Our data demonstrates that LPS stimulation significantly decreases the amount of total TLR4 expression when compared to unstimulated cells. This observation was also shown at the surface level using FACs analysis. However, although total TLR4 was decreased by LPS, it appeared that the longer variant increased when compared to unstimulated cells. This is interesting as it perhaps points to the regulation of TLR4, in that after activation of the cell expression of the longer variant continues, creating an additional level of miR regulation, to help resolve inflammation after the initial phase of the response. However, this preliminary experiment was only carried out on 3 samples, and therefore requires repetition before any conclusions can be drawn.

Future experiments could still be designed in which specific PCR primers would amplify only specific variants of TLR4 from macrophage DNA. These products could then be sequenced, allowing identification of the length of the 3'UTR within these cells. This data may prove important as studies have previously demonstrated that LPS stimulation of murine monocytes and macrophages, unlike in this current study, results in the upregulation of a truncated TLR4 isoform, which functions as a negative regulator of inflammation by preventing NF $\kappa$ B activation and TNF $\alpha$  production [415]. This is important as Jaresova et al have shown a failure to up-regulate this variant in human monocytes from cystic fibrosis patients, resulting in a pro-inflammatory phenotype of these cells within

disease. These studies indicate that identifying expression isoforms may prove important in understanding inflammatory responses within responsive cells under both health and disease states [416].

It would therefore be interesting to examine expression of TLR4 variants after miR-125a manipulation, as although miR125a may not have a measurable effect on total TLR4, it may regulate expression of the longer TLR4 variant, indicating a role for the resolution of inflammation. Additionally, as LPS decreased TLR4 surface expression in our macrophage cultures (Figure 5.3), it would be also be interesting to investigate whether there are other miRs involved in TLR4 down-regulation that is contributes to the formation of LPS tolerance.

The activation of TLR4 and the consequent signalling pathway is important in the activation of the transcription factor NF $\kappa$ B, essential for the production of pro-inflammatory cytokines. We identified the signalling molecule TRAF6 as a potential target of miR-125a. TRAF6 is an E3 ubiquitin ligase molecule which is essential for LPS induced production of TNF $\alpha$  and IL-6 by macrophages [417]. Due to the key role of these cytokines in inflammation, it is important that their production is regulated. We therefore hypothesised that miR-125a played a key role in this regulation by targeting of TRAF6 post LPS stimulation. Inhibition of miR-125a would therefore remove this level of regulation, resulting in a pro-inflammatory phenotype. We demonstrated by luciferase reporter assay that TRAF6 appears to be a direct target of miR-125a. However, this preliminary assay was only performed once and therefore needs to be repeated in the future, to definitively validate this target.

The potential of miR-125a to target TRAF6 was further demonstrated by overexpression studies within human macrophages. Overexpression of miR-125a in resting human macrophages results in significantly decreased TRAF6 mRNA expression, suggesting miR-125a is directly repressing this molecule. However, there is no reciprocal increase in expression at the mRNA level when miR-125a is inhibited in these cells. The latter could be explained by multiple reasons. Data demonstrated by Guo et al who have shown that miR-125a directly targets TRAF6 at the protein level, by WB in osteoclast cells, where overexpression causes a downregulation of the protein, whilst blocking miR-125a causes an increase [370]. Interestingly, it has been documented that miR target regulation does not

always result in a measurable change in mRNA levels, therefore future experiments should be undertaken to examine the protein level of TRAF6 by WB in these cells [418].

Additionally, it would be important for any future work to examine the expression of TRAF6 after manipulation of miR-125a at a number of time points post LPS stimulation, as it is possible that the lack of alteration at the mRNA level is due to the 24 hour time point that we have chosen to examine. A study by Nahid et al has shown that miR-146a, another miR which directly targets TRAF6, demonstrates its regulatory effect on protein expression in macrophages at as early as 5 hours post LPS stimulation [419]. Therefore, it is possible that at 24 hours TRAF6 expression has returned to resting levels in the macrophages.

We can therefore hypothesize that miR-125a may target TRAF6 early in the response to LPS to prevent overactive signalling, and pro-inflammatory cytokine production. Interestingly, when we examined early time-point mRNA in miR-125a sponge expressing cells, we showed that after 6 hours, TRAF6 mRNA is downregulated in these cells when compared to the reporter control. Due to the environment of engineered cells, it may be possible that other miRs may be functionally compensating for the loss of miR-125a, resulting in increased regulation of TRAF6. It would be interesting to examine levels of other miRs within these cells, which have been shown to target TRAF6, such as miR-146a.

In addition to signalling molecules, we wanted to examine the potential of miR-125a to directly regulate pro-inflammatory cytokines themselves. There is evidence within the literature that miR-125b, a homologue of miR-125a, targets TNF $\alpha$  directly, resulting in its downregulation [296]. Therefore we hypothesised that miR-125a targets TNF $\alpha$ , with inhibition removing this layer of regulation. However as our luciferase reporter assay demonstrated variable results, we do not believe that miR-125a directly targets TNF $\alpha$ . This result again demonstrates that although miRs within the same family may possess identical seed regions, they do not necessarily regulate the same molecules.

Next, we identified the chemokine CCL4 as a potential target of miR-125a. In this case there was a more convincing knock down of luciferase activity within the reporter assay, however this knock down again was not significant. In

order to confirm targeting via luciferase reporter assay it may be important to optimise the concentration of mimic used to witness knock down, as it is possible that there is interference in this assay with endogenous miRs and mRNAs.

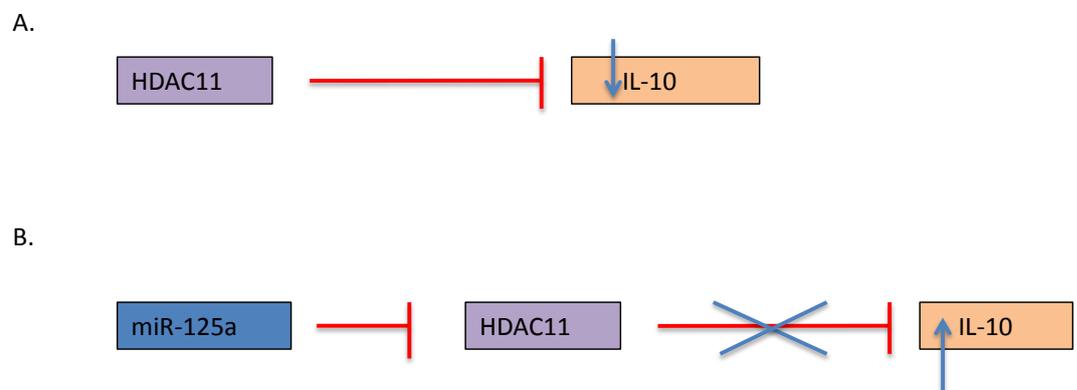
However, we were able to demonstrate that differentiated miR-125a sponge cells express significantly higher CCL4 mRNA than reporter cells, in line with the higher production of CCL4 protein witnessed in these cells by ELISA. This suggests that miR-125a indeed regulates CCL4. However, more studies are required to dissect the exact nature of interaction between miR-125a and CCL4 in human macrophages. This includes use of MRE protector that will allow us to distinguish the direct effect of miR-125a on CCL4 mRNA from the effect of miR-125 on LPS signalling pathway.

CCL4 mRNA was significantly downregulated only in resting macrophages in which miR-125a was overexpressed. A downregulation was also demonstrated after overexpression in LPS stimulated cells. This difference was however not significant, as with CCL4 protein by ELISA in these cells. We could therefore investigate a broader range of time-points in the future.

As miRs can target many different mRNAs it is important to view these molecules as wide regulators of pathways, and not just concentrate on the interaction between one miR and one specific molecule, at a given time. It was beyond the scope of this study to investigate and validate all potential targets of miR-125a involved in TLR4 signalling, however the number of these potential targets within the one pathway suggests an important role for miR-125a in regulating this pro-inflammatory signalling pathway. It is also probable that the list we have identified is not exhaustive, and that further *in-silico* analysis and use of sequencing technology, such as microarray would identify further potential targets within this, and related pathways.

We can therefore hypothesis that miR-125a endogenously functions in an anti-inflammatory manner, by regulating members of the TLR4 signalling pathway post LPS stimulation resulting in the regulation of the production of pro-inflammatory cytokines.

In addition to this regulation of pro-inflammatory cytokines and chemokines, we have identified a role for miR-125a in regulating the production of anti-inflammatory IL-10. Although we were unable to functionally test and validate predicted mRNA targets which could account for this regulation, extensive literature searching identified a potential candidate for this expression pattern. miR-125a is predicted to target histone deacetylase 11 (HDAC11), with 2 predicted sites conserved between humans and chimpanzee (TargetScan). HDAC11 is a recently discovered deacetylase which compacts chromatin around the IL-10 promoter, preventing the binding of transcription factors such as STAT3, and the production of IL-10 by macrophages (Figure 5.20) [420].



**Figure 5.20 Potential miR-125a regulation of IL-10 production through inhibiting HDAC11.** miR-125a potentially targets HDAC11. This HDAC molecule inhibits expression of IL-10. Therefore miR-125a regulation of HDAC11 potentially leads to higher IL-10 production in human macrophages.

A previous study by Lin et al has demonstrated targeting of HDAC11 by another miR, miR-145. They have shown that miR-145 promotes IL-10 production after LPS stimulation, through the targeting of HDAC11, releasing its gene silencing effect on IL-10. They were also able to show a secondary effect on IL-12 production, which was increased by these cells post miR-145 inhibition, as we have demonstrated with an inhibition of miR-125a [421]. We can therefore hypothesise that miR-125a functions in a similar manner in relation to IL-10, and promotes its expression through the targeting of HDAC11, and this would be an interesting hypothesis to test in the future. Additionally it would be interesting to measure levels of HDAC11 in the cohort of patients discussed in Chapter 3 (Figure 3.7), in which we demonstrated higher levels of miR-125a in PB CD14+ cells of RA patients who respond well to conventional therapy. We could

hypothesis that higher levels of miR-125a in these cells results in lower HDAC11 expression, allowing increased production of anti-inflammatory IL-10.

Although we have proposed an anti-inflammatory role for endogenous miR-125a in macrophages, overexpression of this miR has also been shown to result in increased TNF $\alpha$  production. It is important to understand this result, as although the loss of miR-125a appears pro-inflammatory, any attempt to use a miR-125a mimic as a potential disease therapy may result in off target effects through the concurrent increase of TNF $\alpha$  production. Therefore, we wished to confirm the potential mRNAs which may be regulated by miR-125a at these supra-physiological levels.

In addition to regulation by the miRs, the activation of pro-inflammatory signalling pathways is negatively regulated by additional molecules that help prevent excessive inflammation, and aid its resolution. We identified TNFAIP3, a negative regulator of NF $\kappa$ B, as a target of miR-125a. This molecule is essential for the termination of TLR4 induced NF $\kappa$ B activity in macrophages. It functions as an ubiquitin editing enzyme, by removing ubiquitin tails from signalling molecules, such as TRAF6, therefore terminating NF $\kappa$ B activity, and aiding in the resolution of the inflammatory response [422]. Polymorphisms within this gene have been associated with many inflammatory diseases including RA and SLE, and mice deficient in TNFAIP3 develop a TLR4-MyD88 dependent destructive polyarthritis, characterised by high serum levels of TNF $\alpha$ , IL-6 and IL-1 $\beta$  [30, 423]. Therefore, due to the function of TNFAIP3 and the phenotype witnessed after its loss, we were interested in the relationship between miR-125a and this molecule within our experimental system.

We have demonstrated that miR-125a directly targets TNFAIP3 by luciferase reporter assay. Although this is only preliminary data, it is supported by existing data by Kim et al, who have shown that miR-125a does in fact directly target TNFAIP3 by this method. Kim et al also showed that overexpression of miR-125a resulted in increased NF $\kappa$ B activity and resistance to apoptosis, thereby enhancing the aggressive phenotype of diffuse large B cell lymphoma cells (DLBCL). However, this study did not demonstrate any functional effect on cytokine production after manipulation of miR-125a expression within these cells [369].

These results have led us to hypothesise that when expressed at supra-physiological levels (after mimic transfection), miR-125a can directly bind TNFAIP3, resulting in protein degradation, preventing negative regulation of the TLR4 pathway, resulting in increased TNF $\alpha$  production by these cells. Our preliminary mRNA and protein data on macrophages overexpressing miR-125a supported this assumption. As some of the experiments were performed only once, it is important to repeat and extend the experimentation including the use of specialised target protectors to protect the miR-125a binding site of TNFAIP3 during transfection. This would identify whether or not targeting of TNFAIP3 is causing the increase of this pro-inflammatory cytokine after transfection.

Therefore, from our data we postulate that under normal endogenous levels miR-125a functions to limit (fine tune) the monocyte and macrophage response to LPS, by regulating members of the TLR4 signalling pathway and pro-inflammatory chemokines themselves. However, under conditions in which the level of miR-125a is increased above physiological expression, such as after mimic transfection, off-target effects are witnessed, and the excess of miR-125a begins to target the anti-inflammatory TNFAIP3 molecule, resulting in increased TNF $\alpha$  production.

## Chapter 6 - Summary and Conclusions

This study set out to identify miR species which were dysregulated in RA macrophages, to identify the functional role of these miRs, and to thereafter determine if they play a role in maintaining the pro-inflammatory nature of macrophages in RA. To my knowledge this is the first miR profile of SF CD14+ cells thus far identified. From this profile, we hypothesised that differentially expressed miR would affect the differentiation and effector function of macrophages within the RA joint.

MiRs have been shown to regulate around 60% of protein coding genes, and as such have been identified as powerful regulators of general cell biology and thus inflammatory and disease-related processes. By corollary, dysregulated expression of miRs is well documented in many inflammatory diseases and cancers. Therefore, it is important to study miRs within the context of RA, as aberrant expression may identify novel disease associated pathways which may not have been previously obvious. Also as one miR can target many mRNAs, usually within the same effector pathway, these molecules may prove an interesting therapeutic targets, leading to the regulation of a whole pathway instead of a single, albeit important molecule, such as TNF or IL-6R. Additionally as many studies continue to search for biomarkers that identify particular disease states, the simplicity of identifying miRs in blood or serum samples demonstrates a potential for a non-invasive clinical application.

One identified miR up regulated in this study in synovial CD14+ cells was miR-511. It is encoded within the CD206 gene and expressed in M2 macrophages. This finding may prove important, as although their potential role via M2 macrophage maturation in RA is under debate, emerging evidence points towards their role in early arthritis. Based on this notion a novel diagnostic technique has been proposed by the company Navidea. They will identify early RA patients by fluorescently labelling CD206 expressing macrophages present in affected joints. Therefore we postulate that high levels of miR-511 within synovial cells could potentially be used as a biomarker to identify early RA patients in the future. By virtue of time and scope constraints however I was required to focus in my current studies on other miRs.

The majority of my study therefore concentrated on the expression and functional role of miR-125a in macrophages. We identified this miR, along with its family member miR-99b to be significantly upregulated in SF CD14<sup>+</sup> cells; the majority of our activities focused on this miR, as its expression pattern suggested it may be the dominant miR within this cluster [395]. In addition, we observed that changes in miR-125a expression are detected in RA PB CD14<sup>+</sup> cells when compared to healthy donors. Indeed, one of the key and novel observations made on my examination of a cross-sectional RA patient cohort was that miR-125a expression is increased in PB CD14<sup>+</sup> cells of patients who respond well to cDMARD therapy when compared to healthy controls, and patients resistant to biologic therapies. In these cells, expression of miR-125a negatively correlates with clinical measures of disease activity, DAS28 ESR, SDAI, CDAI, TJC and SJC, suggesting increased miR-125a expression in patients with lower levels of on-going inflammation. Additionally, miR-125a expression positively correlated with serum levels of the anti-inflammatory cytokine, IL-10. This expression pattern suggests that miR-125a expression may prove protective against inflammation in these cells by forming part of an anti-inflammatory regulatory loop. Moreover in the context of on-going inflammatory disease this may represent an attempted resolution pathway that is functionally incompetent in the context of overwhelming synovial inflammation.

This information is important, as although biologic therapies have revolutionised treatment of RA, there is currently a considerable unmet need in the identification of new therapeutic targets and biomarkers, based upon response to therapy that is moderated when remission becomes the prime focus. Currently only 60-70% of patients respond to anti-TNF $\alpha$  therapy when high hurdle responses are implicated, and this treatment is associated with infectious risk e.g. recurrence of latent TB in some recipients of anti-TNF $\alpha$  treatment [248, 249]. Therefore it is important to find a way to identify patients who will “not” or partially respond to biologic therapies, or who will experience an adverse reaction. Thus, it would be interesting to investigate the potential of miR-125a as a predictive marker of disease progression in a prospective RA cohort. In addition to my studies, the involvement of miR-125a in RA has been recently supported by Murata et al, who suggest that circulating miR-125a levels, measured in the plasma, can be used as a biomarker to identify patients

suffering from RA [396]. However, this study did not take into consideration the treatment regimens of these patients.

To determine the functional role of miR-125a we took advantage of new technology and generated a miR-125a sponge. The miR-125a sponge is a vector containing multiple sites that are complementary to the seed region of miR-125a, stably expressed at high levels in THP-1 cells. This inhibits miR-125a, resulting in a loss of activity. To my knowledge, this is the first study to generate a miR-125a sponge, and use it thereafter to investigate its functional role. This technology, first described by Ebert et al, has been used successfully in the study of other miRs to determine their function within specific cell types [424]. For example a study by Valastyan et al, demonstrated the role of miR-31 in controlling breast cancer metastasis. The authors identified the downregulation of miR-31 in aggressive metastasising tumours, and generated a stably expressing miR-31 sponge breast cancer cell line, to mimic this environment. These miR-31 null cells were injected into mice and formed macroscopic metastases in the lungs, indicating that miR-31 plays a role in controlling breast cancer metastasis [404, 405]. Using the miR-125a sponge vector, we generated a THP-1 cell line that stably expressed this miR sponge and using PMA differentiation we created a macrophage like cell that effectively expressed a miR-125a null environment. These cells exhibited a clear pro-inflammatory phenotype when stimulated with LPS, producing significantly higher levels of TNF $\alpha$ , IL-6, CCL4 and CCL5. A similar phenotype was noted with human macrophages transfected with a miR-125a specific inhibitor. Again, we demonstrated a clear pro-inflammatory phenotype, with significantly higher production of TNF $\alpha$ , IL-12, and CCL4 after LPS stimulation. Interestingly, along with high levels of pro-inflammatory cytokines, these cells produced significantly lower levels of anti-inflammatory IL-10. This result was interesting as miR-125a expression correlates with serum IL-10 concentration in RA patients.

These data suggested that indeed miR-125a plays a regulatory role in human macrophages and the removal of this miR from the system would result in a hyper-inflammatory response to LPS, or other agonistic stimuli, with increased production of pro-inflammatory cytokines and a decrease in IL-10 production.

There are conflicting data within the literature with regards to the role of miR-125a in human macrophages. A study by Graff et al suggests that miR-125a can be used as a marker of M1 macrophage polarisation, with overexpression studies in PMA differentiated THP-1 cells, resulting in increased expression of CXCL9, an M1 marker [368]. On the other hand, a study by Banerjee et al suggests that miR-125a is a marker of M2 macrophages, with overexpression studies in GM-CSF differentiated murine macrophages (GM-BMM), resulting in decreased TNF $\alpha$  and IL-12 production. This study also demonstrates that overexpression of miR-125a in GM-BMMs results in decreased bactericidal properties, but increase phagocytic capabilities, associated with the M2 phenotype [339]. The differences between Banerjee et al and my current study may be accounted for by the different growth factors used for macrophage differentiation, as the current study was performed in M-CSF differentiated macrophages, as opposed to GM-CSF macrophages. Additionally, the problems associated with overexpression studies have now been well documented, with off target effects frequently demonstrated. Indeed, when inhibiting miR-125a in mouse M-CSF differentiated bone marrow macrophages, Banerjee et al demonstrated an increase in TNF $\alpha$ , IL-12, MHC Class II and CD40, in line with the current cytokine profile observed in my own studies.

We identified potential miR-125a targets underlying its anti-inflammatory action using computer based algorithms and functional validation. We identified a novel potential relationship between miR-125a and TLR4 and also miR-125a and CCL4. Additionally, through Targetscan and RNA22 we also identified further potential targets such as TRAF6, a molecule involved in the TLR4 signalling pathway, suggesting that miR-125a plays a key role in the regulation of this pathway.

Based on our data we postulate that, under normal conditions miR-125a fine tunes the macrophage response to LPS/TLR activation, preventing an overactive inflammatory response by regulating NF $\kappa$ B activation, and the production of pro-inflammatory cytokines. This is accomplished by targeting members of the TLR4 signalling pathway and pro-inflammatory chemokines themselves. Additionally miR-125a allows the increased expression of anti-inflammatory IL-10 through targeting of HDAC11, allowing further regulation of inflammation (Figure 6.1 A).

However at sites of chronic inflammation, such as in RA, in macrophages this regulation is impaired. This may be due to the masking of the effect of miR-125a by the increased expression of its targets during chronic inflammation, and the lack of response to IL-10 of macrophages within the RA joint [202, 203]. Additionally miR-125a regulation may be impaired due to the dysregulation of other important miRs within the RA macrophage (Figure 6.1 B).

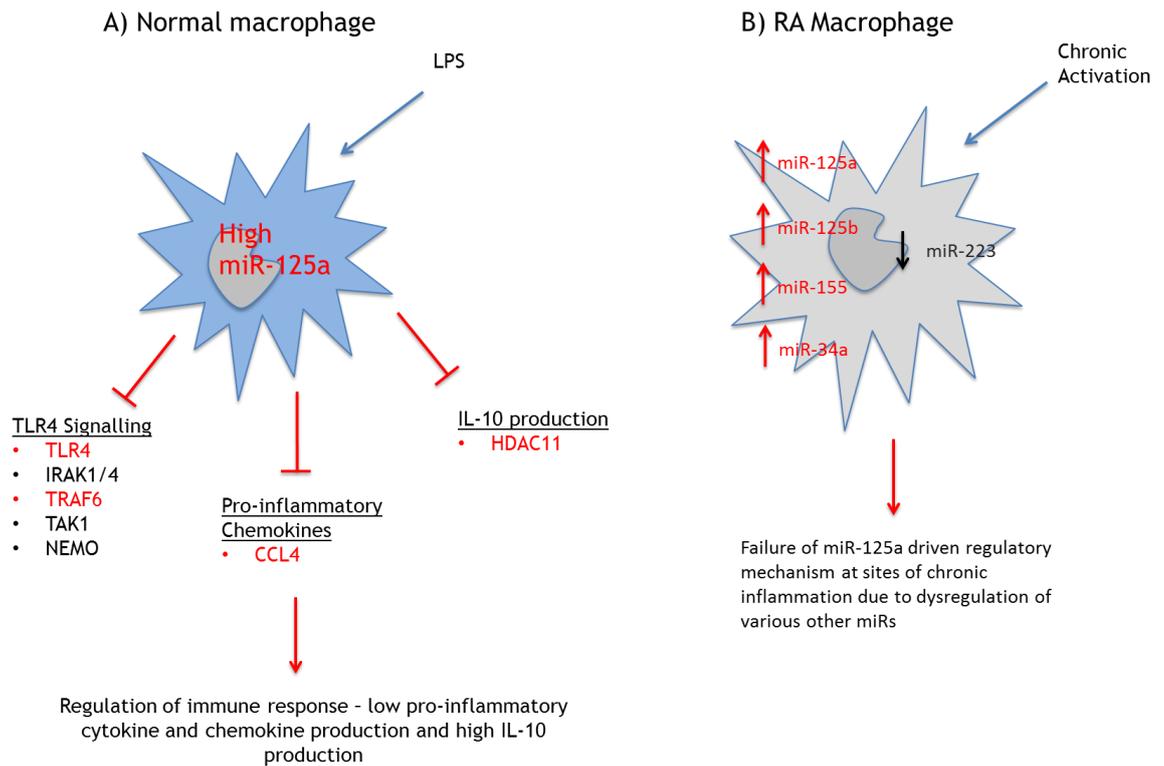
It has been demonstrated by this lab group that miR-155 is upregulated in SF macrophages, where it targets a regulator of pro-inflammatory signalling, SHIP1, allowing constitutive expression of pro-inflammatory cytokines [320]. Also the miR profile identified at the beginning of this study suggests the upregulation of the miR-125a homologue, miR-125b in these cells. This miR has been shown to target TNFAIP3, allowing continual NF $\kappa$ B activation, and also the adoption of an activated phenotype through the targeting of IRF4 [369, 372]. Finally, unpublished data from this lab group, has also demonstrated a downregulation of miR-223 in RA macrophages, which results in increased expression of the NLRP3 inflammasome, a direct miR-223 target, and increased IL-1 $\beta$  production. Therefore the dysregulation of these other miRs may mask the regulatory effect of miR-125a within these cells.

Although the regulatory mechanism of miR-125a appears impaired at sites of chronic inflammation, understanding miR-125a expression may prove important in terms of endotoxin tolerance in monocytes and macrophages. The specific molecules of the TLR4 signalling pathway that are potential miR-125a targets, indicate that this miR may have a specific role in regulating the response of macrophages to LPS. Therefore, it would be interesting to study the functional consequence of miR-125a manipulation under conditions such as bacterial infection and in the context of sepsis.

We believe that endogenous miR-125a acts as a negative regulator of pro-inflammatory signalling pathways in activated macrophages. However, therapeutic alteration of this miR should be considered with caution. In this study we demonstrated, that overexpression of miR-125a results not only in increased IL-10, but also increased TNF $\alpha$ . It has been previously demonstrated that miR-125a can target the inhibitor of NF $\kappa$ B signalling, TNFAIP3. Kim et al have shown that both miR-125a and miR-125b are upregulated in diffuse large B

cell lymphomas (DLBCL), where targeting of TNFAIP3 results in constitutively active NF $\kappa$ B, contributing to the aggressive phenotype of these cells [369]. Additionally, Gomez et al have demonstrated high miR-125a expression in CD34+ cells from MDS patients, with high levels of miR-125a correlating with poor survival [393]. They have proposed a model whereby miR-125a plays dual roles with regards to NF $\kappa$ B activity, depending on cell activation. Inhibition of miR-125a after activation with TLR ligands results in increased NF $\kappa$ B activity, suggesting a fine-tuning mechanism in which miR-125a plays a critical role. We hypothesised that targeting of TNFAIP3 by miR-125a in human macrophages after overexpression is an “off-target” effect of supra-physiological levels of miR (up to 200 fold) after transfection. However, with reports of targeting of this molecule by miR-125a in other cells, it is important to determine if this is the case. Therefore additional experiments should be undertaken to settle these contradictory reports. For example a protector against the miR-125a binding site in TNFAIP3 should be used to ascertain if high levels of TNF $\alpha$  witnessed in these experiments are a result of miR-125a targeting. Also the concentration of mimic could be titred, with TNFAIP3 expression measured by WB, after transfection with different concentrations of mimic. These experiments would then determine if miR-125a targeting of TNFAIP3 in human macrophages is indeed an “off-target” effect.

These studies also highlight how important it is to understand the expression pattern of miRs before they may be used therapeutically, as miRs may play very different roles in depending on cell type. Therefore any attempt to manipulate their expression needs to be cell specific and undertaken with caution.



### Figure 6.1 The role of miR-125a in normal and RA macrophages

Under acute inflammatory conditions miR-125a functions to regulate the immune response to infection, by dampening inflammation. However under chronic inflammatory conditions this regulatory mechanism fails due to high levels of inflammation, and dysregulation of other miRNAs

As previously discussed, future work on this project could be undertaken in many different directions.

Firstly, it would be interesting to undertake a microarray analysis of healthy macrophages which have been transfected with a Ci or miR-125ai. Pathway analysis software could then be used to identify mRNA transcripts increased in the presence of miR-125ai. This may identify novel miR-125a targets which may be responsible for the proinflammatory phenotype witnessed in these cells. Additionally, it would be interesting to determine if the molecules postulated within this work, such as TRAF6, TLR4 and HDAC11 were identified.

It is also important that targets postulated within this work are validated further. As suggested, the transcript variant of TLR4 expressed on LPS stimulated macrophages could be validated, and its 3'UTR sequenced to determine its size, and therefore probability that it is regulated by miR-125a or

other miR species. Along with regulation of proinflammatory molecules, miR-125a regulation of IL-10, through HDAC11 should be confirmed.

As the original observation within this study was the upregulation of miR-125a in SF CD14<sup>+</sup> cells, I believe it will be important to investigate the response of miR-125a to DAMPs present within the joint, such as Tenascin-C. We have hypothesised that within the proinflammatory environment of the joint, upregulation of miR-125a within these cells represents a failed mechanism of regulation, or that the dysregulation of other miRs within these cells prevents miR-125a acting in its usual role. Therefore, in the future, miR-125a levels could be manipulated within SF CD14<sup>+</sup> cells themselves to determine if these observations are proven correct. The long term future direction of this work will therefore depend on the outcome of these suggested experiments.

In summary, this study has generated new evidence with regards to the miR profile of SF CD14<sup>+</sup> cells. It has identified a potential biomarker for response to therapy in RA patients, and a clear regulator of TLR4 signalling in macrophages; however several avenues remain to be explored. Future studies are required to conclusively identify miR-125a targets within the RA synovium and understand the functional consequence of high levels of this miR within macrophages, in RA.

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