Investigating the causes and functional implications of miR-34a dysregulation in rheumatoid arthritis

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Abstract

Rheumatoid arthritis (RA) is a chronic, inflammatory disorder, whereby synovial inflammation ultimately results in joint destruction. Although the joints are the main target tissues affected, RA is also associated with a number of co-morbidities - such as cardiovascular disease. A key cell type involved in the perpetuation of disease pathogenesis is the macrophage, but the mechanisms underlying inflammatory gene expression in these cells are not fully understood. One fascinating and rather novel area of research that could provide insights into the activation of macrophages in RA comprises the biology of microRNA. These small, non-coding RNA molecules are implicated in the post-transcriptional regulation of gene expression.

Here, we show that the expression of one particular microRNA, miR-34a, is increased in synovial fluid CD14+ cells compared to matched peripheral blood cells from RA patients. We have demonstrated that miR-34a expression is increased in synovial tissues from RA patients compared to osteoarthritis comparators, and that a proportion of these miR-34a positive cells are CD68+ macrophages. Of particular interest, miR-34a was also up-regulated in peripheral blood CD14+ cells isolated from multiple drug-resistant RA patients compared to healthy controls.

Using over and under-expression methodologies we were able to demonstrate that miR-34a over-expression reduces toll like receptor-induced cytokine production by macrophages, while miR-34a inhibition enhances cytokine production. The altered cytokine activities included TNFα and IL-6 that are both critically linked to disease pathogenesis, therefore we propose that miR-34a over-expression in RA macrophages represents a failed attempt to attenuate on-going inflammation.

To further explore the mechanism of miR-34a action, a microarray was performed to investigate transcripts that were regulated in response to miR-34a over-expression in monocytes. This study uncovered several pathways, including interferon, metallothionein and chemokine pathways, wherein many members were down-regulated upon miR-34a over-expression. Future work will therefore

aim to dissect the role of these pathways, and their relevance to miR-34a regulated macrophage and dendritic cell biology, and thus to the chronicity of synovitis.

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Author's Declaration

I declare that, except where explicit reference is made to the contribution of others, this thesis is the result of my own work. No part of this thesis has been submitted for any other degree at The University of Glasgow, or any other institution.

Signature……

Printed name……

Abbreviations

3'UTR	3’ un-translated region
7-AAD	7-aminoactinomycin D
ACPA	anti-citrullinated protein antibodies
ACR	American College of Rheumatology
ADCC	antibody-dependant cellular cytotoxicity
AIA	antigen-induced arthritis
APC	antigen presenting cell
APRIL	A PRoliferation Inducing Ligand
ARE	AU-rich element
BAFF	B-cell activating factor
BLyS	B-Lymphocyte Stimulator
C. elegans	Caenorhabditis elegans
CA	cells alone
CD	cluster of differentiation
cDMARD	conventional disease modifying anti-rheumatic drugs
CDP	common DC progenitors
ceRNA	competing endogenous RNA
C_i	control inhibitor
CIA	collagen induced arthritis
C_m	control mimic
CRA	chronic treated rheumatoid arthritis
CRF	central research facility
CRP	C-reactive protein
d0 / 3 / 7	day 0 / 3 / 7
DAMP	damage associated molecular pattern
DAS	disease activity score
DC	dendritic cell
DEPC	diethyl pyrocarbonate
DGCR8	DiGeorge critical region 8
DKK	Dickkopf
DMARD	disease modifying anti-rheumatic drug
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent assay
ESC	embryonic stem cell
ESR	erythrocyte sedimentation rate
EULAR	European League against Rheumatism
FACS	fluorescence-activated cell sorting

FBS	foetal bovine serum
FcR	Fc Receptor
FLS	fibroblast-like synoviocytes
FMO	fluorescence minus one
GM-CSF	granulocyte macrophage colony-stimulating factor
gMFI	geometric mean fluorescence intensity
GPF	glasgow polyomics facility
GWAS	genome-wide association studies
HEK	human embryonic kidney
HMGB-1	high-mobility group box 1
HS CRP	high-sensitivity C-reactice protein
HSC	hematopoietic stem cells
HSP	heat shock protein
IDT	Integrated DNA Technologies
IFITM	interferon-induced transmembrane protein
IL	Interleukin
iOA	inflammatory OA
IP	intra-peritoneal
iPSC	inducible pluripotent stem cells
IRAK	interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factor
ISG	interferon stimulated gene
ITAM	immunoreceptor tyrosine-based activation motif
JAK	janus kinase
KO	Knockout
LB	Luria-Berta
LPS	Lipopolysaccharide
LRP	Lipoprotein
MACS	magnetic-activated cell sorting
MAP3K14	mitogen-activated protein kinase kinase kinase 14
MCP	Metacarpophalangeal
M-CSF	macrophage colony-stimulating factor
MDA-5	Melanoma Differentiation-Associated protein 5
mDC	myeloid dendritic cell
MDP	macrophage / DC progenitors
MEF	mouse embryonic fibroblast
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
miR	microRNA
miR-34a$_i$	miR-34a inhibitor
miR-34a$_m$	miR-34a mimic
miRNA	microRNA

MMP	matrix metalloproteinase
MP	myeloid progenitors
mRNA	messenger RNA
MT	Metallothionein
MTF1	Metal regulatory transcription factor 1
MTP	Metatarsophalangeal
MTX	Methotrexate
MX1	MX dynamin-like GTPase 1
NET	neutrophil extracellular trap
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NIH	National Institute of Health
NIK	NF-κB -inducing kinase
niOA	non-inflammatory osteoarthritis
NK	natural killer
NORA	new-onset untreated rheumatoid arthritis
NSAID	non-steroidal anti-inflammatory drugs
OA	Osteoarthritis
OAS	2'-5'-oligoadenylate synthase
OASF	OA synovial fibroblast
Oct4	octamer-binding transcription factor 4
P. gingivalis	*Porphyromonas gingivalis*
PAD	peptidylarginine deiminase
PAMP	pathogen associated molecular pattern
PB	peripheral blood
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pDC	plasmacytoid DC
PIP	proximal interphalangeal
piRNA	piwi-interacting RNA
PKR	Protein kinase RNA-activated
PNUTS	phosphatase 1 nuclear targeting subunit
RA	rheumatoid arthritis
RANKL	receptor activator of nuclear factor kappa-B ligand
RASF	RA synovial fibroblast
RF	rheumatoid factor
RIG-1	retinoic acid-inducible gene 1
RIN	RNA integrity number
RISC	RNA-induced silencing complex
RMA	robust multichip analysis
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute medium

RT	transfection reagents
SCID	severe combined immunodeficiency
SF	synovial fluid
SHIP	SH2-containing inositol-5'-phosphatase
SIRT	Sitruin
SJC	swollen joint count
SOCS	suppressor of cytokine signalling
SOX	SRY-box 2
SP	serum plasma
SRY	sex determining region Y
SSC	saline-sodium citrate
STAT	signal transducers and activators of transcription
TAE	tris-acetate-EDTA
TG	Transgenic
Tgif	transforming growth factor-β-induced factor
T_h	T helper
TJC	tender joint count
TLR	toll-like receptor
TMB	Tetramethylbenzidine
TNC	tenascin-C
TNF	tumor necrosis factor
TRAF2	TNF receptor-associated factor 2
TSLP	thymic stromal lymphopoietin
TSLPR	thymic stromal lymphopoietin receptor
WNT	Wingless
WT	wild type

Chapter 1 – Introduction

1.1 **Rheumatoid Arthritis**

Rheumatoid arthritis (RA) is a chronic, inflammatory disorder of unknown aetiology. Principally attacking the diarthrodial joints of the body, RA is associated with inflammatory synovitis, where progression of the disease results in the destruction of articular cartilage and bone. This often devastating condition affects around 0.8-1% of the general Caucasian population [1, 2], and in severe cases can cause permanent deformity and loss of function of the affected joints. This in turn promotes substantial personal and societal socioeconomic impact. In consequence, RA comprises one of the major autoimmune mediated causes of disability.

Although the initial causes remain undefined, RA is thought to arise predominantly as a result of genetic susceptibility, upon which certain environmental and stochastic factors operate to drive disease. It is also thought that a transition event may be required to focus the attack to the joints. Although not well understood, this could comprise an infection or local trauma/injury to the joint. The result is a perpetuating cascade of inflammation, with many aspects of the immune system implicated in disease pathogenesis.

Although the joints are the main areas affected by RA, it should be considered a systemic condition, reflected in the presence of a number of co-morbidities, and of decreased life expectancy. These co-morbidities likely reflect systemic inflammatory activity, and can include pulmonary problems, increased cancer risk [3, 4], increased infection rates [5], psychological problems and vascular disease [6, 7] – particularly accelerated atherosclerosis. Over the past decade the treatment of RA has improved remarkably, but disease remission is still rare and almost never achieved without on-going pharmaceutical intervention.

1.1.1 **Diagnosis and Classification**

There is no single test that can confirm or exclude the diagnosis of RA, and instead a number of different criteria are considered when making the diagnosis. These include assessing the number and location of affected joints, measuring acute phase reactants, and the presence or absence of rheumatoid factors (RF) or anti-citrullinated protein antibodies (ACPA) in the serum. RA patients are now broadly divided in to two major serological subsets based on these tests – ACPA positive and ACPA negative. Patients were initially separated by the presence or absence of RF in the blood, and although in established disease ACPA and RF positive patients represent largely overlapping populations, ACPA antibodies are more specific to RA, found in a higher percentage of patients, and shown to correlate with disease severity. These therefore represent a better diagnostic tool.

In order to achieve consistent identification of patients, primarily for clinical trial and cohort design purposes, the American Rheumatism Association developed the 1987 Criteria for Classification of Rheumatoid Arthritis [9]. Seven criteria were considered (listed in Table 1-1), of which the patient must show signs of 4 to be diagnosed with RA. Although these criteria worked well in diagnosing patients with established disease, they didn't help identify patients with early RA – who are the group most likely to benefit from aggressive interventions. Therefore, in 2010, the American College of Rheumatology (ACR) and the European League against Rheumatism (EULAR) collaborated to update these criteria to better aid the diagnosis of early RA patients [10]. This updated criteria set for classification takes more factors into consideration (shown in Table 1-2). Patients are given a point value between 0 and 10, with any patient showing synovitis of otherwise unexplained cause, and achieving a score of 6 or greater classified as having RA.

Criterion	Definition
Morning stiffness	Morning stiffness in and around the joints, lasting at least 1 hour before maximal improvement
Arthritis of 3 or more joint areas	At least 3 joint areas simultaneously have had soft tissue swelling or fluid (not bony overgrowth alone) observed by a physician. The 14 possible areas are right or left PIP, MCP, wrist, elbow, knee, ankle, and MTP joints
Arthritis of hand joints	At least 1 area swollen (as defined above) in a wrist, MCP, or PIP joint
Symmetric arthritis	Simultaneous involvement of the same joint areas on both sides of the body (bilateral involvement of PIPs, MCPs, or MTPs is acceptable without absolute symmetry)
Rheumatoid nodules	Subcutaneous nodules, over bony prominences, or extensor surfaces, or in juxtaarticular regions, observed by a physician
Serum rheumatoid factor	Demonstration of abnormal amounts of serum rheumatoid factor by any method for which the result has been positive in <5% of normal control subjects
Radiographic changes	Radiographic changes typical of rheumatoid arthritis on posteroanterior hand and wrist radiographs, which must include erosions or unequivocal bony decalcification localized in or most marked adjacent to the involved joints (osteoarthritis changes alone do not qualify)

Table 1-1 The 1987 Criteria for Classification of Rheumatoid Arthritis
Taken from [9]. PIP - proximal interphalangeal, MCP metacarpophalangeal - , MTP - metatarsophalangeal.

Criteria	Score
Joint involvement	
1 large joint	0
2-10 large joints	1
1-3 small joints (with or without involvement of large joints)	2
4-10 small joints (with or without involvement of large joints)	3
>10 joints (at least 1 one small joint)	5
Serology	
Negative RF and negative ACPA	0
Low-positive RF or low-positive ACPA	2
High-positive RF or high-positive ACPA	3
Acute-phase Reactants	
Normal CRP and normal ESR	0
Abnormal CRP or abnormal ESR	1
Duration of Symptoms	
< 6 weeks	0
≥ 6 weeks	1

Table 1-2 The 2010 ACR / EULAR classification criteria for rheumatoid arthritis
Taken from [11]. RF – rheumatoid factor, ACPA – anti-citrullinated protein antibodies, CRP - C-reactive protein, ESR – erythrocyte sedimentation rate.

1.1.2 **Risk Factors**

Although the initial cause(s) of RA remain largely unknown, a number of genetic and environmental factors have been identified that are proposed to be associated with susceptibility to, and subsequent progression of disease.

1.1.2.1 Genetic Susceptibility

The link between genetics and RA has been known for many years. A number of different genes have been linked to the development of disease, some affecting different patient subsets. The use of twin studies has led to the proposal that genetic factors account for approximately 50%-60% of an individual's susceptibility to RA [12], with the rest being down to environmental and chance factors. Some of these genetic associations with disease have also led to insights into disease pathogenesis.

HLA alleles were the first genetic factors associated with susceptibility to RA, with a number of different class II (DR/DQ) alleles now known to infer genetic susceptibility. It was initially estimated that HLA alleles account for 50% of an individual's genetic risk susceptibility to RA [13], although some more recent studies suggest it may be slightly lower. Most of the RA-associated alleles contain a common amino acid motif in their ß chain – referred to as the "shared epitope" [14]. This led to the suggestion that major histocompatibility complex (MHC) class II-dependant adaptive immune responses were important in RA.

PTPN22 is another gene which has been linked to susceptibility of various autoimmune disorders, including RA. The intracellular tyrosine phosphatase can associate with adapter proteins, and plays a role in setting the threshold for T cell activation, as well as having roles in B cell signalling. A link between RA and PTPN22 was suggested in 2004 [15], with later studies confirming this as a susceptibility gene for RA [16, 17].

Although many studies have supported a link between certain HLA alleles and PTPN22 polymorphisms and RA, they appear to only infer susceptibility to seropositive disease, having little to no effect on the subset of patients which are negative for ACPA antibodies. The link between these two genetic loci and

RA implicates T and B cells in the pathogenesis of RA, suggesting that adaptive immune responses play a role in the development, or the associated pathology of RA – at least in ACPA positive disease.

It has also long been known that being of the female sex predisposes an individual to the development of RA, with more female than male sufferers at a ratio of approximately 3 : 1 [18]. It has recently been suggested that this is only true for individuals not encoding the shared epitope, with a study showing risk to be equal between the sexes where individuals are homozygous for the shared epitope [19]. The link between females and RA is thought, at least in part, to be due to sex hormones. It has been shown that oestrogens can stimulate the production of inflammatory cytokines by RA macrophages. This also partly explains the fluctuations of disease severity seen in females throughout the menstrual cycle, and the improvement of symptoms during pregnancy [20].

More recently, technological advances have seen the use of genome-wide association studies (GWAS) to assess genetic susceptibility to RA, and have implicated a variety of other immune system genes – including TRAF-1 [21, 22], OLIG3-AIP3 [23] and Signal Transducers and Activators of Transcription (STAT) -4 [24] among many others. Okada *et al* [25] recently performed a comprehensive GWAS meta-analysis using data from over 100,000 subjects of mixed ancestry. They discovered 42 novel RA risk loci, taking the current total to 101 risk loci. They found 377 genes in the associated region of linkage disequilibrium, and using BIOCARTA and Ingenuity pathway analysis softwares they found enrichment of pathways further implicating T cells, B cells and cytokine signalling (including interleukin (IL) 10, IFN and granulocyte macrophage colony-stimulating factor (GM-CSF)) pathways in RA pathogenesis. They used a multi-factorial *in silico* bioinformatics pipeline to prioritise a list of 98 "candidate biological RA risk genes". The authors then proposed that if genetic methods like this were valuable for finding new drug targets, it would highlight therapies which are already used to treat RA. Interestingly, when they looked at what drugs were known to target the protein products associated with their RA risk loci, they found significant enrichment for approved RA drugs. These included the most common biologics used to treat RA, such as those targeting TNF, as well as Toculizimab and Abatacept. Also highlighted by this method were CDK

inhibitors, such as Flavopiridol. Interestingly, this molecule has already been studied in the context of RA. It was shown in the murine collagen-induced arthritis (CIA) model that it ameliorated disease symptoms and severity [26], and the compound is now in early clinical trials for the treatment of RA patients. This study has therefore highlighted the usefulness of such genetic approaches when looking for novel drug targets.

1.1.2.2 Environmental Factors

The best established environmental link with RA is smoking. Some studies have suggested that smoking is only a risk factor for the subset of RA patients which are ACPA positive [27], while having little to no influence on susceptibility in ACPA negative individuals [28]. Importantly, it is thought to have a cumulative dose effect, where the duration and quantity of cigarettes smoked are risk factors for more severe disease outcomes, particularly erosive progression [29, 30]. Smoking is also thought to be a more important risk factor in patients carrying HLA-DR risk alleles [31, 32]. Interestingly smokers are less likely to respond to therapeutics. As well as smoking, a number of other airway exposures have been associated with the development of RA, including silica dust [33], mineral oils [34] and charcoal – which is documented to cause severe RA symptoms in charcoal workers [35].

Another environmental link with the development of RA is infection. Although the exact mechanisms remain unclear, infectious agents have long been implicated in the development and/or persistence of RA. These include Epstein-Barr virus (EBV), cytomegalovirus, Proteus species, *Escherichia coli* and *Porphyromonas gingivalis (P. gingivalis)*.

P. gingivalis is a pathogenic bacterium implicated in the development of many cases of periodontal disease. Numerous studies have reported a link between incidence of periodontal disease and RA [36-38], but it is currently unclear whether there is a true causal link, or this is purely a consequence of the fact these diseases share a number of risk factors. Interestingly though, *P. gingivalis* is the only bacterium known to produce peptidyl arginine deaminase enzymes capable of promoting the citrullination of mammalian proteins [39].

Although the relationship between EBV and RA remains unclear, there are several lines of evidence supporting a link between the two. There is data suggesting that RA patients have elevated serum titres of anti-EBV antibodies [40, 41], and peripheral blood mononuclear cells (PBMCs) isolated from RA patients express far higher levels of EBV deoxyribonucleic acid (DNA) than control cells [42, 43]. The virus has also been detected in synovial fluid from RA patients [44].

In more recent years, the focus has switched from studying a single infectious agent, to looking at the host microbiome in its totality. Technological advances such as culture-independent 18S ribosomal ribonucleic acid (RNA) sequencing and the launch of the National Institute of Health (NIH) Human Microbiome Project in 2007 have led to increased interest in this field. There are now a number of studies looking at faecal samples to study a link between possible gut dysbiosis and RA. One study [45] looked at the different *Lactobacillus* species, and found higher diversity in early RA patients. There were increased numbers of many species, with one – *Lactobacillus mucosae* – being unique to RA patients. In another, more comprehensive study [46], Scher et al performed 16S sequencing on 114 faecal samples derived from healthy controls, psoriatic arthritis, chronic treated rheumatoid arthritis (CRA) and new-onset untreated rheumatoid arthritis (NORA) patients. This study found the presence of one particular species - *Prevotella copri* - correlated highly with NORA patients. This bacterium has been associated with various other inflammatory events. Recently, high levels of *Prevotella* in the gut of healthy individuals was correlated with plasma levels of trimethylamine N-oxide (a substance thought to be predictive of cardiovascular events in humans) [47].

Taken together these studies suggest a clear correlation between changes in the microbiome and development of RA, but evidence is still lacking to elucidate whether there is a true causative effect, or whether changes in the microbiome simply reflect a secondary response to on-going local and systemic inflammation.

1.1.3 **Current therapies**

Over the past 10 to 20 years, the treatment of RA has improved remarkably. Previous treatment regimes involved giving patients non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids and conventional disease modifying anti-rheumatic drugs (cDMARDS) – which were often used with little attention to pathogenic rationale. However, advances towards understanding the mechanisms underlying disease activity have allowed the development of drugs targeting biological pathways known to play key roles in the disease. A list of the most commonly used conventional and biologic DMARDS is shown in Table 1-3.

Therapy	Target	Mode of Action
Conventional DMARDs		
Methotrexate	Folate-dependent enzymes	↓ folate-dependant enzymes results in impaired pyrimidine and purine synthesis and inhibition of lymphocyte proliferation
Hydroxychloroquine	Lysosomes, lysosomal enzymes TLR-9	Lysomotropic properties hinder antigen presentation and also impairs activation of innate immune cells
Sulfasalazine	Folate-dependent enzymes	Same as listed for methotrexate, also appears to block IκB and induce apoptosis of neutrophils and macrophages
Leflunomide	Dehydroorotate dehydrogenase (DHODH)	↓ DHODH results in impaired pyrimidine synthesis and inhibition of lymphocyte proliferation
Biological DMARDs		
Etanercept	TNF-α	Bind TNF-α thereby preventing binding and signalling through its receptor
Infliximab		
Adalimumab		
Cetrolizumab		
Golimumab		
Tocilizumab	IL-6R	Binds IL-6R preventing binding of IL-6 and subsequent signalling
Anakinra	IL-1R	Binds IL-1 type I receptor preventing binding of IL-1 and subsequent signalling
Rituximab	CD20	Binds to and depletes $CD20^{+}$ B Cells
Abatacept	CTLA-4	Blocks T cell co-stimulation

Table 1-3 List of the most commonly used drugs in the treatment of Rheumatoid Arthritis
Adapted from [48]. DMARD - disease modifying anti-rheumatic drug, DHODH - dehydroorotate dehydrogenase, TNF – tumor necrosis factor, IL - interleukin.

Although methotrexate (MTX) is one of the older cDMARDS used to treat RA, it is often still used as the first line of therapy – either alone or in combination with other treatments [49]. MTX is a folate inhibitor, which functions by inhibiting folate dependant enzymes, thereby preventing purine and pyrimidine synthesis and resulting in impaired inflammatory lymphocyte proliferation [50]. Like MTX, sulfasalazine is known to inhibit folate-dependant enzymes leading to impaired lymphocyte function [51, 52]. The beneficial effects of sulfasalazine may also be due to its ability to induce apoptosis of neutrophils and macrophages [53, 54]. The anti-malarial agents chloroquine and hydroxychloroquine (usually the latter) are also used to treat RA. Although their exact anti-inflammatory mechanisms are not fully understood, it is thought they function in part through their ability to enter lysosomes and alter the pH, thereby influencing cell function [55]. They are also thought to negatively impact TLR signalling – particularly TLR-9 [56].

Although MTX and other cDMARDS are effective in treating the symptoms and clinical manifestations of disease for many patients, this does not always suffice to sufficiently control inflammatory disease activity or progressive joint destruction [57]. For this reason investigators turned to disease pathogenesis for clues to develop new biologic therapies.

Tumor necrosis factor (TNF) is known to be present, and critically involved in the pathogenesis of RA. The cytokine is highly expressed in both synovial fluid and tissues derived from RA patients, with expression of its receptors also shown to be up-regulated in the synovium – particularly in areas adjacent to erosions [58-60]. Further evidence for the involvement of TNF in arthritis is seen in hTNFtg mice. These mice are genetically altered to over-express human TNF, and in consequence develop spontaneous RA-like lesions in their joints [61]. Early *in vitro* studies showed that targeting TNF could reduce the expression of other inflammatory cytokines such as interleukin (IL) -1 and GM-CSF. More importantly, targeting IL-1 or GM-CSF did not reduce TNF levels – suggesting TNF as a master regulator in inflammatory cytokine production [62-64]. In order to test the potential therapeutic benefit(s) of targeting TNF, it was blocked in the collagen induced arthritis (CIA) model, where it reduced inflammation in a dose dependant manner [65]. At the same time another group demonstrated that

administering TNF to mice with CIA led to more severe disease symptoms [66]. The first studies of anti-TNF therapy in humans were performed with small numbers of patients, but data confirmed this treatment was capable of dramatically improving both clinical and acute phase reactant measures of disease activity in the majority of patients [67]. Since then five TNF inhibitors have been approved for clinical use (as described in Table 1-3), providing a major improvement in outcomes for many patients. Owing to the success of anti-TNF, and the fact some patients still fail to respond to this treatment, a number of biologic therapies have been developed and licenced to treat RA. Cytokine inhibitors opposing the actions of IL-6 and IL-1 (Tocilizumab and Anakinra respectively), Rituximab that depletes $CD20^{+}$ B cells and Abatacept that interrupts T cell / APC co-stimulation are now all routinely used in the management of disease.

More recently there has been increased interest in deriving small molecule inhibitors that can recapitulate the effects of extra cellular biologic agents. Tofacitinib is a janus kinase (JAK) 1/3 inhibitor that has undergone several phase II [68-71] and phase III [72-75] trials in RA. Many of these studies have demonstrated promising results in the treatment of patients who have currently failed to respond to methotrexate or anti-TNF therapies. After three months a significant improvement in ACR20 and ACR50 responses were found, however, as with many immune-modulating therapies, this was accompanied with reduced neutrophil counts, increased infection rate and an increase in cholesterol. Longer term studies looking more closely at the potential risk benefit profile of the drug are therefore required, but this agent is now licenced and approved for use in several geographical areas. Other biologic agents are also in development. In particular an anti-GM-CSF receptor (mavrilimumab) is currently in phase IIb trials, having demonstrated efficacy in phase IIa studies thus far. Other trials are underway in which monoclonals targeting IL-20, IL-21 and IL-17 are being tested – but it is currently too early to determine the likely success of these approaches. The blockade of IL-17 in particular has been generally disappointing in RA, despite showing remarkable benefits in the treatment of psoriasis patients.

These new medications have provided a step change in disease management, and have led to an improvement in disease outcomes for many patients. However, partial or non-response to these treatments remains common, and drug free remission is rarely achieved. This means that there is still significant unmet clinic need, and new therapies are still required for the treatment of RA.

1.1.4 **The Synovial Lesion**

In a healthy joint, a thin synovial membrane comprising primarily of two main cells types (synovial fibroblasts and resident macrophages), lines the joint and acts as a lubricating tissue to ensure free movement of the joint without friction or discomfort. In RA however, the synovial membrane becomes hyperplastic. There is an increasedleukocytes. Technological advances have allowed detailed analysis of the synovial lesion, which as such, is increasingly well characterised. Among the immune cells found in the joint are innate immune cells such as macrophages, dendritic cells (DCs), neutrophils and mast cells, but there are also components of the adaptive immune system found. These include activated T helper (Th) 1 and Th17 cells and B cells exhibiting somatic hyper-mutation. The resident host tissue cells include fibroblast-like synoviocytes (FLS), chondrocytes and osteoclasts, which do not act as passive bystanders but rather assume a 'pro-inflammatory' phenotype that actively contributes to articular inflammation and damage. The result of this recruitment and proliferation of cells is "pannus" formation, where the proliferating synovial cells penetrate cartilage and bone and ultimately cause joint destruction [76]. A representation of a healthy and RA joint is shown in Figure 1-1.

The immune cells that influx to the RA synovium represent both arms of the immune response, innate and adaptive immunity, both of which are crucial in protecting the host from infection. The innate immune system mounts the first line of defence. It has evolved to detect common molecular patterns expressed by pathogenic organisms, allowing it to respond quickly to infection. The adaptive immune response, on the other hand, provides a slower, but more specific response to a given threat. The adaptive immune response critically offers memory of the given insult, so that a faster response can be mounted if the same insult is encountered again. Under normal circumstances these two arms of the immune system work together to provide the best protection from infections, but when this goes awry and the immune system is triggered unnecessarily or fails to switch off appropriately, this protective immune response can cause damage to self. This is known as autoimmunity, and is considered to lie at the base of the pathogenesis of RA. The potential role of innate, adaptive and resident cells in RA will now be discussed individually.

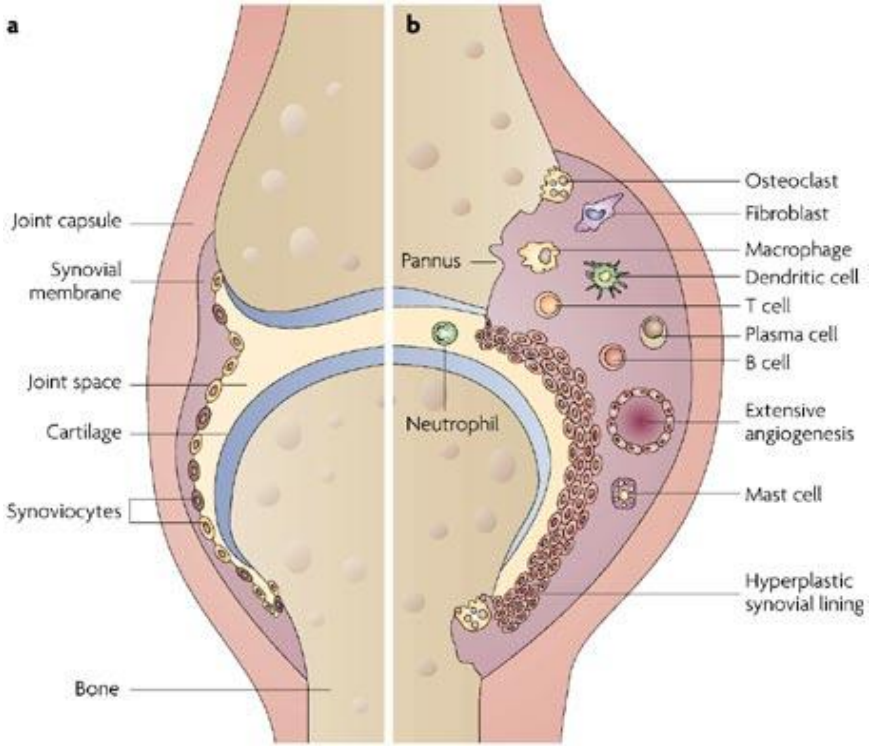


Figure 1-1 Comparative illustration of a healthy and RA joint
The above diagram shows a representative healthy (a) and RA (b) joint. Taken from [77].

1.1.4.1 Innate Immunity in RA

Innate immune cells found in the synovial lesion in RA include monocytes and macrophages – which are the focus of my PhD and will be discussed in section 1.2 – as well as dendritic cells, neutrophils, mast cells and NK cells. Many of these cells influx to the RA synovium and become activated at the sites of inflammation, resulting in the production of molecules which further perpetuate inflammation, and ultimately result in joint damage. One of the ways these cells become activated is by innate immune sensing pathways. These pathways include those initiated by toll-like receptors (TLRs), NOD-like receptors and inflammasome components [78, 79]. These receptors are involved in recognising DAMP (damage associated molecular pattern) and PAMP (pathogen-AMP) products and likely serve to amplify and perpetuate disease.

1.1.4.1.1 Toll-like Receptors

TLRs are the best studied of these pathways. These receptors are crucial for the innate immune response to invading microbial and viral pathogens, and function by sensing PAMPs such as the bacterial cell wall components lipopolysaccharide (LPS) and peptidoglycan. This TLR ligation results in a signalling cascade which ultimately causes the release of pro-inflammatory cytokines and other inflammatory mediators from the cell. Important in the context of RA, however, TLRs can also be activated by a number of endogenous DAMPs, many of which have been found in the inflamed RA synovium. These include heat shock proteins, fibronectin and hyaluronan [80, 81], as well as more recently described TLR ligands such as Tenascin-C (TNC). TNC is an extracellular matrix glycoprotein of which the expression is increased at sites of tissue damage or injury. TNC levels return to normal after the resolution of inflammation, but in conditions like RA, where there is on-going inflammation and tissue remodelling, the expression of TNC is shown to remain elevated [82, 83]. This could result in the persistent stimulation of synovial cells via TLRs. A list of TLRs and their potential ligands in infection and inflammation is given in Table 1-4.

TLR	Location	Pathogen associated molecular patterns	Potential endogenous TLR ligands in RA (DAMPS)	Experimental Ligands used
TLR2 (dimerises with TLR1 or TLR6)	Extracellular	Lipoglycans (Mycobacterium) Lipoteichoic Acids (Gram-positive bacteria) Peptidoglycan (Gram-positive bacteria) Zymosan (Yeast)	HSP 60 / 70, gp96, HMGB-1, Biglycan, Serum amyloid A	Pam3
TLR4	Extracellular	LPS (Gram-negative bacteria) Mannan (Candida) Envelope protein (Virus)	HSP 22 / 60 / 70, EDA fibronectin, Fibrinogen, HMGB-1, Biglycan, Tenascin C	LPS
TLR3	Intracellular	dsRNA	Necrotic cells	Polyl:C
TLR5	Extracellular	Flagellin		
TLR7	Intracellular	ssRNA		Cl097
TLR8	Intracellular		Necrotic cells	
TLR9	Intracellular	CpG motifs		

Table 1-4 Summary of toll-like receptors and their ligands
Adapted from [79]. TLR – toll-like receptor, HSP – heat shock protein, LPS – lipopolysaccharide, HMGB-1 - high-mobility group box 1.

TLRs are present on many cell types, and numerous studies have examined the expression of TLRs on synovial cells in RA. The levels of TLRs 3, 7 and 9 have been shown to be increased in RA cells compared to those derived from OA patients or healthy controls [84-86]. Expression of TLRs on innate immune cells like macrophages and dendritic cells was initially thought to be most functionally relevant, but increased expression of TLRs on RA synovial fibroblasts (RASFs) is now known to be important. One study demonstrated increased TLR-2 and TLR-4 expression on RASFs compared to OASFs [87], and while another study found no significant differences in the TLR expression of these cells, they found that RASFs produced more IL-6, matrix metalloproteinase (MMP) -3 and MMP-13 [84] in response to TLR stimulation. In abstract form, Chamberlain *et al* reported data confirming that TLR-7 is up-regulated in monocytes and macrophages from RA patients, and suggested that TLR-7 ligands are chemotactic for monocytes. They therefore hypothesised that TLR-7 ligation could actually promote monocyte migration to the joint [88].

A role for these TLRs in arthritis is also suggested in rodent models, with mice deficient in TLR2 or TLR4 showing reduced CIA and streptococcal-cell-wall-induced arthritis [89]. *In vitro* cytokine production by cultures of human synovial cells is also dependant on TLR adapter proteins [90].

1.1.4.1.2 Dendritic Cells in RA

DCs are often referred to as the classical antigen presenting cells, and are thought to be the principal cell-type involved in the activation of naive T cells. During T cell priming they play a role in determining the developmental fate of these cells, and influence whether they become a particular T_h subset or develop regulatory function [91]. DCs are also involved in the initiation and maintenance of both central and peripheral tolerance, and are capable of deleting auto-reactive T cells in the periphery [92], as well as expanding regulatory T cell populations. Using models of autoimmune disease, DCs have been shown to be important in the generation of MHC-restricted immune responses [93, 94]. The resulting T cell activation is involved in autoantibody production, and the development/perpetuation of chronic inflammation. The many and varied roles of DCs suggest they could be involved in several aspects of RA disease pathology.

There are two major DC subsets, myeloid (mDCs) and plasmacytoid (pDCs) DCs, both of which are found in the SF of RA patients [95]. Many DCs found in the synovium of RA patients are positive for nuclear RelB, and therefore have a high capacity to stimulate T cells [96, 97]. It has also been shown that DCs and T cells are found in close association in the RA synovium, where it is thought the DCs may be involved in priming and activating the T cells [98]. Synovium-infiltrating DCs also process and present antigen locally, which could sustain local inflammation and perpetuate disease pathogenesis [96, 98, 99].

DCs also have a role in the production of pro-inflammatory cytokines, working in concert with macrophages and other cells within the synovial compartment they produce inflammatory mediators, such as TNF, which can drive the inflammatory pathology seen in RA [94, 100]. In fact, inflammatory DCs have been shown to act as effectors in cartilage destruction via an indirect mechanism involving the production of TNF [101]. It has also been demonstrated that synovial DCs from RA patients produce higher levels of CCL18 – which is a T cell chemo-attractant and can also induce fibroblasts to produce collagen [102]. A recent paper by Segura *et al* studied cells derived from inflammatory fluids, including RA synovial fluid, and found a discrete population of cells which shared many features similar to *in vitro* monocyte-derived DCs, and clearly distinct from inflammatory macrophages. They went on to show that these “inflammatory DCs”, but not

inflammatory macrophages, could promote Th17 cell differentiation, in part through their secretion of IL-17. This study highlights a potential role for DCs in promoting pathogenic Th17 cell differentiation in inflammatory conditions like RA [103].

Recent data suggest an important role for thymic stromal lymphopoietin (TSLP) in DC : T cell interactions in RA. This cytokine plays important roles in DC-induced maturation of T cells, and its levels are significantly higher in SF from RA patients compared to OA-derived samples. mDCs from the SF of RA patients also express significantly higher levels of its receptor (TSLPR). Exposure of mDCs to TSLP resulted in their increased production of CCL17 and macrophage inflammatory protein (MIP) 1α, and also increased their capacity to induce $CD4^+$ T cell proliferation [104]. Administration of TSLP during the CIA model in mice was also found to significantly exacerbate disease severity, while $TSLPR^{-/-}$ mice developed less severe arthritis than their wild-type counterparts. The knockout mice had lower levels of inflammatory cytokines including IL-17, IL-1β and IL-6 [105]. Due to the results of these and other studies, TSLP has been suggested as a potential therapeutic target in RA.

Despite the many possible pro-inflammatory roles of DCs in RA, it has been shown that selective depletion of pDCs in vivo in a murine arthritis model actually increased the severity of articular pathology and both T and B cell autoimmune responses against type II collagen. This suggests that pDCs may in fact offer a net anti-inflammatory effect in this situation [106]. This could, in part, be due to the involvement of DCs in maintaining peripheral tolerance. This occurs where an antigen is encountered in the absence of co-stimulatory signals from APCs [107]. Immature DCs express low levels of the co-stimulatory molecules CD80 and CD86, and produce high levels of IL-10. These cells are often termed “tolerogenic” as it is thought presentation of antigens by these cells induces T cell anergy, thereby promoting peripheral tolerance.

It is possible in inflammatory diseases, like RA, that the balance between DCs as tolerance inducing vs pro-inflammatory cells is swayed towards the latter, inflammation promoting phenotype. For this reason many groups are looking to harness the potential of tolerogenic DCs as an immunotherapeutic tool to induce or restore tolerance. Many different methods are currently being studied, with

the aim to generate autologous, tolerogenic DC populations *in vitro* which can be re-introduced to patients, with the hope that this could reinstate immune tolerance [108].

1.1.4.1.3 Neutrophils in RA

Neutrophils are the most abundant circulating leukocytes found in mammals, and are primarily involved in host defence against bacterial and fungal pathogens. In order to protect us from such infections, neutrophils are armed with a number of antimicrobial proteins, which among other things, are capable of degrading microbial proteins and cell wall components. Unnecessary activation of these cells however, can be detrimental and result in immune mediated damage to host cells. In RA, neutrophils are the most abundant cells found in synovial fluid, and when isolated they show clear signs of activation. This suggests these cells have been primed, and could be partly responsible for the increased expression of cytokines such as IL-1ß, IL-18 and IL-8 seen in the RA synovial environment [109, 110]. The location of these neutrophils also suggests they could be involved in disease pathogenesis. They are often found at the pannus: cartilage junction, which is the location where most tissue damage occurs [111]. Evidence to suggest a role for neutrophils in arthritis pathogenesis is also seen in animal models. Using an anti-Gr1 antibody to deplete these cells protects mice from several experimental models of arthritis, including the CIA and K/BxN serum transfer models [112, 113].

More recently neutrophils have been implicated in the pathogenesis of RA through their formation of neutrophil extracellular traps (NETs). These were first described in 2004 as a new method by which neutrophils could kill bacteria [114]. NETs consist of networks of extracellular fibres, mainly chromatin that are covered with antimicrobial peptides normally present in the granules of neutrophils [114]. Stimulation of neutrophils in vitro with IL-8 or LPS has been shown to result in their release of these NETs, which cause the externalisation of pro-inflammatory, immune-stimulatory molecules [115, 116]. This is particularly relevant in conditions like RA where there is an abundance of neutrophils together with many stimulatory factors that could cause their release of these NETs. Indeed, NET formation is enhanced in both circulating and synovial fluid neutrophils from patients with RA, compared to neutrophils from healthy controls [117].

Another indicator that neutrophil NET formation may be important in RA pathogenesis is the link between citrullination and NET formation. It is reported

that citrullination of histones is an essential step during NET formation, and that citrullinated proteins are detected in NETs. Indeed, RA sera containing ACPA can react with citrullinated histone derived from NETs [118]. These findings suggest that neutrophil NET formation is enhanced in RA, and has the potential to expose other cells in the synovium to citrullinated autoantigens that could lead to ACPA formation.

1.1.4.1.4 Other innate cells implicated in RA pathogenesis

Although monocytes, macrophages, dendritic cells and neutrophils are the most studied innate immune cells in RA, other innate cells and present in the heterogeneous cell milieu and have functions that could be important in RA pathogenesis. These include mast cells and natural killer (NK) cells.

Mast cells are granulocytic cells that are best known for their roles in allergy and anaphylaxis, though they also play important roles in host defence against pathogens and wound healing [119]. In RA, both the numbers of mast cells, and the concentration of many of their mediators are increased in the synovium [120-122]. Mast cells express TLRs 1 through 9 [123-127], and stimulation of blood-derived mast cells with TLR ligands causes their production of cytokines - including GM-CSF, IL-8, MIP-1α and TNF-α. This study went on to show that mast cells also express FcγRII, and stimulation with immune complexes can lead to their production and release of IL-8. IL-8 production by these blood-derived mast cells was further increased by TLR stimulation in the presence of plate-bound IgG, suggesting synergy between FcγR and TLR stimulation, which could be of particular interest in the RA synovium where stimulation of both pathways together is likely [123]. This study highlights the potential roles of mast cells in RA, though further studies looking specifically at primary mast cells rather than those differentiated from blood precursors would be useful to reinforce these findings.

NK cells are another group of innate immune cells that are crucial for host defence, but could play a significant role in the perpetuation of inflammatory diseases when activated unnecessarily. It is reported that a subset of NK cells, CD56 bright cells, are greatly expanded within the synovium of RA patients [128]. As the name suggests, these cells were initially defined by their ability to spontaneously kill tumour and virally infected cells [129, 130]. It is now known these cells express FcγRIIIA and are capable of performing antibody-dependant cellular cytotoxicity (ADCC) [131]. As well as FcγRIIIA, NK cells express many other surface receptors, including NKG2D and CD244, which allow them to recognise and kill stressed or antibody coated cells. Some reports suggest that the expression of these receptors is actually reduced in RA, which could contribute to an impaired NK cell activity in these patients [132]. Another

function of NK cells is cytokine production. One of the key cytokines they produce is IFN-γ. This cytokine is capable of enhancing immune responses by promoting Th1 development and also increasing the expression of MHC class I molecules on other cells, such as APCs. It has also been reported that sub-populations of NK cells can produce many other cytokines, including TNF-α, IL-15 and GM-CSF [131, 133, 134], all of which have previously been implicated in RA pathogenesis. NK cells could therefore contribute to RA disease progression through cell : cell interactions and their production of inflammatory mediators in the joint.

1.1.4.2 Adaptive Immunity in RA

Several strands of evidence support a role for adaptive immunity in RA. GWAS clearly identify a variety of loci that are vital for T cell activation and co-stimulation – notably the strong association with Class II suggests a core role for peptide presentation in pathology at some point. The presence of ACPA and IgG (rheumatoid factors) antibodies, arising often from class switched B cells which rely on T cell help, and also strong evidence from a large number of animal model systems implicate adaptive immune responses in RA. Nevertheless, it is unclear what causes breach of immune tolerance in the offset.

1.1.4.2.1 T cells

As mentioned above, a role for T cells in the pathogenesis of RA is suggested by the genetic association of RA with certain class II MHC alleles and PTPN22. The presence of high numbers of T cells in synovium of RA patients, and the requirement for T cells in many animal models of arthritis also implicates these cells in disease pathogenesis [135]. A number of approaches have been used to target T cells in RA, most with disappointing results [136], but some benefit has been seen with the use of CTLA-4-Ig fusion protein Abatacept [137]. Abatacept can inhibit T cell co-stimulation and subsequent activation, and may also promote T-cell tolerance and regulatory function [138]. *In vitro* studies have also suggested that treatment of cytokine activated T cells with Abatacept can suppress their ability to induce the production of TNF-α and other pro-inflammatory cytokines by macrophages in a co-culture system [139]. The clinical efficacy of this therapy implicates T cells directly in the pathogenesis on RA.

In RA auto-reactive T cells have been found; these include populations specific for ACPA antigens, type II collagen and also cartilage glycoprotein 39, but the significance of these cells *in vivo* is debated. In a recent study Klarenbeek *et al* [140] set out to determine whether the T cell receptors of RA patients displayed an abnormal repertoire. They looked at synovial T cells from early and established RA patients, as well as peripheral blood T cells. The study found that the T cell repertoire of synovial cells from early RA patients in particular, was dominated by highly expanded clones (determined by a frequency of over

0.5%). The group went on to speculate that these clones could be auto-reactive, and that their expansion could be due to the presence of specific auto-antigens in the synovium. Although this is an interesting hypothesis, further studies will be required to find these possible auto-antigens and determine their role in RA pathogenesis.

T cells are also likely to be one of the cell types involved in inflammatory cytokine production in the joint. Naive $CD4^+$ T cells can differentiate into diverse effector phenotypes depending on the cytokine milieu to which they are exposed, such as T helper 1 (T_h1), T_h2, T_h17 and T regulatory (T_{reg}) cells. This in turn determines their pattern of cytokine release.

RA was initially thought to be a T_h1-mediated disorder, with more recent data suggesting T_h17 cells could be the dominant T cell players. Matters are even less clear in early disease in which the first T cell subset to emerge appear to have more of a T_h2 phenotype [141]. One of the key cytokines T_h17 cells produce is IL-17. In recent years, what was originally termed IL-17 is now known as IL-17A, with 5 other members of this family being discovered (IL-17 B through F). Of these, IL-17A and IL-17F are most closely related, sharing around 50% homology [142]. Because of this, it is thought that they exhibit many of the same functions, though IL-17A often appears more potent than IL-17F. Both of these cytokines and their receptors are expressed in the synovium of RA patients, where they likely contribute to disease pathogenesis [143-147]. IL-17A has been implicated in many autoimmune diseases, with a role in RA pathogenesis suggested in many animal models. Here it has been shown that its up-regulation or down-regulation of can worsen or suppress joint inflammation, respectively [148]. It is known that IL-17A can act on synovial cells, including monocytes and neutrophils, driving their activation and subsequent cytokine release. It can also act on synovial fibroblasts, and is involved in their production of prostaglandins and matrix metalloproteinases [149]. As well as IL-17, T_h17 cells are capable of secreting many other inflammatory cytokines, including IL-21, IL-22 and TNF [150]. More recent data, in fact, suggests that the production of GM-CSF by T_h17 cells could be responsible for many of their pathogenic functions [151]. The GM-CSF released by these cells can act on macrophages and dendritic cells increasing their activation state, and also causing their release of cytokines such

as IL-23, which can further enhance T_h17 cell development and maturation – thereby creating a positive feedback loop. As well as RA, GM-CSF produced by T_h17 cells has now been implicated in a number of other autoimmune diseases, including multiple sclerosis and myocarditis [152, 153].

In addition to these "pathogenic" T cells, there is also evidence of regulatory T cell populations expressing FoxP3, CD4 and CD25 in the RA synovium. These regulatory cells are normally involved in the maintenance of tolerance and regulation of immune responses. They can do this in a cell contact dependant manner using molecules such as CTLA-4, but also through their secretion of soluble mediators such as IL-10 and TGF-β. In RA, there is some evidence to suggest that although these cells are present in the synovium of patients; their function may be impaired due to high levels of TNF. While they are able to supress the proliferation of effector cells, their ability to supress pro-inflammatory cytokine production appears defective [154, 155].

1.1.4.2.2 B Cells

The successful use of Rituximab in treating RA has generated a lot of interest in the role of B cells in disease pathogenesis. Rituximab is a CD20+ B cell / plasmablast depleting monoclonal antibody, which provides significant (and in some cases prolonged) clinical benefit in RA patients [156]. This, together with the fact that B cells have been shown crucial in several murine models of arthritis [157-159], has generated a lot of interest in the role of these cells in RA. B cells have many diverse functions though, so could play multiple roles in the pathogenesis of RA. They are the cells that produce antibodies, and could therefore be responsible for the generation of autoantibodies, but they can also contribute to pathogenesis by acting as antigen presenting cells and cytokine producers.

The role of B cells as antibody producers is seen in patients positive for RF and ACPA antibodies. The presence of ACPA antibodies is often seen many years before the onset of disease symptoms, and patients are often subdivided based on the presence of absence of these antibodies. $ACPA^+$ RA patients generally develop a more aggressive condition with a worse prognosis [160], and disease severity has even been correlated to antibody titres. Although not fully understood, it is possible these antibodies contribute to disease pathogenesis. ACPA antibodies are generated against citrullinated self-proteins. Peptidylarginine deiminase (PAD) enzymes are a family of enzymes that can cause the post-translation modification known as citrullination; where arginine residues are converted to citrulline. In RA ACPA have been found that target many self-proteins, including fibrinogen, vimentin, α-enolase and collagen type II. Increasing evidence suggests that these antibodies are not simply a diagnostic tool in RA, but they also play a role in disease pathogenesis. One study demonstrated that immune complexes carrying ACPA could stimulate macrophages via TLR and FC gamma receptor-mediated pathways [161, 162], and they have also been shown to activate complement [163] and promote osteoclastogenesis [164]. One particular study looking at the effect of ACPAs on macrophage activation demonstrated that ACPA-containing immune complexes stimulated the macrophages to produce TNF-α – providing a clear link between the ACPAs found in RA and disease mechanisms [162].

The genetic susceptibility of certain HLA-DR positive individuals to developing RA supports a role for MHC-dependant antigen presentation in the development and / or pathogenesis of RA. Although not likely to be involved in the initial priming of naïve T cells, B cells can act as antigen-presenting cells, and are thought to be important in later T cell responses [165]. They can also express some of the co-stimulatory molecules crucial for T cell activation, including CD80, CD86 and CD40, so it is possible that they could support the generation of autoreactive T cells [166].

B cells are also capable of producing chemokines (including CXCL13 [167]) and pro-inflammatory cytokines such as IFN–γ, IL-4 and IL-6. These cytokines can act on other cells and provide nonspecific help to bystander T cells, further perpetuating the on-going inflammation.

Due to the partial success of Rituximab treatment and the many possible roles of B cells in RA, a number of other B cell targeting therapies have been considered for the treatment of RA patients. These include Belimumab and Atacicept. Belimumab is an anti-BAFF/BLyS (B-cell activating factor/B-lymphocyte stimulator) antibody, while Atacicept blocks both BLyS and APRIL (A PRoliferation Inducing Ligand). These are two factors known to be crucial in promoting B cell function and survival. Both drugs went onto the clinical trial stage for rheumatoid arthritis, but failed to meet expectations. Belimumab showed only modest efficacy, with ACR20 responses being marginally improved compared to the placebo group [168], while Atacicept produced no significant clinical improvement in phase II trials [169, 170]. These therapeutic failures very clearly demonstrate the incomplete understanding that we currently have as to the role of B cells in RA disease pathogenesis.

As well as their possible pathogenic roles, B cells are also demonstrated to have a protective function in autoimmune and inflammatory disorders [171], highlighting a need to determine which populations of B cells may be pathogenic, and which may actually be protective and beneficial in RA. Regulatory B cells exist which produce IL-10, and experiments have shown that the transfer of these IL-10 producing cells into a CIA model reduces disease severity [172].

1.1.4.3 Host tissue cells and joint destruction in RA

Host tissue cells, including fibroblast-like synoviocytes (FLS), chondrocytes and osteoclasts, do not act as passive bystanders in RA, but rather assume a pro-inflammatory phenotype that actively contributes to articular inflammation and damage.

Bone and cartilage destruction are the hallmarks of structural damage in RA – and are both at least partly caused by the inflammatory microenvironment found in RA joints. Evidence from both animal and human studies suggests that several key pro-inflammatory cytokines – e.g. IL-1, IL-17, IL-6 and TNF-α – can drive elements of both cartilage and bone destruction [173, 174]. The key cells involved in the turnover of bone and cartilage, and their possible roles in the destruction observed in RA joints are discussed below.

1.1.4.3.1 Osteoclasts and osteoblasts in RA

Osteoclasts are specialised multi-nucleated cells that are the primary cells responsible for bone resorption [175]. They play a critical role in normal bone remodelling, but they are also involved in the destructive bone erosion seen in RA, which occurs as a result of bone resorption at the joint surface.

Various growth factors and cytokines secreted by both inflammatory and resident cells can promote the development and differentiation of osteoclasts. Mononuclear precursor cells are recruited to the joint, and their differentiation to osteoclasts driven by locally produced macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL). Synovial fibroblasts and activated T cells are thought to be a primary source of these cytokines [176]. Several pro-inflammatory cytokines present in the RA synovium (predominantly TNF, IL-1 and IL-6, although roles are also emerging for other cytokines such as IL-15 and IL-17) can induce RANKL expression, and also up-regulate RANK expression by osteoclast precursor cells, thereby promoting osteoclast formation [177-180]. The inflammatory environment in the synovium of RA patients therefore drives osteoclast formation and differentiation, which in turn causes increased bone resorption, resulting in local bone erosions.

While osteoclasts are the key cells involved in bone resorption, osteoblasts are the cells involved in bone formation. Under normal circumstances when bone erosions occur, osteoblast activity increases, but in RA these repair mechanisms are not seen. This suggests that osteoclast activity 'overpowers' osteoblast activity, or that osteoblast activity is somehow impaired.

One family of molecules involved in regulating bone formation are the wingless (WNT) proteins. These bind to the low density lipoprotein (LRP) 5/6 receptor, initiating a signalling pathway that ultimately drives osteoblastogenesis [181]. Dickkopf (DKK) proteins, particularly DKK-1, are highly expressed in both experimental arthritis models and human RA inflammatory lesions. This family of proteins can be induced by TNF, and regulate WNT-induced osteoblast activity by competing with the WNT proteins to bind LRP5/6. They also bind the negative co-receptor Kremen-1, and therefore prevent WNT induced LRP5/6 signalling [182, 183]. DKK-1 inhibition completely prevents bone erosions occurring in various rodent arthritis models, and results in bone formation. In humans, treatment of RA patients showing increased DKK-1 levels with anti-TNF reduces its expression, suggesting that inflammatory cytokines like TNF can induce the production of molecules which block bone formation.

It was thought that regulating the balance between bone resorbing osteoclasts and bone forming osteoblasts could provide therapeutic potential in the treatment of RA. Treatment with the anti-RANKL monoclonal antibody Denosumab has been shown effective in treating osteoporosis, and it can also reduce inflammation in a rat RA model, but in humans its effects are less clear. Phase II trials of Denosumab have shown a significant improvement in joint destruction and erosions compared to placebo groups, but the treatment doesn't appear to have quite as remarkable an effect on joint space narrowing or the measures of disease activity observed [184]. Thus targeting osteoclasts has not found a place in the current therapeutic armamentarium in the treatment of RA.

1.1.4.3.2 Chondrocytes in RA

Chondrocytes are the only cell type present in cartilage, and along with the matrix components collagen and proteoglycan, they form the joint protecting cartilage structure. In the absence of disease, chondrocyte proliferation is very limited, and the penetration of cartilage by other cell types is restricted. In RA, however, FLS invasion into this normally privileged site occurs.

Some data suggest that chondrocytes themselves may play a role degrading the cartilage matrix in RA. Experiments in early RA have shown proteoglycan loss in zones deep in the cartilage [185, 186], and chondrocytes are capable of producing some proteinases which can degrade cartilage collagens and proteoglycans, such as MMP-1 [187], MMP-3 and MMP-10 [188]. Chondrocytes may also produce inflammatory cytokines which can effect local responses and inhibit normal anabolic repair mechanisms. The destruction of cartilage in RA, however, predominantly occurs near the synovial pannus [189], areas where high numbers of fibroblast-like synoviocytes and macrophages are found. These cells can produce large quantities of MMPs and other proteinases, which play an important role in the degradation of structural proteins in the cartilage. It is now believed, however, that the primary cell type involved in cartilage destruction are the fibroblast-like synoviocytes (FLS).

1.1.4.3.3 Fibroblast-like synoviocytes in RA

In a healthy joint, the intimal lining layer of the synovium is composed a thin layer of cells consisting primarily of two main cell types - macrophages and fibroblasts. In RA, however, these cells are known to greatly expand in number. It is thought that the FLS found in RA develop an unusual, aggressive and invasive phenotype – at least partly as a result of their exposure to the inflammatory environment in the inflamed synovium. These cells can produce a number of pro-inflammatory cytokines and proteolytic enzymes, as well as small molecule mediators of inflammation. Because of this, they can play a role in perpetuating inflammation, and also mediate local damage by degrading the extracellular matrix.

When FLS are cultured from human samples, they spontaneously produce a number of inflammatory mediators, as well as destructive proteinases. This production gradually decreases, but upon exposure to cytokines like TNF or IL-1 (cytokines abundantly present in the RA synovium) this is restored [190, 191]. Further evidence of the destructive behaviour of these cells has been demonstrated in severe combined immunodeficiency (SCID) mice, where human cartilage is co-transplanted with FLS. In these experiments, RA FLS, but not OA or normal FLS, enter and destroy the cartilage [192]. The production of cathepsins and MMPs are thought to be primarily responsible for this cartilage degradation. These molecules are expressed by RA FLS, and their production can be increased *in vitro* by IL-1 and TNF. FLS are also the primary producers of IL-6 in RA joints, and its production is also increased by exposure to IL-1 or TNF [193]. These data suggest that inflammatory cytokines, which are known to be abundantly expressed in the RA synovium, could promote destructive behaviour by FLS.

FLS may also play a role in both recruiting and then promoting the survival of other cell types important in RA pathogenesis, either by secreting soluble molecules or via cell-cell interactions. Upon stimulation with IL-1 and TNF, they are capable of producing a number of macrophage/monocyte recruiting molecules – including IL-1, RANTES, MCP-1, MIP-1α and MIP-1ß. Both T and B cell survival has also been shown to increase when they are incubated with FLS, which can produce SDF-1α – thereby inhibiting T cell apoptosis – and BAFF –

which promotes B cell survival [194, 195]. In expanding joints neovascularisation is a requirement to support the increased cell numbers. Chemokines produced by the FLS such as VEGF, IL-8 and SDF-1 can promote angiogenesis [196].

A body of evidence now suggests that some of these changes observed in RA FLS could be the result of epigenetic alterations. A recent definition of epigenetics states it is "the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states" [197]. Several methods of epigenetic changes in FLS have been studied in the context of RA, including DNA methylation and histone modifications. A global hypomethylation signature is found in RASF compared to those from OA or healthy controls [198, 199]. Hypomethylation was found in specific loci associated with genes that have significant relevance to RA. These include the cartilage glycoprotein GP39, the transcription factor STAT3 and TNF receptor-associated factor 2 (TRAF2) - which is involved in TNF receptor signal transduction [199]. Hypomethylation is generally associated with increased gene expression, so hypomethylation of these targets could be involved in the pathogenetic phenotype witnessed in RASF. Many therapeutics in RA currently target the immune system directly, but due to the common adverse effects of immunosuppression, many studies are now looking at the potential therapeutic benefit of targeting fibroblasts.

1.2 **Monocytes and macrophages**

Monocytes and macrophages are mononuclear phagocytes that play crucial roles in tissue homeostasis and immunity. Since the hypothesis proposed by *van Furths* of the mononuclear phagocyte system in 1968 [200], it was generally accepted that monocytes circulate in the blood, and can then move into peripheral tissues for further maturation and differentiation towards macrophages (process summarised in Figure 1-2).

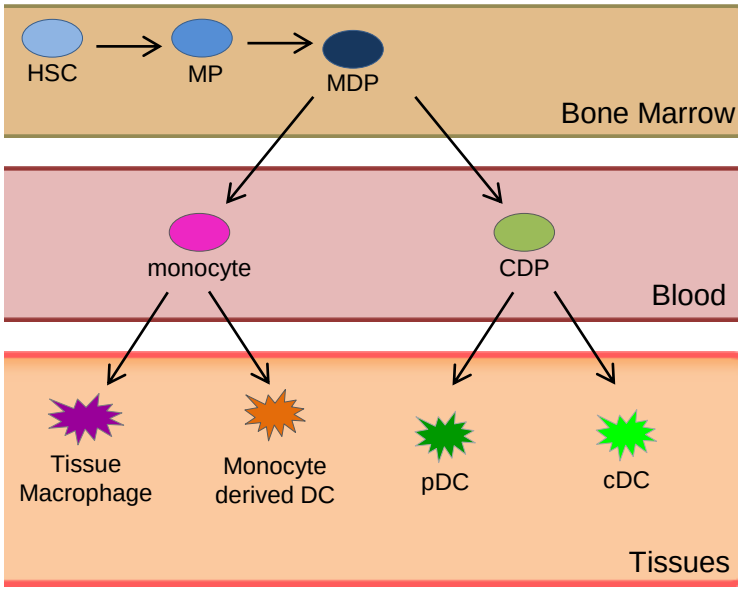


Figure 1-2 Original theory of monocyte, macrophage and dendritic cell differentiation
It is known that hematopoietic stem cells (HSC) give rise to myeloid progenitors (MP) and then macrophage / DC progenitors (MDP). It was originally thought that these MDPs in turn gave rise to monocytes and common DC progenitors (CDP) in the blood, which moved into tissues to differentiate into macrophages, monocyte-derived DCs, plasmacytoid (pDC) or conventional (cDC) DCs.

Technological advances, and the discovery of diverse and unique mononuclear cell populations have questioned this idea, and our understanding of monocyte and macrophage differentiation and function has changed dramatically in recent years. Many of these new ideas are based on experiments carried out in mice. Although monocyte subsets in mice are phenotypically different and carry different surface markers, many experiments suggest at least some similarities between the functions of these cells and their human counterparts, so parallel experimentation in rodents and humans appears warranted on this basis.

Broadly speaking there are two main monocyte populations found in mice that have great similarities to human populations (summarised in Table 1-5). Murine

cells are subdivided based on their expression of Ly6C ($Ly6C^{hi}$ and $Ly6C^{lo/-}$), whereas human monocytes are generally subdivided based on their expression of CD14 and CD16 ($CD14^{hi}$ $CD16^{-}$ and $CD14^{dim}$ $CD16^{+}$). Gene expression profiling of these subsets has suggested that the $CD14^{hi}$ human monocytes are very similar to $Ly6C^{hi}$ murine cells, while $CD16^{+}$ human monocytes may be the counterparts of $Ly6C^{lo/-}$ murine cells [201]. While this approach has shown that the murine and human monocyte subsets are quite broadly conserved, it also highlighted a number of differences, which could have important consequences when considering how to apply findings from murine experimental models directly to a human setting.

Monocyte population	Species	Markers	Primary Functions	Refs
Classical monocytes	Human	$CD14^{hi}$ $CD16^{-}$	Highly phagocytic cells that are recruited to sites of inflammation	[202]
	Mouse	$Ly6C^{hi}$		[203]
Alternative monocytes	Human	$CD14^{dim}$ $CD16^{+}$	Crawl along the inside of the lumen, possible roles in surveying endothelial integrity and responding to local stresses and viral infection	[204]
	Mouse	$Ly6C^{lo/-}$		[205]

Table 1-5 Table of the main monocyte populations in humans and mice

Although many of the first studies comparing monocyte subsets in mice and humans focussed on these two populations, for both mice and humans a third population has been suggested. This is often referred to as the intermediate monocyte population as it may represent an intermediate phase during differentiation - although these cells appear to have their own unique set of marker expression profiles. In humans, the third monocyte population are $CD14^{+}$ $CD16^{+}$ cells [206], while in mice they are Ly6C intermediate cells ($Ly6C^{int}$). Flow cytometry plots of these three populations in both humans and mice are shown in Figure 1-3.

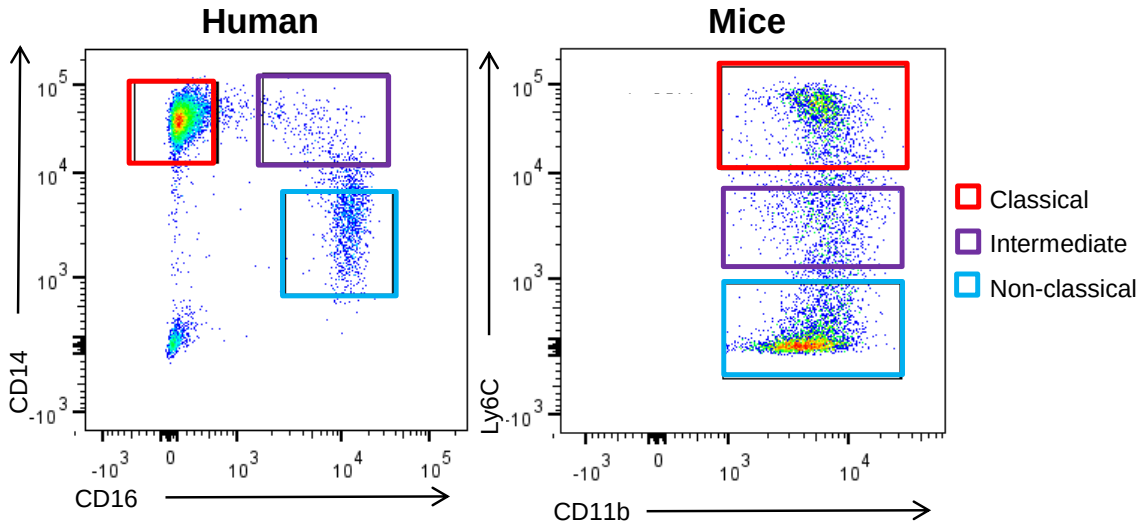


Figure 1-3 FACS representations of monocyte populations in humans and mice
New studies suggest the division of human and murine monocytes into three subsets – classical (red box), intermediate (purple box) and non-classical (blue box) cells. Shown are flow cytometry plots from my own work, which are representative of others in the literature, showing the markers that are suggested to differentiate between the subsets. In humans the monocyte populations can be separated based on their expression of CD14 and CD16 – $CD14^{hi}$ $CD16^{-}$, $CD14^{hi}$ $CD16^{+}$ and $CD14^{dim}$ $CD16^{+}$. In mice however, a single marker (Ly6C) is used to differentiate between the subsets – $Ly6C^{hi}$, $Ly6C^{int}$ and $Ly6C^{lo/-}$.

The phenotype of these intermediate cells is somewhere between the classical and non-classical monocytes, and in time courses of inflammatory disease in mice an increase in intermediate monocytes is followed by an increase in non-classical monocytes. This could suggest that the intermediate cells develop into non-classical monocytes [207].

One of the main changes to our thinking in recent years is that tissue macrophages may not be primarily derived from monocytes. Macrophages were originally thought of as fully differentiated cells with little proliferative capacity. Instead of self-renewal, it was thought that macrophages were replenished by monocyte recruitment into the tissues. For many sites though, macrophage replenishment by self-renewal has now been demonstrated in mice. This includes the highly specialised Langerhans cells in the skin [208, 209] and brain microglia [210], but also classical tissue-resident peritoneal macrophages [211, 212]. However, it has also been demonstrated that bone marrow derived cells are able to replace resident cells after their experimental ablation [212, 213], although it was not determined whether these cells recapitulate all functions of the cells they replace.

Although more recent experiments dispute whether monocytes differentiate into macrophages in the steady state, it is well established that during infection or when tissues are damaged following injury, monocytes are recruited into the tissues where they can differentiate into monocyte-derived macrophages (reviewed in [214, 215]).

The signals these cells receive once in the tissues are thought to determine their mature phenotype. Although there is still controversy over whether these represent distinct populations, or are simply cells responding to their micro-environment, macrophages are generally split into two major subsets – M1 and M2 cells. The M1 macrophages (often referred to as classical or inflammatory macrophages) differentiate in response to inflammatory cytokines such as IFN-y, GM-CSF and TNF-α or TLR stimulation (such as LPS), while M2 macrophages (often referred to as alternative or anti-inflammatory macrophages) are induced upon exposure to IL-4, IL-13 and M-CSF.

Cells recruited to an inflammatory environment therefore differentiate into inflammatory M1 macrophages. These cells are crucial for host defence as they facilitate the clearance of invading micro-organisms, but they can also cause substantial damage, especially where the response is uncontrolled or triggered unnecessarily. When this happens damage can be caused by the macrophages production of inflammatory cytokines, toxic reactive oxygen and nitrogen species, and their promotion of pathogenic T_h1 and / or T_h17 cells [216, 217].

Because of this, macrophages have been implicated in the pathogenesis of a number of autoimmune diseases, including multiple sclerosis, inflammatory bowel diseases and rheumatoid arthritis, where they are thought to be an important source of many of the inflammatory cytokines that drive the on-going inflammation.

1.2.1 **Monocytes and macrophages in rheumatoid arthritis**

Monocytes and macrophages are known to be crucial players in RA disease pathogenesis. Herein they display clear signs of activation, both in the synovium and also in extra-articular locations such as the peripheral blood, which could be important in the systemic complications associated with RA [218]. In healthy individuals, $CD16^+$ monocytes account for just under 10% of the total blood monocyte population, but in RA this is increased to between 11 and 15% [219, 220]. Monocytes from RA patients were also found to have increased expression of TLR2 [221], and $CD16^+$ monocytes produce higher TNF-α levels in response to TLR2-specific ligands [222]. CD16 expression on blood monocytes has also been shown to correlate with disease activity [223]. Together these data suggest that monocytes in the periphery of RA patients have a "pro-inflammatory" phenotype and display clear signs of activation. Moreover, $CD16^+$ monocytes in the periphery and lining layer of synovial tissue display increased CX3CR1 expression. This is the ligand for fractalkine, a receptor found at high levels in the synovial fluid of RA patients and thought to be involved in the recruitment of monocytes into the inflamed synovium [224].

When monocytes arrive in the inflammatory environment of the inflamed synovium, they will mature and differentiate into various effector cell lineages – including macrophages [225, 226]. These cells will become activated by the various inflammatory signals, including cytokines and TLR ligands, and in turn will be stimulated to produce a variety of cytokines that further perpetuate the on-going inflammation. The key cytokines macrophages produce include IL-1, IL-6 and TNF-α. Biologic therapies opposing the actions of these cytokines are all successful to some degree in treating RA patients, further establishing a role for the cytokines and macrophages in RA pathogenesis. Another indication that macrophages are involved in disease pathogenesis is the correlation between the degree of macrophage infiltration in the synovium, therapeutic response and radiological progression of disease in RA patients [227]. The two main ways macrophages are thought to influence other cells and processes in the synovium are via cell-cell interactions or by their release of soluble mediators.

1.2.1.1 Cell-cell interactions

Cell-cell interactions between macrophages and other inflammatory cells, as well as mesenchymal cells, are known to be important in immune function. Interactions between macrophages and synovial fibroblasts are thought to be important in RA, and *in vitro* co-cultures of these cells have shown that they induce much higher cartilage degradation together than either cell type can induce alone [228]. Direct cell contact between macrophages and fibroblasts has also been shown to promote the production of IL-6, GM-CSF and IL-8 [229].

In synovial tissue, macrophages are also found in very close contact with T cells [230]. It is known that T cell contact can stimulate human monocytes and macrophages to produce inflammatory mediators such as IL-1, IL-6 and TNF-α in a cell-contact dependant manner [231, 232].

A recent study by Evans *et al* has shown that $CD14^+$ monocytes isolated from the SF of RA patients, but not from the PB, spontaneously and specifically induce a Th17 phenotype in blood-derived $CD4^+$ T cells. Co-culture of PB $CD4^+$ cells with RA SF $CD14^+$ cells resulted in a higher percentage of IL-17 expressing cells, but similar numbers of IFN-γ and IL-4 expressing cells compared to co-culture with PB $CD14^+$ cells, suggesting specific enhancement of the capacity to expand Th17 responses, but not Th1 or Th2 cells. These data suggest that $CD4^+$ T cells that migrate into the inflamed RA synovium could be driven towards a Th17 cell phenotype by interactions with activated monocytes or macrophages – indicating a crucial role for monocytes and macrophages in the synovium in shaping the phenotype of other cell populations [233].

1.2.1.2 Soluble mediators

Fully differentiated and activated macrophages produce a number of soluble mediators that act in an autocrine fashion, and also on neighbouring cells to contribute to the continual inflammation and joint destruction. These soluble mediators include the numerous inflammatory cytokines as well as a range of CC and CXC chemokines.

Among the chemokines of interest is CXCL16. This is the ligand for CCR6 that is present on RA peripheral blood monocytes, and involved in their recruitment into synovial tissue [234]. Synovial macrophages also produce chemotactic factors for other cells – including CXCL8, CXCL1, CXCL7 and CCL18 - which could be involved in recruiting neutrophils and T cells to the joints [102, 235].

Of the cytokines synovial macrophages produce, TNF-α, IL-1 and IL-6 are among the most important. TNF and IL-1 are thought to be primarily produced by macrophages, while IL-6 is produced by macrophages and FLS. The key roles of these cytokines relevant to RA are outlined in Table 1-6.

Cytokine	Key functions relevant to RA
TNF-α	• activates leukocytes and endothelial cells, causing their production of cytokines, chemokines, adhesion molecules and matrix degrading enzymes • blocks the activity of regulatory T cells • activates osteoclasts • promotes resorption or cartilage and bone
IL-6	• promotes activation of leukocytes and osteoclasts • important role in driving B cell proliferation, differentiation and antibody production • regulates lipid metabolism • important regulator of the acute-phase response
IL-1 α/β	• promotes activation of leukocytes and endothelial cells • induces matrix enzyme production by chondrocytes • activates osteoclasts

Table 1-6 Key functions of TNF-α, IL-6 and IL-1 relevant to RA
Adapted from [236].

Therapies targeting these molecules are validated therapies in the treatment of RA. Treatment of patients with anti-TNF and IL-6 provide marked improvement in disease, but the clinical benefits of anti-IL-1 therapy appear to be modest. TNF is present in most RA synovial biopsies, and its inhibition has been shown to suppress several arthritis models in mice. Anti-TNF therapy is effective in

treating around 70% of patients with established disease, resulting in reduced levels of IL-6 and acute-phase proteins [237], with treatment also thought to help impaired T cells regain their regulatory function [155]. The levels of IL-1 found in the synovial fluid of RA patients has been shown to correlate with inflammation [238]. This cytokine could have numerous roles in disease pathogenesis. It is involved in proteoglycan synthesis and degradation, and was shown to play a role in the breakdown of articular cartilage in an antigen induced arthritis model [239]. The IL-1 type 1 receptor mediates IL-1-induced cell activation and is found on many cells in the RA synovium [240], so IL-1 produced by synovial macrophages could have effects of various other cells within the synovium.

Taken together these data show the many and varied potential ways monocytes and macrophages could contribute to RA pathogenesis, but the mechanisms underlying inflammatory gene expression in these cells are not fully understood. We therefore chose to investigate this area of research in our study.

1.3 **Non-coding RNA**

One fascinating and rather novel area of research that could provide new treatment targets in RA comprises the RNA interactome – especially the potential role of microRNA. Protein coding genes make up less than 2% of the human genome, with the remainder made up of non-protein coding deoxyribonucleic acid (DNA) [241]. Originally thought of as 'junk' DNA, it is now estimated that approximately 75% of the human genome is transcribed [242], so non-coding ribonucleic acid (RNA) accounts for a large proportion of our transcriptional output. Non-coding RNA includes transfer RNA and ribosomal RNA which are important for protein synthesis, as well as the more recently discovered long non-coding RNAs, piwi-interacting RNA (piRNA) and microRNAs (miRNAs/miRs) [243, 244].

1.4 **microRNA**

MicroRNA are a family of small, non-coding RNA species around 22 nucleotides long, which have emerged as key regulators of mammalian gene expression. They were first discovered in *Caenorhabditis elegans* (*C. elegans*) as a small non-protein coding RNA that could negatively regulate the protein level of LIN-14 through binding of an antisense sequence in its 3' un-translated region (3'-UTR) [245]. Since this finding in 1993, hundreds of miRNAs have been discovered which are believed to regulate a large proportion of the human genome [246]. At present there are 1881 suggested mature microRNA sequences in the miRbase database, although some of these are predicted from small RNA deep sequencing experiments, so it is unclear how many of these are bona fide true microRNA with regulatory function. They are involved in the post-transcriptional regulation of target messenger RNA (mRNA) expression; a single miRNA is capable of regulating a number of mRNA – often from the same biological pathway. This renders them appealing therapeutic targets, as it could offer the potential benefit of 'combination therapies' since a single drug (notionally targeting a single miR) could modulate multiple effector pathways (by manipulating multiple mRNAs of a given biological pathway). microRNA are now known to be involved in the regulation of many cellular processes – including cell cycle, apoptosis and inflammatory cytokine production.

1.4.1 **miR biogenesis**

The main steps involved in the muti-step process of miR biogenesis are shown in Figure 1-4. microRNA are primarily transcribed from intergenic sequences, but are also found within the introns of coding genes [247, 248]. In the latter case, microRNA often share a common transcriptional unit with the host gene and therefore show similar expression patterns [249]. miRNA biogenesis begins in the nucleus, where transcription results in the generation of the primary miRNA (pri-miR) [250]. The structure of these pri-miRs allows them to be recognised by the miRNA "microprocessor complex", Drosha and DiGeorge critical region 8 (DGCR8) [251, 252]. Drosha contains two RNase III domains which allow it to cleave the pri-miRNA [253], resulting in a double-stranded stem-loop precursor miRNA (pre-miR) around 60 - 100 nucleotides long. These pre-miRs are then transported to the cell cytoplasm by Exportin 5, where they are processed by

Dicer. This enzyme facilitates removal of the hairpin loop, leaving a small, double stranded miRNA consisting of a "functional" and a "passenger" strand (often referred to as miR*). It was initially thought that the functional strand provided the main biological functionality, but increasing data now support a role of both strands in gene regulation, with many passenger strands now proven to target and suppress their own target genes [254-256]. It is possible that both strands have unique roles, or that depending on the cell type and conditions, the strand that is preferentially loaded to the RNA-induced silencing complex (RISC) may vary. When the selected miRNA strand is loaded to RISC with argonaute proteins it creates a miRISC complex, which can then induce target silencing.

A microRNA recognises its target through complementary base pairing, and the degree of complementarity is thought to be important in determining whether mRNA degradation or repression occurs [244]. Full complementary of a miRNA with its target mRNA can result in direct Argonaute2-catalysed endonucleolytic cleavage of the target [257, 258]. This process is common in plants, but rare in mammals [259]. In mammals complementarity between the microRNAs "seed sequence" and its target mRNA appears to be the primary method for target recognition. The seed sequence is nucleotides 2 to 7/8 in the 5' region of the miRNA [260]. It was initially thought that this reduced complementarity would primarily result in translation inhibition, but several studies looking into the abundance of miRNAs and their respective mRNA targets have shown that they largely inversely correlate with each other [261, 262], suggesting that mRNA degradation is a major mechanism of microRNA-mediated target inhibition in mammals.

Another important finding concerning microRNA is that they can be released from cells in exosomes. These are cell-derived vesicles that can be found in biological fluids such as blood and urine, and also in the medium of cultured cells. Many groups are looking at the potential use of microRNA as biomarkers of disease or response to therapy, so the relative accessibility of blood and urine make microRNA measurement in their vesicles a promising biomedical approach [263, 264]. It has in fact been shown that microRNA derived from exosomes in metastatic prostate cancer differ significantly compared to those derived from non-recurrent cancer patients [265]. This, and many other studies suggest that

microRNA in exosomes could be useful as biomarkers. Possibly a more interesting role of these exosomes though, is the transfer of microRNA between cells. It has been shown *in vitro* that exosomes derived from cells can be taken up by other cells, and microRNA within these exosomes are then functional in a miRNA reporter assay in the host cell [266].

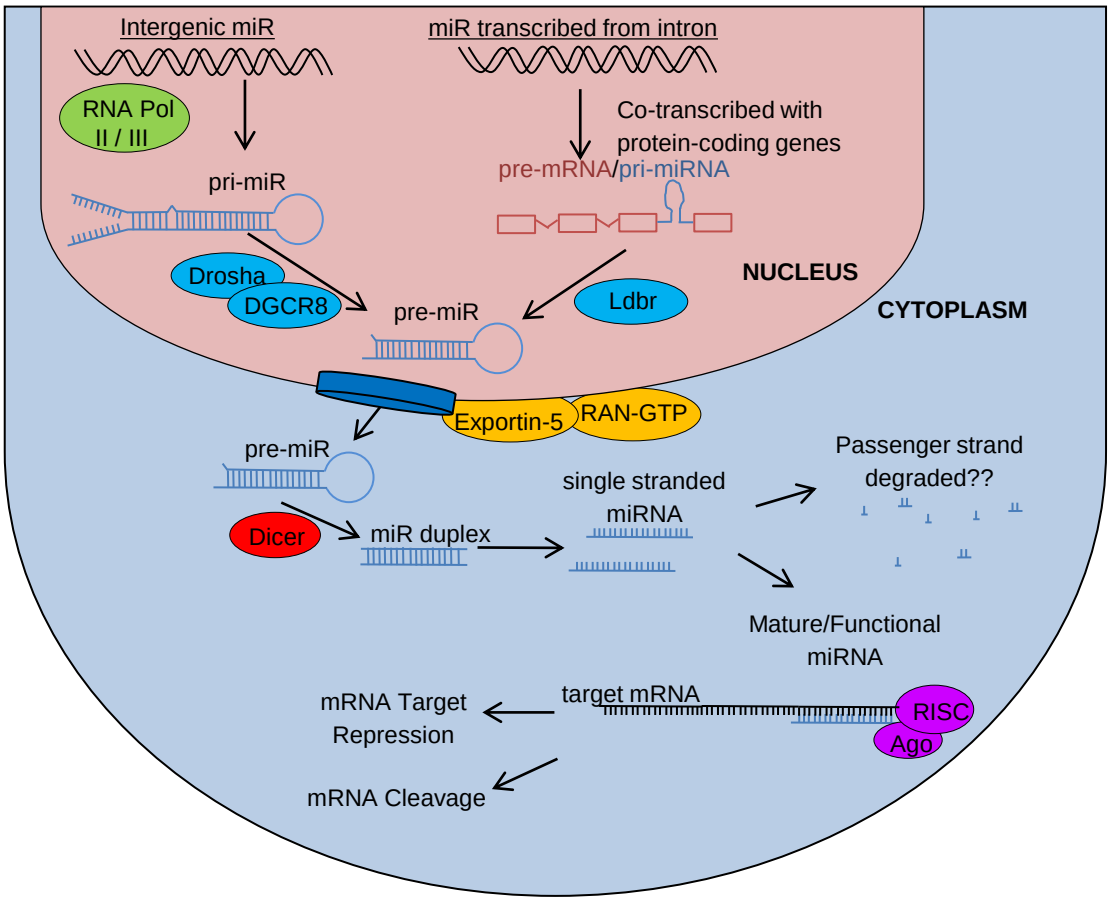


Figure 1-4 Typical microRNA biogenesis pathways
MiRNA are mainly transcribed from specific, miR-encoding, intergenic regions of the host genome, though some can be found in intronic sequences. Intergenic miRs are transcribed by RNA polymerase enzymes to form a primary miRNA (pri-miR) transcript, which is then bound to anchor protein DGCR8 and cleaved by the enzyme Drosha to create the pre-miR. Intronic miRs are co-transcribed with protein-coding genes, then spliced by the lariat debranching enzyme (Ldbr) to form the pre-miR. These pre-miRs are then transported to the cell cytoplasm by Exportin 5, where they are cleaved by Dicer to form a short, double-stranded miRNA molecule. It was initially thought that the passenger strand was degraded, while the functional strand was loaded with argonaute proteins into the RISC complex. The miRISC complex can then induce target mRNA silencing.

1.4.2 **The role of microRNA in development**

The first suggestion that microRNA could play a role in development came with their discovery. The microRNA lin-4 was shown to negatively regulate the expression of lin-14 [245, 267] – a key protein involved in controlling larval development in *C. elegans* [268]. A key role for microRNA in development is also suggested in studies of knockout (KO) mice lacking some of the key microRNA-processing factors. Lack of Dicer, Drosha or Ago2 individually in mice was embryonically lethal [269-271]. So, in order to study the role of microRNA conditional knockouts were made.

Embryonic stem cells (ESCs) are stem cells derived from the undifferentiated inner mass cells of a human embryo, and are the pluripotent cells that have the potential to develop into each of the more than 200 cell types of the adult body under the correct signals. A conditional knockout of dicer-1 in embryonic stem cells alone, results in viable mice, but the cells display severe defects in differentiation [272]. ESCs have been shown to express a unique set of microRNA, including the miR-302 family. This cluster of 5 miRs are not detected in cells at later stages of development or in adult tissues. It is thought that these miRNAs may play an essential role in maintaining pluripotency and aiding the differentiation of ESCs [273, 274].

1.4.3 **miRNA in immune cell regulation**

Several studies have now highlighted the role of microRNA in both the development and function of immune cells. The differentiation of haematopoietic stem cells to committed progenitor cells and then mature effector cells has to be tightly regulated at both the transcriptional and post-transcriptional level. One of the first studies to highlight the importance of microRNA in this process focussed on miR-181. This microRNA is expressed in murine bone marrow cells, and up-regulated specifically during the differentiation of B cells. It was also shown *in vitro* that ectopic expression of miR-181 in haematopoietic progenitor cells promotes B cell differentiation – suggesting that this microRNA is a positive regulator of B cell differentiation [275].

Since then several studies have followed microRNA expression during mammalian haematopoiesis and have shown that many microRNA are differentially expressed throughout differentiation of haematopoietic stem cells to mature, differentiated effector cells [275-277]. This said, few studies have actually identified the targets of these microRNA – so future studies should focus on potential targets that could influence lineage commitment to determine whether microRNA really are important regulators of this process. A regulatory role for microRNA in cell differentiation is, however, implicated in a study of mice deficient in Dicer specifically in myeloid progenitor cells. In this system there was impaired differentiation of myeloid cells, and progenitor cells even regained self-renewal potential [278].

As well as being important for immune cell differentiation, many microRNA are now known to be involved in the activation of immune cells. For example, one study showed that miR-146a, miR-155 and miR-132 were up-regulated after exposure of a human monocytic cell line - THP-1 cells - to LPS [279]. This group then went on to show that microRNA up-regulation was not solely a response to cell activation – but they showed a more specific pattern of expression. miR-146a up-regulation was only induced upon stimulation of TLRs recognising bacterial components (TLR2, 4 and 5) but not intracellular TLRs recognising viral components (TLR3, 7 and 9). Other groups went on to define possible targets of miR-146a and miR-155 that could be involved in cell activation. Current

evidence suggests that while miR-146a up-regulation could be an attempt to attenuate inflammation, increased miR-155 actually promotes inflammation. miR-146a has been shown to target interleukin-1 receptor-associated kinase 1 (IRAK1) and TNF receptor associated factor 6 (TRAF6) – key signalling molecules that can promote inflammation [280]. miR-155, on the other hand, has been shown to target SH2-containing inositol-5'-phosphatase 1 (SHIP-1) [281] and suppressor of cytokine signalling 1 (SOCS-1) [282] – two important negative regulators of the TLR pathway. Increased miR-155 expression could therefore down-regulate SHIP-1 and SOCS-1 expression, in turn increasing inflammatory cytokine production. Indeed, mice deficient in miR-155 show increased expression of both SHIP-1 and SOCS-1, and their macrophages show impaired inflammatory response to LPS stimulation [283].

miR-187 is a further anti-inflammatory miR. Increasing its expression in monocytes reduces their LPS-induced release of TNFα, IL-6, and IL-12p40, while inhibiting miR-187 increases their production. The effects of miR-187 on TNF are direct, but it is thought to down-regulate IL-6 and IL-12p40 indirectly via IκBζ, a master regulator of the transcription of these latter two cytokines [284]

With regards to autoimmune, inflammatory conditions, microRNA-21 is demonstrated to be up-regulated in many diseases, including SLE [285, 286], ulcerative colitis [287, 288] and psoriasis [289]. In psoriasis miR-21 was shown increased in lesional, but not non-lesional skin, where it is thought to play a role in promoting T cell survival by inhibiting apoptosis. Indeed, *in vitro* inhibition of miR-21 resulted in an increase in apoptosis, while the overexpression of miR-21 reduced the apoptosis rate in T cells [289]. Inhibition of miR-21 was also found to ameliorate disease pathology in patient-derived psoriatic skin xenotransplants in mice and in a psoriasis-like mouse model [290].

These studies, along with many others, highlight the role of microRNA in both the positive and negative regulation of inflammation.

1.4.4 **MicroRNA in RA**

It is known that miRNA regulate a wide range of cellular processes, including cell growth, proliferation and death, so it is unsurprising that they have now been implicated in a number of disease states. A large literature base has described their potential roles in a variety of cancers [291, 292], and more recently interest has grown in their potential activities in autoimmunity [293, 294]. In RA, there is an increasing literature describing miRNA dysregulation, and the potential consequence this could have on disease activity.

In 2008, Stanczyk *et al* provided the first report that microRNAs were dysregulated in RA patients [295]. They found that two particular microRNA – miR-155 and miR-146a – were markedly up-regulated in RA synovial fibroblasts (SF) compared to osteoarthritis (OA) SFs. They further found that miR-155 was up-regulated in response to stimulation of these cells with inflammatory cytokines (TNF-α and IL-1ß) and TLR ligands (LPS and polyI:C), but its forced up-regulation resulted in reduced MMP production in response to stimulation. They therefore concluded that the inflammatory microenvironment of the RA synovium could alter the microRNA expression of resident cells, and that the increased miR-155 expression was an attempt to modulate the destructive nature of RASFs. Since this report in 2008, a number of studies have looked at microRNA expression in RA, and many miRs are now known to be up or down-regulated in various cell subsets in the synovium and peripheral blood of RA patients (summarised in Table 1-7), which have been contributed to a variety of pathological consequences. MiRs have been implicated in the regulation of leukocyte activation and subsequent cytokine production, and also in the generation of the activated and invasive phenotype of RA-fibroblast like synoviocytes [295-297].

miRNA	Where	Known targets in RA cells	Known effects of Dysregulation	Refs
miR-155	↑ SF			[298]
	↑ SP			[298]
	↑ PBMC			[299]
	↑ FLS	MMP-1 and 3	Decrease MMP production	[295]
	↑ $CD14^+$ cells	SHIP-1	↑ pro-inflammatory cytokine production	[300]
miR-146a	↑ SF			[298]
	↑ SP			[298]
	↑ PBMC	TRAF-6, IRAK-1	Inhibits osteoclastogenesis	[299]
	↑ FLS			[295]
	↑ $CD4^+$ T cells			[301, 302]
miR-223	↑ $CD4^+$ T cells			[303]
	↑ synovial monocytes & macrophages	Osteoclastogenesis markers	Inhibits osteoclastogensesis	[304]
miR-124a	↓ FLS	CDK-2, MCP-1	Contributes to apoptosis resistance and promotes proliferation and chemokine production	[297, 305]
miR-203	↑ FLS		↑ MMP-1 and IL-6	[306]
miR-34a*	↓ FLS	XIAP	Contributes to apoptosis resistance	[254]
miR-19a/b	↓ FLS	TLR2	Impaired regulation of MMP-3 and IL-6	[307]
miR-23b	↓ FLS	TAB2, TAB3, Ikk-α	Impaired regulation of NFkβ activation and pro-inflammatory cytokine production	[308]
miR-221	↑ FLS			[309]
miR-222	↑ FLS			[309]
miR-323-3p	↑ FLS	B-transducin	Enhanced WNT/Cadherin signalling	[309]

Table 1-7 List of microRNA dysregulated in RA and suspected consequences
↑ - up-regulated, ↓ - down-regulated, SF - synovial fluid, SP - serum plasma, FLS - fibroblast-like synoviocytes, PBMC - peripheral blood mononuclear cells.

MiRNA regulate some of the pro-inflammatory cytokines that play key roles in RA disease pathogenesis – including TNF, although relatively few studies have as yet focussed especially on RA derived tissues. Several miRNA have been shown to promote TNF-α down-regulation, with others increasing its expression. MiR-16 and miR-369-3 both bind to the TNF AU-rich element (ARE), which regulates mRNA stability. miR-16 is required for ARE-mediated degradation of TNF [310], whilst miR-369-3 promotes TNF mRNA translation [311]. miR-125b has also been shown to inhibit TNF while miR-155 enhances its production [300], showing that even for a single cytokine, miRNA regulation is complicated, but an incredibly important mechanism of regulation.

Growing evidence suggests that miRNA networks govern the unique biology of FLS in RA. An over-production of IL-6 by these cells appears to correlate with elevated miR-203 expression [306], whilst reduced levels of miR-124a and miR-34a*, which target a cell cycle progression protein (cyclin-dependent kinase 2) [297] and an inhibitor of apoptosis (X-linked inhibitor of apoptosis) [254] respectively, could explain the increased expansion of FLS in RA joints, and consequent cartilage invasion. On the other hand, high levels of miR-155, which inhibits MMP production, and miR-146a, which targets TLR signalling molecules, may reflect the initiation of inflammation counterbalance mechanisms in these cells [295].

1.4.4.1 MiRs in *in vivo* Animal Models of RA

Much of the research examining a potential role of miRNA in inflammatory arthritis has been undertaken *in vitro*. Although useful for understanding their basic functions and targets, this can in no way elicit the role these complicated RNA species play in a whole organism during disease processes. Our understanding of how miRNA function is further complicated with the idea of competing endogenous RNA (ceRNA) and miRNA networks. The relationship between a miRNA and its target is not linear, but instead is likely to depend upon the levels of other targets of the given miR (other mRNA and pseudogenes), and also the expression level of other miRs that may positively or negatively regulate the given mRNA [312]. Together this means that in order to get a true picture of a miRNAs effects, we must study the consequence of miRNA

dysregulation in an organism as a whole. A number of groups have now looked at the effects of miRNAs *in vivo* in established animal models of arthritis.

Possibly the most extensively studied miRNA in arthritis is miR-155. This miRNA is up-regulated in cells of both the synovium and peripheral blood of RA patients, and is thought to be involved in regulating the production of pro-inflammatory cytokines by macrophages and metalloproteinases by FLS [295, 299, 300]. *In vitro* experiments suggest that over-expression of miR-155 in monocytes/macrophages results in the up-regulation of a number of pro-inflammatory cytokines, including TNF-α and IL-6 [300] – cytokines proven to be involved in RA disease pathogenesis and validated therapeutic targets of the disease. Consistent with a pro-inflammatory role, one of the candidate molecular regulatory targets of miR-155 – at least in myeloid cells – is the inflammatory inhibitor SHIP-1 [281, 300].

To test a possible role of miR-155 *in vivo*, two groups - Kurowska-Stolarska *et al* and Bluml *et al*, used the murine model of collagen-induced arthritis (CIA). Kurowska-Stolarska *et al* showed that, similar to the RA synovium, miR-155 expression was up-regulated in the joints of WT CIA mice compared to control-immunised mice, and in both studies, miR-155 deficiency offered protection from the development of arthritis. After induction of CIA, wild type (WT) mice develop the expected levels of disease, but miR-$155^{-/-}$ mice fail to develop any clinical signs of arthritis [300, 313]. Both groups noted a decrease in joint and systemic inflammation, Th17 cell expansion, and a reduced capacity of cells to produce collagen-specific IgG antibodies in the miR-$155^{-/-}$ mice. Together these studies suggest that miR-155 regulation has an important role in the development of both innate and adaptive arms of immune responses. Although the primary trigger of miR-155 in CIA and RA has not yet been defined, it has been shown that miR-155 is up-regulated upon TLR stimulation [281, 300]. Therefore it is likely that endogenous TLR ligands, generated by tissue damage, may up-regulate miR-155 in monocytes/macrophages, resulting in the production of pro-inflammatory cytokines by myeloid cells, which goes on to drive inflammation and support Th17 cell development. Consistent with this data it has been reported that miR-155 is involved in the development of Th17 cells in other experimental murine diseases [314]. In addition, miR-155 is intrinsically

involved in plasma cell differentiation and B cell class switching by targeting lymphotoxin-alpha and activation induced cytidine deaminase [315-317].

Bluml *et al* also used the K/BxN serum-transfer murine arthritis model to determine whether miR-155 deficiency affects the FcR-mediated innate effector response during arthritis, and found under these circumstances WT and miR-155$^{-/-}$ mice develop comparable arthritis in terms of paw swelling and histological examination. This suggests that miR-155 regulated pathways are not non-redundantly involved in the effector antibody-mediated responses [313]. This supposition is supported by *in vitro* data showing that there was no difference in TNF-α production by WT or miR-155 deficient macrophages upon stimulation with immune-complexes [300]. The miR-155$^{-/-}$ mice did, however, develop fewer bone erosions, thought to be due to a reduced number of osteoclasts. The group went on to show impaired RANK-L-induced osteoclast formation *in vitro* from miR-155$^{-/-}$ murine bone marrow. Thus, unbalanced miR-155 expression in myeloid cells leads to the profound perturbation in their activation and development into osteoclasts. Given that miR-155 appears to be involved in the regulation of many pathways involved in development and progression of arthritis, it may represent a novel and promising therapeutic target in the treatment of autoimmune disease. Its wider biologic activities, however, indicate that such approaches should be made in a cautious manner at this stage, as on target adverse events in other tissues might be predicted.

A critical pathogenic aspect of RA is the expansion and proliferation of FLS. These cells develop an aggressive and invasive phenotype, which allows them to invade and degrade the articular cartilage. miR-15a has been shown to induce cell apoptosis by down-regulating the anti-apoptotic Bcl-2, which has previously been shown up-regulated in RA FLS [318-320]. To investigate whether miR-15a could exert its effects *in vivo*, Nagata *et al* injected the double-stranded microRNA into mice with autoantibody mediated arthritis [321]. In this study, intra-articular injection resulted in the down-regulation of Bcl-2 and an increase in caspase 3 expression compared to the control group. This led to the induction of apoptosis in synovial fibroblasts, thus limiting their invasion into adjacent cartilage. This study provides a promising indication that local administration of miRNA mimics targeting proliferation and survival pathways in synovial

fibroblasts, could be a useful approach to tackle the inflammatory synovitis associated with RA.

Another miRNA of which the function has been studied *in vivo* is miR-146a. This miRNA is highly expressed in RA FLS, and in RA synovial tissue as a whole [295, 296]. It is also overexpressed in PBMCs from RA patients, compared to healthy controls or OA patients [299]. miR-146a is induced in macrophages [279] and synovial fibroblasts [295] upon stimulation with TLR ligands or IL-1ß. Numerous functional *in vitro* studies suggest that miR-146a acts as a negative feedback regulator of innate immune responses by targeting the TLR signalling molecules IRAK1 and TRAF6, as well as cytokine signalling molecules, such as STAT1 [279]. Consistent with these data miR-146a deficient mice spontaneously develop autoimmunity [280]. Interestingly, systemic administration of miR-146a mimics into mice with full-blown CIA prevented cartilage and bone destruction, but not inflammation and synovitis. This group also showed that transfection of PBMCs with miR-146a inhibits osteoclastogenesis *in vitro* [322]. This study suggests that miR-146 may be involved in targeting pathways regulating bone turnover, but its potential contribution to dampening on-going inflammation is still controversial. It is likely that injection of miR-146a mimics into mice with late phase CIA was not able to affect inflammation, as other pathways – such as immune-complexes and cell-cell interactions – are known to be involved in driving inflammation at this stage. Thus, administration of miR-146a mimics before the onset of arthritis, and studies of miR-146$^{-/-}$ mice in different arthritis models would help to understand and validate its potential role in preventing and limiting chronic inflammation.

Most microRNA that are studied in the context of inflammation are found due to their increased expression in disease, but microRNA-23b was found to be down-regulated in inflammatory lesions of lupus and rheumatoid arthritis, as well as in the mouse models of lupus, rheumatoid arthritis and multiple sclerosis. In transgenic mice over-expressing miR-23b, the onset of CIA as well as EAE was delayed and found to be less severe compared to wild-type mice. Also consistent with a protective, anti-inflammatory role, administration of a miR-23b sponge that inhibits the microRNAs function caused more severe disease symptoms and bone erosion during a CIA model. The study went on to identify

TGF-Beta Activated Kinase 1/MAP3K7 Binding Protein (TAB) 2, TAB3 and IkappaB kinase complex (IKK) α as novel miR-23b targets. These molecules act in signalling cascades downstream of inflammatory cytokines. Suggesting that miR-23b regulates inflammatory cytokine-mediated signalling. [308]

1.4.5 **miRNA in Other Joint Diseases**

A role for miRNA in osteoarthritis (OA) has also been established. miR-140 is expressed in normal human articular cartilage, but is significantly reduced in patients suffering from OA. Treatment of chondrocytes in vitro with IL-1ß – a cytokine known to be associated with OA – also results in reduced miR-140 expression [323]. The same group went on to do a serious of *in vivo* experiments, using three different animal models to further elucidate the role of miR-140 in cartilage homeostasis - an aging model, a surgical model, and an antigen-induced arthritis (AIA) model. In the aging model, miR-140$^{-/-}$ mice develop age-related OA symptoms much earlier than WT mice and OA scores are much higher in KO mice than age-matched WT mice. The KO mice also develop more severe proteoglycan loss and demonstrate higher OA scores than WT mice in the surgical model. The AIA model was studied in WT, miR-140$^{-/-}$ and transgenic (TG) mice overexpressing miR-140 in cartilage. While synovial hyperplasia was not affected in any of the mice genotypes, proteoglycan loss was shown to be significantly higher in the miR-140$^{-/-}$, and lower in the TG mice compared to WT [324]. Together, these findings suggest that miR-140 is indispensable for cartilage homeostasis and is protective in OA, possibly by its repression of the critical cartilage-degrading enzyme – Adamts-5.

1.4.6 microRNA-34a

In mammals, miR-34a is one of a family of three microRNA. While miR-34a is transcribed from chromosome 1, miRs-34b and 34c are co-transcribed from a location on chromosome 11. The mature sequence of these three microRNA is shown in Figure 1-5.

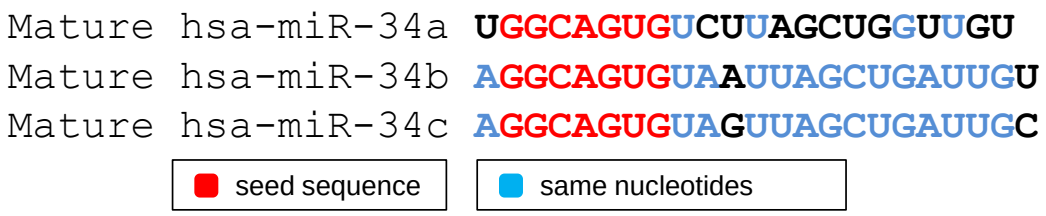


Figure 1-5 The mature microRNA sequence for human miR-34a, miR-34b and miR-34c
Shown is the nucleotide sequence for the mature microRNA-34a, 34b and 34c. Highlighted in red is the seed sequence which is identical for all three microRNA. In blue are the nucleotides shared by at least two of the microRNA.

1.4.6.1 miR-34a in cancer

microRNA-34a is most highly studied in the cancer field, as it is dysregulated in a variety of cancers and known to target many genes involved in cell cycle and apoptosis. miR-34a is completely absent or significantly down-regulated in numerous pancreatic carcinoma cell lines [325], neuroblastoma [326], as well as colon, pancreatic, lung and prostate cancer [327]. Moreover, it has been shown that over-expression of miR-34a in both cancer cell lines and primary cells can lead to cell cycle arrest [328, 329] and even cellular senescence [330].

A mimic of miR-34a – MRX34 – is in fact the first microRNA mimic to enter clinical trials [331]. In 2013 a phase one trial started in patients suffering primary liver cancer or metastatic cancer with liver involvement, where it is hoped restoring miR-34a expression to a more physiological level will restore tumour suppressive function to the liver cells.

The first study published on miR-34a$^{-/-}$ mice highlighted a potential role for miR-34a in regulating somatic cell reprogramming [332]. This is a process whereby differentiated somatic cells are induced to generate pluripotent stem cells (iPSCs) by forced expression of a set of specific transcription factors - octamer-

binding transcription factor 4 (OCT4), SRY (sex determining region Y)-box 2 (SOX2), Krüppel-like factor 4 (KLF4) and MYC [333]. Although this is an inefficient process with slow kinetics, it had previously been shown to be enhanced in $p53^{-/-}$ mice [334, 335]. Given the known relationship between p53 and miR-34a, this group set out to determine whether miR-34a had a role in regulating somatic cell reprogramming. When reprogramming was induced in mouse embryonic fibroblasts (MEFs), miR-34a, b and c were all induced in a p53 dependant manner, with miR-34a being most highly up-regulated. Similarly to $p53^{-/-}$ cells, when this process was induced in MEFs from $miR\text{-}34a^{-/-}$ mice, a higher number of iPSCs were generated. The group also showed that miR-34a directly targeted Nanog, Sox2 and N-Myc – 3 transcription factors involved in maintaining self-renewal and pluripotency of ESCs. As suggested by its role in cancer, this study further declares a role for miR-34a in restraining somatic reprogramming.

1.4.6.2 Other roles of miR-34a

miR-34a has more recently been implicated in cardiac ageing. Expression of miR-34a, and to a lesser extent miRs 34b and c, is increased in heart tissue during ageing in both humans and mice. miR-34a's expression is also further enhanced in a murine model of acute myocardial infarction. This study by Boon *et al* went on to show that treatment with a miR-34a inhibitor could reduce age-associated cardiac cell death and functional decline – at least in part due to its targeting of Phosphatase 1 Nuclear Targeting Subunit (PNUTS) [336], which is known to be involved in DNA repair [337] and apoptosis [338].

A role for microRNA-34a in the regulation of bone turnover has recently been highlighted. Krzeszinski *et al* reported that miR-34a was down-regulated during osteoclast differentiation, and that over and under-expression of this microRNA in vitro resulted in decreased and increased osteoclast formation, respectively. They also demonstrated that miR-34a knockout mice exhibit elevated bone resorption, while transgenic mice overexpressing miR-34a are less susceptible to ovariectomy-induced osteoporosis. Transforming growth factor-ß-induced factor 2 (Tgif2) was identified as a pro-osteoclastogenic target of miR-34a that could be responsible for these phenotypes [339].

miR-34a has also been implicated in metabolic disorders. Its expression is elevated in obese mice, and it has been shown to directly target SIRT1, which is implicated in the prevention of metabolic disorders [340]. A more recent study also suggested that miR-34a could target Nicotinamide phosphoribosyltransferase (NAMPT). This enzyme is proposed to have insulin-mimetic effects, and be capable of lowering blood glucose and improving insulin sensitivity. Its serum levels also correlate with obesity. miR-34a inhibition in obese mice restored NAMPT levels and improved steatosis, inflammation, and glucose intolerance. Therefore miR-34a inhibition could be a potential new target in the treatment of obesity and age-related diseases like steatosis and type 2 diabetes [341]. Thus far there are no data concerning the role of miR-34a in the pathogenesis of RA.

1.5 **Research aims**

Although the treatment of rheumatoid arthritis has improved markedly over the past 10 – 20 years, there remain a considerable number of patients that fail to respond to current therapies – therefore there is still significant unmet clinical need.

microRNA are emerging as an important class of post-transcriptional regulators, with a profound and broad effect on many cellular processes. Accumulating *ex vivo* and *in vitro* studies suggest that miRNA dysregulation could contribute to the development and progression of clinical arthritis, but the complicated interactions that take place between miRNA and their targets make it difficult to define whether a given miR is simply a fine-tuner, or a master switch regulator of the pathways driving arthritis. Recent progress in the field, including the generation of miR deficient mice and development of *in vivo* quality miRNA mimic and inhibitors, will provide an opportunity to understand the hierarchy of miRNA networks, and reveal that interfering with miRNA networks could be a promising approach to target RA pathogenesis. In particular, dissecting the role of microRNA in regulating the inflammatory gene expression of synovial macrophages could provide new insights into disease pathogenesis. Preliminary data from our laboratory have suggested miR-34a may be dysregulated in RA. I wished to follow this observation up and dissect its functional importance in detail. Accordingly, this project has three main aims;

1. To confirm dysregulated expression in the periphery and / or synovium of RA patients

2. To study the functional consequences of miR-34a dysregulation in macrophages – particularly on inflammatory cytokine production

   and

3. To investigate potential pathways miR-34a may regulate in monocytes and macrophages, with an aim to dissect the mechanism of action of any functional changes induced by miR-34a

1.6 **Hypothesis**

Given the previous work carried out in the laboratory looking at microRNA which are differentially expressed in RA, my starting hypothesis is that miR-34a is a microRNA which is up-regulated in monocytes and macrophages in RA, that promotes the pro-inflammatory nature of these cells – therefore contributing to RA disease pathogenesis.

Chapter 2 – Materials and Methods

2.1 General reagents and buffers

2.1.1 Materials and reagents

Basic Chemicals: All chemicals were purchased from Sigma unless otherwise stated.

Plastics: All plastics used for tissue culture were purchased from Corning or Greiner unless otherwise stated.

2.1.2 Buffers and culture media

Complete RPMI: Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat inactivated foetal bovine serum (FBS), Penicillin (100 units/ml), Streptomycin (100 µg/ml) and L-Glutamine (2 nM) (all Invitrogen).

Complete DMEM: Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated foetal bovine serum (FBS), Penicillin (100 units/ml), Streptomycin (100 µg/ml) and L-Glutamine (2 nM).

Wash media: RPMI 1640 supplemented with Penicillin (100 units/ml) and Streptomycin (100 µg/ml).

FACS / MACS buffer: Phosphate buffered saline (PBS) (Invitrogen) supplemented with 2% FBS, Penicillin (100 units/ml) and Streptomycin (100 µg/ml). The only difference between fluorescence-activated cell sorting (FACS) and magnetic activated cell sorting (MACS) buffer is that MACS buffer is kept sterile, as cells are frequently cultured after its use.

ELISA wash buffer: 1x PBS (VWR, diluted from 10x with dH_2O) with 0.05% Tween 20 (Sigma).

Tris-acetate-EDTA (TAE) buffer: A 50x stock solution was made by dissolving 242g of Tris base in 750 ml dH_2O. This was mixed with 57.1 ml glacial acetic acid and 100ml 0.5 M Ethylenediaminetetraacetic acid (EDTA) (pH 8). The final

volume was made up to 1000 ml by the addition of dH_2O. Before use the 50x stock was diluted 1:50 with dH_2O.

Diethyl Pyrocarbonate (DEPC) H_2O: DEPC was used to inhibit nucleases and clean H_2O so it could be used for *in situ* hybridisation buffers. 1 ml DEPC was used per 1 L dH_2O. DEPC was dissolved and mixed in water by magnetic stirring. DEPC-treated dH_2O was then autoclaved to destroy remaining DEPC.

Saline-Sodium Citrate (SSC) buffer: A 20x stock solution was purchased (Sigma), and diluted to the required concentrations in DEPC-treated H_2O.

Tail tip lysis buffer: The alkaline lysis buffer is made up in dH_2O containing NaOH at a final concentration of 25 nM and 0.2 mM EDTA, pH12.

Tail tip neutralising buffer: The neutralising buffer is 40 mM Tris in dH_2O, pH5.

2.2 Collection and culture of human cells and tissues

2.2.1 Clinical samples and controls

All samples were obtained from buffy coats, healthy volunteers or patients who fulfilled the 2010 American College of Rheumatology/European College Against Rheumatism criteria for RA after obtaining written consent.

Ethical approval for the studies was obtained from the West of Scotland Research Ethics committee. They covered the use of human buffy coat samples, and also the use of clinical material from patients with rheumatoid arthritis, psoriatic arthritis, osteoarthritis and matched healthy volunteers as required.

2.2.2 Purification of mononuclear cells from buffy coats and peripheral blood

Peripheral blood mononuclear cells (PBMCs) were purified from blood samples by density gradient centrifugation. Buffy coats were diluted 1 : 1 in wash media, while fresh peripheral blood samples were used neat. 10 mls of the blood preparation was layered carefully on top of 4 mls Histopaque 1077 (Sigma) in 15 ml centrifuge tubes. These were then centrifuged at 2100 RPM for 20 minutes at room temperature. The mononuclear cell layer was carefully removed using a Pasteur pipette into a clean 50 ml tube, and an equal volume of wash medium added. Samples were then spun at 1800 RPM for 10 minutes at room temperature. Cell pellets were re-suspended in 20 mls of cold MACS buffer, and the number of viable cells determined using Trypan Blue and a haemocytometer. Finally, cells were centrifuged at 1800 RPM for 5 minutes at 4°C before being re-suspended at the required concentration.

2.2.3 Purification of mononuclear cells from synovial fluid

Synovial fluid samples were centrifuged at 2000 RPM for 20 minutes to pellet cells. The cell-free fluid was stored in aliquots at -80°C, and the cell pellet was re-suspended in wash medium. This cell preparation was layered on top of 4 mls Histopaque 1077 in a 15 ml tube and centrifuged at 2100 RPM for 20 minutes at room temperature. The mononuclear cell layer was carefully removed using a Pasteur pipette into a clean 50 ml tube, and an equal volume of wash medium

added. Samples were then spun at 1800 RPM for 10 minutes at room temperature. Cell pellets were re-suspended in 20 mls of cold MACS buffer, and the number of viable cells determined using Trypan Blue and a haemocytometer. Finally, cells were centrifuged at 1800 RPM for 5 minutes at 4°C before being re-suspended at the required concentration

2.2.4 **Isolation of CD14$^+$ monocytes and from mixed PBMCs**

CD14$^+$ monocytes were isolated using magnetic-activated cell sorting (MACS) positive bead selection (Miltenyi) on an AutoMACS Pro Separator according to the manufacturer's instructions. We used the Posseld programme, meaning that cells are run through two columns to ensure a higher purity was achieved. After isolation, the purity of cells was determined using flow cytometry (described in section 2.5.1), and was routinely > 95%.

2.2.5 **Culture of CD14$^+$ monocytes**

Unless otherwise stated, CD14$^+$ cells were either classed as immature monocytes and used for stimulation / transfection experiments straight away, or were matured using macrophage colony-stimulating factor (M-CSF) (Peprotech) for 7 days and classed as M-CSF-matured macrophages. In this case, cells were cultured with 50 ng/ml recombinant M-CSF for 3 days before spent media and non-adherent cells were removed and fresh media supplemented with 50 ng/ml M-CSF was added. The cells were then cultured for a further 4 days, and classed as M-CSF-matured macrophages by day 7.

2.2.6 **Monocyte / macrophage stimulation**

Both human immature monocytes and M-CSF matured macrophages were routinely stimulated using the TLR ligands; LPS, poly I:C (both Sigma), Cl097 and Pam3CSK4 (Pam3 - Invivogen) at varying concentrations and durations of stimuli (noted in the results for each experiment).

2.3 **Cell transfection**

In order to determine the consequence of microRNA-34a dysregulation, we used under and over expression experiments. Control and microRNA-34a mimics (Dharmacon) were used to investigate the effects of its increased expression, while control and microRNA-34a inhibitors (Qiagen) were used to look at the effects of decreased expression. Controls routinely run to check effective transfection included measuring the microRNAs expression by PCR and using miRIDIAN microRNA Mimic Transfection Control with Dy547 to check cellular uptake of a labelled mimic.

2.3.1 **Transfection of human monocytes**

For the transfection of $CD14^+$ monocytes freshly isolated from peripheral blood, the N-TER nanoparticle siRNA transfection reagent (Sigma) was used at a final concentration of 20 nM miRNA mimic or inhibitor. It important to mention that, previous studies carried out in our laboratory had concluded that 20 nM is the optimal concentration for the transfection of monocytes using this reagent. Transfection reagents were routinely left in cell cultures for 16-24 hours before being removed and fresh media added for the remainder of the experiment.

2.3.2 **Transfection of M-CSF matured macrophages**

For the transfection of $CD14^+$ cells which were M-CSF matured, and had therefore become adherent, TransIT-TKO® transfection reagent was used (mirus) at a final concentration of 25 nM mimic or inhibitor (as advised in standard reagent protocol). Transfection reagents were routinely left in cell cultures for 16-24 hours before the cells were taken off for further analysis (flow cytometry / ELISA) or stimulated.

2.4 Isolation of murine cells

2.4.1 Mice

Wild type and miR-34a knockout (miR-34a$^{-/-}$) C57BL/6 background breeding pairs were purchased from Jackson Laboratory, and animals were then bred in house. To avoid substrain divergence, miR-34a$^{-/-}$ mice were periodically backcrossed to the parental C57BL/6J strain. All animals were housed in pathogen-free conditions within the central research facility (CRF) at the University of Glasgow. All procedures were carried out in accordance with project licences approved by the United Kingdom Home Office and in accordance with the Animals (Scientific Procedures) Act 1986.

All mice were culled using recommended schedule 1 methods.

2.4.2 PCR genotyping of mice

2.4.2.1 Preparation of tail tips for genotyping

Mice were genotyped from tail tips cut prior to animals being used for experiments. Mice were first anaesthetised with isofluorane before a 5 mm tail-tip was cut and the tail cauterised. The tail tip was then placed in 75 µl tail-tip lysis buffer and incubated at 90ºC for 1 hour. 75 µl of neutralising buffer was then added to the tubes. This was either used straight away or stored at -20ºC until a polymerase chain reaction (PCR) was performed.

2.4.2.2 Primers and cycling conditions

The following primers (as suggested by Jackson) were used for genotyping:

Common primer	5’- ACTGCTGTACCCTGCTGCTT
Wild Type	5’- GTACCCCGACATGCAAACTT
miR-34a KO	5’- GCAGGACCACTGGATCATTT

Primers were ordered from IDT and made to stock concentrations of 100 µm. Working stock solutions were made with all 3 primers together at 2.5 µM and

stored at -20ºC. The 15 µl PCR reaction was made by mixing 7.5 µl Taq (go) Colourless Master Mix (Promega), 1 µl of the genomic tail tip DNA and primers at a final concentration of 250 nM. The PCR product was then amplified using a thermal cycler with the settings shown in Table 2-1.

Temperature	Time	Cycles
94ºC	2 min	-
94ºC **65ºC (-0.5ºC/cycle)** **68ºC**	20 sec 15 sec 10 sec	10 cycles
94ºC **60 ºC** **72 ºC**	15 sec 15 sec 10 sec	28 cycles
72ºC	1 min	-
4ºC	hold	-

Table 2-1 Thermal cycler settings for tail tip DNA amplification

2.4.2.3 Agarose gel electrophoresis

All gels used for genotyping were 2% agarose gels made up in Tris-acetate-EDTA (TAE) buffer. This was heated in a microwave to dissolve the agarose before ethidium bromide (Sigma) was added and mixed in by gentle shaking. The mixture was then poured into a gel cast and left to set for approximately 20 minutes. Once set, the gel was placed in an electrophoresis tank containing TAE buffer. 1.5 µl Blue Juice (Invitrogen) was then added to the 15 µl PCR reaction before it was added to gel wells. 10 µl of 1 kb plus DNA ladder (Invitrogen) was added to one or two wells per gel to estimate the size of PCR products visible. The gel was left to run for approximately 40 minutes at 100v before being visualised under a UV light.

Expected results are;

WT mouse - 1 band at ~471 bp

KO mouse - 1 band at ~180 bp

Heterozygous mouse - 2 bands at ~471 and ~180 bp

2.4.3 **Isolation and preparation of murine cells**

2.4.3.1 Isolation of bone marrow cells

All mice were culled using suggested schedule 1 methods, before the rear legs were cut away and removed. All skin and muscle was cut off and clean tibia and femurs were placed in a bijoux containing cold PBS on ice. The rest of the procedure was carried out in a tissue culture flow hood. Sterile scissors were used to cut the ends of the bones, and bone marrow was flushed out with complete media using a 25 gauge, 5/8th inch needle and a 2 ml syringe. The syringe was then used to create a single cell suspension and cells pelleted by centrifugation at 500 g for 5 minutes. The number of viable cells was counted using Trypan Blue and a haemocytometer before cells were re-suspended at the required concentration.

2.4.3.2 Isolation of splenocytes

Spleens were removed after culling and placed in a bijoux containing complete media on ice. A single cell suspension was then made by using the plunger of a syringe to push the spleen through a 70 µm filter into a 50 ml tube containing ice cold FACS buffer. Cells were pelleted by centrifugation at 500g, 4ºC for 5 minutes. The pellet was then re-suspended in 5 mls red blood cell (RBC) lysis buffer (Stem Cell Technologies) and placed on ice for 10 minutes. Immediately after the incubation, 30 mls of ice cold FACS buffer was added and the cells washed a further 2 times to remove any residual RBC lysis buffer. The number of viable cells was then counted using Trypan Blue and a haemocytometer and cells were re-suspended at the required concentration.

2.4.3.3 Isolation of cells from the blood

Blood was collected by cardiac puncture into lithium heparin tubes immediately after animals were culled. The blood was later transferred to a 15 ml centrifuge tube and mixed with 3 mls of RBC lysis buffer. This was placed on ice for 10 minutes before cells were washed with ice cold FACS buffer. If necessary this process was repeated. The number of viable cells was then counted using Trypan Blue and a haemocytometer and cells were re-suspended at the required concentration.

2.4.4 **Preparation of bone marrow macrophages**

Bone marrow cells were isolated and counted as described in section 2.4.3.1 above. Generally cells from four age and genotype matched mice were grouped together. Cells were then re-suspended in complete media supplemented with 10 ng/ml recombinant murine M-CSF (Peprotech) at a concentration 1 x 10^6 cells/ml. 10 mls in total was cultured per petri dish, and cells were incubated at 37ºC, 5% CO_2. After 3 days, spent medium and non-adherent cells were removed and fresh medium supplemented with 10 ng/ml M-CSF was added. Cells were then incubated for a further 4 days. On day 7 the media and non-adherent cells were removed, and ice cold PBS was added to the petri dishes to aid cell scraping. The number of viable M-CSF matured cells was then counted and cells were plated out at the required concentration for further experiments.

2.4.5 **Induction of peritoneal inflammation by LPS**

Each mouse was injected intraperitoneally with either PBS as a control, or 100 µg of LPS (Sigma). After 4 hours mice were culled using accepted schedule 1 methods. Immediately after death had been established a peritoneal wash was performed and blood taken by cardiac puncture.

The peritoneal cavity was flushed using 2 mls PBS, which was then placed in an eppendorf on ice. This was later centrifuged at 500g for 5 min at 4ºC to pellet cells. The supernatant was aliquoted and stored at -80ºC for future luminex, and the cells were counted and used for flow cytometry.

The blood collected from these mice was centrifuged at 8000g for 30 min at 4°C, after which the pellet was discarded and the serum was stored at -80ºC for future luminex.

2.5 Flow Cytometry

2.5.1 Assessment of cell purity after MACS

All samples isolated by positive selection were checked for purity using flow cytometry. Approximately 1 x 10^5 cells from the positive fraction were added to 2 clean FACS tubes per sample in 100 µl total FACS buffer. 1 µl human Fc block (Human TruStain FcX™, Biolegend) was added per tube, and samples incubated at 4ºC for 15 minutes. After this, the anti-human CD14 antibody was added to one tube and an isotype control added to the other.

Antibody	Isotype	Dilution (µl/100 µl)	Source / Cat No.
PE anti-human CD14	IgG2a	10 µl	Miltenyi / 130-091-242
PE Mouse IgG2a	IgG2a	10 µl	Miltenyi / 130-091-835

Table 2-2 List of antibodies used to determine cell purities

Cells were then incubated at 4ºC for 30 minutes before being washed 3 times with FACS buffer and re-suspended in 200 µl FACS buffer to be analysed on the FACS Calibur (BD Biosciences). When calculating the cell purity, gates were set to ensure < 2% of cells were positive for CD14 in the isotype. Greater than 95% purity was routinely observed.

2.5.2 Assessment of transfection efficiency

During the transfection process some cells were routinely put aside for transfection using the miRIDIAN microRNA Mimic Transfection Control with Dy547. After the 24 hour transfection, cells were taken off and measured for uptake of the fluorescent mimic on a FACS Calibur (BD Biosciences). When calculating the cell purity, gates were set to ensure < 2% of cells were positive for Dy547 in the unlabelled control mimic cells.

2.5.3 Separation of human monocyte subsets using the ARIA cell sorter

In order to separate monocyte subsets from human blood, blood from healthy donors was taken into lithium heparin tubes, and PBMCs isolated as described in section 2.2.2. Cells were then counted and re-suspended at 1 x 10^7 cells /

100µl, and incubated with human Fc block for 15 minutes at 4°C to reduce non-specific binding via Fc receptors.

In order to determine the best concentration for each antibody, titrations were performed, and the concentration which gave the biggest separation between the positive and negative fractions was chosen. This was calculated by taking the mean fluorescence intensity (MFI) of the positive fraction and dividing it by the MFI of the negative fraction. An example is shown in Table 2-3 for one antibody. 1 µl in 100 µl gave the biggest separation so this was the chosen concentration.

	Antibody Dilution (µl/100 µl)			
	0.5	1	2.5	5
MFI +ve	2302	3154	3365	3412
MFI –ve	35.4	36.2	41.9	47.4
MFI +ve ÷ MFI –VE	65.0282	87.1271	80.3103	71.9831

Table 2-3 Calculation used to determine optimal antibody concentration

For sorting of monocyte populations, cells were stained with specific antibodies; CD14, CD16 and HLA-DR, as well as on one channel having several "dump" stains to be removed from analysis before sorting; NKp46, CD56, CD19, CD15 and CD2 (all antibodies and concentrations shown in Table 2-4). These stains were all added together and incubated for 30 minutes at 4°C. Cells were then washed twice in FACS buffer before being re-suspended for sorting. All experiments were routinely controlled with unstained cells, appropriate isotype antibodies and FMO (fluorescence minus one) controls.

Antibody	Clone	Dilution (µl/100 µl)	Source / Cat No.
APC anti-human CD14	HCD14	5 µl	Biolegend / 325608
FITC anti-human CD16	3G8	5 µl	Biolegend / 302006
Pacific Blue anti-human HLA-DR	L243	2.5 µl	Biolegend / 307633
PE anti-human NKp46	9E2	5 µl	Biolegend / 331908
PE anti-human CD56	AF12-7H3	1 µl	Miltenyi / 130-090-755
PE anti-human CD19	HIB19	10 µl	Biolegend / 302208
PE anti-human CD15	W6D3	10 µl	Biolegend / 323006
PE anti-human CD2	TS1/8	10 µl	Biolegend / 309208

Table 2-4 List of antibodies used for cell sorting

10 minutes prior to sorting, 7-aminoactinomycin D (7-AAD; BD Biosciences) was added to each sample for the exclusion of dead cells. Cells were then run on an Aria cell sorter. During the sort cells were initially gated on the basis of FSC : SSC, then single, live cells were gated for on the basis of being positive for HLA-DR and negative for all antibodies in the dump channel. These cells were then sorted for collection into three tubes based on their expression of CD14 and CD16 - $CD14^{hi}$ $CD16^{-}$, $CD14^{hi}$ $CD16^{+}$ and $CD14^{dim}$ $CD16^{+}$. Sorted populations were centrifuged at 500 g for 5 minutes, and then re-suspended in Qiazol (Qiagen) for subsequent RNA extraction.

2.5.4 **Staining of human cells for flow cytometry**

After miR-34a manipulation by transfection, human M-CSF-matured macrophages were stained to assess the expression of HLA-DR, CD16 and CD64 by flow cytometry. After the 24 hour transfection cells were washed with PBS before a cell dissociation solution (Sigma) was added directly to the wells. Tissue culture plates were then placed back in the 37ºC incubator for 15 minutes before gentle pipetting was used to detach adherent cells. The liquid was transferred to FACS tubes and centrifuged at 500g for 5 minutes to pellet cells, which were then re-suspended in 100 µl FACS buffer containing 1 µl human Fc block and incubated at 4ºC for 15 minutes. Antibodies were then added to tubes at the concentrations indicated in Table 2-5, and cells incubated for a further 30 minutes at 4ºC in the dark. After the incubation, cells were washed three times in FACS buffer before being re-suspended in 200 µl and filtered through a cell strainer prior to running on the machine (LSR II, BD Biosciences).

Antibody	Clone	Dilution (µl/100 µl)	Source / Cat No.
Pacific Blue anti-human HLA-DR	L243	2.5 µl	Biolegend / 307633
PerCP/Cy5.5 anti-human CD64	10.1	2 µl	Biolegend / 305024
FITC anti-human CD16	3G8	5 µl	Biolegend / 302006

Table 2-5 List of antibodies used for flow cytometry analysis of human cells

2.5.5 **Staining of murine cells for flow cytometry**

Throughout this project, cells isolated from the blood, spleen, bone marrow and peritoneal cavity of mice were stained to allow assessment of cell populations. Cells were isolated using the aforementioned conditions, and then re-suspended at 1 x 10^6 cells per 100 µl in FACS buffer. 100 µl of each sample was then transferred to a FACS tube, and 1 µl murine Fc block (TruStain fcX™, Biolegend) was added per tube. Cells were then incubated at 4ºC for 15 minutes before the antibodies were added at the concentrations indicated in Table 2-6. Cells were incubated for a further 30 minutes at 4ºC in the dark, after which they were washed three times in FACS buffer before being re-suspended in 200 µl and filtered through a cell strainer prior to running on the machine (LSR II, BD Biosciences).

Antibody	Clone	Dilution (µl/100 µl)	Source / Cat No.
APC anti-mouse CD115	AFS98	1 µl	Biolegend / 135509
Af700 anti-mouse CD11b	M1/70	1 µl	Biolegend / 101222
PE Cy7 anti-mouse Ly6C	HK1.4	1 µl	Biolegend / 128017
APC Cy7 anti-mouse Ly6G	1A8	1 µl	Biolegend / 127623
PerCP anti-mouse F4/80	BM8	1 µl	Biolegend / 123126

Table 2-6 List of antibodies used for flow cytometry analysis of murine cells

2.5.6 **Analysis of flow cytometry data**

All flow cytometry data was analysed using FlowJo, software version V10.

2.6 Cytokine and chemokine analysis of cell supernatants

Cytokine and chemokine concentrations in cell supernatants, and murine serum and peritoneal washes, were measured in singleplex format by Enzyme Linked Immunosorbent assay (ELISA), and / or multiplex format using luminex kits.

2.6.1 Basic ELISA protocol

Enzyme Linked Immunosorbent assay (ELISA) was frequently used to measure the concentration of human and murine TNF-α (Life Technologies cat. no. CHC1753 and R&D Systems cat. no. DY410 respectively).

2.6.1.1 ELISA measurement of human TNFα

All protocols were performed using the manufacturer's instructions, but in brief: high binding microplates (Corning) were coated with the detection antibody diluted in PBS and incubated overnight at 4ºC. Plates were then washed in wash buffer (0.5% Tween / PBS), then blocked for 1 hour in the appropriate assay buffer (0.5% bovine serum albumin in PBS) at room temperature. Plates were then inverted to remove liquid, and blotted on absorbent paper before samples and standards (2000 pg/ml top standard) were added to the appropriate wells. Dilutions for samples were determined depending on the specific experiment. The detection antibody was then diluted in assay buffer and 50 µl added alongside samples and standards for a joint 2 hour incubation. After the detection step, plates were again washed 3 times in wash buffer before streptavidin HRP was added. Plates were then washed again before the addition of 100 µl of 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase (Biosource). Once appropriate colour change in the standards had developed the reaction was stopped with the addition of 100 µl Stop Solution (Biosource) and the intensity read on a microplate reader at the appropriate wavelength

2.6.1.2 ELISA measurement of murine TNFα

The murine TNFα ELISA kit is almost identical to the human kit with a few minor changes. For this kit the assay buffer is 1% BSA in PBS, and rather than using a joint sample, standard and detection antibody step, the samples and standards

are added to the plate for a two hour incubation, then the plates are washed before the detection antibody is added alone for a subsequent two hour incubation.

2.6.2 **Luminex assay**

Multiplex cytokine and chemokine analysis was performed using luminex assays. For the measurement of cytokines in human cell culture supernatants the Cytokine Human 25-Plex Panel from Life technologies (cat. no. LHC0009) was used, capable of measuring IL-1RA, IFN-γ, IL-7, IFN-α, IP-10, Eotaxin, MIG, IL-5, IL-6, IL-10, MIP-1α, IL-17, IL-8, IL-12 (p40/p70), RANTES, MIP-1β, GM-CSF, TNF-α, IL-1β, IL-2, IL-4, IL-2R, IL-15, MCP-1 and IL-13. For the measurement of cytokines in murine peritoneal wash and serum samples the Life Technologies Cytokine Mouse 20-Plex Panel (cat. no. LMC0006) was used, capable of measuring FGF-Basic, IL-1α, MCP-1, MIG, VEGF, KC, MIP-1α, GM-CSF, TNF-α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-17, IP-10, IL-13 and IFN-γ. All protocols were run according to manufacturer's instructions on a Bio-Plex system (Bio-Rad).

2.7 Analysis of gene expression

2.7.1 Purification of RNA from cell culture

In order to extract RNA from cells, cell pellets were re-suspended in 700 µl of Qiazol (Qiagen) to disrupt cell membranes and release RNA. These samples were then stored at -20°C until required for RNA extraction.

RNA was extracted from Qiazol samples using the miRNeasy kit (Qiagen), using the manufacturer's recommended protocol. This kit was chosen as it allows the quantification of both microRNA and standard mRNA using the same sample. In brief, Qiazol samples were vortexed to ensure adequate homogenisation of cells. 140 µl chloroform was then added to samples, which were shaken vigorously before being centrifuged for 15 minutes at 12,000 g, 4°C. This separated the samples into three different layers. The upper (colourless) aqueous phase containing the RNA was transferred to a fresh eppendorf and mixed with 1.5 volumes of 100% ethanol. This was mixed thoroughly before applying to the RNeasy spin column. Columns were centrifuged for 15 seconds at 8000g and the flow through discarded. 700 µl of RWT wash buffer was then applied to the column, centrifuged for 15 seconds at 8000g and the flow through discarded. Next, 500 µl of buffer RPE was applied to the column, which was then centrifuged for 15 seconds at 8000g. This step was repeated with a subsequent 2 minute spin to ensure all contaminants were removed, and the column spun for a further minute at full speed. Finally the RNA was eluted in RNase-free water (30-50 µl depending on the expected RNA yield). The quantity and quality of RNA was determined using a NanoDrop spectrophotometer.

Initially, a DNase step was included in the RNA extraction, but as all primers were designed or known to cross intron / exon boundaries, and therefore should not detect genomic DNA, this step was later removed.

2.7.2 **cDNA Synthesis**

All cDNA was made using commercially available kits. At the beginning of this project one kit was used for microRNA (Life Technologies microRNA RT kit) and another for mRNA (Life Technologies High Capacity cDNA RT Kit). This method was initially used as we found it more sensitive for microRNA detection, and it allowed reliable detection from a very low starting RNA concentration. However, once expression levels were established, Qiagen's miScript II RT kit was used as it allowed for quantification of both miRNA and mRNA from the same cDNA sample, and this method is much more time and cost effective.

2.7.2.1 Life Technologies microRNA RT kit

Originally, when measuring microRNA expression by PCR, we used the TaqMan MicroRNA Reverse Transcription Kit along with the miR specific RT primers from Life Technologies TaqMan microRNA Assays (assays used listed in Table 2-7).

Assay Name	Cat. no	Assay ID
hsa-miR-34a	4427975	000426
hsa-miR-34b	4427975	002102
hsa-miR-34c	4427975	000428
hsa-miR-22	4427975	000398
U6 snRNA	4427975	001973
Let-7a	4427975	000377

Table 2-7 Life technologies TaqMan microRNA Assays used

This allowed the reliable and highly sensitive detection of microRNA from only 10 ng of starting RNA. The components of the 15 µl cDNA reaction are shown in Table 2-8. This reaction was then put in a thermal cycler (Veriti®, Applied Biosystems) using the profile outlined in Table 2-9.

Component	Volume / reaction
100mM dNTPs	0.15 µl
MultiScribe Reverse Transcriptase	1 µl
10x RT Buffer	1.5 µl
RNase Inhibitor	0.19 µl
RNase-free H_2O	4.16 µl
5x RT primers	3 µl
Template RNA (at 2 ng/µl)	5 µl
Total Volume	= 15 µl

Table 2-8 Life technologies microRNA RT kit reaction components

Temperature	Time
16ºC	30 mins
42ºC	30 mins
85ºC	5 mins
4ºC	Hold

Table 2-9 Life technologies microRNA RT kit thermal cycler profile

2.7.2.2 High Capacity cDNA RT kit

As the microRNA RT kit used specific RT primers in the cDNA reaction, a separate cDNA was required for quantification of mRNA in the samples, for this we frequently used the high capacity cDNA kit (Life Technologies). The components of the 20 µl cDNA reaction are shown in Table 2-10. This reaction was then put in a thermal cycler (Veriti®, Applied Biosystems) using the profile outlined in Table 2-11.

Component	Volume / reaction
100mM dNTPs	0.8 µl
MultiScribe Reverse Transcriptase	1 µl
10x RT Buffer	2 µl
RNase Inhibitor	1 µl
RNase-free H_2O	3.2 µl
10x RT Random Primers	2 µl
Template RNA	10 µl
Total Volume	= 20 µl

Table 2-10 Life technologies high capacity cDNA RT kit reaction components

Temperature	Time
25ºC	10 mins
37ºC	120 mins
85ºC	5 mins
4ºC	Hold

Table 2-11 Life technologies high capacity cDNA RT kit thermal cycler profile

2.7.2.3 miScript II RT kit

Once we were confident in the expression level of our microRNA, and were simply using PCR to determine up or down-regulation of miR-34a following transfection of cells with mimics and inhibitors, we switched back to the miScript II RT kit (Qiagen). Here we used the miScript II RT kit with HiFlex buffer as it allows parallel quantification of mature microRNA and mRNA from a single cDNA sample. Depending on the experiment and RNA concentration achieved, between 200 and 600 ng of starting RNA was used per 20 µl PCR reaction. The components of the 20 µl cDNA reaction are shown in Table 2-12. This reaction was then put in a thermal cycler (Veriti®, Applied Biosystems) using the profile outlined in Table 2-13. Prior to using these samples, cDNA was diluted by the addition of 180 µl RNase-free H_2O.

Component	Volume / reaction
miScript HiFlex Buffer	4 µl
10x Nucleics mix	2 µl
RNase-free H_2O	2 µl
miScript Reverse Transcriptase	2 µl
Template RNA	10 µl
Total Volume	= 20 µl

Table 2-12 miScript II RT kit reaction components

Temperature	Time
37ºC	60 mins
95ºC	5 mins
4ºC	hold

Table 2-13 miScript II RT kit thermal cycler profile

2.7.3 **qPCR**

2.7.3.1 Taqman qPCR

To measure microRNA expression the taqman PCR primers from the TaqMan microRNA assays were used. For the measurement of mRNA, Taqman primers were ordered from IDT or Life technologies (all taqman primers used in this project are listed in Table 2-14). For both mRNA and microRNA detection, Taqman Universal Master Mix no UNG (Life Technologies) was used. The primers and RNase/DNase-free H_2O were added to the master mix, which was then pipetted into 96 or 384 well PCR plates before the cDNA was added. The qRT-PCR reaction was performed on Applied Biosystems 7900HT or 7500 machines for 40 cycles according to the assay instructions.

Gene Name	Cat no. / Assay Name	Probe	Company
IRF1	4331182	FAM	Life Technologies
MTF1	4331182	FAM	Life Technologies
TLR7	4331182	FAM	Life Technologies
SIRT6	4331182	FAM	Life Technologies
MT1E	Hs.PT.53.19749017	FAM	IDT
MT1F	Hs.PT.53.19974633	FAM	IDT
MT1G	Hs.PT.53.25205882	FAM	IDT
GAPDH	Hs.PT.53.26895230.g	Cy5	IDT
IRF3	Hs.PT.53.19791480.g	Cy5	IDT
STAT1	Hs.PT.53.26308725	Cy5	IDT
IRF7	Hs.PT.53.23026870	Hex	IDT
DDX58	Hs.PT.53.27193757	Hex	IDT
IFIH1	Hs.PT.53.18912568	Hex	IDT

Table 2-14 List of Taqman primers used for PCR

2.7.3.2 SYBR Green PCR

SYBR green PCR was also used to quantify mRNA expression with Power SYBR green master mix (Life Technologies) and Quantitect primers from Qiagen (Table 2-15). The reaction mix was prepared as shown in Table 2-16, before 10 µl was added in triplicate to a 384 well plate.

Gene Name	Cat no.	Company
IFIT1	QT00201012	Qiagen
IFIT3	QT00100030	Qiagen
IFI35	QT00010402	Qiagen
IFITM1	QT00064246	Qiagen
OAS1	QT00099134	Qiagen
MX1	QT00090895	Qiagen
EIF2AK2	QT00022960	Qiagen
MT1H	QT01004612	Qiagen
MT1M	QT01004626	Qiagen
MT1X	QT00048237	Qiagen

Table 2-15 List of primers used for SYBR PCR

Component	Volume / reaction
Power SYBR green master mix	17.5 µl
Primers	3.5 µl
Template cDNA	1.75 µl
RNase-free H_2O	12.25 µl
Total Volume	= 35 µl

Table 2-16 Power SYBR green PCR reaction components

2.7.4 Generating PCR standards for absolute quantification of microRNA

In order to gain a better understanding of the expression levels of different microRNA, we decided to make PCR standards. For the let-7a control, single stranded RNA template was ordered from Integrated DNA Technologies (IDT). To make template for microRNA, 5 µl stock double stranded microRNA mimic (20 µM) was diluted in 45 µl RNase-free H_2O and denatured at 95ºC for 5 minutes. This was then immediately placed in ice water to cool. 5 µl of this stock was then added to the 15 µl RT reaction, giving $4.013x10^{11}$ copies/µl cDNA.

miRNA copy number was calculated using Avogadro's Constant as follows:

5 µl 20 µM mimic = 0.1 nmoles in a total of 50 µl

Copy Number = Avogadro's Constant x moles

$= (6.022 \times 10^{23}) \times (1 \times 10^{-10})$

$= 6.022x10^{13}$ copies in 50 µl

$= 1.204x10^{12}$ copies/µl

In microRNA cDNA reactions we use 0.6 µl template cDNA per well, so we need to achieve the desired concentration of standard template in 0.6 µl. To obtain a standard curve, standards were serial diluted to give copy numbers in the range of $1x10^9$ to $1x10^3$.

2.7.5 **Microarray**

In order to investigate pathways regulated by miR-34a in monocytes, PB $CD14^+$ cells were transfected with either miR-34a or a control miRNA mimic (20 nM) for 24 hours. RNA was then isolated and sent to the Glasgow Polyomics facility to perform an mRNA microarray (GeneChipHG-u133 plus 2; Affymetrix). Data were analysed using GeneSpring and Ingenuity Pathway analysis softwares. To minimalize systemic non-biological differences, Robust Multichip Analysis (RMA) was used, followed by quantile normalization with baseline transformation to the median of all samples. Entities were filtered based on their signal intensity value and retained if they achieved a value within the range (percentile cut off: 20-100) for at least one sample out of 10. To identify differentially expressed genes, Mann Whitney test and 2 fold cut off was applied. To identify direct miR-34a targets, all genes (regardless of fold change) that were down-regulated in miR-34a mimic transfected cells were ranked and checked against miR-34a predicted targets proposed by TargetScan prediction algorithm.

2.8 **In situ hybridisation**

An *in situ* staining protocol was developed using the miRCURY™ microRNA ISH Optimization Kit (Exiquon) using the manufacturer's guidelines. Detailed below is the final protocol used. Unless otherwise stated reagents were provided with, or recommended for use with the optimisation kit.

2.8.1 **Standard non-fluorescent protocol**

For the staining of tissue sections, samples first had to be deparaffinised in xylene and ethanol as outlined in Table 2-17.

Step	Solvent	Duration
1	Xylene	5 minutes
2		5 minutes
3		5 minutes
4	99.9% Ethanol	Immerse 10 times
5		Immerse 10 times
6		5 minutes
7	96% Ethanol	Immerse 10 times
8		5 minutes
9	70% Ethanol	Immerse 10 times
10		5 minutes
11	PBS	5 minutes

Table 2-17 *In situ* hybridisation - Steps used to deparaffinise tissue sections

Slides were then incubated with 15 µg/ml Proteinase-K for 10 minutes at 37ºC, before being washed twice in PBS and dehydrated as shown in Table 2-18.

Step	Solvent	Duration
1	70% Ethanol	Immerse 10 times
2		1 minute
3	96% Ethanol	Immerse 10 times
4		1 minute
5	99.9% Ethanol	Immerse 10 times
6		1 minute

Table 2-18 *In situ* hybridisation - Steps used to dehydrate slides

Slides were then allowed to air dry for approximately 15 minutes before the double digoxigenin (DIG) labelled LNA™ probe (miR-34a or scrambled) was applied and the slides incubated for a further 1 hour at 55ºC on a heated plate hybridiser. To avoid evaporation of liquids and slides drying out coverslips were

placed over the slides, and these were sealed using fixogum. After the 1 hour incubation the cover slips were removed and slides washed in SSC buffers as outlined in Table 2-19.

Step	Buffer	Duration	Temperature
1	5 x SSC	5 minutes	55ºC
2	1 x SSC	5 minutes	55ºC
3	1 x SSC	5 minutes	55ºC
4	0.2 x SSC	5 minutes	55ºC
5	0.2 x SSC	5 minutes	55ºC
6	0.2 x SSC	5 minutes	RT

Table 2-19 *In situ* hybridisation - Steps used to wash slides

A hydrophobic barrier was then applied to the slides by placing them in PBS, before a blocking solution was applied for 15 minutes at room temperature. The blocking solution is made up by diluting a 10 x blocking solution in 1 x Maleic acid. When the blocking solution is removed, an anti-DIG-alkaline phosphatase (AP) was applied and the slides incubated in a humidified chamber at room temperature for one hour. To help reduce non-specific binding this was diluted 1:800 in blocking solution containing 2% sheep serum. After the one hour incubation, slides were washed three times in PBS-Tween and incubated in AP substrate for a further 2 hours at 30ºC in a humidified chamber in the dark.

Slides were then incubated with KTBT buffer for two 5 minute incubations to stop the reaction before being washed in water. If Nuclear Fast Red was added, it was added for 1 minute at this stage, before slides were washed in tap water. Slides were then dehydrated once again (procedure as detailed in Table 2-18) and mounted using Vectamount hard set.

2.8.2 Double fluorescent *in situ* hybridisation with miR-34a and CD68

When fluorescent *in situ* hybridisation was required to allow double staining with CD68, the protocol was identical until the addition of the anti-DIG antibody. For fluorescent staining at this stage a specific anti-DIG fluoresceine antibody was used (again diluted in blocking solution containing 2% sheep serum to avoid non-specific binding). After one hour slides were washed in PBS-Tween before a

mouse anti-human CD68 or control IgG antibody was added at 1 µg/ml and slides were incubated at 4ºC overnight in the dark.

The following day, slides were washed in PBS-T, and a secondary biotinylated horse anti-mouse antibody was added to the sections for 30 minutes at room temperature (1:200 with blocking solution). Slides were once again washed in PBS-T before Avid D-Texas Red (1:500) was added for a further 30 minutes. To mount slides for viewing, 1 drop of Vectamount hard set containing DAPI was added to each section, a coverslip placed on top and sealed with clear nail varnish.

2.9 **Luciferase Assays**

Luciferase assays were used to determine whether a microRNA was capable of physically binding to a given predicted target.

2.9.1 **Generation of target insert**

For each predicted target, primers were designed that would allow amplification of a section of the 3' untranslated region (3'UTR) containing the potential microRNA binding site. In this project luciferase assays were carried out to assess whether microRNA-34a was capable of binding to its predicted binding sites in Notch1, CSF1-R, IRF1 and MTF1 - which had two separate binding sites that were tested individually (MTF1 (1) and MTF1 (2)). All primers used in this project are shown in Table 2-20. All primers were ordered from IDT and made up to 100 µM in RNase free water. Working stocks were then made with 5 µl of each of the forward and reverse primers and 90 µl RNase-free water. These primers were used to amplify the required insert from a genomic DNA (gDNA) template. An example PCR reaction is shown in Table 2-21, and the thermal cycler profile is shown in Table 2-22.

Target	Forward Primer	Reverse Primer
Notch1	AGTTTAAACGTCTGTGTGCGCTCTGT	AGTCGACGAGCATCTTCTTCGGAACCT
CSF1-R	AGTTTAAACATCAGTTCTGCTGAGGAGTTG	AGTCGACTGAAGGAGGGAAGGAAAGAAAG
IRF1	AGTTTAAACTCTGAAGAACATGGATGCCACCTG	AGTCGACGCCCTGGGATTGGTGTTATGCTT
MTF1 (1)	AGTTTAAACTGCTCAGGGCCAGCCAAGATATTA	AGTCGACAATCCCAGCACTTTGGGAGACTG
MTF1 (2)	AGTTTAAACTTTAGGGAGAGAGGGAAACAGTCC	AGTCGACAAGGGCAAGAGAAAGCCTCTGACT

Table 2-20 Primers used for luciferase assays

Component	Volume / reaction
2 x GoTaq Master Mix (Promega)	25 µl
Primers (working stock)	2.5 µl
gDNA	2 µl
H_2O	20.5 µl
Total Volume	= 50 µl

Table 2-21 PCR reaction components used for generation of candidate 3'UTR amplicons

Temperature	Time	Stage
94ºC	2 mins	1
94ºC	20 secs	2 (Repeat x 35)
56ºC	20 secs	
72ºC	40 secs	
72ºC	10 mins	3

Table 2-22 Thermal cycler profile used for generation of luciferase insert

PCR products were initially run out on 0.8% (W/V) TAE agarose gels to confirm the size and specificity of amplifications. Bands of the correct predicted sizes were excised from the gel and gel purified using the QIAquick Gel Extraction kit (Qiagen). The purified DNA fragment was then inserted into pCR2.1 TOPO vector (Invitrogen) following the manufacturer's instructions. This vector takes advantage of Taq DNA polymerases ability to add non-template dependent terminal deoxy-adenosines to the 3' ends of amplified products.

Reactions were transformed into competent XL1-Blue cells and plated out onto LB agar plates containing 50 µg/ml ampicillin and grown overnight at 37ºC. The following day colonies were picked and used to inoculate 5 ml cultures of LB plus 50 µg/ml ampicillin. The cultures were grown overnight at 37ºC in a shaking incubator. The following day plasmid DNA was prepared from 1.5 mls of bacterial culture using the Qiaprep Spin miniprep kit (Qiagen). Plasmids containing the correct inserts were confirmed by performing a double digest using the restriction enzymes *Pme*I and *Sal*I (New England Biolabs – NEB). Digested plasmids were run out on 0.8% TAE agarose gel next to a 1kB (+) DNA ladder (Invitrogen). This digest served to identify clones with the correct insert, but also generated insert fragments that could be gel purified and cloned into *Pme*I/*Sal*I sites in the pmiRGLO reporter as described below.

2.9.2 **Preparation of luciferase vector**

The pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega) was used as the reporter vector in these experiments. As demonstrated in Figure 2-1, the vector contains a multiple cloning site (MCS) 3' of a firefly luciferase reporter gene (luc2), and also a humanized Renilla luciferase-neomysin resistance cassette (hRluc-neo), which functions as a reporter gene for the normalisation of luciferase activity.

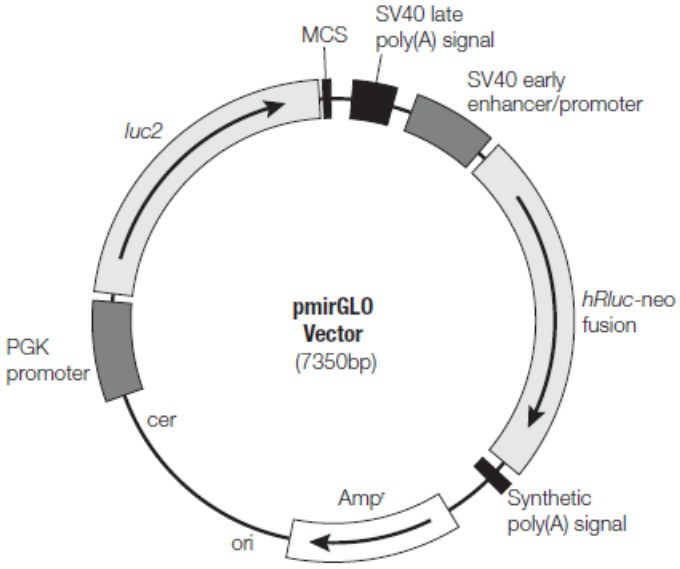


Figure 2-1 pmirGLO Dual-Luciferase miRNA Target Expression Vector

Prior to ligation of the vector and insert, the vector must be linearized with appropriate restriction enzymes. These are chosen depending on the insert, and are used to create complimentary overhangs between the insert and vector to allow later annealing. An example of the reaction used for vector digestion is shown in Table 2-23. This mixture was incubated at 37ºC for 2 hours.

Component	Volume / reaction
10 x Buffer 4 (NEB)	5 µl
plasmid	20 µl
*Pme*I (NEB)	1 µl
*Sal*I (NEB)	1 µl
H_2O	23 µl
Total Volume	= 50 µl

Table 2-23 Reaction setup for digestion of pmirGLO vector for luciferase assay

2.9.3 **Ligation of target insert and luciferase vector**

The next step is ligation of the insert (containing the predicted microRNA binding site) and vector. The components of the ligation mixture are outlined in Table 2-24. The components were all mixed in an eppendorf and incubated at room temperature for 10 minutes, creating the pmirGLO vector with the desired insert containing the predicted microRNA binding site.

Component	Volume / reaction
10 x T4 ligase buffer (NEB)	2 µl
Vector (cut pmirGLO)	3 µl
Insert	14 µl
T4 DNA ligase	1 µl
Total Volume	= 20 µl

Table 2-24 Components of ligation reaction for pmirGLO vector and target insert

2.9.4 **Transformation of competent cells**

In order to transform the ligated pmirGLO vector into competent cells, 3 µl of the assembled product was placed in a microcentrifuge tube, and 50 µl of competent cells added to the tube. This mixture was left on ice for 30 minutes, before cells were heat-shocked at 42ºC for 30 seconds and placed back on ice for 2 minutes. 300 µl of Luria-Berta (LB) broth was then added and the cells allowed to recover for 60 minutes at 37ºC in a shaker (approx. 200 – 250 rpm). 200 µl of the competent cells were then spread on an LB-Amp plate and placed in a 37ºC incubator overnight.

The following day three colonies from each plate were picked and placed in individual universals containing 5 mls LB-AMP broth. These were cultured overnight at 37ºC in a shaker (approx. 200 – 250 rpm). The following day plasmid DNA was extracted from 1.5 mls of the culture using a QIAprep Spin Miniprep Kit (Qiagen). These samples were routinely sent for sequencing to validate correct target insert.

2.9.5 **Transfection of cells with luciferase vector**

Luciferase experiments were performed in Human embryonic kidney (HEK)-293 cells. Shortly before transfection of the cells, they were seeded at 1 x 10^5 cells per well of a 24 well plate in 500µl complete DMEM, and placed under normal growth conditions (37ºC, 5% CO_2) to allow cells to adhere. In the meantime the transfection mix was made using Attractene transfection reagent (Qiagen). Reaction components are shown in Table 2-25. Transfection mixtures were incubated at room temperature for 20 minutes to allow the formation of

transfection complexes. After this time the mixture was added drop-wise onto cells to assure uniform distribution of complexes.

Component	Volume / reaction
DNA (200 ng/µl)	2 µl
5 µM control or microRNA mimic	2.8 µl
Attractene	1.5 µl
Optimem (Invitrogen)	60 µl
Total Volume	66.3 µl

Table 2-25 Attractene mixture components for luciferase assay

After 24 hours adherent cells were washed with PBS, then 200 µl 1 x passive lysis buffer was added per well (5 x lysis buffer provided with Promega's Dual-Luciferase® Reporter Assay, diluted in distilled water). The plate was left to gently rock for 15 minutes at room temperature, before the lysates were diluted 1 in 200 in the 1 x passive lysis buffer. 50 µl of the diluted lysate was added in triplicate to white 96 well flat bottomed plates (Greiner). 50 µl of diluted luciferase assay substrate was then added to each well, and firefly luciferase activity measured on a luminometer. 50µl of Stop & Glo® Reagent was then added to each well, and Renilla luciferase activity measured on the luminometer. (all reagents and buffers provided in Promega's Dual-Luciferase® Reporter Assay kit).

2.9.6 **Calculating the results**

miRNA knock down was calculated as a percentage of normalised miRNA treated sample compared to scrambled control. In brief the luciferase activity of all samples were normalised to the renilla activity by dividing luciferase/renilla. The normalised value obtained for the specific miRNA treated samples were then expressed as a percentage of the scrambled control samples.

2.10 **Statistical Analysis**

All statistical analyses were performed using GraphPad Prism 5, except correlation statistics which were carried out in Minitab 17 statistical software. All results are displayed as mean ± standard error of the mean.

The particular statistical test used depended on the given experiment, and is indicated in the figure legend for each graph. Where there was an $n \geq 8$, a normality test was used (D'Agostino-Pearson normality test) to determine whether data displayed a Gaussian distribution.

A p value of < 0.05 was considered statistically significant. Where significance was reached asterisk on the graph indicate the level of significance. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Chapter 3 – microRNA-34 Family Expression and Regulation

3.1 Introduction and Aims

Rheumatoid arthritis is a chronic inflammatory disorder, whereby synovial inflammation ultimately results in joint destruction and impaired function of affected joints. Although disease processes are not fully understood, one cell type known to be a key player in the perpetuation of this inflammation is the macrophage. These cells are recruited to the synovium in large numbers, where they are capable of producing substantial quantities of inflammatory cytokines; including TNF-α, IL-6 and IL-1ß. The mechanisms underlying this inflammatory gene expression in macrophages are yet to be fully dissected, but a better understanding of this could allow the development of new treatments targeting known, as well as completely novel disease-driven pathways.

One area of research which could provide such new treatment targets comprises the RNA interactome – especially the potential role of microRNA. These are a recently discovered class of post-transcriptional regulators known to be involved in the regulation of many cellular processes – including cell cycle, apoptosis and inflammatory cytokine production. They are demonstrated to be dysregulated in a variety of diseases. Since they can be readily detected in cells and in the soluble phase, they could be useful as biomarkers, or even in the generation of new therapies. Certainly since they cross regulate a variety of functionally linked pathways, their elucidation is likely to prosper our understanding of the underlying pathogenetic events subserving the target lesion in the RA synovium.

Previous experiments from our laboratory set out to determine the differential transcript expression between $CD14^+$ cells derived from the peripheral blood (PB) and synovial fluid (SF) of RA patients. In order to achieve this, a microarray was performed on RNA isolated from patient samples. As well as developing an mRNA signature for these cells, the array also highlighted a few microRNA that were differentially expressed. As demonstrated in Figure 3-1, the microarray data showed that miR-155 and miR-34a were expressed higher in the SF $CD14^+$ cells, while miR-223 was significantly lower in the SF cells. Our group have already generated data examining the expression and regulation of miR-155 [300] and miR-223 [342], and I set out to establish a similar potential role for miR-34a in the same context.

microRNA-34a is already known to have a role in several disease states, and in particular has been extensively studied in cancer and heart disease. In mouse models of heart disease, it has been shown that miR-34 inhibition attenuated cardiac remodelling and improved systolic function [343]. Furthermore, in the cancer field many studies have shown that miR-34a acts as a tumour-suppressor by targeting numerous genes related to proliferation, apoptosis and invasion (reviewed in [344]). miR-34a replacement therapy is already in phase I clinical trials for the treatment of liver cancer [331]. Given the many confirmed roles of miR-34a in cellular regulation, its differential expression in RA could be significant, so I sought to dissect its possible role in the inflammatory phenotype seen in monocytes and macrophages in RA. In this chapter I examined the expression of this microRNA in both the periphery and synovium of RA patients, and also set out to establish those factors that regulate miR-34a expression.

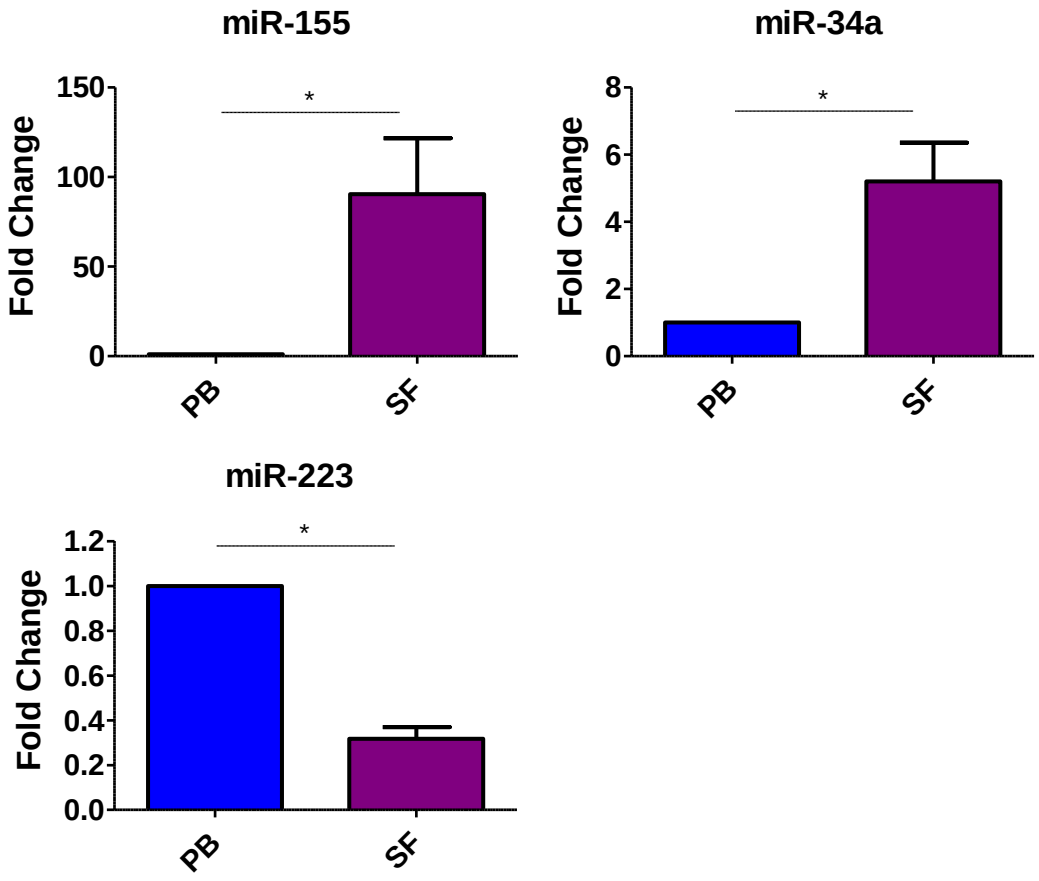


Figure 3-1 Expression of miR-155 and miR-34a is up-regulated, while miR-223 is down-regulated in CD14$^+$ cells isolated from the synovial fluid of RA patients compared to matched peripheral blood samples

CD14$^+$ cells were isolated from the peripheral blood (PB) or synovial fluid (SF) of RA patients before a microarray was used to compare transcript expression between these populations. As well as generating an mRNA signature for the cells, the microarray highlighted three microRNA that were differentially expressed – miR-155, miR-34a and miR-223. The PB expression for each donor was normalised to 1, with SF expression shown as relative fold change. Data presented as mean plus standard error of the mean. Wilcoxon matched-pairs signed rank test; * $P < 0.05$. $n = 7$. This microarray was analysed by my supervisor – Dr Kurowska-Stolarska – prior to the beginning of my PhD, and served as the pilot data on which my project was based.

3.2 Expression of the microRNA-34 family in health and disease

In order to validate the results from the microarray, fresh samples were obtained from RA patients. $CD14^+$ cells were isolated from the matched PB and SF samples and Taqman PCR performed to measure the expression of miR-34a. As shown in Figure 3-2, these experiments show that miR-34a is expressed at significantly higher levels in the $CD14^+$ cells isolated from RA SF compared to PB samples. As such they confirmed the preliminary analysis on which my project was predicated and served to encourage further investigation. As miR-34a belongs to a family of microRNA, we also measured the expression of the other family members – miR-34b and miR-34c – which were not differentially expressed between the PB and SF $CD14^+$ cells.

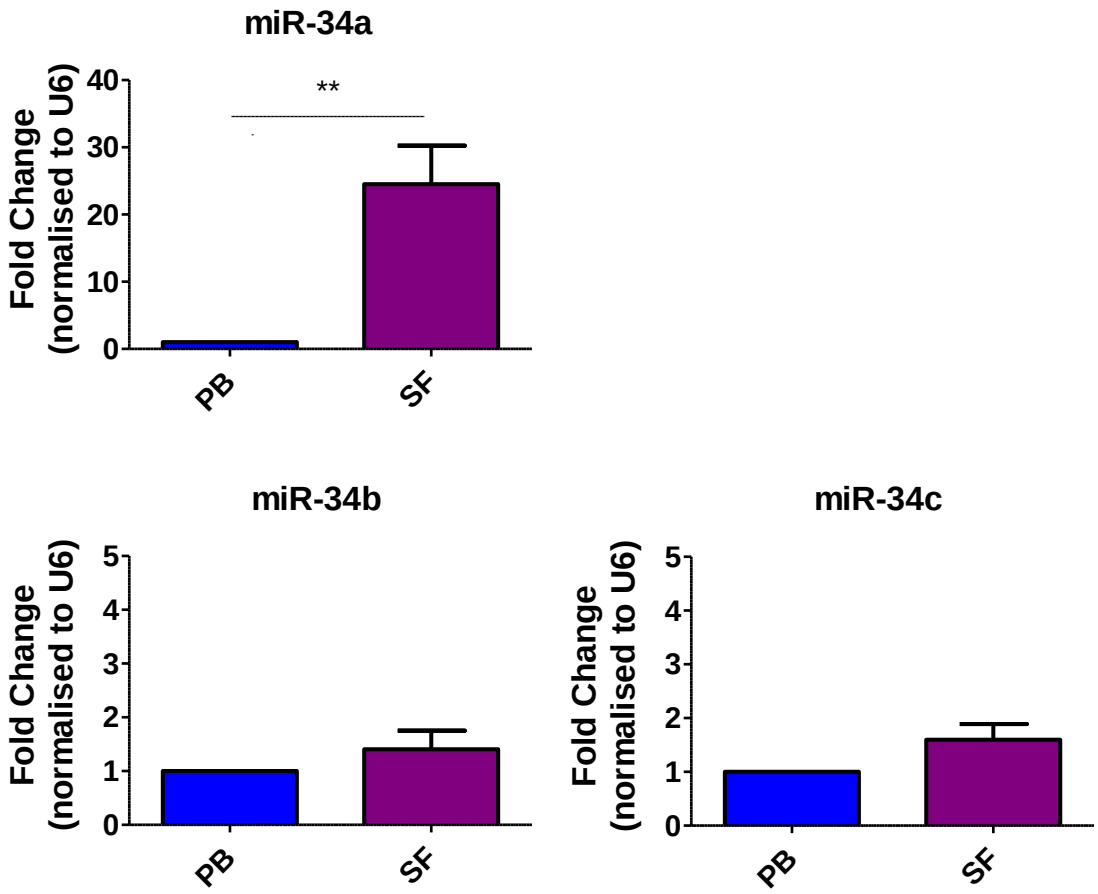


Figure 3-2 miR-34a, but not miRs 34b/c, is up-regulated in CD14$^+$ cells isolated from the synovial fluid of RA patients compared to matched peripheral blood samples
CD14$^+$ cells were isolated from the peripheral blood (PB) or synovial fluid (SF) of RA patients before Taqman PCR was used to quantify microRNA levels compared to the housekeeping control U6. The expression of microRNA-34a, b and c was measured. The PB expression for each donor was normalised to 1, with SF expression shown as relative fold change. Data presented as mean plus standard error of the mean. Paired t test; ** $P < 0.01$. $n = 10$.

3.2.1 **Developing standards to quantify miR-34a expression**

Although the expression of miR-34b and miR-34c is equivocal in the PB and SF $CD14^+$ cells, it was important to gain a better understanding of their expression levels. miRs-34b and 34c share the same seed region as miR-34a, and as a consequence share a number of the same targets. I therefore set out to determine whether they are present in our cells and likely to interfere with future experiments. In order to do this we aimed to develop PCR standards. For several reasons, at this stage we decided to switch from our previous PCR housekeeping (small nuclear RNA U6), to let-7a. U6 is larger in size than miRNA, and we were also unsure which part of U6 was amplified by our TaqMan PCR primers – making it difficult to design standards. After searching the literature we found that let-7a microRNA was often used as a house-keeping control [345, 346]. We therefore sought to determine and compare the expression levels of U6 and let-7a in our cells, and ensure their expression was stable. We chose to check the expression during the M-CSF maturation of $CD14^+$ cells, and also after stimulation of macrophages with various TLR ligands. Taqman PCR was used to measure U6 and let-7a expression where samples had been normalised to the same starting RNA concentration in each reaction. Figure 3-3 shows the CT values for U6 expression, and confirms that it's expression is indeed stable during both the M-CSF maturation of $CD14^+$ cells and their subsequent stimulation via TLR ligands. Figure 3-4A demonstrates that let-7a expression remains constant in $CD14^+$ cells freshly isolated from buffy coat samples, and after M-CSF maturation for 3 or 7 days. Let-7a expression also remains stable after stimulation of M-CSF matured macrophages with various TLR ligands (Figure 3-4B). We therefore concluded that it was a suitable housekeeping control for normalisation of transcript expression in future experiments.

PCR standards for let-7a and miRs 34a, b and c were developed as described in section 2.7.4 in the materials and methods. Figure 3-5 shows a representative standard curve and amplification plot for the miR-34a standards, and show successful detection of the miRNA between 10^3 and 10^9 copies per PCR reaction. Thus we continued to run these standards and use absolute quantification for future microRNA PCR.

Using these standards, Taqman PCR was repeated on the same PB and SF samples from RA patients used in Figure 3-2. As demonstrated in Figure 3-6, again miR-34a is expressed at a higher level in the SF $CD14^+$ cells of RA patients compared to matched PB cells. Also clear is that miR-34b and miR-34c are barely detectable, or expressed at very low levels in both the PB and SF cells, and there was no difference in the magnitude of expression between PB and SF cells. This gave us confidence that these microRNA would not interfere with our future results, and from this point onwards we chose to focus solely on miR-34a.

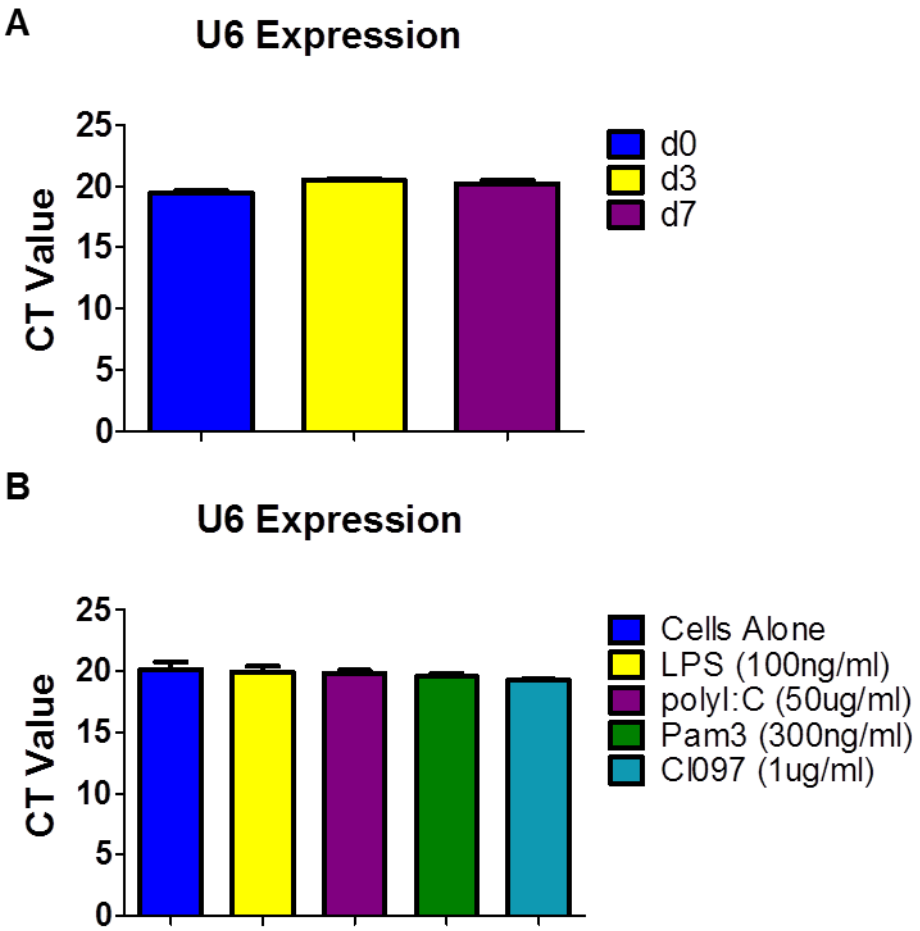


Figure 3-3 U6 expression is stable across monocyte maturation and subsequent TLR ligand stimulation

As we have used U6 as a housekeeping control for the normalisation of transcript expression after PCR, we sought to determine whether its expression was stable across our frequently used conditions. $CD14^+$ cells were isolated from buffy coat samples and their U6 expression measured at day 0 (d0), and after 3 (d3) and 7 (d7) days 50 ng/ml M-CSF-induced maturation. Panel A shows the CT values for each condition, where experiments were normalised for the same starting concentration of RNA. The CT values are also shown after stimulation of these 7 day M-CSF matured cells with various TLR ligands (B). Data presented as mean plus standard error of the mean. n = 3.

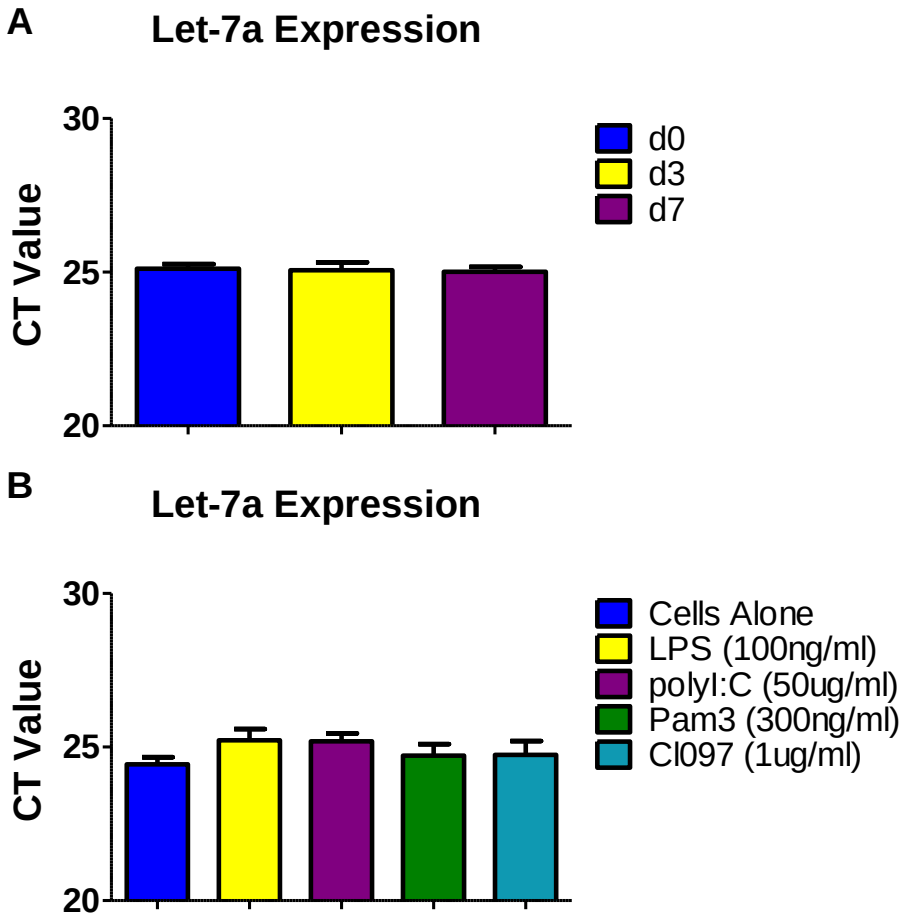


Figure 3-4 Let-7a expression is stable across monocyte maturation and subsequent TLR ligand stimulation

Before using let-7a as a house keeping control for PCR normalisation, we sought to determine whether its expression was stable across our frequently used conditions. $CD14^+$ cells were isolated from buffy coat samples and their let-7a expression measured at day 0 (d0), and after 3 (d3) and 7 (d7) days 50 ng/ml M-CSF-induced maturation. Panel A shows the CT values for each condition, where experiments were normalised for the same starting concentration of RNA. The CT values are also shown after stimulation of these 7 day M-CSF matured cells with various TLR ligands (B). Data presented as mean plus standard error of the mean. n = 3.

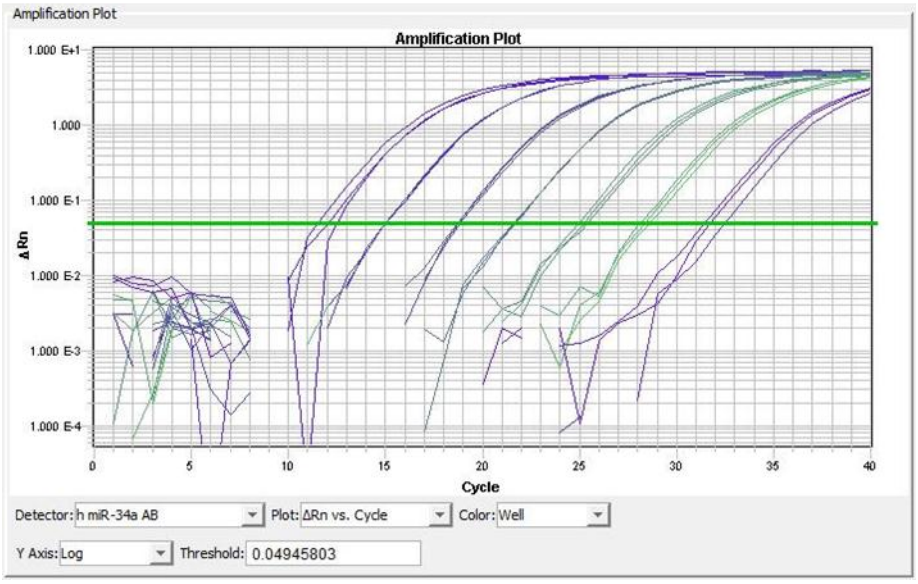


Figure 3-5 Double stranded microRNA mimics can be used to develop PCR standards for microRNA

microRNA standards were generated from double stranded microRNA mimics as described in the materials and methods. The figure shows a representative standard curve and amplification plot for 10^3 to 10^9 miR-34a copies / reaction.

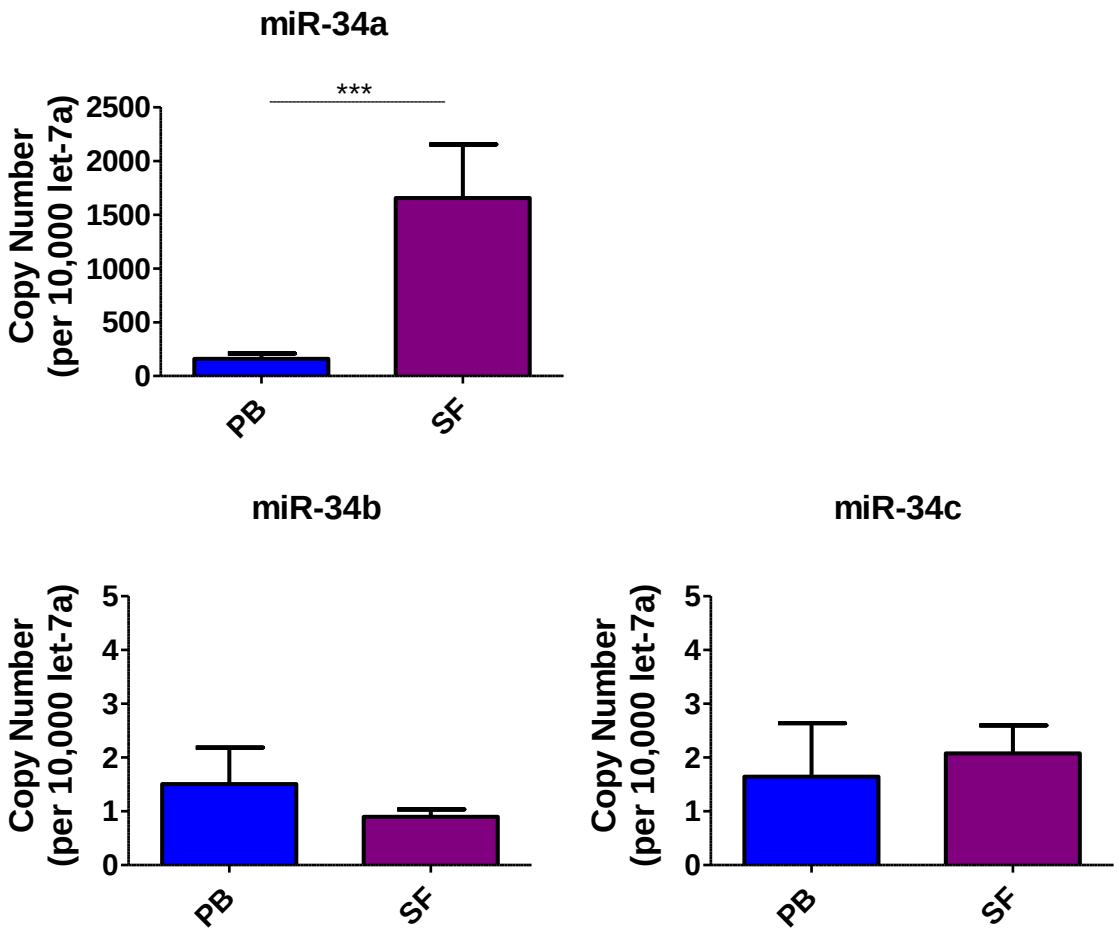


Figure 3-6 miR-34a, but not miRs 34b/c, is highly expressed in SF CD14$^+$ cells
CD14$^+$ cells were isolated from the peripheral blood (PB) and synovial fluid (SF) of RA patients, and PCR standards were used to determine the absolute copy number of microRNA-34a, b and c by TaqMan PCR. Data presented as mean plus standard error of the mean. Wilcoxon matched-pairs signed rank test; *** $P < 0.001$. $n = 11$.

3.2.2 **miR-34a expression in synovial tissues**

Next, we set out to determine whether microRNA-34a was differentially expressed in the synovial tissue of RA patients. To investigate this possibility, we performed *in situ* hybridisation for miR-34a in synovial tissues derived from RA patients and compared it to tissues from inflammatory (iOA) and non-inflammatory (niOA) osteoarthritis patients. This *in situ* staining protocol was originally worked up in the lab by Stefano Alivernini, who also performed a lot of the tissue staining. Figure 3-7 shows representative staining pictures for one RA (A) and one niOA (B) tissue stained using a miR-34a probe, as well as an RA tissue stained with a scramble microRNA probe (C). A staining score was developed based on the percentage of cells positive for miR-34a, and an average from four fields of view taken per section. As shown in Figure 3-8, RA tissues contain a significantly higher percentage of miR-34a positive cells compared to both iOA and niOA.

In order to establish whether any of these miR-34a positive cells were macrophages, we performed *in situ* staining for miR-34a, followed by fluorescent staining for CD68. The orange staining in Figure 3-9 demonstrates that a proportion of cells in the RA synovium are positive for both miR-34a and CD68, suggesting macrophages in the RA joint express miR-34a. Equally a significant number of cells were miR-34a positive but CD68 negative, and thus are likely not macrophages. The focus of my project, however, was the role of miR-34a in macrophage activation and for this reason I chose to continue to examine the biology of miR-34a in this lineage.

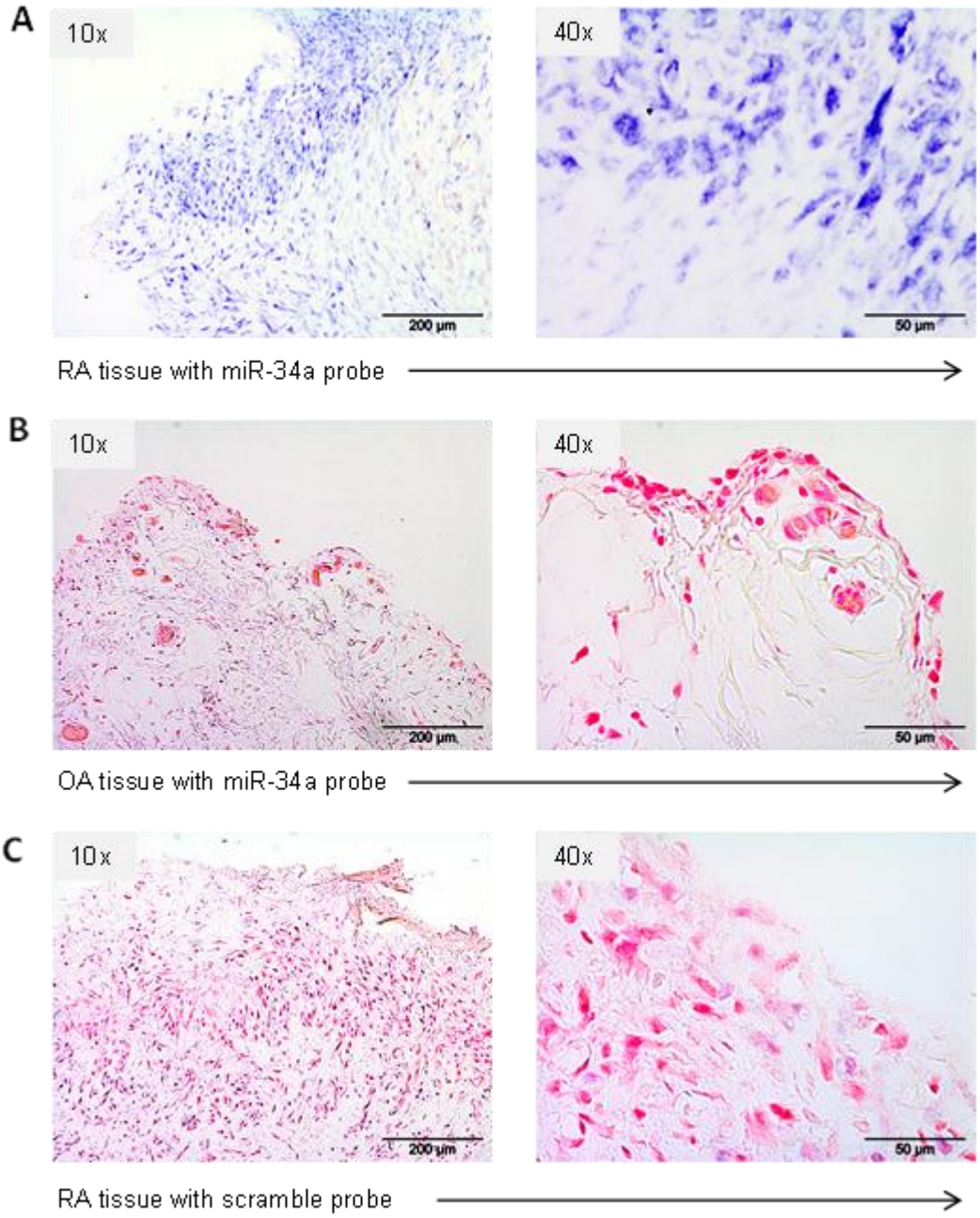
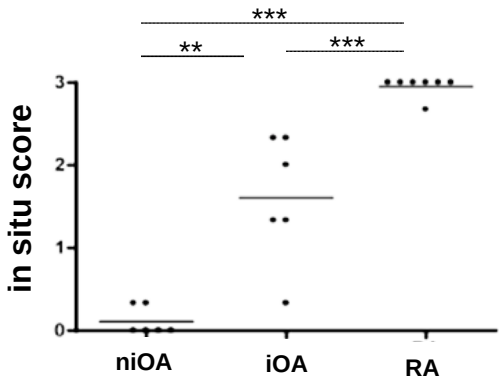


Figure 3-7 *In situ* hybridisation staining for miR-34a in RA and OA synovial tissues
Synovial tissues from rheumatoid arthritis (RA) and osteoarthritis (OA) tissues were stained for miR-34a expression using *in situ* hybridisation. Representative staining is shown here for one RA (A) and one OA (B) sample. Also shown is an RA tissue stained using a miRNA scramble probe (C). In the OA tissue and where the scramble probe was used, nuclear red was used for clarity of tissue integrity. For each tissue one 10x magnification and one 40x magnification image is shown (annotated on pictures).



STAINING SCORE

- **0**: no positive cells
- **1**: <10% positive cells
- **2**: 10-50% positive cells
- **3**: >50% positive cells

Figure 3-8 miR-34a *in situ* hybridisation score in RA and OA synovial tissues
Synovial tissues from rheumatoid arthritis (RA) and both non-inflammatory (niOA) and inflammatory (iOA) osteoarthritis tissues were stained for miR-34a expression using *in situ* hybridisation. The level of staining was quantified using the staining score shown. Each dot on the graph represents the average staining score given for a particular sample. Data points represent the average taken from four fields of view, line represents the average of all samples. One-way ANOVA with Bonferroni post test; ** $P < 0.01$, *** $P < 0.001$. n = 6/7 samples per group. Scoring of tissues performed by Stefano Alivernini.

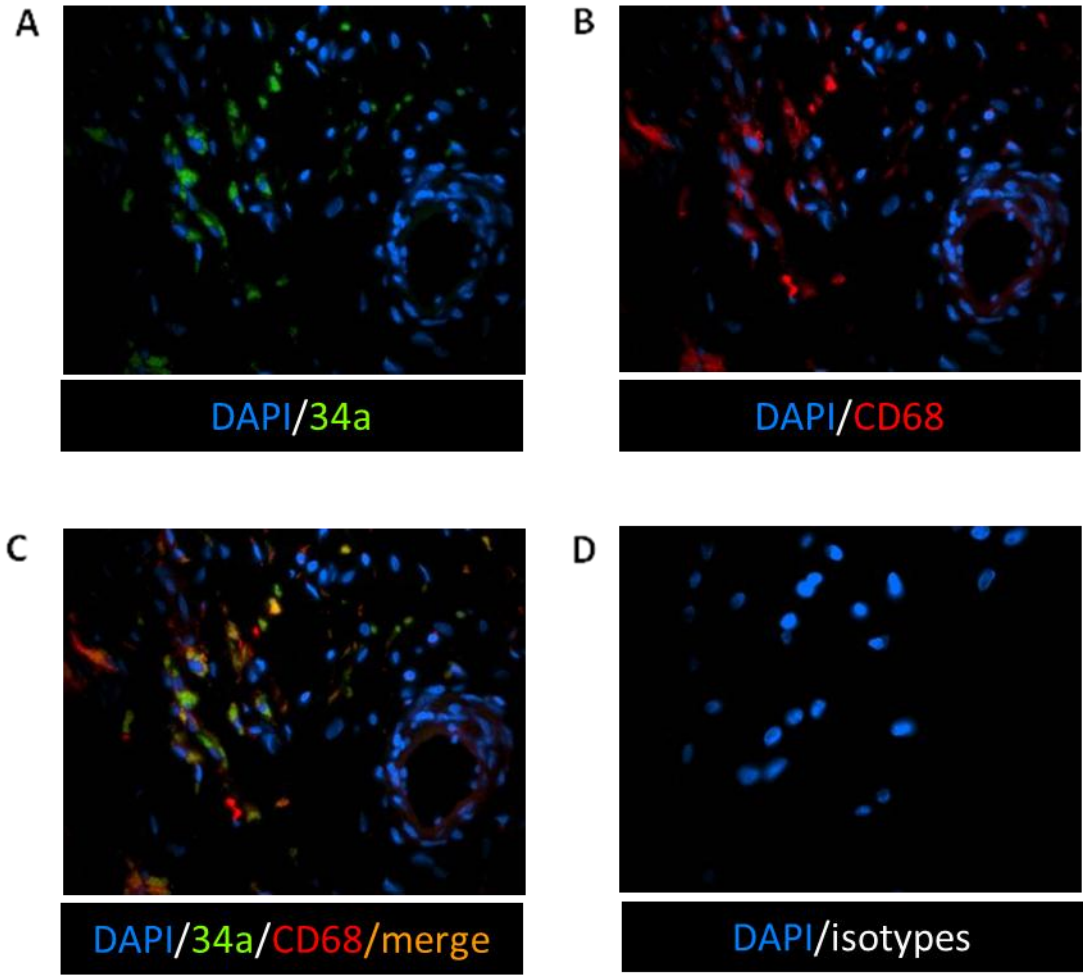


Figure 3-9 Double fluorescent staining shows macrophages in the RA synovium express miR-34a
Fluorescent *in situ* hybridisation staining for miR-34a (green) was performed, followed by fluorescent staining for CD68 (red). DAPI was also used to stain the nuclei (blue). The orange staining visible in figure (C) shows the overlap of red and green, representing miR-34a positive, CD68-expressing macrophages in the synovium.

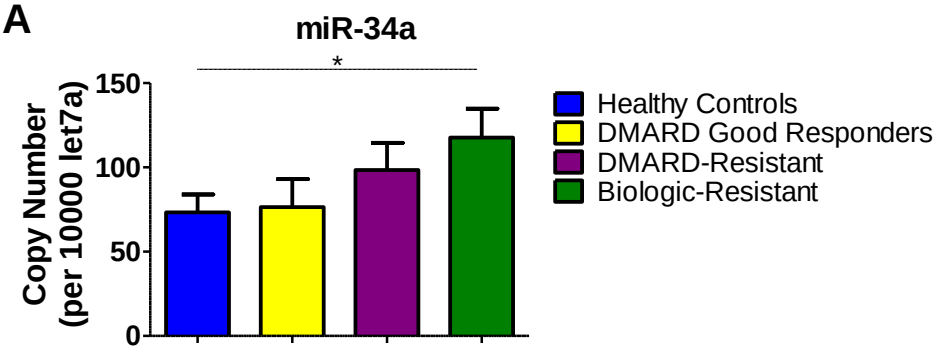
3.2.3 **miR-34a expression in peripheral blood CD14$^+$ cells**

We next wished to compare the expression of miR-34a in the peripheral blood compartment of RA patients versus healthy controls. To achieve this, cells were isolated from the blood of healthy controls, as well as three different RA patient subgroups: those responding well to cDMARD treatment (DMARD good responders), those currently not responding to cDMARDs (DMARD-resistant) and patients who had now failed multiple conventional and biologic therapies (biologic resistant). Taqman PCR was then used to quantify miR-34a expression. As shown in Figure 3-10A, those patients which were classed as "biologic-resistant" have significantly higher miR-34a expression in their PB CD14$^+$ cells compared to healthy controls. Figure 3-10B shows the patient demographics; although age and smoking status did vary between groups, these had no effect on miR-34a expression levels (Figure 3-10C and D, respectively).

We went on test whether miR-34a expression correlated with disease duration or other clinical markers of disease activity; RF titre (IU/ml), HS CRP (mg/dl), ESR, DAS28 ESR, tender joint count (TJC) and swollen joint count (SJC). These correlations are shown in Figure 3-11, and demonstrate that only the SJC of patients showed a positive correlation with miR-34a expression. Given multiple comparisons it is not clear whether this carries any significance, and future replication will be important. We also wanted to determine whether miR-34a expression was simply a marker of the degree of on-going inflammation. To test this we performed a luminex on the serum of all blood donors, and checked to see if any of the cytokines measured correlated with miR-34a expression. Figure 3-12 shows the correlations for cytokines that were detectable in the majority of samples. miR-34a expression did not correlate with the serum expression of IL-1RA, IL-2R, IL-7, IL-12, IFN-α, MCP-1, MIP-1β or MIG.

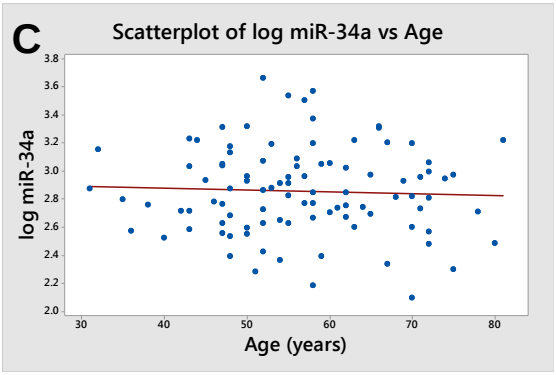
Thus, I have identified the differential expression of a microRNA in circulating monocytes in RA patients that suggests the state of disease in a cohort of multiple drug resistant patients may be altered, despite otherwise similar levels of intercurrent inflammation. If macrophages are a critical perpetuator cell lineage in RA then this would be compatible with the hypothesis that miR-34a dysregulation is associated with chronic synovial inflammation and that the peripheral monocyte pool is not 'normal'.

A



B

		Healthy Controls	DMARD Responders	DMARD Resistant	Biologic Resistant	P value
Number	n=	26	19	23	30	
Age (years)	Mean (StDev)	48.38 (7.74)	62.95 (10.55)	58.35 (10.19)	58.67 (10.45)	0.000
Gender	F:M	22:4	12:7	18:5	26:4	0.211
Height	Mean (StDev)	1.6350 0.0776	1.6389 0.1104	1.6691 0.0990	1.6276 0.0847	0.410
Weight	Mean (StDev)	73.73 (14.53)	75.47 (23.64)	80.30 (18.02)	68.39 (13.98)	0.106
BMI	Mean (StDev)	27.75 (6.10)	27.88 (7.85)	28.84 (6.08)	25.91 (5.72)	0.406
Smoking Status	Never:Ex: Smoker	18:3:5	10:5:4	6:8:9	22:4:4	0.025



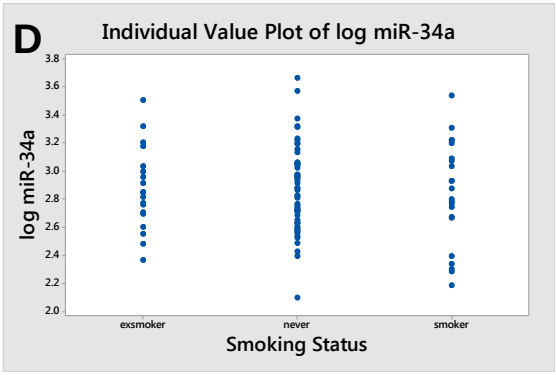


Figure 3-10 miR-34a is expressed higher in CD14$^+$ cells isolated from the peripheral blood of biologic-resistant RA patients compared to controls

miR-34a expression was measured in CD14$^+$ cells isolated from the peripheral blood of healthy controls and three different RA patient subgroups: those responding well to cDMARD treatment (DMARD good responders), those currently not responding to cDMARDs (DMARD-resistant) and patients who had now failed multiple conventional and biologic therapies (biologic resistant). miR-34a expression is shown in panel A. Data shown as mean plus standard error of the mean. Kruskal-Wallis test with Dunn's multiple comparison; * $P < 0.05$. n = 19 – 30 / group. (B) Patient demographics show age and smoking status were not comparable between the groups (P value highlighted by a red circle on the table), but these do not influence miR-34a expression levels, (C) and (D) respectively.

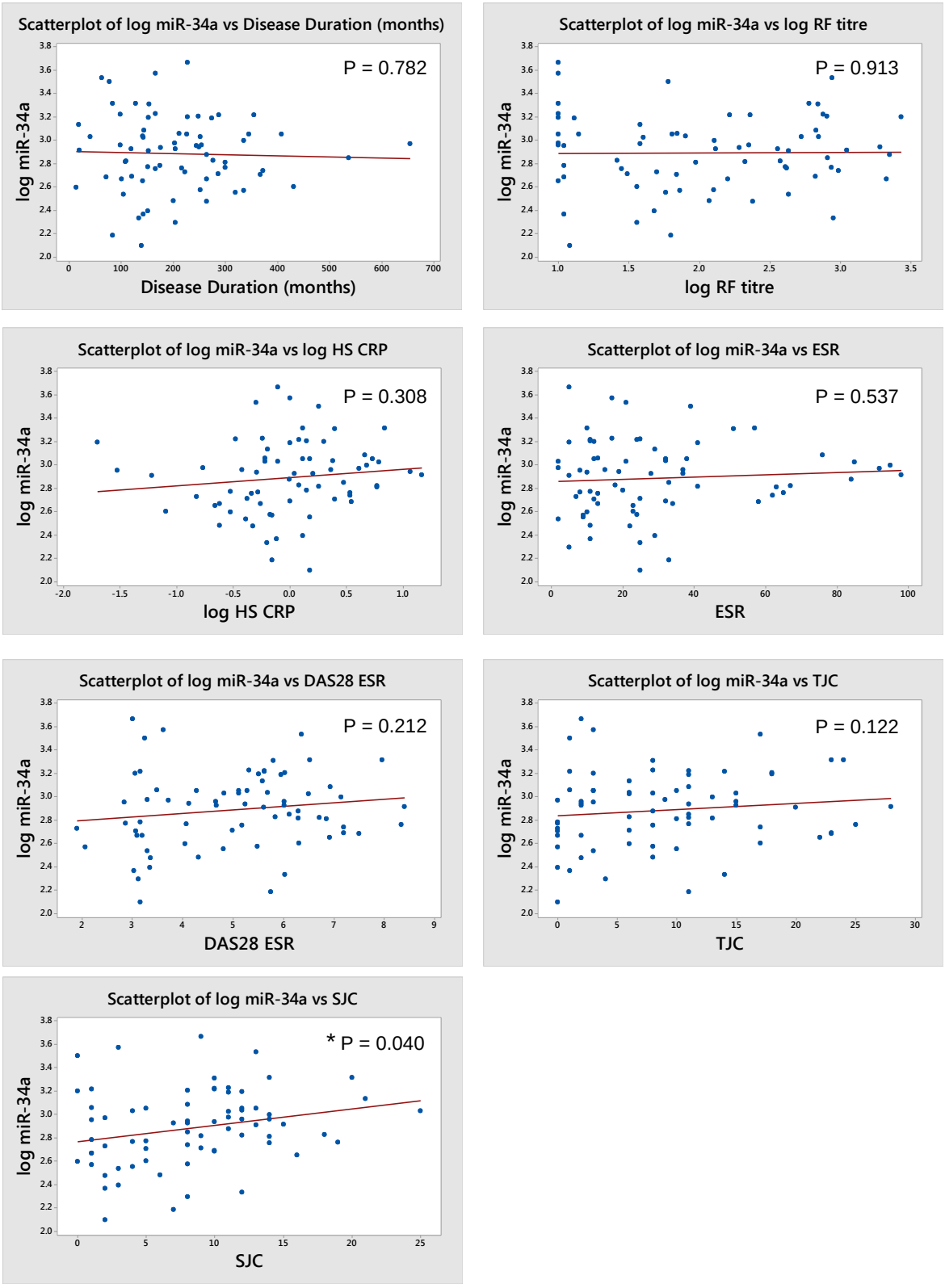


Figure 3-11 miR-34a expression correlated with clinical measures of disease
$CD14^+$ cells were isolated from the blood of RA patients and miR-34a expression measured by PCR. Correlations were then performed to determine whether increased miR-34a expression could be influenced by disease state. Scatterplots are shown for miR-34a expression (raw data was per 10,000 let-7a) against disease duration or other clinical markers of disease activity; rheumatoid factor (RF) titre (IU/ml), HS CRP (mg/dl), ESR, DAS28 ESR, tender joint count (TJC) and swollen joint count (SJC).

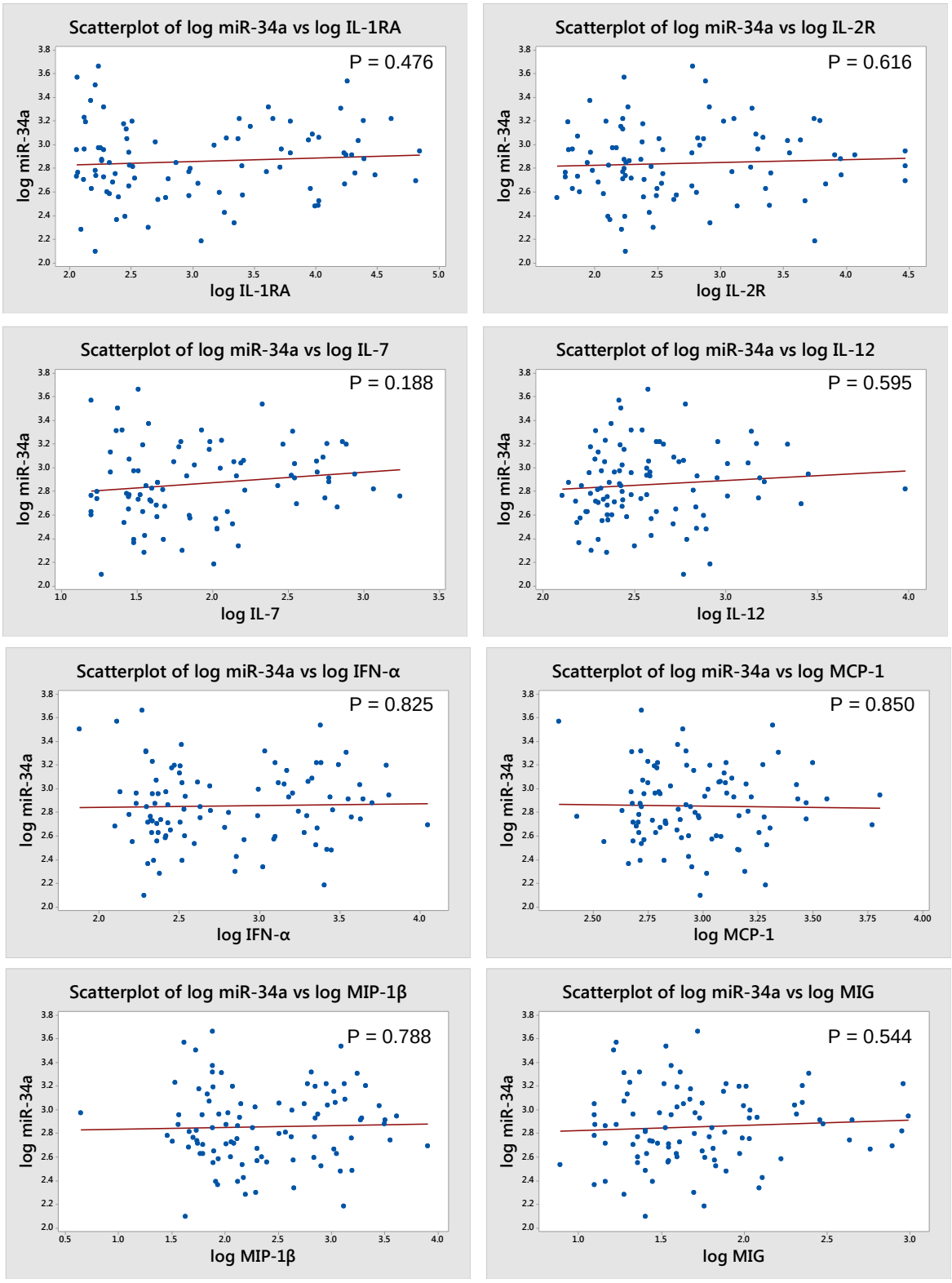


Figure 3-12 miR-34a expression correlated with serum cytokine levels
$CD14^+$ cells were isolated from the blood of RA patients and miR-34a expression measured by PCR. From the same samples, serum was stored for luminex analysis to measure cytokine expression. Correlations were then performed to determine whether increased miR-34a expression could be influenced by the general peripheral inflammatory state. Scatterplots are shown here for miR-34a expression (raw data was per 10,000 let-7a) against IL-1RA, IL-2R, IL-7, IL-12, IFN-α, MCP-1, MIP-1β and MIG.

3.2.4 **miR-34a expression in monocyte subsets**

When measuring miR-34a expression in the PB of RA patients and healthy controls, it would have been useful to further phenotype the $CD14^+$ cells. It is already well documented that monocytes from the peripheral blood of RA patients show clear signs of activation. For example, they produce increased quantities of cytokines such as IL-1 and IL-6 [347, 348], and a greater proportion of $CD14^+$ cells from RA patients also express CD16 [219, 349]; with more recent data suggesting they also have increased expression of CD56 [350]. This population of $CD14^+$ cells produce more TNF-α and IL-23 compared to that of $CD56^-$ monocytes, so we wished to check if different monocyte populations expressed different levels of miR-34a – which could be in part responsible for the differential miR-34a expression seen in RA patients. To do this we obtained healthy peripheral blood samples, and sorted the monocytes on an Aria FACS machine. Figure 3-13 shows the gating strategy used, which was based on that used by Cros *et al* [204]. Live, single cells were gated based on HLA-DR positivity, and being negative for other cellular markers – namely CD2, CD15, CD19, CD56 and NKp46. The remaining cells were gated into the three major monocyte populations based on their expression of CD14 and CD16 – $CD14^{hi}$ $CD16^-$, $CD14^{hi}$ $CD16^+$ and $CD14^{dim}$ $CD16^+$. The miR-34a expression of these cells was then measured by Taqman PCR, and showed no significant differences between the expression of miR-34a in these cell subsets.

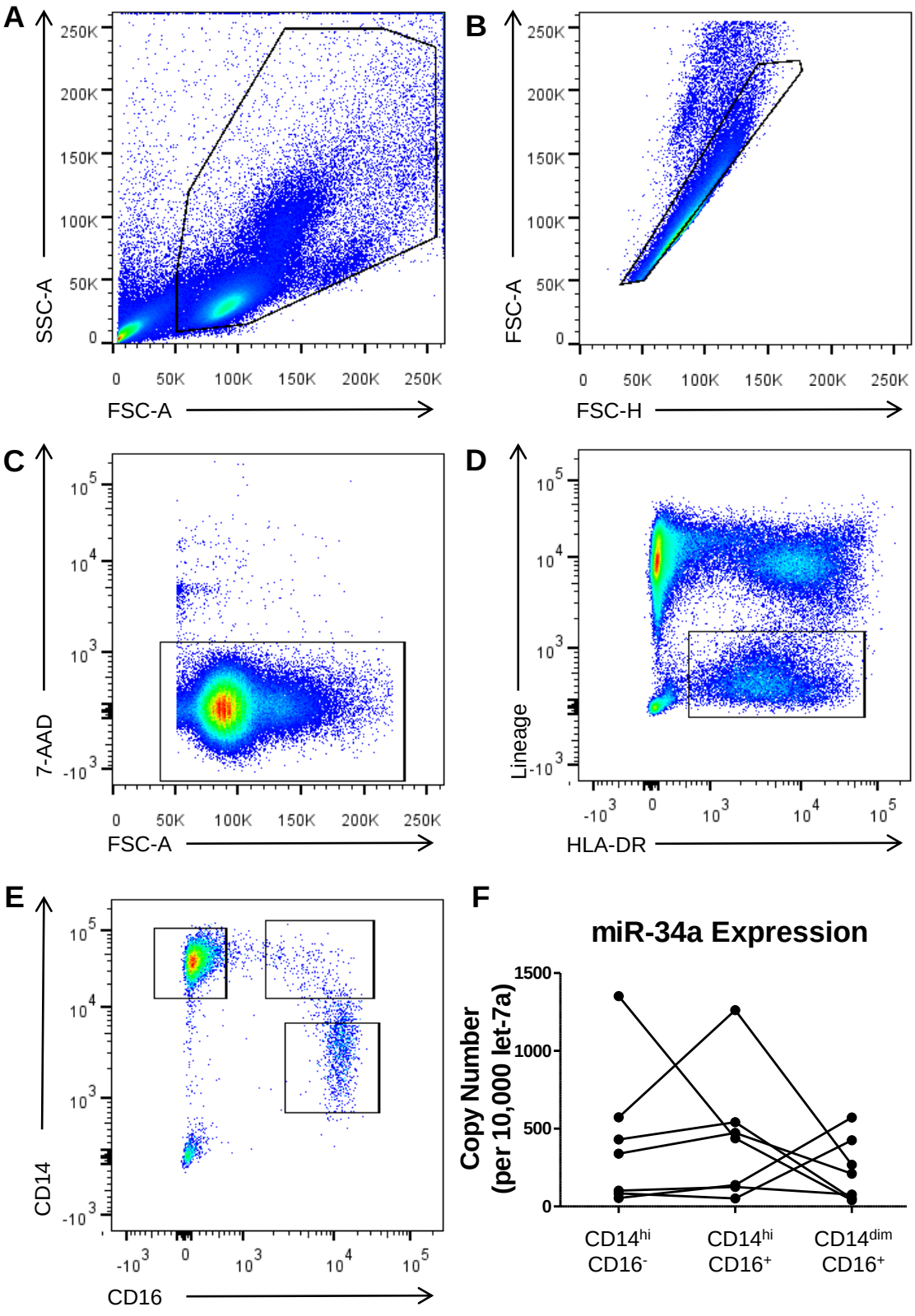


Figure 3-13 Gating strategy and miR-34a expression in sorted monocyte populations from healthy peripheral blood

PBMCs were isolated from healthy blood donors and stained for FACS. Cells were first gated on forward / side scatter (A), before doublets (B) and dead cells (C) were excluded. Next, cells were gated for HLA-DR positivity, and negative for other lineage markers (CD2, CD15, CD19, CD56 and NKp46) (D). The three major monocyte populations were then sorted based on their expression of CD14 and CD16 – $CD14^{hi}$ $CD16^{-}$, $CD14^{hi}$ $CD16^{+}$ and $CD14^{dim}$ $CD16^{+}$ (E). RNA was extracted from these cells and PCR used to quantify miR-34a expression (F). Data points represent the miR-34a expression of a single healthy donor; data points from the same donor are linked by a line.

3.3 **Regulation of microRNA-34a expression**

Having established miR-34a up-regulation in monocytes and macrophages in both the joint and periphery of RA patients, we next wished to determine what the possible causes of this up-regulation could be. First, we wanted to determine whether the basal maturation state could have an effect on miR-34a expression. In order to evaluate this, miR-34a expression was measured by PCR in $CD14^+$ cells freshly isolated from buffy coats, and compared to the levels seen after 3 and 7 days M-CSF or GM-CSF maturation (50 ng/ml). As seen in Figure 3-14, miR-34a expression was up-regulated during maturation with M-CSF, and also to a lesser extent with GM-CSF. In order to attain a more accurate time scale for this up-regulation, cells were cultured with M-CSF and miR-34a expression checked at the earlier time points of 2, 6 and 24 hours. As seen in Figure 3-14C, there was no change in expression up until 6 hours, with a trend towards increased expression seen after 24 hours. Significant up-regulation of miR-34a, however, was not seen until the 72 hour time point, suggesting that there are indirect regulatory steps required to drive its expression.

Due to the constraints of working with primary cells, particularly in terms of manipulation of gene expression, I next wished to assess whether a monocytic cell line (THP-1 cells) could be used to investigate miR-34a regulation. Firstly, we wanted to establish whether these cells also up-regulate miR-34a during maturation, so cultured them in M-CSF for 3 or 7 days. As seen in Figure 3-15A, no change in miR-34a expression was observed across the time points. Next, we used the well-established method of maturing THP-1 cells with PMA, but again saw no increase in miR-34a expression (Figure 3-15B), whereas differences were observed in another maturation induced microRNA – miR-22 (Figure 3-15C). These experiments established that only primary cells would permit addressing the questions of relevance, and I proceeded with these alone.

We hypothesised that the increased relative expression of miR-34a seen in SF $CD14^+$ cells was due to exposure of monocytes to the inflammatory microenvironment in the inflamed synovium, leading to maturation and presumably also an enhanced activation state. In order to test this, $CD14^+$ cells isolated from buffy coats were cultured for 24 hours in media alone, or with 10% SF extracted from 6 different RA patients. As shown in Figure 3-16A, when miR-

34a levels were averaged across the different SF cultures, a trend towards higher miR-34a expression was seen, but when the expression was measured individually for each SF (Figure 3-16B) it is obvious that not all synovial fluids stimulate monocyte miRNA expression in the same way. This likely reflected the distinctive composition of individual patient derived SFs, and in this sense was unsurprising.

We next wished to determine if stimulation of monocytes or macrophages could alter miR-34a expression. In order to establish this we took $CD14^+$ cells isolated from buffy coats and stimulated them with various TLR ligands. We also performed the same stimulations with cells which had been matured with M-CSF for 7 days prior to addition of the stimuli. Cells were stimulated for 2, 6 and 24 hours, and miR-34a expression was later measured by Taqman PCR. The supernatants from the 24 hour time point were also taken to establish TNF-α levels by ELISA as a measure of successful stimulation. As shown in Figure 3-17, no difference was seen in the miR-34a expression of stimulated monocytes (A) or M-CSF-matured macrophages (C) although cells displayed increased TNF-α production (B – monocytes, D - M-CSF matured macrophages). We therefore concluded that stimulation of myeloid cells by endogenous TLR ligands in the RA synovium was unlikely to be the cause of their increased miR-34a expression.

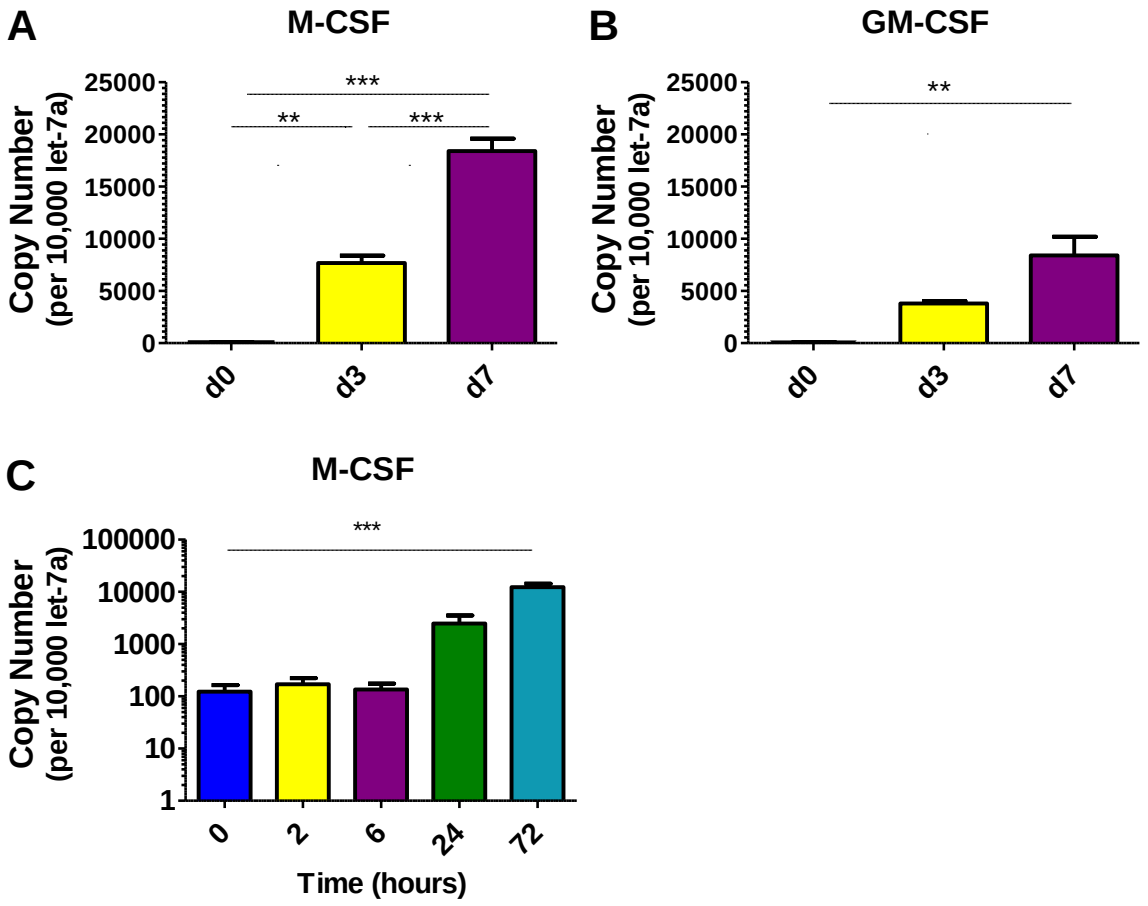


Figure 3-14 miR-34a expression is increased in CD14$^+$ cells from buffy coats during M-CSF and GM-CSF maturation

To determine the effect of maturation on miR expression, miR-34a was measured in freshly isolated CD14$^+$ cells (d0), and compared to cells after 3 or 7 days maturation with M-CSF or GM-CSF (50 ng/ml) – panel A and panel B, respectively. In order to determine a more precise timescale for miR-34a up-regulation, cells were cultured with M-CSF and taken off at earlier time points. Data shown as mean plus standard error of the mean. n = 4, Repeated Measures ANOVA with Bonferroni's multiple comparison test; ** $p < 0.01$, *** $p < 0.001$.

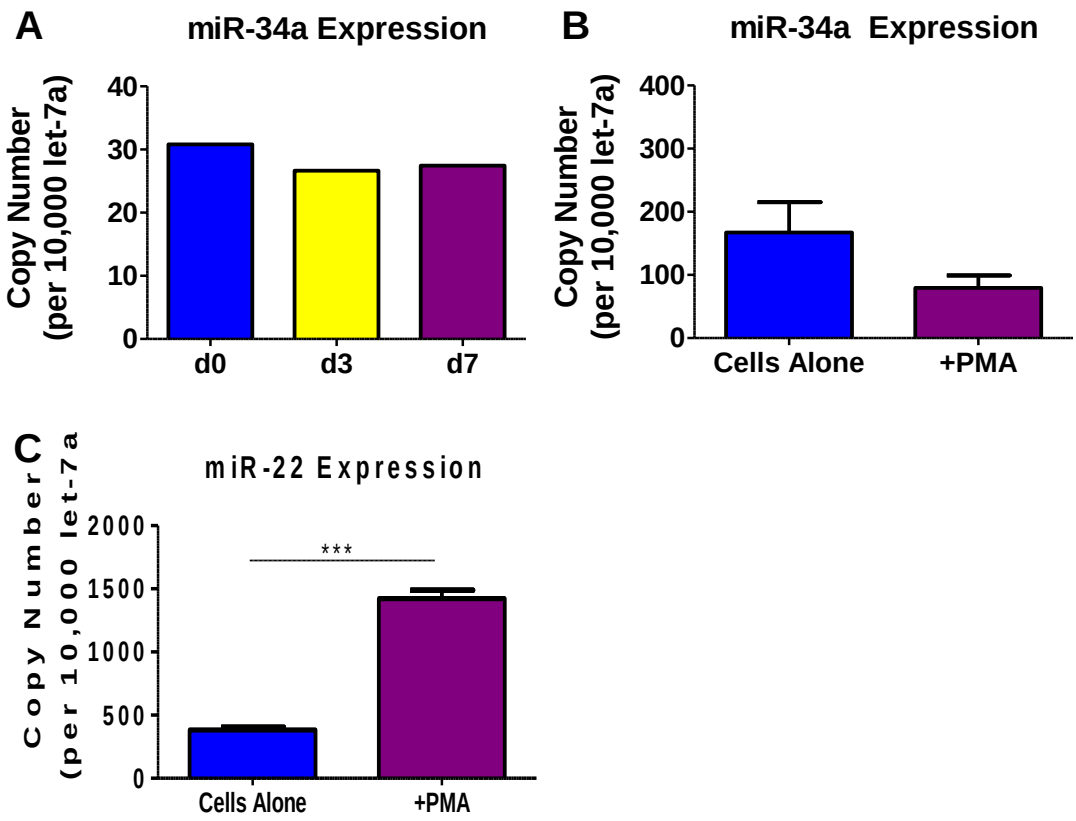


Figure 3-15 miR-34a expression is not up-regulated by THP-1 cells during maturation
miR-34a expression was measured after culture of THP-1 cells with 50 ng/ml M-CSF (A - n = 2 independent experiments) and after 100 ng/ml PMA had been used for 3 days to induce differentiation and adherence (B). Data are also shown for the expression of miR-22 – another microRNA known to be regulated by maturation (C). Data presented as mean plus standard error of the mean. Paired T test; *** $P < 0.001$.

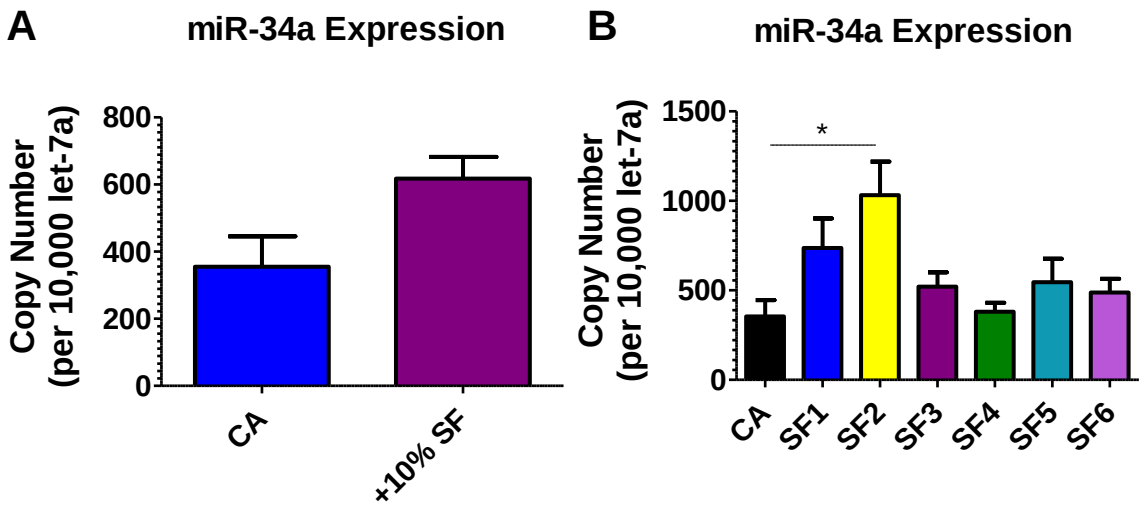


Figure 3-16 miR-34a is up-regulated after culture of CD14$^+$ cells with some synovial fluid samples

CD14$^+$ cells were isolated from buffy coats and cultured alone (CA) or with 10% synovial fluid derived from 6 different RA patients (SF1-6). After 24 hours miR-34a expression was measured by PCR. Panel A represents data where the average of the 6 SF cultures was taken, while panel B shows the data for each individual SF separately. Data shown as mean plus standard error of the mean. Kruskal-Wallis test with Dunn's multiple comparison; * $P < 0.05$.

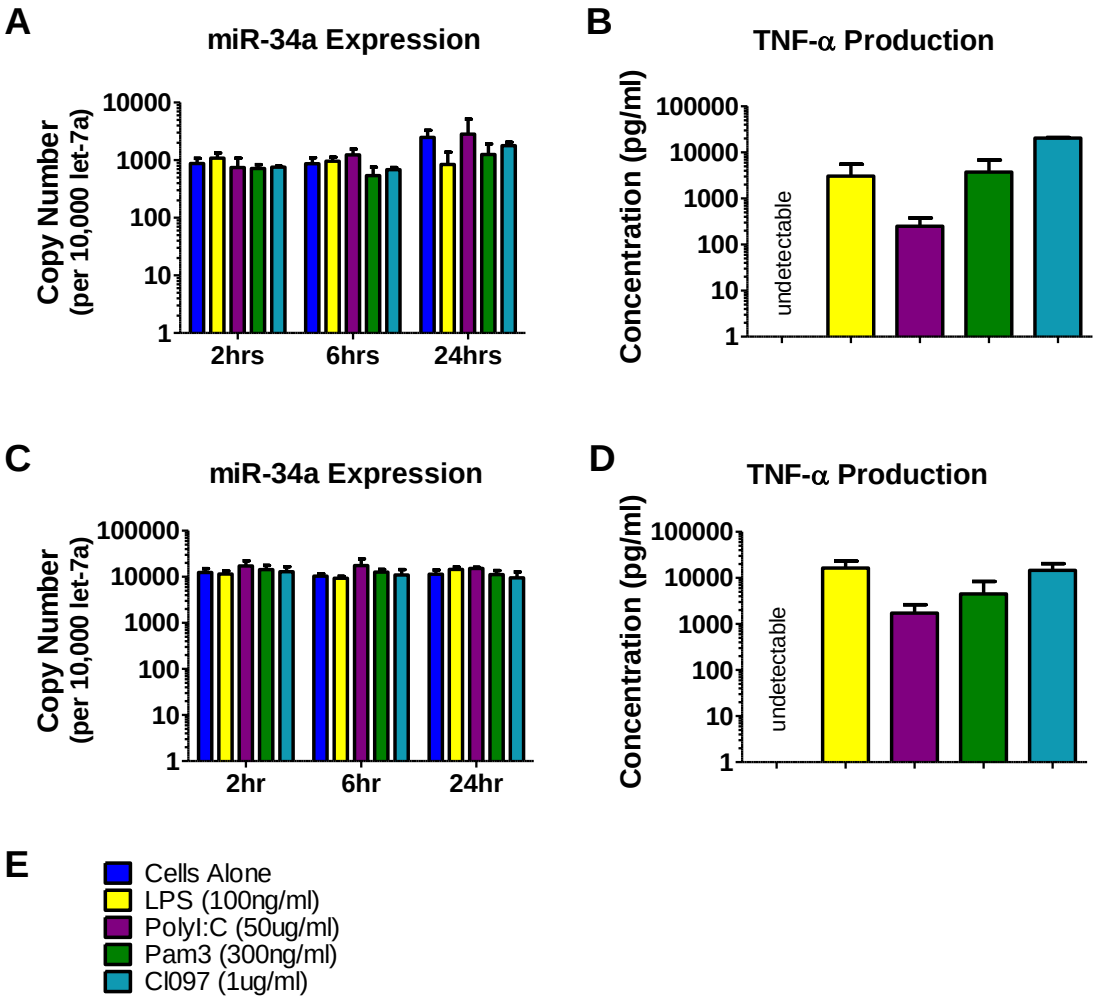


Figure 3-17 miR-34a expression is not regulated by TLR stimulation
$CD14^+$ cells were isolated from peripheral blood samples and stimulated with various TLR ligands for 24 hours before miR-34a expression was measured by qPCR (A). As a measure of activation, TNF-α levels were measured in the supernatants after the 24 hour stimulation by ELISA (B). Cells were also M-CSF matured for 7 days prior to stimulations (C), and again TNF-α production after the 24 hour stimulation used to confirm successful stimulation (D). Shown in (E) is the colour code used for all graphs. Data shown as mean plus standard error of the mean. n = 3.

3.4 **Discussion**

Previous data from our laboratory suggested that miR-34a was increased in $CD14^+$ monocytes isolated from the SF of RA patients compared to matched PB cells. Therefore, this study set out to confirm this observation, and having achieved this, determine whether miR-34a was differentially expressed in RA samples compared to controls. Another key part of this investigation was to determine the potential factors driving this dysregulated miR-34a expression.

My first key observation is that synovial fluid and tissue macrophages do indeed express miR-34a. Moreover, circulating peripheral blood monocytes from biologic-resistant RA patients express higher levels of microRNA-34a than were observed in matched healthy controls. These data have some fundamental implications. The key biologic activities of miR-34a thus far include the regulation of cell cycle, which proved to be important in miR-34a anti-oncogene activity [351]; and it is also associated with accelerated cell aging [336, 352]. The finding that miR-34a is indeed elevated in the context of synovial tissue in RA therefore suggests this as a novel pathway in RA, potentially regulating the chronic contribution of macrophages to pathology.

There were however some obvious limitations to this part of my study. I was hindered by the inability to obtain certain samples from healthy tissue controls. Healthy controls could be used when looking at microRNA expression in peripheral blood samples, but when looking at synovial fluid cells or synovial tissue this was not possible, and appropriate controls had to be determined depending on the given sample type. To look at sites of inflammation in the joint, although not ideal, we considered the best control to be patients with non-inflammatory and inflammatory joint disease (as determined by H&E staining), as these tissues were easily accessible in the lab. Thus, our interpretation cannot truly demonstrate disease specificity. That said the presence of this pathway in itself is pathogenetically informative. In particular, we recognise the challenges inherent in using OA as a control – for this reason I have chosen to segregate these tissues into inflammatory and non-inflammatory subgroups based on histological appearances. An intriguing side observation is therefore that in inflamed OA tissues the presence of miR-34a may have

significance – this is outwith the scope of my thesis but will be followed up by the host laboratory in due course with Dr Carl Goodyear and Prof William Ferrell.

The increased miR-34a expression seen in cells isolated from the peripheral blood of drug-resistant patients is of particular interest, as it suggests the microRNAs increased expression is not solely due to activation and maturation at the sites of synovial inflammation. This said, it is well documented that PB monocytes are primed or activated in RA, but the similar miR-34a expression seen in the monocyte populations sorted from peripheral blood also suggests miR-34a expression in monocytes is not simply a result of maturation status. A study looking at microRNA expression in early RA patients would be useful to determine whether these patients have constitutively higher miR-34a expression, which could be used as a potential biomarker of non-response to therapy, or whether miR-34a levels change in response to the on-going, unopposed inflammatory activity in these patients.

The fact that miR-34a does not correlate with any peripheral measures of inflammation, but does correlate with the SJC of patients is also interesting. Swollen joints are one of the most characteristic features of RA, and the number of swollen joints is directly linked to disease severity. This could suggest that miR-34a expression in PB $CD14^{+}$ cells is altered in response to localised inflammation in the joint, as opposed to the overall cumulative systemic inflammatory state. This said, we were concerned about statistical errors introduced by multiple comparison testing – so further validatory analyses would be helpful in this regard. Potentially this could be performed in the PEAC consortium cohort of which Glasgow is a part. An alternate view is that the elevated levels of miR-34a in circulating monocytes of patients with drug-resistant disease are a feature of the chronicity in such patients, rather than intercurrent disease activity. This in one sense makes miR-34a more interesting as a molecular factor that could play a fundamental role in maintaining the cellular events driven by monocytes.

The increase in miR-34a expression seen during M-CSF maturation, and lesser increase in GM-CSF matured cells is intriguing. This suggests that miR-34a may play a pivotal role in the differentiation and activation of different subtypes of macrophages, and potentially dendritic cells. The latter is currently being

studied by others in the lab, so I focused on dissecting the role of miR-34a in the biology of M-CSF driven macrophages.

The increase in miR-34a expression seen in biologic resistant patients, as well as the increase in healthy cells cultured in SF suggests that exposure to inflammatory stimuli could result in increased miR-34a expression, although the lack of response to TLR ligands could suggest otherwise. It has previously been suggested that LPS stimulation actually down-regulates miR-34a expression in macrophages, although these experiments were performed in RAW264.7 cells [353], and a subsequent study on human monocyte derived macrophages also failed to find a significant difference compared to controls [354]. Alternatively, M-CSF and / or GM-CSF derived from the sites of inflammation may be responsible for the changes seen in miR-34a expression in some RA patients, and in the healthy donor monocytes cultured with SF.

This study, to my knowledge, provides the first evidence that miR-34a is up-regulated in monocytes and macrophages in both the periphery and sites of inflammation in RA. Future experiments will therefore focus on what affect altering miR-34a expression has on monocytes and macrophages, as unravelling the pathways regulated by miR-34a in these cells could give clues to why it is differentially expressed in RA.

Chapter 4 – Investigating the functional and phenotypic consequence of miR-34a manipulation in macrophages

4.1 **Introduction and Aims**

Having established in the previous chapter that miR-34a expression is increased in peripheral blood monocytes and synovial macrophages in RA, and knowing it is also up-regulated during monocyte to macrophage differentiation, we next set out to determine the possible functional role(s) of miR-34a in macrophage biology. The primary roles of macrophages include production of a wide range of cytokines, reactive oxygen and nitrogen intermediates and also phagocytosis of cellular debris, microorganisms and infected, damaged or antibody-coated cells. RA is characterised by the chronic dysregulated activation of macrophages, such that they could contribute to pathogenesis by several or all of these mechanisms. We therefore wished to investigate whether altering miR-34a expression could affect the ability of macrophages to produce cytokines in response to TLR ligands, or change their expression of various surface markers involved in macrophage activation and the promotion of phagocytosis.

4.2 **Effect of miR-34a manipulation on cytokine output**

In order to assess the effect of altering miR-34a levels on the cytokine output of macrophages, $CD14^+$ cells were first isolated from buffy coats and then M-CSF-matured for 7 days. These cells were then transfected with a control mimic, miR-34a mimic, control inhibitor or miR-34a inhibitor for 24 hours prior to stimulation with 10 ng/ml LPS for a further 24 hours. The microRNA mimic used here is transfected as a double-stranded RNA molecule, where exclusion of the passenger strand (through proprietary mechanisms undisclosed by the company) ensures that only the strand of interest is active. This is intended to "mimic" native microRNAs, and allow overexpression of microRNA to aid the discovery of potential functional roles of individual microRNA. The microRNA inhibitors are single-stranded RNA molecules, which bind and inhibit endogenous microRNA, thereby revealing any consequence of the microRNAs down-regulation / absence.

We initially measured only the TNF-α production by the transfected and stimulated macrophages, as this cytokine is known to play a key role in the pathogenesis of RA; moreover it is known to be primarily produced by macrophages [235, 355]. TNF-α is also thought to have a hierarchical role in the RA cytokine milieu, illustrated by the fact that treatment of cultures of synovial cells from RA patients with anti-TNF therapy reduces the levels of IL-1, IL-6, IL-8 and GM-CSF synthesis [62, 356]. It is also known that TNF-inhibitors represent some of the most successful biologic therapies currently available in the treatment of RA.

The TNF-α concentration in the supernatants of transfected and stimulated cells was measured by ELISA. As demonstrated in Figure 4-1, cells transfected with miR-34a mimic produced significantly less TNF-α when compared to control mimic transfected cells. miR-34a inhibition, however, had no effect on the measured TNF-α concentration. In order to determine whether the over and under-expression of miR-34a was successful, PCR was performed to measure miR-34a expression in the transfected cells. As seen in Figure 4-1C, transfection of cells with a miR-34a mimic results in the desired increase in miR-34a expression. Transfection of day 7 M-CSF matured cells with a miR-34a inhibitor, however, produced no significant change in miR-34a expression detectable by

PCR. We therefore concluded that miR-34a inhibitors had been unsuccessful in these cells, and decided to explore other approaches to miR-34a inhibition.

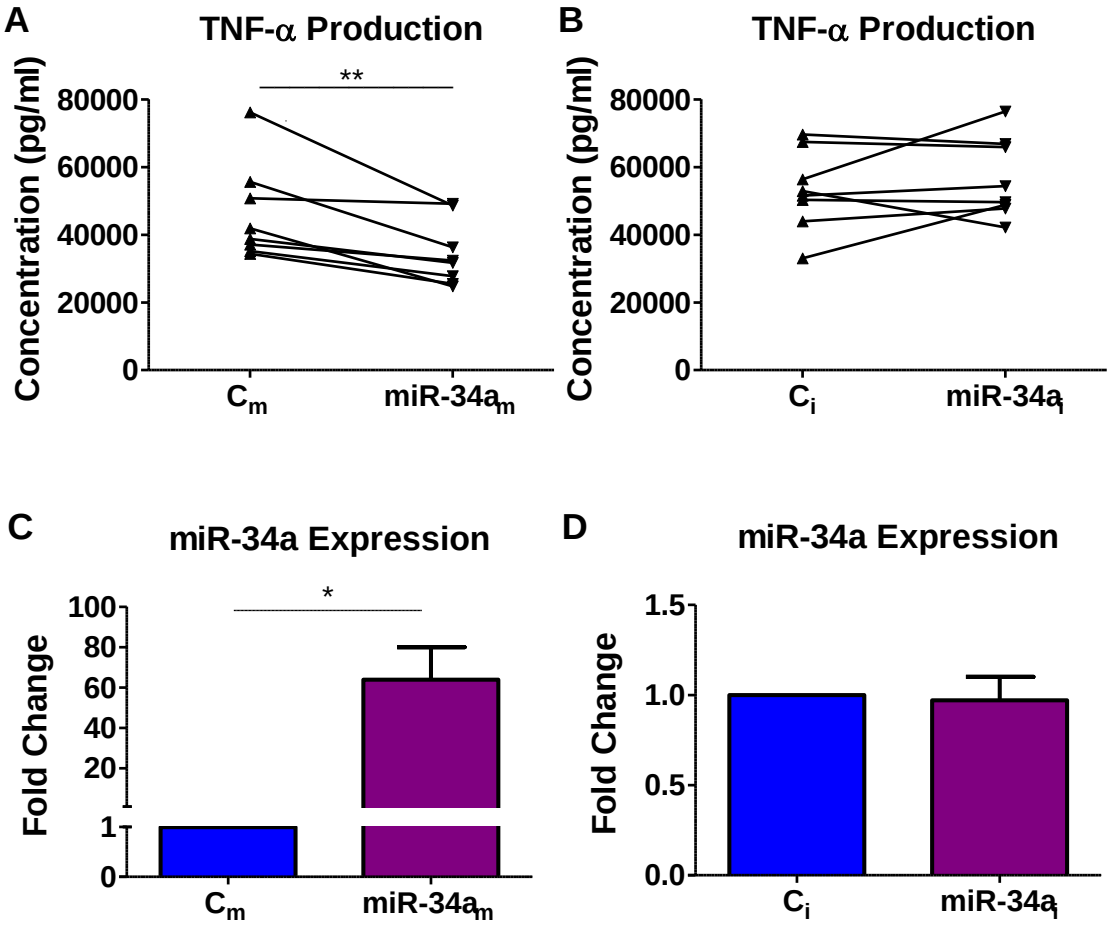


Figure 4-1 Effect of miR-34a manipulation on TNF-α production by 7 day M-CSF-matured macrophages

$CD14^+$ cells were isolated from buffy coat samples and matured with 50 ng/ml M-CSF for 7 days. These cells were then transfected with 25 nM control (C_m) or miR-34a (miR-34a_m) mimics or inhibitors (C_i or miR-34a_i) 24 hours before stimulation with 10 ng/ml LPS for a further 24 hours. The TNF-α concentration in the supernatants of transfected and stimulated cells was then measured by ELISA. Panel A shows the TNF-α concentration of cells transfected with C_m or miR-34a_m, while panel B shows the TNF-α concentration of cells transfected with C_i or miR-34a_i. Shown in panels C and D is the miR-34a expression measured by PCR after transfection with control and miR-34a mimics and inhibitors, respectively. The miR-34a expression of control mimic or control inhibitor transfected cells was normalised to 1, with the other conditions being expressed as fold change. Data shown as mean plus standard error of the mean. Paired t test; * $P < 0.05$. ** $P < 0.01$, $n = 8$.

In chapter 3 we demonstrated that miR-34a expression increases significantly during the M-CSF maturation of $CD14^+$ cells. For this reason we hypothesised that the miR-34a levels achieved after 7 days maturation in M-CSF were too high, and our inhibitor could not effectively reduce its expression at the manufacturer's suggested concentration. As $CD14^+$ cells already achieve an increased level of miR-34a and an adherent macrophage-like morphology after 3 days in M-CSF, and previous experiments in our group have shown their cytokine response to culture with RA synovial fluid or cytokine-activated T cells is equivalent to that achieved by cells cultured in M-CSF for 7 days (Jagtar Nijjar, PhD thesis), we decided to try using miR-34a mimics and inhibitors in 3 day M-CSF-matured cells. As previously, $CD14^+$ cells were isolated from buffy coats, but this time they were M-CSF-matured for 3 days prior to 24 hour transfection with control or miR-34a mimics or inhibitors. RNA was isolated from these cells and the miR-34a expression determined by PCR. As shown in Figure 4-2, miR-34a was significantly increased in miR-34a mimic transfected cells, and this time significant down-regulation of endogenous miR-34a was also observed with the use of miR-34a inhibitors.

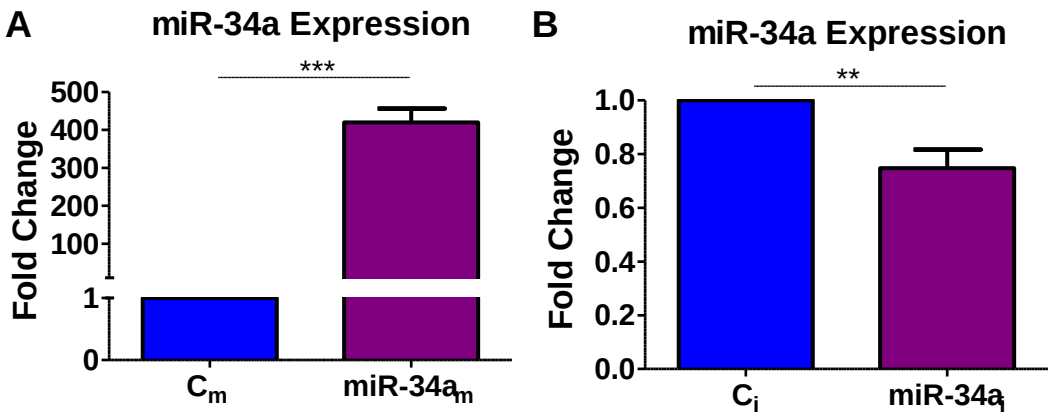


Figure 4-2 miR-34a expression of 3 day MCSF-matured macrophages after transfection with miR-34a mimics and inhibitors

$CD14^+$ cells were isolated from buffy coat samples and matured with 50 ng/ml M-CSF for 3 days. These cells were then transfected with 25 nM control (C_m) or miR-34a ($miR\text{-}34a_m$) mimics or inhibitors (C_i or $miR\text{-}34a_i$) for 24 hours. The miR-34a expression of mimic and inhibitor transfected cells are shown in panel A and B, respectively. For each donor the control mimic or inhibitor expression was normalised to 1, with the other conditions being expressed as fold change. Data shown as mean plus standard error of the mean. Paired t test; ** $p < 0.01$, *** $p < 0.001$. $n = 8$.

We therefore decided to use this transfection scheme in all further experiments. Accordingly, these 3 day M-CSF-matured cells were left in transfection reagents for 24 hours, and then stimulated for 24 hours with the addition of TLR ligands. We chose two stimuli, namely LPS and CL097, to demonstrate the effects of both extracellular and intracellular stimuli on macrophage cytokine production, and also because these two TLRs can utilise different signal transduction pathways to mediate their effects.

Figure 4-3A and Figure 4-3B show the TNF-α concentration after stimulation with 1 or 10 ng/ml LPS, respectively. In both instances, significant down-regulation of TNF-α was observed with miR-34a over-expression, and a significant up-regulation observed upon miR-34a inhibition. As human cytokine production is highly variable from donor to donor, we also normalised the data so that the control (mimic or inhibitor) for each donor was set to 100%, with the experimental condition shown as a percentage. Both the raw data calculation and percentage change from control were similar between the 1 and 10 ng/ml LPS stimulation conditions. LPS stimulation of miR-34a over-expressing cells resulted in a mean (SEM) reduction of 18% (±3.89%) in TNF-α production, while in miR-34a inhibited cells an increase of 45% (±7.79%) TNF-α production was observed.

miR-34a mimic and inhibitor transfected cells were also stimulated with the TLR7/8 ligand Cl097. Cells were transfected using the aforementioned conditions, then stimulated with 1 μg/ml Cl097 for a further 24 hours. **Figure 4-4**A shows the TNF-α concentration in the supernatants as measured by ELISA. As with the LPS-stimulated cells, Cl097-stimulated cells demonstrate a significant down-regulation of TNF-α with miR-34a over-expression, and a significant up-regulation with miR-34a inhibition. Again data are also displayed as percentage change. Cl097 stimulation exhibited 24% (±11.03%) reduction in TNF-α production following miR-34a over-expression (**Figure 4-4**B), and a 68% (±18.75%) increase in TNF-α production following miR-34a inhibition (**Figure 4-4**C). These data demonstrate that miR-34a has a likely role in the negative regulation of TNF-α production in response to both LPS and Cl097 stimulation.

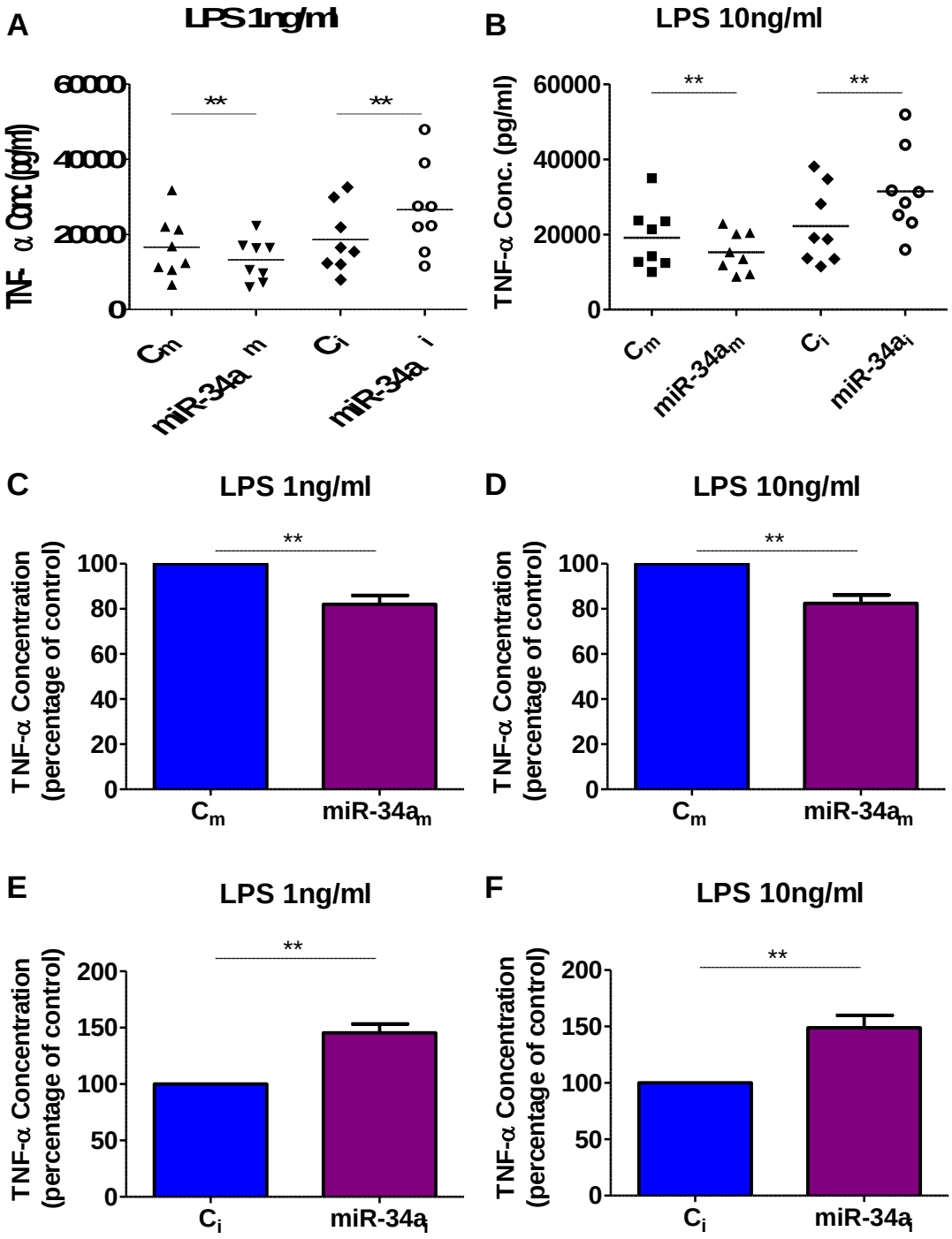


Figure 4-3 Effect of miR-34a manipulation on LPS-induced TNF-α production of 3 day M-CSF-matured macrophages

$CD14^+$ cells were isolated from buffy coats and matured for 3 days in 50 ng/ml M-CSF. Cells were then transfected with 25 nM control (C_m) or miR-34a mimic (miR-34a_m) or inhibitors (C_i or miR-34a_i) prior to a 24 hour stimulation with LPS. The concentration of TNF-α in the cell supernatants was then measured by ELISA. Panel A and B show the TNF-α concentration after stimulation with 1 or 10 ng/ml LPS, respectively. Wilcoxon matched-pairs signed rank test; ** $P < 0.01$. $n = 8$. Also shown are the normalised TNF-α production values for the same samples. For each donor the control mimic or inhibitor was normalised to 100%, with miR-34a being shown as a percentage of control. The miR-34a over-expression data are shown for 1 ng/ml and 10 ng/ml LPS stimulated cells in panels C and D respectively, while inhibitor data are shown in E and F. Data shown as mean plus standard error of the mean. Paired t test; ** $P < 0.01$. $n = 8$.

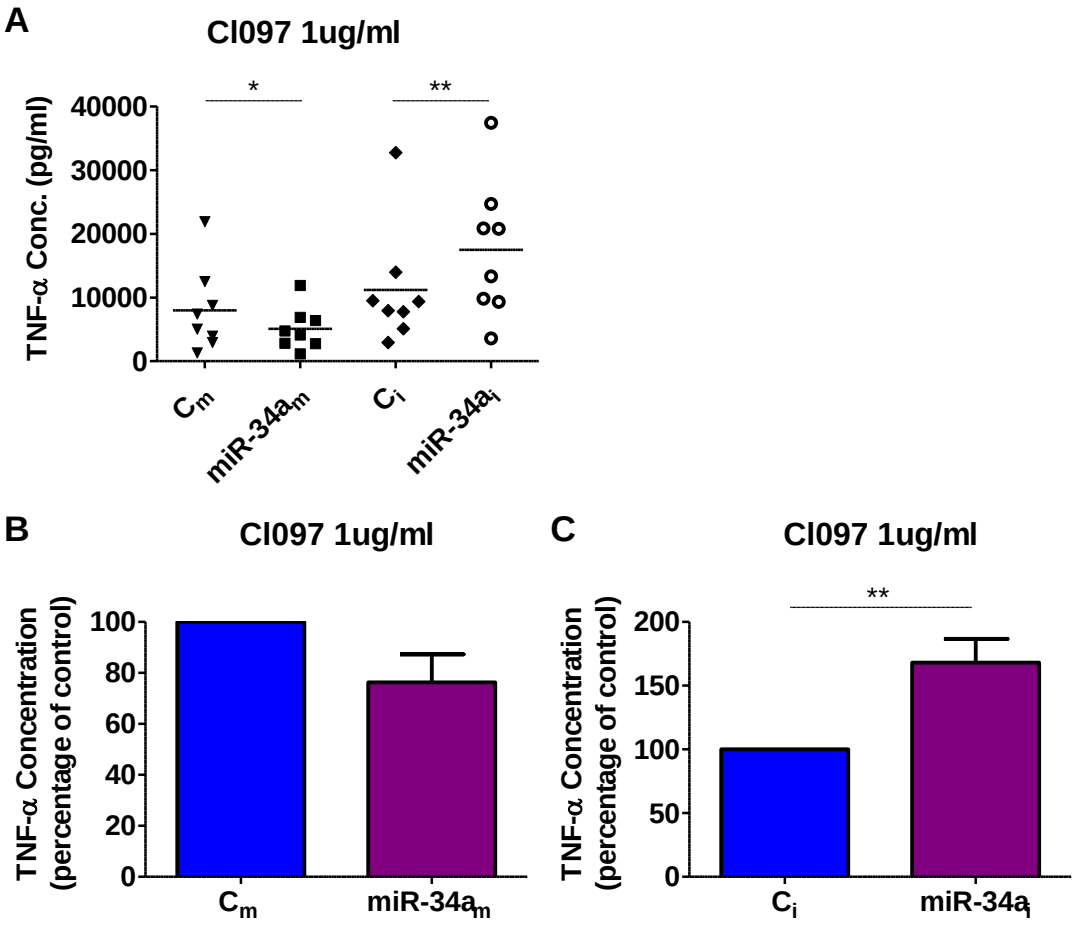


Figure 4-4 Effects of miR-34a manipulation on Cl097-induced TNF-α production of 3 day M-CSF-matured macrophages

$CD14^+$ cells were isolated from buffy coats and matured for 3 days in 50 ng/ml M-CSF. Cells were then transfected with 25 nM control (C_m) or miR-34a mimic (miR-$34a_m$) or inhibitors (C_i or miR-$34a_i$) prior to a 24 hour stimulation with Cl097. The concentration of TNF-α in the cell supernatants was then measured by ELISA. Panel A shows the TNF-α concentration after stimulation with 1 ug/ml Cl097. Wilcoxon matched-pairs signed rank test; * $P < 0.05$, ** $P < 0.01$. n = 8. Also shown are the normalised TNF-α production values for the same samples. For each donor the control mimic or inhibitor was normalised to 100%, with miR-34a being shown as a percentage of control. Panel B shows the normalised data for miR-34a over-expression, while C shows the effect of miR-34a inhibition on TNF-α production. Data shown as mean plus standard error of the mean. Paired t test; ** $P < 0.01$. n = 8.

Next, in an attempt to gain a better understanding of the effects of altering miR-34a expression on macrophage cytokine production, we decided to perform a multiplex luminex assay. This allowed us to determine the concentration of many cytokines – both pro- and anti-inflammatory. Although the TNF-α production was similar between cells stimulated with 1 or 10 ng/ml LPS, we chose to use the 10 ng/ml LPS stimulation for the luminex, as other cytokines may require a higher level of stimulation to generate detectable cytokine output. The samples from transfected cells stimulated with 10 ng/ml LPS and those stimulated with 1 μg/ml Cl097 were assayed by a luminex which was capable of measuring IL-1RA, IFN-γ, IL-7, IFN-α, IP-10, Eotaxin, MIG, IL-5, IL-6, IL-10, MIP-1α, IL-17, IL-8, IL-12 (p40/p70), RANTES, MIP-1β, GM-CSF, TNF-α, IL-1β, IL-2, IL-4, IL-2R, IL-15, MCP-1 and IL-13. All analytes that were detectable and in range for all samples are shown in the graphs below.

For LPS stimulated cells, other than TNF-α, only IL-6 showed reciprocal changes upon miR-34a manipulation. Thus we observed a significant reduction with miR-34a over-expression and an increase with miR-34a inhibition. For IL-1β we observed only a trend towards reduced expression with miR-34a over-expression but we did see a significant increase in expression with miR-34a inhibition (graphs shown in Figure 4-5). Of interest, several cytokines showed significant down-regulation in miR-34a over-expressing cells, but no change where miR-34a had been inhibited. As demonstrated in Figure 4-6 these included IFN-γ, MIG, IL-7, IL-15 and IL-1RA. Figure 4-7 shows all the analytes whose expression remained consistent between all four conditions; namely IL-10, IL-12, IL-2R and IFN-α.

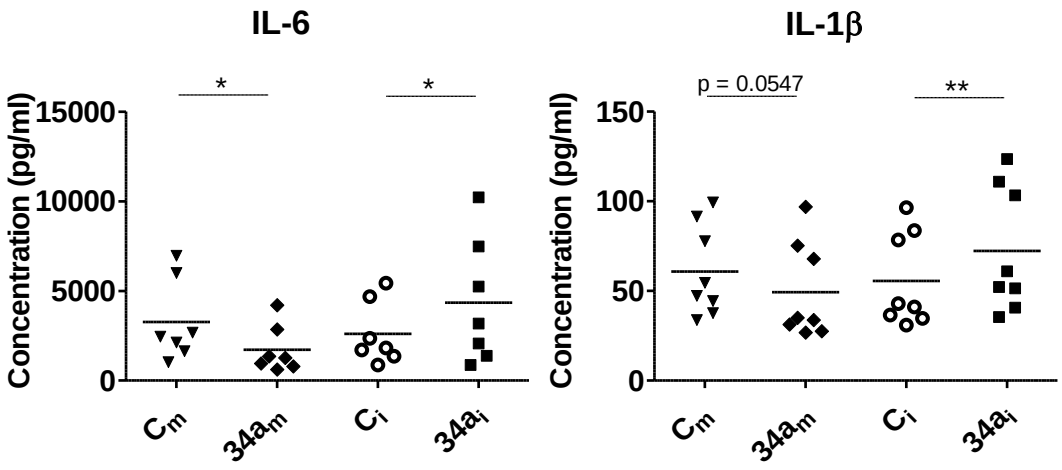


Figure 4-5 Analytes whose LPS-induced production was influenced by miR-34a over and under expression in macrophages

$CD14^+$ cells were isolated from buffy coats and matured for 3 days in 50 ng/ml M-CSF. Cells were then transfected with 25 nM control (C_m) or miR-34a mimic ($34a_m$) or inhibitors (C_i or $34a_i$) prior to a 24 hour stimulation with LPS. The concentration of cytokines in the supernatant was then measured by luminex. Shown are the analytes whose expression was detectable in all samples, and changes were observed with the use of miR-34a mimics and inhibitors. Wilcoxon matched-pairs signed rank test; * $p < 0.05$, ** $p < 0.001$. $n = 8$.

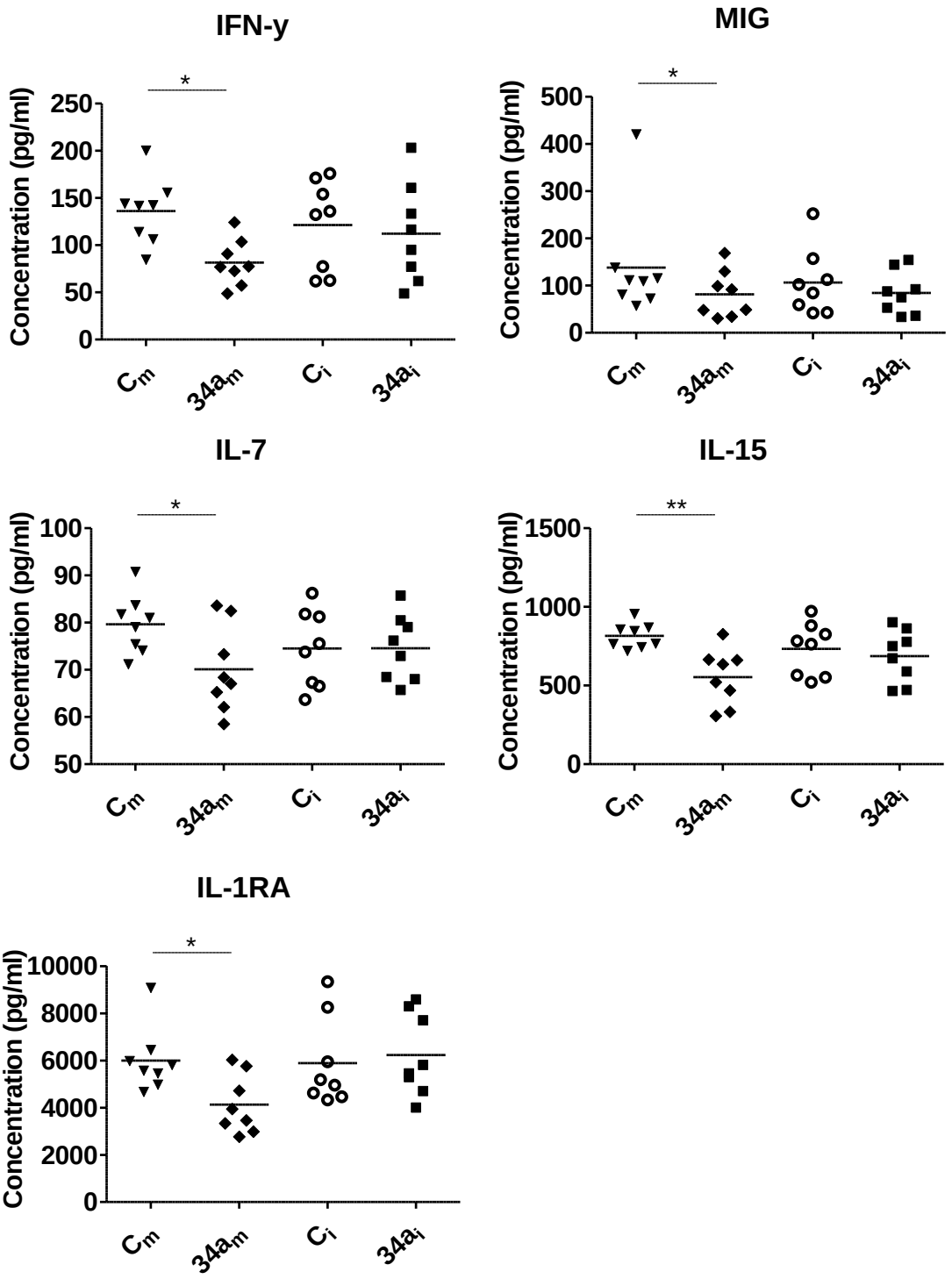


Figure 4-6 Analytes whose LPS-induced production was influenced by miR-34a over expression in macrophages

$CD14^+$ cells were isolated from buffy coats and matured for 3 days in 50 ng/ml M-CSF. Cells were then transfected with 25 nM control (C_m) or miR-34a mimic ($34a_m$) or inhibitors (C_i or $34a_i$) prior to a 24 hour stimulation with LPS. The concentration of cytokines in the supernatant was then measured by luminex. Shown are the analytes whose expression was detectable in all samples, and changes were observed with the use of miR-34a mimics but not inhibitors. Wilcoxon matched-pairs signed rank test; * $p < 0.05$, ** $p < 0.001$. n = 8.

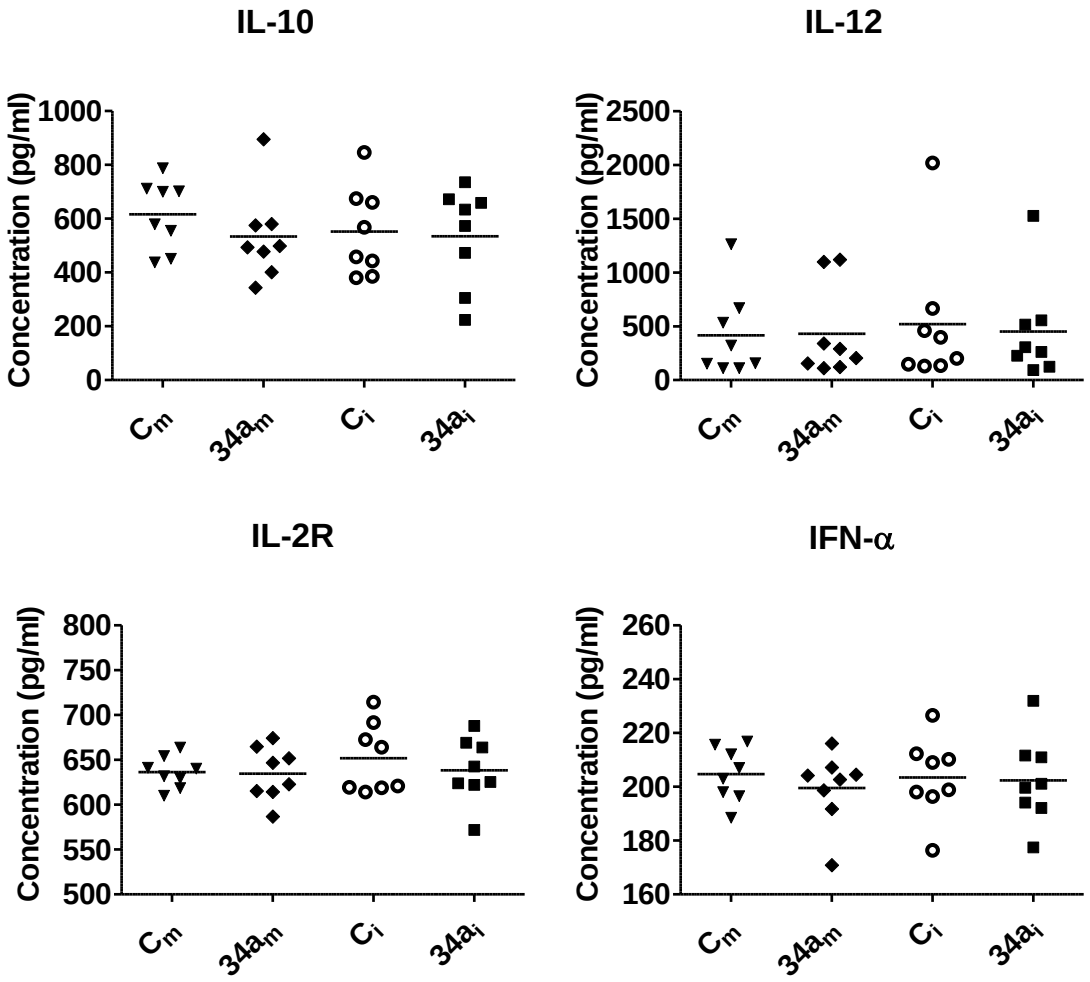


Figure 4-7 Analytes whose LPS-induced production was not influenced by miR-34a over or under expression in macrophages

$CD14^+$ cells were isolated from buffy coats and matured for 3 days in 50 ng/ml M-CSF. Cells were then transfected with 25 nM control (C_m) or miR-34a mimic ($34a_m$) or inhibitors (C_i or $34a_i$) prior to a 24 hour stimulation with LPS. The concentration of cytokines in the supernatant was then measured by luminex. Shown are the analytes whose expression was detectable in all samples, but no significant differences were observed by Wilcoxon matched-pairs signed rank test. n = 8.

When transfected cells were stimulated with Cl097, the range of cytokines that demonstrated reciprocal changes upon miR-34a manipulation was broader, and included IL-1ß, IL-6, IL-7, IL-13, IL-15, RANTES and GM-CSF. These were all significantly down-regulated in miR-34a mimic transfected cells, and up-regulated in miR-34a inhibitor transfected cells (shown in Figure 4-8). As with LPS, Cl097 stimulation also elicited a group of cytokines whose expression was affected by miR-34a over-expression, but not miR-34a inhibition, including IL-1RA, IL-2R, IL-12 and IFN-α (Figure 4-9). Figure 4-10 shows the analytes whose expression remained consistent in all 4 conditions after Cl097 stimulation. Of those that could be detected namely IL-10 and IP-10. In summary, miR-34a seems to inhibit overall macrophage activation, manifest by the altered production of a broad range of cytokines, particularly in response to stimulation with the intracellular TLR, TLR7.

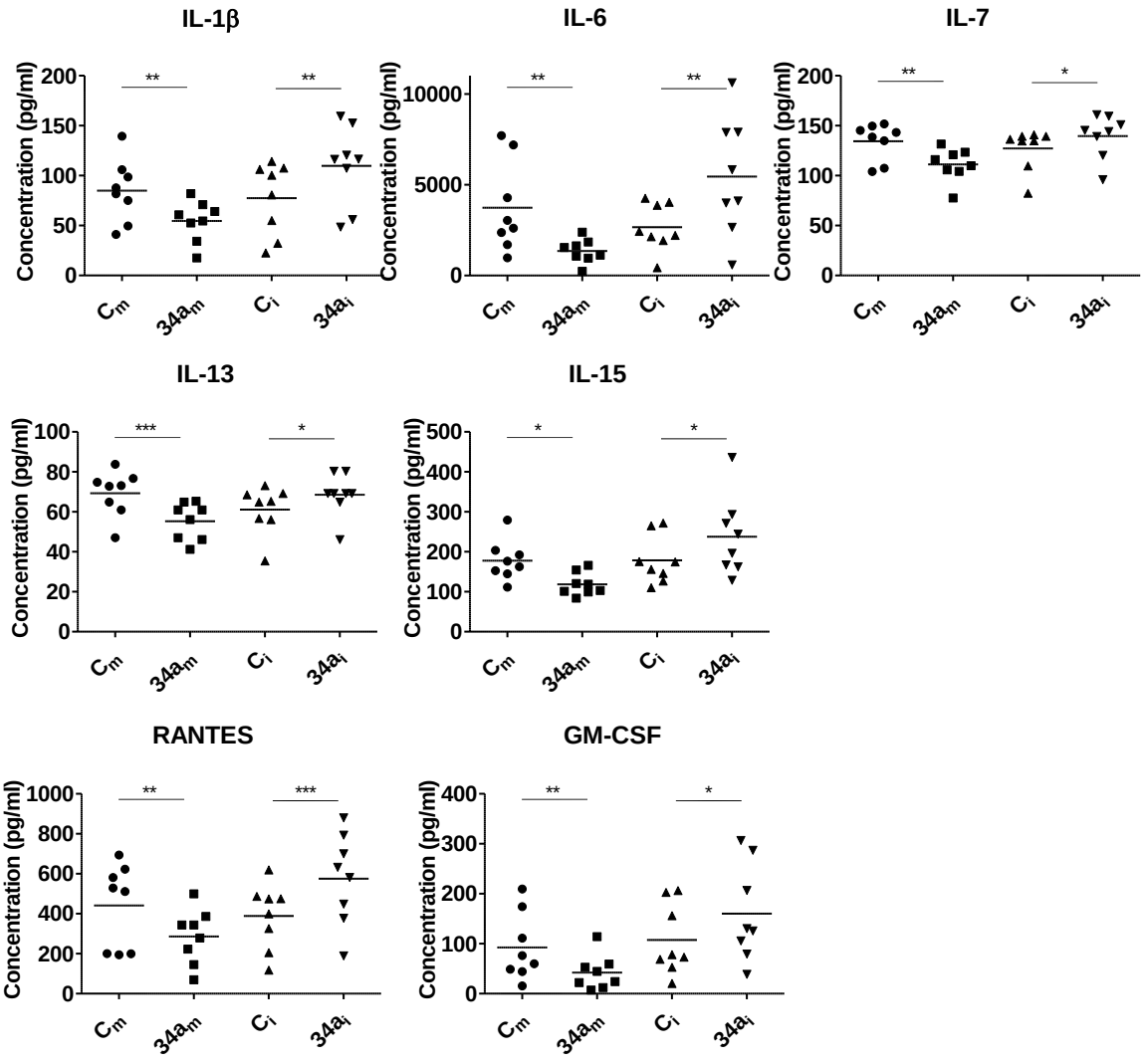


Figure 4-8 Analytes whose Cl097-induced production was influenced by miR-34a over and under expression in macrophages

$CD14^+$ cells were isolated from buffy coats and matured for 3 days in 50 ng/ml M-CSF. Cells were then transfected with 25 nM control (C_m) or miR-34a mimic ($34a_m$) or inhibitors (C_i or $34a_i$) prior to a 24 hour stimulation with Cl097. The concentration of cytokines in the supernatant was then measured by luminex. Shown are the analytes whose expression was detectable in all samples, and changes were observed with the use of both miR-34a mimics and inhibitors. Wilcoxon matched-pairs signed rank test; * $p < 0.05$, ** $p < 0.001$. $n = 8$.

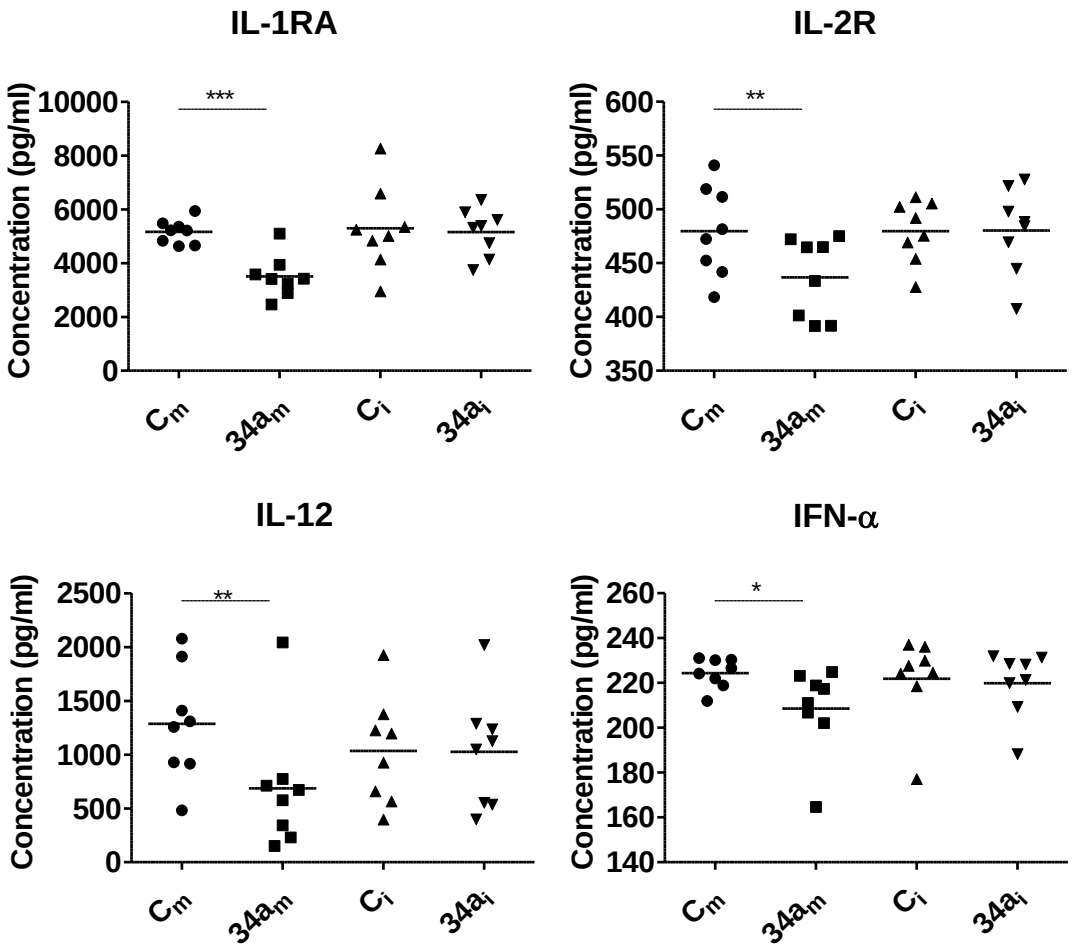


Figure 4-9 Analytes whose Cl097-induced production was influenced by miR-34a over expression in macrophages

$CD14^+$ cells were isolated from buffy coats and matured for 3 days in 50 ng/ml M-CSF. Cells were then transfected with 25 nM control (C_m) or miR-34a mimic ($34a_m$) or inhibitors (C_i or $34a_i$) prior to a 24 hour stimulation with Cl097. The concentration of cytokines in the supernatant was then measured by luminex. Shown are the analytes whose expression was detectable in all samples, and changes were observed with the use of miR-34a mimics but not inhibitors. Wilcoxon matched-pairs signed rank test; * $p < 0.05$, ** $p < 0.001$. $n = 8$.

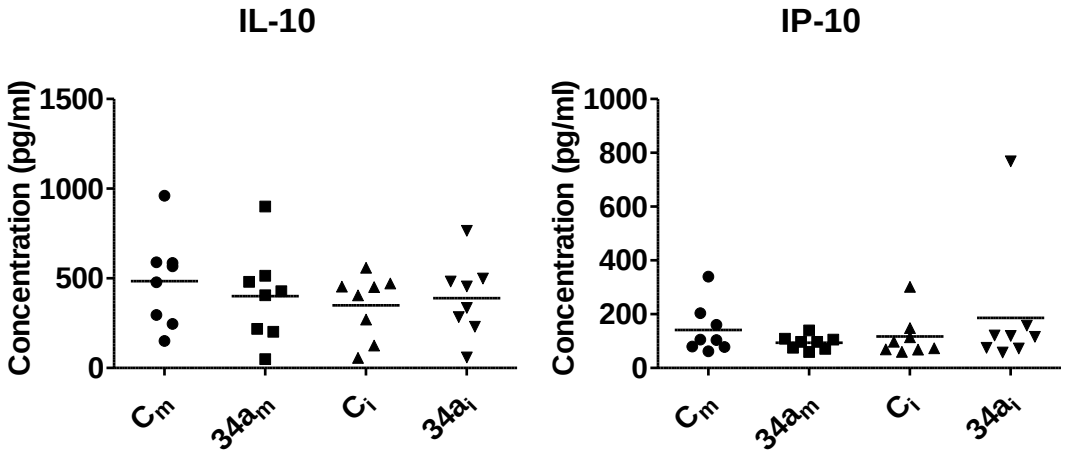


Figure 4-10 Analytes whose Cl097-induced production was not influenced by miR-34a over or under expression in macrophages

$CD14^+$ cells were isolated from buffy coats and matured for 3 days in 50 ng/ml M-CSF. Cells were then transfected with 25 nM control (C_m) or miR-34a mimic ($34a_m$) or inhibitors (C_i or $34a_i$) prior to a 24 hour stimulation with Cl097. The concentration of cytokines in the supernatant was then measured by luminex. Shown are the analytes whose expression was detectable in all samples, but no significant differences were observed by Wilcoxon matched-pairs signed rank test. n = 8.

Both we (to be discussed in chapter 5) and others have demonstrated that miR-34a can target the MCSF receptor CSF1R [357]. There are several studies suggesting that M-CSF can induce TNF-α mRNA and protein expression, and even act synergistically with LPS stimulation to cause increased TNF-α production [358-361]. For this reason, and because we supplement our cultures with M-CSF (potentially acting as a "co-stimulant" with TLR agonism), we wanted to test whether reduced CSF1-R expression by our miR-34a mimic transfected cells could be responsible for the reduced TNF-α expression. To test this, as previously described, $CD14^+$ cells were isolated from buffy coats and cultured with M-CSF for 7 days, however, when changing the media of these already differentiated cells prior to transfection, they were replenished with fresh complete medium with no M-CSF supplementation, or with 5 or 50 ng/ml M-CSF. Cells were transfected for 24 hours as before, then stimulated with 10 ng/ml LPS for a further 24 hours. The TNF-α concentration in the supernatants was then measured by ELISA. Figure 4-11 shows the results, and while data are variable (as expected with human cells) and from only three donors, regardless of the M-CSF concentration, the LPS-induced TNF-α production was consistently lower in the miR-34a mimic transfected cells. Thus miR-34a targeting of CSF1R is unlikely to explain the reduced TNF production we observed in miR-34a mimic transfected cells.

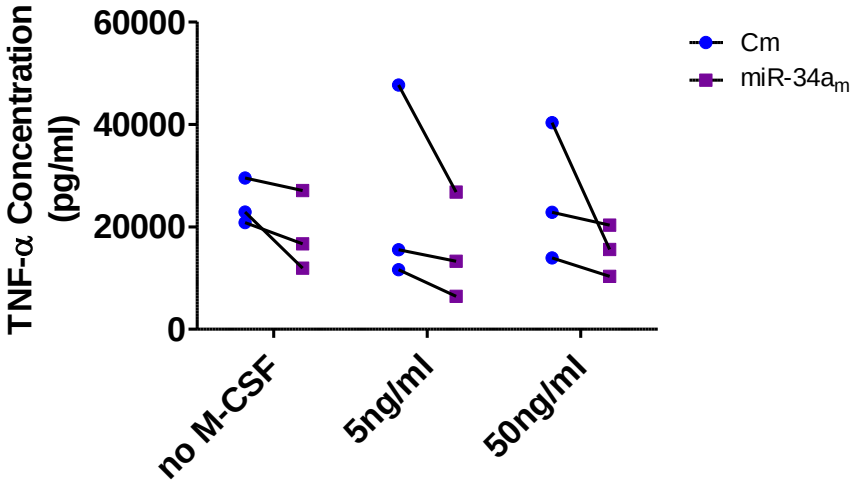


Figure 4-11 The effect of different M-CSF concentrations on LPS-induced TNF-α production by miR-34a-transfected macrophages

$CD14^+$ cells were isolated from buffy coat samples and matured with 50 ng/ml M-CSF for 7 days. These cells were then transfected with 25 nM control (C_m) or miR-34a (miR-$34a_m$) mimics in the absence of M-CSF, or in the presence of 5 or 50 ng/ml M-CSF for 24 hours prior to a further 24 hour stimulation with 10 ng/ml LPS. The TNF-α concentration in supernatants was measured by ELISA. Data from cells from the same donor in the same M-CSF condition are linked by a line. n = 3.

4.3 Effect of miR-34a manipulation on macrophage phenotype

We next wanted to determine whether miR-34a regulates monocyte to macrophage differentiation and subsequent activation. Thus we tested whether over-expression or inhibition of miR-34a affected some of the key surface markers found on macrophages. $CD14^+$ cells were isolated from buffy coats and matured with M-CSF for 3 days. These cells were then transfected with a control mimic, miR-34a mimic, control inhibitor or miR-34a inhibitor for 24 hours before being harvested and stained for FACS analysis. We chose to look at CD16, CD64 and HLA-DR.

CD64 and CD16 are Fc receptors (FcRs); FcγRI and FcγRIII respectively. FcγRs bind IgG in many forms, including monomeric IgG, immune complexes and antibody-coated or opsonized particles or cells. These two specific receptors associate with the FcR common γ-chain that contains immunoreceptor tyrosine-based activation motifs (ITAMs). Upon binding of the receptors by IgG, these ITAMs activate signalling cascades, with subsequent functional outcomes depending on the type of IgG bound. Binding of opsonized pathogens or infected cells results in increased uptake by phagocytosis, ultimately resulting in pathogen killing or antibody-dependant cell-mediated cytotoxicity (ADCC), whereas binding of immune complexes can increase cytokine production and alter the activation state of the macrophage. These markers are also often altered during macrophage differentiation and activation. CD64 for example is up-regulated upon macrophage activation, and is frequently used as an "M1" macrophage marker [362, 363]. Fc receptors are also important in the context of RA, where ACPA containing immune-complexes binding to FcγRs are thought to be one of the mechanisms responsible for the activation of synovial macrophages. HLA-DR is an MHC class II molecule, whose primary role is to present antigens to T cells, which is often up-regulated during cell activation [364]. We therefore chose to measure the expression of these three cell surface markers as changes in their expression could indicate differences in the maturation or activation state of the miR-34a mimic or inhibitor transfected cells.

Figure 4-12 shows representative histograms for cells from one donor stained with these markers. The majority of cells in all conditions stained positive for all markers, but no differences were seen between the control or miR-34a mimic or inhibitor transfected cells. Figure 4-13 shows the percentage of positive cells compared to the fluorescence minus one (FMO) control and also the geometric mean fluorescence intensity (gMFI). These data suggest that miR-34a does not regulate the expression of macrophage cell surface markers during maturation in an obligate manner.

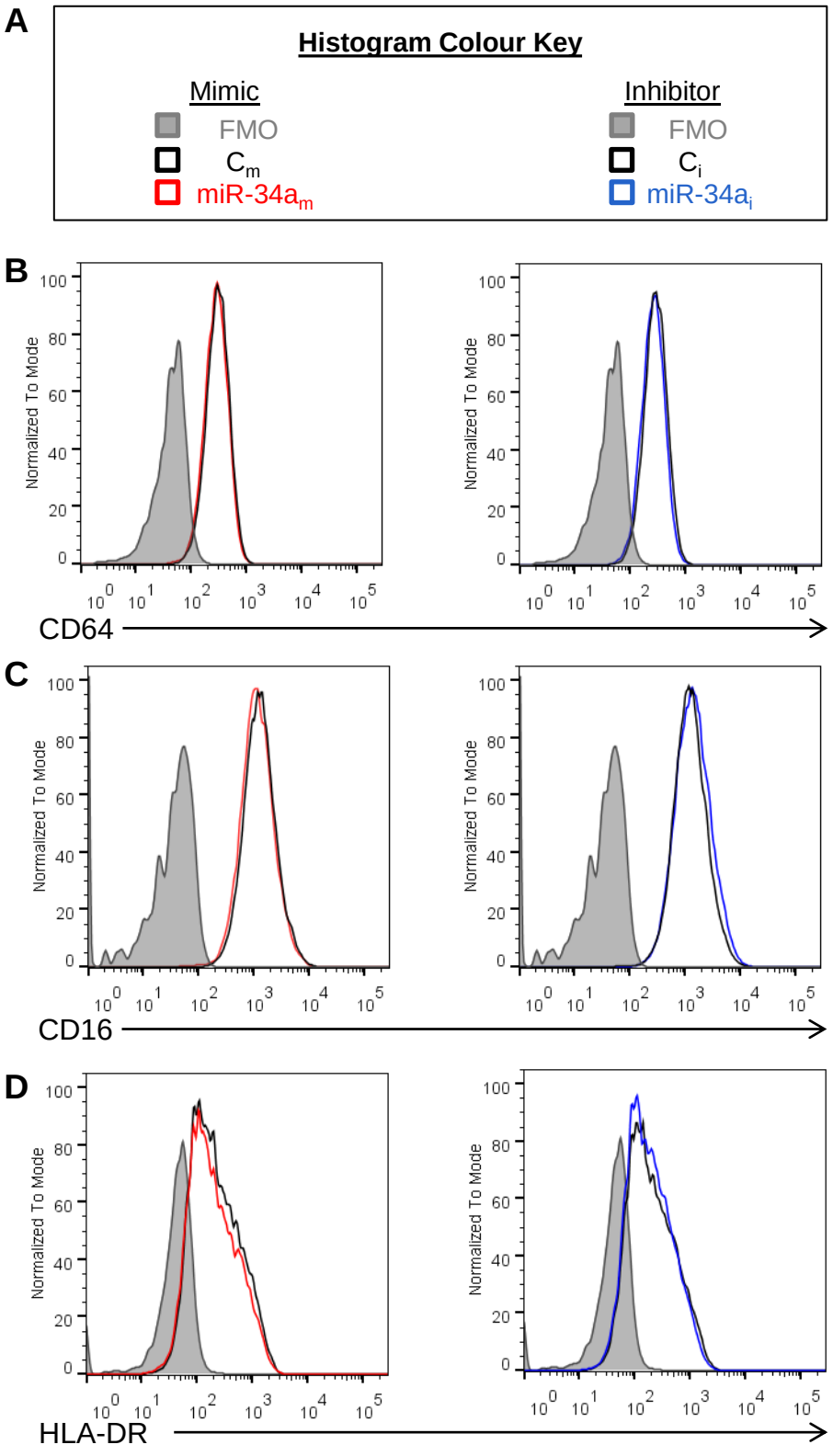


Figure 4-12 Histograms showing representative FACS staining of miR-34a mimic and inhibitor transfect macrophages

$CD14^+$ cells were isolated from buffy coats and matured for 3 days in 50 ng/ml M-CSF. Cells were then transfected with 25 nM control (C_m) or miR-34a mimic (miR-$34a_m$) or inhibitors (C_i or miR-$34a_i$) and cells taken for FACS staining 24 hours later. Shown in panel A is the colour key used for all histograms. Panels B, C and D show representative histograms for one donor stained with CD64, CD16 and HLA-DR respectively.

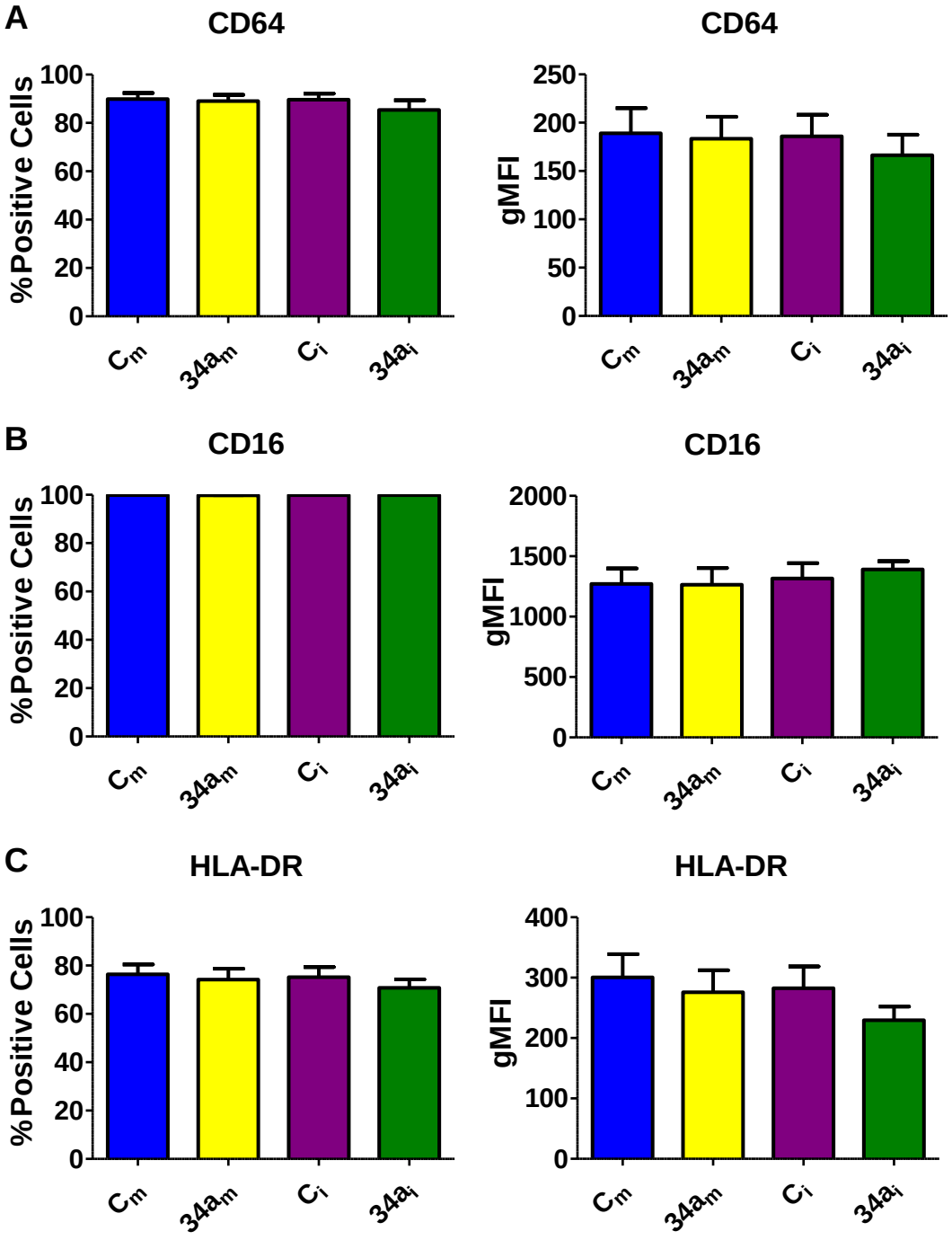


Figure 4-13 Analysis of CD64, CD16 and HLA-DR expression in miR-34a mimic and inhibitor transfected macrophages

$CD14^+$ cells were isolated from buffy coats and matured for 3 days in 50 ng/ml M-CSF. Cells were then transfected with 25 nM control (C_m) or miR-34a mimic ($34a_m$) or inhibitors (C_i or $34a_i$) and cells taken for FACS staining 24 hours later. Shown are the percentage of positive cells and geometric mean fluorescence intensity (gMFI) for CD64 (A), CD16 (B) and HLA-DR (C). Data shown as mean plus standard error of the mean. n = 4.

4.4 **Discussion**

We have previously shown that the expression of miR-34a is increased in the synovium of RA patients, and also in circulating monocytes from patients with drug resistant disease. Therefore, this part of the study set out to determine the possible functional consequences of dysregulated miR-34a expression.

My first key observation is that even in 7 day M-CSF-matured macrophages, whose miR-34a expression is quite high (shown in chapter 3), further up-regulation of this microRNA can cause a significant decrease in the TNF-α production in response to LPS stimulation. However, possibly due to the high levels of miR-34a found in 7 day M-CSF-matured cells, miR-34a inhibition was not bioactive in these cells. We therefore chose to look at over and under-expression of miR-34a in $CD14^+$ cells matured with M-CSF for 3 days because these cells express intermediate levels of miR-34a, and as such represented a system in which the reciprocal activities of miR-34a mimic and inhibitors could be formally tested. Moreover, while we have demonstrated that synovial fluid $CD14^+$ cells from RA patients show increased miR-34a expression, these levels are not as high as those observed in cells matured with M-CSF for 7 days. Finally, as noted earlier, 3 day M-CSF matured cells do not differ phenotypically or functionally (at least in terms of cytokine production) from the standard 7 day matured cells.

When day 3 M-CSF-matured cells were examined, a significant reduction in TNF with miR-34a over-expression, and a reciprocal increase with miR-34a inhibition was found in both LPS and Cl097 stimulated cells. Consistent with this finding, another paper was recently published subsequent to my performing this work showing that miR-34a affects TNF-α production by human M-CSF-matured macrophages [354]. Interestingly, while we only studied the "functional" miR-34a strand (miR-34a-5p), this paper looked at the effect of both the 5p and 3p strands of miR-34a on TNF production. They demonstrated that over-expression of both strands individually could reduce TNF mRNA and protein, while over-expression of both strands together resulted in a greater reduction, suggesting synergistic effects. While the authors suggested that miR-34a-5p affected TNF production indirectly, the 3p strand actually targeted TNF mRNA directly; which they proved using a luciferase reporter assay. In the initial stages of this project

we actually observed increased expression of both strands of miR-34a in synovial fluid $CD14^+$ cells from RA patients, but at the time chose to focus on what was termed the "functional" strand. However, in the past two years miR-34a-3p has been proven to be a functional microRNA in its own right. In this study its role in TNF production of monocyte-derived macrophages is highlighted. In an earlier study miR-34a-3p expression was found to be reduced in fibroblasts from RA patients where it could promote the increased expression of an inhibitor of apoptosis, thereby aiding the expansion of FLSs [254]. These studies suggest that the increased miR-34a-3p expression in RA SF $CD14^+$ cells could indeed be important, and warrant further study.

In order to obtain a clearer picture of what was going on in our cells after miR-34a manipulation, we performed a luminex assay to observe a broader cytokine profile. The most interesting results are those that move in reciprocal directions with the use of microRNA-34a mimics and inhibitors. There are many cytokines whose expression was unchanged in either condition, or that changed only with miR-34a mimics. The latter results are harder to interpret on their own, because the supraphysiological levels of microRNA achieved after transfection with microRNA mimics likely causes targeting of pathways that would not occur under physiological conditions. Therefore, microRNA mimics are useful for understanding the potential of what a microRNA has the capability of targeting, but microRNA inhibitors give a more accurate representation of the role of a given microRNA in the cell at that time [365]. For this reason we are more engaged with the notion that cytokines that were reciprocally regulated represent functionally relevant output from this experimental series.

The only cytokine, other than TNF-α, which was significantly down-regulated with miR-34a over-expression, and up-regulated with miR-34a inhibition under both LPS and Cl097 stimulation conditions was IL-6. IL-1β, however, was significantly altered by Cl097 stimulation in both conditions, and significantly up-regulated after LPS stimulation of miR-34a inhibited macrophages, with a trend towards lower expression seen in the miR-34a mimic transfected cells. This is intriguing since TNF-α, IL-6 and IL-1 are considered three of the most important cytokines in RA disease pathogenesis. Treatments opposing their actions are licenced for the treatment of RA, and all three are up-regulated in

both the serum and SF of RA patients where they correlate with various parameters of disease activity [366-369]. They are all involved in multiple levels of regulation of immune responses and have many over-lapping and distinct functions [370].

Interestingly, miR-34a inhibited the production of a broader range of cytokines in the presence of CL097 stimulation. These include GM-CSF, IL-15 and IL-7. GM-CSF is known to regulate the production, differentiation and activation of myeloid cells. It is found at high levels in the synovium of RA patients, where it is thought to play a role in driving local inflammatory responses. Increased GM-CSF in RA could be involved in promoting an inflammatory phenotype in macrophages [371], and also play a role in promoting the generation and survival of pathogenic Th17 cells [153]. IL-15 is also increased in expression in both the serum and synovial fluid of RA patients, where it correlates with disease severity [372]. Initially described as T cell growth factor, it is now known that IL-15 is involved in regulating the activation and proliferation of T cells and NK cells, and promoting the survival of memory T cells [373]. It is also capable of promoting osteoclastogenesis [374], while blocking IL-15 activity reduced the destruction of cartilage and bone in the murine CIA model [375]. The expression of IL-7 in the serum of RA patients is still controversial, with conflicting reports suggesting it could be up or down-regulated compared to controls, but it is known to be up-regulated in the synovial fluid of RA patients compared to OA patients [376, 377]. IL-7 has been shown to promote osteoclastogenesis in RA $CD14^+$ cells [378], and like IL-15, it also has a role preventing apoptosis of naïve T cells [379] and promoting the survival and expansion of memory T cells [380, 381]. The fact that many of these cytokines are up-regulated in the serum and synovial fluid of RA patients, while miR-34a is up-regulated in monocytes and macrophages in the periphery and synovium could suggest that miR-34a is actually unsuccessfully trying to dampen the on-going inflammation.

The results from these experiments leave two particularly interesting questions; what causes miR-34a's negative regulation of macrophage cytokine production, and why are the differences seen with Cl097 stimulation more profound than those seen with LPS stimulation.

Firstly, what causes miR-34a's negative regulation of macrophage cytokine production? As mentioned previously, miR-34a overexpression down-regulates CSF1-R, which is a predicted and verified target of miR-34a. M-CSF is crucial for macrophage differentiation and can synergise with other stimuli to promote macrophage activation. Thus, one possible hypothesis we had was that miR-34a targeting of CSF1R had a negative effect on macrophage activation and therefore reduced cytokine production in response to TLR stimulation (by diminution of the combinatorial effect of CSFR1 and TLR signalling). This did not appear to be the case, however, as all macrophage maturation markers tested were not affected by miR-34a manipulation, and altering the M-CSF concentration didn't affect the reduced TNF-α production in miR-34a over-expressing cells in response to LPS stimulation. Though to formally exclude autocrine M-CSF release mediating this effect it would be necessary to add a neutralising anti-MCSF antibody to the culture.

When using ingenuity pathway analysis and using predicted miR-34a targets to look for other potential ways by which miR-34a could negatively influence TNF-α production, interleukin-1 receptor-associated kinase (IRAK) -2 and IRAK-4 came up as interesting potential miR-34a targets. The IRAK family has four members, IRAK-1, 2, 3 and 4, which are implicated in the downstream signalling of the TLR and IL-1R family molecules. Both IRAK-2 and IRAK-4 have two potential miR-34a binding sites in their 3'-UTR, but to my knowledge no studies have confirmed miR-34a targeting of these mRNAs [382]. Another interesting potential miR-34a target that could be involved in altering cytokine production after miR-34a over-expression and inhibition is mitogen-activated protein kinase kinase kinase 14 (MAP3K14), also known as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) -inducing kinase (NIK). MAP3k14 is crucial in the non-canonical NF-κB pathway. This serine/threonine protein-kinase binds to TNF receptor-associated factor 2 (TRAF2) and stimulates NF-κB activity. It participates in an NF-κB-inducing signalling cascade common to the TNF and IL-1 type 1 receptors [383]. These three predicted targets therefore represent novel and interesting pathways by which miR-34a could regulate macrophage cytokine production.

The second question this study poses is why are the differences seen with Cl097 stimulation more profound than those seen with LPS stimulation. As well as

TNF-α, IL-1β and IL-6, in the Cl097-stimulated cells IL-7, IL-13, IL-15, RANTES and GM-CSF are also significantly down-regulated following miR-34a mimic transfection and up-regulated with miR-34a inhibition. LPS activates cells by binding and signalling through TLR-4, while Cl097 does so via TLR-7/8 signalling. These data could suggest that manipulation of miR-34a has more profound effects on TLR-7/8 induced cytokine production than TLR-4 induced cytokine production. Interestingly, IRAK-2 and IRAK-4 are only involved in MyD88-dependent TLR signalling – which is the only pathway utilised by TLRs 7 and 8. TLR-4, on the other hand, can signal through both MyD88-dependant and independent pathways. miR-34a targeting of IRAK-2 and IRAK-4 could, therefore, explain the more profound differences seen here with Cl097 stimulation versus LPS stimulation.

There was no time to further explore these hypotheses within this body of work, but given more time there are several experiments that would help determine whether IRAK-2, IRAK-4 or MAP3k14 do indeed target miR-34a, and whether this is in part responsible for the differences in cytokine production described here. Firstly, a luciferase assay could confirm whether miR-34a is capable of actually binding to the 3'-UTR of these potential targets. The next step would be to use target protectors and see if protecting the binding sites in one or more of these mRNA molecules prevented the changes in cytokine production.

The main limitation in this study is the inability to say without doubt what these results infer about the altered miR-34a expression in RA synovial fluid $CD14^+$ cells. These RA cells are neither peripheral blood $CD14^+$ cells, nor M-CSF matured macrophages. microRNA targeting experiments are very susceptible to differences in the mRNA expression in a cell at the given time, therefore, if given the extra time I would have liked to perform similar experiments on patient derived synovial fluid $CD14^+$ cells.

In summary, this chapter demonstrated that miR-34a negatively regulates TLR-induced cytokine production by human primary monocyte-derived macrophages from the PB of healthy donors. Further studies in the lab will be conducted to test whether miR-34a exhibits the same potential in RA synovial fluid macrophages and RA monocyte derived macrophages.

Chapter 5 – Identification of targets and pathways regulated by microRNA-34a in monocytes

5.1 Introduction and Aims

microRNA mediate their effects on protein expression by binding to target mRNA molecules, ultimately resulting in translation inhibition or mRNA degradation. In plants, miRNA : mRNA binding usually involves perfect complementarity upon binding, which often results in direct Argonaute2-catalysed endonucleolytic cleavage of the target [257, 258]. In mammals however, direct complementarity is rare, and more often miRNAs recognise and bind their targets through the "seed sequence". This is generally contained in nucleotides 2 – 8 in the 5' region of the microRNA – which is often the most conserved region of microRNA [384, 385]. In most cases it is thought that this seed sequence binds complementary sequences in the 3'-UTR of target mRNA molecules. An example of these complementary sequences is presented for miR-34a and one of its well-established targets, Sirtuin (SIRT) 1, in Figure 5-1. Shown is a section of the SIRT1 3'-UTR which contains a six nucleotide sequence complementary to six nucleotides of the miR-34a seed sequence.

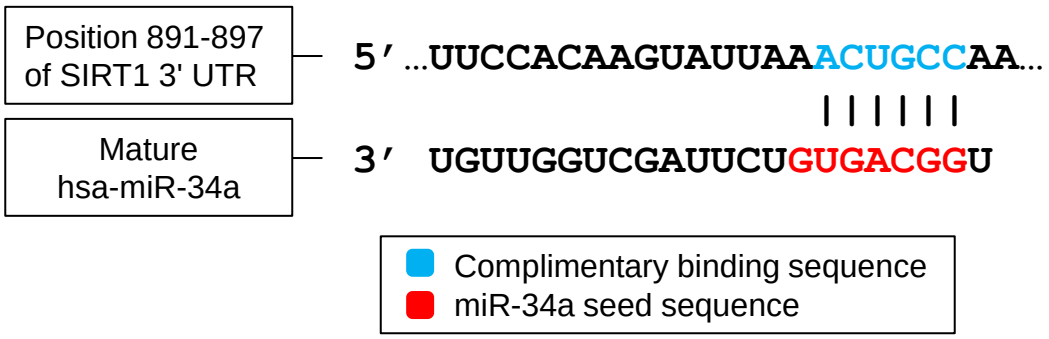


Figure 5-1 Mature hsa-miR-34a binding to the 3'-UTR of SIRT1
The diagram above shows a small portion of the SIRT1 3'-UTR, which contains a binding site (in blue) for miR-34a. Also shown is the mature sequence of miR-34a, with the seed sequence highlighted in red. Complementary binding sites between the miR-34a seed sequence and SIRT1 3'-UTR are linked by a line.

A number of prediction programmes are now freely available to aid the discovery of potential microRNA targets; these include TargetScan, PicTar, RNA22 and miRanda, among others. These programmes use algorithms to detect potential microRNA binding sites, primarily based on the presence of complementary binding sites. The targets of a given microRNA, however, are likely to depend on a number of factors. These include, but are not restricted to, the expression

level of the particular microRNA, the expression level of other microRNA that could target the same mRNA, and also the expression level of competing endogenous mRNA within a cell at the given time. Many of these factors cannot be taken into account using these simple prediction algorithms, therefore it becomes clear that the use of prediction algorithms alone to find targets would result in a significant number of false positive and false negative results. For these reasons, although prediction algorithms are useful in the search for candidate targets, experimental techniques and target validation are required for confirmation of potential miRNA targets [386, 387].

There are a number of experimental techniques frequently used to help elucidate the potential role of particular microRNA, and work out the targets whereby such function is mediated (reviewed in [388]). These include under and over expression of the candidate microRNA of interest. This is generally followed by PCR or microarray to look at changes at the mRNA level, and/or western blot or large-scale proteomics to look at changes in protein expression. These strategies can generate a list of potential targets of interest, but does not give an insight into whether these candidates are direct targets of the microRNA, or are differentially regulated as a result of indirect effects. For this reason luciferase reporter assays are often used. In this technique the candidate sequence containing the potential miRNA binding site is cloned into an expression vector downstream from a luciferase reporter gene. This is then transfected into cells in the presence or absence of the targeting microRNA, and luminescence is measured to determine whether binding occurs - which in consequence will modulate the level of luciferase expression, hence reporting functional interactions [389].

Here we have used some of the techniques documented above to investigate the potential role(s) of microRNA-34a in myeloid cell biology.

5.2 **Pathway Discovery**

The first question we wanted to address was what pathways could be regulated by miR-34a in monocytes? Understanding this profile should build an understanding of the potential consequences of miR-34a up-regulation in RA. In order to address this, we chose to use an overexpression system. $CD14^+$ cells were isolated from the peripheral blood of healthy donors, then transfected with a control or synthetic miR-34a mimic. We chose to use monocytes for this part of the study, as this could replicate what happens in RA when peripheral blood monocytes (which we have shown express relatively low levels of miR-34a) migrate to the sites of inflammation and up-regulate miR-34a (possibly due to interactions with synovial tissue enriched for M-CSF / GM-CSF expression). 24 hours after transfection of monocytes with a control or miR-34a mimic, RNA was purified, and a microarray (GeneChip® Human Genome U133 Plus 2.0 Arrays) performed to determine differentially expressed transcripts.

We chose to perform a microarray as it would allow the simultaneous measurement of thousands of transcripts. This technology also adopted experimental and analysis techniques that were already well established in the laboratory. Early microRNA research suggested that a proteomic approach may be better for looking at microRNA targets, but increasingly the field is showing that most of the protein reductions (recently estimated at 84% [390]) caused by microRNA are a consequence of mRNA destabilisation – giving us the confidence to move forward with this method [391, 392].

Following some initial problems with low transfection efficiency and high cell death of transfected monocytes, the conditions were optimised for the best cell density, time of transfection and FBS concentration in the culture. Once these conditions were determined, cells from 8 different donors were transfected and a number of factors considered before 5 donors were chosen for the microarray analysis (discussed below).

5.2.1 Viability and transfection efficiency of monocytes following transfection with a miR-34a mimic

Firstly, we wanted to determine cell viability after the 24 hour transfection. To do this, cells were stained with 7-AAD and analysed by flow cytometry. Figure 5-2A shows a representative flow cytometry plot with dead cells staining positive for 7-AAD. This plot also shows the gating strategy used to quantify the percentage of live cells in the culture. The graph in Figure 5-2B shows the viability for all the different transfection conditions and 8 different transfected blood donors. As shown on the graph no significant differences were seen in cell viability after monocytes were cultured for 24 hours in complete media, with transfection reagents, or with the addition of control or miR-34a microRNA mimics.

Next, we wanted to ensure successful transfection of the cells was achieved. For this a labelled control mimic (Cm547) was transfected into cells from each donor. Uptake of Cm547, as measured by increased fluorescence, was then analysed by flow cytometry and used to estimate the transfection efficiency. Figure 5-3A shows a representative histogram of cells transfected using the labelled mimic, compared to the standard unlabelled control mimic, while Figure 5-3B shows the percentage of cells which have taken up the labelled mimic for each donor.

This data concerning cell viability and transfection efficiency, together with the RNA concentration and RNA Integrity Number (RIN), were used to choose samples from 5 of the 8 donors for microarray analysis.

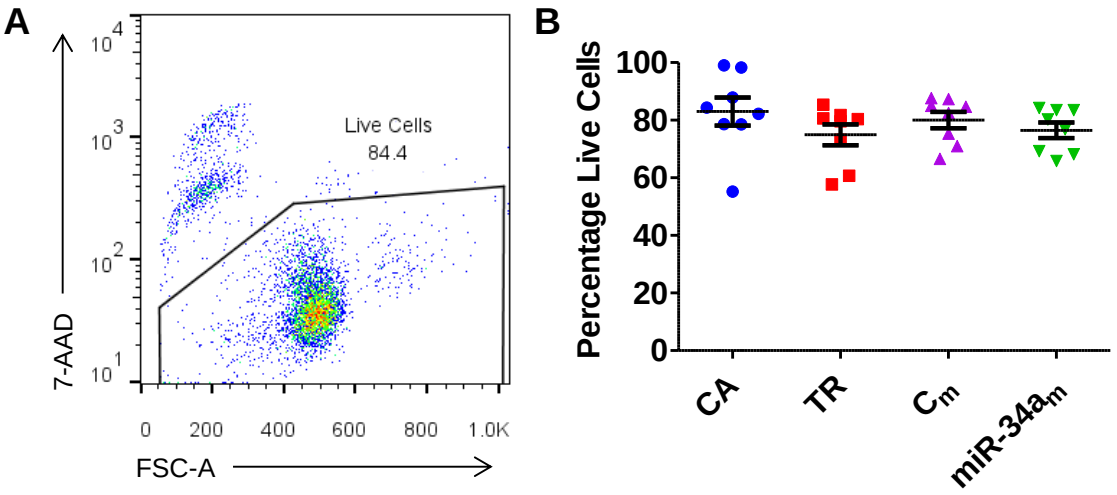


Figure 5-2 Monocyte cell viability after transfection
Monocytes were isolated from buffy coat samples and cultured for 24 hours in complete media alone (CA), with transfection reagents (TR), or with the control (C_m) or miR-34a (miR-34a_m) microRNA mimics before flow cytometry was performed to assess cell viability. Panel A shows a representative flow cytometry plot for cells stained with 7-AAD. The gate used to determine the percentage of live cells is also shown. The graph shown in panel B displays the cell viability for all conditions with the 8 individual donors. Each data point represents one donor. Data shown as mean plus standard error of the mean.

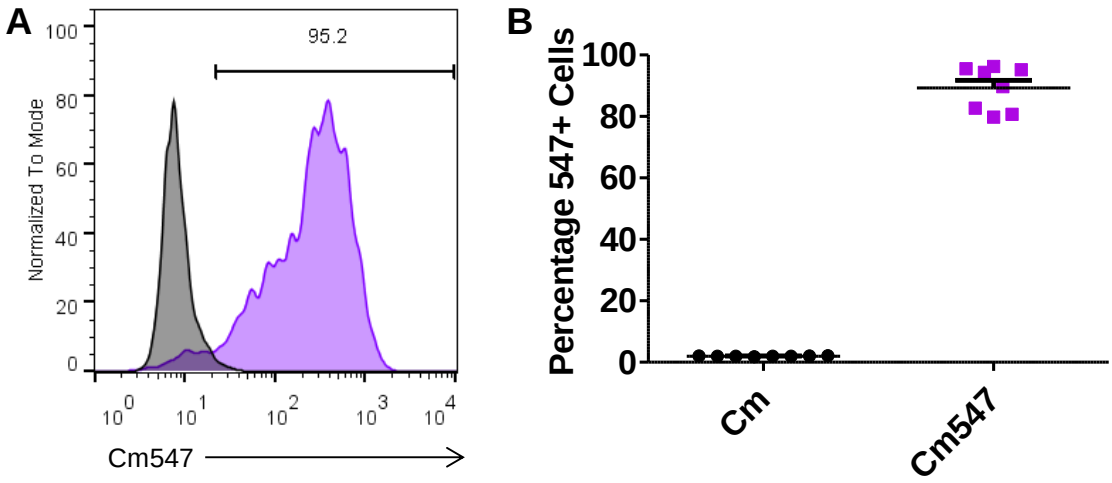


Figure 5-3 Transfection efficiency for monocytes as determined by uptake of a fluorescent-labelled mimic

$CD14^+$ monocytes were isolated from buffy coat samples and transfected with a regular control (Cm) or labelled control (Cm547) microRNA mimic. After 24 hours the cells were analysed via flow cytometry to measure uptake of the labelled mimic by the cells. Panel A shows a representative histogram where cells transfected with the unlabelled mimic are represented in grey and Cm547 transfected cells are represented in purple. The gate used to determine the percentage of transfected cells (Cm547+) is also shown. The graph shown in panel B displays the transfection efficiency for all 8 donors. Each data point represents one donor. Data shown as mean plus standard error of the mean.

5.2.2 Microarray and pathway analysis of control mimic and miR-34a mimic transfected monocytes.

RNA from the 10 chosen samples (5 control mimic transfected and 5 miR-34a mimic transfected) was sent to the Glasgow Polyomics Facility (GPF) where a microarray was performed to determine differentially expressed transcripts (GeneChip® Human Genome U133 Plus 2.0 Arrays were used).

The data generated by the GPF (CEL files) was analysed using GeneSpring. First we wanted to determine potential genes and pathways regulated by miR-34a. We performed Mann-Whitney test analysis that resulted in 4482 differentially expressed genes with p value less than 0.05 between control mimic and miR-34a mimic transfected cells. To narrow this down we chose to look at all transcripts greater than 2 fold up or down regulated in miR-34a transfected cells compared to control mimic transfected cells. This created a list of 117 differentially expressed transcripts. These transcripts are shown in the heat map in Figure 5-4, which was generated through unbiased clustering based on both the entities (genes) and conditions. This heat map groups genes and conditions based on the similarity of their expression profile and shows that indeed conditions (miR-34a mimic vs control mimic) segregate separately. Table 5-1 lists all of these transcripts and shows the exact fold change determined by the microarray analysis.

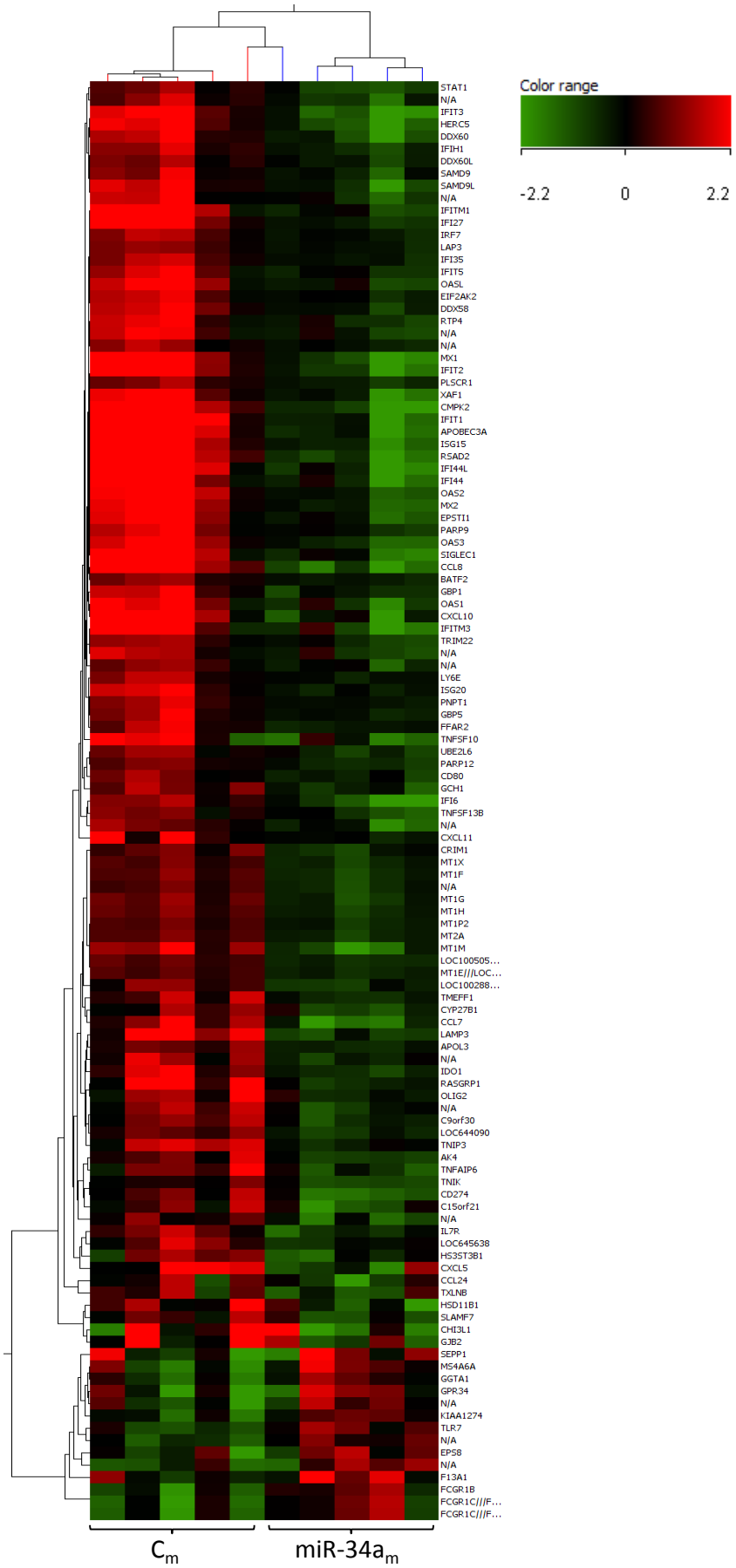


Figure 5-4 Heat map of transcripts differentially expressed after miR-34a over-expression
$CD14^+$ cells from 5 buffy coat donors were isolated and transfected with a control (C_m) or miR-34a ($miR\text{-}34a_m$) mimic for 24 hours. A microarray was performed on the isolated RNA, and a heat map was generated for all transcripts up or down-regulated 2 fold or greater (heat map was generated in GeneSpring using Pearson un-centred correlation with unbiased clustering).

Symbol	Entrez Gene Name	Fold Change
IFI44L	interferon-induced protein 44-like	-15.169
RSAD2	radical S-adenosyl methionine domain containing 2	-14.631
CCL8	chemokine (C-C motif) ligand 8	-13.250
IFIT1	interferon-induced protein with tetratricopeptide repeats 1	-12.865
CMPK2	cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial	-11.966
APOBEC3A	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3A	-10.449
MX1	MX dynamin-like GTPase 1	-9.731
IFIT2	interferon-induced protein with tetratricopeptide repeats 2	-8.401
CXCL10	chemokine (C-X-C motif) ligand 10	-7.744
IFIT3	interferon-induced protein with tetratricopeptide repeats 3	-7.564
ISG15	ISG15 ubiquitin-like modifier	-6.560
HERC5	HECT and RLD domain containing E3 ubiquitin protein ligase 5	-6.260
IFI44	interferon-induced protein 44	-6.207
IFITM1	interferon induced transmembrane protein 1	-5.520
OAS2	2'-5'-oligoadenylate synthetase 2, 69/71kDa	-5.418
CCL7	chemokine (C-C motif) ligand 7	-5.360
SIGLEC1	sialic acid binding Ig-like lectin 1, sialoadhesin	-5.166
IFI27	interferon, alpha-inducible protein 27	-5.123
MX2	MX dynamin-like GTPase 2	-5.106
OAS3	2'-5'-oligoadenylate synthetase 3, 100kDa	-4.925
LAMP3	lysosomal-associated membrane protein 3	-4.765
XAF1	XIAP associated factor 1	-4.731
MT1M	metallothionein 1M	-4.715
IFITM3	interferon induced transmembrane protein 3	-4.677
OAS1	2'-5'-oligoadenylate synthetase 1, 40/46kDa	-4.502
RASGRP1	RAS guanyl releasing protein 1 (calcium and DAG-regulated)	-4.491
EPSTI1	epithelial stromal interaction 1 (breast)	-4.265
DDX60	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60	-4.171
IFI6	interferon, alpha-inducible protein 6	-4.062
SAMD9L	sterile alpha motif domain containing 9-like	-4.042
TNFSF10	tumor necrosis factor (ligand) superfamily, member 10	-3.988
OASL	2'-5'-oligoadenylate synthetase-like	-3.823
CXCL5	chemokine (C-X-C motif) ligand 5	-3.691
IDO1	indoleamine 2,3-dioxygenase 1	-3.267
DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	-3.241
GBP1	guanylate binding protein 1, interferon-inducible	-3.200
CD274	CD274 molecule	-3.156
RTP4	receptor (chemosensory) transporter protein 4	-3.103
PARP9	poly (ADP-ribose) polymerase family, member 9	-2.941
ISG20	interferon stimulated exonuclease gene 20kDa	-2.932
IFIT5	interferon-induced protein with tetratricopeptide repeats 5	-2.836
TNIP3	TNFAIP3 interacting protein 3	-2.834
TNFAIP6	tumor necrosis factor, alpha-induced protein 6	-2.803
HSD11B1	hydroxysteroid (11-beta) dehydrogenase 1	-2.735
IFIH1	interferon induced with helicase C domain 1	-2.655
CHI3L1	chitinase 3-like 1 (cartilage glycoprotein-39)	-2.650
IL7R	interleukin 7 receptor	-2.611
STAT1	signal transducer and activator of transcription 1, 91kDa	-2.609
GCH1	GTP cyclohydrolase 1	-2.592
CXCL11	chemokine (C-X-C motif) ligand 11	-2.578
AK4	adenylate kinase 4	-2.549
MT1G	metallothionein 1G	-2.494
EIF2AK2	eukaryotic translation initiation factor 2-alpha kinase 2	-2.477
TRIM22	tripartite motif containing 22	-2.459
MSANTD3	Myb/SANT-like DNA-binding domain containing 3	-2.448

Table continued on next page…

Symbol	Entrez Gene Name	Fold Change
HMGN2P46	high mobility group nucleosomal binding domain 2 pseudogene 46	-2.445
GBP5	guanylate binding protein 5	-2.418
HS3ST3B1	heparan sulfate (glucosamine) 3-O-sulfotransferase 3B1	-2.406
SAMD9	sterile alpha motif domain containing 9	-2.391
TMEFF1	transmembrane protein with EGF-like and two follistatin-like domains 1	-2.381
IFI35	interferon-induced protein 35	-2.364
MT1F	metallothionein 1F	-2.351
GJB2	gap junction protein, beta 2, 26kDa	-2.345
TNFSF13B	tumor necrosis factor (ligand) superfamily, member 13b	-2.320
FFAR2	free fatty acid receptor 2	-2.319
MT1H	metallothionein 1H	-2.287
CYP27B1	cytochrome P450, family 27, subfamily B, polypeptide 1	-2.277
UBE2L6	ubiquitin-conjugating enzyme E2L 6	-2.274
IRF7	interferon regulatory factor 7	-2.261
PLSCR1	phospholipid scramblase 1	-2.249
LOC100288911	uncharacterized LOC100288911	-2.247
LOC644090	uncharacterized LOC644090	-2.242
OLIG2	oligodendrocyte lineage transcription factor 2	-2.220
MT1E	metallothionein 1E	-2.194
MT2A	metallothionein 2A	-2.181
PNPT1	polyribonucleotide nucleotidyltransferase 1	-2.179
CRIM1	cysteine rich transmembrane BMP regulator 1 (chordin-like)	-2.162
CCL24	chemokine (C-C motif) ligand 24	-2.157
DDX60L	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60-like	-2.155
MT1X	metallothionein 1X	-2.146
APOL3	apolipoprotein L, 3	-2.121
PARP12	poly (ADP-ribose) polymerase family, member 12	-2.118
WFDC21P	WAP four-disulfide core domain 21, pseudogene	-2.081
BATF2	basic leucine zipper transcription factor, ATF-like 2	-2.076
LY6E	lymphocyte antigen 6 complex, locus E	-2.060
SLAMF7	SLAM family member 7	-2.052
CD80	CD80 molecule	-2.050
TNIK	TRAF2 and NCK interacting kinase	-2.042
TXLNB	taxilin beta	-2.040
LAP3	leucine aminopeptidase 3	-2.021
MT1HL1	metallothionein 1H-like 1	-2.005
EPS8	epidermal growth factor receptor pathway substrate 8	2.040
SEPP1	selenoprotein P, plasma, 1	2.055
PALD1	phosphatase domain containing, paladin 1	2.069
GGTA1P	glycoprotein, alpha-galactosyltransferase 1 pseudogene	2.072
F13A1	coagulation factor XIII, A1 polypeptide	2.205
FCGR1A	Fc fragment of IgG, high affinity Ia, receptor (CD64)	2.243
FCGR1B	Fc fragment of IgG, high affinity Ib, receptor (CD64)	2.302
TLR7	toll-like receptor 7	2.327
FCGR1C	Fc fragment of IgG, high affinity Ic, receptor (CD64), pseudogene	2.334
MS4A6A	membrane-spanning 4-domains, subfamily A, member 6A	2.514
GPR34	G protein-coupled receptor 34	2.881

Table 5-1 List of transcripts differentially expressed after miR-34a over-expression
$CD14^+$ cells from 5 buffy coat donors were isolated and transfected with a control or miR-34a mimic for 24 hours. A microarray was performed on the isolated RNA, and the table above shows all transcripts which were differentially expressed two fold or greater in the miR-34a mimic transfected cells compared to control mimic transfected cells. Pathway analysis was performed, and those transcripts which are in the highly regulated pathways are highlighted. (green – interferon signalling pathway; blue – metallothionein pathway; red – toll like receptor and pattern recognition receptor pathways; purple - chemokines)

This list of differentially expressed transcripts was then further analysed in GeneSpring and Ingenuity pathway analysis software programmes, which came up with 3 pathways where it appears several members were differentially expressed. One of these was the interferon signalling pathway, where the transcription factor STAT1, and also several other IFN-inducible genes were down-regulated (highlighted in green in Table 5-1).

In mammalian cells there are hundreds of IFN-inducible or IFN-stimulated genes (ISGs), and while many of these have known roles in inhibiting viral replication, the exact function(s) of many of these ISGs remain to be fully elucidated. OAS1 is one of a family of 2-5A synthetase enzymes, which are involved in the activation of latent RNase L. Upon its recognition of viral RNA within a cell this enzyme becomes activated and results in the degradation of all RNA in the cell [393]. IFITM1 is an IFN-γ induced gene that plays a key role in the anti-proliferative action of IFN-γ [394], and MX1 has been shown to inhibit the transcription and replication of a number of viruses, including influenza A and hepatitis B [395-397]. Although the precise roles of many of the ISGs aren't fully understood, they are all up-regulated in response to stimulation of cells with IFNs, and thought to be involved in the anti-viral response. The pathway map generated in Ingenuity showing the differentially regulated transcripts is shown in Figure 5-5.

In order to validate these findings, PCR was performed on the same 5 samples used for the microarray, as well as samples obtained from an additional 6 donors. Figure 5-6 shows the results from the PCR, and confirms the microarray findings that these transcripts are all significantly down-regulated upon miR-34a over-expression in monocytes.

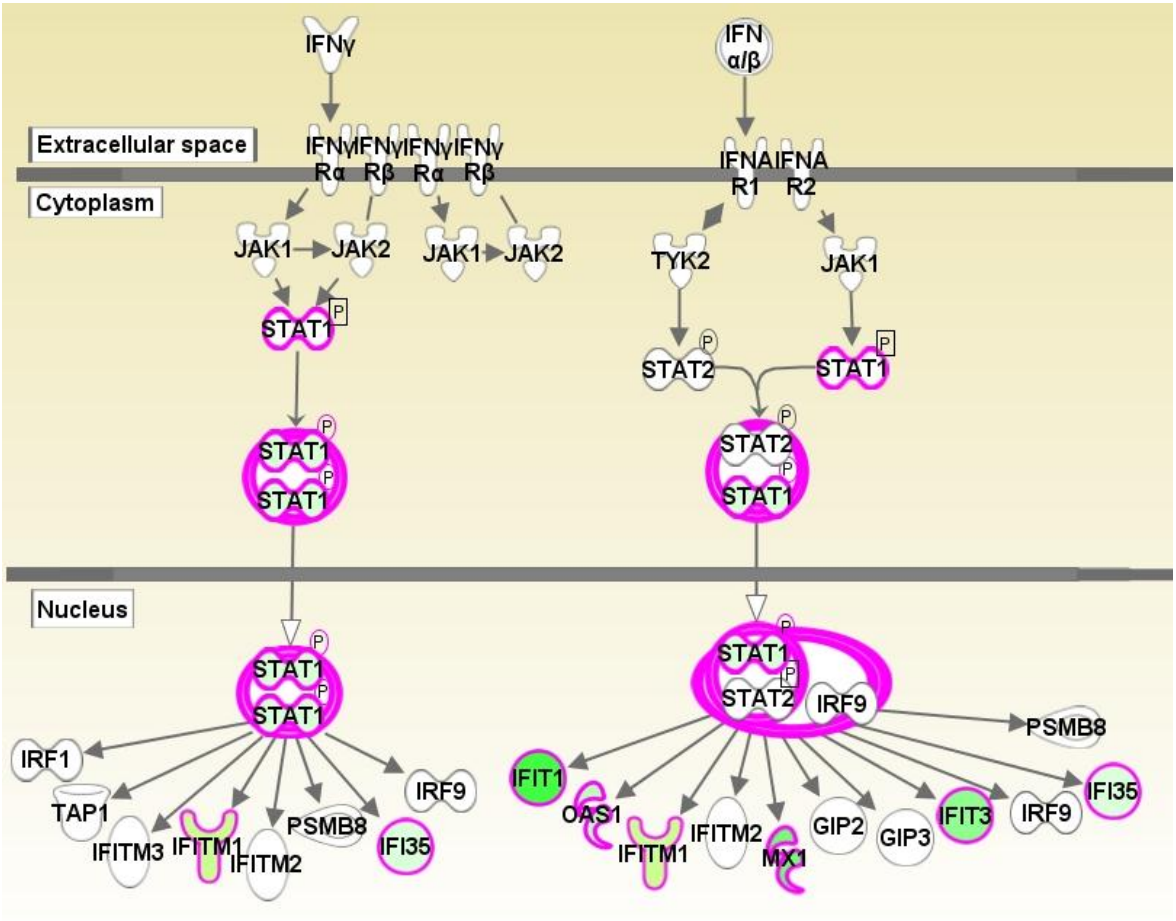


Figure 5-5 Many members of the interferon signalling pathway are differentially regulated upon miR-34a over-expression

$CD14^+$ cells were isolated from buffy coat samples and transfected with a control or miR-34a mimic for 24 hours. A microarray was performed on RNA samples from 5 donors and differentially expressed transcripts were analysed in Ingenuity pathway analysis software. The interferon signalling pathway was highlighted as a pathway with a number of differentially expressed members. Transcripts whose expression was down-regulated two fold or greater are outlined in pink.

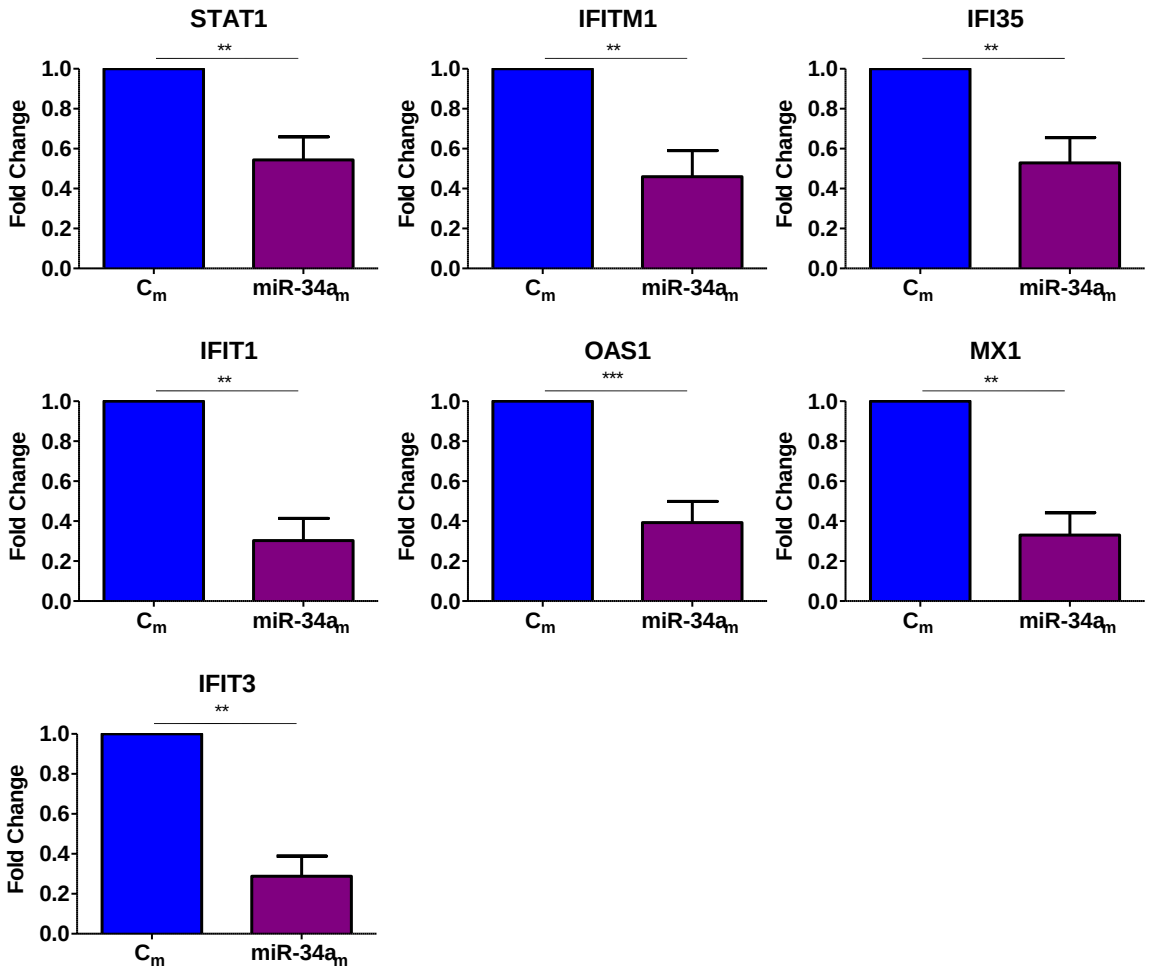


Figure 5-6 PCR validation of the differential expression of members of the IFN pathway upon miR-34a over-expression

$CD14^+$ cells were isolated from buffy coat samples and transfected with a control (C_m) or miR-34a (miR-$34a_m$) mimic for 24 hours. A microarray performed on RNA samples from 5 donors highlighted the differential expression of a number of IFN-inducible molecules. To validate these results PCR was performed on the same samples, plus those from an additional 6 donors. For each donor the transcript expression level of the control mimic transfected cells was normalised to 1, with miR-34a-transfected cells being shown as average fold change. Data shown as mean plus standard error of the mean. Paired t test or Wilcoxon matched-pairs signed rank test depending on whether data were normally distributed (determined by D'Agostino and Pearson omnibus normality test); ** $P < 0.01$, *** $p < 0.001$. n=11.

When analysing the differentially expressed transcripts in Ingenuity, another interesting pathway that was highlighted was the TLR signalling pathway (highlighted in red in Table 5-1 and pathway map shown in Figure 5-7). As mentioned in the introduction, pattern recognition receptors, such as TLRs, are found on the surface or in the cytoplasm of immune cells. These receptors are crucial for the innate immune response to invading microbial and viral pathogens. They recognise and respond to the presence of PAMPs such as the bacterial cell wall components lipopolysaccharide (LPS) and peptidoglycan, and also viral DNA and RNA. Herein, we show that following miR-34a over-expression, TLR7 appears up-regulated, while a number of other pattern recognition receptors are down-regulated. TLR7 is involved in the recognition of single stranded RNA, while RIG-1, MDA-5, PKR and OAS are involved in the recognition of double stranded RNA [398].

Once again, differentially expressed transcripts from the microarray were measured in samples obtained from 11 donors by PCR to validate these findings. The results are shown in Figure 5-8. IRF3 was initially measured as upon first glance it appeared highlighted in the pathway map in Figure 5-7. Upon closer inspection, however, it was clear IRF3/7 was highlighted due to down-regulation of IRF7, while IRF3 itself was unchanged. So here IRF3 serves as a negative control. These PCR results clearly demonstrate the differential expression of all other transcripts, confirming the results observed from the microarray analysis.

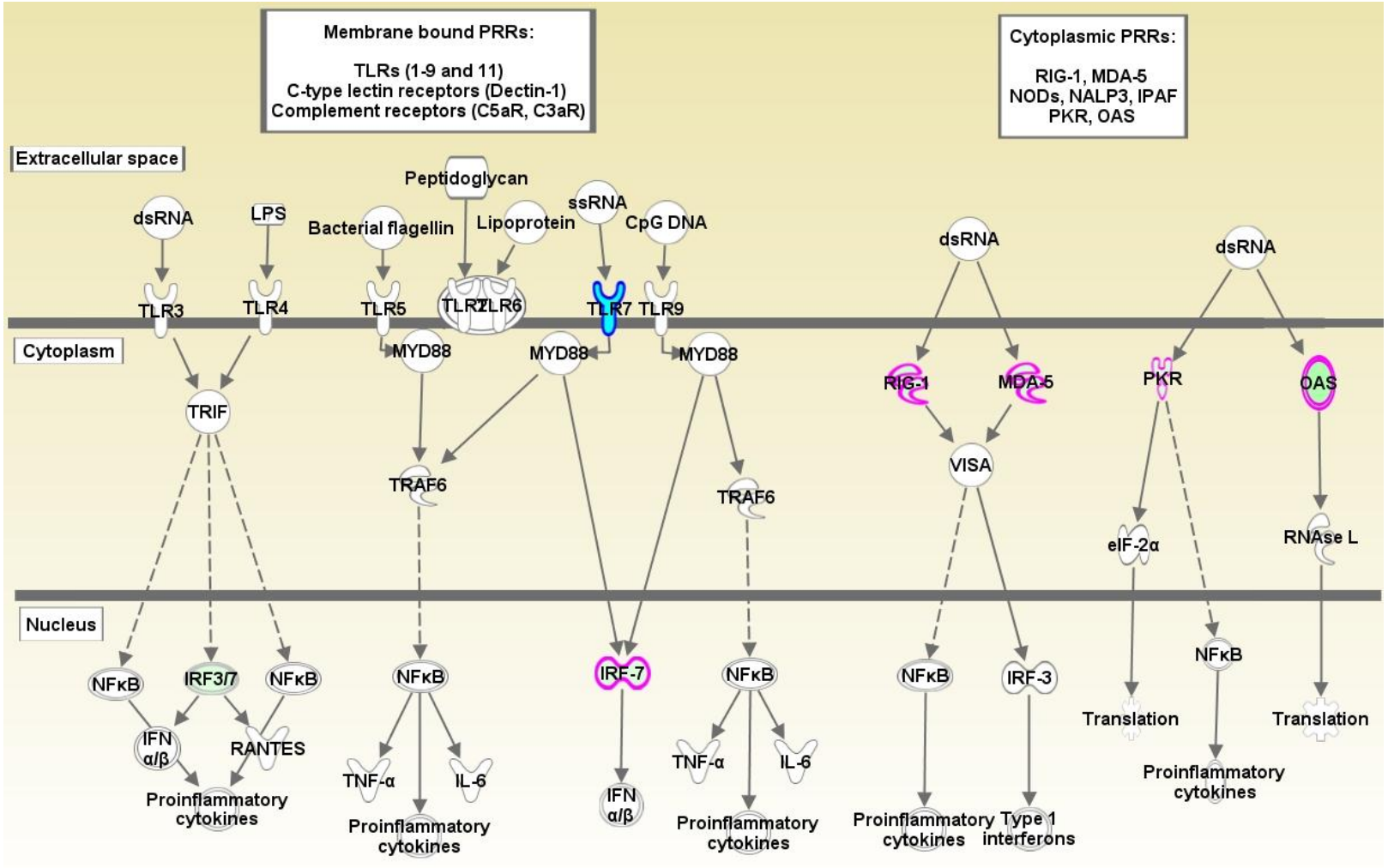


Figure 5-7 Many members of the toll-like receptor (TLR) signalling pathway are differentially regulated upon miR-34a over-expression
$CD14^+$ cells were isolated from buffy coat samples and transfected with a control or miR-34a mimic for 24 hours. A microarray was performed on RNA samples from 5 donors and differentially expressed transcripts were analysed in Ingenuity pathway analysis software. The TLR signalling pathway was highlighted as a pathway with a number of differentially expressed members. Transcripts whose expression was down-regulated two fold or greater are outlined in pink, while transcripts up-regulated two fold or great are highlighted and circled in blue.

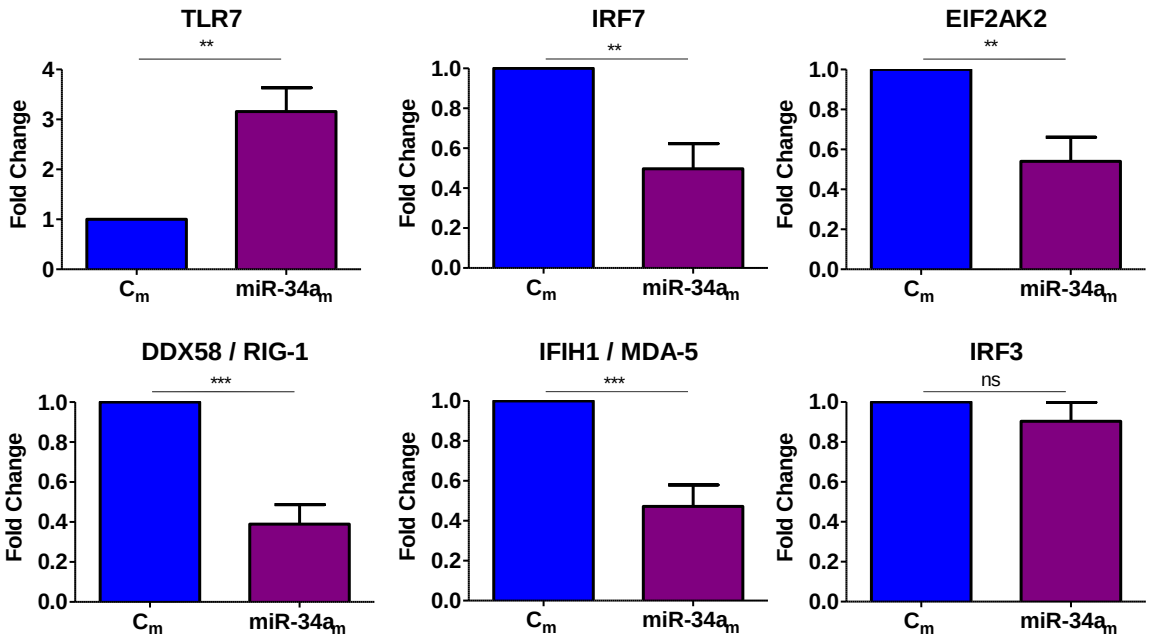


Figure 5-8 PCR validation of the differential expression of members of the TLR signalling pathway

$CD14^+$ cells were isolated from buffy coat samples and transfected with a control (C_m) or miR-34a (miR-$34a_m$) mimic for 24 hours. A microarray performed on RNA samples from 5 donors highlighted the differential expression of a number of members of the TLR signalling pathway. To validate these results PCR was performed on the same samples, plus those from an additional 6 donors. For each donor the transcript expression level of the control mimic transfected cells was normalised to 1, with miR-34a-transfected cells being shown as average fold change. Data shown as mean plus standard error of the mean. Paired t test or Wilcoxon matched-pairs signed rank test depending on whether data were normally distributed (determined by D'Agostino and Pearson omnibus normality test); ** $P < 0.01$, *** $p < 0.001$. n=11.

The list of differentially expressed transcripts was also analysed on GeneSpring pathway analysis, where the metallothionein pathway was highlighted. The pathway map with transcripts whose expression was down-regulated two fold or greater highlighted in blue is shown in Figure 5-9. Metallothioneins (MT) are a family of heavy-metal binding proteins whose primary roles are thought to be in maintaining heavy metal homeostasis [399-401] and protection from free radicals [402-404]. This said, their pathophysiological roles remain to be fully dissected and a number of studies are now suggesting potential roles in the regulation of immune responses.

Confirmatory PCR was performed on the same and additional samples in order to validate the findings from the microarray (graphs are shown in Figure 5-10). The results clearly demonstrate that all six metallothionein members measured are significantly down-regulated in miR-34a over-expressing monocytes, which confirms the results observed from the microarray analysis.

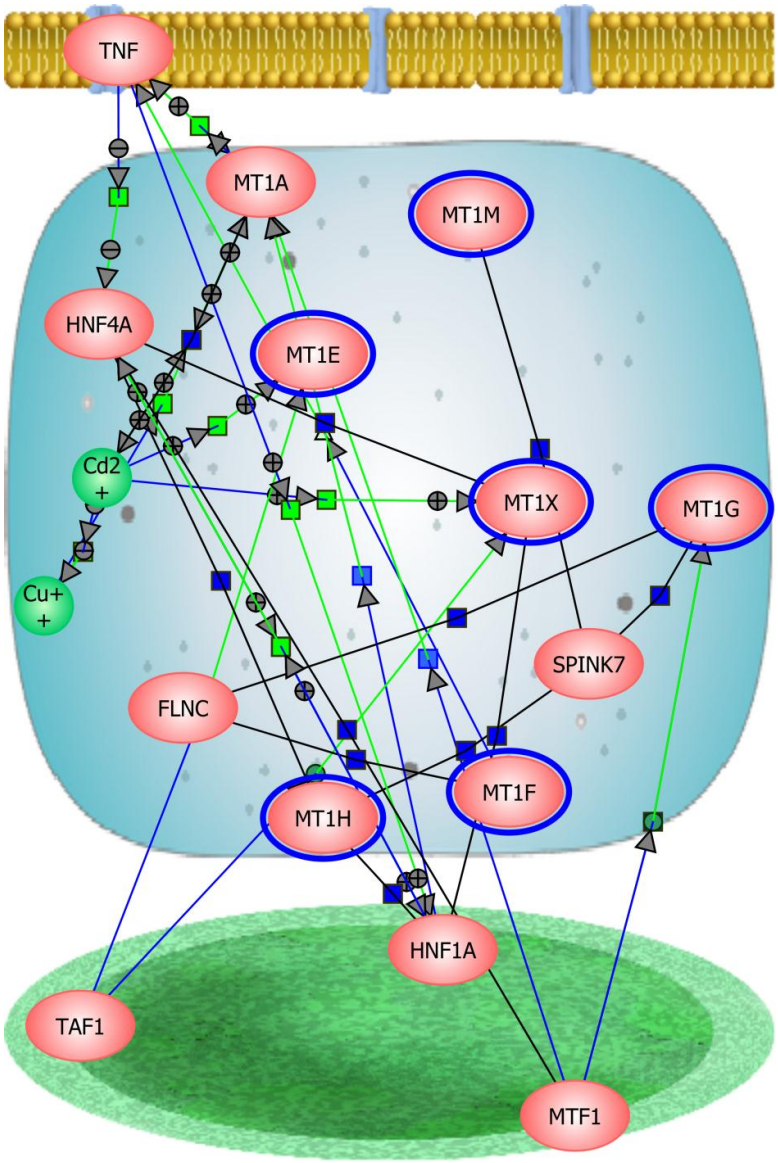


Figure 5-9 Many members of the metallothionein family are differentially regulated upon miR-34a over-expression

$CD14^+$ cells were isolated from buffy coat samples and transfected with a control or miR-34a mimic for 24 hours. A microarray was performed on the isolated RNA from samples from 5 donors and differentially expressed transcripts were analysed in GeneSpring pathway analysis. The metallothionein pathway was highlighted as a pathway with a number of differentially expressed members. Transcripts whose expression was down-regulated two fold or great are outlined in blue.

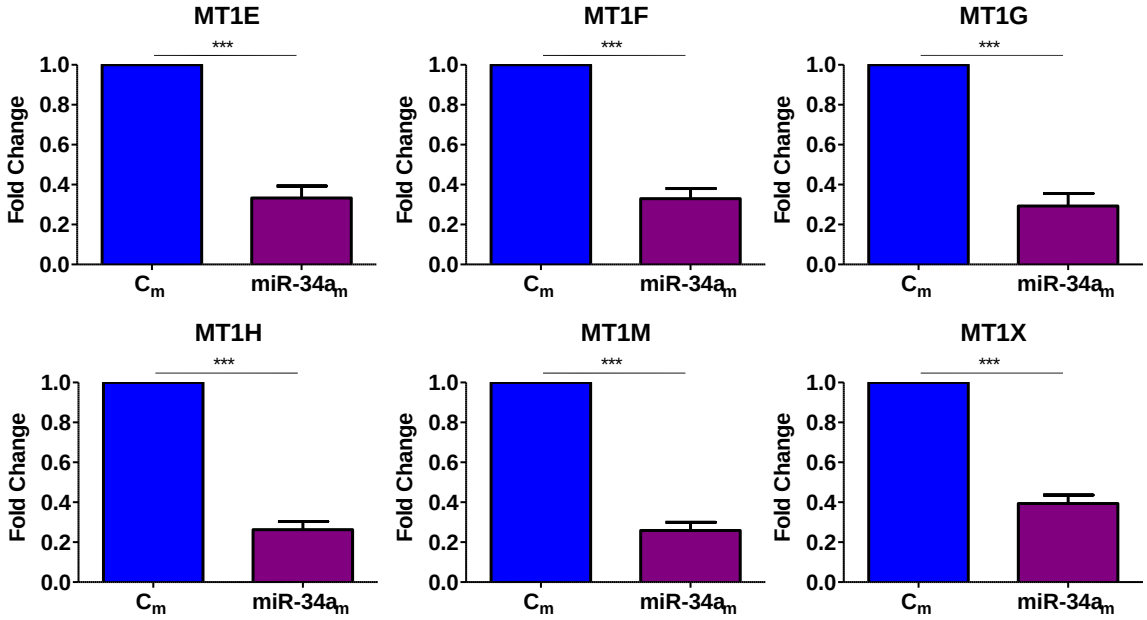


Figure 5-10 PCR validation of the differential expression of members of the metallothionein pathway

$CD14^+$ cells were isolated from buffy coat samples and transfected with a control (C_m) or miR-34a ($miR\text{-}34a_m$) mimic for 24 hours. A microarray performed on RNA from 5 donors highlighted the differential expression of a number of members of the metallothionein pathway. To validate these results PCR was performed on the same samples, plus those from an additional 6 donors. For each donor the transcript expression level of the control mimic transfected cells was normalised to 1, with miR-34a-transfected cells being shown as average fold change. Data shown as mean plus standard error of the mean. Paired t test; *** $p < 0.001$. n=11.

In addition to the pathways highlighted above, we also observed significant down-regulation of 6 members of the chemokine family (CCL8, CCL7, CCL24, CXCL5, CXCL10 and CXCL11 – highlighted in purple in Table 5-1) upon miR-34a up-regulation. This is of particular interest in the context of monocyte migration and/or retention at site of inflammation such a synovial tissue. Due to time constraints I was unable to investigate this fully but this lead will be followed up by other members of the lab.

5.3 Using the microarray to look for direct miR-34a targets in monocytes

Our analysis has demonstrated several pathways and molecules which appear to be differentially expressed upon miR-34a up-regulation. However, this type of analysis does not distinguish between direct miR-34a targets and indirect effects mediated via downstream effects upon inhibition of upstream regulatory mRNAs. In order to establish the direct targets of miR-34a, the microarray data were re-analysed. This time looking at only ranked down-regulated transcripts, and using a cut-off point of Mann Whitney P value lower than 0.05. We chose this method as many miRNA targets, such as transcription factors, do not need to be changed dramatically in expression to have significant effects on the cells. This analysis provided us with 2657 down-regulated transcripts. We then looked at the list of predicted miR-34a targets on TargetScan, and found that there were 61 transcripts which are down-regulated upon miR-34a over-expression that have predicted miR-34a binding sites. Figure 5-11A shows a Venn diagram displaying the overlap of down-regulated transcripts containing predicted miR-34a binding sites, while Figure 5-11B shows a heat map of those 61 transcripts.

Once again we wished to validate the microarray findings, so chose candidate transcripts of interest to measure by PCR on the samples. We chose MTF1 and IRF1 because of their link with the dysregulated pathways discussed above (IFN and metallothionein pathways, respectively) as well as CSF1R and SIRT6. We were interested in CSF1R as in chapter 3 we demonstrated that miR-34a expression was increased during the M-CSF maturation of peripheral blood $CD14^+$ cells, and because of its obvious importance in monocyte and macrophage biology. SIRT6 stood out as a potential molecule of importance as it has been implicated in regulating NFκB-mediated immune responses [405], and SIRT6 over-expression in murine CIA resulted in reduced disease severity and a lower production of both local and systemic pro-inflammatory cytokines [406, 407]. As demonstrated in Figure 5-12, of these four candidate transcripts, IRF1, MTF1 and CSF1R were significantly down-regulated by PCR, while the expression of SIRT6 appeared unchanged.

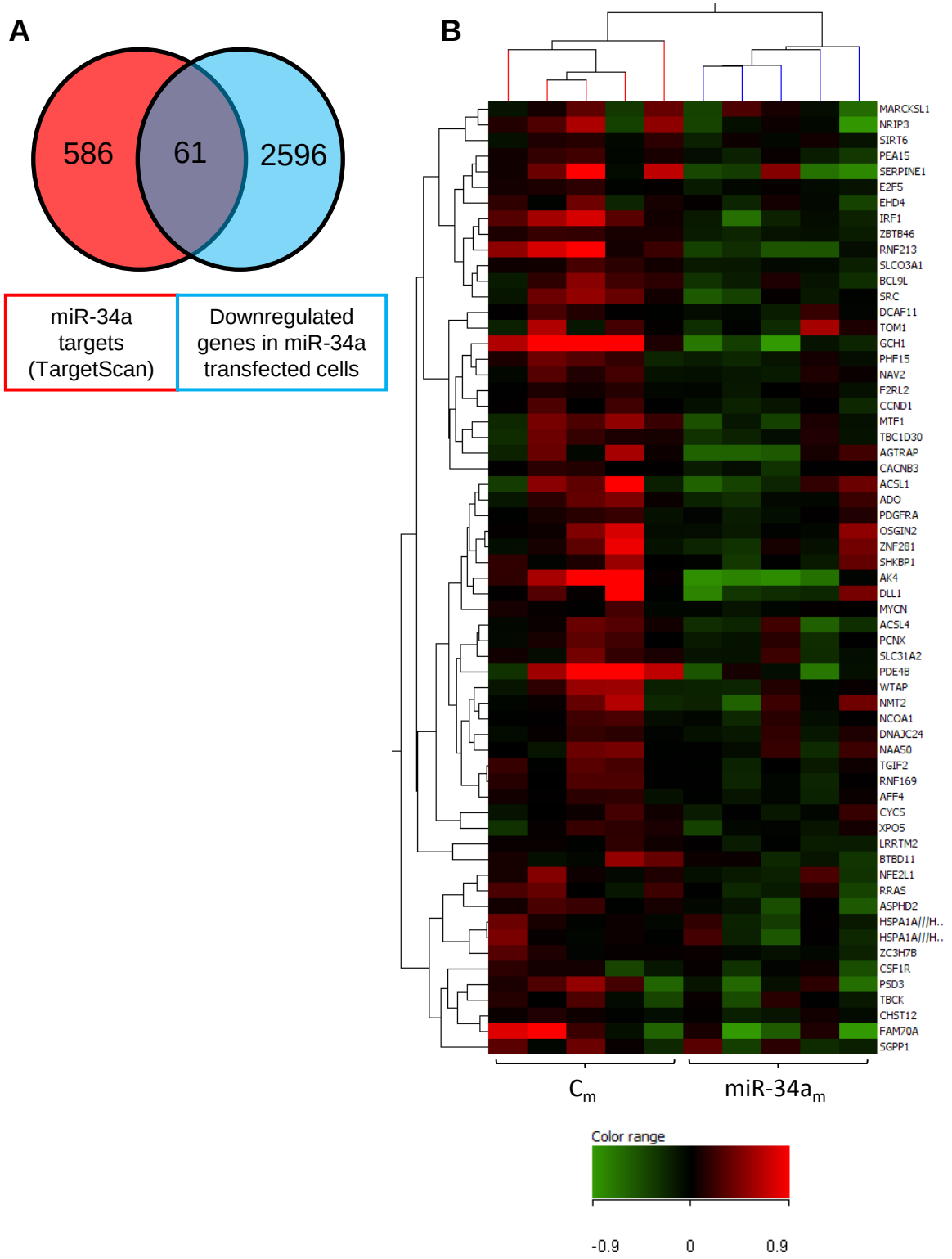


Figure 5-11 61 transcripts significantly downregulated in monocytes upon miR-34a upregulation that contain potential miR-34a binding sites

CD14^{+} cells were isolated from buffy coat samples and transfected with a control (C_m) or miR-34a (miR-34a_m) mimic for 24 hours. A microarray was performed on the RNA samples from 5 donors and the list of down-regulated transcripts compared to the list of potential miR-34a targets on TargetScan. Panel A shows a Venn diagram displaying the overlap of down-regulated transcripts containing a predicted miR-34a binding site, while panel B is a heat map of those 61 transcripts.

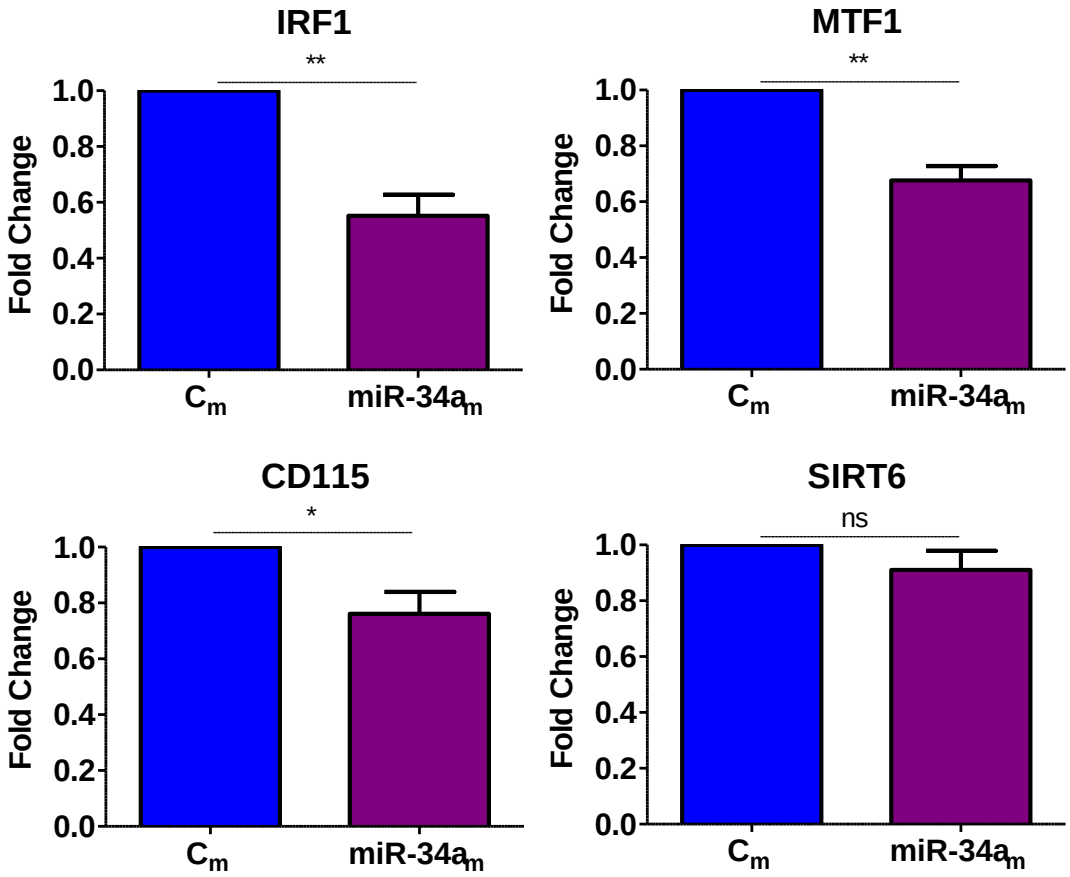


Figure 5-12 PCR validation of potential miR-34a targets
$CD14^+$ cells were isolated from buffy coat samples and transfected with a control (C_m) or miR-34a (miR-$34a_m$) mimic for 24 hours. A microarray was performed on the RNA samples, and down-regulated transcripts compared to the list of potential miR-34a targets on TargetScan. This gave a total of 61 transcripts which contained potential miR-34a binding sites and were also down-regulated upon miR-34a up-regulation. Above is the PCR validation of 4 of these transcripts. For each donor the transcript expression level of the control mimic transfected cells was normalised to 1, with miR-34a-transfected cells being shown as average fold change. Data shown as mean plus standard error of the mean. Paired t test; * $p < 0.05$, ** $p < 0.01$. n=5.

Microarray, PCR data and prediction algorithms suggest that IRF1, MTF1 and CSF1R could be direct targets of miR-34a. To validate this functionally, we performed luciferase assays. For each potential target, a section of the 3'UTR (containing the predicted binding site for miR-34a) was cloned into expression vectors downstream from a luciferase gene. These constructs were then transfected into HEK 293 cells along with 25 nM control or miR-34a mimic, and luciferase activity measured 24 hours later to determine whether binding occurs. Where miR-34a binds its predicted target a drop in luciferase activity is observed. TargetScan suggests CSF1R and IRF1 have one potential miR-34a binding site in their 3'UTR, while MTF1 has two – so two separate plasmids were generated for MTF1, each containing one of the binding sites. A luciferase assay was also performed for Notch1 as a positive control, as this has previously been validated as a miR-34a target by luciferase assay in several other studies [408-410]. The results from the luciferase assays are shown in Figure 5-13, and demonstrate significant down-regulation of luciferase activity by miR-34a when co-transfected with the Notch1 and CSF1R plasmids, but not with IRF1 or MTF1. These data suggest that IRF1 and MTF1 are not genuine direct miR-34a targets, while CSF1R and Notch1 most likely are.

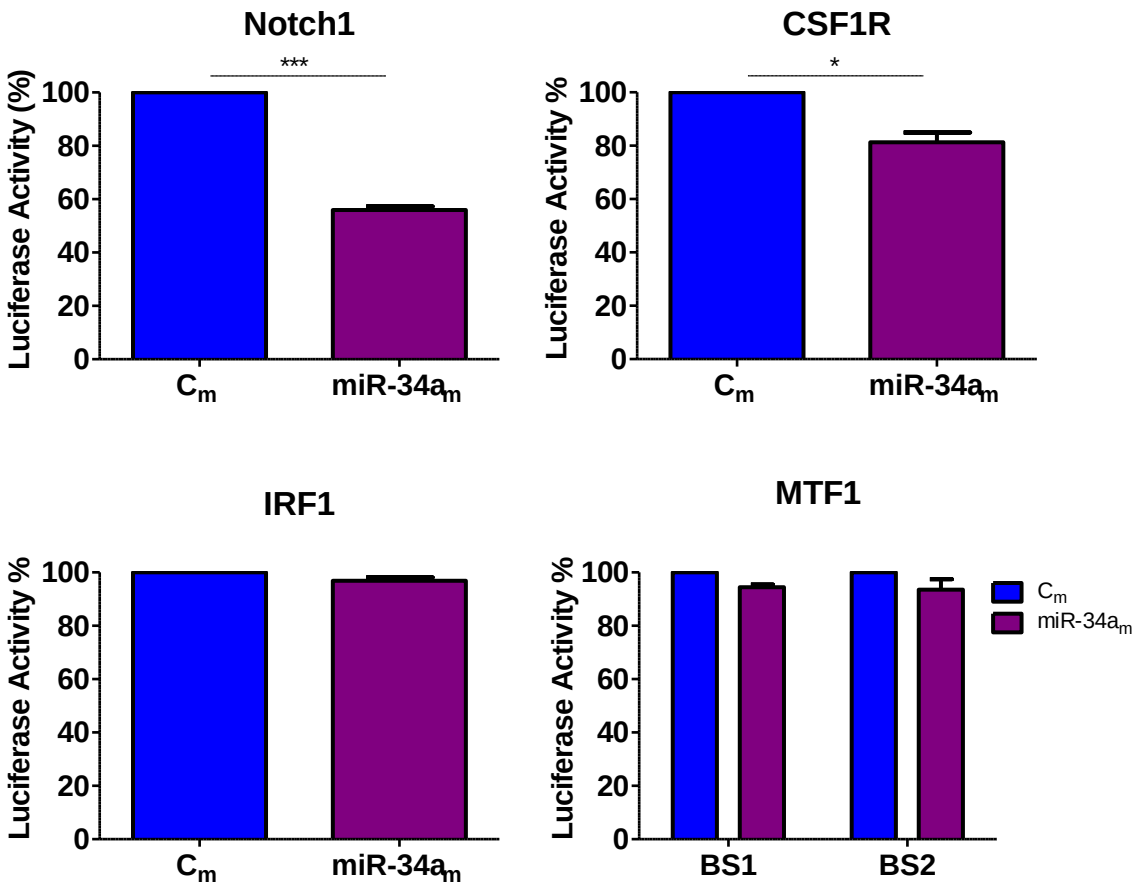


Figure 5-13 Luciferase validation of potential miR-34a targets
Luciferase assays were performed to determine whether miR-34a binds its predicted targets; Notch1 (positive control), CSF1R, IRF1 or MTF1 (which contains two potential miR-34a binding sites – BS1 and BS2). For each target, a section of the 3'UTR containing the biding site(s) for microRNA-34a was cloned into an expression vector downstream from a luciferase gene. These constructs were then transfected into HEK 293 cells along with 25 nM control (C_m) or miR-34a (miR-34a_m) mimic. Luciferase activity was then measured and normalised to the endogenous control (renilla). Data represents mean plus standard error of the mean. n = 3 technical replicates. Paired t test; * $P < 0.05$, *** $P < 0.001$.

5.4 Discussion

The main aim of this part of the study was to gain an insight into the potential pathways regulated by miR-34a in monocytes, and see if we could infer from this the functional impact of the increased miR-34a expression we have observed in myeloid cells in the periphery and synovium of RA patients, or to other important role(s) for miR-34a in monocyte and macrophage function.

We choose to use a miR-34a over-expression system, because we aimed to mimic high levels of miR-34a in the RA synovial fluid $CD14^+$ cells, and in monocytes upon M-CSF stimulation. Also, many studies, including one study looking at miR-140 in cartilage development [411], have shown that over-expression of microRNA using microRNA mimics achieves a greater change in target gene expression than the use of microRNA inhibitors. So we felt this was the optimal approach for novel pathway discovery, particularly given the small sample size.

The key finding from this section of work was that upon miR-34a overexpression in monocytes, we can identify novel pathways that are regulated by the microRNA. Of particular interest, upon miR-34a over-expression we observed possible regulation of IFN, PRR and metallothionein pathways. These pathways could also be involved in the activation of monocyte-derived macrophages and dendritic cells – which will be further explored by other members of the laboratory in due course as time precluded my exploring all of these in the context of my thesis.

5.4.1 Possible implications of dysregulation of PRR and IFN pathways

The down-regulation of many PRRs and ISGs could render cells less capable of detecting and responding to viral infections. Due to the significant regulation of these pathways, we were interested in studying the effect of miR-34a dysregulation on the response of cells to viral infection. Some preliminary experiments were performed (collaboration with Dr Clive McKimmie using Semliki forest virus), however, we encountered some technical problems when trying to infect cells which had previously been transfected with microRNA mimics. Consistent with our hypothesis, miR-34a has recently been linked to

viral infection. Its expression is induced during Epstein-Barr virus (EBV) infection of primary human B cells, while its inhibition resulted in impaired growth of EBV-transformed cells [412]. The potential role of miR-34a in the regulation of cell response to viruses will be further investigated in the laboratory in miR-34a$^{-/-}$ mice.

STAT1, the key signalling molecule involved in up-regulating genes in response to IFNs, and IRF7, a key regulator of the type I IFN (IFNα/ß) response, are both down-regulated in response to miR-34a overexpression. This would suggest that the response of these cells to IFNs, and possibly IFNα and ß production by miR-34a over-expressing cells may be impaired. Consistent with this, in the previous chapter we demonstrated decreased IFNα production in response to Cl097 stimulation of miR-34a over-expressing macrophages, but saw no difference in IFNα when miR-34a was inhibited. For this reason I would like to repeat these experiments using a range of inhibitor concentrations. This would control for inefficient miR-34a inhibition that could mean we do not see the full effect of its knockdown.

It is well documented that IFNs up-regulate MHC class II and co-stimulatory molecules on APCs, facilitating their activation of antigen-specific T cells [413, 414]. Interestingly, alongside STAT1 and IRF7, my data also demonstrated down-regulation of CD80. However, the flow cytometry data on human macrophages transfected with miR-34a mimics and inhibitors in the previous chapter showed no effect of miR-34a manipulation on MHC class II or FcγR expression. It would therefore be interesting to repeat these experiments and stimulate cells with IFNs and/or TLR ligands, and include markers to look at the expression of co-stimulatory molecules, such as CD80 and CD86.

Another key role of IFNs is to induce specific chemokine signatures. In the array here, we demonstrate down-regulation of several chemokines upon miR-34a over-expression; including CCL8, CXCL10, CCL7, CXCL5, CXCL11 and CCL24, many of which are IFN-inducible [415-419]. Taken together these data suggest a key role for miR-34a in the induction of IFNs, and the subsequent immune responses, including chemokine-mediated inflammatory cell recruitment. Therefore, some of this work will be studied in a murine setting where these hypotheses can be further explored more easily.

Also interesting in this set of results is the up-regulation of TLR7 in miR-34a mimic transfected cells. In chapter 4 we demonstrated that transfection of cells with a miR-34a mimic actually results in reduced cytokine production in response to simulation with the TLR7/8 agonist Cl097. These data suggest that it is downstream effectors, such as signalling molecules that cause miR-34a's negative effect on cytokine production, rather than an effect on TLR expression itself. However, data from another member of our lab have shown that miR-34a over-expression increases TLR7 expression on monocyte-derived dendritic cells, which may in part be involved in the increased production of TNF-α upon TLR7 stimulation of these cells. These date indicate that miR-34a might have different target engagements depending on the on the particular cell type, even between monocyte-derived macrophages and dendritic cells.

Interestingly, increased expression of STAT1 and IFN inducible genes is well documented in RA [420-422]. There is an increasing literature base looking at the relationship between the IFN signature and the response to therapy in patients, with contradictory findings. There are studies suggesting that a high IFN signature in patients is predictive of a poor response to treatment with rituximab [423] and infliximab [424], while another study found that an elevated type I IFN signature predicted a good response to anti-TNF therapy. This is interesting, as in chapter 3 we demonstrated increased miR-34a levels in our multiple drug resistant patients. Thus, it would be interesting to look at the IFN signature in peripheral blood monocytes of these patients.

5.4.2 Possible implications of targeting metallothionein pathway dysregulation

As mentioned previously, metallothioneins are a family of heavy-metal binding proteins that are thought to be primarily involved in maintaining heavy metal homeostasis [399-401], and protection from free radicals [402-404]. However, a number of more recent studies suggest an important role for MT in immune responses. MT expression is induced by a number of pro-inflammatory cytokines, including IL-1, IL-6 and IFN-γ [425-427], and $MT^{-/-}$ mice fair worse in a number of models of inflammatory disease. One example of this is in an LPS-induced model of acute lung injury, where $MT^{-/-}$ mice had a greater degree of lung edema and neutrophil infiltration to the lung [428]. Relevant to the context of rheumatoid

arthritis, one group decided to test the effect of MT on the murine CIA model. They found that administration of two different isoforms of MT - MTI and MTII - dramatically reduced both the clinical and histological symptoms of disease, and that expression of the pro-inflammatory mediators TNF-α and Cox-2 were significantly lower in the injected joints of MT-injected mice compared to un-injected controls [429]. A mutation in the metallothionein gene has been associated with diabetes and its cardiovascular complications [430], and most importantly recent meta-analysis reveal a strong association between a SNP in the 3'UTR of MTF-1 and RA [25]. The down-regulation of many metallothionein family members by miR-34a up-regulation in monocytes, therefore, could also suggest a pro-inflammatory effect on cells, although this does not agree with our monocyte derived-macrophage cytokine data. The link between miR-34a and the metallothionein pathway will be investigated further in the lab in the context of monocyte-derived dendritic cells activation.

5.4.3 **Targets**

In an attempt to find direct miR-34a targets among genes down-regulated in monocytes overexpressing miR-34a, we compared down-regulated transcripts with predicted miR-34a targets found on TargetScan. This method resulted in a list of 61 transcripts which are down-regulated upon miR-34a over expression and have predicted miR-34a binding sites in their 3'UTRs. Supporting the use of this method, many of the molecules highlighted in these experiments are already verified miR-34a targets – including PDGFRA [431, 432], ACSL1 [433], CCND1 [434], MYCN [351] and DLL1 [435].

Of the 61 transcripts highlighted using this method, MTF1 (a master transcription factor regulating the expression of all members of the MT family), IRF1 (a transcription factor first recognised for its ability to regulate IFNβ expression), SIRT6 (a histone deacetylase linked to the regulation of NFκB-induced inflammation) and CSF1R (the receptor for M-CSF, which is involved in macrophage differentiation and function) were of particular interest in the context of myeloid cell biology and the pathways we found to be regulated by miR-34a over-expression in monocytes. We therefore initially chose these four to validate by PCR and luciferase assays. Of these 4, the only one which was down-regulated by PCR validation, and also gave a positive luciferase result was

CSF1-R. Although this receptor is targeted by miR-34a, we provide data in chapter 4 showing that altering the miR-34a expression of 3 day M-CSF matured human PB $CD14^+$ cells had no effect on CD16, CD64 or HLA-DR expression 24 hours post transfection. It is however possible that an effect on macrophage phenotype and maturation would manifest later than 24 hours after miR-34a manipulation, so given more time it would be interesting to examine the consequences of increased and decreased miR-34a expression at later time points.

The negative luciferase result for MTF1 and IRF1 mean that while we observe significant regulation of interferon and metallothionein pathways following miR-34a over-expression in monocytes, we are currently unsure what miR-34a targets are responsible for these changes. One possibility is that the changes in MT and IFN pathways may result from a direct targeting of IFNß by miR-34a [436]. Given more time I would investigate this by repeating the miR-34a over-expression experiments followed by PCR, but this time also using target protectors to prevent miR-34a targeting of IFNß.

One of the key limitations to this study was the use of miR-34a mimics to pull out potential targets of miR-34a in monocytes. Although microRNA mimics are widely used in microRNA research, they do have a number of potential flaws. It is estimated that the majority of microRNA fluctuate in expression by around 20-30%, but the levels of expression reached after transient transfection with mimics are much higher than this. These supraphysiological levels of expression could lead to false-positive results, where the artificial levels of expression allow a microRNA to target low affinity targets that would not be regulated under normal conditions [365]. This said, it is unlikely that much of the transfected microRNA is actually incorporated into RISC complexes allowing it to become functionally active. There are actually a number of current studies supporting the use of microRNA mimics followed by microarray analysis to find pathways and targets regulated by microRNA. One of these studies transfected a miR-124 mimic into HeLa cells and found that 76% of down-regulated transcripts contained potential miR-124 seed matches in their 3' UTR. When the experiment was repeated using a miR-124 mimic with a mutated seed region, no such enrichment of potential binding sites was found [261]. We therefore chose

to proceed with this method, while being aware of the potential flaws and knowing that additional methods would be required for proper validation.

Another problem was the choice of monocytes as the cell type for transfection. While these are the best cells to study miR-34a over-expression, as they have low endogenous levels, we have seen on several occasions during this project that microRNA targets are very cell and time specific. This means these results are useful for our first aim – to discover possible pathways regulated when miR-34a expression is increased in monocytes, but the data may not give an insight into possible pathways responsible for the difference in cytokine production we observed in Chapter 4 when miR-34a expression was manipulated in macrophages.

Given more time it would therefore be useful to perform similar profiling experiments, but this time use miR-34a inhibitors in monocyte-derived macrophages to see the direct effects of down-regulating endogenous miR-34a levels. This may provide increased confidence in the transcript over-expression data, and provide clues to the cause(s) of the cytokine changes observed in macrophages.

Chapter 6 – Analysis of miR-34a knockout mice

6.1 Introduction and Aims

Although major advances have been made towards understanding microRNA biology, many of their physiological and pathophysiological roles remain largely unknown. In an attempt to better understand the roles of microRNA *in vivo*, many investigators have chosen to study loss of function in microRNA knockout mice. To examine the role of microRNA as a whole, mice lacking some of the key microRNA-processing factors were generated, but lack of Dicer, Drosha or Ago2 individually in mice was embryonically lethal [269-271], so conditional knockouts had to be generated. When dicer-1 was knocked down in embryonic stem cells alone, although viable, cells displayed severe defects in differentiation [272]. The deletion of either Drosha or Dicer in T cells only resulted in spontaneous inflammatory disease, while deletion specifically in the regulatory T cell lineage caused defective Foxp3 induction, and impaired the cells suppressive capabilities [437]. These studies highlight the importance of microRNA in general in cell differentiation and function, but do not help determine which microRNA are responsible.

To look at the potential roles of specific microRNA a number of individual microRNA or microRNA family mice strain knockouts have been generated. Some have no obvious phenotype (including miR-182, miR-208b and miR-499 [438, 439]), while others result in partial embryonic lethality (miR-1-2 and miR-126 [440-442]). There are also cases where microRNA have been shown to exhibit redundancy between family members that share identical or highly similar seed regions. This is the case for miR-133a-1 and miR-133a-2. Deletion of either of these microRNA alone has no obvious phenotype, but deletion of both simultaneously results in embryonic lethality in approximately 50% of mice [443]. There are also some examples of remarkable and very obvious phenotypes in single microRNA knockout mice, suggesting important immune-regulatory functions of particular microRNA. For example, miR-146a null mice spontaneously develop autoimmunity, indicating the importance of this miRNA in the broad regulation of immune responses [280]. It may be reasonably considered that some microRNA function as master regulators, while others are simply fine tuners of the mRNA and processes they regulate.

In the case of microRNA-34a, two individual groups have successfully developed knockout mice. In both reports mice were born at the expected Mendelian ratio, with no obvious developmental abnormalities [332, 444]. Because of the link between miR-34 microRNAs and p53 in human studies, one group focussed on the effect of miR-34 ablation on p53-dependant responses. Concepcion *et al* show no obvious effects of miR-34 knockout on three well-characterised p53-dependant processes – response to DNA damage, replicative senescence or response to oncogene activation. A number of independent groups had reported a key role for miR-34 in the p53 pathway, but this report suggests, at least in mice, that miR-34a is not completely necessary for p53 function *in vivo* [444].

The other group focused on another process that is also reported to implicate p53 – somatic cell reprogramming [332]. This is a process whereby differentiated somatic cells are induced to generate pluripotent stem cells (iPSCs) [182]. This process has previously been shown to be enhanced in p53 null mice [183, 184], and this study demonstrated that somatic reprogramming is also enhanced in cells from miR-34a knockout mice. They went on the show direct targeting by miR-34a of 3 transcription factors involved in maintaining the self-renewal and pluripotency of ESCs - Nanog, Sox2 and N-Myc.

Neither of these groups focussed on immune cell phenotype or function, so given the effect of miR-34a on human macrophage activation described in Chapter 5, we decided to obtain miR-34a knockout mice and perform some studies looking from an immunological view point. In this chapter we will look at monocytes and macrophages in the unchallenged mice, and also go on to challenge miR-34a null cells *in vitro* to see if complete absence of miR-34a in murine macrophages resembles the results we achieved upon artificial manipulation of miR-34a in human macrophages. The knockout mouse will also allow us to subject the mouse to immunological stresses and observe any effect of absent miR-34a in the organism as a whole *in vivo*.

6.2 Quantity and phenotype of monocytes in resting WT and miR-34a knockout mice

Before subjecting murine cells to any stimuli we wanted to check whether there were any underlying differences in the number or phenotype of the basic monocyte populations in the spleen, blood or bone marrow of the miR-34a knockout mice compared to wild type mice. To do this we chose to analyse the cells by flow cytometry using a gating strategy similar to that frequently used by the Geissmann group [445]. Figure 6-1 shows a representative gating strategy used for one sample. Firstly, a gate was drawn around the cells of interest (most importantly this gate excludes cellular debris and includes monocytes), then doublets and dead cells were excluded before a gate was drawn to enumerate the percentage of all monocytes that are positive for both CD115 (CSF1R) and CD11b. Finally, Figure 6-1E shows the gating strategy used to determine the proportion of the two main murine monocyte sub-populations: $Ly6C^{-}$ and $Ly6C^{hi}$ cells. As demonstrated in Figure 6-2, the total number of $CD11b^{+}$ $CD115^{+}$ monocytes in the blood, spleen and bone marrow does not differ between WT and miR-34a knockout mice. Next, we went on to look at the proportion of the two main monocyte sub-populations found in these locations. We took the $CD11b^{+}$ $CD115^{+}$ cells and divided them based on their Ly6C expression to $Ly6C^{-}$ and $Ly6C^{hi}$. The $Ly6C^{hi}$ monocytes or "inflammatory monocytes" are short-lived cells which are recruited to inflamed or infected tissues where they can differentiate into antigen-presenting cells and produce TNF, nitric oxide and reactive oxygen species. The $Ly6C^{-}$ monocytes or "patrolling monocytes" migrate over the resting vasculature, and are thought to be the precursor of some resident myeloid cells [203]. Figure 6-3 shows the proportion of $Ly6C^{-}$ and $Ly6C^{hi}$ cells as a percentage of total $CD11b^{+}$ $CD115^{+}$ monocytes in the blood, spleen and bone marrow of WT and miR-34a knockout mice. Here we have shown that despite targeting of CD115 by miR-34a (chapter 5 and [357]), no differences are found in the total percentage of monocytes, or in the percentage of $Ly6C^{-}$ or $Ly6C^{hi}$ cells in the blood, spleen or bone marrow of WT and miR-34a knockout mice.

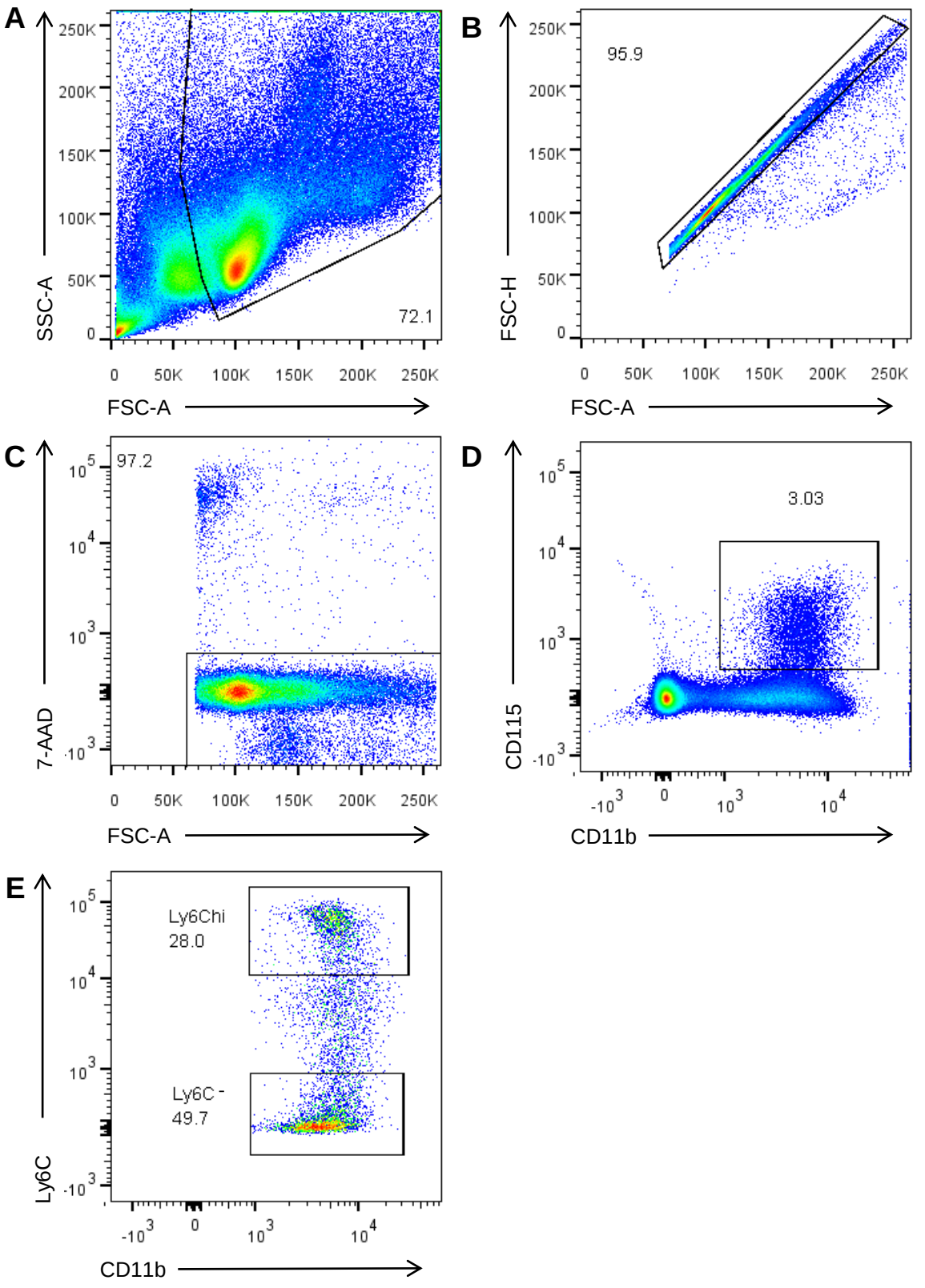


Figure 6-1 Gating strategy used to quantify the percentage of monocytes in murine blood, spleen and bone marrow

Cells were isolated from the blood, spleen and bone marrow of mice and this gating strategy was used to quantify the total number of monocytes, and also the percentage of Ly6C^{hi} and Ly6C^{-} cells. Firstly, a gate was drawn around the cells of interest (A), then doublets and dead cells were excluded (C and D, respectively) before a gate was drawn to quantify the percentage of CD115^{+} CD11b^{+} monocytes (D). The CD115^{+} CD11b^{+} monocytes were then subdivided based on their expression of Ly6C (E).

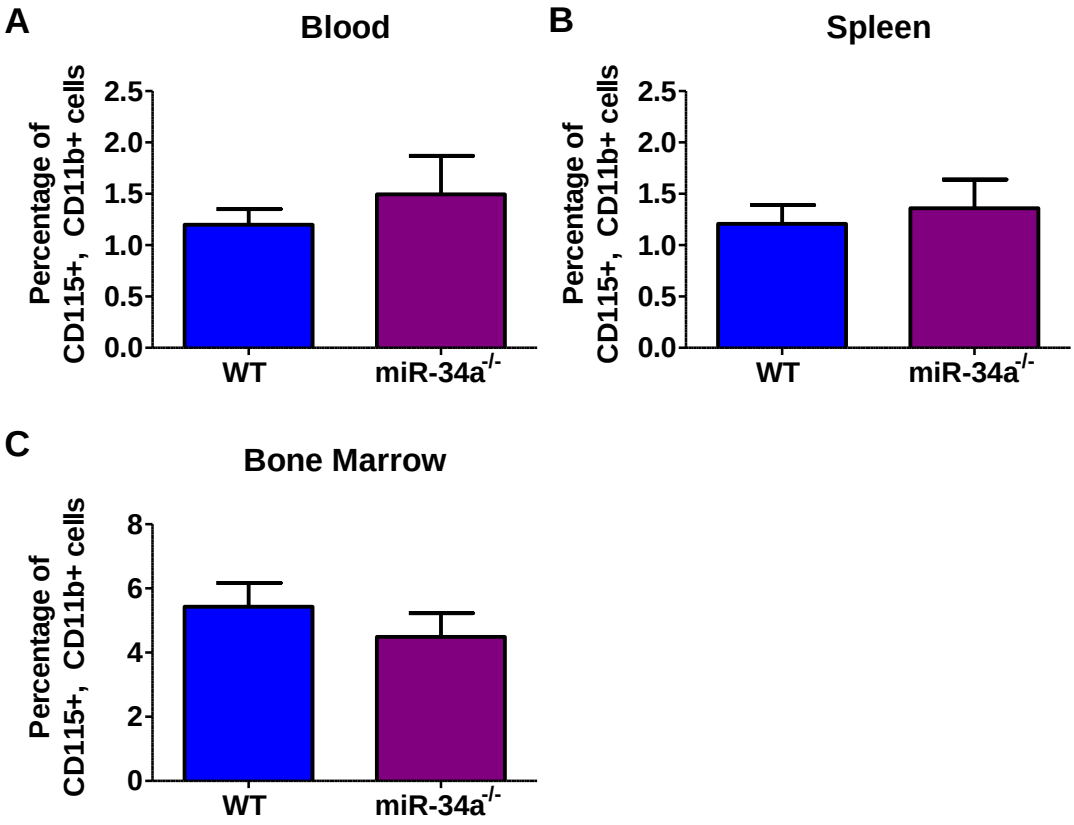


Figure 6-2 Percentage of CD115$^+$ CD11b$^+$ monocytes in the blood, spleen and bone marrow of wild type and miR-34a knockout mice

Cells were isolated from the blood, spleen and bone marrow of wild type (WT) and miR-34a knockout (miR-34a$^{-/-}$**)** mice and analysed by flow cytometry to quantify the percentage of single, live cells that were CD115$^+$ CD11b$^+$ monocytes in these locations. These graphs show the total percentage of monocytes in the blood (A), spleen (B) and bone marrow (C) of the mice. n = 7 mice per group over 2 independent experiments. Data are shown as mean plus standard error of the mean. No significant differences were observed using an unpaired t test.

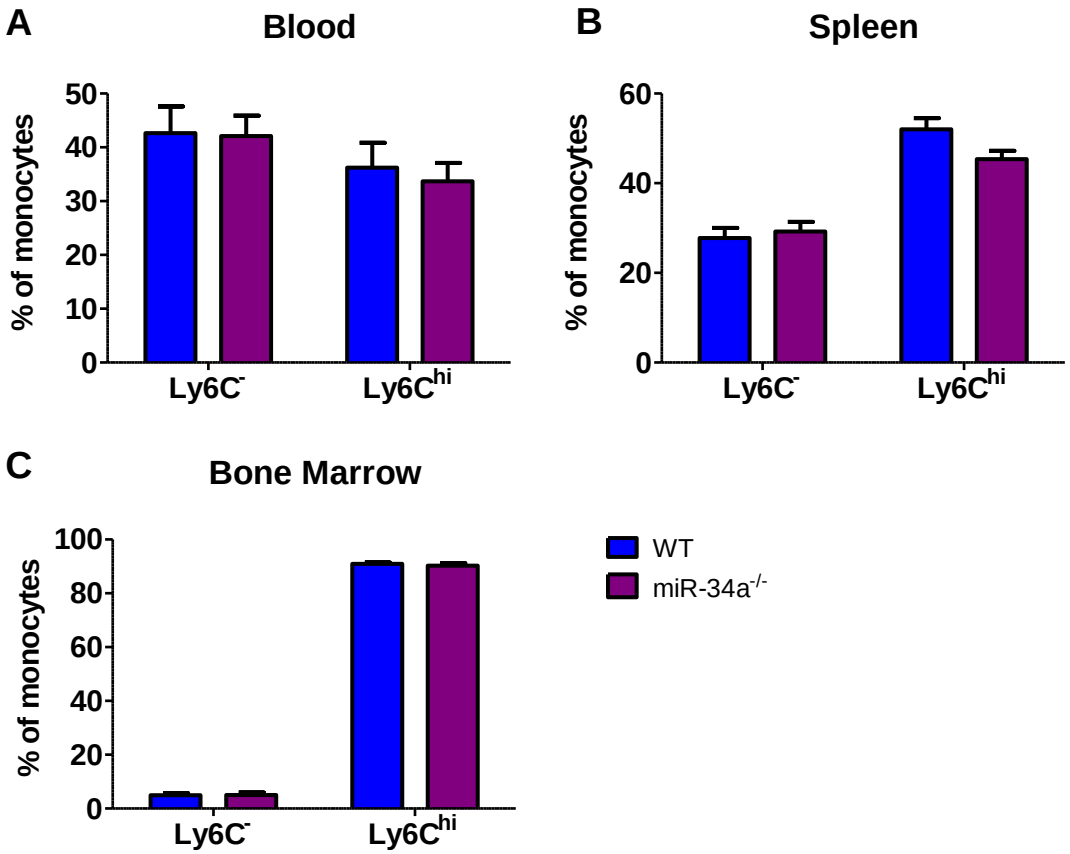


Figure 6-3 Percentage of Ly6C^{hi} and Ly6C^{-} monocytes the blood, spleen and bone marrow of wild type and miR-34a knockout mice

Cells were isolated from the blood, spleen and bone marrow of wild type (WT) and miR-34a knockout (miR-34a$^{-/-}$) mice and analysed by flow cytometry to quantify the percentage of CD115^{+} CD11b^{+} monocytes that were Ly6C^{hi} or Ly6C^{-}. The graphs show percentage of these monocyte subpopulations in the blood (A), spleen (B) and bone marrow (C) of the mice. n = 7 mice per group over 2 independent experiments. Data shown as mean plus standard error of the mean. No significant differences were observed using an unpaired t test.

6.3 TNF production by WT and miR-34a$^{-/-}$ macrophages upon stimulation

In chapter 4 I showed that miR-34a regulates cytokine production by human M-CSF-matured monocyte-derived macrophages. So we next wished to determine whether this was also true in murine cells. Bone marrow was isolated from WT and miR-34a knockout mice and cells matured with M-CSF toward macrophages for 7 days. These macrophages were then left un-stimulated, or stimulated with 1 ng/ml LPS or 1 µg/ml Cl097 for a further 24 hours. The concentration of TNF-α in the supernatants was measured by ELISA, and as shown in Figure 6-4, no differences in TNF-α concentration were seen between macrophages derived from WT or miR-34a knockout mice. The TNF-α concentration in the supernatants of un-stimulated cells was also measured – but these values were undetectable. Here, we demonstrate that unlike with human M-CSF-matured macrophages, knock down of miR-34a seems to have no effect on TNF-α production by murine M-CSF-matured macrophages *in vitro* following LPS or Cl097 stimulation.

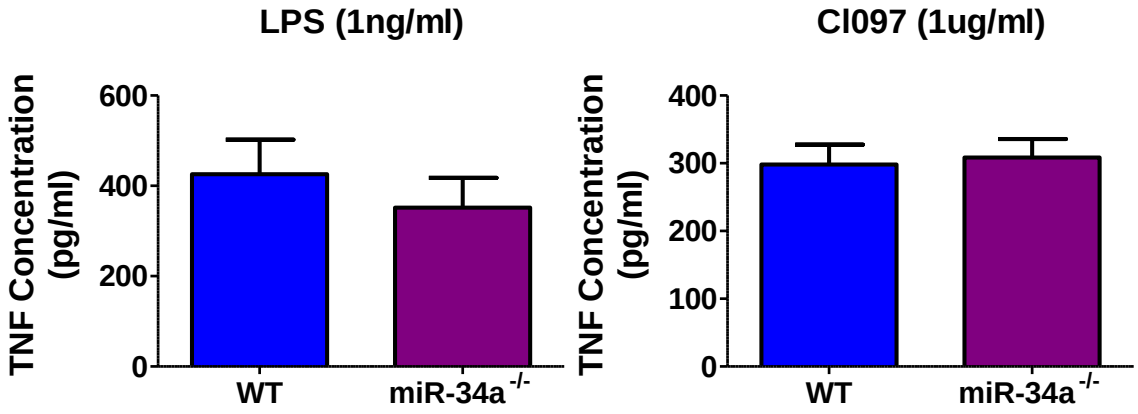


Figure 6-4 TNF production by bone marrow macrophages from WT and miR-34a knockout mice after TLR stimulation
Bone marrow cells were isolated from WT and miR-34a$^{-/-}$ mice and M-CSF matured for 7 days to generate bone marrow macrophages. These cells were then stimulated for 24 hours with 1 ng/ml LPS or 1 µg/ml Cl097. A TNF ELISA was performed on the supernatants and results are shown in the graphs above. Data shown as mean plus standard error of the mean. Bone marrow from 4 mice was grouped for each experiment, n = 3 individual experiments. Data shown as mean plus standard error of the mean. No significant differences were observed using an unpaired t test.

6.4 *In vivo* stimulation of WT and miR-34a knockout mice

Next, we wanted to subject the mice to stress to see if there was any difference in the ability of miR-34a knockout mice to respond to immune challenge *in vivo*. Sterile peritonitis models are often used to study the acute inflammatory response, which is characterised by the coordinated recruitment of immune cells in response to inflammatory cytokines and chemokines – the production of many of which is initiated by resident peritoneal macrophages. We chose to use an LPS peritonitis model, and collect peritoneal lavage 4 hours after the LPS intra-peritoneal (IP) injection. This would allow the collection of the peritoneal exudate that can be used to simultaneously analyse the presence of inflammatory cytokines and chemokines, and also the cellular infiltrate. These models are often used to phenotype transgenic mice as they are a good way to detect any defect or enhancement in the inflammatory response involving multiple cell types.

Accordingly, mice were injected IP with 100 µg LPS per mouse, and a peritoneal lavage performed with 2 mls PBS 4 hours later. 1.5 mls of fluid was retrieved, which was then centrifuged to separate the cells from the fluid. The lavage fluid was then frozen so a luminex could be performed at a later date, and the cells were immediately counted and stained for analysis by flow cytometry.

6.4.1 Flow cytometry analysis of peritoneal exudate cells 4 hours after LPS injection

Figure 6-5 shows the initial gating strategy used to phenotype cells in the peritoneal exudate. First of all a gate was drawn around total leukocytes, then single and live cells were gated for their expression of Ly6C and Ly6G. Figure 6-5D shows the gate used to quantify the percentage of single, live Ly6C/Ly6G double positive cells, which are primarily neutrophils. Panel E and F show the percentage of $Ly6C^{+}$, $Ly6G^{+}$ cells in PBS-injected control mice and the LPS-injected mice respectively. As expected, the LPS-injected mice have a much higher percentage of neutrophils than the control mice, but no differences are seen between the wild type and miR-34a knockout mice.

We next analysed the percentage of $Ly6G^{-}$ cells, which were $F4/80^{hi}$ macrophages in the peritoneal exudate (gating shown in Figure 6-6). As expected, control mice have a higher percentage of macrophages compared to LPS-injected mice, and again we found no differences between the wild type and miR-34a knockout mice.

We also tried to look at monocyte recruitment into the peritoneal cavity using CD11b and CD115 expression but found few cells. Whereas 4 hours post IP stimulation is when neutrophil numbers in the peritoneal cavity peak, this time point is rather early for monocyte recruitment, so a later time point, such as 24 hours after injection would have been required for this purpose [446]. Available mouse numbers precluded such an approach.

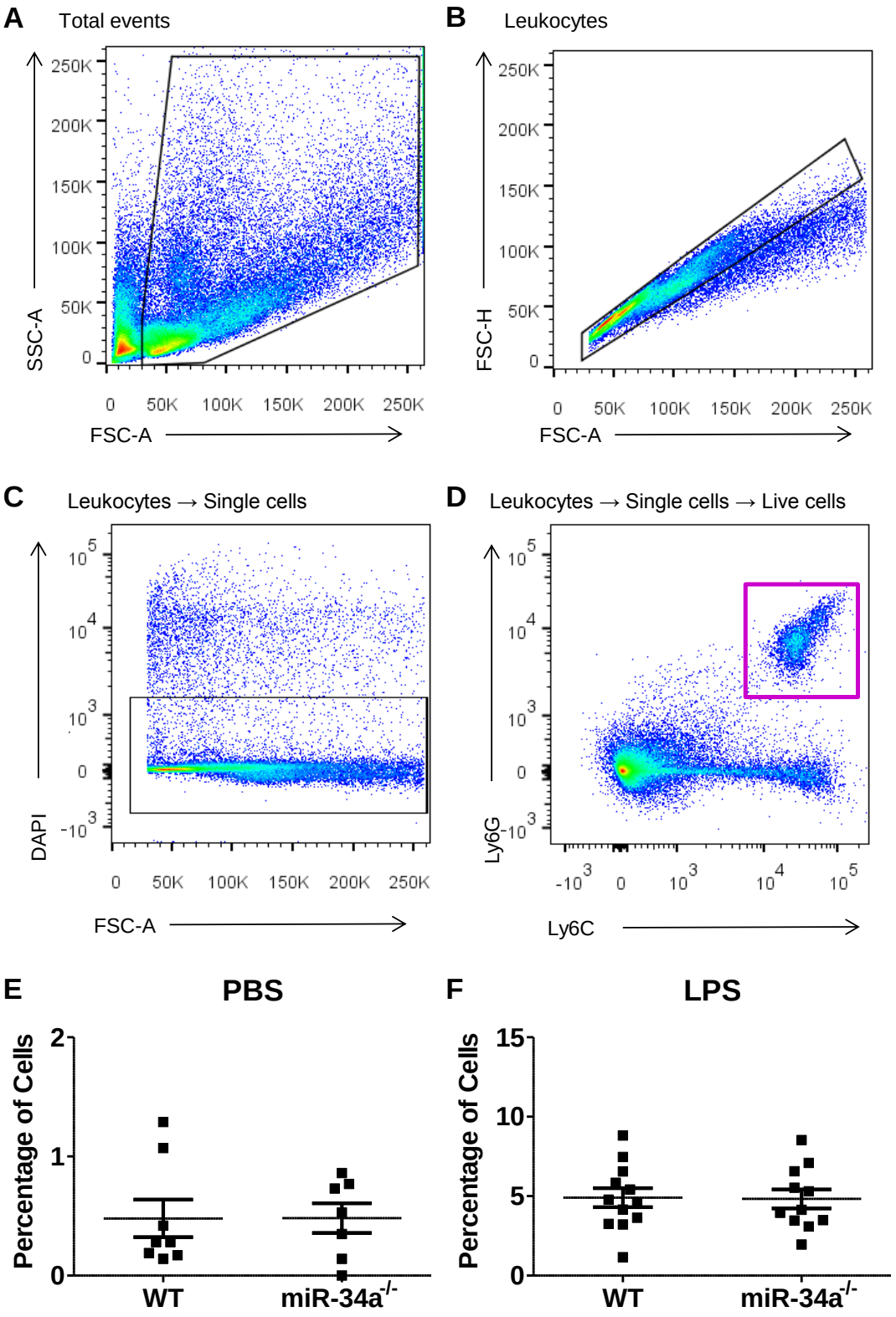


Figure 6-5 The percentage of Ly6C+, Ly6G+ neutrophils in the peritoneal cavity of WT and miR-34a-/- mice after LPS-induced peritonitis

10 week old WT and miR-34a$^{-/-}$ mice were injected intraperitoneally with LPS (100 µg/mouse). 4 hours later the peritoneal cavity was washed with 2 mls PBS. The cells in this exudate were then stained and analysed via flow cytometry. First a gate was drawn around cells of interest (A), then doublets (B) and dead cells (C) excluded before cells were analysed for their expression of Ly6C and Ly6G (D). The pink gate shown in panel D was used to quantify the percentage of Ly6C^{+}, Ly6G^{+} cells. The graphs shown in panel E and F show the percentage of these cells in PBS injected controls and LPS-injected mice respectively. n = 12 (LPS) and 8 (PBS) mice over 3 independent experiments. Line on graphs represents the mean plus standard error of the mean. No significant differences were observed using an unpaired t test.

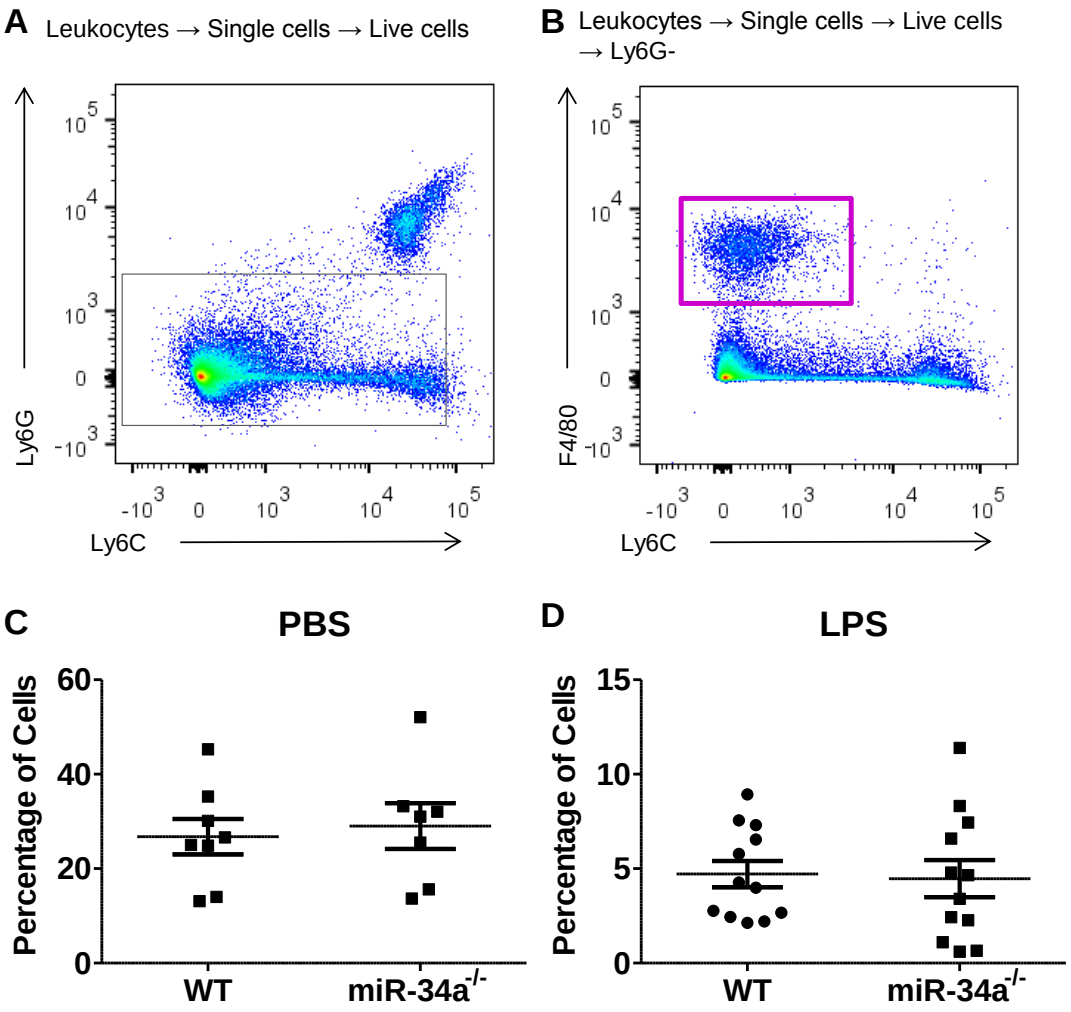


Figure 6-6 The percentage of F4/80^{+} macrophages in the peritoneal cavity of WT and miR-34a$^{-/-}$ mice after LPS-induced peritonitis

10 week old WT and miR-34a$^{-/-}$ mice were injected intraperitoneally with LPS (100 µg/mouse). 4 hours later the peritoneal cavity was washed with 2 mls PBS. The cells in this exudate were then stained and analysed via flow cytometry. Live, single cells were gated on Ly6G- (A), then analysed for their expression of F4/80 (B). The graphs in panel C and D show the percentage of cells positive for F4/80 in the PBS control group and LPS injected mice respectively. n = 12 (LPS) and 8 (PBS) mice over 3 independent experiments. Line on graphs represents the mean plus standard error of the mean. No significant differences were observed using an unpaired t test.

6.5 Luminex analysis of peritoneal exudate and serum of mice following LPS-induced peritonitis

While performing the LPS-induced peritonitis experiments in WT and miR-34a knockout mice the peritoneal exudate was stored for luminex analysis. During these experiments we also collected blood from all mice, and separated and stored the serum to measure systemic cytokine concentrations in response to IP LPS. This would allow us to investigate the acute local cytokine production, primarily by resident peritoneal macrophages, and also the peripheral cytokine responses by multiple cell types. The luminex assays were performed using the Invitrogen cytokine mouse 20-plex kits.

The concentrations for all analytes which were measurable in the majority of samples are shown in Figure 6-7. The results show no significant differences in the measured cytokines, and suggest that the absence of miR-34a has no effect on the local acute inflammatory cytokine production in response to LPS injection. Also measured was the cytokine expression in mice injected with PBS as a control, which was undetectable for all analytes in most samples.

The cytokine measurements in the serum of mice are shown in Figure 6-8 and Figure 6-9. Figure 6-8 shows the results for all analytes which were measurable in the majority of samples where no significant differences were found between WT and miR-34a knockout mice. For the serum, however, significant differences were found between the concentrations of IL-12, GM-CSF, the IFN driven chemokines: IP-10 and KC and also the T cell survival factor IL-2. In all cases the miR-34a knockout mice appear to have lower expression of these cytokines than the wild type mice (shown in Figure 6-9). Although these results achieve statistical significance, the concentrations in most mice are fairly similar, with a few miR-34a null mice having lower cytokine concentrations across the board. Further repetition would therefore be required to see if this really is a true phenomenon, or simply experimental variation. It would also be useful to study longer time points to allow for the full systemic response to develop.

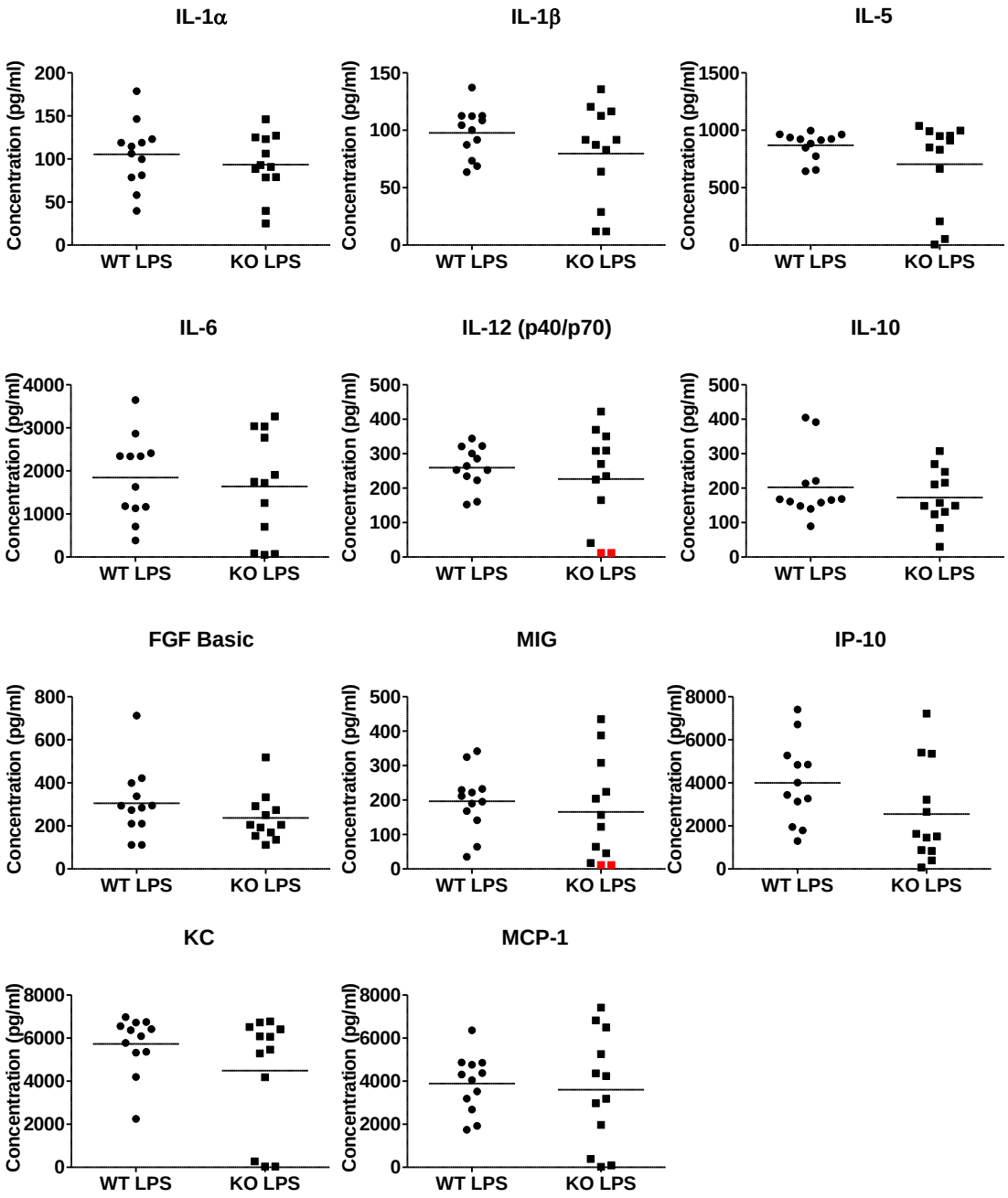


Figure 6-7 Cytokine luminex on the peritoneal wash of mice after LPS-induced peritonitis
10 week old WT and miR-34a$^{-/-}$ mice were injected intraperitoneally with LPS (100 µg/mouse). 4 hours later a peritoneal lavage was performed with 2 mls PBS. The cytokine concentrations in this peritoneal lavage fluid were then measured by luminex, and all analytes which were measurable in the majority of samples are shown above. Where a few samples were below the standard curve the value of half the bottom standard is given and data points are highlighted in red. Each data point represents peritoneal exudate from a single mouse with the line showing the average concentrations. n=12 mice from 3 independent experiments. No significant differences were observed using an unpaired t test or Mann-Whitney U test (test used determined by outcome of D'Agostino and Pearson omnibus normality test).

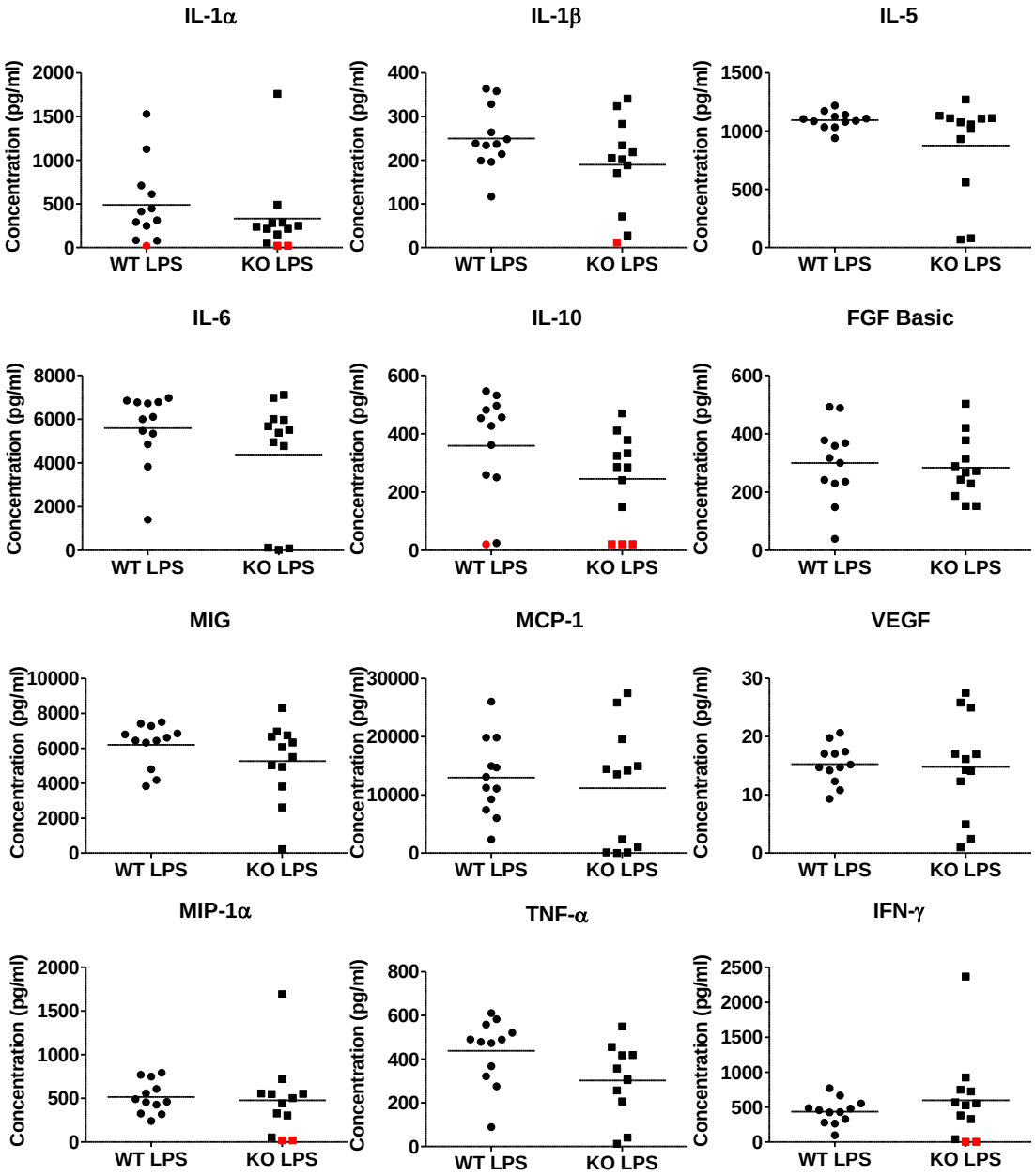


Figure 6-8 Cytokine luminex on the serum of mice after LPS-induced peritonitis
10 week old WT and miR-34a$^{-/-}$ mice were injected intraperitoneally with LPS (100 µg/mouse). 4 hours later blood was collected by cardiac puncture. The cytokine concentrations in the serum were then measured by luminex, and all analytes which were measurable in the majority of samples are shown above. Where a few samples were below the standard curve the value of half the bottom standard is given and data points are highlighted in red. Each data point represents the serum sample from a single mouse with the line showing the average concentrations. n=12 mice from 3 independent experiments. No significant differences were observed using an unpaired t test or Mann-Whitney U test (test used determined by outcome of D'Agostino and Pearson omnibus normality test).

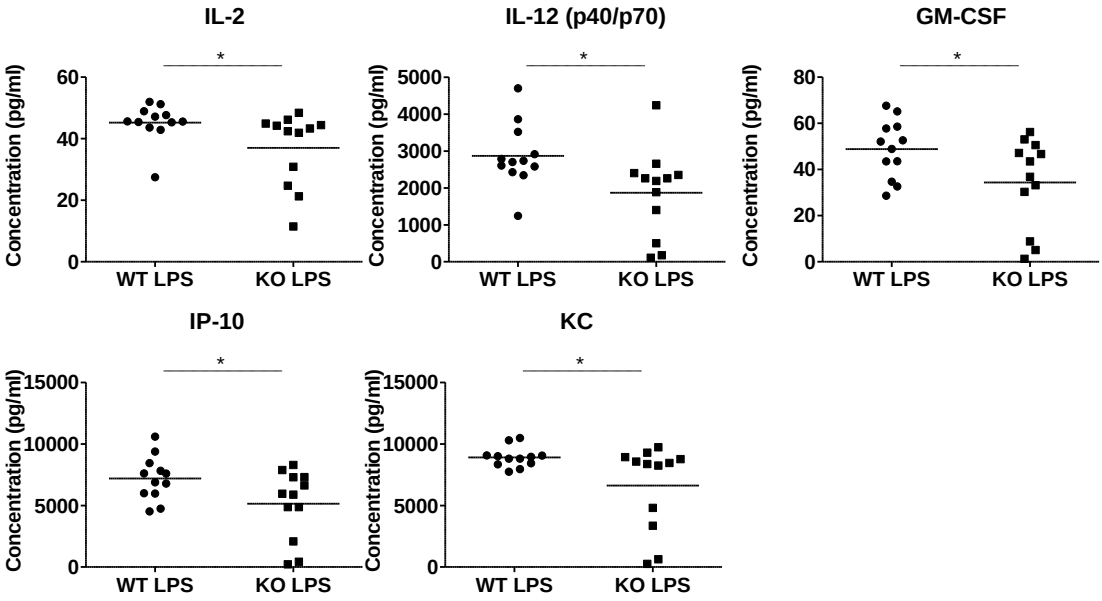


Figure 6-9 Cytokine luminex on the serum of mice after LPS-induced peritonitis
10 week old WT and miR-34a$^{-/-}$ mice were injected intraperitoneally with LPS (100 µg/mouse). 4 hours later blood was collected by cardiac puncture. The cytokine concentrations in the serum were then measured by luminex, and all analytes which were measurable in the majority of samples with significant differences are shown above. Each data point represents the serum sample from a single mouse with the line showing the average concentrations. n=12 mice from 3 independent experiments. Unpaired t test or Mann-Whitney U test; * $p < 0.05$ (test used determined by outcome of D'Agostino and Pearson omnibus normality test).

6.6 **Discussion**

Having already shown that manipulation of miR-34a in human monocyte-derived macrophages results in significant changes in cytokine production, this part of the study set out to determine the effects of total loss of miR-34a on innate immune system function using miR-34a knockout mice.

The first key finding of this work was that there is no difference in the total number of monocytes, or in the percentage of the $Ly6C^{hi}$ or $Ly6C^{-}$ monocyte subsets in miR-34a knockout mice compared to wild type mice. This is interesting, as our work on human cells demonstrated that miR-34a targets the MCSF receptor CD115, and a recent study by Riepsaame *et al* [357] showed that miR-34a also targets CD115 in the mouse. This group went on to show that miR-34a inhibition during DC maturation resulted in an increased expression of CD115, and a decrease in the percentage of bone marrow-derived $MHCII^{hi}$ $CD86^{hi}$ differentiated DCs. These data suggest that miR-34a may not be involved in monocyte development, or in the regulation of CD115 expression in monocytes, but perhaps plays a role in the final stages of cell maturation. For this reason, if given more time I would like to investigate the expression of mature macrophage and DC markers between wild type and miR-34a knockout mice in various tissues.

The next part of the murine study set out to investigate whether loss of miR-34a in the mouse resembled the clear phenotype we saw in the previous chapter when miR-34a was inhibited in human macrophages. While in human MCSF-matured macrophages we saw significant up-regulation of TLR-induced TNF-α production when miR-34a was actively inhibited, here we saw no difference in the TNF-α production between wild type and miR-34a knockout M-CSF-matured bone marrow-derived macrophages. This could be explained in a number of ways. Perhaps whole organism loss of miR-34a results in compensatory mechanisms regulating cytokine production so that miR-34a is no longer required for this role, or perhaps the miR-34a targets responsible for the changes in cytokine production in human cells are not regulated in the murine system. Indeed, the binding sites in some of our predicted targets that could be involved in cytokine production, such as IRAK2 and IRAK4, are not conserved between humans and mice.

Next, we set out to determine the potential role of miR-34a in inflammatory processes, so chose to initially study an acute model of inflammation. The LPS-induced peritonitis model would allow us to study the inflammatory cell infiltrate, and also look at the production of cytokines and chemokines locally by the peritoneal cells, and peripherally in the serum. When studying the cells found in the peritoneal exudate, no significant differences were found in neutrophil infiltration, or in the percentage of macrophages between the wild type and miR-34a knockout mice. This was initially intended to be a pilot study, however, and to be certain there were no differences in the cellular infiltrate the experiment would need to be repeated using a more extensive list of antibodies; including CD3 (T cells), B220 (B cells) and Siglec F (eosinophils). This would allow more certainty over the exact cell types being measured, and the addition of other markers would also let us observe potential differences in cell subsets. We also wanted to look at monocyte recruitment to the sites of inflammation, but at the 4 hour time point this is limited. Although later time points could not be carried out during this body of work, these experiments are currently ongoing in the lab.

The luminex data give an insight into the local and peripheral cytokine production in response to IP LPS in the wild type and miR-34a knockout mice. While there were no significant differences found in the local cytokine production, the possible differences seen in the serum warrant further study to determine whether there are true differences in some of the miR-34a knockout mice. If this turned out to be the case, it may suggest that although there is no difference in the response of resident peritoneal cells (mainly macrophages) to infections, resident immune cells in other parts of the body (spleen, lymph nodes) are affected by the lack of miR-34a. Interestingly, this could agree with work being carried out by other members of the lab, where it has been shown that the cytokine production by bone marrow-derived DCs in response to TLR stimulation is different between wild type and miR-34a knockout mice. It would therefore be interesting to isolate differentiated macrophages and DCs, as well as other cell subsets from various tissues to test their response to stimulation, rather than studying precursor-derived *in vitro* matured cells or the whole organism response. This may help identify in which cells cytokine production could be affected by lack of miR-34a.

Access to the miR-34a knockout mice came quite late into my PhD, so by the time our colonies had been backcrossed and bred in house we were not left enough time to do all of the planned experiments. Given increased expression of miR-34a in RA tissues was where this project started, we wanted to perform an arthritis model. Initially we performed preliminary experiments using the CIA model, but the miR-34a knockout mice are on a C57BL/6 background, and these animals are relatively resistant to this particular model [447]. Studies looking at this model, and others are therefore currently ongoing in the laboratory.

In conclusion, this short study into miR-34a knockout mice has shown that the number of monocytes and monocyte sub-populations is not altered in the blood, spleen or bone marrow of mice lacking miR-34a. We have also shown that unlike in human cells, lack of miR-34a seems to have no effect on the TNF-α production of MCSF-matured macrophages. We then used an acute LPS-induced peritonitis model to demonstrate that lack of miR-34a doesn't affect local cytokine production, or the early recruitment of neutrophils to the peritoneal cavity, while systemic cytokine production requires further study.

Chapter 7 – Discussion

At the beginning of this study, preliminary data from our laboratory suggested that miR-34a expression was up-regulated in SF $CD14^+$ cells compared to matched PB samples. The first aim of this body of work, therefore, was to confirm this finding, and to proceed thereafter to look at the expression of miR-34a in RA compared to control samples. I was able to confirm that miR-34a expression was significantly up-regulated in SF $CD14^+$ cells, while the other members of the miR-34 family – miRs 34b and 34c – were not. My work must be considered in the general context of an evolving field of miR mediated regulation of gene expression, and also that of my laboratory in which a number of miRs are being examined for their potential functional impact on RA disease pathogenesis. Specifically, we have been trying to generate an overall picture of how microRNA expression changes when monocytes arrive in the inflammatory, maturation-inducing environment of the synovium, with the aim to dissect the role of these changes to monocyte and macrophage activation and function.

We are currently studying a number of microRNA that are summarised in Figure 7-1, including miR-155 which is up-regulated in synovial monocytes, and miR-223 which is down-regulated. Extensive published functional data implicate both of these microRNA in the regulation of synovial macrophages – however many elements of the gene expression matrix in these cells cannot be accounted for by these miRs alone, and hence other pathways are of current interest. The role of my PhD was to focus on dissecting the possible causes and functional implications of miR-34a dysregulation in monocytes and macrophages.

As well as being increased in SF $CD14^+$ cells compared to PB cells, we were able to demonstrate that miR-34a expression is increased in synovial tissues from RA patients compared to OA samples, and that some of the miR-34a positive cells were $CD68^+$ macrophages. Of particular interest, miR-34a was also up-regulated in PB $CD14^+$ cells of biologic-resistant RA patients compared to healthy controls. Among the potential stimuli present in the synovial microenvironment (as depicted in Figure 7.1) we discovered that M-CSF and GM-CSF, but not TLR ligands, triggered the expression of miR-34a in monocytes. Dissecting a functional role for this microRNA was crucial for determining the possible consequence(s) of its dysregulation in RA.

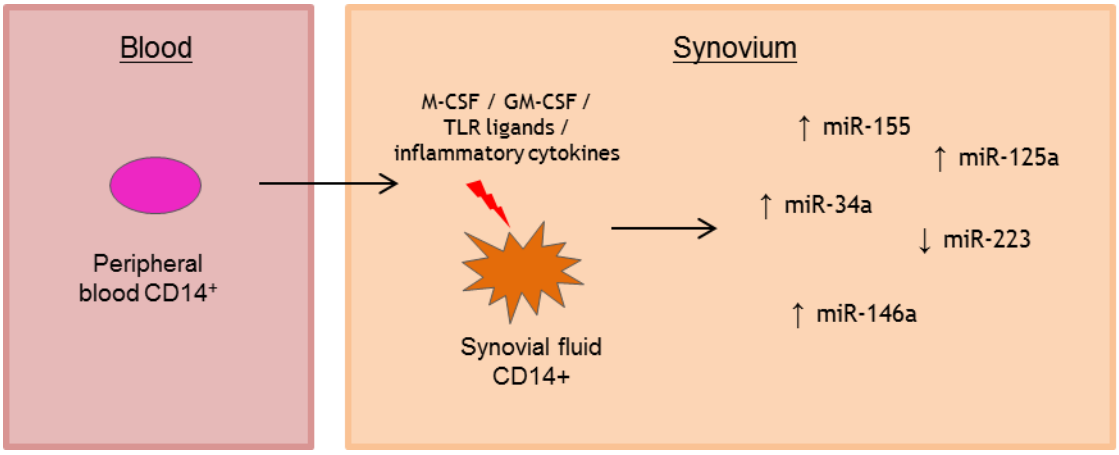


Figure 7-1 Schematic of microRNA changes in PB vs SF CD14$^+$ cells
Studies in our laboratory have shown that the microRNA expression profile of PB CD14$^+$ cells significantly differs from SF CD14$^+$ cells. We propose that upon entering the synovial microenvironment, contact with various mediators (including M-CSF, GM-CSF and TLR ligands) causes changes in microRNA expression. We have demonstrated differential expression of many microRNA – including miR-155, miR-34a, miR-223 and miR-146a.

Using over and under-expression systems to look at miR-34a function in MCSF-matured macrophages, we found that over-expressing miR-34a reduced TLR-induced cytokine production, while inhibiting miR-34a increased cytokine production. This should mean that increased miR-34a expression in RA has a predominantly anti-inflammatory effect, and thereby could assist in promoting the resolution of inflammation. However, we know that monocytes in RA display an activated, pro-inflammatory phenotype – or at least that a predominant subset are considered to play a pro-inflammatory role. I interpret my observations as reflecting a failure of miR-34a to mediate a normally homeostatic regulatory arm in terms of cellular activation. This failure of miR-34a to dampen inflammation could be down to several factors. One of these could be the altered levels of several other microRNA in the synovial myeloid cells. There are several microRNA known to be differentially expressed in RA SF $CD14^+$ cells, some of which exert pro-inflammatory effects on the cells, and some of which have an anti-inflammatory effect. There is therefore likely a balancing act between these counteracting mechanisms. We know that miR-155 is over-expressed in monocytes and macrophages in RA, and this microRNA targets SHIP-1, an inhibitor of TLR and PI3/Akt kinase pathways, as well as SOCS1. Increased expression of this microRNA therefore in general has an inflammation promoting effect on the cells [300]. We also know that miR-223 is down-regulated in RA monocytes. This microRNA targets NLRP3, thereby limiting the production of IL-1 family members, so its down-regulation in RA also has an inflammation promoting effect on cells [342]. Perhaps in monocytes in RA, the effects of altered microRNA that promote inflammation (like miR-155 and miR-223) dominate, thereby limiting the true 'potential' effects of miR-34a over-expression. Alternatively, it is possible that miR-155 triggers the acute pro-inflammatory activation of macrophages, which is negatively fine-tuned by constitutively high levels of miR-34a, likely in collaboration with other anti-inflammatory miRs such as miR-146.

Studies have been conducted that demonstrate microRNA hierarchy where two microRNA with opposing actions are over-expressed simultaneously. One particular study in Treg cells provided data to show that over-expression of miR-155 in Tregs results in their increased production of TNF, IFNγ and IL-17, while over-expression of miR-146a resulted in reduced production of all three

cytokines. Over-expression of both microRNA together, however, resulted in a cytokine profile mimicking that observed when miR-146a alone was over-expressed. This study suggested that the effects of miR-146a were dominant over miR-155 in the Treg cells [448]. In our case we propose that the pro-inflammatory effects of increased miR-155 and reduced miR-223 dominate over the anti-inflammatory effects of increased miR-34a and 146a in monocytes in the synovium (Figure 7-2), and therefore promote the pro-inflammatory phenotype witnessed in these cells. In fact, mechanistic experiments looking at the effects of simultaneous over-expression of miR-155 and miR-34a in monocytes are currently being undertaken in the laboratory – results are awaited with considerable interest. Long-term experimental plans include simultaneous overexpression of these miRs in DICER specific human monocyte/macrophage deficient cells (depleted from all other miRs) to dissect the details of miR-155 and miR-34a interactions in these cells. It would also be interesting to determine whether more profound anti-inflammatory effects are observed when miR-34a is overexpressed and miR-155 inhibited simultaneously. In addition, similar experiments will be conducted in monocyte and monocyte derived macrophages from RA patients, which show high levels of both miRNAs.

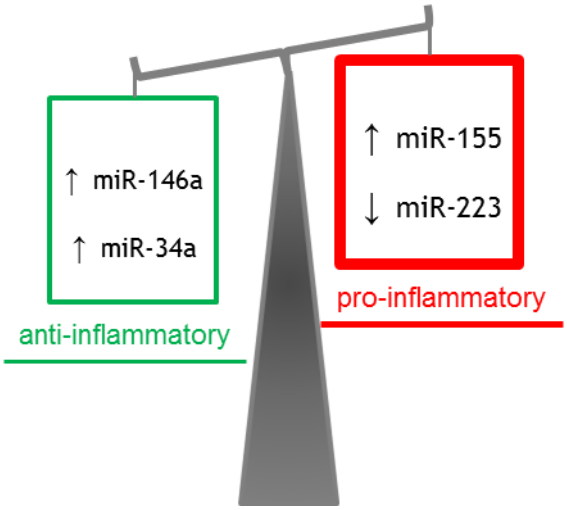


Figure 7-2 microRNA balancing act in synovial fluid CD14$^+$ cells
Several microRNA are known to be differentially expressed in monocytes in RA. These include alterations which should have an anti-inflammatory effect on cells (increased miR-146a and miR-34a), as well ones which will have a pro-inflammatory effect (increased miR-155 and decreased miR-223). We therefore propose that in RA, although some microRNA are altered in an attempt to restore balance and dampen the on-going inflammation, the effects of decreased miR-223 and / or increased miR-155 dominate, and add to the pro-inflammatory phenotype observed in these cells.

The anti-inflammatory effect of miR-34a in macrophages was initially surprising to us, as data from another member of the lab had already shown that miR-34a overexpression in monocyte-derived DCs actually resulted in increased LPS-induced TNF-α production. Since these experiments were carried out, however, another group have reported similar data on the effect of miR-34a manipulation on macrophage cytokine production [354].

Supporting the differences observed between monocyte-derived macrophages and dendritic cells, one target we have demonstrated that plays a key role in miR-34a function in DCs is Axl. Axl is a member of the TAM (TYRO3, Axl and Mer) family of receptor tyrosine kinases. These receptors are involved in dampening immune responses, and are capable of inhibiting TLR signalling through the induction of SOCS1 and SOCS3. Data from our lab suggests that miR-34a, by inhibiting Axl, facilitates pro-inflammatory cytokine production by DCs, and also promotes Th17 cell differentiation upon TLR stimulation. Interestingly, although Axl is highly expressed by DCs, a recent study actually demonstrated that macrophages express very low levels of Axl compared to DCs [449]. This differential expression of Axl, and perhaps other mRNA targets of miR-34a, could explain the different role for miR-34a in these cells. miR-34a has a number of verified and / or predicted targets that could have a pro-inflammatory effect on cells (Axl and SOCS3), but also several that would have an anti-inflammatory effect (Notch1, IRAK2, IRAK4 and MAP3k14). We therefore hypothesise that the differential expression of potential target transcripts between macrophages and DCs could account for the differential effect of miR-34a in these cells. Our hypothesis for the distinct role of miR-34a in macrophages and DCs is summarised in Figure 7-3.

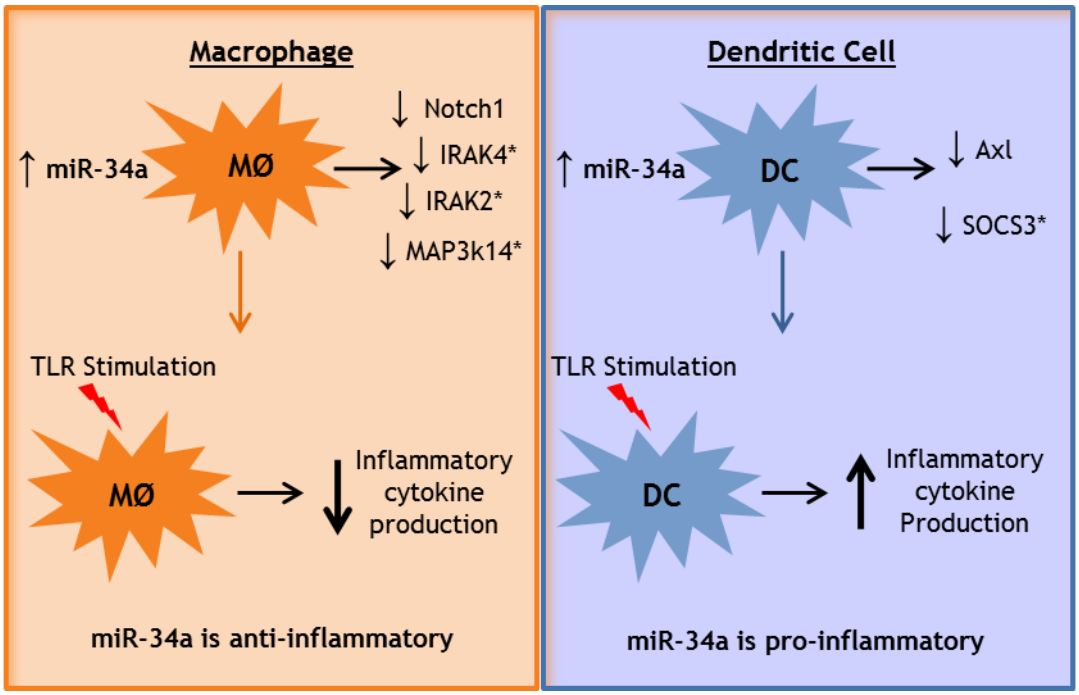


Figure 7-3 Current hypothesis of the role of miR-34a in macrophages and dendritic cells
Experimental manipulation of miR-34a in macrophages and dendritic cells has shown differential regulation of TLR-induced cytokine production. When miR-34a expression is increased in macrophages it could target several transcripts – possibly including Notch1, IRAK2, IRAK4 and MAP3k14. This could explain the reduced cytokine response observed in response to TLR stimulation of miR-34a over-expressing cells. In dendritic cells, however, when miR-34a is upregulated it targets Axl and possibly other transcripts like SOCS3, which could explain the increased cytokine production observed upon TLR stimulation of miR-34a over-expressing DCs.* = predicted according to TargetScan, but not yet experimentally validated.

The key differences between the effect of miR-34a manipulation on macrophages and dendritic cells clearly highlights the importance of taking the utmost care when interpreting or comparing data between cell types. The role of microRNA is very dependent on the expression of target transcripts in the cell at that particular time. This could be particularly important when studying cell lines (which could confound observations) with the aim to detect and predict the role of a given microRNA in primary cells. In light of this work, we plan to repeat some of these experiments using patient-derived synovial fluid $CD14^+$ cells. Although here we have dissected a functional role for miR-34a in macrophages, it will be interesting and important to determine what effect(s) manipulating miR-34a has on freshly isolated patient cells.

In the context of miR-34a's role in broad macrophage biology, it would be interesting to test the effect of miR-34a not only in the acute inflammatory response presented in this thesis, but also in LPS tolerance and innate immune memory. It has been shown that another inhibitory / anti-inflammatory microRNA, miR-146, is critical for the development of endotoxin induced tolerance [450]. Finally, given more time I would also test the role of miR-34a in the development and activation of GM-CSF primed, inflammatory macrophages. These cells are considered more prone to the production of pro-inflammatory stimuli than M-CSF differentiated macrophages [451], so it would be interesting to determine whether miR-34a had an inflammation promoting or dampening role in these cells.

In this study we also used a microarray to investigate, at a transcriptional level, pathways that could be regulated by miR-34a. We found several pathways, including particularly the interferon, metallothionein and chemokine pathways, where many members were down-regulated upon over-expression of miR-34a in monocytes. However, none of the targets we hypothesise are involved in the reduced cytokine production following miR-34a over-expression in macrophages were highlighted. Since monocytes strongly up-regulate miR-34a during differentiation towards dendritic cells, we are planning to investigate whether identified novel pathways underlie the function of miR-34a in these cells. For example, we speculate that inhibition of the IFN pathway by miR-34a may be

relevant to the pro-inflammatory action of this miR in DCs, as the miR-34a target Axl requires IFN signalling for the induction of SOCS in DCs [449].

In light of our recent observation that microRNA targeting is extremely cell specific, we aim to perform miR over and under-expression experiments in macrophages and dendritic cells. This would allow us to determine whether any of these pathways highlighted in monocytes are also relevant in macrophages and / or DCs, and also look more specifically at the potential targets responsible for the altered TLR-induced cytokine production following miR-34a manipulation.

Taken together, this study and several others demonstrate a complex role for miR-34a in the regulation of myeloid cell biology. In this study I have shown that miR-34a is increased in monocytes following culture with M-CSF or GM-CSF, while *Krzeszinski* et al demonstrated that miR-34a is down-regulated during the differentiation of osteoclasts with M-CSF and RANKL [339]. A key role for miR-34a in human monocyte to DC differentiation and subsequent DC function (as manifested by developing high levels of MHC II expression and phagocytic properties) has already been supported by other studies [357, 452], and we have shown that miR-34a inhibits TLR-induced cytokine production by monocyte-derived macrophages, but promotes TLR-induced cytokine production by monocyte-derived DCs. Both human and murine studies have also shown that miR-34a targets transforming growth factor-b-induced factor 2 (Tgif2), thereby inhibiting osteoclastogenesis and bone resorption [339]. These data highlight the importance of miR-34a in myeloid cell differentiation and function, but could mean that harnessing its potential as a therapeutic in inflammatory disease may be difficult. While miR-34a over-expression could dampen the inflammatory response by macrophages and perhaps reduce bone erosions by inhibiting osteoclastogenesis, it could act on DCs to promote cytokine production and inflammation.

In summary, this study is the first to demonstrate increased miR-34a expression in monocytes and macrophages in RA. It also agrees with other studies [354] in demonstrating that miR-34a reduces TLR-induced cytokine production in macrophages – and therefore has an anti-inflammatory effect on these cells. We propose that miR-34a over-expression in monocytes and macrophages in RA is an

attempt to attenuate inflammation, which fails due to the strong pro-inflammatory milieu, including the dysregulation of other inflammation promoting microRNA. Future work in the lab will therefore focus on looking at the effects of manipulating multiple microRNA simultaneously, to explore the hierarchy and dominance of microRNA in synovial fluid cells.

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