Identification of targets for the prognosis and/or treatment of vascular pathology associated with inflammatory arthritis.



By Katherine Sime BSc(Hons)

This thesis is submitted to Cardiff University in fulfilment of the requirements for the Degree of Doctor of Philosophy in the School of Medicine.

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This thesis is dedicated to the loving memory of

Javier Uceda Fernández

(1992-2018)

Your smile lit up every single day I spent with you in Cardiff. I could not have asked for a better friend or colleague, miss you everyday.

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DECLARATION

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SUMMARY

Premature deaths in rheumatoid arthritis (RA) patients are linked to the development of cardiovascular pathologies. One mechanism that can influence vascular function during health and disease is mediators released from the perivascular adipose tissue (PVAT). However it is not known if PVAT morphology and/or function are altered during RA, contributing to the development of CVDs, or if this is a mechanism that can be therapeutically targeted.

The collagen-induced arthritis (CIA) model was used to characterise alterations in PVAT morphology. An increase in total cell number and percentage macrophages in PVAT during CIA was accompanied by a decrease in adiposity. This thesis discovered that galectin-3 was specifically elevated in PVAT during arthritis and was subsequently selected as a PVAT-associated macrophage marker of vasculopathy during CIA. It is not known if elevated galectin-3 expression during inflammatory arthritis is associated with vascular dysfunction or if therapeutical intervention (e.g. with etanercept) affects galectin-3 expression. RA patients receiving etanercept have a reduced CV risk. Treatment with etanercept reduced the incidence of CIA and improved vascular function. Etanercept therapy reduced galectin-3 expression in PVAT during CIA but not systemically. Therefore the effect of direct galectin-3 minibition on the vascular dysfunction associated with CIA was determined. Galectin-3 was inhibited in PVAT and in plasma by GB1107 during CIA. This novel therapy decreased the severity of CIA but did not prevent onset. Vascular function was partially restored in GB1107 treated mice, more so than in etanercept treated CIA mice.

For the first time, this thesis showed that CIA altered PVAT morphology during CIA. Galectin-3 was highlighted as a marker of PVAT-associated vasculopathy, with inhibition of galectin-3 during CIA improving vascular function. Furthermore, this thesis demonstrated that CIA could be used as a platform to investigate the effects of therapeutic intervention on the associated vascular dysfunction that is linked to inflammatory arthritis.

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ABBREVIATIONS

AAA	Abdominal aortic aneurism
ADCC	Antibody-dependent cytotoxicity
AI	Arthritic index
AIA	Antigen induced arthritis
AOI	Area of interest
APCA	Anti-citrullinated protein antibodies
ApoE	Apolipoprotein E
Arg-1	Arginase-1
ATGL	Adipose triglyceride lipase
ATM	Adipose tissue macrophage
BAT	Brown adipose tissue
BSA	Bovine serum albumin
CCL	C-C Motif chemokine ligand
CD	Cluster of differentiation
cDNA	Complentary deoxyribonucleic acid
CFA	Complete Freud's adjuvant
CIA	Collagen induced arthritis
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
CV	Cardiovascular
CVD	Cardiovascular disease
DAB	3,3'-diaminobenzidine
DCIR	Dendritic cell immunoreceptor
dH ₂ O	Distilled water
DR3	Death receptor 3
cGMP	Cyclic guanosine monophosphate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme- linked immunosorbent assay
FcγR	Fc gamma receptor
FLS	Follicular-like structures
Folr2	Folate receptor 2 (Beta)
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H&E	Haematoxylin and Eosin
HRP	Horseradish peroxidase
HSL	Hormone-sensitive lipase
ICAM1	Intercellular Adhesion Molecule 1
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
IMS	Industrial methylated spirit
iNOS	Inducible nitric oxide synthase
K _{CA}	Calcium-activated potassium channels
LC/MS/MS	Liquid chromatography/mass spectrometry/mass spectrometry
LPS	Lipopolysaccharide
mABs	Monoclonal antibodies
MCP	Modified citrus pectin
MerTK	MER tyrosine kinase
MHC	Major histocompatibility complex

mLDL	Modified low density lipoprotein
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
Msr1	Macrophage scavenger receptor 1
NADPH	Nicotinamide adenine dinucleotide phosphate
NBFS	Neutral buffered saline
ΝϜκΒ	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
NOS	Nitric oxide synthase
NT-proBNP	N-terminal pro-hormone brain natriuretic peptide
OD	Optical density
PBS	Phosphate buffered saline
PDCF	PVAT-derived contractile factors
PET	Positron emission tomography
PGE ₂	Prostaglandin E2
PGI ₂	Prostaglandin I2
PI3K	Phosphoinositide 3-kinase
PRR	Pattern recognition receptor
PVAT	Perivascular adipose tissue
qPCR	Quantitative polymerase chain reaction
RA	Rheumatoid arthritis
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
RF	Rheumatoid factor
Rmax	Maximum contractile response
RNA	Ribonucleic acid
RNA-Seq	Ribonucleic acid sequencing
RQ	Relative quantification
RT	Real time
RTU	Ready to use
SCID	Severe combined immunodeficiency
STAT	Signal transducer and activator of transcription
TAGs	Triacylglycerol
TCR	T cell receptor
TGF-β	Transforming growth factor Beta
Т _н	T helper cell
Tip-DCs	TNF and NO producing dendritic cells
ТМВ	Tetramethylbenzidine
TNF	Tumour necrosis factor
TRAF1	TNF Receptor Associated Factor 1
TRAP	Tartrate-resistant acid phosphatase
Treg	T regulatory cell
ТХА	Thromboxane A
VCAM1	Vascular cell adhesion molecule 1
VSMC	Vascular smooth muscle cell
WAT	White adipose tissue
	•

Chapter 1

General Introduction

1.1 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is an autoimmune disease where the clinical phenotype arises due to the breakdown of cartilage and bone resulting in pain, restricted movement and joint destruction. RA develops from a diverse range of pathways (e.g. genetic, environmental and immunological factors) that variably overlap across individual patients (Firestein, 2014). From the evidence produced from the last 80 years, RA is associated with stromal tissue dysregulation that causes chronic inflammation and articular destruction (Firestein and McInnes, 2017). RA affects more than 400,000 people in the UK, approximately 1% of the population, and can affect people of any age (National Rheumatoid Arthritis Society, 2017). Studies have shown that women are 3 times more likely to develop RA compared to men (Alpizar-Rodriguez and Finckh, 2017). The reason for the risk of RA being greater in women than men is not yet understood, however studies have ruled out the risk being linked to the chromosome X (Frisell et al., 2016). There is a strong link between RA and cardiovascular disease (CVD), accounting for approximately 50% of premature mortalities in RA patients (Symmons and Gabriel, 2011). To understand the mechanisms underpinning the development of RA-associated CVDs, we first need to understand each pathology separately and investigate overlapping factors that influence the progression of both diseases.

1.1.1 RA Pathogenesis

RA has three phases: pre-clinical RA, clinically evident RA and chronic established RA, which are summarised in Figure 1.1. The normal synovial membrane is 2-3 cells thick, comprising of stromal tissue containing macrophages, follicular-like structures (FLS) and sparse blood vessels (McInnes and Schett, 2011). After the onset of clinically evident RA the synovial membrane expands to 10-12 cells thick, mainly comprising of synoviocytes, FLS and cellular infiltrate containing both innate (e.g. macrophages and neutrophils) and adaptive (e.g. T cells and B cells) immune cells (McInnes and Schett, 2011). Two main cells shape normal bone metabolism: the osteoblasts, located in the periosteum and endosteum of bone, are involved in bone production and the osteoclasts, located on the bone surface, are involved in the breakdown of bone (Green and Deodhar, 2001). Bone metabolism is tightly regulated by signalling pathways (e.g. bone morphogenetic proteins), transcription factors (e.g. runt-related transcription factor 2) and cytokines (e.g. receptor activator of nuclear factor- κ B Ligand (RANKL), tumour necrosis factor- α (TNF- α) and interleukin-1 (IL-1)) (Shahi et al., 2017, Vitale and Ribeiro Fde, 2007). During RA there is an upset in the balance of bone metabolism due to an increase in pro-inflammatory cytokines including TNF- α and IL-1 (Hofbauer et al., 1999). The production of RANKL from osteoblasts in the bone and immune cells in the synovium (e.g. T cells) is increased in RA patients (Hofbauer et al., 2004). RANKL binds to the RANK receptor on osteoclast precursor cells causing them to mature into and increase the number of bone resorbing osteoclasts within the joint, leading to bone destruction (Boyle et al., 2003).

1.1.1.1 Genetic background and RA

There are over 100 loci associated with increased RA risk and progression (Okada et al., 2014). The most common genetic risk allele is in the major histocompatibility complex (MHC) class II locus (HLA-DR4), which accounts for approximately 40% of the genetic influence in RA. The variants of this allele (DRB*0401, DRB*0404, DRB*0101 and DRB*1402) are found in approximately 90% of RA patients. Individuals' positive for these alleles have a 5:1 chance of developing RA at some point in their life (Weyand et al., 1992, Firestein and McInnes, 2017). Other risk alleles associated with RA risk and progression are involved in T-cell activation (e.g. co-stimulatory molecules CD28 and CD40) and in NF-κB dependent signalling (e.g. TRAF1) (Kurreeman et al., 2007). Although these percentages underpin the genetic involvement in RA, the percentages are relatively low therefore is not



Figure 1.1 Factors influencing RA risk and progression

Rheumatoid arthritis progression can be spilt into three phases in which a multitude of factors can influence the pathology of RA in an individual. The presence of genetic risk alleles and environmental stimuli of an individual are strongly implicated in RA risk and progression. Each of the factors outlined in the purple box can influence RA risk and progression at each phase of disease. During the pre-clinical RA phase an increase in immune mediators including auto-antibodies and pro-inflammatory cytokines can be detected in the serum. This phase precedes the clinically evident RA phase and can last, undetected, for years. During the clinically evident RA phase patients receive aggressive treatment strategies. However, in some patients, the disease pathogenesis will evolve into chronic, established RA which is characterised by chronic inflammation, tissue remodelling and articular damage. Adapted from: (McInnes and Schett, 2017, Firestein and McInnes, 2017)

the sole influencing factor in RA risk, progression and severity. Other influencing factors include environmental stimuli and immunological pathways (Firestein and McInnes, 2017).

1.1.1.2 Environmental exposures and RA

Environmental factors that increase an individual's risk of developing RA include inhaled pollutants and nutritional habits. Tobacco smoke is an inhaled pollutant that is a major risk for the development of RA; risk is increased by approximately 40% in smokers versus non-smokers in the general population (Sugiyama et al., 2010). The mechanism by which smoking increases RA risk is not well understood; elevated levels of citrullinated proteins in the lungs of RA patients may be a contributory factor. Citrullinated proteins are linked with the development of autoantibodies in seropositive RA (Kallberg et al., 2007, Klareskog et al., 2006). Exposure to inhaled pollutants such as traffic pollution and silica dust also increase RA risk (Alpizar-Rodriguez and Finckh, 2017). Mechanism(s) for this association have not been proposed or studied. The nutritional habit of patients with RA is controversial in terms of its role in modulating an individual's risk of developing the disease. For example, high salt consumption more than doubled the risk for RA in smokers compared non-smokers in a population-based prospective case-control study (Sundstrom et al., 2015). On the other hand, and perhaps surprisingly, a meta-analysis of 9 studies showed a protective effective of low dose alcohol consumption (less than 70 g per week) in Rheumatoid Factor (RF) positive patients compared to non-drinker RF⁺ patients (Scott et al., 2013). Additional to the involvement of the environmental and genetic factors described here, the immune system plays an essential role in the onset and progression of RA.

1.1.1.3 Immunological factors in RA

A number of immune cells have been implicated in aiding the pathogenesis of RA including macrophages, mast cells, neutrophils, dendritic cells, T cells and B cells. Figure 1.2 summarises the complex interactions between these cells within an arthritic joint. Through the release of inflammatory mediators, e.g. chemokines, cytokines, matrix

metalloproteinases (MMPs) and reactive nitrogen and oxygen species by these cells, a positive regulatory feedback loop is formed within the affected joint and results in an acute and persistent inflammation. A selection of these mediators that are studied and/or discussed in subsequent chapters are summarised in Table 1.1 (macrophage-associated mediators) and Table 1.2 (mediators associated with other immune cells).

In addition to the release of these inflammatory mediators, macrophages have additional roles in RA pathogenesis including the phagocytosis of immune complexes, which results in the production of reactive oxygen species and collagenases leading to further immune activation and degradation of collagen within the joint, and the presentation of antigens (e.g. rheumatoid factor and self antigens) in order to prime T cells (Arleevskaya et al., 2011, Rodriguez-Fernandez, 2013). The roles of macrophages in RA will be described in greater detail in section 1.1.2. Interactions between T cells and other immune cells present in the inflamed synovium are critical in the progression of RA to its chronic state. Plasmacytoid dendritic cells are present in vast numbers during RA and have important roles in T cell activation and antigen presentation (Lebre et al., 2008). T cells also provide B-cell help during RA resulting in the over production of antibodies. During the pre-RA stage, the serum levels of autoantibodies (e.g. rheumatoid factor and anti-citrullinated protein antibodies (APCAs)) gradually increase and peak at the time of clinical onset (Deane et al., 2010). The percentage of RA patients who are positive for APCAs is 80-90%. The sheer number of immune cells and pathways involved in RA pathogenesis, alongside the genetic and environmental influencing factors, highlights the complexity of this disease.

1.1.2 Involvement of macrophages in RA

Macrophages are critically involved in the pathogenesis of RA, where they have multiple functions within the synovium that help drive the disease (Udalova et al., 2016). Cytokines produced by the macrophages are important for the recruitment and

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Figure 1.2 Summary of immune interactions occurring in an RA joint

A complex network of immune interactions from both the innate and adaptive arms are implicated within the synovial membrane of the joint during RA. These interactions include antigen presentation and the production of pro-inflammatory cytokines, amines and prostaglandins which will recruit and activate additional immune cells driving the inflammatory response. Through T cell help, B cells produce auto-antibodies, for example against citrullinated proteins, which drives the autoimmune response seen in RA patients. Adapted from: (McInnes and Schett, 2011)

Inflammatory mediator	Cell produced by	Function in RA pathogenesis	Reference
CCL3 (MIP-1α)	Macrophages, dendritic cells	Responsible for the trafficking of innate effector cells into the synovium and activates neutrophils.	(Loetscher and Moser, 2002, Maurer and von Stebut, 2004)
GM-CSF	Macrophages, mast cells, T cells, endothelial cells, B cells	Enhances the maturation of monocytes into innate effector cells (e.g. macrophages).	(Avci et al., 2016, Marston et al., 2010)
ΙL-1β	Macrophages, dendritic cells	Promotes TH17 differentiation, suppresses Treg differentiation, stimulates MMP production, a strong stimulator of bone resorption via the upregulation of RANKL which in turn activates osteoclastogenesis.	(Behrens et al., 2007, Burrage et al., 2006, Ruscitti et al., 2015)
IL-6	Macrophages, monocytes, dendritic cells, T cells, B cells	Promotes TH17 differentiation, survival and proliferation, activates B cell maturation, promotes angiogenesis and involved in leukocytes recruitment.	(Behrens et al., 2007, Jones et al., 2018, Maruotti et al., 2006, Pistoia, 1997)
IL-12	Macrophages, B cells	Involved in leukocyte migration into the joint and promotes TH17 differentiation.	(Cornelissen et al., 2009, Pope and Shahrara, 2013, Marston et al., 2010)
IL-18	Macrophages, T cells	Activates TH1 differentiation, promotes angiogenesis and induces the production of TNF- α by macrophages as well as GM-CSF and NO production.	(Gracie et al., 1999, Maruotti et al., 2006)
IL-23	Macrophages, dendritic cells	Promotes TH17 differentiation and in conjugation with IL-17, IL-23 has been shown to promote angiogenesis, leukocyte migration and bone erosion during RA.	(Zaky and El-Nahrery, 2016, Pope and Shahrara, 2013)
MMPs	Macrophages, synovial cells, chondrocytes	MMPs are enzymes that degrade extracellular matrix. In RA MMP1 and MMP13 degrade collagen within the joint whereas MMP2, MMP3 and MMP9 degrade non-collagen matrix components of the inflamed joint.	(Burrage et al., 2006)
NOS	Macrophages, T cells, osteoclasts, osteoblasts, fibroblasts, neutrophils	Nitric oxide species are involved in T cell activation and TH1 differentiation, inducing apoptosis and is involved in macrophage activation and polarisation towards an M1 phenotype.	(Nagy et al., 2010, Martinez and Gordon, 2014)
TGF-β	Macrophages, dendritic cells, T cells, B cells	Promotes TH17 cell differentiation and angiogenesis within the joint as well as stimulating synovial fibroblasts.	(Behrens et al., 2007, Gonzalo-Gil et al., 2013, Pistoia, 1997)
TNF-α	Macrophages, monocytes, T cells, B cells, fibroblasts, neutrophils	Induces the production of other pro-inflammatory cytokines (e.g. IL-1, IL-6, IL-8, MMPs and GM-CSF), stimulates fibroblasts to express adhesion molecules (e.g. ICAM1), increases the proliferation of macrophages, T cells, B cells and synovial cells resulting in synovitis and promotes angiogenesis in the joint.	(VASANTHI et al., 2007, Butler et al., 1995, Choy and Panayi, 2001, Cascao et al., 2010)

 Table 1.1 The role of macrophage-associated inflammatory mediators secreted in an RA joint

Inflammatory mediator	Cell produced by	Function in RA pathogenesis	Reference
CCL2 (MCP-1)	Fibroblasts, stromal cells,	Recruits monocytes, memory T cells and dendritic cells to the synovium from the	(Avci et al., 2016, Loetscher and
	endothelial cells, T cells	bone marrow and surrounding vasculature.	Moser, 2002)
CCL18	Neutrophils	Promotes T cell trafficking into the inflamed joint.	(Auer et al., 2007)
CD40	T cells	Interaction with its ligand CD40L results in T cell contact-mediated activation of	(McInnes et al., 2000)
		macrophages and fibroblasts.	
ICAM1	T cells	Involved in T cell contact-mediated activation of macrophages and fibroblasts.	(McInnes et al., 2000)
IL-13	T cells, mast cells	Promotes angiogenesis through the upregulation of VCAM1 on synovial cells.	(Rifas and Cheng, 2003)
	Fibroblasts	Promotes B cell survival through the upregulation of BAFF and VCAM1, activates	(Park et al., 2011, Benito-Miguel et al.,
IL-15		T cells, promotes angiogenesis of the synovium and promotes	2012, Maruotti et al., 2006)
		osteoclastogenesis through the upregulation of PLD and RANKL.	
IL-17	T cells, mast cells	Induces bone destruction via interrupting the RANKL:OPG bone turnover ratio	(Shen et al., 2005, Shahrara et al., 2009)
		and promotes the recruitment of monocytes and neutrophils into the synovium.	

Table 1.2 The role of inflammatory mediators secreted immune cells in an RA joint.

polarisation of T cells: IL-12 and TNF- α drive T_H1 polarisation, whereas IL-17 drives T_H17 (Rossol et al., 2012). As described above, macrophages play an important role the activation of osteoclasts, resulting in an increase in bone erosion within the inflamed joint. The secretion of IL-8 and CCL2 results in the recruitment of monocytes and neutrophils (Wright et al., 2014). Macrophages within the synovium are also activated by other immune cells present, e.g. T cell-contact mediated, cytokines produced by T cells or via auto-antibodies produced by synovial B cells (McInnes and Schett, 2011). Overall macrophages are instrumental in driving RA pathogenesis through a complex network of interactions.

1.1.3 Co-morbidities associated with RA

Patients with RA are at a high risk of developing co-morbidities; including cardiovascular diseases, pulmonary conditions, depression, infectious diseases and cancer (Dougados et al., 2014). The link and pathological mechanisms that drive these systemic comorbidities are not fully eluded, however it is suspected that they are more prevalent in RA sufferers compared to the general population because of their medication (e.g. glucocorticoids), traditional risk factors (e.g. smoking) and the chronic inflammatory environment generated during RA (Wotton and Goldacre, 2012, Liao and Solomon, 2013, Solomon et al., 2010). Pulmonary conditions associated with RA include chronic obstructive pulmonary disease (COPD), respiratory infection and asthma, with approximately 5-7% of RA patients developing one of these conditions (Dougados et al., 2014). Common infectious diseases including influenza, Epstein-Barr virus, hepatitis B and hepatitis C are more prevalent in RA patients compared to the general population, however these comorbidities are considered lower risk and rarely result in mortality due to strict vaccination routines for RA patients (Kamphuis et al., 2005, Dougados et al., 2014). However, the most prevalent co-morbidity associated with RA is cardiovascular disease (CVD) and this will be a main focus of this thesis.

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Epidemiologic data has shown an increased risk of CVD in RA patients, with one meta-analysis detailing a 50% increase in cardiovascular mortality in RA patients compared with the general population (Avina-Zubieta et al., 2008, Symmons and Gabriel, 2011). Both RA and CVD share some traditional risk factors (e.g. smoking and hypertension) however current evidence demonstrates the important role of inflammation in the pathogenesis linking these diseases (Lauper and Gabay, 2017). The cardiovascular pathologies associated with RA will be described in greater depth in section 1.2.4 of this General Introduction.

1.1.4 Treatments for RA

Current RA therapies and the aggressive treatment plans either treat patient symptoms or slow down disease progression – there are no curative or preventative treatments for RA. Providing treatment to RA patients costs the NHS approximately £560 million per year (National Rheumatoid Arthritis Society, 2017). However, the advances in RA therapeutics over the last two decades has not only allowed the improvement of clinical care and clinical outcome for RA patients, but has helped begin to unravel the complex molecular, cellular and inflammatory networks associated with RA. The therapies available to RA patients currently target different immunological factors involved in disease pathogenesis (e.g. cytokines and co-stimulatory molecules). These are summarised in Table 1.3.

Several RA therapeutics including methotrexate, anti-TNF biologics (e.g. etanercept) and rituximab have been able to be protective against associated cardiovascular pathologies by reducing the total cardiovascular events and myocardial inflammation and improving overall cardiovascular function in patients on these antirheumatic biologics (Ntusi et al., 2018, Sattin and Towheed, 2016, Kim et al., 2015, Novikova et al., 2016).

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Drug	Type of therapy	Mode of action during RA	Reference
Methotrexate	Low-dose therapy	The mode of action of methotrexate during RA is not fully understood, however it has been postulated that it works through the inhibition folate-dependent processes, decrease of adhesion molecule expression, reduction of MMP expression, modification of cytokine expression, stimulation of adenosine signalling and increase of reactive oxygen species production.	(Brown et al., 2016, Gubner et al., 1951)
Infliximab Etanercept Golimumab Adalimumab Certolizumab	Anti-TNF therapy	Infliximab, golimumab and certolizumab block TNF- α signalling during RA by neutralising soluble and transmembrane forms of the cytokine preventing binding to the TNF receptors. Etanercept is a dimeric soluble form of the TNF receptor that binds to two soluble TNF preventing effective binding to the TNF receptors, blocking the downstream processes of TNF- α during RA. Adalimumab blocks the pro-inflammatory effects of TNF- α through binding to soluble TNF and preventing binding to the TNF receptors.	(Izquierdo et al., 2009, Canete et al., 2009, Buch et al., 2008)
Tocilizumab Sarilumab	IL-6 inhibitors	Inhibits IL-6 activity by blocking its binding the cognate receptor expressed on the cell surface and the soluble receptor found in the circulation and synovial fluid.	(Raimondo et al., 2017, McInnes and Schett, 2017)
Secukinumab Brodalumab	IL-17 inhibitors	Secukinumab binds to IL-17A preventing the cytokine from binding to the IL-17 receptor (IL-17R) inhibiting the ability of IL-17 to trigger inflammatory responses. Brodalumab blocks IL-17 signalling during RA by binding to the IL-17R preventing cytokine interaction.	(Kunwar et al., 2016)
Anakinra	IL-1 inhibitor	Anakinra inhibits the activity of both IL-1 α and IL-1 β by binding to the IL-1 type I receptor, preventing IL-1 signalling in RA patients.	(McInnes and Schett, 2017)
Abatacept	CD28-CD80/86 inhibitor	Abatacept targets the co-stimulatory interactions that occur between T-cells, dendritic cells and macrophages by inhibiting CD28-CD80/86 co-stimulatory binding. This results in reduced downstream cytokine signalling, B cell infiltration and osteoclastogenesis during RA.	(Bonelli et al., 2016)
Rituximab	CD20 inhibitor	Rituximab causes B-cell depletion during RA via a number of mechanisms including complement- mediated lysis, antibody dependent cell-mediated cytotoxicity, or apoptosis.	(Schioppo and Ingegnoli, 2017)
Tofacitinib	JAK1/3 kinase inhibitor	Treatment with tofacitinib results in the suppression of synovial T cells, B cells and fibroblast-like synoviocytes either directly by apoptosis or via inhibition of cytokine-dependent feedback loops. A reduction in MMP and chemokine (CCL2, CXCL10, CXCL13) expression is also seen in patients receiving tofacitinib as well as diminished STAT-1 and STAT-3 phosphorylation.	(Boyle et al., 2015, Hodge et al., 2016)

Table 1.3 Mode of action of RA therapeutics

1.1.5 Animal models of RA

The value of animal models in studying the kinetics of RA pathogenesis is the similarity to human pathology, the ability to investigate the pre-arthritis phase and for design and testing of potential therapeutics (Benson et al., 2017). Several species can be used in models of RA with the most common being murine (e.g. DBA/1 mice); others include swine and rat. There several are different types of murine RA models based on their mechanism of induction: spontaneous, adjuvant induction and passive immunisation. These models are summarised in Table 1.4.

Collagen-induced arthritis (CIA) is the gold standard model for pre-clinical RA studies due to the parallels in disease pathogenesis seen between this model and RA. These include the generation of autoantibodies (e.g. rheumatoid factor), inflammation of the synovial membrane, swelling and erosion of the joints, involvement of susceptible alleles (e.g. the HLA alleles) and the loss of peripheral tolerance resulting in systemic complications (e.g. vascular dysfunction) (Luross and Williams, 2001, Reynolds et al., 2012). DBA/1 mice are the strain most commonly used in CIA due to their highly susceptibility of disease onset. Unlike in RA, gender does not affect the susceptibility of developing CIA in mice (Brand et al., 2007). The CIA was the model selected for this thesis due to its close resemblance to the human RA pathology and a stable model was already established within the laboratory (Sime et al., 2017, Williams et al., 2016, Williams et al., 2004, Reynolds et al., 2012, Nowell et al., 2009). The CIA model mimics the systemic nature of RA (summarised in Table 1.5) therefore provides an excellent platform for investigating the cardiovascular events associated with inflammatory arthritis. To date, this laboratory has utilised the CIA model to uncover contractile impairments in CIA mice compared to healthy controls (Reynolds et al., 2012, Williams et al., 2016). Contractile impairment during CIA was linked to an increase in macrophages in the perivascular adipose tissue (PVAT) surrounding the aorta (Williams et al., 2016). Furthermore, the detrimental and protective effects of death

Model	Model type	Brief description	Reference
TNF-Tg	Spontaneous	Mice that overexpress human TNF develop a spontaneous polyarthritis that resembles human RA.	(Li and Schwarz, 2003)
SKG mouse	Spontaneous	A point mutation in the ZAP70 gene results in chronic autoimmune arthritis in the presence of environmental cues.	(Rehaume et al., 2014)
F759	Spontaneous	Mice expressing a variant of gp130 (involved in IL-6 signalling) develop micro- bleeding and joint damage.	(Benson et al., 2017)
Adjuvant arthritis	Adjuvant	A single injection of an adjuvant (e.g. Freund's adjuvant and pristane) with onset occurring between days 10-45.	(Gowayed et al., 2015)
Antigen-induced Arthriti (AIA)	s Adjuvant	A local mBSA challenge within a knee joint that has been pre-exposed to an antigen results in joint inflammation and damage confined to that joint.	(Jones et al., 2015)
Collagen-induced Arthriti (CIA)	s Immunisation	Immunisation with type II collagen results in the generation of autoantibodies triggering synovitis.	(Brand et al., 2007)
K/BxN serum-transfer	Immunisation	Serum from K/BxN mice induces inflammatory arthritis in various mouse strains (e.g. C57BL/6).	(Christensen et al., 2016)

Table 1.4 Murine models of inflammatory arthritis

receptor 3 (DR3) in the aorta and PVAT to vascular constriction during CIA has been characterised (Williams et al., 2016). The methodology of CIA induction in DBA/1 mice is described in full in Chapter 2 of this thesis.

1.2 Cardiovascular system

The cardiovascular system consists of the heart and two main types of vessels (arteries and veins) that allow the transport of oxygen, nutrients and immune cells throughout the body. The structure of major arteries comprises of a three-layered wall: the innermost layer is the tunica intima that is surrounded by the muscular tunica media layer and the outer layer is called the adventitia (Figure 1.3). Surrounding the adventitia is the PVAT, which is a unique deposit of adipose tissue with the ability to influence vascular function (see section 1.3) (Szasz and Webb, 2012). The structure of the vasculature is essential in maintaining efficient vascular function.

1.2.1 Normal vascular function

The main function of the vascular network is the delivery of oxygen and nutrients around the body. Importantly it is also used as a trafficking system for immune cells and cytokines to ensure immune homeostasis and to clear infections at any site within the body(Ross, 1999). Through vascular constriction and vasorelaxation responses the cardiovascular system regulates blood pressure and vascular tone. The constriction response occurs when agonists including serotonin, angiotensin II and norepinephrine interact with their partner receptors on vascular smooth muscle cells (VSMC), resulting in the contraction of the vascular smooth muscle (Benoit and Taylor, 1997). The relaxation of the blood vessel is also tightly regulated. One mechanism vasorelaxation is mediated through is the release of nitric oxide (NO) from the endothelium, in turn increasing the levels of cyclic guanosine monophosphate (cGMP) and through kinase activation lowers the vascular tone (Sausbier et al., 2000). However in states of chronic inflammation, the

Systemic complication	Brief description	Reference
Vascular dysfunction	Mice induced with CIA developed an impaired contractile response compared to non-CIA	(Reynolds et al., 2012,
	controls. An increase in macrophages in the perivascular adipose tissue surrounding the	Williams et al., 2016,
	aorta was linked to vascular dysfunction in CIA mice. CIA has also been used to identify	Denys et al., 2016)
	VCAM-1 as a potential early marker of vascular inflammation.	
Pulmonary inflammation	Following CIA induction mice developed inflammation in the lungs, to a similar extent as	(Schurgers et al., 2012)
	to RA. Treatment with anti-TNF therapy resolved this inflammation.	
Cerebrovascular events	CIA induction impairs the integrity of the blood brain barrier, leading to the development	(Nishioku et al., 2010)
	of neurological complications.	
Secondary metastasis in	An increase in secondary metastases to the lungs and bone were seen when CIA was	(Roy et al., 2011)
cancer models	induced in a strain of mice that spontaneously develop breast cancer.	

Table 1.5 The use of murine models to study systemic complications associated with inflammatory arthritis



Figure 1.3 Structure of the arterial vessel wall

The normal structure of an artery has three main layers: the tunica intima, the tunica media that is a thick layer of elastin and muscle cells, and the outer adventitia layer. Surrounding the adventitia is perivascular adipose tissue (PVAT). There is no anatomical barrier separating the adventitia and PVAT, allowing the easy passage of cells and molecules (e.g. cytokines) from PVAT to the vessel wall. Separating the intima and lumen of the vessel is a thin endothelial lining which allows the trafficking of cells and molecules into the vessel wall from the blood.

vasculature network has the ability to enable a local inflammatory event to turn systemic and advance to other sites in the body. During inflammatory conditions dysregulation of these mechanisms can result in the onset of CVD.

1.2.2 Vascular inflammation

Inflammation in the major blood vessels results in increased vasoconstriction or vasodilation, an increase in vascular permeability due to disruption of the endothelial tight junctions and reduced vascular flow (Sprague and Khalil, 2009). An increase in systemic pro-inflammatory cytokines (for example during RA) can influence vascular function in many ways: through immune cell proliferation (e.g. IL-1, IL-6, IFN- γ , TNF- α , GM-CSF), induction of vascular cell growth and migration (e.g. TNF- α and IL-6), apoptosis (e.g. TNF- α), upregulation of adhesion molecules (e.g. IL-4, IL-17, TNF- α) and remodelling of the extracellular matrix (e.g. IL-1 β , IL-1 β , IFN- γ , TNF- α , MMP-9) (Sprague and Khalil, 2009, Zhang, 2008, Kofler et al., 2005). Vascular inflammation precedes many CVDs including atherosclerosis (build up of plaque in the arteries causing them to harden and narrow), myocardial infarction (heart attack), hypertension (high blood pressure due to an increase in vasoconstriction) and coronary artery disease (the hardening or narrowing of the arteries that supply the heart) (Willerson and Ridker, 2004).

1.2.3 Involvement of macrophages in CVD

Atherosclerosis is a chronic inflammatory disease in which the initiation and progression is dependent on the inflammatory response and lipid accumulation in the artery vessel wall. The inflammatory factors present in the vessel wall contribute to both the progression and resolution of atherogenesis (Ley et al., 2011). Both M1 and M2 macrophages are found within atherosclerotic lesions and have important roles within the disease (Colin et al., 2014). M1 macrophages found in the lesion site have differentiated from Ly6C⁺ CCR2⁺ CX₃CR1^{Io} monocytes that have been recruited into the vessel wall from the blood. Ly6C+ monocytes are commonly found located on parts of the vasculature that

are prone to develop lesions, where they can undergo trans-endothelial migration into the vessel wall and differentiate into inflammatory macrophages or Tip-DCs, showing the importance of the macrophage in atherosclerosis from the start of the disease (Ley et al., 2011). In atherosclerosis one of the first characteristic events is the formation of the foam cell. Macrophages that enter the atherosclerotic lesion begin to phagocytose modified lipids and lipoproteins present at the site and transform into foam cells. This is a regulated process that controls the balance between the uptake of modified LDL (mLDL) and release of cholesterol (Ley et al., 2011). Foam cells will likely differentiate from one of the infiltrating monocytes being recruited into the lesion. The uptake of mLDL, e.g. oxidised LDL, is mediated by scavenger receptors which are expressed on the cell surface the macrophage, e.g. SR-A, CD36 and LOX-1 (Ley et al., 2011, Crucet et al., 2013). Another role of macrophages, mainly M2 macrophages, in the lesion during early atherosclerosis is the clearance of dying cells and other cellular debris deposited during the inflammatory events within the site (Ley et al., 2011). Many of the foam cells formed during the early stages will undergo apoptosis after engulfing lipids present in the lesion. Neighbouring macrophages will clear the apoptotic foam cells and this is associated with regression of atherosclerosis as the levels of pro-inflammatory cytokines will decrease and the chances of a necrotic core forming are also reduced (Tabas, 2010). However, clearance of a large number of apoptotic cells leads to oxidative ER stress within the macrophages and ultimately they fail to keep clearing the debris as the disease progresses. Accumulation of foam cells within the lesion leads to the formation of the necrotic core. As the number of foam cells increase, the larger the lesion will become, ultimately leading to the possible rupture of the fibrous cap and the necrotic core being released into the bloodstream (Tabas, 2010, Ley et al., 2011).

1.2.4 Cardiovascular pathologies associated with RA

Among the CVDs, the most common associated with RA are myocardial infarction, hypertension, stroke, vasculitis and atherosclerosis (Solomon et al., 2006, Kishore et al.,
2017). Of the CVDs listed atherosclerosis is mostly studied. Immune mechanisms and inflammation occurring in RA have been associated with accelerated atherosclerosis (Gualtierotti et al., 2017). This is due to the systemic activation of the pro-inflammatory cytokines (IL-18, IL-33 and TNF- α) that in turn generate a pro-thrombotic environment and endothelial dysfunction (Cugno et al., 2010, Ahmed et al., 2016). This results in upregulation of adhesion molecules and increased infiltration of immune cells into the adventitia in RA patients, accelerating the formation of an atherosclerotic plaque (Ahmed et al., 2016). Comparatively, very little focus has been given to the early changes to vascular structure and function that precede atherosclerosis during RA. This thesis will focus on the characterisation of inflammation-induced changes to the aorta during arthritis in an experimental model CIA.

With increased CVD risk appearing early in RA pathogenesis, even in newly diagnosed patients, it is imperative that the underlying mechanisms linking these two diseases are fully understood in order to reduce cardiovascular-associated mortality and improve the patient's clinical care and outcome. In the general population models have been developed to estimate cardiovascular risk (e.g. Framingham Score), which are currently used to monitor the CV risk of RA patients. Due to chronic inflammation contributing to CVD development, assessment of RA patients cardiovascular risk score this factor will be multiplied by 1.5 (van den Oever et al., 2017). Suppression of RA-associated inflammation by therapeutics (e.g. methotrexate and etanercept) results in a reduction of cardiovascular events and a lower risk score (Choy et al., 2014, Steiner and Urowitz, 2009).

Recently, PVAT was implicated in cardiovascular pathologies including hypertension and atherosclerosis (Fernandez-Alfonso et al., 2017, Oriowo, 2015). Due to the systemic nature of RA, this thesis will investigate the effect on inflammatory arthritis on the cellular and structural composition of PVAT and the potential impact of these alterations on vascular function in the CIA model.

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1.3 Perivascular adipose tissue

Adipose tissue is now recognised as a key organ comprised of many depots. It has important roles in structural support, lipid storage, can respond to multiple stimuli, including temperature, nutritional and immune signals (Szasz and Webb, 2012). Perivascular adipose tissue (PVAT) is a unique depot of adipose tissue mainly due to its location, surrounding systemic blood vessels (Fig 1.3). PVAT differs in abundance, composition and function depending on the type of vasculature it is surrounding. For example, murine large arteries, such as the aorta, have a high abundance of PVAT and a mixed composition of brown and white adipose tissue (Szasz et al., 2013). On the other hand, PVAT sites in the murine peritoneal cavity, such as renal PVAT and mesenteric PVAT are comprised of white adipose tissue (WAT) only (Gao, 2007). The same is true for PVAT in humans (Ozen et al., 2015). PVAT is comprised of a variety of cells including adipocytes, adipocyte precursor cells, fibroblasts, macrophages, T cells and endothelial cells (Raajendiran et al., 2016). The function and characteristics of each PVAT-associated cell type is summarised below in Table 1.6.

Metabolically active PVAT secretes biologically active substances called adipokines, as well as cytokines and gaseous molecules (Szasz and Webb, 2012). Adipokines can be classified as pro-inflammatory (e.g. leptin) or anti-inflammatory (e.g. adiponectin) and can act in an endocrine or paracrine manner (Szasz and Webb, 2012). These substances are produced by adipocytes, the main cell found within PVAT. There are three types of adipocytes; white, brown and beige, each differing in structure, function and origin (Fig 1.4) (Cinti, 2005).

Microarray data has shown the ability of adipocytes to express both pro- and antiinflammatory genes, illustrating their importance as mediators of an immune response (Chatterjee et al., 2013). The ability of adipocytes to regulate their balance between proand anti-inflammatory actions are not fully understood (Omar et al., 2014). However, this

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Type of cell	Role in PVAT	Reference
Adipocyte	PVAT consists of both white and brown adipocytes. The primary function of white adipocytes is the	(Cinti, 2005, Cedikova et al., 2016,
	accumulation and release of fatty acids, which is controlled by the adipokine leptin. Brown adipocytes	Giralt and Villarroya, 2013)
	expend energy through thermoregulation.	
Adipocyte precursor cell	Adipocyte precursor cells can proliferate and differentiate into either white or brown adipocytes in	(Berry et al., 2016)
	order to replenish adipocyte numbers after cell death.	
Fibroblast	Maintain adipose tissue structure through regulation of the extracellular matrix.	(Lee and Lee, 2014)
Macrophage	Adipose tissue macrophages (ATMs) are essential for the uptake of lipids, lipoproteins and	(Zeyda and Stulnig, 2007)
	glycoproteins in PVAT as well as the clearance of apoptotic cells.	
T cell	T regulatory cells are present in PVAT under non-inflammatory conditions to maintain homeostasis.	(Szasz and Webb, 2012)
B cell	B cells present in PVAT release athero-protective IgM antibodies.	(Srikakulapu et al., 2017)

Table 1.6 Role of resident cells in PVAT

balance is key to maintaining homeostasis within PVAT and if an imbalance occurs this has the potential to lead to alterations in PVAT function.

Adipocytes perform a key process called lipolysis that enables them to release fatty acids. Lipolysis involves the hydrolysis of triacylglycerols (TAGs) by specific enzymes into glycerol resulting in the production of fatty acids at each stage (Raajendiran et al., 2016). Multiple mediators tightly regulate lipolysis: lipases (e.g. adipose triglyceride lipase), prolipolytic hormones (e.g. epinephrine) and anti-lipolytic hormones (e.g. insulin) (Raajendiran et al., 2016). This process of releasing stored fatty acids in required in times of increased energy demand, in states of fasting and during exercise (Grant and Stephens, 2015).

1.3.1 Normal PVAT function

PVAT has important functions in regulating vasculature tone and function. One mechanism in which it regulates vascular tone is through alteration of the constriction and relaxation of the blood vessel (Villacorta and Chang, 2015).

The most well known role of PVAT is its ability to induce vasodilation, causing the vessel wall to relax via the release of anti-contractile mediators (e.g. adiponectin, members of the angiotensin family and prostacyclin) (Galvez-Prieto et al., 2012). Adiponectin reduces vascular tone through the activation of calcium-activated potassium channels (K_{CA}) (Lynch et al., 2013). Angiotensin 1-7 produced from PVAT causes the vessel wall to relax in an endothelium-dependant manner (Lee et al., 2011). Angiotensin interacts with Mas receptor resulting in the production of nitric oxide (NO) from the endothelium (Lee et al., 2011), which in turn activates the K_{CA} channels inducing an anti-contractile effect on the surrounding vasculature (Lee et al., 2009). Prostacyclin acts as a powerful anti-contractile factor, causing effect on through interaction with the prostacyclin receptors on the VSMCs (Egan et al., 2004). The predominant mechanism in which the anti-contractile mediators released from PVAT work is through the activation of potassium channels. This has been illustrated through blockage of potassium channels (Aghamohammadzadeh et al., 2012).

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Figure 1.4 The differences between white, brown and beige adipocytes

White adipocytes comprise of one large lipid droplet, a nucleus and few mitochondria. In contrast brown adipocytes, which are smaller in size, have few, small lipid droplets and many mitochondria. The structure of the adipocytes is essential for their respective functions. Beige adipocytes represent a structural intermediate of white and brown adipocytes. Adipocytes have distinct precursors, with white and beige adipocytes arising from either Myf- or Myf+ cells, whereas brown adipocytes only differentiate from Myf+ cells. Key markers, detailed above, can be used to distinguish between adipocytes.

Studies have also shown calcium-dependent potassium channels also contribute to the anti-contractile ability of PVAT (Weston et al., 2013).

PVAT also regulates vascular constriction; multiple mechanisms are involved (e.g. the production of PVAT-derived contracting factors (PDCFs), noradrenaline and prostaglandin) (Villacorta and Chang, 2015). Catecholamines have been shown to be present in certain rat PVAT depots that can induce the release of noradrenaline from PVAT causing the vessel wall to constrict (Ayala-Lopez et al., 2014). Another study in rats also showed constriction through a COX-2 dependent manner (Mendizabal et al., 2013). COX activity in PVAT has also been shown to impact the vasculature constriction in mice in response to serotonin (Meyer et al., 2013).

1.3.2 PVAT under inflammatory conditions

PVAT-dependent anti-contractile responses are compromised by states of oxidative stress and inflammation (Villacorta and Chang, 2015). This loss of function is observed in several diseases including diabetes, hypertension and obesity (Greenstein et al., 2009). It is attributed to a variety of factors that include increased mass of PVAT, infiltration of the tissue by immune cells (e.g. macrophages and T cells) and altered adiponectin levels (Szasz et al., 2013, Lynch et al., 2013). In mice on a high-fat diet it was reported that a decrease in prostacyclin was reported (Chang et al., 2012). This underlines the importance of production of anti-contractile mediators from PVAT in order to maintain vascular function.

The increase in pro-inflammatory cytokines during inflammation (e.g. TNF- α , IL-1 β , IL-6, IL-17 and IFN- γ) stimulates lipolysis in adipocytes (Grant and Stephens, 2015). The storage of lipids in adipocytes is protective against the development of metabolic disease (Khan et al., 2009). The release of pro-inflammatory cytokines, through stimulating lipolysis, causes insulin resistance and leads to adipose tissue dysfunction (Grant and Stephens, 2015).

1.3.3 PVAT and RA

The impact of RA on PVAT morphology and function is not known. Recent studies described an increase in adipose tissue macrophages and pro-inflammatory cytokines in subcutaneous and articular adipose tissue (Giles et al., 2017, Plebanczyk et al., 2011). This was associated with systemic inflammation and autoantibody production during RA. To my knowledge, we are the first group to investigate the alterations that occur in PVAT during inflammatory arthritis (Sime et al., 2017).

1.3.4 PVAT and CVD

PVAT is implicated in the pathogenesis of multiple CVDs, e.g. hypertension, atherosclerosis and abdominal aortic aneurysms (AAA) (Fernandez-Alfonso et al., 2017, Oriowo, 2015). The primary site of inflammation in hypertension is within the PVAT and adventitia where the release of contractile factors (e.g. PDCFs and noradrenaline) causes the vessel to constrict and results in increased blood pressure (Mikolajczyk et al., 2016). The development of high blood pressure increases the risk of the individual suffering from a heart attack or stroke. During the development of hypertension immune cells infiltrate into PVAT, increasing the production of pro-inflammatory cytokines and chemokines, and suppresses immunoregulatory cells and anti-inflammatory adipokines (Nosalski and Guzik, 2017). Only recently PVAT-associated inflammation been implicated in the pathogenesis of atherosclerosis. Perivascular inflammation has been shown to have important roles at various stages of atherosclerosis development, e.g. recruitment of immune cells into the injury site and the production of pro-inflammatory cytokines and adipokines which polarise infiltrating cells (Nosalski and Guzik, 2017). In some instances PVAT inflammation precedes the plaque formation, endothelial dysfunction and oxidative stress which is indicative of atherosclerosis, suggesting it could be a driving factor of this CVD (Skiba et al., 2017). Abdominal aortic aneurysms (AAA) is an inflammatory disease associated with cellular compositional alterations within the aorta and PVAT (Nosalski and Guzik, 2017). During AAA immune cells are recruited into PVAT and contribute to an increase in proinflammatory cytokine release and the production of proteases (e.g. cathepsins) that cause the degradation of the aortic wall (Folkesson et al., 2017).

1.4 Macrophages

Elie Metchnikoff first discovered macrophages in 1882. The name macrophage means 'big eaters' due to their ability to phagocytose (engulf) surrounding cells and cellular debris. As previously outlined in this chapter macrophages have been implicated in the pathogenesis of both RA and CVD. Therefore alterations to the macrophage population in PVAT may be a possible link to the onset of cardiovascular pathologies during inflammatory arthritis.

1.4.1 Macrophage subsets and functions

Macrophages polarise to different subsets depending on the signals present in their microenvironment. Traditionally macrophages were split into two distinct subsets: M1 proinflammatory macrophages or M2 anti-inflammatory macrophages. However, it is now accepted that macrophages are capable of adapting their surface marker expression, secretory profile and therefore function according to alterations in their environment, e.g. cytokines present (Martinez and Gordon, 2014) and has resulted in the establishment of multiple subsets that macrophages move between (summarised in Figure 1.5).

Pro-inflammatory M1 macrophages have important roles in killing of intracellular pathogens via phagocytosis and mediating T_H1 responses (Italiani and Boraschi, 2014). Traditional anti-inflammatory M2 macrophages have now been subdivided into M2a, M2b and M2c subsets based on their polarisation (summarised in Figure 1.6). M2a macrophages are required for clearing parasitic infections and mediating T_H2 responses to combat allergens (Italiani and Boraschi, 2014). M2b macrophages also mediate T_H2 responses but are also critical for immunoregulation (Sironi et al., 2006). M2c macrophages are classed as 'wound-healing' macrophages due to their roles in tissue remodelling (Sindrilaru and Scharffetter-Kochanek, 2013). Over the last few years new subsets have been defined. M4 macrophages are classed as pro-inflammatory macrophages due to their roles in cytotoxicity, monocyte recruitment and have implicated in plaque instability during atherosclerosis (Erbel et al., 2015, Domschke and Gleissner, 2017). Mhem macrophages are anti-inflammatory macrophages that are resistant to lipid accumulation (Chinetti-Gbaguidi et al., 2015). Mox macrophages are pro-inflammatory macrophages that have been found to have a reduced phagocytic capability compared to M1 macrophages (Chinetti-Gbaguidi et al., 2015). The functions of these 'new' subsets are not yet fully understood and more subsets will be established over the coming years. From here on out macrophages will be referred to by their traditional terms in this thesis: M1 are pro-inflammatory and M2 antiinflammatory.

1.4.2 Adipose tissue macrophages

In PVAT one of the key players with roles in homeostasis and inflammation are the resident adipose tissue macrophages. Adipose tissue macrophages (ATMs) have the surface characteristics of a typical M2 macrophage (Zeyda and Stulnig, 2007). It is established that human perivascular ATMs express the M2 surface markers CD163, CD200, CD206, CD209, CD1b, CD1c and integrin αvβ5 (Zeyda et al., 2007). Studies have shown murine perivascular ATMs express M2 macrophage genes *Ym1, arginase, IL-1Ra* and *IL-10* (Todoric et al., 2006, Lumeng et al., 2007a). Although ATMs appear like M2 macrophages based on surface expression, the have the ability to secrete both pro-and anti-inflammatory cytokines. Other immune cells, such as T regulatory cells and eosinophils, present in the PVAT secrete anti-inflammatory cytokines including IL-10 which polarises the resident ATMs towards their M2 phenotype (Vieira-Potter, 2014). Due to their high expression of scavenger receptors and increased endocytic activity, ATMs are functionally important for the uptake of lipids, lipoproteins, apoptotic cells and glycoproteins (Zeyda and Stulnig, 2007). Other important



Figure 1.5 The spectrum of macrophage polarisation

Factors present in the microenvironment will determine the polarisation of macrophages down a specific lineage. This will induce the expression of a specific gene profile that will determine the secretory profile of the macrophage and in turn their function. Alterations to signals within the microenvironment can result in a macrophage polarising from one lineage to another. This allows the immune system to effectively attack an inflammatory insult and resolve the inflammation to a homeostatic state.

functions of ATMs include the regulation of T-cell function through antigen presentation and maintaining adipose tissue homeostasis through balancing cytokine and adipokine levels (Boutens and Stienstra, 2016, Morris et al., 2013). However ATMs have been implicated in fuelling adipose tissue inflammation during a chronic inflammatory insult, e.g. the development of obesity (Lumeng et al., 2007b). The role of ATMs in inflammatory arthritis-associated cardiovascular pathologies has not yet been investigated.

1.5 Summary

There is a high rate of cardiovascular-associated mortalities in RA patients, where greater knowledge of mechanisms that link the two diseases is required and a clinical need for new/improved therapeutics are needed to reduce this mortality rate. RA is a disease with systemic characteristics that affects multiple areas of the body, not just the joints. Recent studies have shown during RA adipose tissue becomes inflamed. In adipose tissue, for example PVAT, there are resident ATMs as well as adipocytes, other immune cells and structural cells. Macrophages have been implicated in the pathogenesis of both RA and CVD; therefore it was hypothesised that alterations to PVAT-associated macrophages during inflammatory arthritis could trigger early vascular dysfunction leading to the onset of cardiovascular pathologies.

1.6 Thesis aims

The aims of this thesis were four-fold:

- To investigate morphological alterations in adipose tissues and phenotypic changes in the ATM population during CIA.
- To identify a specific molecular marker of vascular pathology in PVAT during inflammatory arthritis.
- To investigate the impact of TNF on ATMs and vascular function during CIA through inhibiting TNF- α *in vivo* with etanercept.
- To characterise and assess the effects of galectin-3 inhibition on CIA, vascular function and ATMs.

Chapter 2

Materials and Methods

2.1 Materials

All chemicals were purchased from Sigma-Aldrich (unless stated otherwise). Solvents were supplied by Fisher Scientific (unless stated otherwise). Phosphate Buffered Saline (PBS) pH7.2 was supplied by Life Technologies Ltd (10010-023). All plastic-ware was obtained from Greiner Bio-One Ltd. A Millipore Milli-Q system produced dH₂O that was used to prepare buffers, reagents and stains.

2.1.1 Murine collagen-induced arthritis (CIA) reagents

Animals and housing

Male DBA/1 mice (sourced from Envigo, UK) were used for the CIA model as previous studies have shown they display high incidence, 80-100%, of developing CIA (Brand et al., 2007). Mice were bought in at 8 weeks old and housed in Joint Biological Service Unit (Cardiff University) for 1-2 weeks before experiments were started and throughout procedures. All animals were kept in conventional housing with stable temperature, on a 12 h light/dark cycle and with food and water *ad libitum*.

1M Acetic Acid

In a class II fume hood, 5.74 mL of glacial acetic acid was added to 94.6 mL of dH₂O.

Type II chicken collagen solution

Type II chicken collagen (C9301-5MG, Sigma Aldrich, UK) was prepared at the stock concentration of 5 mg/mL by adding 10mM acetic acid (2475 μ L sterile dH₂O + 25 μ L 1M acetic acid). The collagen was left to dissolve overnight at 4 °C.

Complete Freund's adjuvant (CFA)

100 mg heat-killed *M. Tuberculosis* (strain H37Ra, 231141, BD Biosciences, UK) was ground to a fine powder using a glass mortar and pestle. Using a glass syringe fitted with a 19 gauge needle, 20 mL of incomplete Freud's adjuvant (F5506- 10X10ML, Sigma Aldrich, UK) was mixed thoroughly with the powdered adjuvant (final concentration = 5mg of *M*. *Tuberculosis* per ml of IFA). This complete Freud's adjuvant (CFA) suspension was aliquoted and stored -20 °C until needed.

Type II chicken collagen emulsion

On day 0, CFA was defrosted and re-suspended. CFA (2.5 mL) was added to the Type II chicken collagen solution in acetic acid (2.5 mL). An emulsion was formed by passing liquid through a glass syringe (fitted with 19 gauge needle) at least 20 times. The final concentration of the emulsion was 1 mg of Type II chicken collagen and 2.5 mg of *M. Tuberculosis* per mL of emulsion.

Anti-TNF treatment

Enbrel stock (50 mg/mL), sourced from Pfizer, was diluted in PBS to a working concentration of 2.5 mg/kg.

Galectin-3 inhibitor

Galectin-3 inhibitor GB1107 – Lot 03 (Galecto Biotech) was dissolved in the vehicle to make a working concentration of 10 mg/kg. The vehicle comprised of 10% solutol in PEG300, both sourced from Sigma Aldrich, UK.

2.1.2 Histology reagents

Throughout this thesis, alcohol for histology was prepared using Industrial methylated spirit

(ThermoFisher Scientific, UK) unless stated otherwise.

10% neutral buffered formalin saline (NBFS)

10% NBFS solution was sourced from Sigma Aldrich, UK.

Ethylenediaminetetracetic acid (EDTA) buffer

EDTA is used to sequester metal ions, including calcium (Ca²⁺) and iron (Fe³⁺), in an aqueous solution. EDTA buffer was made by dissolving 70 g EDTA and 5 PBS tablets in 900 mL of distilled H_2O , before adjusting pH to 7.1 by the addition of sodium hydroxide.

Sectioning

R35 blades (JD-0300-00A, Cell Path, UK) were used for sectioning joints and S35 blades (JD-

0100-00A, Cell Path, UK) for sectioning soft tissue.

Haematoxylin

Haematoxylin stain was sourced from VWR UK (EM1.05175.0500).

Scott's Tap Water

Scott's Tap water was made by diluting 100 mL of 10x Scott's Tap solution (S5134-6X100ML, Sigma Aldrich, UK) in 900 mL dH₂O.

1% Eosin

1% Eosin stain was sourced from Thermo Scientific Raymond Lamb Products (LAMB-100-D).

TRAP preparation buffer

The preparation buffer for TRAP staining was made by dissolving 0.82 g sodium acetate and

0.58 g tartaric acid in 50 mL dH₂O.

TRAP stain

Sodium hydroxide pellets were added to TRAP preparation buffer until a PH of 5.0 reached.

To this 0.5 mg/mL napthol AS-MX phosphate (855-20ML, Sigma Aldrich, UK) and 1.1 mg/mL

fast red TR salt (F3381, Sigma Aldrich, UK) was added.

2.1.3 Immunohistochemistry reagents Trypsin-EDTA

0.05% Trypsin-EDTA was sourced from Gibco by Life Technologies, UK (25300054).

Citrate buffer

Citrate buffer was made by dissolving 2.94 g Tri-sodium citrate in 1 L dH $_2$ O and pH adjusted

to 6.0 with 1 M hydrochloric acid (HCl).

Tris/EDTA buffer

Tris/EDTA buffer (10 mM Tris base, 1mM EDTA, 0.05% Tween20) was made by dissolving

1.21g of Tris, 0.37g EDTA and 0.5 mL of Tween20 into 1 L dH₂O, pH was adjusted to 9.0.

Blocking Reagents

A 3% H_2O_2 block was made by diluting 30% H_2O_2 1:10 in PBS. An avidin and biotin blocking kit was sourced from Vector Labs (SP-2001). Serum was sourced from Vector Labs and diluted 1:10 with PBS (Goat: S-1000, Rabbit: S-5000-20).

Immunohistochemistry antibodies

All antibodies used in immunohistochemistry are detailed in Table 2.1 (mouse) and Table 2.2 (human).

Detection Reagents

Ready to use (RTU) streptavidin HRP was sourced from Vector Labs (SA-5704-100). DAB solution was made using the DAB kit (SK-4100, Vector Labs): 1 drop buffer, 2 drops DAB, 1 drop H_2O_2 diluted in 2.5 mL dH₂O.

Human Sections

Sections of human aorta and PVAT samples from RA patients and healthy controls were obtained through collaboration with Dr Ivana Hollan and Dr Agata Burska (Leeds University). Biopsy samples of aortic and adventitial tissue were collected RA and random control subjects. All of the patients gave written informed consent. The regional Ethics Committee for Medical Research Norway approved the study (Ahmed et al., 2016).

2.1.4 Quantitative polymerise chain reaction reagents

RNA Later was sourced from Life Technologies, UK (AM7020). TRI reagent was sourced from Life Technologies, UK (AM9738). RNAse/DNAse free water was sourced from Gibco by Life Technologies, UK (4387936). A high capacity RNA-cDNA kit was sourced from Applied Biosystems, UK (4387406). SYBR green was scoured from Life Technologies, UK (4385618).

Antigen Retrieval	Trypsin-EDTA @ 37°C	Trypsin-EDTA @ 37°C	Trypsin-EDTA @ 37°C	Citrate Buffer pH6 @ 95°C	Citrate Buffer pH6 @ 95°C	Tris/EDTA Buffer @ 95°C
Serum	Goat	Goat	Goat	Goat	Goat	Goat
Working dilution	10%	10%	10%	10%	10%	10%
Primary Antibody	F4/80	F4/80	F4/80	CD68	FcyR1	Galectin-3
Supplier	GeneTex	AbD Serotech	Biolegend	AbD Serotech	antibodies-online	Abcam
Cat. No.	GTX26640	MCA497G	123101	MCA1957	ABIN2000303	ab76245
Species	Rat	Rat	Rat	Rat	Rabbit	Rabbit
Specificity	Mouse	Mouse	Mouse	Mouse	Mouse	Mouse
Туре	Monoclonal	Monoclonal	Monoclonal	Monoclonal	Monoclonal	Monoclonal
Stock concentration	1 mg/mL	0.5 mg/mL	0.5 mg/mL	1 mg/mL	0.37 mg/mL	0.46mg/mL
Working concentration	10 μg/mL	5 μg/mL	20 µg/mL	10 µg/mL	1 μg/mL	1.84µg/mL
Working dilution	1:100	1:100	1:25	1:100	1:370	1:250
Diluent	PBS	PBS	PBS	PBS	PBS	PBS
Isotype Control	Rat IgG2b	Rat IgG2b	Rat IgG2b	Rat IgG2a	Rabbit IgG	Rabbit IgG
Supplier	Santa Cruz	Santa Cruz	Santa Cruz	Biolegend	GeneTex	GeneTex
Cat. No.	SC-3884	SC-3885	SC-3886	400503	GTX35035	GTX35035
Stock concentration	100 tests in 2mL	100 tests in 2mL	100 tests in 2mL	0.5 mg/mL	1 mg/mL	1 mg/mL
Working dilution	1:5	1:10	1:5	1:100	1:1000	1:1000
Secondary Antibody	Goat anti-Rat IgG Biotinylated	Goat anti-Rat IgG Biotinylated	Goat anti-Rat IgG Biotinylated	Goat anti-Rat IgG Biotinylated	Goat anti-Rabbit IgG Biotinylated	Goat anti-Rabbit IgG Biotinylated
Supplier	Vector	Vector	Vector	Vector	Vector	Vector
Stock concentration	1.5 mg/mL	1.5 mg/mL				
Working dilution	1:750	1:750	1:750	1:750	1:750	1:750

Table 2.1 Details of mouse immunohistochemistry antibodies concentrations and dilutions

Antigen Retrieval	Citrate Buffer pH6 @ 95°C	Citrate Buffer pH6 @ 95°C
Serum	Rabbit	Rabbit
Working dilution	10%	10%
Primary Antibody	CD68	Galectin-3
Supplier	DAKO	Abcam
Cat. No.	M081401-2	ab2785
Species	Mouse	Mouse
Specificity	Human	Human
Туре	Monoclonal	Monoclonal
Stock concentration	185 μg/mL	1 mg/mL
Final concentration	3.7 µg/mL	10 μg/mL
Working dilution	1:50	1:100
Diluent	PBS	PBS
Isotype Control	Mouse IgG	Mouse IgG
Supplier	Abcam	Abcam
Cat. No.	ab37355	ab37355
Stock concentration	5 mg/mL	5 mg/mL
Working dilution	1:1000	1:500
Secondary Antibody	Rabbit anti-Mouse IgG Biotinylated	Rabbit anti-Mouse IgG Biotinylated
Supplier	DAKO	DAKO
Stock concentration	1.5 mg/mL	1.5 mg/mL
Working dilution	1:200	1:200

Table 2.2 Details of human immunohistochemistry antibodies concentrations and dilutions

2.1.5 ELISA reagents Mouse Galectin-3 ELISA

A DuoSet mouse galectin-3 ELISA kit was sourced from R&D Technologies, UK (DY1197).

Mouse NT-proBNP ELISA

A mouse NT-proBNP (N-terminal prohormone brain natriuretic peptide) sandwich ELISA was purchased from Source Bioscience, UK (LS-F23107-1).

Wash Buffer

10 PBS tablets and 1 mL 0.05% Tween20 were dissolved in 2 L dH $_2 O.$

Reagent Diluent (1% BSA)

Reagent diluent was made by dissolving 1 PBS tablet and 2 g Bovine Serum Albumin

(A2153, Sigma Aldrich, UK) in 200 mL dH_2O .

Substrate solution

Substrate solution consisted of 10 mL citrate buffer pH 3.95, 100 μL TMB solution and 10 μL

 H_2O_2 .

Stop solution

1 M HCl was used for stop solution.

Plate reader

A Thermo Labsystems Multiskan Spectrum plate reader was used to measure the optical

density (OD) of samples.

2.1.6 Multiplex assay reagents

Mouse T_{H} cytokine panel (740005) and mouse inflammation panel (740150) LEGENDplex

assays were purchased from BioLegend UK, and reagents set up following manufacturer's

instructions. Target details of each panel are listed in Table 2.3.

Flow Cytometer

Analysis was carried out on the BD FACSCanto[™] using DIVA software.

Mouse TH	Mouse inflammation	
cytokine		
IFN-γ	CCL2	
TNF-α	GM-CSF	
IL-2	IFN-β	
IL-4	IFN-γ	
IL-5	TNF-α	
IL-6	IL-1α	
IL-9	IL-1β	
IL-10	IL-6	
IL-13	IL-10	
IL-17A	IL-12	
IL-17F	IL-17A	
IL-21	IL-23	
IL-22	IL-27	

Table 2.3 LEGENDplex assay targets for analysis in mouse plasma samples

2.1.7 Myography reagents Krebs buffer

Krebs buffer pH 7.4 contents are detailed in Table 2.4, was made up in 2 L dH₂O.

High potassium Krebs buffer

High potassium Krebs buffer (60mM) pH7.4 contents are detailed in Table 2.5, was made

up in 1 L dH_2O .

5-HT solution

10 mM 5-HT solution (H5923, Sigma Aldrich, UK) was made by dissolving 2.13 mg of 5-HT in

 $1 \text{ mL } dH_2O.$

Gas Cylinder

For myography a gas supply of 95% oxygen and 5% carbon dioxide (BOC, UK) is required to

each bath.

Galectin-3 inhibitor

Galectin-3 inhibitor GB1107 – Lot 03 (Galecto Biotech) was dissolved in dimethyl sulfoxide (DMSO; 67-68-5, Fisher Scientific, UK) to a working concentration of 10 mM.

Reagent	mM	Weight (g)
NaCl	109.17	12.76
KCI	2.68	0.4
KH ₂ PO ₄	1.18	0.32
MgSO ₄ .7H ₂ O	1.22	0.6
NaHCO ₃	25	4.2
Glucose	10.99	3.96
CaCl ₂ .H ₂ O	1.71	0.44

Table 2.4 Krebs buffer for myography pH7.4

Reagent	mM	Weight (g)
NaCl	39.36	2.3
KCI	59.99	4.472
KH ₂ PO ₄	1.18	0.16
MgSO ₄ .7H ₂ O	1.22	0.3
NaHCO ₃	25	2.1
Glucose	10.99	1.98
CaCl ₂ .H ₂ O	1.71	0.22

Table 2.5 High potassium Krebs buffer for myography pH7.4

2.2 Methods

2.2.1 Murine collagen-induced arthritis (CIA)

Collagen-induced arthritis (CIA) is a very well established model in our group (Nowell et al., 2009, Bull et al., 2008, Reynolds et al., 2012) and is the key model to this thesis. All animal care and experimental procedures were approved by Local Research Ethics Committee, were carried out under personal licence ICF1A6C95 and complied with the United Kingdom Animals (Scientific Procedures) Act 1986 in accordance with project license 30/2928.

2.2.1.1 Induction of CIA

CIA is induced in mice by injecting an emulsion made of Type II chicken collagen and completes Freud's adjuvant (CFA) (See 2.1.1) at the base of the tail on day 0 with a second injection given on day 21 to boost the immune response.

2.2.1.2 Injection protocol

The emulsion of Type II chicken collagen in CFA was administered to each mouse by the intradermal route. Injections (100 μ L in total) were given at the base of the tail on day 0 and day 21. Injections were given at two or three sites on each occasion in order to avoid adjuvant-induced ulceration. The procedure is summarised in Fig 2.1.

2.2.1.3 Anti-TNF therapy

On day 21, animals in the anti-TNF therapy group received intravenous injections of 100 μ L etanercept solution, before their booster intradermal injection of TII chicken collagen in CFA. The vehicle control CIA mice received an intravenous injection of 100 μ L PBS.



Figure 2.1 Summary of CIA injection protocol

Male DBA/1 mice were injected intradermally with 50 μ L of an emulsion containing Type II chicken collagen in CFA on day 0 (right flank) and on day 21 (left flank). Animals were weighed, health checks were performed and arthritis progression was monitored daily from day 21 until termination. Mice received analgesia in their drinking water from day 20 (Buprenorphine solution (400 mg/L)) until the end of each experiment, normally day 28 to day 36.



Figure 2.2 Representative images of non-arthritic and arthritic paws in DBA/1 mice Representative images for daily paw scoring during arthritis progression: front and hind paws scoring 0, 1 and 4.

2.2.1.4 Galectin-3 inhibition therapy

From day 21 to day 29 animals in the GB1107 therapy group received daily oral gavage of 10 mg/kg. Control CIA animals received daily oral gavage of the vehicle.

2.2.1.4 Determining the severity of arthritis

From day 21 onwards, all four paws in each mouse was monitored daily for onset of arthritis and CIA progression. Paw diameter, paw score, clinical score were measured and body weight recorded as indicators of arthritis severity. Joint swelling was measured by taking a paw diameter reading across the mid foot of each hind paw in each mouse using a calliper. Arthritis progression was scored in all four paws using and established scale that ranged from 0 to 5 (Fig 2.2), the criteria for each score is summarised in Table 2.6. Clinical score was the cumulative score for all 4 paws, Table 2.7. Adverse effects and control measures outlined in the project licence were in place until the animals reached the licensed humane endpoint for this procedure. These included a single paw score of 5, an accumulative clinical score of 14 or more and weight loss below 20% of weight at day 21. If any of these adverse effects were met the animal would be culled.

2.2.1.5 Tissue harvest

At the end of each experiment animals were killed by inhalation of rising CO₂ concentration, death was confirmed by palpitation. Adipose tissues: thoracic perivascular adipose tissue (PVAT), abdominal PVAT, renal white adipose tissue (WAT), gonadal WAT and inter-scapular brown adipose tissue (BAT), the thoracic and abdominal aorta, heart and spleen were harvested at termination (Fig 2.3). Skin was removed from hind limbs (left and right) by making an incision at the back of the ankle and pulling the skin towards the abdomen until the hip joint was revealed. The same method was used to remove the skin from the hind paw, pulling the skin towards the toes. Hind limbs were removed at the hip joint.

Paw score	Pathological features
0	Normal (No erythema and swelling)
1	Mild/Moderate erythema and swelling
2	Severe swelling covering entire paw
3	Three joints affected by arthritis
4	All joints affected by arthritis
5	Deformed paw/ankylosis

Table 2.6 Criteria for determining paw score

Clinical score	Severity of arthritis
0	No Arthritis
1-5	Mild
6-9	Moderate
10-14	Severe

Table 2.7 Determination of severity of arthritis

2.2.1.6 Plasma

Whole blood was collected by cardiac puncture after death when each mouse was killed. It was transferred into an individually labelled vacutainer containing 5.4mg EDTA (BD Bioscience, UK). The whole blood was kept on ice for approximately 1 hour until all samples were harvested and until plasma was separated by centrifugation. Plasma was isolated from whole blood after spinning at 5000 g for 15 mins, was transferred into labelled Eppendorfs and stored at -20 °C until required for assessment by ELISA.

2.2.2 Histology

All samples harvested for histological or immunohistochemical analysis were fixed prior to processing. Fixation of tissues by 10% neutral buffered formalin saline (NBFS) was carried out to ensure the preservation of tissue structure and protein expression through cross-linking of amino acid side chains, to halt autolysis and degradation and to harden tissue for sectioning. Fixation with 10% NBFS is the most common for histology due to its good preservation and ease of use (Hewitson et al., 2010).

2.2.2.1 Fixation and decalcification of joints

Hind limbs were removed from the hip joint, as described in section 2.2.1.4, and cut halfway between the ankle and knee joints. Muscle surrounding the tibia and ankle joints (left and right hind limbs) were scored to allow penetration of the fixative through to the bone and were fixed in 10% NBFS for 48 h. Ankle joints were decalcified in EDTA buffer for 3 weeks, changing buffer every 3 days, to remove calcium deposits which is essential for good embedding procedure and sectioning. To ensure ankle joints were fully calcified they were checked via x-ray for any calcium deposits, Fig 2.4.



Figure 2.3 Tissue harvest from CIA and non-CIA mice.

Adipose tissues, heart, spleen and skeletal material were harvested at termination. Perivascular adipose tissue (PVAT) from the thoracic aorta and the abdominal aorta, white adipose tissue (WAT) from kidney and gonads (A.) and brown adipose tissue (BAT) from the inter-scapular region (B.) were collected.



Figure 2.4 Decalcification of bone by EDTA

Hind limbs (left and right) were decalcified in ETDA for 3 weeks post fixation in order to remove all calcium deposits. To determine if all calcium deposits were removed, hind limbs were checked via x-ray. **A.** Hind limb that has not undergone decalcification by EDTA: bones were clearly visible on the x-ray due to the presence of calcium. **B.** Hind ankle joint that has been decalcified in EDTA for 3 weeks: no bones detected due to removal of calcium deposits.

2.2.2.2 Fixation of soft tissue

Soft tissue refers to any tissue harvested that does not contain bone or calcium deposits therefore did not require decalcification with EDTA. Soft tissue samples were fixed in 10% NBFS for 48 h to preserve tissue structure and to retain antigens before being transferred to 70% ethanol for storage until processing or indefinitely. Samples were transferred to 70% ethanol as alcohol is also a fixative for histology and is not carcinogenic like NBFS therefore is safer for handling and storage (van Essen et al., 2010). One batch of soft tissue samples underwent fixation with only 70% ethanol. Fixation with alcohols is safer, does not cross-link antigens, therefore antigen retrieval is not required, and is required for some antibodies to produce successful immunohistochemistry.

2.2.2.3 Histological processing of tissue

Soft tissues and joint specimens were processed through to paraffin wax using a Shandon Tissue processor to allow for thin sectioning. Two different established protocols were used, depending on tissue type, processing samples through serial cycles of methanol, to dehydrate the tissue in order to remove water from the specimen and allow the infiltration of wax, and xylene, to clear the sample of methanol as both water and methanol are immiscible in wax, however xylene is not. Samples then underwent permeation with paraffin wax to allow thin, easy sectioning. The cycles used for both tissue types are summarised in Table 2.8.

2.2.2.4 Sectioning

Processed samples were embedded into paraffin to make wax blocks using a Shandon Histocentre. Tissue sections (7µm) were cut using a microtome and serial sections were mounted onto SuperFrost Plus (ThermoFisher Scientific, UK) slides (2 sections per slide). Slides were placed vertically in a rack, heated overnight at 60°C to remove excess wax and then stored in slide boxes until required for staining.

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Reagent	Soft tissue cycle time (h)	Joint cycle time (h)
70% Methanol (v/v)	01:30	01:00
90% Methanol (v/v)	01.:30	02:00
100% Methanol (v/v)	01:00	02:00
100% Methanol (v/v)	01:00	02:00
100% Methanol (v/v)	01:00	02:00
100% Methanol (v/v)	01:00	02:00
100% Methanol (v/v)	01:00	02:00
Xylene	01:30	02:00
Xylene	02:00	02:00
Xylene	02:00	02:00
Wax	02:00	02:00
Wax	01:30	02:00
Wax	01:30	02:00
Wax	01:00	02:00

Table 2.8 Shandon Tissue Processor cycles for soft tissue and joint samples

2.2.2.5 Haematoxylin and Eosin Staining

Haematoxylin and Eosin (H&E) was used to observe structural and cellular characteristics of different adipose sites and joints from CIA and non-CIA mice. The protocol used is detailed in Table 2.9.

2.2.2.6 TRAP Staining

TRAP staining was used to visualise osteoclast in the hind paws of non-CIA, CIA and treated CIA mice. Rehydration was carried out as outlined in the protocol for H&E staining (Table 2.9) before incubating slides in TRAP preparation buffer overnight at room temperature. The buffer was tapped off before incubating the slides in TRAP stain for 6 hours at 37 °C for 6 hours. Slides were washed in dH₂O for 5 mins, before counterstaining with haematoxylin for 30 secs and dehydrated as outlined in Table 2.9.

2.2.2.7 Microscopy

Images of sections were visualised using light microscopy on the Zeiss Axio Observer 1 and acquired using Zen Pro 2 computer package (ZEISS, UK).

2.2.2.8 Quantification of total cell counts in adipose tissue and the aorta

Total cells/mm² was quantified in thoracic and abdominal PVAT, renal and gonadal WAT, inter-scapular BAT and the thoracic and abdominal aortas. To calculate total number of cells per mm² areas of interest (AOI) of 100 µm x 100 µm were drawn over the adipose area using the rectangle tool in ImageJ. Approximately 70% of the adipose area was covered in AOIs, averaging at 12-14 AOIs for PVAT and 16-18 AOIs for WAT and BAT. A pixel: millimetre ratio was required for drawing AOIs in ImageJ. In order to do this an image of a haemocytometer was taken, at the same magnification as the samples being analysed. As the length of the haemocytometer counting grids are known (250 µm per small square), a pixel/mm ratio was calculated in ImageJ by measuring the number of pixels in 250 µm and multiplying by 4, Fig 2.5. A scale was then set for each image using this ratio, therefore all measurements were then in millimetres. AOIs of 100 µm x 100 µm x 100 µm were drawn, and

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Step	Reagent	Time
Rehydration, Removal of	Xylene 100% (v/v)	5 mins (x3)
excess wax.		
Rehydration, Removal of	Alcohol 100% (v/v)	3 mins (x2)
excess wax.		
Rehydration, Removal of	Alcohol 90% (v/v)	3 mins
excess wax.		
Rehydration	dH ₂ O	5 mins
Cell nucleus stain	Haematoxylin	45 secs
Removal of excess stain	Running tap water	Until water is clear
Removal of excess stain	dH ₂ O	1 min
Bluing of Haematoxylin	Scott's Tap water	30 secs
Wash	Running tap water	1 min
Cytoplasmic stain	Eosin 1% (v/v)	20 secs
Removal of excess stain	Running tap water	Until water is clear
Dehydration, Removal of	Alcohol 90% (v/v)	3 mins
excess stain		
Dehydration, Removal of	Alcohol 100% (v/v)	3 mins (x2)
excess stain		
Dehydration	Xylene 100% (v/v)	5 mins (x3)
Protection of stain	DPX and Coverslip	-

Protection of stain DPX and Coverslip

Table 2.9 Haematoxylin and Eosin staining protocol.



Figure 2.5 Calculating a pixel: millimetre ratio in Image J

A pixel: millimetre ratio is required to be calculated in order to set a scale for accurately drawing AOIs. An image of a haemocytometer is taken at the same magnification as stained samples, here it is at x20. The length of each small box on the counting grid is $250 \mu m$. This distances was measured in pixels on ImageJ using the measure command (Ctrl + M). This distance in pixels was multiplied by 4 to equal the number of pixels/mm at this magnification.
each cell nuclei was counted using the Cell Counter plugin in ImageJ. An average of total cells counted in the AOIs was taken and multiplied by 100 to give total cells/mm², Fig 2.6. Due to the vessel wall having a smaller area to quantify, approximately 8-10 AOIs of 50 μ m x 50 μ m were drawn on the aorta, average cells calculated as previously described and multiplied by 200 to give cells/mm², Fig 2.6.

2.2.2.9 Measuring the thickness of the aorta

The thickness of the aorta was calculated using ImageJ by measuring the distance from the lumen to the adventitia using the line and measure tools, Fig 2.6. Twelve lines were drawn, at regular intervals, around the full circumference of the aorta. As the pixel/mm scale had previously been set all distances were given in millimetres.

2.2.2.10 Quantification of vacuolarity in adipose tissue

Adobe Photoshop Elements 6 package was used to determine the vacuolarity of adipose sites in CIA and non-CIA mice, summarised in Fig 2.7. The term vacuolarity refers to the size of the lipid droplets in the adipocytes. The full area of adipose tissue was selected using the Lasso tool. The colour select histogram window was opened and displayed the total number of pixels present in the area highlighted, this was recorded. Using the + colour select dropper the background and vacuole areas were selected. The number of pixels of these colours in the highlighted area were then displayed in the histogram window. The positive number of pixels was then divided by the total pixels and multiplied by 100 to give the percentage area of the tissue that was vacuolar.

2.2.2.11 Arthritis Index

Arthritis index (AI) is used to examine the severity of arthritis in the hind ankle joints based on an in-house scoring system. H&E stained ankle joints were scored based on synovial hyperplasia (0-3), joint exudate (0-3), synovial infiltrate (0-5) and bone erosion (0-3). Results are displayed as arthritis index (0-14), made up from the total of these four parameters.



Figure 2.6 Quantifying total cells/mm² and vessel wall thickness

To quantify total cells/mm² in adipose tissue (e.g. perivascular adipose tissue (PVAT) illustrated here), areas of interest (AOIs) of 100 μ m x 100 μ m (shown in black) were drawn over the adipose tissue area. Cells in AOIs were counted, an average taken and multiplied by 100 in order to give cells/mm². Boxes of 50 μ m x 50 μ m (shown in yellow) were drawn on the aorta, average cells calculated and multiplied by 200 for cells/mm². The thickness of the aorta was measured in millimetres (white arrow) at 12 intervals around the full vessel circumference and an average was taken.





Adobe Photoshop Elements 6 was used to quantify the vacuolarity of H&E stained sections. The lasso tool was used to select an area of PVAT (**A**.). Opening the histogram window, the total number of pixels in the lassoed adipose tissue area was displayed (**B.-C.**). The colour picker tool was then selected and using the '+ dropper' pixels in the vacuolar area of the adipose tissue were selected (**D.-E.**). The number of pixels in the vacuolar regions were then displayed in the histogram window (**F.**). This number was then divided by the total pixel number of the selected area in order to quantify percentage vacuolarity of adipose tissue.

2.2.3 Immunohistochemistry

A schematic of the immunohistochemistry protocol used is summarised in Fig 2.8.

2.2.3.1 Rehydration

Sections required rehydration and removal of excess wax to allow the tissue to be miscible in water-soluble stains. Sections were cycled through xylene for 5 mins (x3), 100% (v/v) IMS for 3 mins (x2), 90% (v/v) IMS for 3 mins and distilled H_2O (d H_2O) for 5mins.

2.2.3.2 Antigen retrieval

Antigen retrieval was performed to remove any cross-linking of antigens that may have occurred during 10 % NBFS fixation. Different primary antibodies required different methods of antigen retrieval as described in the manufacturer's data sheet.

2.2.3.2.1 Trypsin-EDTA antigen retrieval

Trypsin-EDTA antigen retrieval was required for staining with F4/80 primary antibodies. Slides were incubated in 0.05% Trypsin-EDTA at 37 °C for 1h.

2.2.3.2.2 Citrate buffer antigen retrieval

Citrate buffer antigen retrieval was required for staining with CD68 and FcyR1 primary antibodies. Plastic slide chambers were filled with 40 mL citrate buffer and 20 μ L Tween20. Chambers were pre-heated at 95 °C for 15 mins in a water bath before slides are placed inside the chambers. Slides are then incubated at 95 °C for 40 mins before being left to cool at room temperature for 20 mins.

2.2.3.2.3 Tris/EDTA buffer antigen retrieval

Tris/EDTA buffer was required for staining with the galectin-3 primary antibody. Plastic slide chambers were filled with 40 mL of Tris/EDTA buffer and pre-heated at 95 °C for 15 mins. Slides were incubated at 95 °C for 30mins and left to cool at room temperature for 20mins.

2.2.3.3 Blocking

Blocking of all exogenous molecules and proteins that could interfere with the antibody complex is vital to ensure a false positive does not occur. Slides were incubated in $3\% H_2O_2$ for 10 mins at room temperature, before undergoing a 5 mins PBS wash. Slides were incubated with the avidin block for 15 mins at room temperature, followed by a 5 mins PBS wash. Slides were then incubated with the biotin block for 15 mins at room temperature, followed by a second 5 mins PBS wash. Then slides were incubated with 10% serum for 1 h at room temperature.

2.2.3.4 Primary and isotype antibodies

After blocking the primary and isotype antibodies were applied to sections and incubated overnight at 4 °C. The primary antibody bound to any cell presenting its specific antigen.

2.2.3.5 Secondary antibody

Sections were washed in PBS for 15 mins before secondary antibody incubation at room temperature for 1 h. The biotinylated secondary antibody attached to the primary and isotype antibodies.

2.2.3.6 DAB substrate

Sections were incubated with R.T.U streptavidin horseradish peroxidase, which made a complex with the biotin attached to the secondary antibody. Sections were incubated with the DAB solution until sections turned visibly brown, this was monitored under a light microscope. Sections were submerged in dH₂O to stop the reaction and left for 5 mins.



Figure 2.8 Summary of immunohistochemistry protocol

Sections underwent rehydration and antigen retrieval steps in order to allow the penetration of water soluble stains and to reveal cross-linked antigens. **A. 1**) A series of blocking steps were performed in order to block any exogenous peroxidase, avidin and biotin activity which could result in non-specific staining. **2**) Sections were incubated with the primary antibody, which binds to the target antigen, or isotype control antibody overnight at 4°C. **3**) The biotinylated secondary antibody binds to both the primary and isotype antibodies. **4**) Streptavidin HRP makes a complex with the biotin on the secondary antibody. The addition of the DAB substrate reacts with this complex and is oxidised, resulting in a brown colour changes. **B.** Cells expressing the target antigen were identified as brown cells. Sections were counterstained with haematoxylin therefore cells not expressing the target antigen were blue.

2.2.3.7 Counterstain and dehydration

Sections were counterstained in haematoxylin for 45 secs. Excess stain was removed by running sections in water and blued in Scott's Tap water as previously described in section 2.2.2.5. Sections were then cycled through 90% (v/v) IMS for 3 mins, 100% (v/v) IMS for 3 mins (x2) and xylene for 5 mins (x3) for dehydration and preservation of stain. Slides were preserved using DPX mountant and cover slips.

2.2.3.8 Quantification of positive cells

The same method of calculating cells/mm² in Image J was used here as previously described in section 2.2.2.7. Firstly the total cells/mm² were calculated using this technique and recorded. Positive (brown) cells in each box were then counted, averaged and multiplied using the same method to quantify positive cells/mm². The number of positive cells was divided by the total cells and multiplied by 100 to give percentage positive cells.

2.2.4 Identification of potential PVAT-associated molecular markers

Professor Phil Taylor identified potential targets of macrophage-associated genes from microarray data available on an online database called The Immunological Genome Project (www.immgen.org). This database was used to identify adipose macrophageassociated markers that are expressed at heightened levels in adipose tissue in comparison to other sites, e.g. spleen. This database has samples from WAT and BAT regions but not PVAT samples; therefore this study will add novel findings to this database. Relevant PVATassociated macrophage markers for vasculopathy during inflammatory arthritis were identified by setting specific criteria for a literature review on targets identified by Professor Taylor: 1) markers must be expressed in adipose tissue, 2) markers must be expressed on or secreted by macrophages, 3) markers must be implicated in the pathogenesis of inflammatory arthritis and 4) markers must be implicated in a cardiovascular pathology. Potential markers identified are listed in Table 2.10, with markers highlighted with * being taking forward for investigation in Chapter 4.

2.2.5 Quantitative polymerise chain reaction

Quantitative polymerise chain reaction (qPCR) was carried out to investigate the expression of target genes in the PVAT, WAT and BAT of CIA and non-CIA mice.

2.2.5.1 RNA extraction

Thoracic and abdominal PVAT (aorta excluded), renal and gonadal WAT and interscapular BAT were harvested from CIA and non-CIA mice and immediately stored in RNA Later at 4 °C. Samples were transferred to 5 mL screw top tubes containing 1 mL TRI Reagent which aids the release of RNA by breaking up the cell wall. Tissue was homogenised at full speed until no tissue visually remained. The homogenised samples in TRI Reagent were transferred to 1.5 mL Eppendorfs containing 200 µL Chloroform, vortexed for 15 secs to mix well and centrifuged at 14,000 rpm at 4 °C for 15 mins. This split the nucleic acids into layers and carefully the top, RNA containing layer was removed and transferred to a new labelled Eppendorf. Isopropanol, 500 μ L, was added to each Eppendorf, vortexed and stored at -20 °C overnight. Samples were centrifuged at 14,000 rpm at 4 °C for 15 mins. The isopropanol was carefully discarded, without disturbing the white pellet at the bottom of the Eppendorf. The pellet was then washed twice with 1 mL 75% ethanol, centrifuged at 7,500 rpm at 4 °C for 5 mins after each wash. All ethanol was carefully discarded and the pellet left to air dry for 15 mins. Once dry 10 μ L of RNAse/DNAse free water was added to each sample and stored at -80 °C. RNA concentration was determined using 1 µL of each sample on a NanoDrop 1000 spectrophotometer (ThermoFisher Scientific, UK). The concentration of RNA (ng/mL), 260/280 and 260/230 ratios were recorded.

GENE	PROTEIN	PRESENT ON	PRESENT	IMPLICATED	REFERENCE
		MACROPHAGE	IN	IN CVD	
			ADIPOSE		
* FOLR2	Folate receptor 2	Y	Y	Y	(Jager et al., 2012)
* MRC1	Macrophage	Y	Y	Y	(Haase et al., 2014,
(CD206)	mannose Receptor				Fujisaka et al., 2011)
*CD11C		Y	Y	Y	(Haase et al., 2014)
VSIG4	Complement Receptor Ig	Y	Y	Ν	(Zheng et al., 2014, He et al., 2008)
* MSR1	Macrophage	Y	Y	Y	(Qian et al., 2014, Dai et
(CD204)	scavenger Receptor 1				al., 2013, Neyen et al., 2009)
CD209A	DC-SIGN	Y	Y	Ν	(Fujisaka et al., 2011, Ochoa et al., 2008)
CD209B	SIGNR1	Y	Y	Ν	(Fujisaka et al., 2011)
CD86	Co-	Y	Y	Y	(Edwards et al., 2006)
	stimulatory molecule				
* MERTK	Mer	Y	Y	Y	(Anwar et al., 2009, van
	Tyrosine Kinase				der Meer et al., 2014)
* FCGR1	Fc gamma R	Y	Y	Y	(Nino et al., 2014,
(CD64)					Hernandez-Vargas et al., 2006)
TIMD4	Tim4	Y	Y	Ν	(Chihiro Nishi, 2014,
					Thornley et al., 2014)
MGL2	Mgl2	Y	Y	Ν	(Fink et al., 2014, Denda- Nagai et al., 2010)
CLEC4A1	DCIR4	Y	Y	Ν	(Bloem et al., 2014, Kaifu
					et al., 2011, Ma et al.,
					2010)
* CLEC4A2	DCIR	Y	Y	Y	(Ma et al., 2010, Kaifu et
					al., 2011, Bloem et al., 2014)
CLEC4A3	DCIR3	Y	Y	Ν	(Bloem et al., 2014, Ma
					et al., 2010, Kaifu et al.,
					2011)

Table 2.10 Potential markers for identifying vasculopathy during inflammatory arthritis selected from literature review

2.2.5.2 Reverse Transcription Polymerise Chain Reaction

In order to generate cDNA from the RNA extracted Reverse Transcription Polymerise Chain Reaction (RT-PCR) was carried out. RNA was diluted to a stock concentration of 500 ng/µL with RNAse/DNAse free water. A master mix of 2xRT Buffer (10 µL per sample) and enzyme (1 µL per sample) from the high capacity RNA-cDNA kit was made and distributed into PCR strip tubes (11 µL per tube). To each tube 7 µL of RNAse/DNAse free water and 2 µL of RNA was added to give a total reaction volume of 20 µL per sample. A water (W) control consisting of 10 µL 2xRT Buffer, 1 µL enzyme and 9 µL RNAse/DNAse free water was ran in each RT-PCR reaction as well as a genomic DNA control (-RT) which consists of 10 µL 2xRT Buffer, 2 µL RNA and 8 µL RNAse/DNAse free water. Samples were heated at 37 °C for 60 mins on a thermocycler (Applied Biosystems, UK) to allow the reverse transcription to occur before being heated to 95 °C to stop the reaction. The cDNA samples are stored at -20 °C.

2.2.5.3 Primer design

Murine genes of interest were identified on the database The European Bioinformatics Institute (www.ebi.ac.uk) and exported to Ensembl to view the gene sequence. The protein encoding transcript was selected from the transcript table and the exon sequence highlighted and copied into a Microsoft Word document. Exons were then separated by hyphens and copied into the source input box of Primer3 Input (www.primer3.ut.ee). Parameters generic and RODENT_AND_SIMPLE were selected and product size set to 85-125 before pressing 'pick primers'. Primers were then checked in the word document to ensure at least one of them was exon spanning. Reverse Compliment (http://www.bioinformatics.org/sms/rev_comp.html) was used to identify the complementary forward sequence of the reverse primer in order to check if it was exon spanning. Desalted primers were then ordered from Invitrogen using their custom primers tool. All primers used are listed in Table 2.11. Each primer was diluted with RNAse/DNAse free water to make a 100 μ M stock solution.

2.2.5.4 Real Time- Quantitative Polymerise Chain Reaction (RT-qPCR)

The Fast SYBR method of RT-qPCR was used to determine the expression of selected target genes. Primers were diluted 1:10 from stock and cDNA 1:5 for working concentrations. A master mix for each gene was made up containing 10 μ L SYBR Green, 0.8 μ L forward primer, 0.8 μ L reverse primer and 4.4 μ L RNAse/DNAse free water per well. To each appropriate well on a 96-well plate, 16 μ L of master mix and 4 μ L of cDNA were added. The plate was sealed and centrifuged at 7,500 rpm for 1 min. For each sample the housekeeping gene β -actin was subjected to PCR alongside the test primer set. All samples were plated in triplicate. The Viia7 qPCR machine (Applied Biosystems) was used to analyse each plate summarised in Fig 2.9.

2.2.5.5 Delta-Delta CT analysis

The $\Delta\Delta$ CT method was used to analyse RT-qPCR data, summarised in Fig 2.10. Therefore results reported are relative to the house keeping gene as well as to a control sample.

2.2.6 ELISA

Enzyme-linked immunosorbent assays (ELISA) were used to detect the concentration of target antigens in the plasma of non-arthritic, arthritic and therapy treated DBA/1 mice. The kinetics of a sandwich ELISA are summarised in Fig 2.11.

2.2.6.1 Mouse galectin-3 ELISA

The capture antibody was diluted to the working concentration of 2 μ g/mL and a 96-well plate coated with 50 μ L in each well overnight at room temperature. The plate was aspirated and washed three times with wash buffer. Non-specific binding was blocked by incubated each well with 300 μ L reagent diluent at room temperature for 1 h before being aspirated and washed with wash buffer three times. The standards of known concentration

Gene	Forward Primer	Reverse Primer		
β-actin	TGGCACCACACCTTCTACAA	AGGTCTCAAACATGATCTGGGT		
Folr2	TGGGATCACTGTGGCAAGAT	CAACTCTGGTCCACTTGCTG		
Mrc1	GTTCAGCTATTGGACGCGAG	AGTTGCCGTCTGAACTGAGA		
Msr1	GCAAAGCAACAGGAGGACAT	ACGTGCGCTTGTTCTTCTTT		
Mertk	ACGGGAGATCGAGGAGTTTC	TACACACGCCTAGAAGTCGG		
Fcgr1	TCGGTGGGGAAGTGGTTAAT	AGGCCCCTCACACCATAAAG		
DCIR	AATCTCCTGCAGCTCCCAG	CAGGAGAAGTAGCATCAGGGA		
CD11c	ACGCTTACCTGGGTTACTCC	AAGATGACAACCTTCCCCGT		
iNOS	TATTTTCAGGGCTTGCGTGG	CCACCTCTCCTGGCTTGATG		
Arg1	ACAAGACAGGGCTCCTTTCA	TGCCGTGTTCACAGTACTCT		
PAT2	CCTTCCTGAGAGTGCCAAGA	TGTCTGGAACCCGGTTATGC		
Asc-1	ACAGGCCAGGATCTAAGGTG	CCTCGTGGGGTCTCAACATA		
MMP9	GAGTCTTTGAGTCCGGCAGA	CTTCCAGTACCAACCGTCCT		
Galectin-3	ACCCAACGCAAACAGGATTG	TGTCCTGCTTCGTGTTACAC		
TNF-α	AATGGCCTCCCTCTCATCAG	GTGGTTTGCTACGACGTGG		
CCL2	AGGTCCCTGTCATGCTTCTG	GGGCGTTAACTGCATCTGG		
COL6A1	GGATACCGAGGTGATGAGGG	CATCCTCACCTCTCTACCC		
Table 2.11 Primer sequences used in RT-qPCR				



Figure 2.9 ViiA7 qPCR machine SYBR Green protocol

Complementary DNA samples were ran on the ViiA7 qPCR machine (Applied Biosystems) in order to determine the expression of target genes. Samples were heated to 95°C and then cycled 40 times through 95°C for 1 second and 60°C for 30 seconds rotation for the annealing and extension of the primers along the cDNA. Data on the melt curve was also collected to ensure the primers were amplifying the cDNA.

listed in Table 2.12, were made in 2-fold serial dilutions in reagent diluent: The first standard was made at a dilution of 1:90 from stock provided. Standards (100 μ L) were added to assigned wells and reagent diluent (100 μ L) was used as a blank. Plasma samples from -CIA mice were asaayed at dilutions 1:80 and 1:160 and +CIA samples at 1:160 and 1:320. Samples (50 µL) were added to assigned wells. All standards and samples were run in duplicate. Standards and samples were incubated for 2 h at room temperature before being aspirated and washed three times with wash buffer. The detection antibody was diluted to a working concentration of 200 ng/mL and 50 μ L was added to each well for 2 h at room temperature. The plate was aspirated and washed three times with wash buffer. Streptavidin-HRP was diluted 1:200 from stock and 50 µL added to each well for 20 mins at room temperature. The aspirate and wash step was repeated. Substrate solution (50 µL) was added to each well and incubated avoiding direct light until sufficient colour change in standards is seen. Once this point is reached 25 µL of stop solution is added to each well and the O.D. readings taken. A standard curve is made with the average O.D. readings from standard wells and the slope, intercept and R-squared values of the curve were worked out. The concentration of galectin-3 in each sample was then calculated by dividing (O.D. – intercept) by the slope and then multiplying by the dilution factor.

2.2.6.2 Mouse NT-proBNP ELISA

The microtiter plate supplied in the kit was pre-coated with a target specific capture antibody. Standards of known concentrations, listed in Table 2.12, were made in a series of 2-fold serial dilutions: the first standard was made by reconstituting 1 tube of lyophilised standard in 1 mL of sample diluent. Standards (100 μ L) were added to assigned wells and sample diluent (100 μ L) was used as a blank. Plasma samples were assayed neat and at a 1:10 dilution. Samples (100 μ L) were added to assigned wells. All standards and



Figure 2.10 Summary of $\Delta\Delta C\tau$ qPCR analysis

After samples were analysed on the qPCR machine, the raw data was exported as a Microsoft Excel file. This contained the CT values from all genes analysed in all samples. As samples were ran in triplicate an average of the CT values was taken. To calculate the Δ CT the average CT value of the house keeping gene, a gene which is constitutively expressed across all samples, was subtracted away from each target gene CT in every sample. The $\Delta\Delta$ CT was calculated by subtracting the Δ CT of a non-arthritic sample away from the Δ CT of all other samples of the same tissue type, including the other non-arthritic samples. Finally the RQ value was calculated which details whether there was a fold increase, fold decrease or no change within each sample. This was calculated using the formula $2^{-\Delta\Delta$ CT}.



Figure 2.11 Kinetics of a sandwich ELISA used to determine the concentration of target antigens in plasma samples

A 96-well plate was blocked for endogenous antigens. 1) The plate was then coated with the capture antibody which has specific binding regions for the target antigen. 2) Plasma samples, containing the target antigen, were added to assigned wells allowing the binding of the antigen and antibody to occur. Standards of known concentration were added to assigned wells at this stage also. 3) A biotinylated detection antibody, also specific for the target antigen, was added and binds to a different part of the target antigen which is not interacting with the capture antibody. 4) The avidin section of the streptavidin-HRP complex binds to the biotin attached to the detection antibody. 5) Addition of the substrate TMB initiated a colour change reaction through interaction with the HRP, which allowed optical density (O.D) readings to be obtained in order to work out the concentration of each sample against the standards.

samples were ran in duplicate and incubated at 37°C for 90 mins. Wells were aspirated, but not washed before adding 100 μ L of 1x biotinylated detection antibody to each well and incubated for 1 h at 37°C. The plate was aspirated and washed three times with wash buffer. To each well 100 μ L of 1x HRP conjugate was added and incubated at 37°C for 30 mins. The plate was aspirated and washed five times with wash buffer. TMB solution (90 μ L) was added to each well and incubated at 37°C for 15 mins, monitoring periodically to ensure optimal colour development was reached. The reaction was stopped by adding 50 μ L of stop solution to each well and the O.D. determined by the plate reader. The same method previously described was used to determine the sample concentration of NTproBNP.

2.2.7 Multiplex Assays

LEGENDPlex assay kits were purchased from BioLegend, UK (740005 and 740150). In a V-bottom 96-well plate, 25 μ L Matrix B, 25 μ L of mixed beads and 25 μ L of detection antibodies were added to each assigned standard well. The standard cocktail supplied was serial diluted 1:4 with assay buffer and 25 μ L added to appropriate wells. In wells assigned to samples, 25 μ L assay buffer, 25 μ L mixed beads, 25 μ L detection antibodies and 25 μ L sample, diluted 1:1 in assay buffer, was added to appropriate wells. All standards and samples were ran in duplicate. The plate was sealed, wrapped in aluminium foil and placed on a plate shaker at 600 rpm for 2 hours at room temperature. Without washing, 25 μ L of SA-PE was added to each well, re-sealed and covered, and placed back on the plate shaker at 600 rpm for 30 mins at room temperature. The plate was centrifuged at 1,000 x g for 5 mins. Supernatant was removed using a multichannel pipette, ensuring not to remove the pellet of beads. 200 μ L of wash buffer was added to each well, the beads re-suspended and the plate centrifuged at 1,000 x g for 5 mins. The supernatant was removed using a multichannel pipette and each well re-suspended with 200 μ L wash buffer. Samples were then ran on the BD FACSCanto flow cytometer using FACSDiva software.

Standard	Concentration (pg/mL)
1	1000
2	500
3	250
4	125
5	62.5
6	31.25
7	15.625

Table 2.12 Known concentrations of standards for mouse galectin-3 ELISA and mouse NTproBNP kits Forward (FSC) and side (SSC) parameters were adjusted until two clear bead populations were seen when running the set up Raw beads. The FITC and PE settings were adjusted so that the FITC and PE signals for the majority of beads were between 1x10¹ and 1x10². The APC settings were adjusted so that the APC fluorescence intensities of all bead populations were between 1x10² and 5x10⁴. Samples were run on a low flow rate for approximately 4,000 events. FCS files were exported and analysed using the LEGENDplex Data Analysis Software and Dongle provided.

2.2.8 Myography

The myograph was used to determine the constriction response in non-arthritic, arthritic and therapy treated mice. Details of myograph set up are explained in Fig 2.12. Krebs buffer was bubbled with 95% O_2 and 5% CO_2 prior to start of harvest.

Post schedule 1 and cardiac puncture the base of the abdominal aorta is vented and the heart perfused with 1 mL of oxygenated Krebs buffer to flush the thoracic and abdominal aorta. The thoracic aorta is carefully removed and placed in a universal of oxygenated Krebs buffer. Under a dissection microscope, in a Petri dish of Krebs buffer, the PVAT was carefully removed without causing damage to the vessel wall. Rings of 2 mm in length were cut and mounted on the prongs in each bath. Two rings were cut from each aorta and ran side by side. Baseline tension was set by leaving the rings at 0 mN (millinewtons) for 20 mins to equilibrate before increasing the tension 0.5 mN every minute until the settle at 5 mN. After leaving the rings to settle at this tension the vascular smooth muscle cells were excited by replacing the Krebs buffer in each well with 5 mL high potassium Krebs buffer. Rings were left until the tension plateaued before the high potassium Krebs buffer was removed and each well was washed three times with Krebs buffer. Whilst the aortic rings were left to settle again the 5-HT solutions were made (Fig 2.13). The volumes listed in Table 2.13 were added in sequence to each well, allowing the rings to plateau before the next addition of 5-HT solution. Tension in mN was continually

recorded on the MyoDaq 2.01 Multi+ program and the time of the addition of each 5-HT solution highlighted. The program MyoData 2.02 was used to identify the change in tension of the aortic rings at each 5-HT addition time point.



Figure 2.12 Set up of the myograph before measuring constriction responses

The myograph is turned on and the heated plate set at 37°C. Krebs buffer (5 mL) was added to each bath. The gas supply (95% O2, 5% CO2) was turned on and using valves at the back of each well the flow was controlled to ensure a steady stream of bubbles was present in each bath. After harvest the aortic rings are mounted onto the two prongs submerged in oxygenated Krebs buffer.



Figure 2.13 Serial dilutions of 5-HT used to constrict aortic rings

5-HT induces the constriction of blood vessels. Solutions of 5-HT were made by adding 300 μ L of 10mM 5-HT into 2.7 mL of Krebs buffer and conducting serial dilutions of 500 μ L into 4.5 mL Krebs buffer.

Volume	Concentration	Concentration in Well (mM)
50 μL	1x10 ⁻⁷	1x10 ⁻⁹
100 μL	1x10 ⁻⁷	3x10 ⁻⁹
35 μL	1x10 ⁻⁶	1x10 ⁻⁸
100 μL	1x10 ⁻⁶	3x10 ⁻⁸
35 μL	1x10 ⁻⁵	1x10 ⁻⁷
100 μL	1x10 ⁻⁵	3x10 ⁻⁷
35 μL	1x10 ⁻⁴	1x10 ⁻⁶
100 μL	1x10 ⁻⁴	3x10 ⁻⁶
35 μL	1x10 ⁻³	1x10 ⁻⁵
100 μL	1x10 ⁻³	3x10 ⁻⁵

Table 2.13 Order of the volume and concentration of 5-HT solutions added into eachmyograph bath

Chapter 3

Characterisation of changes to perivascular adipose tissue morphology during collageninduced arthritis

3.1 Introduction

The impact of inflammatory arthritis on the morphology of adipose tissue, specifically thoracic perivascular adipose tissue (PVAT), to my knowledge has not yet been investigated. However as previously described in section 1.1.2, there is a well-established link between rheumatoid arthritis (RA) and cardiovascular disease (CVD) and also evidence that PVAT can directly influence vasculature tone and function (del Rincon et al., 2001, Villacorta and Chang, 2015).

Adipose tissue is a key regulatory organ comprised of many depots throughout the body. PVAT is the primary site of interest in this thesis because of its important functions in regulating vascular tone and function by altering constriction and relaxation responses in the blood vessel (Villacorta and Chang, 2015). The release of PVAT-derived contracting factors (PDCFs) such as adrenaline and prostaglandin induce constriction responses in the surrounding vasculature and the release of anti-contractile mediators, for example adiponectin, members of the angiotensin family and prostacyclin induce vasorelaxation (Villacorta and Chang, 2015, Lynch et al., 2013). These studies underline the complexity of PVATs involvement in vasoconstriction and vasorelaxation via multiple mechanisms, previously described in section 1.2.1. Although the impact of inflammatory arthritis on PVAT morphology and function has not been investigated, studies have implicated the production of auto-antibodies and systemic inflammation present during arthritis with an increase in adipose tissue macrophage (ATM) numbers and pro-inflammatory cytokine production in subcutaneous and articular adipose tissue (Giles et al., 2017, Plebanczyk et al., 2011). For this reason, and to allow comparison of multiple adipose sites during inflammatory arthritis, three additional, non-vascular associated adipose depots were selected for analysis: renal, gonadal and inter-scapular adipose tissue.

Renal and gonadal adipose tissue are classified as white adipose tissue (WAT) due to the fact they comprise mainly of white adipocytes (Cinti, 2005). Renal WAT is located

around the outer layer of the kidney and gonadal WAT surrounds the testes. The function of WAT is the accumulation and release of energy as fatty acids. On the other hand, interscapular adipose tissue is classed as brown adipose tissue (BAT) because it is made up of brown adipocytes (Cinti, 2005). The main function of BAT is heat production through thermogenesis. These specific sites were selected for analysis based on them being anatomically distinct from each other and PVAT, not associated to the vascular system and due to them being classed as either WAT or BAT (whereas PVAT contains both white and brown adipocytes).

Both white and brown adipocytes perform lipolysis, however this process is more instrumental for the release of fatty acids from white adipocytes, as this is their primary function (Raajendiran et al., 2016). As previously described in section 1.3.3, lipolysis is stimulated by pro-inflammatory cytokines. Studies have shown that the blood lipid profile of RA patients differs to that of non-RA patients, suggesting an increase in lipolysis (Chimenti et al., 2013). This has been associated with accelerated atherosclerosis and endothelial dysfunction (Dessein et al., 2002). However, the impact of inflammatory arthritis on adipose tissue morphology and adipocyte lipolysis in PVAT, WAT or BAT sites has not yet been investigated.

As well as adipocytes, there are many other cell types present in adipose tissue. These have been termed the "stromal vascular cell fraction" containing macrophages, T cells, eosinophils, fibroblasts and endothelial cells (Garg et al., 2014, Vieira-Potter, 2014). Of these, the macrophage is an innate immune cell that is implicated in both cardiovascular disease and rheumatoid arthritis (Frantz and Nahrendorf, 2014, Haringman et al., 2005, Ley et al., 2011). The adipose tissue macrophages were therefore considered to be a priority cell-type for investigation in this Chapter.

Macrophages are typically divided into M1 and M2 subsets based on their surface marker expression, secretome and function, previously described in section 1.4.1. Typical

pro-inflammatory M1 ATMs are identified by their expression of iNOS and CD11c, whereas the anti-inflammatory and tissue remodelling M2 ATMs can be identified by their expression of arginase 1 and CD206 (Mosser and Edwards, 2008, Fujisaka et al., 2011, Ferrante and Leibovich, 2012). These markers have been selected to investigate the impact of inflammatory arthritis on the polarisation of the ATM population.

Much of what is known about changes to PVAT morphology and ATMs during inflammation is derived from studies in obesity and diabetes. Rodent obesity models have shown that this increase in total ATM number is likely in response to the increased requirement for phagocytosis of apoptotic adipocytes (Cinti et al., 2005). The mechanisms by which ATMs recruit to the PVAT during obesity is not fully understood, however there is evidence suggesting MCP-1 (CCL2) is involved (Cancello et al., 2005). Murine models of obesity have be used to successfully show the shift from M2-like to M1-like phenotypes in ATMs (Zeyda and Stulnig, 2007, Lumeng et al., 2007a). These activated pro-inflammatory macrophages produce TNF- α , IL-6 and CCL2, which have been linked to the onset of type-2 diabetes, and iNOS, which in turn has been associated with the onset of various cardiovascular diseases (Xu et al., 2003, Lumeng et al., 2007a). It is not known if these alterations occur in PVAT, WAT or BAT during inflammatory arthritis. This therefore warrants the investigation of the effect of arthritis on ATM number and polarisation and in turn the potential impact they may have on the cardiovascular system.

The acquisition of PVAT samples and samples of aorta from RA patients and healthy controls is challenging from a technical and ethical perspective because the procedure is extremely invasive and without direct patient benefit. For these practical reasons, the collection of human tissues was not justified for this proof-of-concept study. Instead, this thesis used an animal model namely; collagen-induced arthritis. CIA is an experimental model of RA that mimics the systemic, polyarticular nature of the arthritis seen in human patients. The CIA model in mouse is established, well-characterised and

induced reproducibly in our laboratory (Brand et al., 2007, Williams et al., 2016, Reynolds et al., 2012, Nowell et al., 2009). Recent data from this laboratory also demonstrated the applicability of the CIA model for assessing arthritis-associated vascular function in conjunction with vasculopathology (Williams et al., 2016). Therefore, the CIA model provides an ethically justifiable and mechanistically strong platform to address the novel questions relating changes in adipose tissue morphology and ATMs caused by inflammatory arthritis.

Hypothesis: Acute CIA leads to morphological alterations in adipose tissue defined by cell ingress, increased adiposity and polarisation of ATMs.

3.2 Aims and objectives

The aim of this chapter was to investigate morphological alterations in adipose tissues and phenotypic changes in the ATM population during CIA.

The following objectives were identified in order to achieve the aim of this Chapter:

- To reproducibly initiate CIA in male DBA/1 mice with an incidence greater than 90% and a time course of arthritis progression that mirrors the established protocol used by successive doctoral candidates in the Rheumatology laboratory.
- To establish a robust quantification method to assess morphological alterations through H&E staining in PVAT and aortas from thoracic and abdominal depots, WAT from renal and gonadal depots and BAT from the inter-scapular depot based on total cell number, vacuolarity of adipocytes and thickness of blood vessel during CIA.
- To investigate the impact of CIA on the expression of WAT marker Asc-1 and BAT marker PAT2 in adipose tissue via quantitative RT-PCR.
- To characterise macrophage populations by measuring M1 and M2 markers in adipose tissues from non-CIA mice via qPCR to compare their expression during CIA.
- To investigate the expression of factors involved in tissue remodelling (e.g. MMP9 and collagen VI) and early inflammation (e.g. TNF-α and CCL2) in PVAT, WAT and BAT during CIA.

3.3 Results

3.3.1 Collagen-induced arthritis

Collagen-induced arthritis was initiated with 100% incidence in male DBA/1 mice (Fig 3.1A). At arthritis onset (normally Day 25), a robust inflammatory response was observed in both hind and front paws. Weight loss occurred at the onset of arthritis. Mice that progressed to severe arthritis lost significantly more weight (p<0.001) than mice with mild CIA (Fig 3.1B). No animal reached the severity limit defined by PPL 30/2928, namely weight loss greater than 20%. As CIA progressed it was characterised by polyarthritis that affected all four paws in each mouse. Swelling of hind paws was monitored throughout the time course of CIA progression by measuring the diameter of the mid-foot. Paw diameters were increased in mice with severe CIA compared to mice with mild arthritis (Fig 3.1C). Hind paw diameters were significantly increased (p<0.001) in both mild and severe CIA compared to the non-immunised controls. Mice that progressed to severe arthritis had a significantly higher (p<0.001) average paw score (average of all four paws) at termination than mice in the mild group (Fig 3.1D). Paw score in the severe group had faster progression of arthritis than mice in the mild group. Clinical score (cumulative paw score for each mouse) also increased at a more rapid rate in mice that developed severe arthritis than mice that developed mild arthritis (Fig 3.1E). Mice with mild arthritis were culled earlier (Day 28-31) than those in the severe group to prevent progression onto moderate or severe arthritis. Mice with severe arthritis were culled when the clinical score reached 14 (the severity limit defined by Home Office project licence; PPL 30/2928) or by Day 36 and whichever factor was reached first.

Histological assessment of hind paws from non-arthritic and mice with mild CIA and severe CIA revealed substantial changes to inflammatory parameters and the micro-





A. Arthritis was induced in 100% of DBA/1 mice where the CIA protocol was initiated. All animals were weighed daily. **B.** Weight change compared to age-matched non-CIA mice at termination reported (n=20 per group), dashed line represents severity limit defined by PPL 30/2928. **C.** Paw diameter was measured daily to monitor swelling. Dashed line represents paw diameters from non-arthritis mice at termination (n=20 per group). **D.** Paw score (0-5) was measured daily from day 21, all non-arthritic mice scored 0 (n=20 per group). **E.** Clinical score (0-14) was calculated as a measure of arthritis progression, all non-arthritis mice scored 0 (n=20 per group). Data expressed mean \pm SEM. Unpaired *t* test or One-way ANOVA with Bonferroni's post multiple comparison test. ** = p<0.01, *** = p<0.001.



Figure 3.2 Arthritis Index of hind paws

Arthritis index (AI, cumulative range 0 to 14) in non-arthritic, mild and severe (n=8 per group) hind paws was assessed by histology and measured by assessing cellular infiltration (0 to 5), synovial hyperplasia (0 to 3), bone erosion (0 to 3) and cellular exudate (0 to 3). **A.** Representative image of a non-arthritic ankle joint, AI=0. **B.** Representative image of a CIA ankle joint, AI=14. *M*: 1st metatarsal, *N*: navicular, *T*: talus, *JS*: joint space, *PF*: pannus formation, *E*: bone erosion, *CI*: cellular infiltrate. *SH*: synovial hyperplasia. Scale bar = 200 µm. Data expressed mean ± SEM. One-way ANOVA with Bonferroni's post multiple comparison test. ** = p<0.01, *** = p<0.001. architecture of the joints as arthritis progressed (Fig 3.2). Infiltration of immune cells into synovial tissues, bone erosion, cellular exudate into joint spaces and synovial hyperplasia were measured (Fig 3.2B). The composite score for these characteristic pathological features of inflammatory arthritis provided an arthritis index for each joint. Arthritis Index was significantly higher in mild mice (p<0.01) and in severe mice (p<0.001) compared to non-immunised controls (Fig 3.2C). Indeed, the arthritis features listed were absent (arthritis index=0) in a healthy joint (Fig 3.2A).

3.3.2 Characterisation of adipose tissue morphology in CIA mice and nonimmunised control mice without arthritis

Adipose tissue morphology was determined in several depots (PVAT, WAT and BAT) by staining tissue sections with H&E staining. Total cell number per mm², percentage vacuolarity of adipose tissue and vessel cell number per mm² and thickness of the aorta was measured in each section as was appropriate for the sample type.

3.3.2.1 Thoracic aorta

Representative images of thoracic PVAT and the thoracic aorta in animals with no, mild or severe arthritis are shown in Figure 3.3A.

In thoracic PVAT cell number (cells/mm²) was significantly increased in mice with mild arthritis (3388±190, p<0.01) and mice with severe arthritis (3800±125, p<0.001) compared to non-immunised control mice (1948±222) (Fig 3.3B). Percentage vacuolarity of thoracic PVAT significantly decreased in severe mice (11.4±2.9, p<0.01) compared to non-CIA mice (33.2±4.3) (Fig 3.3B). This decrease is due to the lipid droplets being smaller in size, as shown in the representative images (Fig 3.3A).

Severe CIA also increased total cell number (cells/mm²) in the blood vessel of the thoracic aorta (2733±124, p<0.001) compared to mildly arthritic mice (2147±51) and the non-arthritic controls (1741±144) (Fig 3.3C). During an inflammatory insult, circulating immune cells traffic through the vasculature in order to reach surrounding tissue. An

increase in vessel thickness (μ m) of the thoracic aorta was also seen in severe CIA mice (67.05±5.4, p<0.05) compared mice in the mild CIA group (46.88±2.1) (Fig 3.3C).

In order to assess if morphological changes observed in the thoracic aorta of mice during CIA were site specific, the abdominal aorta was then analysed.

3.3.2.2 Abdominal aorta

Representative images of abdominal PVAT and the abdominal aorta in animals with no, mild or severe arthritis are shown in Figure 3.4A.

There was a significant increase in total cell number (cells/mm²) of abdominal PVAT in mild (3028±162, p<0.01) and severe (3862±199, p<0.001) groups compared to the non-arthritis controls (1849±38) (Fig 3.4B), similar to what was seen in thoracic PVAT. However, contrary to what was seen in thoracic PVAT, there was no significant difference in percentage vacuolarity of abdominal PVAT between groups (Fig 3.4B).

An increase in cell number (cells/mm²) in the abdominal aorta was also seen in severe mice (2540±132) compared to both mild (1988±120, p<0.05) and non-immunised (1863±16, p<0.01) groups (Fig 3.4C). Contrary to the significant increase in vessel thickness (μ m) seen in the thoracic aorta during severe CIA, no significant difference in vessel thickness was seen in the abdominal aorta (Fig 3.4D). The increase in total cell number in thoracic and abdominal PVAT is similar in both mild (1.6-1.7 fold) and severe (1.92.0 fold) mice compared to the non-immunised controls.

To investigate whether the morphological alterations seen in the thoracic and abdominal aortae during CIA were unique to PVAT or true for all adipose depots, nonvasculature-associated sites were analysed: renal WAT, gonadal WAT and inter-scapular BAT.





Histological assessment of H&E stained sections from the PVAT-intact thoracic aortae from non-arthritic (n=4), mild CIA (n=4) or severe CIA (n=4) revealed morphological changes that were consistent with localized inflammation and tissue remodelling. **A.** Representative images of thoracic aorta (PVAT intact) from non-arthritic, mild and severe arthritic mice. **B.** Thoracic PVAT, cell number and vacuolarity reported (mean \pm SEM). **C.** Aorta (blood vessel only), cell number and vessel thickness presented (mean \pm SEM). Scale bar = 200 µm. Field of view per section = 4. One-way ANOVA with Bonferroni's post multiple comparison test applied. * = p<0.05, ** = p<0.01, *** = p<0.001.



Figure 3.4 Morphological changes in the abdominal PVAT and aorta in CIA mice

Histological assessment of H&E stained sections from the PVAT-intact abdominal aortae from non-arthritic (n=4), mild (n=4) and severe (n=4) mice revealed morphological changes that were consistent with localized inflammation and tissue remodelling. **A.** Representative images of abdominal aorta (PVAT-intact) from non-arthritic, mild and severe mice. **B.** Abdominal PVAT, cell number and vacuolarity reported (mean \pm SEM). **C.** Aorta (blood vessel only), cell number and vessel thickness presented (mean \pm SEM). Scale bar = 200 µm. Field of view per section = 4. Data expressed mean \pm SEM. One-way ANOVA with Bonferroni's post multiple comparison test. * = p<0.05, ** = p<0.01, *** = p<0.001.
3.3.2.3 Renal WAT

Representative images of renal WAT in animals with no, mild or severe arthritis are shown in Figure 3.5A. Total cell number (cells/mm²) of renal WAT was significantly increased during severe CIA (3562±104) compared to mild CIA (2498±329, p<0.05) and non-CIA (1783±54, p<0.001) groups. Therefore the increase in total cell number was not specific to PVAT during CIA. Similar to abdominal PVAT, there was no significant differences in percentage vacuolarity between groups (Fig 3.5B).

3.3.2.4 Gonadal WAT

Representative images of gonadal WAT in animals with no, mild or severe arthritis are shown in Figure 3.6A. Gonadal WAT was selected because it is located away from major blood vessels. In line with previous sites analysed, total cell number (cells/mm²) was significantly increased in mild (1670±150, p<0.05) and severe (2570±178, p<0.001) arthritis compared to non-immunised controls (978±94) (Fig 3.6B). Similar to abdominal PVAT and renal WAT there was no significant difference in percentage vacuolarity of gonadal WAT between treatment groups (Fig 3.6B)

3.3.2.5 Inter-scapular BAT

Representative images of inter-scapular BAT in animals with no, mild or severe arthritis are shown in Figure 3.7A. A significant increase of total cell number (cells/mm²) was also seen in inter-scapular BAT during mild (3773±196, p<0.01) and severe (4273±261, p<0.001) CIA compared to non-arthritic controls (2593±155) (Fig 3.7B). Therefore total cell number increases in PVAT, WAT and BAT sites located adjacent to and away from major blood vessels during CIA. Similar to thoracic PVAT, there was a significant decrease in vacuolarity in inter-scapular BAT of mild (5.5±0.9, p<0.01) and severe (4.7±1.4, p<0.01) groups compared to non-immunised controls (20.2±4.2) (Fig 3.7B).





Histological assessment of H&E stained sections from renal WAT in non-arthritic (n=4), mild (n=4) and severe (n=4) mice revealed morphological changes that were consistent with localized inflammation and tissue remodelling. **A.** Representative images of renal WAT from non-arthritic, mild and severe mice. **B.** Renal WAT, cell number and vacuolarity reported (mean \pm SEM). Scale bar = 200 μ m. Field of view per section = 4. Data expressed mean \pm SEM. One-way ANOVA with Bonferroni's post multiple comparison test. * = p<0.05, ** = p<0.01, *** = p<0.001.





Histological assessment of H&E stained sections from gonadal WAT in non-arthritic (n=4), mild (n=4) and severe (n=4) mice revealed morphological changes in CIA mice. **A.** Representative images of gonadal WAT from non-arthritic, mild and severe mice. **B.** Gonadal WAT, cell number and vacuolarity reported (mean \pm SEM). Scale bar = 200 μ m. Field of view per section = 4. Data expressed mean \pm SEM. One-way ANOVA with Bonferroni's post multiple comparison test. * = p<0.05, ** = p<0.01, *** = p<0.001.





Histological assessment of H&E stained sections from inter-scapular BAT in non-arthritic (n=4), mild (n=4) and severe (n=4) mice revealed morphological changes in CIA mice. **A.** Representative images of inter-scapular BAT from non-arthritic, mild and severe mice. **B.** Inter-scapular BAT, cell number and vacuolarity reported (mean \pm SEM). Scale bar = 200 μ m. Field of view per section = 4. Data expressed mean \pm SEM. One-way ANOVA with Bonferroni's post multiple comparison test. * = p<0.05, ** = p<0.01, *** = p<0.001.

3.3.2.6 Assessment of adipose tissue browning during CIA

Adipose tissue is made up of white, brown and beige adipocytes depending on the depots location, with white adipocytes being larger than brown and being adipocytes due to the size of the lipid droplet (Smorlesi et al., 2012). Browning of white adipose tissue occurs during states of low-grade inflammation correlating with the increase in total cell number within the site, reducing the space available for the adipocytes (Spalding et al., 2008).

Browning of adipose tissue during CIA was determined by analysing the expression of WAT marker Asc-1 (Fig 3.8A) and BAT marker PAT2 (Fig 3.8B). As a proof of concept study and being mindful of the 3Rs (Replacement, Reduction and Refinement) the number of animals used to obtain tissues for each experimental group was kept to the absolute minimum ($n\leq3$).

3.3.2.6.1 Asc-1

In thoracic PVAT, expression of Asc-1 (RQ value) was significantly decreased in mild $(0.25\pm0.01, p<0.001)$ and severe $(0.25\pm0.05, p<0.001)$ arthritic mice compared to the nonimmunised controls (0.96 ± 0.05) . Similarly, Asc-1 expression was also significantly decreased in abdominal PVAT of mild $(0.34\pm0.01, p<0.001)$ and severe $(0.49\pm0.03, p<0.001)$ mice compared to non-arthritic controls (1.01 ± 0.04) . Analysis of Asc-1 expression in nonvascular associated adipose sites revealed no change between CIA groups in renal and gonadal WAT. Asc-1 expression was significantly reduced in inter-scapular BAT during mild $(0.22\pm0.09, p<0.01)$ and severe $(0.39\pm0.13, p<0.05)$ CIA compared to non-CIA controls (1 ± 0.02) .

3.3.2.6.2 PAT2

Analysis of BAT marker PAT2 in thoracic PVAT and renal WAT determined there was no difference in expression (RQ value) between groups. In abdominal PVAT, the expression of PAT2 was significantly decreased in severe (0.8±0.03, p<0.05) CIA compared



Figure 3.8 Adipose tissue expression of WAT and BAT markers in CIA and non-CIA mice Expression of **A.** WAT marker Asc-1 (n≤3 per group) and **B.** BAT marker PAT2 (n≤3 per group) in thoracic PVAT, abdominal PVAT, renal WAT, gonadal WAT and inter-scapular BAT in non-arthritic, mild and severe mice was determined through qPCR analysis. Data expressed as relative quantification (RQ) values calculated in relation to a non-arthritic control. One-way ANOVA with Bonferroni's post multiple comparison test. * = p<0.05, ** = p<0.01, *** = p<0.001.

to mild CIA (1.22±0.07). A significant decrease in PAT2 expression was also seen in gonadal WAT of mild (0.84±0.05, p<0.05) and severe (0.42±0.03, p<0.05) mice compared to non-immunised controls (0.92±0.08). However, in inter-scapular BAT the expression of PAT2 was significantly increased in mild (1.8±0.02, p<0.05) and severe (2.18±0.17, p<0.01) arthritic mice compared to the non-arthritic controls (0.98±0.08).

3.3.3 Identification of macrophages in PVAT, WAT and BAT

In order to investigate if the increase in total cell number in adipose tissue depots during CIA was caused by the infiltration of immune cells, an immunohistochemistry protocol was optimised for adipose tissue.

F4/80 was initially selected as a pan macrophage marker for this thesis because immunohistochemistry protocols had already been established for joint tissue in several of our experimental models of arthritis. Three antibodies were tested for optimisation in adipose tissue staining protocol. Several protocols were tested either with or without antigen retrieval. At best, only weak, non-specific and 'washed out' positive staining was observed by light microscopy (Table 3.1). Results determined that antigen retrieval and fixation in formalin, rather than alcohol, was required in order to obtain any positive staining in adipose tissues (Fig 3.9). F4/80 antibody supplied by GeneTex produced the best results (Fig 3.10).

An alternative pan macrophage marker (CD68) was subsequently tested. It was reported to stain adipose tissue-associated macrophages in the mouse in the literature (Barros et al., 2013). An initial staining protocol was established in mouse tissue sections. Inflamed synovium from a knee joint affected by antigen-induced arthritis (Fig 3.11A) and the spleen (Fig 3.11B) were used as reference macrophage-positive (by F4/80 IHC) tissue standards. CD68 staining produced strong positive staining that was specific (by comparison against an appropriate IgG control antibody) in both joint and spleen tissue samples. The standard was sufficient for accurate quantification by light microscopy.

	AbD Serotech	GeneTex	BioLegend
No antigen retrieval	-	-	-
Trypsin EDTA antigen retrieval	+/-	+	+/-
Archived Aorta + PVAT Sections (Ethanol fixed)	-	-	-
Knee Joints (Formalin fixed)	+	+	+
Ankle Joints (Formalin fixed)	+/-	+	+
Ankle Joints (Ethanol fixed)	_	-	_
Adipose Tissue (Formalin fixed)	N/A	+/-	N/A

Table 3.1 Optimisation of F4/80 antibodies in adipose tissue produced washed out, non-specific staining (+/-).



Figure 3.9 Fixation of tissues with formalin is required for positive staining using F4/80 antibodies

To assess the effect of different tissue fixation methods on positive F4/80 staining immunohistochemistry was carried out in **A.** an ethanol fixed ankle joint, **B.** a formalin fixed ankle joint and **C.** a formalin fixed knee joint in immunised mice. Representative images shown were stained with the GeneTex primary antibody, inserts are IgG isotype controls. Scale bar = 50 µm.



Figure 3.10 F4/80 IHC in PVAT, WAT and BAT produced weak, non-specific staining

Formalin fixed adipose sites **A.** Thoracic PVAT and aorta, **B.** Renal WAT and **C.** Inter-scapular BAT were stained with the GeneTex F4/80 primary IHC antibody in order to identify macrophages within these sites for quantification. Inserts are IgG isotype controls. Scale bar = 50 µm.



Figure 3.11 Immunohistochemistry with CD68 primary antibody produces clear and specific positive staining in murine inflamed synovium and spleen. Identification of macrophages via CD68 immunohistochemistry in formalin fixed **A.** inflamed synovium of murine knee joint and **B.** murine spleen produced clear positive staining. Inserts are IgG isotype controls. Scale bar = 200 µm.

3.3.4 The number of macrophages in PVAT, WAT and BAT significantly increase during CIA

Macrophages are one of the first immune cells to respond in the event of an inflammatory insult. PVAT, WAT and BAT sites were analysed by immunohistochemistry in order to investigate if the total cell increase seen across adipose tissue during CIA was due to an increase in the number of macrophages (CD68⁺ cells).

3.3.4.1 Thoracic aorta

Representative images of $CD68^+$ cells in the thoracic PVAT are shown in Figure 3.12A and of the blood vessel in Figure 3.12C.

In thoracic PVAT the percentage of CD68⁺ cells was significantly increased 2-fold in mice with mild (13.08 \pm 0.7, p<0.001) and severe (12.49 \pm 0.9, p<0.001) arthritis compared to the non-immunised controls (6.2 \pm 0.7) (Fig 3.12B). Therefore macrophages, at least in part, account for a proportion of the increased total cell number described previously in thoracic PVAT.

A significant increase of percentage $CD68^+$ cells was also noted in the thoracic aorta of mice with mild arthritis (12.33±0.9, p<0.05) compared to non-immunised controls (7.04±1.1), but not during severe CIA (Fig 3.12D).

3.3.4.2 Abdominal aorta

Representative images of CD68⁺ cells in the abdominal aorta are shown in Figure 3.13A and of the blood vessel in Figure 3.13C.

In abdominal PVAT there was a significant 1.5 fold increase in percentage CD68⁺ cells in the mild mice (12.51±0.8, p<0.05) compared to the non-immunised controls (8.1±0.6), however there was no significant difference between the severe and no-immunised mice (Fig 3.13B). Macrophage number was only increased during mild CIA in



Figure 3.12 CD68 IHC identified an increase in macrophages in the thoracic aorta during CIA

Immunohistochemistry analysis with CD68 primary antibody identified an increase in macrophages within the thoracic aorta of mice during CIA. **A.** Representative images identifying CD68 positive cells (\checkmark) in thoracic PVAT (n=8 per group). **B.** Thoracic PVAT total cell number, number of CD68 positive cells and percentage positive cells reported (mean ± SEM). **C.** Representative images identifying CD68 positive cells (\checkmark) in the blood vessel (n=8 per group). **D.** Blood vessel total cell number, number of CD68 positive cells and percentage positive cells reported (mean ± SEM). **D.** Blood vessel total cell number, number of CD68 positive cells and percentage positive cells reported (mean ± SEM). Inserts are IgG isotype controls. Scale bar = 25 µm. Field of view per section = 4. One-way ANOVA with Bonferroni's post multiple comparison test. * = p<0.05, ** = p<0.01, *** = p<0.001.

abdominal PVAT, whereas in thoracic PVAT increased number was maintained through to severe CIA.

Unlike in the thoracic aorta, there was no significant difference in the percentage of CD68⁺ cells between groups in the abdominal aorta (Fig 3.13D).

3.3.4.3 Renal WAT

Quantification of percentage $CD68^+$ cells in renal WAT (Fig 3.14A) determined a significant 2-fold increase in mild (11.39±1.1, p<0.001) and severe (11.6±0.8, p<0.001) mice compared to non-immunised controls (5.61±0.5) (Fig 3.14B). Therefore the increase in macrophage number was not specific to vasculature-associated adipose depots.

3.3.4.4 Gonadal WAT

Similar to renal WAT, significant 2-fold increase of percentage CD68⁺ cells was seen in gonadal WAT in both mild (11.39 \pm 0.7, p<0.001) and severe (12.46 \pm 1.2, p<0.001) groups compared to the no arthritis controls (5.97 \pm 0.4) (Fig 3.15A+B).

3.3.4.5 Inter-scapular BAT

In inter-scapular BAT (Fig 3.16A) there was a significant 1.5-fold increase in the percentage of CD68⁺ cells in mild (15.7 \pm 1.1, p<0.001) and severe (15.31 \pm 0.6, p<0.001) arthritic mice compared to the non-arthritic controls (9.9 \pm 0.7) (Fig 3.16B). Therefore the number of macrophages present in PVAT, WAT and BAT were significantly increased during CIA.

3.3.5 The expression of M2 macrophage markers were significantly increased in adipose tissue during CIA

To determine the effect of CIA induction on the phenotype of macrophages in adipose tissue between CIA and non-CIA mice, M1 and M2 macrophage markers were examined using qPCR analysis in PVAT, WAT and BAT.



Figure 3.13 An increase in percentage CD68 positive cells is present in abdominal PVAT during CIA

Immunohistochemistry to identify macrophages (CD68⁺ cells) was carried out in the abdominal aorta of non-immunised, mild and severe CIA mice. **A.** Representative images identifying CD68 positive cells (\checkmark) in abdominal PVAT (n=8 per group). **B.** Abdominal PVAT total cell number, number of CD68 positive cells and percentage positive cells reported (mean ± SEM). **C.** Representative images identifying CD68 positive cells (\checkmark) in the blood vessel (n=8 per group). **D.** Blood vessel total cell number, number of CD68 positive cells and percentage positive cells reported (mean ± SEM). **C.** Scale bar = 25 µm. Field of view per section = 4. One-way ANOVA with Bonferroni's post multiple comparison test. * = p<0.05, ** = p<0.01, *** = p<0.001.





CD68 immunohistochemistry was carried out in renal WAT in order to assess macrophage number during CIA. **A.** Representative images identifying CD68 positive cells (\checkmark) in renal WAT (n=8 per group). **B.** Renal WAT total cell number, number of CD68 positive cells and percentage positive cells reported (mean ± SEM). Scale bar = 25 µm. Field of view per section = 4. One-way ANOVA with Bonferroni's post multiple comparison test. ** = p<0.01, *** = p<0.001.





An increase in CD68 positive cells in gonadal WAT during CIA was determined through immunohistochemistry. **A.** Representative images identifying CD68 positive cells (\checkmark) in gonadal WAT (n=8 per group). **B.** Gonadal WAT total cell number, number of CD68 positive cells and percentage positive cells reported (mean ± SEM). Scale bar = 25 µm. Field of view per section = 4. One-way ANOVA with Bonferroni's post multiple comparison test. * = p<0.05, ** = p<0.01, *** = p<0.001.





3.3.5.1 M1 markers

Inducible nitric oxide synthase (iNOS) is a macrophage marker classically used to identify an M1 population within all tissue sites, whereas CD11c has previously been used specifically to identify this population in adipose tissue (Fujisaka et al., 2011, Mosser, 2003). In thoracic and abdominal PVAT, gonadal WAT and BAT, there was no significant difference in M1 markers CD11c (Fig 3.17A) and iNOS (Fig 3.17B) in mild and severe CIA mice compared to the non-immunised controls. In renal WAT, iNOS expression (RQ value) was significantly increased in severe (2.7±0.5) mice compared to mild (1.39±0.1, p<0.01) and non-arthritic (0.95±0.1, p<0.001) controls. There was no significant difference between groups in CD11c expression in renal WAT. The significant increase in iNOS in renal WAT suggests that changes in polarisation of macrophage phenotype may be dependent on environmental cues present within each adipose depot.

3.3.5.2 M2 markers

Similar to the M1 markers, a classical (Arg1) and an adipose specific (Mrc1) M2 marker were selected for qPCR analysis. Analysis of M2 marker Mrc1 (CD206) showed no significant difference across adipose sites between CIA and non-CIA controls (Fig 3.18A). The expression (RQ value) of M2 marker Arg1 (Fig 3.18B) was significantly increased in thoracic PVAT in mild (5.21±0.6, p<0.01) and severe (4.84±1.3, p<0.01) mice compared to non-immunised controls (1.11±0.1). Expression was not increased in abdominal PVAT, thus the environmental stimuli between different adipose depots of the same type impact macrophage polarisation. Arg1 expression was also significantly increased in renal WAT of severe animals (2.1±0.4, p<0.05) compared to non-CIA controls (1.05±0.06), and in BAT of severe mice (2.54±0.6) compared to mild (0.74±0.2, p<0.01) and non-arthritic (0.94±0.1, p<0.05) controls. There was no significant increase in expression in gonadal WAT between groups.

Overall, both M1 and M2 macrophage phenotypes are present in PVAT, WAT and BAT sites during CIA, with a substantial increase in M2 macrophages in thoracic PVAT.

3.3.5.3 Expression of TNF- α and CCL2 in adipose tissue during CIA

TNF- α and CCL2 were analysed by qPCR in order to assess if inflammationassociated recruitment of monocytes in adipose tissue is occurring during CIA. During the initial stages of a pro-inflammatory immune response macrophages release TNF- α to activate surrounding and infiltrating immune cells, e.g. monocytes. This makes TNF- α a strong candidate marker of early inflammation in PVAT, WAT and BAT during CIA (Gordon and Taylor, 2005). There was no significant difference in expression of TNF α (Fig 3.19A) across adipose sites, however there was a trend of increased TNF α expression in thoracic PVAT of mildly arthritic (4.68±1.8) animals compared to the non-immunised controls (1.03±0.1).

The main chemoattractant that facilitates the ingress of circulating monocytes into an inflamed tissue, including PVAT, is CCL2 (Chow et al., 2011). Therefore CCL2 is a critical marker to use for assessment of monocyte ingress in PVAT, WAT and BAT during CIA. A non-significant trend of increased expression (RQ value) CCL2 (Fig 3.19B) was also seen in the thoracic PVAT of mild mice (3.58±1.4) compared to non-CIA controls (0.95±0.2). CCL2 expression was significantly decreased in gonadal WAT of severe mice (0.42±0.1, p<0.05) compared to non-immunised controls (1.35±0.3).

3.3.6 Markers of remodelling were not upregulated in PVAT during CIA

Adipose tissue remodelling is defined as alterations in the number/size of adipocytes and changes in the number and function of stromal and immune cells present which in turn directly effects the function of the adipose tissue as an endocrine organ



Figure 3.17 Adipose tissue expression of M1 markers in CIA and non-CIA mice The expression of M1 macrophage markers **A.** CD11c (n=8 per group) and **B.** iNOS (n≤7 per group) in thoracic PVAT, abdominal PVAT, renal WAT, gonadal WAT and inter-scapular BAT in non-arthritic, mild and severe mice was analysed using qPCR. Data expressed as relative quantification (RQ) values calculated in relation to a non-arthritic control. One-way ANOVA with Bonferroni's post multiple comparison test. ** = p<0.01, *** = p<0.001.



Figure 3.18 Adipose tissue expression of M2 markers in CIA and non-CIA mice The expression of M2 macrophage markers **A.** Mrc1 (n=8 per group) and **B.** Arg1 (n≤7 per group) in thoracic PVAT, abdominal PVAT, renal WAT, gonadal WAT and inter-scapular BAT in non-arthritic, mild and severe mice was assessed by qPCR. Data expressed as relative quantification (RQ) values calculated in relation to a non-arthritic control. One-way ANOVA with Bonferroni's post multiple comparison test. * = p<0.05, ** = p<0.01.

(Choe et al., 2016). Next, the expression of tissue remodelling markers matrix metalloproteinase 9 (MMP9) and collagen VI were investigated. This was because the primary function of M2 macrophages is tissue remodelling and an increased expression of M2 macrophage marker Arg1 was observed in thoracic PVAT during CIA.

3.3.6.1 MMP9

MMP9 is one member of the matrix metalloproteinase family with functions in tissue remodelling by degrading denatured collagen (Nagase et al., 2006). In thoracic PVAT, gonadal WAT and inter-scapular BAT there was no significant difference in MMP9 expression (RQ value) between CIA and non-CIA mice (Fig 3.20A). MMP9 expression was significantly decreased in abdominal PVAT of mild (0.31±0.11, p<0.01) and severe (0.27±0.11, p<0.01) mice compared to non-immunised controls (0.99±0.13) and also in renal WAT of mild mice (0.51±0.1, p<0.01) compared to non-arthritic controls (1.04±0.06).

3.3.6.2 Collagen VI

In animal models of obesity a significant increase in the collagen content of adipose tissue, particularly, collagen VI is observed (Khan et al., 2009). There was a significant increase in collagen VI expression (RQ value) (Fig 3.20B) in renal WAT of mice with severe CIA (2.89±0.5, p<0.05) compared to the non-immunised controls (1.07±0.14). In interscapular BAT collagen VI expression was significantly decreased in mild (0.44±0.11, p<0.01) and severe (0.15±0.01, p<0.001) mice with CIA compared to the non-arthritic controls (1.05±0.08). There was no significant difference in collagen VI expression in PVAT or gonadal WAT between groups.



Figure 3.19 Adipose tissue expression of TNF- α and CCL2 markers in CIA and non-CIA mice The expression of **A.** inflammatory marker TNF α (n=4 per group) and **B.** ingress marker CCL2 (n=4 per group) in thoracic PVAT, abdominal PVAT, renal WAT, gonadal WAT and inter-scapular BAT in non-arthritic, mild and severe mice was analysed using qPCR. Data expressed as relative quantification (RQ) values calculated in relation to a non-arthritic control. One-way ANOVA with Bonferroni's post multiple comparison test. * = p<0.05.



Figure 3.20 Adipose tissue expression of tissue remodelling markers in CIA and non-CIA mice

The expression of remodelling markers **A.** MMP9 ($n\leq7$ per group) and **B.** collagen VI (n=4 per group) in thoracic PVAT, abdominal PVAT, renal WAT, gonadal WAT and inter-scapular BAT in non-arthritic, mild and severe mice were analysed by qPCR. Data expressed as relative quantification (RQ) values calculated in relation to a non-arthritic control. One-way ANOVA with Bonferroni's post multiple comparison test. * = p<0.05, ** = p<0.01, *** = p<0.001.

3.4 Discussion

This chapter described changes in the morphology of adipose tissues during CIA with specific focus on macrophage-associated changes in in PVAT, WAT and BAT. A proportion of the proof-of-concept results from in this Chapter were recently subjected to peer review and published (Sime et al., 2017). To my knowledge, this is the first detailed account of the morphological alteration to adipose tissues during CIA. Tissue changes to the PVAT compartment adjacent to the thoracic aorta in particular may have potentially important implication for causing early vascular pathology and dysfunction during inflammatory arthritis if translated to human disease. The possible clinical utility of the observations from this Chapter are identified and studied in greater detail in Chapters 4 and 5.

100% of immunised male DBA/1 mice developed CIA characterised by swelling in the joints and redness of paws. Histological analysis of paws from mice with CIA revealed bone erosions, pannus formation and synovial inflammation, which are all pathological features of RA (McInnes and Schett, 2011). In this chapter CIA mice were separated by severity into groups of mild or severe arthritis. Mice in the severe group developed arthritis (clinical score) quickly whereas the mild group developed arthritis at a slower rate when compared on the same day, e.g. Day 26. This mimics what is seen in RA patients as they can be split into groups of rapid progression or slow progression (Mansson et al., 1995). Patients who experience rapid progression and therefore more severe RA have a higher cardiovascular risk score than slow progressing patients (Myasoedova et al., 2016). Therefore we ask the question whether mice that develop a slower, mild arthritis undergo similar or different morphological alterations in PVAT than those who develop a rapid, severe arthritis. This will be discussed later in this section.

In addition to studying mechanisms of joint pathology and testing disease modifying therapies against arthritis, the CIA model has also been used by the Cardiff

laboratory to successfully investigate vasculopathology associated with inflammatory arthritis. The role of MMP9 and death receptor 3 (DR3) in modulating impaired constriction responses during CIA are published (Reynolds et al., 2012, Williams et al., 2016). During Year 1 of this project Williams et al made the initial observation that cell numbers in PVAT were increased by CIA in PVAT and that the ingress was associated with an increase in the number of both macrophages and neutrophils (Williams et al., 2016). The focus of the Williams study was investigating the involvement of PVAT and DR3 on vascular constriction responses during CIA. Although morphological alterations to the PVAT and aorta were observed during CIA this study did not characterise these changes in depth. The novel results of this Chapter agree with, compliment and build upon the initial findings by Reynolds and Williams. Here and for the first time alterations to adipose tissue characterised by total cell number, ATM number and polarisation, vacuolarity and adipocyte phenotype are reported. This study also identifies CIA as a suitable model for future studies focussed on the discovery and development of drugs and diagnostic tools that target CVD associated with inflammatory arthritis.

Tissue morphology is defined as the cells and structural components that form an organ. The adipocyte is the main cell composing adipose tissue; pre-adipocytes, ATMs, endothelial cells, fibroblasts, mesenchymal cells, leukocytes and lymphocytes are also present (Szasz and Webb, 2012). There was an increase in total cell number in vasculature-associated adipose tissue (PVAT) and distant adipose depots (WAT and BAT) during CIA. Several possible reasons for this increase in total cell number include: adipocyte hyperplasia, expansion of resident ATMs and/or infiltration of leukocytes and lymphocytes into the adipose tissue. This hyper-cellular phenotype has previously been described in animal models of obesity, where it was reported that the generation of a pro-inflammatory environment within adipose tissue resulted in both adipocyte hyperplasia and an increase in infiltrating immune cells, in turn causing a shift in the balance of adipokines, cytokines

and gaseous molecules being secreted from the adipose tissue (Apostolopoulos et al., 2016, Hoffstedt et al., 2010, Acosta et al., 2016). This expansion of metabolically active adipose tissue causes systemic inflammation, increased blood pressure, lipid alteration and insulin resistance, which lead to endothelial dysfunction and the onset of cardiovascular diseases (Taube et al., 2012). The link between RA and cardiovascular disease is well characterised, therefore in the CIA model the alterations in PVAT morphology may have an impact on the surrounding vasculature, increasing the chance of cardiovascular pathologies developing in CIA mice.

Initial experiments to identify macrophages in PVAT, WAT and BAT proved challenging even though Williams et al had already reported a protocol for F4/80 IHC in PVAT (Williams et al., 2016). F4/80 is a pan macrophage marker used in immunohistochemistry for the identification of macrophages (Hume D.A, 1984). Only weak, non-specific staining for macrophages by F4/80 IHC was achieved on archived sections known to produce strong positive staining by Williams et al 2016 despite several attempts to re-optimise the original protocol with the new batch of F4/80 from the same supplier and F4/80 from different sources. The fixation method for tissue samples is a major consideration when selecting appropriate antibodies for IHC. After thorough optimisation it was conclusive that the weak, non-specific staining was not due to technical error and a second pan macrophage marker, CD68, was selected and positive identification of ATMs achieved.

During CIA, there was a significant increase in the number of macrophages in PVAT, WAT and BAT, matching what was previously described in Williams et al. By comparing the increase in number of ATMs to the increase in total number of cells in PVAT, WAT and BAT, it was apparent that the increase in ATMs only partly accounts for the increase in total cells. It is already known that an increase in neutrophils is seen in PVAT during CIA (Williams et al., 2016). Expansion of adipocytes and fibroblasts present in the adipose

tissue and/or infiltrating of other immune cells, e.g. T cells and B cells, may also account towards the increase in total cell number. Further experiments either by immunohistochemistry or flow cytometry would answer this question. An increase in ATMs was reported during obesity and associated to the development of hypertension, impaired glucose and lipid metabolism, the onset of type II diabetes and increased risk of CVDs (Weisberg et al., 2003, Zeyda and Stulnig, 2007). In order to determine if the increase in ATMs during CIA is associated with hypertension and/or impaired glucose and lipid metabolism further studies including the measurement of blood pressure (e.g. using a Harvard mouse tail photoplethysmography pressure monitor), a glucose tolerance test (GTT) and analysing lipid profiles using LC/MS/MS (liquid chromatography/mass spectrometry/mass spectrometry) in CIA and non-CIA mice are required (Fu et al., 2016, Bowe et al., 2014, Kramer and Remie, 2005).

In indication of whether the increase in macrophage number in adipose tissue during CIA was caused by the infiltration of monocytes/macrophages from the circulation into adipose depots was assessed by measuring TNF- α and CCL2 as two molecular markers of early inflammation. A study using rat aortic VSMCs demonstrated that there is a well-established relationship between TNF- α -induced CCL2 recruitment of circulating monocytes/macrophages into the vessel wall and surrounding tissues and the onset of early cardiovascular pathologies including atherogenesis (Chen et al., 2004). TNF- α released during RA is thought to contribute to the systemic complications associated with RA (e.g. endothelial dysfunction) and more specifically correlate with an increased risk of developing cardiovascular pathologies (e.g. atherosclerosis) (Ferraccioli and Gremese, 2004, Sarzi-Puttini et al., 2005b). The local expression of TNF- α was unchanged across adipose sites during CIA at protocol termination. However, as TNF- α secreted at high levels early in disease onset in RA patients (Ma and Xu, 2013), the peak levels of TNF- α may have dropped by the time mice were sacrificed, hence the trend of increased expression in

thoracic PVAT but no significant difference during CIA. There was also a trend of increased expression of CCL2 in thoracic PVAT during CIA. Molecular analysis of TNF- α and CCL2 in a time course CIA experiment where mice would be sacrificed on the day of arthritis onset and on each day post onset for four days would address the question of whether the peak in TNF- α and CCL2 expression was missed by the time the mice were sacrificed. During CIA an increase in total cell number was seen in both the thoracic and abdominal aortas. There was also an increase in vessel thickness of the thoracic aorta during severe CIA. An increase in cell number within the vessel wall is associated with TNF- α -induced CCL2 migration of circulating monocytes out of the bloodstream and through the vasculature wall (Chen et al., 2004). Therefore the morphological alterations in adipose tissue, on a cellular level, during CIA are at least in part caused by an ingress of cells (e.g. macrophages) into PVAT, WAT and BAT.

Subpopulations of macrophages, M1 and M2, are key players in maintaining the homeostatic environment of tissues through the balance of pro-inflammatory and antiinflammatory responses (Mosser, 2003). Disruption to this balance can result in detrimental and damaging effects to a tissue or organ. This can happen through the establishment of a chronic inflammatory environment, caused by the over production of pro-inflammatory cytokines (e.g. IL-6 and TNF- α) from M1 macrophages, or by inducing tissue remodelling and fibrosis, caused by the over expression of anti-inflammatory cytokines (e.g. IL-10 and TGF- β) from M2 macrophages (Edwards et al., 2006).

Inducible nitric oxide synthase (iNOS) is a classical M1 macrophage marker commonly used in conjunction with Arg-1 when studying macrophage polarisation. Overall there was no significant elevation in iNOS expression during CIA, expect from renal WAT in severe mice. An increase in M1 macrophages is typically associated to the generation of pro-inflammatory state in adipose tissue (Boutens and Stienstra, 2016). However, the lack of increased iNOS expression during CIA does not mean a pro-inflammatory environment is

not established in adipose tissue during CIA as a recent study in an experimental model of endotoxin shock showed iNOS deficiency resulted in a more enhanced M1 phenotype resulting increasing the severity of the inflammatory environment (Lu et al., 2015). The expression of M2 marker Arg1 was upregulated during CIA in thoracic PVAT, renal WAT and inter-scapular BAT. One key function of M2 macrophages is tissue remodelling, as previously described in section 1.4.1, which through activation of MMPs and members of the collagen family (e.g. MMP9 and collagen VI) can induce tissue fibrosis (Karsdal et al., 2016, Ye, 2006). Fibrosis in PVAT and the adventitia has been associated with development of cardiovascular pathologies (e.g. hypertension) (Majesky, 2015). The mechanism of the iNOS/Arg-1 polarisation pathway is further investigate in Chapter 5. A trend of increased expression of both M1 marker CD11c and M2 marker Mrc1 (CD206) was also seen in thoracic PVAT during CIA. These selected markers for determining alterations in adipose tissue macrophage phenotype during CIA were not exhaustive but sufficiently showed the effect of CIA on macrophage polarisation, and highlights the diverse roles ATMs could potentially have in CIA-associated vasculopathology.

To determine if CIA caused tissue remodelling in PVAT, WAT and BAT, MMP9 and collagen VI expression was analysed by qPCR. Previous studies carried out in collageninduced arthritis reported an increase in MMP9 protein in the plasma and aorta of the CIA mice compared to controls, and demonstrated incubation of naïve aortas with MMP9 resulted in impaired contractile responses (Reynolds et al., 2012). However, analysis of MMP9 expression in adipose tissue showed no significant increase. One possible explanation is that the effect of MMP9 on tissue remodelling resulting in vascular dysfunction is specific to the aorta and not in PVAT during CIA, and another being due to the fact that MMP9 is produced in a pro-enzymatic form and requires cleavage before being activated (Woessner, 1991). Therefore as the CIA is an acute model of arthritis it may be that the cleavage of pro-MMP9 has not occurred yet. To address this question, analysis

of adipose sites and aortas from a chronic CIA model recently established in this laboratory (Data not published) for tissue remodelling markers is required or primers for pro-MMP9 designed. Among the many members of the collagen family, collagen VI has been found to be upregulated in adipose tissue compared to other tissue sites during state of metabolic stress and associated with an increase in adipose tissue fibrosis (Khan et al., 2009). Here, collagen VI expression was only upregulated in renal WAT during severe CIA. These data reiterates the hypothesis that a chronic model of CIA is required to thoroughly investigate the potential remodelling roles ATMs during inflammatory arthritis due to the short time frame of the current acute CIA model used here.

Morphological alterations of PVAT, WAT and BAT during CIA was also assessed based on adiposity, by measuring vacuolar space, and adipocyte phenotype, via qPCR analysis of WAT marker Asc-1 and BAT marker PAT2. Measuring adiposity is an indirect method of assessing whether lipolysis is increased or decreased within an adipose tissue based of the size of the adipocytes lipid droplet in the vacuolar space. During CIA a significant decrease in the adiposity, determined through measuring vacuolarity, was seen in thoracic PVAT and inter-scapular BAT, but not in abdominal PVAT or renal and gonadal WAT. This data suggests that lipolysis is upregulated in thoracic PVAT and BAT. Studies showed an increase of lipolysis in adipose tissue macrophages (Kosteli et al., 2010). Therefore the increase in ATM number (CD68+ cells) during CIA may be an indirect indication that lipolysis may be increase but in order to determine this mRNA expression (by qPCR) and protein expression (by western blot) of hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL), which are the enzymes known to hydrolyse triglycerides in murine and human adipose tissue, in PVAT, WAT and BAT are required.

To investigate the impact of CIA on adipocyte phenotype, the expression of WAT marker Asc-1 and BAT marker PAT2 were analysed in PVAT, WAT and BAT. During CIA, WAT

marker Asc-1 was significantly down regulated in thoracic PVAT, abdominal PVAT and interscapular BAT compared to non-immunised controls, providing evidence of CIA-associated alterations to adipocytes. PVAT is neither white nor brown adipose tissue, it contains both white and brown adipocytes. A full genome DNA microarray carried out in C57BL6/J mice revealed the gene expression profile of adipocytes in thoracic PVAT are extremely similar to adipocytes present in inter-scapular BAT (Fitzgibbons et al., 2011). This may explain why throughout this chapter we have seen similar trends between PVAT and BAT. Brown and beige adipocytes have smaller lipid vacuoles than white adipocytes, therefore a second possible explanation for the decrease in vacuolarity in thoracic PVAT and BAT, described above, could be due to the "browning" of adipocytes during CIA. The expression of PAT2 in thoracic PVAT was unchanged during CIA. A recent murine study by Gan et al demonstrated that LPS-induced inflammation reduced the browning of WAT and this was associated with a decrease in the adipokine leptin (Gan et al., 2017). Measuring the mRNA expression of leptin by qPCR in CIA and non-CIA mice would address the question of whether the inflammatory response generated during CIA is inhibiting the browning of WAT. Although the n-number for this study using Asc-1 and PAT2 was low, the data was deemed sufficient to conclude alterations to the adipocyte population were occurring during CIA. To gain a greater insight on the impact of CIA of adipocyte phenotype a CIA experiment, with a higher n-number per group should be carried out and additional markers for white adipocytes (e.g. HOXC8), brown adipocytes (e.g. UCP-1) and beige adipocytes (e.g. TBX1 and TMEM26) analysed (Wu et al., 2012, Walden et al., 2012).

This chapter set out to characterise the morphological changes in PVAT during inflammatory arthritis. The findings of this chapter are summarised in Fig 3.21. In line with the aims and objectives the CIA model was reproduced with 100% incidence, matching previously published data from this laboratory (Nowell et al., 2009, Reynolds et al., 2012, Williams et al., 2016). As there was no clear differences between adipose tissue

morphology between mild and severe groups throughout this chapter from here on in these groups will be combined into a +CIA group and compared to non-CIA (-CIA) controls. For the first time this chapter describes changes to adipose tissue morphology during inflammatory arthritis based on total cell number, vacuolarity, adipocyte markers, as well as macrophage number and polarisation.

In conclusion, CIA causes morphologically alterations on a cellular: increase in total cells and ATMs, and structural: decrease in vacuolarity and Asc-1 expression, level in adipose tissue, particularly thoracic PVAT. This chapter identifies the macrophage as a potential key functional player in the onset and development of CIA-associated vasculopathology, however also as a potential prognostic, diagnostic or therapeutic target for cardiovascular pathologies associated with inflammatory arthritis. For this reason, further investigation into potential ATM targets was warranted and addressed in the next chapter.



Figure 3.21 Summary of findings for Chapter 3: Characterisation of changes to perivascular adipose tissue morphology during collagen-induced arthritis

An increase in adipose tissue macrophage (ATM) number partially accounts for an increase in total cell number in adipose tissue during CIA. A trend of increased TNF- α and CCL2 expression in PVAT may result in an ingress of monocytes and other immune cells out of the bloodstream and into the surrounding adipose tissue. An increase in cells trafficking out of the bloodstream may account for the increase in vessel wall thickness. Decrease vacuolarity in PVAT may be due to an increase in lipolysis and/or related to adipocyte hyperplasia caused by an increase in total cell number and TNF- α expression. An increase in Arg1 expression explains the non-significant increase in iNOS expression as they compete for the same substrate – a mechanism that is discussed later in this thesis. An increase in ATMs can impair adipogenesis and may explain the unchanged expression of PAT2 in PVAT. An increase in lipolysis and thus a decrease in vacuolarity may account for the decrease in ASC-1 expression as adipocytes undergoing morphological alterations will phenotypically be different from the 'classic' white adipocyte.

Chapter 4

Identification of a novel PVATassociated molecular marker of vasculopathy during inflammatory arthritis
4.1 Introduction

CVD is responsible for the mortality of approximately 50% of RA patients (Kelly and Hamilton, 2007, Symmons and Gabriel, 2011). The underlying mechanisms linking RA and cardiovascular pathologies are not yet fully understood. There are currently no specific tests for identifying CVD early in RA patients or treatments available for RA patients at high risk of developing a CVD. Data from Chapter 3 identified macrophages as an important cell to target for the identification of a novel PVAT-associated molecular marker of vasculopathy during inflammatory arthritis. This was due to the increased number of ATMs in PVAT during CIA. As outlined in the General Introduction (Section 1.4), macrophages have been strongly implicated in the pathogenesis of both RA and CVDs. There is a current clinic need for early therapeutic intervention of CVDs in RA patients. Therefore the aim of this chapter was to identify a PVAT-associated macrophage molecular marker of vasculopathy during inflammatory arthritis.

The method in which a panel of potential markers was selected is outlined in Materials and Methods section 2.2.4. Briefly, a literature review investigating functional roles in inflammatory arthritis and CVD was carried out on a selection of macrophageassociated genes. This list of potential target genes, compiled by Professor Phil Taylor, had increased expression in ATMs compared to other macrophage populations. From this panel five targets were selected for further investigation based on the literature review criteria including: folate receptor 2, macrophage scavenger receptor, MER tyrosine kinase, Fcgamma receptor and dendritic cell immunoreceptor (Table 4.1). A sixth marker, galectin-3, was subsequently selected for investigation as a novel PVAT-associated molecular marker of vasculopathy during inflammatory arthritis. Galectin-3 is a cardiac fibrosis marker expressed on M2 macrophages that has been implicated in both inflammatory arthritis and CVDs (Table 4.2) (MacKinnon et al., 2008, Ezzat et al., 2011, Nayor et al., 2016). Molecular expression of the six selected targets in PVAT during inflammatory arthritis has never been investigated before.

Identification of a PVAT-associated molecular marker of vasculopathy during inflammatory arthritis will provide a step towards meeting the clinical need for therapeutic intervention of cardiovascular pathologies associated with RA. This marker could be used for the development of a new diagnostic, prognostic or therapeutic tool dependant on when and where it is expressed during disease. For example, a diagnostic marker is used to determine whether a patient has a disease or not, therefore early expression of a PVATassociated molecular marker of vasculopathy during inflammatory arthritis would identify them as a patient at risk of developing CVD and allow early clinical intervention within these patients (Albrecht and Zink, 2017). A prognostic marker is used to predict the clinical course of disease therefore a molecular marker of vasculopathy that increases in expression in PVAT during inflammatory arthritis could be used to track CVD progression and determine when and what type of intervention is required. If the expression of a PVATassociated molecular marker of vasculopathy during inflammatory arthritis is upregulated early in disease onset and maintained throughout disease progression the development of a therapeutic strategy against this marker could potentially treat CVD symptoms in RA patients. The identification of a prognostic, diagnostic or therapeutic marker of vascular dysfunction during inflammatory arthritis will be instrumental in the development of new clinical strategies for the treatment of CVD in RA patients.

Hypothesis: A novel molecular marker of inflammatory arthritis-associated vasculopathy may be identified by analysis PVAT during CIA.

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Target	Implication in inflammatory arthritis	Reference	Implication in CVD	Reference
Folate receptor 2 (Folr2)	Folr2 is expressed on activated macrophages, predominantly M2, upregulated on macrophages in the synovium of RA joints. Depletion of Folr2 in a rat model of AIA improved the joint swelling, histological score of inflammation and cartilage and bone destruction in treated rats compared to the non-immunised controls.	(Jager et al., 2012, Paulos et al., 2006, Nagai et al., 2012)	Folr2 is expressed on activated macrophages in human atherosclerotic plaques and has been identified as a good indicator of plaque vulnerability.	(Jager et al., 2014)
Macrophage scavenger receptor 1 (Msr1)	Genetic deficiency of Msr1 in K/BxN TCR transgenic mice decreased the incidence and severity of arthritis because of decreased autoantibody production.	(Haasken et al., 2013)	Msr1 is implicated in a diverse range of CVDs, e.g. foam cell formation and atherogenesis.	(Dai et al., 2013)
MER tyrosine kinase (MerTK)	In RA patients MerTK is expressed on macrophages in the synovium. A study using the CIA model demonstrated that injecting mice with adenoviruses over expressing these its ligands Gas6 and protein S ameliorated inflammatory arthritis through increased MerTK signalling.	(van den Brand et al., 2013, Bassyouni et al., 2017)	In atherosclerosis, MerTK expression is associated with increased plaque necrosis and defective resolution in both human disease and animal models.	(Cai et al., 2017, Anwar et al., 2009)
Fc gamma receptor 1 (FcγR1)	Analysis of copy number variations of FcyR genes in RA patients identified an association between the expression of FcyR1, FcyR2a and FcyR3b with the development and progression of arthritis. Moreover, FcyR1 expression on macrophages in RA patients results in a higher production of TNF- α and MMPs which in turn exacerbate the disease.	(Blom et al., 2003, Franke et al., 2016)	FcγR1 has multiple roles including phagocytosis, cell degranulation and cytokine and chemokine release. Evidence has shown that the loss of FcγR1 limits the development and progression of atherosclerosis implicating its involvement within the disease.	(Nino et al., 2014, Hernandez-Vargas et al., 2006)
Dendritic cell immunoreceptor (DCIR)	Short nucleotide polymorphisms in the human DCIR locus have been associated with the development of RA. It has also been shown that mice deficient of DCIR have an increased response to CIA, experiencing increased paw swelling and clinical score compared to the wild type controls	(Lorentzen et al., 2007, Bloem et al., 2013)	DCIR is a pattern recognition receptor (PRR) that is expressed at high levels on ATMs. A recent study shows macrophages in human atherosclerotic plaques express high levels of DCIR.	(Wang and Yi, 2015)†

Table 4.1 The implication of potential targets in the pathology of inflammatory arthritis and CVD

⁺ Human data in process of publication. Data was presented at 7th Munich Vascular Conference 2017. Presentation can be viewed at https://mac-conference.com/wp-content/uploads/2017/12/01_Monaco-130 MAC-2017-v12.pdf

Target	Implication in inflammatory arthritis	Reference	Implication in CVD	Reference
Galectin-3	Galectin-3 has been found at increased levels in the synovial fluid and serum of RA patients, and correlates with disease severity and progression in patients with juvenile idiopathic arthritis.	(Ezzat et al., 2011, Ohshima et al., 2003)	Increased serum levels of galectin-3 has been directly linked to mortality in CVD patients. Previous studies have also linked galectin-3 with driving an acute inflammatory state within the vasculature into chronic inflammation, with knockout mice showing a reduced fibrotic phenotype.	(Nayor et al., 2016, Henderson et al., 2006)

Table 4.2 The implication of galectin-3 in the pathology of inflammatory arthritis and CVD

4.2 Aims and objectives

The aim of this chapter was to identify a specific molecular marker of vascular pathology in PVAT during inflammatory arthritis.

In order to achieve the aim of this chapter, the following objectives were targeted:

- To quantify the expression by qPCR of Folr2, Msr1, MerTK, FcγR1, DCIR and galectin-3 in thoracic and abdominal PVAT, renal and gonadal WAT and interscapular BAT of non-arthritic and age-matched CIA mice.
- To select one or more targets based on the qPCR results outlined above for further investigation as potential markers of vasculopathy during inflammatory arthritis.
- To validate this selection of a PVAT-associated molecular marker of vasculopathy during CIA by molecular biology and quantify expression at the protein level by immunohistochemistry in thoracic and abdominal PVAT, renal and gonadal WAT and inter-scapular BAT in CIA and non-CIA mice.

4.3 Results

A panel of relevant ATM targets were identified from Immunological Genome Project and results cross-referenced against published function in RA and CVD. The molecular expression of selected targets (FoIr2, Msr1, MerTK, FcyR1, DCIR and Gal-3) was analysed in PVAT (thoracic and abdominal) of non-CIA and CIA mice by qPCR, and protein expression analysed by IHC (see Chapter 2 sections 2.2.3 and 2.2.5 for methodology). In addition to PVAT, the expression of targets was also analysed in non-vascular associated adipose sites (renal and gonadal WAT and inter-scapular BAT).

4.3.1 Induction of CIA had no effect of Folr2 expression in PVAT, WAT and BAT

The expression of Folr2 (Fig 4.1) was not significantly different in PVAT, WAT and BAT in CIA mice compared to non-CIA controls. Therefore Folr2 was excluded from further selection as a PVAT-associated molecular marker of vasculopathy during inflammatory arthritis.

4.3.2 The expression of Msr1, MerTK, FcγR1 and DCIR were significantly increased in PVAT, WAT and BAT during CIA

During CIA MerTK was significantly increased in thoracic PVAT (1.98±0.2, p<0.01) compared to non-CIA controls (1.0±0.07) (Fig 4.2A). Expression of MerTK was also significantly increased in renal (2.4±0.2, p<0.001) and gonadal (1.97±0.2, p<0.001) WAT compared to non-arthritic mice (1.1±0.07 and 0.98±0.06 respectively). Inter-scapular BAT followed the same trend where +CIA (1.7±0.2, p<0.01) mice expressed significantly higher levels of MerTK compared to –CIA (0.98±0.05) mice. MerTK expression was unchanged in abdominal PVAT during CIA. The greatest significant increase of MerTK expression during CIA was seen in renal and gonadal WAT compared to PVAT and BAT sites.



Figure 4.1 Folr2 expression was not affected by CIA induction in adipose tissue

The expression of Folr2 was analysed by qPCR in CIA (+CIA, n = 16) and non-CIA (-CIA, n = 8) mice in thoracic PVAT, abdominal PVAT, renal WAT, gonadal WAT and inter-scapular BAT. Data expressed as relative quantification (RQ) values calculated in relation to a non-arthritic control. Two-tailed Mann Whitney test with Gaussians Approximation.

The expression of Msr1 (Fig 4.2B) was significantly increased in thoracic PVAT (2.2 \pm 0.2, p<0.01) and abdominal PVAT (1.8 \pm 0.3, p<0.05) in CIA mice compared to non-CIA controls (0.98 \pm 0.1 and 0.94 \pm 0.06 respectively). Expression of Msr1 was also significantly increased in renal (3.4 \pm 0.5, p<0.01) and gonadal (2.1 \pm 0.3p<0.05) WAT during CIA compared to naïve controls (0.97 \pm 0.08 and 0.96 \pm 0.07 respectively). A significant increase was also seen in inter-scapular BAT of CIA mice (2.9 \pm 0.3, p<0.001) compared to non-arthritic controls (1.1 \pm 0.1). Although a significant increase in Msr1 expression was seen across all adipose sites analysed in CIA mice compared to non-CIA controls, the most significant increase was seen in inter-scapular BAT.

The expression of DCIR (Fig 4.2C) was significantly increased in thoracic (2.6 \pm 0.3, p<0.01) and abdominal (2.1 \pm 0.3, p<0.05) PVAT in CIA mice compared to non-arthritis controls (1.05 \pm 0.04 and 0.95 \pm 0.05). A significant increase was also seen in renal (3.6 \pm 0.4, p<0.001) and gonadal (2.3 \pm 0.4, p<0.05) WAT in arthritic mice compared to naïve controls (1.04 \pm 0.1 and 1.05 \pm 0.06 respectively). DCIR was significantly increased in inter-scapular BAT of +CIA mice (2.8 \pm 0.3, p<0.001) compared to the matched –CIA controls (0.98 \pm 0.04). Therefore DCIR expression was significantly increased during CIA in PVAT, WAT and BAT.

Molecular analysis of FcyR1 showed a significant increase of expression in thoracic PVAT (2.7±0.3, p<0.001) and abdominal PVAT (1.9±0.4, p<0.001) in CIA mice compared to non-CIA controls (1.07±0.07 and 1.03±0.1 respectively) (Fig 4.2D). Expression was also significantly increased in renal WAT (3.1±0.3, p<0.001) and gonadal WAT (2.7±0.5, p<0.05) in arthritic mice compared to non-immunised controls (0.92±0.07 and 0.98±0.06 respectively). The expression of FcyR1 was significantly increased in inter-scapular BAT of +CIA mice (2.6±0.4, p<0.01) compared to –CIA mice (1.02±0.07). Similar to Msr1 and DCIR, the expression of FcyR1 was significantly elevated across all adipose sites analysed in CIA versus non-CIA mice, the greatest increase was seen in thoracic and abdominal PVAT.



Figure 4.2 Msr1, MerTK, FcyR1 and DCIR were significantly increased in PVAT, WAT and BAT during CIA

The expression of **A**. MerTK, **B**. Msr1, **C**. DCIR and **D**. Fc γ R1 was analysed by qPCR in CIA (+CIA, n = 16) and non-CIA (-CIA, n = 8) mice in thoracic PVAT, abdominal PVAT, renal WAT, gonadal WAT and inter-scapular BAT. Data expressed as relative quantification (RQ) values calculated in relation to a non-arthritic control. Two-tailed Mann Whitney test with Gaussians Approximation, * = p<0.05, ** = p<0.01, *** = p<0.001.

4.3.3 Galectin-3 expression was significantly higher in PVAT from CIA mice versus non-CIA mice

Cardiac fibrosis marker galectin-3, which is produced by macrophages, was significantly increased in thoracic PVAT (10.4 ± 2.2 , p<0.01) and abdominal PVAT (1.7 ± 0.2 , p<0.05) in CIA mice compared to the non-arthritic controls (0.99 ± 0.06 and 1.04 ± 0.07 respectively) (Fig 4.3). The increase in galectin-3 during CIA was split into two groups; the low galectin-3 expressing group developed a milder arthritis compared to the higher expressing group. This is the first time an increase in galectin-3 was described in PVAT during CIA. There was no significant difference in galectin-3 expression between CIA and non-CIA mice in renal and gonadal WAT and inter-scapular BAT. Therefore the increase in galectin-3 during CIA was specifically localised to PVAT.

The expression of MerTK, Msr1, DCIR and FcyR1 was significantly increased in PVAT, WAT and BAT during CIA. FcyR1 was selected for protein expression analysis as the difference in expression was most significant between CIA and non-CIA mice, in both thoracic and abdominal PVAT sites, compared to MerTK, Msr1 and DCIR (Table 4.3). Galectin-3 was also selected for protein expression analysis alongside FcyR1 due to the increase in galectin-3 expression being specific to PVAT in CIA mice compared to the non-arthritic controls.

	Thoracic PVAT	Abdominal	Renal	Gonadal	Inter-scapular
		PVAT	WAT	WAT	BAT
MerTK	**	ns	***	***	**
	p = 0.0027		p = 0.0006	p = 0.0010	p = 0.0096
Msr1	**	*	**	*	***
	p = 0.0017	p = 0.0332	p = 0.0030	p = 0.0166	p = 0.0009
DCIR	**	*	***	*	***
	p = 0.0010	p = 0.0308	p = 0.0002	p = 0.0288	p = 0.0009
FcγR1	***	***	* * *	*	**
	p = 0.0009	p = 0.0006	p = 0.0003	p = 0.0131	p = 0.0031

Table 4.3 Comparison of MerTK, Msr1, DCIR and FcyR1 p-values from qPCR analysis



Figure 4.3 Galectin-3 expression was significantly increased in PVAT during CIA

The expression of galectin-3 was analysed by qPCR in CIA (+CIA, n = 11) and non-CIA (-CIA, n = 8) mice in thoracic PVAT, abdominal PVAT, renal WAT, gonadal WAT and inter-scapular BAT. Data expressed as relative quantification (RQ) values calculated in relation to a non-arthritic control. Two-tailed Mann Whitney test with Gaussians Approximation. * = p<0.05, ** = p<0.01

4.3.4 FcyR1 positive cells are increased in PVAT, WAT and BAT during CIA

To assess if the increase of FcyR1 expression on a molecular level seen during CIA was translated to an increase in protein expression of FcyR1, IHC was carried out in thoracic and abdominal PVAT, renal and gonadal WAT and inter-scapular BAT of non-arthritic and arthritic mice.

Representative images of $Fc\gamma R1^+$ cells in the thoracic PVAT and blood vessel are shown in Figure 4.4 A and C respectively. IHC analysis of $Fc\gamma R1$ in thoracic PVAT showed a significant elevation in percentage $Fc\gamma R1^+$ cells in CIA mice (11.6±0.8, p<0.001) compared to non-CIA controls (5.1±0.9) (Fig 4.4B), matching the molecular data previously described. There was also a significant increase in percentage $Fc\gamma R1^+$ cells in the thoracic blood vessel between CIA (9.9±1.0, p<0.05) and non-CIA (5.6±1.5) mice (Fig 4.4D).

Analysis of $Fc\gamma R1^+$ cells in abdominal PVAT showed a significant increase in $Fc\gamma R1^+$ cells in CIA mice (14.2±0.8, p<0.001) compared to non-CIA controls (8.7±0.5) (Fig 4.5 A and B). There was also a significant increase in $Fc\gamma R1^+$ cells in the abdominal aorta blood vessel in mice induced with CIA (11±0.9, p<0.01) compared to the matched, naïve controls (7.0±0.7) (Fig 4.5 C and D). This showed the increase in molecular expression of $Fc\gamma R1$ described in Figure 4.2D is translated into an increase in $Fc\gamma R1$ protein expression in both thoracic and abdominal PVAT.

Figure 4.6A shows representative images of $Fc\gamma R1$ positive cells in renal WAT of non-CIA and CIA mice. In renal WAT the percentage of $Fc\gamma R1^+$ cells was significantly higher in CIA mice (13.3±0.6, p<0.001) compared to non-CIA mice (7.1±0.8) (Fig 4.6B). This mirrored the significant increase of $Fc\gamma R1$ molecular expression in renal WAT measured by qPCR previously described in this chapter.



Figure 4.4 FcyR1 positive cells are increased in thoracic aortic PVAT during CIA

A. Representative images identifying FcyR1 positive cells (\checkmark) in thoracic PVAT in CIA and non-CIA mice. **B.** Percentage FcyR1 positive cells in thoracic PVAT in CIA (n=16) and non-CIA mice (n=8). **C.** Representative images identifying FcyR1 positive cells (\checkmark) in the thoracic aorta in CIA and non-CIA mice. **D.** Percentage FcyR1 positive cells in the thoracic aorta in CIA (n=16) and non-CIA mice (n=8). Scale bar = 25 µm. Field of view per section = 4. Data expressed mean ± SEM. Two-tailed Mann Whitney test with Gaussians Approximation, * = p<0.05, *** = p<0.001.



Figure 4.5 An increase in FcyR1 positive cells is seen in abdominal aortic PVAT during CIA

A. Representative images identifying FcyR1 positive cells (\checkmark) in abdominal PVAT in CIA and non-CIA mice. **B.** Percentage FcyR1 positive cells in abdominal PVAT in CIA (n=16) and non-CIA mice (n=8). **C.** Representative images identifying FcyR1 positive cells (\checkmark) in the abdominal aorta in CIA and non-CIA mice. **D.** Percentage FcyR1 positive cells in the abdominal aorta in CIA (n=16) and non-CIA mice (n=8). Scale bar = 25 µm. Field of view per section = 4. Data expressed mean ± SEM. Two-tailed Mann Whitney test with Gaussians Approximation, ** = p<0.01, *** = p<0.001.



Figure 4.6 FcyR1 positive cells are increased during CIA in renal WAT

A. Representative images identifying FcyR1 positive cells (\checkmark) in renal WAT in CIA and non-CIA mice. **B.** Percentage FcyR1 positive cells in renal WAT in CIA (n=16) and non-CIA mice (n=8). Scale bar = 25 μ m. Field of view per section = 4. Data expressed mean ± SEM. Two-tailed Mann Whitney test with Gaussians Approximation, *** = p<0.001. Representative images of FcyR1 stained gonadal WAT in –CIA and +CIA mice are shown in Figure 4.7A. The percentage of FcyR1⁺ cells was significantly greater in gonadal WAT of +CIA mice (13.2±0.8, p<0.001) compared to gonadal WAT of –CIA (7.9±0.5) mice (Fig 4.7B). Therefore the increase in molecular expression of FcyR1 in CIA mice measured by qPCR was translated to a significant rise in percentage FcyR1 positive cells in both PVAT and WAT.

The percentage $Fc\gamma R1^+$ cells, determined by IHC, in inter-scapular BAT was increased during CIA (10.9±0.8, p<0.001) compared to the non-immunised controls (4.7±0.6) (Fig 4.8 A and B). Once again, along with the other PVAT and WAT sites analysed, this mirrored the significant increase in FcγR1 molecular expression that was previously described in this chapter. Therefore FcγR1 is significantly increased at both molecular and protein levels during CIA in PVAT, WAT and BAT.

4.3.5 Protein analysis of galectin-3 determined an increase in galectin-3 positive cells in thoracic but not abdominal PVAT

As there was no difference in molecular galectin-3 expression, determined via qPCR analysis, in WAT and BAT sites between CIA and non-CIA mice, protein analysis of galectin-3 by IHC was carried out in PVAT only.

Representative images of galectin-3 positive cells in the thoracic PVAT and blood vessel of arthritic mice and naïve controls are shown in Figure 4.9 A and C respectively. A significant increase in galectin-3⁺ cells was seen in thoracic PVAT in CIA mice (20.4 \pm 1.3, p<0.01) compared to the non-CIA controls (12.4 \pm 1.0) (Fig4.9B). This matched the molecular data previously acquired in this chapter. There was no significant difference in percentage galectin-3⁺ cells in the thoracic aorta blood vessel between +CIA and –CIA mice (Fig4.9D).

Analysis of galectin-3⁺ cells by IHC in abdominal PVAT showed no significant difference between CIA and non-CIA mice (Fig4.10A and B). Similarly, there was no



Figure 4.7 FcyR1 positive cells are increased in gonadal WAT in CIA mice

A. Representative images identifying FcyR1 positive cells (\checkmark) in gonadal WAT in CIA and non-CIA mice. **B.** Percentage FcyR1 positive cells in gonadal WAT in CIA (n=16) and non-CIA mice (n=8). Scale bar = 25 µm. Field of view per section = 4. Data expressed mean ± SEM. Two-tailed Mann Whitney test with Gaussians Approximation, *** = p<0.001.



Figure 4.8 FcyR1 positive cells are increased in inter-scapular BAT in CIA mice

A. Representative images identifying Fc γ R1 positive cells (\checkmark) in inter-scapular BAT in CIA and non-CIA mice. **B.** Percentage Fc γ R1 positive cells in inter-scapular BAT in CIA (n=16) and non-CIA mice (n=8). Scale bar = 25 μ m. Field of view per section = 4. Data expressed mean ± SEM Two-tailed Mann Whitney test with Gaussians Approximation, *** = p<0.001.

significant difference in galectin-3⁺ cells in the blood vessel of the abdominal aorta between CIA and non-CIA mice (Fig4.10C and D). Therefore the increase in galectin-3⁺ cells was specific to thoracic PVAT during CIA.



Figure 4.9 Galectin-3 positive cells are increased in thoracic PVAT but not the thoracic aorta during CIA

A. Representative images identifying galectin-3 positive cells (\checkmark) in thoracic PVAT in CIA and non-CIA mice. **B.** Percentage galectin-3 positive cells in thoracic PVAT in CIA (n=16) and non-CIA mice (n=8). **C.** Representative images identifying galectin-3 positive cells (\checkmark) in the thoracic aorta in CIA and non-CIA mice. **D.** Percentage galectin-3 positive cells in the thoracic aorta in CIA (n=16) and non-CIA mice (n=8). Scale bar = 25 µm. Field of view per section = 4. Data expressed mean ± SEM. Two-tailed Mann Whitney test with Gaussians Approximation, ** = p<0.01.



Figure 4.10 Galectin-3 positive cells are not increased in abdominal PVAT and aorta during CIA

A. Representative images identifying galectin-3 positive cells (\checkmark) in abdominal PVAT in CIA and non-CIA mice. **B.** Percentage galectin-3 positive cells in abdominal PVAT in CIA (n=16) and non-CIA mice (n=8). **C.** Representative images identifying galectin-3 positive cells (\checkmark) in the abdominal aorta in CIA and non-CIA mice. **D.** Percentage galectin-3 positive cells in the abdominal aorta in CIA (n=16) and non-CIA mice (n=8). Scale bar = 25 µm. Field of view per section = 4. Data expressed mean ± SEM. Two-tailed Mann Whitney test with Gaussians Approximation.

4.4 Discussion

The aim of this chapter was to identify a novel PVAT-associated macrophage molecular marker of vascular dysfunction during inflammatory arthritis. An in depth literature review identified six potential targets (Folr2, MerTK, Msr1, DCIR, FcyR1 and galectin-3). They were taken forward for molecular analysis by qPCR in thoracic and abdominal PVAT, renal and gonadal WAT and inter-scapular BAT sites in CIA and non-CIA mice. Several adipose tissue sites were selected for analysis to achieve insight into potential differences gene expression profiles between vascular-associated adipose sites (thoracic and abdominal PVAT) and non-vascular associated adipose sites (WAT and BAT). Two targets (FcyR1 and galectin-3) were subsequently selected for protein expression analysis by IHC on the basis of their molecular expression being greatly increased in PVAT of CIA mice compared to the non-arthritic controls.

FcyR1 was selected as a potential target of early vascular dysfunction during inflammatory arthritis as expression was increased in thoracic and abdominal PVAT, renal and gonadal WAT and inter-scapular BAT during CIA compared to the non-immunised controls. Although the increase in FcyR1 was not specific to PVAT, one benefit of selecting a target that is globally increased in adipose tissue is that PVAT is not easily accessible for the acquisition of adipose tissue biopsies by clinicians for prognostic or diagnostic testing, however WAT and BAT, which are easily accessed, can be used to investigate what is happening within PVAT as the expression patterns of FcyR1 are the same. Subcutaneous abdominal adipose tissue biopsies are currently used for the diagnosis of systemic amyloidosis, in addition to providing samples for research into increasing the understanding of diseases such as obesity and cancer for example and aid the discovery of new treatment strategy within these diseases (Alderete et al., 2015, Bogov et al., 2008, Mutch et al., 2009, Campbell et al., 2009). Currently there are no publications detailing the use of a WAT biopsy from patients (e.g. subcutaneous abdominal WAT) to gain insight into

potential alterations at other adipose sites (e.g. PVAT) or any other organs, therefore biopsies may not be the best diagnostic or prognostic approach for determination of vascular dysfunction based on a PVAT-associated maker during inflammatory arthritis.

A second diagnostic and prognostic approach that will visualise the expression of a target (e.g. FcyR1) without taking a tissue sample would be immuno-positron emission tomography (immuno-PET). Immuno-PET uses radiolabelled monoclonal antibodies (mAbs) that bind to a specific target to visualise the presence and/or track the location of a population of cells (van Dongen et al., 2007). For example, ¹⁸F-fluorodeoxyglucose is used in immuno-PET for imaging metabolic activity in an atherosclerotic plaque, as it is engulfed by plaque macrophages and provides insight into the level of plaque inflammation (Bucerius et al., 2014). Macrophages have also been targeted for imaging in RA patients. One example of this is non-invasive imaging of the inflamed joints using the tracer ¹¹C-(R)-PK11195 which binds to peripheral benzodiazepine receptors on macrophages (Put et al., 2014). The uptake of this tracer was significantly higher in severely inflamed joints therefore is useful in detection of synovitis in RA patients and for monitoring disease progression during therapy (van der Laken et al., 2008). As previously mentioned, FcyR1 expression was significantly increased in thoracic and abdominal PVAT, renal and gonadal WAT and inter-scapular BAT during CIA. FcyR1 expression is not specific to adipose tissue; it is expressed in multiple organs including the intestines, liver, lymph node and spleen (Cheeseman et al., 2016, Tuijnman et al., 1993). Therefore FcyR1 would not be an effective target for use in immuno-PET as all tissues where FcyR1 is expressed would fluoresce. This would make it extremely difficult to accurately identify and quantify populations FcyR1⁺ cells in PVAT and give no insight into the presence and/or progression of vasculopathy during inflammatory arthritis. For this reason FcyR1 was ruled out as a potential PVATassociated molecular marker of vascuolpathy during inflammatory arthritis.

Cardiac fibrosis marker galectin-3 was the second potential target selected after molecular analysis due to its specific increase in expression in thoracic and abdominal PVAT during CIA compared to the non-immunised controls. Protein analysis by IHC revealed the increase in galectin-3 was translated in thoracic PVAT but not abdominal PVAT. Therefore galectin-3 was a PVAT-specific maker, unlike FcyR1, that is upregulated during CIA making it a strong candidate for immuno-PET analysis. To my knowledge imaging strategies targeting galectin-3 have not been developed for use as a prognostic or diagnostic tool in CVD or RA. However, imaging agents targeting galectin-3 are currently being used to improve diagnostic screening in a variety of cancers. Galectin-3 aids tumour growth and metastases, as well as its role in CVD and inflammatory arthritis previously described (Inohara and Raz, 1995). For example a study in thyroid cancers has used preoperative immunocytohistochemistry of galectin-3 for identifying which patients require surgery referral with an 88% success using this method alone (Bartolazzi et al., 2008). Recently, immuno-PET techniques using galectin-3 labelled with zirconium for imaging thyroid tumours in vivo has been developed and is being used successfully in preclinical models of thyroid cancer (Bartolazzi et al., 2018). In vivo studies in human breast tumour-bearing SCID mice used galectin-3 as an imaging target for potential use in breast cancer diagnostic techniques (Kumar and Deutscher, 2008). This shows promising potential for the development of a galectin-3 imaging agent for the use in identifying and/or monitoring the progression of arthritis-associated vasculopathy within CIA, with hope of translation to human studies.

Galectin-3 has also been identified as a potential therapeutic target within cardiovascular pathologies and RA separately, but not for use as a treatment strategy for the development of CVD in RA patients (Xie et al., 2018, Suthahar et al., 2018). There are several galectin-3 inhibitors commercially available for use *in vivo* with the most common being modified citrus pectin (MCP) (Martinez-Martinez et al., 2015, Streetly et al., 2010). MCP has successfully been used to reduce atherosclerotic lesions in apoE-knockout mice,

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inhibit galectin-3 associated cardiac inflammation and fibrosis in models of hypertension and to prevent adipose tissue remodelling in murine models of obesity (Martinez-Martinez et al., 2015, Martinez-Martinez et al., 2016, MacKinnon et al., 2013). Local knockdown of galectin-3 by intra-articular injection of lentiviral vectors encoding galectin-3 small hairpin RNA into the ankle joints of CIA rats significantly reduced arthritic index and histological scores compared to CIA rats receiving the control vector (Wang et al., 2010). However, to my knowledge, the effect of systemically inhibiting galectin-3 on arthritis severity and the associated vascular dysfunction has not been investigated. Therefore galectin-3 is an excellent candidate for a potential macrophage PVAT-associated molecular marker of vasculopathy during inflammatory arthritis as it has the potential to be developed as a diagnostic tool for the identification of vascular dysfunction during inflammatory arthritis, a also as a potential therapeutic target for the prevention and treatment of vascular dysfunction during inflammatory arthritis.

Advances in transcriptome profiling has resulted in methods like RNA-Seq to become more readily available (Wang et al., 2009). RNA-Seq allows the identification of differentially expressed genes to uncover phenotypic variations between treatment groups (Costa-Silva et al., 2017). Therefore performing RNA-Seq on PVAT from non-CIA and CIA mice would have unveiled all potential markers that were upregulated during CIA. Although this method was not used in this thesis, RNA-Seq would highlight additional markers, other than galectin-3, that will provide greater insight into the potential mechanisms that contribute that vascular dysfunction during inflammatory arthritis.

The selected macrophage marker of PVAT-associated vascular dysfunction during inflammatory arthritis, galectin-3, is expressed on M2 macrophages, which as described in the previous chapter are increased in PVAT during CIA based on their expression of Arg-1 (Henderson and Sethi, 2009). Chapter 3 showed an increase in ATM number and TNF- α

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expression in PVAT during CIA. Currently, the most common treatment strategy in RA patients, anti-TNF therapy (see section 1.1.3), significantly reduces the number of circulating monocytes in RA patients and macrophages in abdominal adipose tissue of psoriasis patients (Schotte et al., 2004, Motolese et al., 2017). Studies have shown that anti-TNF treatment is not only beneficial for controlling joint damage and relieving symptoms of RA, but is effective in reducing the cardiovascular morbidity and mortality associated with RA (Jacobsson et al., 2005). However the mechanisms behind anti-TNF therapy on reducing CV risk are not fully understood. Therefore Chapter 5 will investigate the impact of anti-TNF therapy on ATM number and polarisation, galectin-3 expression and vascular function during CIA.

Chapter 5

Assessing the effect of TNF on ATMs and vascular function during collagen-induced arthritis in mice

5.1 Introduction

During CIA there is an increase in ATM number, systemic expression of proinflammatory cytokine TNF- α and expression of cardiac fibrosis marker galectin-3 in PVAT (Chapters 3 and 4). To investigate the impact of TNF- α on ATMs and vascular function during CIA, TNF- α was inhibited *in vivo*. Anti-TNF agents have been used to treat RA for over 15 years and were the first 'true' molecular target used in RA therapy (Yamanaka, 2015). This therapy improves the clinical outcome of RA patients through inhibiting joint destruction, lessening the symptoms of the disease and improving their overall quality of life (Nam and Emery, 2010). For this reason anti-TNF biologics are the most commonly used therapy in RA (Yamanaka, 2015).

Anti-TNF biologics fall into two therapeutic categories: TNF monoclonal antibodies (e.g. infliximab, adalimumab and golimumab) and a fusion protein of the TNF receptor (e.g. etanercept) (Yamanaka, 2015). These biologics are discussed in greater detail in the General Introduction, section 1.1.4. Etanercept was first approved for clinical use in Europe in 2000; its efficacy was displayed in RA patients in multiple clinical studies by an improvement in physical function, symptoms and reduced bone destruction (Klareskog et al., 2004). Etanercept was selected as a research tool to block TNF in the animal studies described in this Chapter because its higher comparative safety profile (low risk of lung infections, ADCC and development of antagonising antibodies against monoclonal antibody formulations) in humans.

Etanercept reduces the cardiovascular morbidity and mortality associated with RA in addition to controlling the progression of RA and relieving the symptoms of arthritis (Jacobsson et al., 2005). There are several reasons why the inhibition of TNF might reduce CV risk in RA patients but the actual mechanisms are not yet established. Firstly, TNF- α propagates the pathogenesis of both RA and atherosclerosis (Cacciapaglia et al., 2014). Blockade of TNF- α for RA therapy should therefore protect a patient against the associated

development and/or progression of atherosclerosis. Secondly, TNF- α regulates vascular homeostasis; it is an impelling cause of inflammation-associated arterial stiffness (Sattar et al., 2003) and reduces vascular endothelial function (Galarraga et al., 2009). Finally, TNF blockers like etanercept suppress the levels of pro-inflammatory cytokines (e.g. TNF- α , IL- 1β , IL-6 and IFN- γ) in the systemic circulation, which are all associated with CVD onset and progression (Schotte et al., 2004). Treatment of RA by blocking TNF using etanercept is therefore likely to improve vascular function and reduce CV risk for a combination of reasons. It is not known if TNF has a role in the development vasculopathy at the initiation of inflammatory arthritis because it is an inherently difficult phase of arthritis to study, nor is it clear how these early inflammation-induced vascular events underpin the development of CVDs like atherosclerosis. Here the CIA model is used to establish the role of TNF in initiating damage to the aorta and controlling aortic constriction responses during the early stage of inflammatory arthritis. Published data demonstrated that etanercept improved the clinical signs of CIA when administered subcutaneously, intravenously and via intraperitoneal injection (Zhang et al., 2013, Huang et al., 2012, Wang et al., 2013). To my knowledge this is the first study that aims to determine the impact of TNF, or indeed treatment of arthritis with etanercept, on the structure and morphology of the aorta, levels of systemic cytokines and vascular function. The CIA model was used for this proof of concept study.

Data from Chapters 3 and 4 of this thesis, together with the published literature discussed there in, showed that Arg1⁺ ATMs and galectin-3 levels in PVAT were increased during CIA. Etanercept significantly reduced the number of circulating monocytes in RA patients and decreased macrophage number in abdominal adipose tissue of psoriasis patients (Schotte et al., 2004, Motolese et al., 2017). To my knowledge the effect of etanercept or indeed TNF blockade upon ATM number and polarisation has never been investigated in CIA or in RA. In this chapter the role of TNF in regulating macrophage

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infiltration of PVAT, WAT and BAT will be determined and the relative expression of iNOS (M1 marker) and Arg1 (M2 marker) was measured in mice with and without CIA. This chapter will investigate potential mechanisms whereby TNF controls ATM polarisation by measuring specific cytokines like TNF- α (M1 polarising) and TGF- β and IL-10 (M2 polarising), and also by assessing the expression of galectin-3, IL-4R α and CD98, which are also involved in M2 polarisation. To my knowledge there are no studies investigating the impact of anti-TNF therapy on galectin-3 expression, the selected marker of vasculopathy from Chapter 4. In addition to driving M2 polarisation, galectin-3 is a systemic marker of CVD however is not used in clinic to assess CV risk in RA patients (Nayor et al., 2016). Currently one method CV risk is assessed by in RA patients is measuring systemic levels of N-terminal pro-hormone of brain natriuretic peptide (NT-proBNP) (Biskup et al., 2018). To assess whether galectin-3 could be used as systemic marker of early vascular changes during inflammatory arthritis, plasma concentrations of both galectin-3 and NT-proBNP were measured by ELISA in CIA and non-CIA mice.

Hypothesis: Treatment with etanercept during CIA will reduce the number and alter polarisation of ATMs and also protect against TNF-associated vascular dysfunction during CIA.

5.2 Aims and objectives

The aim of this chapter was to investigate the impact of TNF on ATMs and vascular function during CIA through inhibiting TNF- α *in vivo* with etanercept.

The following objectives were identified in order to achieve these aims:

- To successfully inhibit TNF-α *in vivo* during CIA through intravenous injection of etanercept.
- To assess the impact of TNF-α on arthritis severity through paw score, paw diameter and arthritic index previously used in Chapter 3.
- To evaluate the effect of TNF-α on vascular constriction in response to serotonin
 (5-HT) using the myograph.
- To characterise the alterations in macrophage number and polarisation in adipose tissue following TNF inhibition with etanercept during CIA through IHC of macrophage marker CD68 and qPCR analysis of iNOS, Arg1, TNF-α, TGF-β and IL-10.
- To investigate differences in plasma concentrations of pro- and anti-inflammatory between non-arthritic, PBS treated and etanercept treated CIA mice through multiplex assays.
- To investigate the impact of TNF on local and systemic galectin-3 levels during CIA by carrying out qPCR and IHC on PVAT samples and an ELISA on plasma samples in non-arthritic, PBS treated and etanercept treated mice.

5.3 Results

5.3.1 Intravenous injection of etanercept on day 21 of CIA protocol inhibits TNF-α expression

Animals in the anti-TNF group received an intravenous injection of etanercept at 2.5 mg/kg on day 21, with control CIA mice given 100 μ L PBS intravenously. The effect of etanercept on TNF- α expression during CIA was analysed in blood plasma by ELISA (section 2.2.6) and in PVAT, WAT and BAT by qPCR (section 2.2.4). Plasma concentration of TNF- α (Fig 5.1A) was significantly elevated in PBS treated arthritic mice (p<0.001) compared to the non-immunised controls and etanercept treated mice, which were below the detection limit. Therefore systemic plasma TNF- α concentration was increased during CIA but was successfully suppressed through etanercept treatment. Molecular analysis of proinflammatory cytokine TNF- α showed there was a significant increase locally in TNF- α expression in thoracic PVAT in PBS treated mice (1.58±0.3, p<0.05) compared to the nonimmunised controls (0.88 \pm 0.1) (Fig 5.1B). TNF- α expression was also significantly increased in the inter-scapular BAT of PBS treated mice compared to the non-CIA controls (p<0.05). Mice that received anti-TNF therapy showed a significant decrease in TNF- α expression (0.49±0.1, p<0.001) in thoracic PVAT compared to the PBS treated controls. This pattern of decreased TNF- α expression was also seen in renal WAT and inter-scapular BAT of etanercept treated mice compared to the non-arthritic controls (p<0.05), therefore treatment with etanercept supressed local TNF- α expression in adjpose tissue in addition to plasma TNF- α levels.

5.3.2 Anti-TNF therapy supresses the onset and progression of collageninduced arthritis

CIA mice treated intravenously with etanercept on day 21 had a supressed onset of arthritis with incidence reaching a maximum of 40% on day 28 compared to the PBS control



Figure 5.1 Intravenous injection of etanercept on Day 21 of CIA protocol inhibits TNF- α expression

TNF- α was inhibited by anti-TNF therapy in systemic blood plasma (**A**.) measured by ELISA compared to PBS controls (n=14 per group) and locally in adipose tissue (**B**.) measured by qPCR at termination of CIA compared to PBS controls (n=8 per group). Data expressed mean ± SEM. One-way ANOVA with Bonferroni's post multiple comparison test. * = p<0.05, *** = p<0.001.

mice, that reached 100% incidence on day 28 (Fig 5.2A). Additionally, treatment with etanercept had a positive impact on percentage weight loss, clinical score, paw score and paw diameter compared to the PBS controls.

There was a significant decrease (p<0.001) in percentage weight loss in CIA mice (PBS treated) compared to the non-CIA controls (Fig 5.2B), matching previous data (Chapter 3). Percentage weight loss in etanercept treated mice was significantly greater (p<0.001) than PBS treated mice, but still significantly lower (p<0.001) than the non-immunised controls (Fig 5.2B). Therefore treatment with etanercept during CIA partially reduces the weight loss associated with this model. There was a significant decrease in paw score (p<0.001) and clinical score (p<0.001) in etanercept treated mice compared to the PBS controls at termination and throughout arthritis progression (Fig 5.2C-D). Paw diameter was also significantly decreased (p<0.001) in treated mice compared to the PBS controls throughout arthritis progression (Fig 5.2E). Overall, anti-TNF therapy during CIA supressed the onset and progression of arthritis compared to the PBS controls. Although etanercept treatment was not protective against arthritis induction, treated mice developed a much milder arthritis compared to the PBS controls. Histological analysis of arthritic index on hind paws (Fig 5.3) showed treatment with etanercept during resulted in a significant decrease (p<0.001) in joint destruction and hyperplasia associated with CIA compared to the PBS treated controls.

5.3.3 Anti-TNF therapy partially restores the constriction response lost during CIA

Published data from this laboratory demonstrated the loss of the vascular constriction response to 5-HT in arthritic mice compared to non-arthritic controls (Reynolds et al., 2012, Williams et al., 2016). To examine the impact of anti-TNF treatment during CIA on vascular function, PVAT-denuded aortas were harvested from non-arthritis, PBS control

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Figure 5.2 Anti-TNF treatment during CIA supresses the onset and progression of arthritis A. Treatment with etanercept (n = 20) (an anti-TNF biologic) resulted in a reduction of mice under protocol developing CIA compared to the PBS treated CIA controls (n =20). **B.** Weight loss associated with CIA induced was partially rescued in etanercept treated mice. Clinical score (**C**.), paw score (**D**.) and paw diameter (**E**.) were all significantly decreased in etanercept treated mice throughout the onset and progression of arthritis, with treated mice displaying a milder arthritis compared to the PBS controls. Data expressed mean \pm SEM. Mann-Whitney test or One-way ANOVA with Bonferroni's post multiple comparison test. * = p<0.05, ** = p<0.01, *** = p<0.001.



Figure 5.3 Anti-TNF treatment significantly reduces the arthritic index of hind paws during CIA

Arthritis index (AI) of hind paws was assessed in – CIA mice (n = 8), PBS treated CIA mice (n = 10) and etanercept treated CIA mice (n = 10) in order determine cellular infiltrate, synovial hyperplasia, bone erosion and cellular exudate within each group. **A.** Representative image of a non-arthritic ankle joint, AI=0. **B.** Representative image of an ankle joint from PBS treated CIA mice, AI = 9. **C.** Representative image of an ankle joint from etanercept treated CIA mice, AI = 4. **D.** Quantification of AI. Data expressed mean ± SEM. One-way ANOVA with Bonferroni's post multiple comparison test. ** = p<0.01, *** = p<0.001.
and etanercept treated mice. Aortas were denuded to ensure PVAT had no influence on the constriction response *ex vivo*. Aortic rings were cut and the constriction response measured on the myograph (Fig 5.4) (section 2.2.8). In line with the published data mentioned above, there was a significant decrease in the Rmax (maximum constriction response) of PBS treated mice (4.04 ± 0.16 , p<0.001) compared to the non-arthritic controls (6.77 ± 0.17), demonstrating the loss of vascular constriction during CIA. The constriction response to 5-HT was partially, but not fully, restored in etanercept treated mice (5.32 ± 0.14), as the Rmax was significantly higher that the PBS treated mice (p<0.001) but still significantly lower than the non-immunised controls (p<0.001). There was a negative correlation between clinical score and vascular function in mice not treated with anti-TNF (Fig 5.5), therefore mice with a more severe arthritis had greater vascular dysfunction.

5.3.4 Anti-TNF therapy significantly decreases the percentage of ATMs in PVAT and BAT during CIA

Results from Chapter 3 and published data described an increase in adipose tissue macrophage number and percentage in PVAT, WAT and BAT in CIA mice compared to nonimmunised controls (Sime et al., 2017). To characterise the impact of anti-TNF therapy on the percentage of ATMs present in adipose tissue for the first time during CIA CD68 IHC was performed and quantified (section 2.2.3).

5.3.4.1 Thoracic aorta

A representative images of CD68⁺ cells in the thoracic PVAT and blood vessel from non-arthritic, PBS treated and etanercept treated mice are shown in Figure 5.6. In line with previous data, there was a significant increase in percentage CD68⁺ cells in CIA PBS treated mice (12.93±0.7, p<0.001) compared to the non-immunised controls (5.97±0.6) (Fig 5.6A). Treatment with etanercept resulted in a significant decrease in percentage CD68⁺ cells (8.18±0.7, p<0.001) compared to the PBS treated controls (Fig 5.6A). In the blood vessel, there was a significant increase in percentage CD68⁺ cells in PBS treated mice (13.63±0.7,



Figure 5.4 Anti-TNF therapy partially restores the constriction response lost during CIA To determine the effect of anti-TNF therapy on vascular function during CIA the constriction response of thoracic aortas in response to serotonin (5HT) was measured on a myograph in nonarthritic, PBS treated and etanercept treated mice (n = 8 per group). Data expressed mean \pm SEM. One-way ANOVA with Bonferroni's post multiple comparison test. *** = p<0.001.



Figure 5.5 There is a negative correlation between clinical score and vascular function in mice not receiving etanercept therapy

To investigate the relationships between clinical score and vascular function (Rmax determined via myography) with (n = 8) and without (n = 16) anti-TNF therapy correlation plots were generated. **A.** Rmax vs. clinical score in non-CIA and PBS treated mice. **B.** Rmax vs. clinical score in etanercept treated mice. Pearson's correlation.



Β.



Figure 5.6 Anti-TNF therapy decreases the percentage of ATMs in thoracic PVAT during CIA

In order to assess the effect of anti-TNF therapy on the ATM number in thoracic PVAT (A.) and the thoracic aorta (B.) CD68 IHC was carried out and percentage CD68⁺ cells (\checkmark) quantified in non-CIA, PBS treated and etanercept treated mice (n = 12 per group). Isotype shown in insert. Scale bar = 100 μ m. Field of view per section = 4. Data expressed mean ± SEM. One-way ANOVA with Bonferroni's post multiple comparison test. ** = p<0.01, *** = p<0.001.

p<0.01) compared to the non-arthritic controls (9.6±1), however there was no significant difference when compared to etanercept treated mice (Fig 5.6B).

5.3.4.2 Abdominal aorta

There was a significant increase in percentage CD68⁺ cells in the abdominal PVAT of PBS treated mice (11.31±0.9, p<0.001) compared to the non-immunised control (7.08±0.5) (Fig 5.7A). This matched previous data from Chapter 3. Treatment with etanercept resulted in a significant decrease in percentage CD68⁺ cells (8.19±0.5, p<0.05) compared to the PBS treated CIA controls. A significant increase in percentage CD68+ cells was also noted in the abdominal blood vessel in PBS treated mice (11.19±0.7, p<0.05) compared to the naïve controls (8.7±0.5) (Fig 5.7B).

5.3.4.3 Renal WAT

In renal WAT there was a significant increase in percentage CD68⁺ cells in PBS treated (10.87 \pm 0.7, p<0.001) and in etanercept treated (9.27 \pm 0.8, p<0.05) mice compared to non-arthritic controls (6.08 \pm 0.6) (Fig 5.8). Therefore treatment with Etanercept during CIA does not affect percentage CD68⁺ cells in renal WAT.

5.3.4.4 Gonadal WAT

There was a significant increase in the percentage $CD68^+$ cells in gonadal WAT in PBS treated mice (9.74±0.6, p<0.001) compared to non-arthritic controls (6.58±0.4) (Fig 5.9), however there was no significant difference in comparison to gonadal WAT from etanercept treated mice.

5.3.4.5 Inter-scapular BAT

Representative images of $CD68^+$ cells in inter-scapular BAT from non-CIA, PBS treated and etanercept treated mice are shown in Figure 5.10. There was a significant increase in the percentage $CD68^+$ cells in inter-scapular BAT in PBS treated mice (12.68±0.9,



Figure 5.7 Anti-TNF therapy decreases the percentage of ATMs in abdominal PVAT during CIA

In order to assess the effect of anti-TNF therapy on the ATM number in abdominal PVAT (**A**.) and the abdominal aorta (**B**.) CD68 IHC was carried out and percentage CD68⁺ cells (\checkmark) quantified in non-CIA, PBS treated and etanercept treated mice (n = 12 per group). Isotype shown in insert. Scale bar = 100 µm. Field of view per section = 4. Data expressed mean ± SEM. One-way ANOVA with Bonferroni's post multiple comparison test. ** = p<0.01, *** = p<0.001.



Figure 5.8 Anti-TNF therapy has no effect on the percentage of ATMs in renal WAT during CIA

In order to assess the effect of anti-TNF therapy on the ATM number in renal WAT, CD68 IHC was carried out and percentage CD68⁺ cells (\checkmark) quantified in non-CIA, PBS treated and etanercept treated mice (n = 12 per group). Isotype shown in insert. Scale bar = 100 µm. Field of view per section = 4. Data expressed mean ± SEM. One-way ANOVA with Bonferroni's post multiple comparison test. * = p<0.05, *** = p<0.001.



Figure 5.9 Anti-TNF therapy has no effect on the percentage of ATMs in gonadal WAT during CIA

In order to assess the effect of anti-TNF therapy on the ATM number in gonadal WAT, CD68 IHC was carried out and percentage $CD68^+$ cells (\checkmark) quantified in non-CIA, PBS treated and etanercept treated mice (n = 12 per group). Isotype shown in insert. Scale bar = 100 µm. Field of view per section = 4. Data expressed mean ± SEM. One-way ANOVA with Bonferroni's post multiple comparison test. *** = p<0.001.



Figure 5.10 Anti-TNF therapy decreases the percentage of ATMs in inter-scapular BAT during CIA

In order to assess the effect of anti-TNF therapy on the ATM number in inter-scapular BAT, CD68 IHC was carried out and percentage CD68⁺ cells (\checkmark) quantified in non-CIA, PBS treated and etanercept treated mice (n = 12 per group). Isotype shown in insert. Scale bar = 100 µm. Field of view per section = 4. Data expressed mean ± SEM. One-way ANOVA with Bonferroni's post multiple comparison test. ** = p<0.01, *** = p<0.001.

p<0.001) compared to the non-CIA controls (7.01 \pm 0.6). There was a significant decrease in percentage CD68+ cells in inter-scapular BAT in etanercept treated mice (9.21 \pm 0.5, p<0.01) compared to the PBS controls, demonstrating a similar pattern as to what was seen in PVAT of etanercept treated mice.

5.3.5 Anti-TNF therapy reduces the expression of M2 marker Arg1 during

CIA

As there was a significant decrease in percentage CD68⁺ cells during anti-TNF therapy in CIA, qPCR was used to investigate the impact of etanercept treatment on the expression of M1 marker iNOS and M2 marker Arg1 in non-arthritic, PBS treated and etanercept treated mice (Fig 5.11) (section 2.2.4). Upstream factors that influence the M1/M2 balance were also analysed including pro-inflammatory cytokine TNF- α and anti-inflammatory cytokines TGF- β and IL-10. Thoracic and abdominal PVAT, renal and gonadal WAT and inter-scapular BAT sites were analysed from each mouse.

Molecular analysis of M1 marker iNOS in PVAT, WAT and BAT revealed no difference between treatment groups during CIA (Fig 5.11A). However, M2 marker Arg1 was significantly increased in thoracic PVAT of PBS treated mice (5.41 ± 1.9 , p<0.05) compared to non-CIA controls (1.06 ± 0.2) (Fig 5.11B), in line with data from previous chapters. In thoracic PVAT there is a trend of decreased Arg1 expression in etanercept treated mice (1.33 ± 0.2) compared to the PBS controls, however this was not significant due to the large variation of Arg1 expression in the PBS treated group.

As shown in Figure 5.1B, etanercept significantly decreased TNF- α expression in adipose tissue. Molecular analysis of anti-inflammatory cytokine TGF- β , which is involved in promoting polarisation of macrophages towards an M2 phenotype, showed a significant decrease in TGF- β expression in thoracic PVAT, abdominal PVAT, gonadal WAT and interscapular BAT in both PBS treated and etanercept treated mice (p<0.001) compared to the



Figure 5.11 Anti-TNF therapy reduces the expression of M2 marker Arg1 during CIA

To investigate the impact of anti-TNF therapy on macrophage polarisation M1 macrophage marker iNOS (**A**.), M2 marker Arg1 (**B**.) and anti-inflammatory cytokines TGF- β (**C**.) and IL-10 (**D**.) expression was analysed by qPCR in non-arthritic, PBS treated and etanercept treated mice (n = 8 per group). Data expressed mean ± SEM. One-way ANOVA with Bonferroni's post multiple comparison test. * = p<0.05, *** = p<0.001.

non-immunised controls (Fig 5.11C). IL-10, also involved in M2 polarisation, expression was significantly decreased in gonadal WAT of etanercept treated mice (p<0.05) compared to non-immunised controls. There was a trend of decreased IL-10 expression across PVAT, WAT and BAT in both PBS and etanercept treated mice compared to the non-immunised controls (Fig 5.11D). Therefore, the increase in Arg1 expression in PVAT during CIA (without anti-TNF treatment) is not due to the local presence of anti-inflammatory cytokines.

5.3.6 Anti-TNF therapy during CIA alters the systemic plasma concentrations of pro- and anti- inflammatory cytokines

In order to assess the impact of CIA induction and anti-TNF therapy during CIA on the systemic concentration of cytokines (TNF- α , IFN- γ , CCL2, GM-CSF, IL-1 β , IL-6, IL-12p70, IL-17A, IL-23, IL-27, IFN- β and IL-10), multi-target LEGENDplex assays were carried out on plasma samples from non-CIA, PBS treated and etanercept treated mice (Fig 5.12) (section 2.2.7). Several cytokines analysed were not in the detectable range of this assay (Supplementary Fig 1, Appendix II).

As shown in Figure 5.1A, etanercept significant inhibited the concentration of plasma TNF- α during CIA compared to PBS controls. Pro-inflammatory cytokines IFN- γ (Fig 5.12A) and IL-6 (Fig 5.12E) were significantly higher in CIA mice that received PBS compared to the non-CIA controls (p<0.05). Mice that underwent anti-TNF therapy showed a trend of increased expression of IFN- γ and IL-6, however there was no significant difference between this group and the PBS group and non-immunised mice. Plasma concentration of CCL2 was significantly increased during CIA (p<0.05, Fig 5.12B). CCL2 was significantly elevated in etanercept treated mice compared to both PBS treated (p<0.001) and non-CIA controls (p<0.001). A similar pattern of increased expression during CIA and elevated higher with etanercept treatment was also seen for pro-inflammatory cytokines GM-CSF, IL-1 β , 1L-23 and IFN- β (Fig 5.12C, D, H and J). IL-27, a cytokine with both pro- and anti-



Figure 5.12 Systemic plasma levels of pro- and anti- inflammatory cytokines during CIA in PBS and etanercept treated mice To determine to effect of CIA induction, with and without anti-TNF therapy, on the systemic level of pro- and anti- inflammatory cytokines LEGENDplex assays were carried out on plasma samples. **A.** IFN-γ **B.** CCL2 **C.** GM-CSF **D.** IL-1β **E.** IL-6 **F.** IL-12p70 **G.** IL-17A **H.** IL-23 **I.** IL-27 **J.** IFN-β **K.** IL-10. Dotted line represents detection limit n = 14 per group. One-way ANOVA with Bonferroni's post multiple comparison test. * = p<0.05, ** = p<0.01, *** = p<0.001.

inflammatory properties, was significantly increased in plasma from Etanercept treated mice compared to the non-immunised controls (p<0.001, Fig 5.12I).

5.3.7 Anti-TNF therapy significantly decreases local galectin-3 expression in PVAT

IHC was used to investigate the local expression of galectin-3 in the thoracic and abdominal aortas in non-arthritic, PBS and etanercept treated CIA mice (section 2.2.3). Molecular analysis of galectin-3 in Chapter 4 showed no difference in galectin-3 expression between CIA and non-CIA mice in renal and gonadal WAT and inter-scapular BAT. For this reason, protein expression of galectin-3 was not analysed in WAT and BAT sites in this chapter.

5.3.7.1 Thoracic aorta

There was a significant increase in percentage galectin-3⁺ cells in the thoracic PVAT of PBS treated mice (18.73±0.8, p<0.001) compared to the non-arthritic controls (7.34±0.6) (Fig 5.13A), matching data discussed in previous chapters. Percentage galectin-3⁺ cells was significantly decrease in CIA mice treated with etanercept (9.11±0.6, p<0.001) compared to PBS CIA controls. A significant increase in percentage galectin-3⁺ cells was also seen in the thoracic blood vessel of PBS treated mice (21.96±1.0, p<0.001) compared to non-immunised controls (13.03±1.1) (Fig 5.13B). There was a significant decrease in percentage galectin-3⁺ cells in the thoracic blood vessel in etanercept treated mice (14.51±0.5, p<0.001) compared to the PBS control mice. Therefore anti-TNF therapy reduced local expression of galectin-3 in the thoracic aorta during CIA.

5.3.7.2 Abdominal aorta

Representative images of galectin-3⁺ cells in abdominal PVAT and the abdominal blood vessel from non-immunised, PBS treated and etanercept treated mice are shown in Figure 5.14. There was a significant increase in percentage galectin-3⁺ cells in abdominal



в.



Figure 5.13 Anti-TNF therapy decreases the percentage of galectin-3 positive cells in the thoracic PVAT and aorta during CIA

In order to assess the effect of anti-TNF therapy on the percentage galectin-3 positive cells (ψ) in thoracic PVAT (A.) and the thoracic aorta (B.) galectin-3 IHC was carried out and percentage galectin- 3^+ cells quantified in non-CIA, PBS treated and etanercept treated mice (n = 12 per group). Isotype shown in insert. Scale bar = 100 μm. Field of view per section = 4. Data expressed mean ± SEM. Oneway ANOVA with Bonferroni's post multiple comparison test. *** = p<0.001.



Figure 5.14 Anti-TNF therapy decreases the percentage of galectin-3 positive cells in the abdominal PVAT during CIA

In order to assess the effect of anti-TNF therapy on the percentage galectin-3 positive cells (\checkmark) in abdominal PVAT (**A**.) and the abdominal aorta (**B**.) galectin-3 IHC was carried out and percentage galectin-3⁺ cells quantified in non-CIA, PBS treated and etanercept treated mice (n = 12 per group). Isotype shown in insert. Scale bar = 100 µm. Field of view per section = 4. Data expressed mean ± SEM. One-way ANOVA with Bonferroni's post multiple comparison test. * = p<0.05, *** = p<0.001.

PVAT of PBS control CIA mice (8.51±0.6, p<0.001) compared to non-arthritic controls (5.97±0.3) (Fig 5.14A). However the percentage of galectin-3⁺ cells in the abdominal PVAT of PBS control mice (8.51±0.6) is dramatically lower that the percentage of galectin-3⁺ cells in the thoracic PVAT in the same mice (18.73±0.8). Similar to the thoracic PVAT, there was a significant decrease in percentage galectin-3⁺ cells in abdominal PVAT of etanercept treated mice (6.71±0.3, p<0.05) compared to the PBS control CIA mice. There was a significant increase in percentage galectin-3⁺ cells in the abdominal blood vessel of PBS control mice (14.36±1.1, p<0.05) compared to non-CIA controls (11.22±0.7) (Fig 5.14B). Anti-TNF therapy did not affect the percentage galectin-3⁺ cells during CIA.

5.3.8 Molecular analysis revealed anti-TNF therapy significantly decreases local galectin-3 expression in thoracic PVAT during CIA

To provide further insight into a mechanism of galectin-3 activation during CIA, qPCR was used to analyse the molecular expression of galectin-3, its upstream receptor IL-4R α and its downstream receptor CD98 in thoracic and abdominal PVAT (Fig 5.15). Molecular expression of galectin-3 was significantly increased in the thoracic PVAT of PBS control mice (10.65±2.6, p<0.01) compared to the non-CIA controls (0.89±0.1) (Fig5.15A). This increase in galectin-3 expression is comparable to the increase of galectin-3 previously described in thoracic PVAT in Chapter 4. Galectin-3 expression was significantly decreased in the thoracic PVAT of etanercept treated mice (1.13±0.3, p<0.001) compared to the PBS control CIA mice. There was no change in galectin-3 expression in abdominal PVAT across experimental groups (Fig 5.15A); therefore the local increase of galectin-3 expression was specific to thoracic PVAT and inhibited by anti-TNF therapy. The expression of IL-4R α , a receptor that activates galectin-3 production, was significantly increased in the thoracic PVAT of PBS treated mice (1.57±0.2, p<0.01) compared to non-arthritic controls (0.85±0.1) (Fig 5.15B). IL-4R α expression was significant decreased in the thoracic PVAT of CIA mice that received etanercept (1.03±0.1, p<0.05) compared to the PBS treated controls. Similar





To investigate the impact anti-TNF therapy on local galectin-3 expression (**A**.) in PVAT, WAT and BAT, qPCR was carried out in non-arthritic, PBS treated and etanercept treated mice (n = 8 per group). To provide further insight upstream target IL-4R α (**B**.) and downstream target CD98 (**C**.) were also analysed. Data expressed mean ± SEM. One-way ANOVA with Bonferroni's post multiple comparison test. * = p<0.05, ** p<0.01, *** = p<0.001.

to galectin-3 expression, the expression of IL-4R α was unchanged in the abdominal PVAT of non-arthritic, PBS and etanercept treated mice (Fig 5.15B). The expression of downstream receptor CD98 showed a trend of increased expression in the thoracic PVAT of PBS control mice, with anti-TNF therapy decreasing CD98 expression during CIA (Fig 5.15C). This pattern matches that described above for the expression of galectin-3 and IL-4R α , however was not significant.

5.3.9 Galectin-3 is a better marker of early vasculopathy and aberrant constriction response in CIA mice compared to NT-proBNP

To investigate the potential use of galectin-3 as an early marker of vascular dysfunction during inflammatory arthritis ELISAs performed on plasma samples (from non-arthritic, PBS treated and etanercept treated mice) were used to quantify the concentration of an established CV risk marker, NT-proBNP, and galectin-3 (Fig 5.16). NT-proBNP level in plasma from non-arthritic mice was 381.1±39.9. A significant decrease in NT-proBNP was measured in CIA mice given PBS (128.9±17.8) or treated with anti-TNF (88.2±12.2) compared to the non-arthritic mice. Unlike NT-proBNP, galectin-3 is not a traditional CV risk marker. There was a significant increase in plasma galectin-3 level in the CIA PBS treated controls (76.26±10.9, p<0.01) compared to the non-immunised controls (32.44±2.9). Plasma galectin-3 levels were also significantly increased in etanercept treated mice (74.36±7.3, p<0.01) compared to the non-immunised controls. Anti-TNF treatment did not alter plasma galectin-3 expression in CIA mice. Therefore galectin-3 may be an earlier marker of vasculopathy in CIA compared to NT-proBNP.

5.3.10 High clinical score and plasma galectin-3 levels correlate with vascular dysfunction during CIA

To investigate the relationship between arthritis severity (clinical score), vascular dysfunction (Rmax) and plasma galectin-3 concentration correlation plots were generated (Fig 5.17). Figure 5.5 showed a negative correlation between clinical score and vascular



Figure 5.16 Galectin-3 is a better CV risk systemic marker during early inflammatory arthritis compared to NT-proBNP

To investigate the use of galectin-3 as a systemic marker of cardiovascular dysfunction during early inflammatory arthritis ELISAs on the current CV risk marker used to assess CV risk in RA patients, NT-proBNP (n = 10 per group) and galectin-3 (n = 8 per group) in non-immunised, PBS treated and etanercept treated mice. Data expressed mean \pm SEM. One-way ANOVA with Bonferroni's post multiple comparison test. ** = p<0.01, *** = p<0.001



Figure 5.17 A high clinical score and plasma galectin-3 concentration correlates with having an impaired vascular function.

To investigate the relationships between clinical score, plasma galectin-3 concentration and vascular function (Rmax determined via myography) with (n = 8) and without (n = 16) anti-TNF therapy correlation plots were generated. **A.** Plasma galectin-3 vs. clinical score in non-CIA and PBS treated mice. **B.** Plasma galectin-3 vs. clinical score in etanercept treated mice. **C.** Rmax vs. plasma galectin-3 in non-CIA and PBS treated mice. **D.** Rmax vs. plasma galectin-3 in etanercept treated mice. Pearson's correlation.

function, meaning the more severe the arthritis the greater the vascular dysfunction. In animals that did not receive anti-TNF therapy, a high concentration of plasma galectin-3 correlated with a high clinical score ($R^2 = 0.5507$, p<0.001) and a low Rmax ($R^2 0.2808$, p<0.01) (Fig 5.17A and C). Therefore animals with a high clinical score and elevated plasma galectin-3 levels have impaired vascular function. There were no correlations between clinical score, plasma galectin-3 and Rmax in mice that received anti-TNF therapy (Fig 5.17B and D).

5.3.10 There is a positive correlation between galectin-3 expression and Arg1 expression in thoracic PVAT

In order to evaluate if the increase in M2 macrophages in thoracic PVAT during CIA was a potential source of our selected target galectin-3, the RQ values for galectin-3 expression in thoracic PVAT of non-arthritic and CIA + PBS mice were correlated with the respective RQ values for Arg1 expression (Fig 5.18). There was a positive correlation between galectin-3 expression and Arg1 expression (p=0.0052, $R^2 = 0.4389$) therefore galectin-3 is potentially involved in driving the M2 phenotype of ATMs during CIA.



Figure 5.18 Galectin-3 expression positively correlates with an increase in Arg1 expression in thoracic PVAT

To investigate the relationship between Arg1 expression and galectin-3 expression in thoracic PVAT in non-arthritic and CIA + PBS mice (n=16) Pearson's correlation analysis was used.

5.4 Discussion

The aim of this chapter was to investigate the impact of TNF on ATMs and vascular function during CIA through inhibiting TNF- α *in vivo* with etanercept. Intravenous administration of etanercept on day 21 of the CIA protocol resulted in a significant decrease of TNF- α concentration systemically in blood plasma and local expression of TNF- α in adipose tissue. Treatment with etanercept significantly reduced the incidence of CIA in mice developing arthritis by approximately 60% compared to the PBS CIA controls. Onset of arthritis in mice treated with etanercept was delayed by 3 days and developed at a reduced severity as there was a significant decrease in paw score and swelling at termination compared to the PBS administered CIA controls. This matched previously published CIA experiments where etanercept was administered as they also reported a decrease in arthritis severity but not full attenuation of disease in DBA/1 mice (Wang et al., 2013, Huang et al., 2012, Zhang et al., 2013).

As mentioned in the introduction to this chapter, an increase in systemic proinflammatory cytokines was linked to early cardiovascular events in RA patients, which predisposed them to the onset of CVDs (Cacciapaglia et al., 2014, Sattar et al., 2003). In this chapter, CIA mice given PBS displayed a significantly impaired constriction response to 5-HT compared to the non-immunised controls. This matched previously published work by members of this laboratory (Williams et al., 2016, Reynolds et al., 2012). Treatment with etanercept on day 21 of the CIA model resulted in a significantly improved constriction response to 5-HT compared to the CIA controls that received PBS. However the constriction response was not fully restored to the level of non-arthritic mice. This is the first time the CIA model was used to mimic the response to anti-TNF therapy on vascular dysfunction that has been described in RA patients (Jacobsson et al., 2005). This underpined the importance of using the CIA model as a platform to investigate the mechanisms underlying the link between RA and the main co-morbidity it is linked to: cardiovascular disease. Unravelling these mechanisms would identify new potential therapeutic targets to improve the treatment strategies currently available, with the aim of preventing the onset of cardiovascular pathologies in RA patients to reduce mortality rates and improve their lifestyle.

Through the inhibition of TNF- α , this chapter uncovered three potential factors that contributed to the partial restoration of the constriction response in CIA mice. Firstly, during CIA, plasma concentrations of pro-inflammatory cytokines TNF- α , IFN- γ , CCL2, GM-CSF and IL-6 were significantly upregulated compared to non-CIA controls, mirroring what was described in the plasma of RA patients (Avci et al., 2016, Zhang et al., 2015, Schotte et al., 2004). This observation reinforces the idea that CIA is an appropriate model for studying the mechanisms that underpin damage to the aorta in RA and potentially during the early stages of disease pathogenesis, when it is currently undetectable in humans. Treatment of CIA with etanercept lowered the plasma levels of several cytokines (e.g. TNF- α , IL-6 and IFN- γ), once again mirroring published data in RA patients (Schotte et al., 2004). RA patients treated with etanercept exhibit reduced inflammation-associated arterial stiffness (Pearson et al., 2003, Daien et al., 2012). Arterial stiffness is an independent CV risk factor in RA patients. It is conceivable that the reduced constriction response of the aorta to 5HT during CIA was attributable to arterial stiffness, however this was not tested. It is also possible that etanercept restored 5HT-induced constriction responses in the thoracic aorta of mice with CIA by reducing cytokine-associated inflammatory responses in the vessel wall and/or PVAT, specifically those that are regulated by TNF- α , IFN- γ and/or IL-6. This idea is supported by the fact that macrophage numbers were lower in the aorta and PVAT of mice with CIA that were treated with etanercept versus mice with CIA that received PBS.

Not all the cytokine levels measured in the plasma of mice with CIA were reduced by etanercept. Levels of CCL2 and GM-CSF were elevated in etanercept treated mice with

CIA compared to CIA mice that received PBS and the non-CIA controls. Raised plasma levels of CCL2 normally correlate with poor disease control in RA that is contrary to our results in CIA. Bystrom et al recently found that the GM-CSF was elevated in the plasma of RA patients after one month of treatment with a TNF inhibitor and peripheral T cells were reported to be the source (Bystrom et al., 2017). There was no change in CCL2 concentration following anti-TNF therapy in this study. However it is documented that GM-CSF increases CCL2 production by T cells (Owen et al., 2007). As CIA is a T-cell dependent model, the observed increase in plasma CCL2 in CIA mice treated with etanercept was potentially regulated by T-cell derived GM-CSF (Brand et al., 2007). In order to test this hypothesis T cells and monocytes isolated from whole blood from non-CIA, CIA and etanercept treated CIA mice on harvest should be run on the Image Stream which allows the visualisation of co-localised markers on single cells. This would determine whether CD3⁺ T cells and/or CD68⁺ monocytes are the source of increased GM-CSF and CCL2 plasma concentration following etanercept treatment during CIA.

Treating CIA with etanercept reduced the number of ATMs as well as the expression of Arg1 and galectin-3 in thoracic PVAT, an observation that has not previously been reported. Arg1 is a classic marker of M2 macrophages and expression was increased in thoracic PVAT during CIA (Sime et al., 2017). Anti-TNF therapy reduced the number of M2 macrophages in thoracic PVAT, as there was decrease in Arg1 expression in etanercept treated CIA mice compared to PBS controls. As iNOS expression in thoracic PVAT was unchanged between treatment groups, it can be concluded that the decrease in Arg1 expression following etanercept treatment was due to a reduction in overall macrophage number and not altered polarisation from M2 to M1 phenotype. M2 macrophages are involved in tissue remodelling and collagen deposition which are essential for vascular development, remodelling and maintaining vascular function (Naito, 2008). However, an increase in M2 macrophages can result in remodelling that can be damaging towards the

vasculature by increasing arterial thickness, impairing the ability of the vasculature to constrict (Ogle et al., 2016). Local galectin-3 expression in thoracic PVAT positively correlates with Arg1 expression during CIA. Galectin-3 promotes the polarisation of a macrophage towards an M2 phenotype by activating PI3K signalling. The activation of PI3K signalling in a macrophage induces Arg1 expression and blocks iNOS expression (Fig 5.18) (MacKinnon et al., 2008). Therefore during CIA local galectin-3 expression in thoracic PVAT drove an M2 macrophage phenotype that induced vascular dysfunction through increased tissue remodelling. Treatment with etanercept decreased galectin-3 expression, which in turn reduced the M2 Arg1⁺ population in thoracic PVAT, accounting for partially restoring vascular function.

In addition to a local, tissue-associated increase in galectin-3 expression in thoracic PVAT during CIA, systemic plasma concentration of galectin-3 was also seen in CIA mice compared to non-CIA controls. A high plasma galectin-3 concentration and a high clinical score positively correlated with impairment of vascular function in CIA mice. Previous studies have determined that increased levels of galectin-3 drive a chronic inflammatory environment and fibrotic state within vasculature, ultimately leading to vascular dysfunction and the development of CVD (Henderson et al., 2006, Henderson and Sethi, 2009). More recently, elevated concentrations of galectin-3 in the serum of CVD patients has been linked to increased mortality rates (Nayor et al., 2016). Therefore plasma galectin-3 concentration may be an earlier indicator of vascular dysfunction during inflammatory arthritis. Galectin-3 concentration was upregulated in the plasma of CIA mice compared to non-CIA controls, however treatment with etanercept did not affect the systemic levels of galectin-3 during CIA. This may account for why a full restoration of the constriction



Figure 5.18 Activation of galectin-3 in macrophages and the downstream effects

Interaction of IL-4 or IL-13 with the IL-4R α receptor activates the release of galectin-3 from the macrophage. Galectin-3 can in turn then interact with CD98 on surrounding macrophages which causes activation of phosphoinositide 3-kinase (PI3K) signalling pathway. This results in the promotion of M2 markers, including Arg1, and therefore in turn blocks iNOS activity.

response is not seen following etanercept treatment during CIA. Therapeutic blockade of galectin-3 may result in complete restoration of the constriction response to 5-HT during CIA because galectin-3 would be inhibited both systemically in the plasma and locally within the PVAT. This will be addressed in the following chapter.

In conclusion, treatment with etanercept during CIA significantly reduced the progression and severity of inflammatory arthritis, improved the vascular constriction response and resulted in a decrease of tissue-remodelling associated M2 ATMs compared to CIA mice that received PBS. Although administration of etanercept was protective against CIA and the associated vascular dysfunction within this model, it was not curative. This chapter also reinforced the selection of galectin-3 as a PVAT-associated molecular marker of vasculopathy during CIA as it has uncovered, for the first time, two potential mechanisms by which galectin-3 has the ability to induce vascular dysfunction during CIA: systemically in plasma and locally in PVAT. Anti-TNF therapy with etanercept inhibited galectin-3 expression locally in thoracic PVAT but not systemically in blood plasma. Etanercept treatment only partially restored the constriction response during CIA. Therefore it was hypothesised that complete blockade of galectin-3, both locally and systemically, could fully restore vascular function during CIA. Following this, the next chapter will investigate the pharmaceutical blockade of galectin-3 during CIA focussing on the effect of galectin-3 inhibition on arthritis progression and severity, vascular function and the ATM population.

Chapter 6

The effect of galectin-3 inhibition on arthritis-associated vascular pathology in mice

6.1 Introduction

Thus far in this thesis, alterations in PVAT morphology during CIA have been characterised and galectin-3 selected as a molecular marker in PVAT of vascular dysfunction that is associated with inflammatory arthritis. The effect of TNF inhibition (a current RA therapy) on vascular function and galectin-3 expression during CIA was determined in Chapter 5. Treatment with etanercept during CIA resulted in a local but not systemic inhibition of galectin-3 and only partial restoration of vascular function. Studies have shown elevated systemic galectin-3 levels in CVD patients have been directly linked to increased mortality and galectin-3 knockout murine models have shown a significant reduction in arterial-associated inflammation (Nayor et al., 2016, Henderson et al., 2006). Therefore we hypothesise complete blockade of galectin-3 during CIA may fully restore vascular function.

There are currently no licenced therapies that target galectin-3 for human use, although galectin-3 has been identified as a potential therapeutic target in multiple inflammatory diseases, including hypertension, liver fibrosis, inflammatory arthritis, psoriasis and pulmonary fibrosis (Taniguchi et al., 2012, Pugliese et al., 2015). There are currently several galectin-3 inhibitors in clinical trial. For example, GR-MD-02 is a galectin-3 inhibitor that has passed phase I, and currently in a phase IIa trial. It is deemed safe and well tolerated at several intravenous doses for the treatment of fatty liver disease and liver fibrosis (Traber and Zomer, 2013). It was also successfully used to treat patients who displayed moderate-to-severe plaque psoriasis. An average reduction of 51.9% in plaques was achieved across all patients 30 days post a 24-week treatment programme (Ritchie et al., 2017). Phase Ib/IIa trials on TD139, an inhaled, small molecule inhibitor of galectin-3, was completed recently and was used successfully to treat Idiopathic Pulmonary Fibrosis (IPF) (Maher, 2017). There are currently two studies on going at Massachusetts General Hospital that use a non-specific inhibitor of galectin-3, modified citrus protein (MCP), as a

dietary supplement to treat hypertension and osteoarthritis respectively. In the pilot, double blind, randomised study, osteoarthritis patients received either MCP or the placebo control twice daily for 12 weeks (Clinical trial identifier: NTC01960946). Hypertension patients were required to take 5g of MCP or the placebo three times per day (Clinical trial identifier: NCT02800629). Both studies are expected to finish in before the end of 2018 and we eagerly await the results from these trials.

Galecto Biotech is a company that specialises in the production of potent inhibitors of galectins including TD139 (mentioned above) and two oral inhibitors that are in the preclinical testing phase (Gal-300 for liver fibrosis and Gal-400 for immuno-oncology). An additional inhibitor, Gal-200, is currently about to enter pre-clinical testing for treating ocular fibrosis. Finally they have additional galectin-3 inhibitors in the discovery phase (e.g. GB1101 and GB1107) (https://galecto.com/products/) (Henderson et al., 2006, MacKinnon et al., 2008, MacKinnon et al., 2016, Dang et al., 2012). Inhibitors produced by Galecto Biotech work at a higher efficacy than commonly used MCP (Garber, 2013). A collaboration between our laboratory at Cardiff University and Galecto Biotech was established after the expression data (reported in Chapter 3 and 4) was presented to the company. On the basis of these preliminary findings, Galecto Biotech agreed to supply the galectin-3 inhibitor GB1107 for *in vivo* experimental use our CIA model. Galecto Biotech instructed that daily, oral administration of GB1107 at a dose of 10 mg/kg would be sufficient, safe and well tolerated in these mice.

To my knowledge this is the first study designed to assess the impact of *in vivo* galectin-3 inhibition on inflammatory arthritis progression, severity and the associated vascular dysfunction. This chapter will also characterise the effect of GB1107 on the systemic profile of both pro- and anti-inflammatory cytokines in plasma during CIA. In line with the outcomes, the systemic and local expression of galectin-3, CIA measurements from previous chapters, the aortic constriction response, ATM number and polarisation

were determined. Our pre-clinical assessments in mice would not provide definitive evidence of the likely impact of GB1107 in human arthritis and/or arthritis-associated vascular pathology even if favourable outcomes were reported from the CIA model. It is not yet known if the expression of galectin-3 is increased in the aorta or PVAT of RA patients. As a first step towards clinical translation, a collaboration with Dr Ivana Hollan (Revmatismesykehuset, Lillehammer, Norway) and Dr Agata Burska (University of Leeds, UK) was established. This team provided precious human aorta and PVAT samples from RA patients and age matched healthy controls for IHC analysis of CD68 and galectin-3. This chapter provides the first insight into the potential impact of galectin-3 on RA-associated vascular disease by measuring the expression of the protein in vasculature tissues.

Hypothesis: Inhibiting galectin-3 by GB1107 administration during CIA will reduce arthritis incidence and severity and restore vascular function.

6.2 Aims and objectives

The aim of this chapter was three-fold. The first aim of this chapter was to characterise to effect of galectin-3 inhibition by GB1107 on arthritis progression, severity and vascular associated dysfunction during CIA. The second aim was to assess the impact of GB1107 treatment on the local and systemic expression of galectin-3 and ATM number and polarisation. The final aim was to determine if the increase in the expression of galectin-3 in the thoracic PVAT and aorta of CIA mice was also seen in the PVAT and aorta of human RA patients.

In order to meet these aims the following objectives were set:

- To quantify alterations in local and systemic galectin-3 expression induced by GB1107 treatment in CIA via ELISA, qPCR and IHC.
- To determine the effect of GB1107 on arthritis severity and progression during CIA through the measurement of clinical score, paw score, paw diameter, arthritic index and number of osteoclast in non-arthritic, vehicle treated and GB1107 treated CIA mice.
- To investigate the impact of GB1107 on vascular function during CIA by assessment of the constriction of aortic rings on the myograph from non-CIA, vehicle and GB1107 mice in response to 5-HT
- To assess the impact of GB1107 treatment during CIA on ATM number and polarisation through quantification of CD68+ cells (by IHC) and qPCR on targets: iNOS, Arg1, TNF-α, TGF-β and IL-10.
- To quantify the expression of CD68 and galectin-3 in the aorta and PVAT of RA patients and healthy controls via IHC.

6.3 Results

6.3.1 Treatment with GB1107 during CIA significantly decreases galectin-3 expression systemically in blood plasma and locally in PVAT

To assess the impact of GB1107 on *in vivo* galectin-3 inhibition during CIA systemic plasma galectin-3 concentration was measured by ELISA (section 2.2.6) in non-arthritic, vehicle treated and GB1107 treated CIA mice. Local expression of galectin-3 in the thoracic and abdominal PVAT and aorta was determined by qPCR (section 2.2.5) in non-arthritic, vehicle treated and GB1107 treated CIA mice. There was a significant increase in plasma galectin-3 in vehicle treated CIA mice (61.6±6.8, p<0.001) compared to the non-arthritic controls (18.7±1.6) (Fig 6.1A). Plasma galectin-3 concentration was significantly decreased in GB1107 treated CIA mice (28.3±4.3, p<0.001) compared to the vehicle CIA controls. Although treatment with galectin-3 inhibitor GB1107 decreased the plasma galectin-3 concentration during CIA, it did not completely block plasma galectin-3. Galectin-3 expression was significantly increased in the thoracic and abdominal PVAT and the thoracic blood vessel of vehicle treated mice with CIA compared to the non-immunised controls (Fig 6.1B), matching previous data from Chapter 4. Treatment with GB1107 resulted in a significant decrease in galectin-3 expression in the thoracic and abdominal PVAT and the thoracic blood vessel compared to the vehicle controls (Fig 6.1B).

6.3.2 *In vivo* inhibition of galectin-3 during CIA significantly decreased the percentage galectin-3⁺ cells in PVAT

To investigate the impact of GB1107 treatment on the protein expression of galectin-3 in the thoracic and abdominal aortas of non-arthritic, vehicle treated and GB1107 treated mice, galectin-3 IHC was carried out and percentage positive cells quantified.





A. To assess the effect of GB1107 on systemic galectin-3 expression plasma galectin-3 concentration was measured by ELISA in non-arthritis (n=14), vehicle treated CIA (n=13) and GB1107 treated CIA mice (n=13). **B.** To assess the effect of GB1107 on local galectin-3 expression qPCR was carried out on thoracic and abdominal PVAT and aorta samples in non-arthritic vehicle and GB1107 mice (n = 8 pre group). Data expressed mean \pm SEM. One-way ANOVA with Bonferroni's post multiple comparison test. ** = p<0.01, *** = p<0.001.
6.3.2.1 Thoracic aorta

Representative images of galectin-3⁺ cells in the thoracic PVAT and blood vessel from non-CIA, vehicle CIA and GB1107 treated CIA mice are shown in Figure 6.2. There was a significant increase in the percentage galectin-3⁺ cells in the thoracic PVAT of vehicle treated CIA mice (14.2±0.9, p<0.001) compared to the non-arthritic controls (6.1±1.0) (Fig 6.2A). Percentage galectin-3⁺ cells in the thoracic PVAT of GB1107 treated mice (2.5±0.4) were significantly decreased compared to both the vehicle treated (p<0.001) and non-CIA controls (p<0.05). Treatment with GB1107 (4.0±0.5) also caused a significant decrease in the percentage galectin-3⁺ cells in the thoracic blood vessel compared to the vehicle treated (16.7±0.6, p<0.001) and non-immunised controls (8.5±0.7, p<0.001) (Fig 6.2B).

6.3.2.2 Abdominal aorta

Percentage galectin-3⁺ cells were significantly increased in the abdominal PVAT of vehicle treated mice (11.7±0.8, p<0.001) compared to non-immunised controls (5.4±0.5) (Fig 6.3A). Treatment with GB1107 (1.7±0.3) resulted in a significant decrease in the percentage galectin-3⁺ cells in abdominal PVAT compared to the vehicle treated (p<0.001) and non-immunised controls (p<0.001) (Fig 6.3A). There was no significant difference in percentage galectin-3⁺ cells across experimental groups in the abdominal blood vessel (Fig 6.3B).

Therefore treatment with GB1107 during CIA decreased the percentage of galectin-3 postive cells in both thoracic and abdominal PVAT and the thoracic blood vessel.

6.3.3 Treatment with GB1107 during CIA delayed arthritis onset and reduced severity in DBA/1 mice

To investigate the role of galectin-3 during the onset and progression of arthritis, galectin-3 was inhibited *in vivo* through oral dosage of GB1107 daily from day 21 until termination. Control CIA mice received the vehicle daily via oral gavage for the same time period. Arthritis was assessed daily by clinical score, paw score, paw diameter and weight



Figure 6.2 GB1107 significantly reduces the percentage of galectin-3 positive cells in the thoracic aorta

To determine the impact of GB1107 treatment during CIA on the percentage of galectin-3⁺ cells (\checkmark) in the thoracic PVAT (**A**.) and the thoracic blood vessel (**B**.) IHC was carried out and percentage galectin-3⁺ cells quantified in non-immunised (n=8), vehicle treated (n=6) and GB1107 treated (n=7) CIA mice. Scale bar = 100 µm. Field of view per section = 4. Data expressed mean ± SEM. One-way ANOVA with Bonferroni's post multiple comparison test. * = p<0.05, *** = p<0.001.



Figure 6.3 GB1107 significantly reduces the percentage of galectin-3 positive cells in the abdominal PVAT

To determine the impact of GB1107 treatment during CIA on the percentage of galectin-3⁺ cells (\checkmark) in the abdominal PVAT (**A**.) and the abdominal blood vessel (**B**.) IHC was carried out and percentage galectin-3⁺ cells quantified in non-immunised (n=8), vehicle treated (n=6) and GB1107 treated (n=7) CIA mice. Scale bar = 100 µm. Field of view per section = 4. Data expressed mean ± SEM. One-way ANOVA with Bonferroni's post multiple comparison test. *** = p<0.001.

in non-arthritic, vehicle treated and GB1107 treated CIA mice.

CIA mice that received GB1107 had a delayed onset of arthritis compared to the vehicle treated controls (p<0.05 at day 25), however both groups reached 100% incidence by day 29 (Fig 6.4A). Therefore inhibition of GB1107 did not attenuate disease pathology in CIA. Although GB1107 did not prevent the onset of arthritis during CIA the severity, measured by clinical score, was significantly decreased (p<0.001) compared to the vehicle treated CIA controls at termination (Fig 6.4C). Individual paw score was also significantly decreased (p<0.001) in GB1107 treated mice compared to the vehicle CIA controls (Fig 6.4D). There was no significant difference in the swelling of hind paws, measured by paw diameter, between vehicle and GB1107 treated CIA mice (Fig 6.4E). Histological analysis of hind paws by H&E staining revealed increased synovial hyperplasia and synovial infiltration in both vehicle and GB1107 treated mice compared against non-CIA mice. This no doubt accounted for the increased in paw swelling (Fig 6.5A-C). Two parameters associated with swelling (synovial hyperplasia and infiltrate) were increased in both groups. However, the combined score for all arthritis parameters (arthritic index (AI)) was significantly decreased in GB1107 treated mice compared to the vehicle treated controls (p<0.001)(Fig 6.5D). Al was calculated based on the extent of the cellular infiltrate, synovial hyperplasia, bone erosion and cellular exudate within each group. The significant decrease in AI in GB1107 mice compared to vehicle controls was due to a decrease in bone erosion during CIA. During arthritis, increased numbers and stimulation of osteoclasts cause bone erosion. To test this TRAP staining (Fig 6.6A) was carried out on hind paw sections to assess the number of osteoclasts. The number of osteoclasts in the mid section of the hind paws was significantly increased in vehicle treated mice compared to the non-CIA controls (p<0.001) (Fig6.6B). Treatment with GB1107 during CIA caused a significant decrease in the number of osteoclasts in the joint (p<0.001), however the number of osteoclasts in GB1107 treated mice was still significantly higher than non-arthritic mice (p<0.05). Therefore GB1107



Figure 6.4 Inhibition of galectin-3 during CIA delayed arthritis onset and severity

A. Inhibition of galectin-3 with GB1107 during CIA (n=23) delayed the onset of arthritis compared to the vehicle treated CIA controls (n=23). **B.** Percentage weight change in –CIA (n=28), vehicle and GB1107 mice from day 21 to termination. **C.** Clinical score **D.** Paw score and **E.** Paw diameter were assessed throughout arthritis progression and at termination. Data expressed mean \pm SEM. Mann-Whitney test or One-way ANOVA with Bonferroni's post multiple comparison test. * = p<0.05, ** = p<0.01, *** = p<0.001.





Arthritis index (AI) of hind paws was assessed in – CIA mice (n = 8), vehicle treated CIA mice (n = 16) and GB1107 treated CIA mice (n = 16) in order determine cellular infiltrate, synovial hyperplasia, bone erosion and cellular exudate within each group. **A.** Representative image of a non-arthritic ankle joint, AI=0. **B.** Representative image of an ankle joint from vehicle treated CIA mice, AI = 14. **C.** Representative image of an ankle joint from GB1107 treated CIA mice, AI = 8. **D.** Quantification of AI. Data expressed mean \pm SEM. One-way ANOVA with Bonferroni's post multiple comparison test. *** = p<0.001





Osteoclast numbers were assessed in the hind paws of non-arthritic, vehicle and GB1107 mice by performing a TRAP stain **A.** Representative images of –CIA, vehicle treated and GB1107 treated hind paws, osteoclasts identified by red stained cells (TRAP⁺). **B.** Quantification of TRAP+ cells. **C.** Red box indicates area of hind paw shown in representative images. Data expressed mean \pm SEM. One-way ANOVA with Bonferroni's post multiple comparison test. * = p<0.05, *** = p<0.001

decreased arthritis severity during CIA and reduced the number of bone-resorbing osteoclasts in the joints of mice with CIA.

6.3.4 GB1107 partially restored vascular function during CIA

Measuring the constriction response of aortic rings from non-arthritic, vehicle treated and GB1107 treated mice assessed the effect of GB1107 treatment during CIA on vascular function (Fig 6.7). Vehicle treated CIA mice had a significantly impaired constriction response to 5-HT (4.2 ± 0.1 , p<0.001) compared to the non-immunised controls (9.4 \pm 0.2). Mice that received GB1107 showed partially restored vascular function (7.9 \pm 0.2) as this group's maximum constriction was significantly higher than the vehicle treated mice, but still significantly lower than the non-CIA controls.

To ensure the partial restoration of vascular function in GB1107 treated mice was due to the inhibition of galectin-3, and not a direct action of the drug on the vasculature, aortic rings from non-CIA male DBA/1 mice were incubated *ex vivo* with and without GB1107 prior to measuring the constriction response to 5-HT (Fig 6.8). Analysis of aortic rings that were incubated with either Krebs buffer only (-CIA), Krebs buffer + DMSO (-CIA + DMSO) or Krebs buffer + DMSO + GB1107 (-CIA + GB1107) showed no significant difference in vascular function. Therefore the partial restoration in vascular function in GB1107 treated mice during CIA was due to the inhibition of galectin-3.

6.3.5 Treatment with GB1107 during CIA significantly decreases plasma IFNγ and IL-17A

Plasma was isolated from whole blood of non-arthritic and mice with CIA that had been given either the vehicle or GB1107. LEGENDplex assays were used to measure the levels of inflammation-associated and T-cell derived cytokines in plasma. The effect of *in vivo* galectin-3 inhibition during CIA on the concentration of systemic pro- and antiinflammatory cytokines was determined and reported here for the first time. Several of the



Figure 6.7 GB1107 treated CIA mice have improved vascular function compared to vehicle CIA controls

Myography was used to assess vascular function by measuring the constriction response of aortic rings to 5-HT from non-arthritic, vehicle and GB1107 mice (n=16 per group). Data expressed mean \pm SEM. One-way ANOVA with Bonferroni's post multiple comparison test. *** = p<0.001.



Figure 6.8 Incubation of aortic rings with GB1107 prior to addition of 5-HT had no effect on the constriction response

To assess if GB1107 had a direct response on vascular constriction, aortic rings from non-arthritic DBA/1 mice were incubated with KREBS buffer only (n=8), or with the addition of DMSO only (n=4) or DMSO + GB1107 (n=4) before the addition of 5-HT. Data expressed mean \pm SEM. One-way ANOVA with Bonferroni's post multiple comparison test.

cytokines analysed were not in the detectable range of this assay, see Supplementary Fig 2, Appendix II.

Plasma concentration of pro-inflammatory cytokines TNF- α (Fig 6.9A) and IL-6 (Fig 6.9F) were significantly increased in vehicle treated CIA mice (p<0.05 and p<0.01respectively) compared to the non-immunised controls. Although there was a trend of increased concentration of TNF- α and IL-6 in plasma from GB1107 treated mice, this trend was not significant in comparison to non-CIA controls. However both TNF- α and IL-6 showed a trend of decreased concentration in plasma of mice that received GB1107 when compared to vehicle treated mice, but again this was not significant. Plasma concentrations of pro-inflammatory cytokines IFN-y (Fig 6.9B) and IL-17A (Fig 6.9H), which have the pathological function during inflammatory arthritis (e.g. to drive activation of fibroblast-like synoviocytes), were also increased in the vehicle treated mice compared to the non-CIA controls (p<0.001). IFN-y and IL-17A plasma concentrations were significantly decreased in GB1107 treated CIA mice compared to the vehicle treated controls (p<0.01 and p<0.001 respectively). Plasma concentrations of pro-inflammatory cytokines CCL2, GM-CSF, IL-1β, IL-23, IL-27 and IFN- β (Fig 6.9C-E and I-K) were significantly increased in vehicle CIA controls. Plasma levels in GB1107 treated CIA mice were also significantly elevated compared to the non-immunsied controls and were comparable to CIA mice that received vehicle.

6.3.6 Treatment with GB1107 resulted in a decrease in CD68⁺ cells in PVAT during CIA

To assess the impact of galectin-3 inhibition during CIA on the number of ATMs in PVAT, IHC was used to determine the percentage of CD68⁺ cells in the thoracic and abdominal PVAT and aorta of non-CIA, vehicle treated and GB1107 treated CIA mice.





To determine to effect of *in vivo* inhibition of galectin-3 through dosing with GB1107 on the systemic level of pro- and anti- inflammatory cytokines LEGENDplex assays were carried out on plasma samples from non-arthritic (n=14), vehicle treated CIA (n=12) and GB1107 treated CIA mice (n=13). **A.** TNF- α **B.** IFN- γ **C.** CCL2 **D.** GM-CSF **E.** IL-1 β **F.** IL-6 **G.** IL-12p70 **H.** IL-17A **I.** IL-23 **J.** IL-27 **K.** IFN- β **L.** IL-10. Dotted line represents detection limit. Data expressed mean ± SEM. One-way ANOVA with Bonferroni's post multiple comparison test. * = p<0.05, ** = p<0.01, *** = p<0.001.

6.3.6.1 Thoracic aorta

Representative images of CD68⁺ cells in the thoracic PVAT and blood vessel from non-arthritic, vehicle treated and GB1107 treated mice are shown in Figure 6.10. The percentage of CD68⁺ cells was significantly increased in thoracic PVAT of vehicle treated CIA mice (13.7±0.9, p<0.001) compared to the non-arthritic controls (6.7±0.5) (Fig 6.10A). The percentage CD68⁺ cells in the thoracic PVAT of CIA mice treated with GB1107 (7.9±0.4) was significantly lower (p<0.001) than in vehicle treated CIA mice. In the thoracic blood vessel, the percentage of CD68⁺ cells was significantly increased in both vehicle treated (11.9±1.1, p<0.01) and GB1107 treated (10.4±0.3, p<0.05) CIA mice compared to the non-immunised controls (7.8±0.6) (Fig 6.10B).

6.3.6.2 Abdominal aorta

Percentage of CD68⁺ cells was significantly increased in the abdominal PVAT of vehicle treated CIA mice (13.2±0.8, p<0.001) compared to the non-CIA controls (6.1±0.3) (Fig 6.11A). Treatment with GB1107 resulted in a significant decrease (7.6±0.6, p<0.001) in the percentage CD68⁺ cells in adominal PVAT compared to the vehicle treated controls (Fig 6.11A). The percentage of CD68⁺ cells in the abdominal blood vessel was not significantly different across experimental groups (Fig 6.11B).

Therefore *in vivo* inhibition of galectin-3 by oral administration of GB1107 results in a significant decrease of ATMs in both the thoracic and abdominal PVAT during CIA.

6.3.7 GB1107 alters ATM polarisation during CIA

In order to determine whether treatment with GB1107 effected the polarisation of macrophages in the thoracic and abdominal aorta the expression of iNOS, Arg1, TNF- α , TGF- β and IL-10 was measured by qPCR in non-immunised, vehicle treated and GB1107 treated mice (Figure 6.12).



Figure 6.10 Treatment with GB1107 significantly decreases the number of macrophages in thoracic PVAT during CIA

In order to assess the effect of galectin-3 inhibition on the ATM number in thoracic PVAT (**A**.) and the thoracic aorta (**B**.) CD68 IHC was carried out and percentage CD68⁺ cells (\checkmark) quantified in non-arthritic (n=8), vehicle (n=6) and GB1107 (n=7) mice. Isotype shown in insert. Scale bar = 100 µm. Field of view per section = 4. Data expressed mean ± SEM. One-way ANOVA with Bonferroni's post multiple comparison test. * = p<0.05, ** = p<0.01, *** = p<0.001.



Figure 6.11 Treatment with GB1107 decreased CD68⁺ cells in abdominal PVAT but not the abdominal aorta during CIA

In order to assess the effect of galectin-3 inhibition on the ATM number in abdominal PVAT (**A**.) and the abdominal aorta (**B**.) CD68 IHC was carried out and percentage CD68⁺ cells (\checkmark) quantified in non-arthritic (n=8), vehicle (n=6) and GB1107 (n=7) mice. Isotype shown in insert. Scale bar = 100 μ m. Field of view per section = 4. Data expressed mean ± SEM. One-way ANOVA with Bonferroni's post multiple comparison test. *** = p<0.001.

The expression of M1 marker iNOS (Fig 6.12A) was significantly increased in the thoracic PVAT and thoracic aorta of GB1107 treated mice compared to the non-arthritic controls (p<0.05). This was the first time in this thesis an increase in M1 macrophages in thoracic PVAT and aorta has been described. However, the expression of pro-inflammatory cytokine TNF- α , which is one factor that promotes iNOS expression, was significantly decreased in both the thoracic and abdominal PVAT and blood vessel of GB1107 treated mice (Fig 6.12C). Therefore TNF- α was not responsible for the induction of iNOS expression at these sites following GB1107 administration. Treatment of CIA with GB1107 resulted in a significant increase in the expression of M2 marker Arg1 in the thoracic aorta (PVAT and blood vessel) compared to both the vehicle CIA controls and non-arthritic controls (Fig 6.12B). The expression of anti-inflammatory cytokine TGF- β , a cytokine involved in driving M2 polarisation, was also significantly increased in both the thoracic aorta and the abdominal aorta (PVAT and blood vessel at each site) of GB1107 treated mice compared to the vehicle treated, as well as the non-immunised controls (Fig 6.12D). The expression of the anti-inflammatory cytokine IL-10 was significantly increased in the thoracic PVAT and the abdominal aorta of mice that received GB1107 compared to both vehicle CIA and non-CIA controls (Fig 6.12E). Therefore modulation of cytokine expression (increased TGF- β and IL-10) by GB1107 may, in part explain the polarisation of macrophages in the thoracic aorta (PVAT and blood vessel) towards an Arg1⁺ M2 phenotype.

6.3.8 Inhibition of galectin-3 in the thoracic aorta did not affect the expression of IL-4Rα during CIA

As previously shown in Figure 1B, treatment with GB1107 resulted in a significant decrease of galectin-3 expression in the thoracic and abdominal PVAT and thoracic aorta during CIA. To provide insight into potential mechanisms galectin-3 is activated during CIA, the effect of GB1107 treatment on the molecular expression of galectin-3 upstream receptor IL-4R α and its downstream receptor CD98 in the thoracic and abdominal aortas,



Figure 6.12 GB1107 treatment alterations macrophage polarisation through modulation of cyotkines in PVAT

To investigate the impact of galectin-3 inhibition on macrophage polarisation, M1 macrophage marker iNOS (A.), M2 marker Arg1 (B.), pro-inflammatory cytokine TNF- α (C.) and anti-inflammatory cytokines TGF- β (D.) and IL-10 (E.) expression was analysed by qPCR in non-arthritic, vehicle treated and GB1107 treated mice (n = 8 per group). Data expressed mean ± SEM. One-way ANOVA with Bonferroni's post multiple comparison test. * = p<0.05, ** = p<0.01, *** = p<0.001.

qPCR was carried out and quantified in non-arthritic, vehicle treated and GB1107 treated mice (Fig 6.13).

A trend of increased IL-4R α expression was seen in the thoracic and abdominal PVAT and blood vessel of CIA mice given the vehicle compared to non-CIA mice, however this increase was only significant in the thoracic aorta (p<0.05) (Fig 6.13A). IL-4R α expression was significantly increased across all thoracic and abdominal sites analysed in GB1107 treated mice compared to non-arthritic controls (Fig 6.13A). Therefore it is possible GB1107 blocked galectin-3 downstream of IL-4R α , or there are other cytokines present in GB1107 treated mice that interact with this receptor. Treatment with GB1107 during CIA had no effect on CD98 expression in the thoracic aorta, however CD98 expression was significantly decreased in the abdominal PVAT and blood vessel compared to vehicle treated controls (Fig 6.13B).

6.3.9 The percentage of CD68⁺ and galectin-3⁺ cells were significantly increased in the aorta and PVAT of human RA patients

To ensure galectin-3 was an appropriate therapeutic target for translation from the CIA model to RA patients, IHC staining for CD68 and galectin-3 was carried out and quantified in the aorta and PVAT samples from RA patients and age-matched healthy controls.

Representative images of CD68⁺ cells in the aorta and PVAT of RA and control samples are shown in Figure 6.14. The total cell number and number of CD68 positive cells were quantified from each section and the percentage CD68⁺ cells determined. The percentage of CD68⁺ cells was significantly increased in the aorta and PVAT of RA patients compared to the healthy controls (p<0.001) (Fig 6.14A and B). Therefore macrophage numbers are increased in the vasculature and PVAT during RA, mirroring what was described in this thesis during CIA. Representative images of galectin-3 positive cells in the aorta and PVAT of RA and control samples are shown in Figure 6.15. The percentage of



Figure 6.13 GB1107 does not affect the expression of upstream receptor IL-4R α To assess to effect of GB1107 treatment on the expression of galectin-3 upstream receptor IL-4R α (**A**.) and downstream receptor CD98 (**B**.) qPCR analysis was carried out in the thoracic and abdominal PVAT and blood vessel of non-CIA, vehicle treated and GB1107 treated CIA mice (n = 8 per group). Data expressed mean ± SEM. One-way ANOVA with Bonferroni's post multiple comparison test. * = p<0.05, ** = p<0.01.



Figure 6.14 Percentage $CD68^+$ cells is increased in the human aorta and PVAT of RA patients

To determine if the alteration in ATM number seen the thoracic aorta during CIA are seen in RA patients, CD68 IHC (\checkmark) was carried out and quantified in the aorta (**A**.) and PVAT (**B**.) from RA patients and age-matched healthy controls (n = 8 per group). Scale bar = 100 µm. Field of view per section = 3. Data expressed mean ± SEM. Mann-Whitney test. *** = p<0.001.

galectin-3⁺ cells was significantly increased in the aorta and PVAT of RA patients compared to the healthy controls (p<0.01) (Figure 6.15A and B). To my knowledge this is the first time the protein expression of galectin-3 was quantified in the aorta and PVAT of RA patients, and again matched data previously described during CIA.



Figure 6.15 Percentage Galectin-3⁺ cells is increased in the human aorta and PVAT of RA patients

To determine if the increase in percentage galectin-3⁺ cells seen the thoracic aorta during CIA are seen in RA patients, CD68 IHC (\checkmark) was carried out and quantified in the aorta (**A**.) and PVAT (**B**.) from RA patients and age-matched healthy controls (n = 8 per group). Scale bar = 100 µm. Field of view per section = 3. Data expressed mean ± SEM. Mann-Whitney test. ** = p<0.01.

6.4 Discussion

For the first time, this chapter showed administration of GB1107 during CIA inhibited local galectin-3 expression in the thoracic and abdominal PVAT and in the thoracic blood vessel. Furthermore, treatment with GB1107 during CIA caused a significant decrease in systemic blood plasma galectin-3 concentration, however it did not cause a complete blockade. Animals treated with GB1107 showed a decrease in arthritis severity and vascular dysfunction associated with CIA compared to mice given vehicle. Treatment with GB1107 drove the polarisation of ATMs towards a tissue-remodelling associated M2 phenotype via increased IL-10 and TGF- β concentration within the PVAT. Additionally, this chapter described an increase in macrophages (CD68⁺ cells) and galectin-3⁺ cells in the aorta and PVAT of RA patients compared to healthy controls for the first time, mirroring what was described in this thesis during CIA. Therefore the CIA model has been used here as a platform to identify a potential therapeutic target (galectin-3) for vasculopathy associated with CIA that can be translated into human RA studies.

Treatment with GB1107 in CIA mice delayed the onset of arthritis, and although an incidence of 100% was reached, there was significant reduction in disease severity (determined by clinical score) in mice that received the inhibitor compared to the vehicle CIA controls. In addition to a decreased clinical score, CIA mice given with GB1107 daily from day 21 had a significant decrease in osteoclast number (TRAP⁺ cells) and a decrease in bone-erosion present in hind paws which was determined during arthritis index scoring. GB1107 treatment had no effect on paw swelling, which was determined by measuring the diameter of the mid-foot daily, or synovial hyperplasia, which was the assessment of cellular infiltrate scored on histological sections of hind paws (AI), during CIA. Overall, this suggests that GB1107 functionally decreased disease severity through inhibiting bone erosion during CIA. However, treatment with GB1107 had little or no effect on the systemic infiltration or expansion of the immune cells in the synovium, which drives disease

pathology, therefore the mice still develop CIA. One potential mechanism by which GB1107 supressed bone erosion during CIA was via significantly decreasing IL-17A plasma concentration. Previous studies investigating the impact of galectin-3 down regulation on the expression of pro-inflammatory cytokines also showed a significant decrease in the production of IL-17A from T cells (Jiang et al., 2009, Chen et al., 2015). Pro-inflammatory cytokine IL-17A is produced by Th17 cells and synovial fibroblasts within a joint (Stockinger and Veldhoen, 2007). High levels of IL-17A during inflammatory arthritis activate fibroblast-like synoviocytes and RANKL production (e.g. by osteoblasts and T cells). Over production of RANKL activates osteoclasts within the joints and results in increased bone erosion (Kato et al., 2013, van den Berg et al., 2007).

To determine the effect of galectin-3 inhibition on vascular function during CIA, myography was carried out on non-arthritic, vehicle control CIA and GB1107 treated CIA mice. The constriction response to 5-HT of aortic rings from these experimental groups concluded that treatment with GB1107 during CIA partially restored vascular function during CIA. This was the first time it has been reported that in vivo inhibition of galectin-3 improves vascular function during inflammatory arthritis. Analysis of plasma galectin-3 concentration by ELISA, molecular expression of galectin-3 in vasculature by qPCR and protein expression of galectin-3 in the PVAT and blood vessel by IHC showed that treatment with GB1107 successfully inhibits galectin-3 systemically and locally during CIA but does not induce a full blockade. From this study it cannot be determined if a full attenuation of galectin-3 can be achieved. Galecto Biotech selected the dose of 10mg/kg GB1107 daily. Conducting an in vivo dose range experiment with GB1107 in CIA (e.g. at 10, 15 and 20 mg/kg) would provide a platform to answer the following questions: can GB1107 induce complete blockade of galectin-3 during CIA, and can GB1107 treatment fully restore vascular function which is lost during inflammatory arthritis. However, full restoration of vascular function might not be achievable within CIA as the blood vessel may already be

damaged beyond repair, even early in disease pathology. Currently there are no therapies available to fully reverse vascular damage associated with CVD (Esselstyn et al., 2014). Recently, a group generated transgenic mice that had increased expression of the vascular protective molecule prostaglandin I₂ (PGI₂) by enzymatically engineering cyclooxygenase-1 (COX-1) and PGI₂ synthase in order to block the production of pro-inflammatory PGE₂ and thromboxane A₂ (Ling et al., 2018). These mice displayed an increased resistance to vascular assault (e.g. carotid arterial blockage and acute thrombotic stroke) and also resistance to non-prostanoid-related vasoconstrictive damage (Ling et al., 2018). Therefore if increasing the dose of GB1107 does not further or fully restore vascular function during CIA, perhaps enzymatic engineering could be a useful tool to block galectin-3 production completely.

To assess the impact of GB1107 administration on ATM number and polarisation during CIA, CD68 IHC quantified the number of macrophages in the thoracic and abdominal aortas and polarization of the macrophages at these sites analysed based on molecular expression of iNOS and Arg1. Galectin-3 promotes macrophage polarisation towards an M2 phenotype through the induction of Arg1 expression by activated PI3K (MacKinnon et al., 2008), which can modulate vascular function as previously described in Chapter 1 (Ghosh et al., 2015, Zeyda and Stulnig, 2007). Treatment with GB1107 during CIA resulted in a decrease in ATM number in thoracic and abdominal PVAT compared to the vehicle treated controls. In line with this data, recent studies have shown that macrophage infiltration was significantly reduced in the myocardium of galectin-3 KO mice and also in the abdominal aorta of mice treated with MCP (Mosleh et al., 2018, Fernandez-Garcia et al., 2017). Published data from this laboratory and data from this thesis has linked an increase in macrophage number in the thoracic aorta with CIA-associated vascular dysfunction (Williams et al., 2016). Therefore the decrease in CD68^{*} macrophages described in CIA mice given GB1107 could, in part, play a role in the partial restoration of

the constriction response. The previous chapter of this thesis positively correlated M2 macrophage marker Arg1 expression with galectin-3 expression in the thoracic PVAT during CIA. Although galectin-3 was significantly decreased at both molecular and protein levels following treatment with GB1107, Arg1 expression was significantly increased in the thoracic PVAT of these mice. There are other factors in addition to galectin-3 that drives Arg1 expression in macrophages, e.g. anti-inflammatory cytokines TGF- β and IL-10 (Rath et al., 2014). The expression of TGF- β and IL-10 was significantly increased in the thoracic PVAT of GB1107 treated CIA mice compared to both control groups. Therefore the increase in anti-inflammatory cytokines in PVAT following treatment with GB1107 maintained the M2 phenotype previously described during CIA. A study showed Galectin-3 KO mice have an increased expression of IL-10, in line with the data described in this chapter (Jiang et al., 2009). However the role of galectin-3 in the regulation of TGF- β is more complex, with opposing roles described in the literature; one study suggested that TGF- β controls galectin-3 expression, where another described galectin-3 as a mediator of pro-fibrotic roles of TGF- β (Tian et al., 2016, He et al., 2017). An increase in TGF- β and IL-10 expression is characteristic of immune suppression; therefore treatment with GB1107 may modulate and dampen the pro-inflammatory immune response during CIA (Yoshimura and Muto, 2011). TGF- β has also been shown to promote the expression of upstream receptor for galectin-3, IL-4Ra, in macrophages (Zhou et al., 2012). IL-4Ra expression was significantly elevated in the vasculature of GB1107 treated mice, therefore the increase in TGF- β expression in GB1107 treated mice explains why there is an increase in IL-4R α expression in these mice but no increase in galectin-3 expression. Aside from inducing the production of galectin-3, IL-4R α is involved in e.g. promoting and priming a Th2 T cell response (Hurdayal and Brombacher, 2017).

For the first time, to my knowledge, this chapter reported an increase in the percentage of CD68 and galectin-3 positive cells in the human aorta and PVAT of RA

patients compared to age-matched healthy controls. This underpins and justifies the selection of galectin-3 as a molecular marker for in PVAT of vascular dysfunction that is associated with inflammatory arthritis as it shows translation from the mouse model to human disease. However translation of GB1107 into clinical trials is a long way off. Before clinical trials can take place, additional *in vivo* experiments with GB1107 are required to gain a greater insight into the potential mechanisms galectin-3 is involved in, both in inflammatory arthritis and cardiovascular pathologies, as well as dose range experiments to find the best dose for use during CIA described above.

This is the first study where galectin-3 has been inhibited successfully *in vivo* with GB1107; therefore all observations described are preliminary, with no comparisons in published literature to refer to. Treatment with GB1107 during CIA resulted in pathophysiological improvements in arthritis severity and vascular function, potentially through the modulation of cytokines including IL-17A and TGF-β. Although disease severity was reduced and vascular function improved following GB1107 administration during CIA, this therapy at the current dose was not curative. This chapter has identified galectin-3 as a successful, novel therapeutic target for vasculopathy during inflammatory arthritis. Further investigation into the potential mechanisms by which galectin-3 inhibition improves vascular function during CIA is required. Furthermore, this chapter has demonstrated the CIA model can be used as a platform to identify potential PVAT-associated targets that can be translated into human disease for the first time. Going forward this model will be a key tool for uncovering the pathological kinetics that link inflammatory arthritis and cardiovascular pathologies.

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Chapter 7

General Discussion

There is an established link between rheumatic diseases and cardiovascular pathologies as epidemiological studies have determined approximately 50% of premature deaths in RA patients are due to CVD (Symmons and Gabriel, 2011). Therefore there is a current clinical need for therapeutically targeting RA-associated CVDs in order to improve patient outcomes and reduce this mortality rate. However, the underlying mechanisms that link these two pathologies are not fully understood. There are many mechanisms and factors that can influence vascular function in health and disease: one of which is perivascular adipose tissue. PVAT, which surrounds all major blood vessels, can induce vasorelaxation and vasoconstriction through the release of soluble mediators (e.g. adiponectin and noradrenaline respectively) (Villacorta and Chang, 2015). A loss of function in PVAT-dependent anti-contractile responses has been observed in several diseases including hypertension, diabetes and obesity (Greenstein et al., 2009). However it is not yet known if the systemic nature of RA can induce morphological and/or function alterations in PVAT that in turn could influence vascular function in these patients. Furthermore, this leads to the question if there are vascular-associated PVAT alterations during RA, is there a mechanism in which we can target in order to prevent/reduce the vascular dysfunction associated with rheumatic disease. To address these questions, the CIA model was used in this thesis. Previous studies from this laboratory have identified the CIA model as a tool for investigating mechanisms related to the impaired vascular function that is associated with inflammatory arthritis (Reynolds et al., 2012, Williams et al., 2016).

In order to help answer these questions, four aims were set out at the start of this thesis: to investigate the potential morphological alterations in adipose tissues and phenotypic changes in the ATM population during CIA, to identify a specific molecular marker of vascular pathology in PVAT during inflammatory arthritis, to investigate the impact of TNF on ATMs and vascular function during CIA through inhibiting TNF- α in vivo

with etanercept, and to characterise and assess the effects of galectin-3 inhibition on CIA, vascular function and ATMs.

This thesis, for the first time, identified and described morphological alterations in PVAT during CIA. These were characterised on a cellular (an increase in total cell number and an increase in M2 ATMs) and structural level (decrease in adiposity). Due to the increase in ATMs, the macrophage was identified as a potential key functional player in the development of CIA-associated vasculopathy. To try and address the current need for early therapeutical intervention of CVDs in RA patients, this thesis aimed to identify a PVATassociated macrophage molecular marker of vasculopathy during CIA. Chapter 4 identified galectin-3 as the best marker out of the potential targets that were selected from a literature review. Galectin-3 is expressed by M2 macrophages, which are elevated in PVAT during CIA, and is associated with an increased mortality rate in CVD patients (Nayor et al., 2016). In RA patients, treatment with an anti-TNF therapy (e.g. etanercept) reduced the cardiovascular risk associated with rheumatic diseases (Lim et al., 2014). Treatment with etanercept during CIA significantly reduced the severity and progression of arthritis compared to the PBS CIA control mice. In CIA mice given etanercept there was a partial, but not full, restoration of the constriction response, which was lost in untreated CIA mice. Administration of etanercept during CIA also a decreased the number of tissue remodelling-associated M2 macrophages in PVAT. Furthermore, treatment with etanercept in CIA mice resulted in the local suppression of galectin-3 in PVAT, but not systemically in blood plasma. The concentration of galectin-3 in the plasma of CIA mice correlated with an increased severity of arthritis (high clinical score) and reduced vascular function (low Rmax). Therefore it was hypothesised that full attenuation of galectin-3, both locally in PVAT and systemically in blood plasma, could further restore vascular function during CIA. Galectin-3 was inhibited during CIA via oral administration of GB1107 at 10mg/kg daily, which was supplied by Galecto Biotech. This resulted in a decrease in galectin-3 expression

locally in PVAT and systemically in the blood plasma. Treatment with GB1107 reduced the severity of CIA and the level of bone erosion compared to the CIA controls that received the vehicle. However, GB1107 did not prevent disease onset as all mice that received this treatment developed CIA. GB1107 treatment during CIA also resulted in a reduction of ATM numbers in PVAT, however these were still mainly M2 macrophages. Similar to anti-TNF therapy, treatment with GB1107 during CIA partially restored vascular function.

GB1107 was a novel therapeutic strategy used in CIA for the first time in this thesis, whereas etanercept is an established therapy for the treatment of RA. This allowed us to compare the outcomes of our novel therapeutic strategy to an established therapy. Treatment with GB1107 during CIA delayed arthritis onset by two days with all mice developing CIA, however treatment with etanercept during CIA delayed onset by 3 days and only 40% of mice developed CIA. At termination both therapies had significantly reduced the severity of arthritis, however only etanercept treatment reduced severity of CIA throughout the protocol after arthritis onset. Daily measurements of paw diameter and analysis of arthritis index at the termination of the CIA protocol determined that etanercept treatment reduced paw swelling and cellular infiltrate, however GB1107 treatment did not. Therefore treatment with etanercept was more favourable for the treatment of inflammatory arthritis symptoms compared with GB1107 treatment during CIA. Both etanercept and GB1107 treatment during CIA resulted in the partial restoration of constriction response. Mice that received etanercept during CIA restored vascular function to 79% of the non-CIA controls. Treatment with GB1107 during CIA restored vascular function by 84%. Therefore administration of GB1107 during CIA was marginally more efficient at restoring vascular function compared with etanercept treatment. In this thesis myograph was conducted on denuded aortas. Williams et al 2016 determined that conducting myography with PVAT intact aortic rings did not alter the constriction response measured on the myograph compared to denuded aortic rings from the same animal. An

increase in macrophage number, TNF- α and galectin-3 concentrations are all individually linked to the onset of vascular dysfunction (Gui et al., 2012, Fernandez-Garcia et al., 2017, Sarzi-Puttini et al., 2005a). Treatment with etanercept during CIA reduced the number of ATMs in PVAT, inhibited TNF- α locally in PVAT and systemically in blood plasma, however only decreased the expression of galectin-3 locally. Therefore it could be hypothesised that the partial restoration in vascular function following etanercept treatment was due to a reduction in ATMs and inhibition of TNF- α locally in PVAT and systemically in plasma. Daily administration of GB1107 during CIA reduced the number of ATMs present in PVAT, inhibited galectin-3 expression locally in PVAT and partially in blood plasma, however the systemic concentration of TNF- α was comparable to CIA mice given the vehicle. Therefore it could be hypothesised that the partial restoration in vascular function following GB1107 treatment was due to a reduction in ATMs and inhibition of galectin-3. Thus, etanercept and GB1107 treatments partially restore vascular function through suppression of different mechanisms during CIA.

This thesis has reinforced the use of the CIA model as a tool to investigate the vascular dysfunction associated with inflammatory arthritis. Furthermore, it identified the CIA model as a platform to investigate the potential impact of alterations in PVAT has on vascular function. In addition, the CIA model was also used as a tool for the identification of PVAT-associated markers of vasculopathy that can be translated into human disease for the first time. Through the use of the CIA model and immunohistological analysis of PVAT from RA patients this thesis established for the first time that morphological alterations occur in PVAT during inflammatory arthritis. Identification of galectin-3 as a PVAT-associated marker of vasculopathy during inflammatory arthritis highlighted one potential mechanism that could be targeted to improve the mortality rate associated with RA and CVD. Although therapeutic inhibition of galectin-3 reduced the vascular dysfunction associated with CIA, it was not as effective as the established therapy, etanercept, at improving the incidence and

severity of arthritis in the CIA model. Therefore the question of identifying a possible therapeutic strategy that targets both inflammatory arthritis and the associated vascular dysfunction remains, and the question of whether galectin-3 is the best target of PVATassociated vasculopathy during inflammatory arthritis has arisen.

If time had permitted there are three experimental approaches I would have taken to answer these questions. Firstly, an in vivo dose range experiment with GB1107 would be conducted to establish if there is a more efficient, but still tolerated, dose of this novel therapeutic that would reduce the incidence and severity of CIA and further improve the vascular constriction response in these mice. As this thesis identified the restoration of the constriction response following etanercept and GB1107 treatment were due to the inhibition of different mechanisms, a combination therapy run with administration of both etanercept and GB1107 during CIA would be carried out. Hypothetically this combination therapy would significantly reduce the incidence and severity of arthritis and further restore vascular function during CIA compared to the individual therapy runs conducted in this thesis. In order to investigate if there are additional PVAT-associated macrophage molecular markers of vasculopathy during CIA, other than galectin-3, RNA-seq analysis of PVAT from CIA and non-CIA mice would be carried out. This would identify all potential molecular markers of vasculopathy during inflammatory arthritis that could be therapeutically targeted, rather than the short list produced from the literature review carried out in chapter 4.

In addition to M2 macrophages, neutrophils and T cells also express galectin-3. Studies have determined galectin-3 is a key positive regulator of neutrophil recruitment to inflamed sites through the upregulation of adhesion molecules (e.g. P-selectin glycoprotein ligand-1), which bind to E-selectin on the endothelium of the blood vessels (Gittens et al., 2017). Endogenous galectin-3 is key to activating the apoptosis and clearance of neutrophils (Wright et al., 2017). As galectin-3 was elevated during CIA, an increase in

neutrophil trafficking into the vasculature could be occurring. Neutrophils increase endothelial stress in the vasculature via the release of granule-based proteins, which in turn promote inflammation and is linked to the formation of foam cells that drive atherosclerosis (Sanda et al., 2017). Therefore an increase in neutrophil numbers in the PVAT and blood vessel during CIA could be an additional mechanism contributing to vascular dysfunction associated with this model. Immunohistochemical analysis of Ly6G⁺ cells in the PVAT and vasculature from CIA and non-CIA mice would determine if neutrophil numbers are increased at these sites during CIA. As CIA is a T cell dependent model of RA, T cells could be a key source of galectin-3. Studies have determined galectin-3 has roles in T cell apoptosis, activation and migration (de Oliveira et al., 2015). In RA patients T cells in the synovium overexpress galectin-3, which in turn increases the production of proinflammatory mediators (e.g. IL-6, GM-CSF, TNFα and CCL2) from synovial fibroblasts (Filer et al., 2009). A population of cytotoxic CD4+CD28- T cells, which are directly linked to cardiovascular mortality, are elevated systemically in RA patients (Broadley et al., 2017). To my knowledge the potential roles of galectin-3 expressing T cells in PVAT during inflammatory arthritis has not been investigated. Characterising any alterations to the T cell population, and indeed the neutrophil population, in PVAT and the vasculature during, as was done with macrophages in this thesis, will also allow the potential identification of mechanisms by which PVAT contributes to vascular dysfunction, not only during arthritis, but in other diseases including diabetes and obesity (Fernandez-Alfonso et al., 2013, Lastra and Manrique, 2015).

In summary, this thesis characterised morphological alterations in PVAT during inflammatory arthritis for the first time. Furthermore, it identified galectin-3 as a potential novel therapeutic target of vasculopathy during CIA. Findings of this thesis have already contributed to this field of research and have been peer reviewed in publications and presentations (summarised in Appendix I).

Appendix II

Supplementary Figures


Supplementary Figure 1 Systemic plasma levels of pro- and anti- inflammatory cytokines that were below detection limit in non-CIA, PBS and Etanercept treated mice

To determine to effect of CIA induction, with and without anti-TNF therapy, on the systemic level of pro- and anti- inflammatory cytokines LEGENDplex assays were carried out on plasma samples. **A.** IL-1α **B.** IL-2 **C.** IL-4 **D.** IL-5 **E.** IL-9 **F.** IL-13 **G.** IL-17F **H.** IL-21 **I.** IL-22. Dotted line represents detection limit n = 14 per group. One-way ANOVA with Bonferroni's post multiple comparison test.



Supplementary Figure 2 Systemic plasma levels of pro- and anti- inflammatory cytokines that were at detection limit in non-CIA, PBS and GB1107 treated mice

To determine to effect of *in vivo* inhibition of galectin-3 through dosing with GB1107 on the systemic level of pro- and anti- inflammatory cytokines LEGENDplex assays were carried out on plasma samples from non-arthritic (n=14), vehicle treated CIA (n=12) and GB1107 treated CIA mice (n=13). **A.** IL-1α **B.** IL-2 **C.** IL-4 **D.** IL-5 **E.** IL-9 **F.** IL-13 **G.** IL-17F **H.** IL-21 **I.** IL-22. Dotted line represents detection limit. Data expressed mean ± SEM. One-way ANOVA with Bonferroni's post multiple comparison test.

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