Macrophage phenotypes in giant cell arteritis

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The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

Giant cell arteritis (GCA) is the commonest form of vasculitis that affects individuals over 50 years of age, predominantly occurring within medium and large-sized arteries. Without immediate glucocorticoid treatment, GCA can culminate in blindness and stroke. T-cells and macrophages are found to infiltrate through the arterial wall and are intricately involved in disease pathogenesis, from arterial destruction to neointimal hyperplasia resulting in tissue ischemia. Macrophages perform an array of different functions in GCA arteries however disease heterogeneity, along with poor characterisation of macrophage phenotypes has hindered studies into the role of macrophages. I hypothesise that the heterogeneity of histological and clinical manifestations seen between individuals with GCA is in part due to the phenotypic heterogeneity of macrophages found within the artery wall of different patients.

A THP-1 cell line model was developed to enable identification of phenotype-specific macrophage markers which were confirmed at the RNA and protein level. M(LPS, IFNγ) markers ANKRD22 and GBP5 were used to characterise M1 macrophages and M(IL-4) marker MRC1, and M(IL-10) marker CD163 were used to characterise M2 macrophages in temporal artery biopsies using immunohistochemistry.

M1 and M2 macrophages were found within each layer of the artery wall layer (adventitia, media and intima) and inter-individual variation in macrophage infiltration patterns was observed. M1 macrophages correlated with media destruction and greater expression of ANKRD22 was significantly associated with increased arterial inflammatory infiltration. Greater MRC1 staining was significantly associated with patients who reached 5mg of glucocorticoids sooner.

Identification of all markers within each artery layer suggested different macrophage phenotypes co-exist. Arterial expression of ANKRD22 and MRC1 may identify different groups of patients who require different treatment strategies. Macrophage phenotype heterogeneity and downstream immunological processes may therefore in part explain the variation seen between patients in terms of their clinical presentation, long-term sequelae and response to treatment.

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List of Abbreviations

°C	degrees Centigrade
AECA	Anti-endothelial cell antibodies
ALOX15	Arachidonate 15-Lipoxygenase
ANKRD22	Ankyrin repeat domain 22
APC	Antigen presenting cell
ATLO	Artery tertiary lymphoid organ
BAFF	B-cell activating factor
bp	base pair
BMDM	Bone marrow-derived macrophage
BSA	Bovine serum albumin
BSR	British Society for Rheumatology
C1QA	Complement C1q A chain
CCL	Chemokine ligand
CCR, CXCR	Chemokine receptor
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CMV	Cytomegalovirus
CRP	C-reactive protein
CSF	Colony stimulating factor
CTLA	Cytotoxic T-lymphocyte-associated protein
CXCL	Chemokine (C-X-C) ligand
CXCR	Chemokine (C-X-C) receptor
DAMP	Danger associated molecular pattern
DC	Dendritic cell

DNA	Deoxyribonucleic acid
DPBS	Dulbecco's Phosphate Buffered Saline
DPX	Distyrene/plasticiser/xylene
DTT	Dithiothreitol
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
EEL	External elastic laminae
ESR	Erythrocyte sedimentation rate
ET	Endothelin
FBS	Fetal bovine serum
FFPE	Formalin fixed and paraffin embedded
GBP5	Guanylate binding protein 5
GC	Glucocorticoid
GCA	Giant cell arteritis
gDNA	Genomic deoxyribonucleic acid
GM-CSF	Granulocyte-macrophage colony-stimulating factor
gp	Glycoprotein
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
GWAS	Genome-wide association study
H & E	Haematoxylin and eosin
HIF-1	Hypoxia-inducible factor-1
HLA	Human leukocyte antigen
hMDM	Human monocyte-derived macrophage
HOMER2	Homer scaffolding protein 2
IEL	Internal elastic laminae
IFNɣ	Interferon gamma
IHC	Immunohistochemistry

IL	Interleukin
IL-6R	IL-6 receptor
sIL-6R	Soluble IL-6 receptor
INHBA	Inhibin beta A subunit
iNOS	inducible nitric oxide synthase
IRF	Interferon-regulatory factor
JAK	Janus Kinase
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
M0	Mature macrophage
M1, 2(a,b,c)	Macrophage phenotype 1, 2, 2a, 2b, 2c
MAPK	Mitogen-activated protein kinase
MARCO	Macrophage receptor with collagenous structure
MDM	Monocyte-derived macrophage
MGC	Multinucleated giant cell
MHC	Major histocompatibility complex
MRC1	Mannose receptor C-type 1
MMP	Metalloproteinases
mRNA	Messenger ribonucleic acid
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
nGRE	Negative glucocorticoid response element
NICE	National institute for health and care excellence
NK	Natural killer cell
NO	Nitric oxide
NT	Neurotrophin
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline

PBS-BSA	Phosphate-buffered saline with bovine serum albumin
PCR	Polymerase chain reaction
PD	Programmed death
PD-L	Programmed death-ligand
PDGF	Platelet-derived growth factor
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PMR	Polymyalgia rheumatica
PPAR	Peroxisome proliferator-activated receptor
PRR	Pattern recognition receptor
PTEN	Phosphatase and tensin homolog
REC	Leeds East Research Ethics Committee
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RT	Reverse transcriptase
SAA	Serum amyloid A
SCID	Severe combined immunodeficiency
SLC16A1	Solute carrier family 16 member 1
SEPP1/SELENOP	Selenoprotein P
SERPING1	Serpin family G Member 1
SLE	Systemic lupus erythematosus
SMA	Smooth muscle actin
SOCS	Suppressor of cytokine signalling
ST	Suppression of tumorigenicity
STAT	Signal transducer and activator of transcription

TAE	Tris-Acetate-EDTA
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween
TCR	T-cell receptor
Tfh	T follicular helper cell
TGFβ	Transforming growth factor beta
TGM2	Transglutaminase 2
Th(1, 2, 9, 17, 21)	T helper cell type 1, 2, 9, 17, 21
TIMP	Tissue inhibitors of MMPs
TLR	Toll-like receptor
TMIGD3	Transmembrane and immunoglobulin domain containing 3
TNF	Tumour necrosis factor
TNFAIP6	TNF alpha induced protein 6
Treg	Regulatory T-cell
v/v	volume to volume
VCAM	Vascular cell adhesion molecule
VD_3	1,25-dihydroxyviamin D_3
vDC	Vascular Dendritic cell
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell
w/v	weight to volume

Chapter 1. Introduction

1.1 Giant cell arteritis

Giant cell arteritis (GCA) is a chronic vasculitis which affects large and medium-sized arteries, namely the aorta, the external carotid artery and their branches. The disease is associated with local vessel inflammation which culminates in ischaemia as a result of luminal occlusion, as well as a systemic inflammatory response when mediators are released into vascular circulation. GCA is the most common form of vasculitis worldwide, affecting 1 in 4,500 people in the UK annually (Smeeth *et al.,* 2006), with an increased incidence in women over the ages of 50 years of age and occurring most commonly between ages 75 and 85 (Wang *et al*., 2017). Subjects present with symptoms associated with vessel inflammation and occlusion including headache, scalp tenderness, jaw claudication, and systemic inflammatory response which manifests as weight loss, fever, night sweats and malaise. If left untreated, GCA leads to luminal occlusion of the vessel resulting in ischaemia of downstream organs leading to, for example, vision loss, stroke and jaw claudication.

A classification criterion was published by Hunder *et al.* in 1990 which enabled differentiation of GCA from other forms of vasculitis for research studies, with the presence of 3 or more criteria out of 5 being required to confirm GCA. One of these criteria includes an abnormal artery biopsy, however a “gold standard” diagnosis requires temporal artery biopsy and histological examination to determine a positive or negative diagnosis. A negative diagnosis does not indicate a lack of disease however, due in part to the presence of skip lesions as well as the large heterogeneity that is seen within the histology of patients. This highlights the importance of identifying 3 or more criteria to confirm GCA. Until recently, the treatment of GCA relied on high dose glucocorticoids as the standard treatment for all patients and in approximately two thirds of those patients, glucocorticoids were not able to result in remission, with patients relapsing at least once after follow-up (Kermani *et al*., 2015). From July 2018, Tocilizumab has been licenced by the National Institute for Health and Care Excellence (NICE) for use in patients with resistant and refractory disease.

1.2 Epidemiology of GCA

1.2.1 Ageing

Ageing results in predisposition to various diseases including cancer and autoimmune diseases as well as infections. This is in part due to the ageing of the immune system, resulting in its dysregulation, decreasing its ability to recognise self and maintain homeostasis, preventing the onset of chronic inflammation and autoimmunity (Niccoli and Partridge, 2012). Dysregulation of the immune system in the elderly has been thought to give rise to persistent chronic inflammation, termed “inflammaging” (Sanada *et al.*, 2018). This is a consequence of a lack of antigen-specific responses, resulting in senescence of T-cells and consistent secretion of pro-inflammatory cytokines, including IL-6 (Campisi and d'Adda di Fagagna, 2007). One reason for this altered response in the elderly is a lack of naïve T-cell production within the thymus, described as age-associated thymic involution (Palmer, 2013). This results in a decreased circulating T-cell repertoire, altered T-cell phenotypes with altered functions and altered expression of activatory receptors, such as MHC class II (Moro-García *et al.*, 2013). Furthermore, ageing has also been associated with immunosenescence, where dysregulation of the immune system results in autoimmunity and irregular tissue repair (Shaw *et al.*, 2013). Patients with GCA, however, have been described to lack features of immunosenescence *(*Wen *et al.*, 2017). In GCA, changes to vDC receptor expression and chemokine secretion, preventing homing to lymph nodes has been found to result in the induction of an immune response within the artery wall (Krupa *et al*., 2002). In addition to adaptive and innate immune ageing, changes to vascular tissue in the ageing population has also been described, contributing to the inflammatory response. Changes to the structure of the vessel wall has been found to occur over time, with increased collagen accumulation (Fukushima *et al*., 2005), thinning of the media and increased stiffness of the artery wall (Watanabe *et al.*, 1996), as well as increased secretion of reactive oxygen species (Li and Fukagawa, 2010) contributing to tissue damage.

1.2.2 Genetics

A number of genetic studies have identified an association with a range of immune-related processes as well as endothelial functions. The major histocompatibility complex (MHC) class II region, including leukocyte antigen (HLA)-DRB1 has been found to have a very strong association with the susceptibility of GCA, particularly *HLA-DRB1*04*, in a number of different studies

(Carmona *et al*., 2017; Carmona *et al*., 2015; Mackie *et al*., 2015) as well as visual loss in GCA patients (González-Gay *et al*., 2000). In addition to the MHC locus, a large number of candidate gene studies have reported associations with *IL-10, TNFα, IL-4 and IL-18* genes (Palomino-Morales *et al*., 2010; Amoli *et al*., 2003; Boiardi *et al*., 2006; Mattey *et al*., 2000), however, these have not been validated in recent large scale genetic studies performed on both the Immunochip (Carmona *et al.*, 2015) and genome wide platform (Carmona *et al.*, 2017). A number of non-HLA associated genes have been identified in these two studies which have been linked to genes involved in T cell function (Carmona *et al*., 2015) as well as neoangiogenesis and tissue remodelling, such as *VEGF* and *MMP-9* (Carmona *et al.*, 2017) which may have an impact on the progression of GCA. Indeed, these studies have known limitations, such as the low number of patients involved in the studies and therefore the power to identify gene variants, as well as the restriction of the geographical location of recruited patients to European countries and the resultant lack of genetic diversity. Furthermore, the contribution of genetic variants, epigenetics and environmental factors to the risk of developing GCA and of which the human immune system is highly susceptible to (Brodin *et al*., 2015), may be overlooked.

1.2.3 Gender

As GCA is more commonly seen in women, with an increased incidence and prevalence of between 2- and 4-fold, a link with GCA and gender has been suggested (Nordborg and Bengtsson B, 2009), with women also exhibiting an increased inflammatory response within the artery (Narvaez *et al*., 2002), the mechanism of which is unknown. As age increases, however, the bias seen in women decreases (Smeeth *et al.,* 2006).

1.2.4 Environment

Variations in the season, with a peak onset of GCA identified in May and June, have been associated with the onset of GCA and have been attributed to an infectious trigger (Bas-Lando *et al*., 2007; Salvarani *et al*., 1995; Elling *et al*., 1996). Elling *et al.* (1996) analysed patient data across Denmark over a 12-year period and identified simultaneous incidence peaks across the country, with additional close occurrence of GCA with epidemics of various viral infections. However, other studies, although they found a trend for a monthly peak in incidence during the summer months, did not find a statistically significant trend in seasonal onset (Gonzalez-Gay *et al*., 2001; Narváez *et al*., 2000; De Smit *et al*., 2017; Kisza *et al*., 2013). Differences seen may be due to the number of

cases that were analysed and the length of the period over which these cases were taken. Furthermore, more recent studies have followed the ACR 1990 criteria for inclusion of cases, whereas older studies were found to include cases of polymyalgia rheumatic (PMR) (Brekke *et al*., 2017) which may have implications in their findings.

Additionally, geographical location has been suggested as having an impact on GCA incidence. The incidence of GCA has been found to be greater in northern Europe, especially within the Scandinavian population (Baldursson *et al*., 1994; Brekke *et al*., 2017). The increased incidence of GCA during the early summer months and in populations with reduce sun exposure, may implicate exposure to sunlight and levels of vitamin D to GCA onset. Nevertheless, migration of Scandinavian individuals to Minnesota, USA, did not alter incidence of GCA within this population (Chandran *et al*., 2015), suggesting a greater genetic component contributing to the incidence of GCA in Scandinavian patients than an environmental component. Additionally, Mackie *et al*. (2011) identified socio-economic deprivation as a correlating factor for ischaemic complications, whilst Smeeth *et al.* (2006) showed those living in southern areas of the UK had an increased prevalence for GCA. This, however, may be in result of under-diagnosis in other areas of the UK. Furthermore, worldwide studies found those within the northern hemisphere were more likely to develop GCA (Gonzalez-Gay *et al.,* 2001).

1.3 Pathogenesis of GCA

Advances have been made in understanding the pathogenesis of GCA however, the mechanisms resulting in the onset and progression of GCA are still largely unknown.

Pathogenesis of GCA is a multistage process with DCs, T-cells and macrophages being the main drivers of both the destructive and healing phases of the disease. The initial trigger of GCA remains elusive, however, based on evidence that DCs control tolerance and prevent autoimmunity, it is thought that immune-privilege is broken in result of DC activation within the arterial wall, triggering an immune response that gives rise to an influx of inflammatory cells, tissue destruction and excess wound healing (Han *et al.*, 2008). Ultimately this causes systemic inflammation, intimal hyperplasia, luminal occlusion and clinical manifestations associated with the disease. A schematic of the process can be seen in Figure 1.1. Both autoimmune components and components of the innate immune system contribute to the cause and development of GCA, including various immune and vascular cells, and cellular dysregulation. Furthermore, susceptibility and severity factors including age, gender, genetics and the environment may play a role in the development of the disease.

1.3.1 Immunopathology

1.3.1.1 Dendritic cells

DCs are antigen presenting cells (APCs) which play an important role within the innate immune response. They express an array of pattern recognition receptors (PRRs), including toll-like receptors (TLRs), which makes them excellent patrolling and antigen-sensing cells. DCs are able to initiate an immune response through the recognition of danger signals, the engulfing of foreign bodies and presentation of antigen to T-cells, and the secretion of a range of cytokines and chemokines.

Vessel specific DCs (vDCs) are positioned at the adventitial-media border of temporal arteries in healthy subjects and display an immature phenotype. They are believed to carry out a sentinel role in this immune privileged site, preventing the activation of T-cells. Interestingly, vDCs found in different vascular areas express different TLR repertoires, suggesting each region has specialised to detect specific danger signals, subsequently leaving these regions susceptible to invading pathogens which they are not specialised to detect (Pryshchep *et al.*, 2008).

In GCA, vDCs become activated, as evident by their expression of CD83, enabling these cells to initiate an inflammatory response by generation of chemokines gradients, such as C motif ligand (CCL)19, CCL20 and CCL21 which then recruits T-cells via the vasa vasorum vascular network, found in the adventitia. It has been shown that these vDCs, due to their expression of the chemokine receptor C motif receptor (CCR)7 and their secretion of CCL19 and CCL21 (which are ligands for CCR7), remain trapped within the artery wall (Krupa *et al.*, 2002). These trapped vDCs instead of migrating towards lymph nodes to prime T-cells, initiate T-cell recruitment and activation within the artery wall leading to an adaptive immune response and granuloma formation at the adventitial-media border (Krupa *et al.*, 2002). vDCs are also thought to induce different patterns of arterial wall inflammation depending on the initial danger signal they encounter. Using human temporal artery-severe combined immunodeficiency (SCID) mouse chimeras, Deng *et al*. (2010) identified vDCs which, when they encountered TLR4 ligands, resulted in the recruitment of specific CCR6+ Th17 cells via upregulation and secretion of CCL20, leading to a pan-arteritis pattern of inflammation. In contrast, TLR5 ligands were found to recruit T-cells which localised to the adventitia only.

The removal of vDCs from the arterial wall of GCA affected arteries in a SCID mouse chimera model has been shown to abolish vascular inflammation (Ma-Krupa *et al*., 2004) which provides evidence for their fundamental role in initiating and propagating the disease process.

1.3.1.2 CD4+ T-cells

CD4+ T-cells are professional antigen recognition cells, that identify specific antigens which causes their activation and differentiation into various effector T-cell subsets, including T helper (Th)1, Th2, Th17, Th9 and regulatory T-cells (Treg), all of which express different cytokine profiles and carry out various functions. T-cells are recruited to the adventitia by vDCs and are activated by vDCs through the presentation of antigen via HLA class II molecules to the T-cell receptor (TCR) on T-cells, along with binding of the co-stimulatory receptor CD86, which is only expressed on a subset of activated vDC cells within the artery lesion, resulting in T-cell priming (Han *et al.,* 2008; Ma-Krupa *et al.*, 2004). Concurrent stimulation with different cytokines secreted by vDCs, results in the differentiation of T-cells towards different inflammation states.

T-cells are believed to be the main drivers of inflammation within the arterial wall in GCA; their depletion in mouse chimera models of GCA results in the complete abrogation of inflammation in the transplanted human artery tissue (Brack *et al.*,

1997). T-cells from left and right temporal arteries have also been found to be clonally expanded, expressing the same TCRs (Weyand *et al.*, 1994a), which suggests a common antigen that induces vDC activation and is recognised by these selective T-cells. The formation of granulomas in the artery wall (which is commonly seen in diseases triggered by pathogens) (Saunders and Cooper, 2000) suggests an infectious agent or autoantigen, such as elastin, may be the trigger. Although a range of antigens have been suggested, including autoantigens, viruses and other infectious agents (Gilden *et al.*, 2016; Schmits *et al.*, 2002) these results have not been confirmed by other groups (Rhee *et al*., 2017; Muratore *et al.*, 2017). More recent work carried out by Watanabe *et al.* (2017) suggests the initiation and progression of GCA is driven by defects in various immune checkpoints and subsequent cell recruitment and therefore GCA may not be an antigen-specific disease (discussed in more detailed in Section 1.3.3).

CD4+ T-cells are the most common T-cell subtype within the GCA arterial wall (Deng *et al*., 2010) as well as being elevated within the blood (Terrier *et al.*, 2012). CD4+ T-cells are most commonly found as Th1 and Th17 subsets, and represent two arms of the disease, where Th1 cells promote chronic inflammation and Th17 cells promote the acute inflammatory response (Deng *et al.*, 2010), however, other subsets have also been identified, albeit in much lower numbers.

1.3.1.2.1 Th1 cells

Th1 cells are differentiated from CD4+ T-cells through stimulation with cytokines interferon (IFN)γ and interleukin (IL)-12 and are involved in the inflammatory response against invading pathogens. Differentiation is regulated by the Th1-specific transcription factor, T-bet (Szabo *et al*., 2000). In GCA, recruited T-cells are thought to be activated by vDCs, differentiating into Th1 cells which secrete pro-inflammatory cytokines IFNγ and IL-12 within the adventitia (Wagner *et al*., 1996; Terrier *et al*., 2012; Weyand *et al*., 1994b; Weyand *et al.*, 1997; Deng *et al*., 2010).This is thought to promote the recruitment of further CD4+ T-cells and their differentiation towards a Th1 subset, as well as recruiting circulating monocytes (Weyand *et al*., 2011). IFNγ produced by Th1 cells has a multitude of functions, including the maturation, activation and differentiation of macrophages towards an inflammatory phenotype, as well as activating other cell types such as endothelial cells and dendritic cells (Deng *et al.*, 2010).

Th1 cells are able to migrate through all layers of the artery wall, forming granulomas and contributing to the tissue destruction of vessel wall structures across all layers of the vessel wall. They are found in increased numbers in artery

biopsies as well as within the circulation of GCA patients when compared to healthy controls (Terrier *et al.*, 2012). Th1 cells belong to one of two arms of the inflammatory response in GCA, producing cytokines and other mediators that promote chronic inflammation. Interestingly, this arm of the disease is resistant to glucocorticoid treatment, with levels of IFNγ+ Th1 cells, and their associated cytokines remaining elevated after high dose glucocorticoid treatment regimens (Deng *et al.*, 2010). Samson *et al.* (2012), however, described a significant decrease in the circulating levels of $IFN\gamma^+$ Th1 cells after glucocorticoid treatment, yet this combined both GCA and PMR patients, where PMR patients show no T cell infiltration and a lack of IFNγ mRNA in temporal arteries (Weyand and Goronzy, 2003; Weyand *et al*., 1994b). Differences in the dose of glucocorticoid may have implications on the results of these studies. Furthermore, the low numbers recruited to both these studies, (biopsy pre-treatment, n=26 and biopsy post-treatment, n=23 in the Deng *et al.* (2010) study) and n=22 in the Samson *et al.* (2012) study, impact the power of these results.

1.3.1.2.2 Th2 cells

The cytokine IL-4 is required for the differentiation of CD4+ T-cells into a Th2 cell subset, via induction of the Th2 specific transcription factor, GATA-3, which stimulates downstream regulation of Th2 cytokines IL-4, IL-13 and IL-15 (Zheng and Flavell, 1997). Th2 cells are implicated in the humoral immune response fighting bacterial and parasitic infections as well as promoting allergic responses. Th2 cells also play a role in suppressing inflammation by inhibiting the pro-inflammatory cytokine release of Th1 cells as well as Th1 differentiation, via IL-10 secretion (Fiorentino *et al.*, 1989). In GCA, IL-4 has been described as being consistently absent within the arterial wall at the mRNA level (Weyand *et al*., 1994b; Weyand *et a*l., 1997). Although these studies are old, more recent studies into mRNA expression of cytokines within the inflamed artery wall have corroborated these findings (Watanabe *et al*., 2017; Ciccia *et al.*, 2015; Ciccia *et al.*, 2013; Terrier *et al*., 2012). However, despite Ciccia *et al*. (2015) detecting no mRNA expression of IL-4 within the artery wall, in the same study they found overexpression of IL-4 in the same temporal artery biopsies, using an immunohistochemical approach. To my knowledge, this is the only immunohistochemical study into IL-4 expression within GCA artery biopsies. As mRNA expression levels do not necessarily predict the level of protein (Maier *et al.*, 2009), this may suggest IL-4 is found within GCA arteries, albeit, only at the protein level. Further immunohistochemical studies are required to confirm IL-4

expression at the protein level. Despite these findings, however, no Th2 cells have been found in GCA arteries.

1.3.1.2.3 Th17 cells

Th17 cells are another subset of T-cells which protect against fungal and bacterial infections, as well as covering gaps in protection not provided by Th1 and Th2 cells (Korn *et al*., 2009). Th17 cells express an IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-26, and IL-8 cytokine profile (Liang *et al*., 2006; Wilson *et al*., 2007), cytokines that can promote an inflammatory reaction as well as cell proliferation and cell survival (Fouser *et al*., 2008). Furthermore, similarly to Th1 and Th2 cells, Th17 cells require the lineage specific transcription factor, RORγt, for their differentiation (Ivanov *et al.*, 2006). Th17 cells which recognise autoantigens can induce severe inflammatory responses and failure to control these Th17 cells and their highly pathogenic functions results in autoimmune disease (Tabarkiewicz *et al*., 2015). Th17 cells have been implicated in numerous autoimmune diseases, including rheumatoid arthritis, psoriasis and systemic lupus erythematosus, with increased levels of IL-17 found in the serum and blood of patients with these diseases (Shah *et al.*, 2010; Rosu *et al.*, 2012; Kagami *et al*., 2010).

Like Th1 cells in GCA, Th17 cells are also polarised from naïve T-cells by vDCs, however, differentiation requires stimulation with IL-1β and IL-6. Concurrent exposure to IL-23 and/or IL-6 are thought to enhance the effect of Th17 cell differentiation by IL-1β (Acosta-Rodriguez *et al*., 2007). Th17 cells belong to the other arm of the inflammatory response in GCA, contributing to the acute phase of the disease. Th17 cell numbers are increased when compared to healthy controls with their cytokine, IL-17, being found in high quantities within the blood, serum and within the temporal artery wall (Deng *et al*., 2010; Samson *et al*., 2012). In contrast to Th1 cells, Th17 cells have been found to be highly sensitive to glucocorticoids, with Th17 numbers and levels of IL-17 returning to healthy control levels after treatment (Deng *et al.*, 2010). The lack of subset-specific transcription-factors to define each T cell in this study, however, decreases the validity of this study, as $CD8^+$ T cells are also known to secrete IFNγ and IL-17 (Srenathan *et al*., 2016). Furthermore, as mentioned previously, the low number of patients recruited impacts the results of this study.

Th17 cells have a multitude of effects on a wide range of cells and tissues in GCA due to the extensive expression of receptors for its comprehensive cytokine profile. Th17 derived cytokines IL-17, IL-6, IL-1β, TGF-β and IL-21 have been identified within the artery wall. These cytokines have been shown to stimulate the induction of pro-inflammatory cytokines and chemokines, as well as

destructive mediators such as metalloproteinases, from a range of different cell types, including immune cells and tissue, such as endothelial cells, fibroblasts and VSMCs (Terrier *et al*., 2012; Unizony *et al*., 2012; Weyand *et al*., 2011).

Both Th1 and Th17 represent two different immune responses in GCA and their effector functions result in distinct patterns of disease, both locally within the affected artery wall and circulation.

1.3.1.2.4 Treg cells

In contrast to Th17 cells, differentiation of Tregs only requires exposure to TGF-β. Tregs are responsible for carrying out anti-inflammatory functions, curtailing pro-inflammatory T-cell responses through their secretion of cytokines TGF-β and IL-10 thereby preventing chronic inflammation, as well as preventing autoimmune responses through the induction of tolerance. There is evidence to suggest, from *in vitro* stimulation of human T-cells and *in vivo* mouse models, that Treg and Th17 cells can switch polarisation states via exposure to different stimuli (Koenen *et al*., 2008; Osorio *et al*., 2008; Gagliani *et al*., 2015). The shared requirement of TGF-β for their differentiation has implicated this cytokine in their ability to switch phenotypes (Bettelli *et al*., 2006; Gagliani *et al*., 2015) along with other phenotypes, such as Th2 and Th9 cells (Veldhoen *et al*., 2008). These studies however do not represent *in vivo* disease conditions, where a complex microenvironment may have different effects on Treg and Th17 polarisation.

In GCA, the increase in Th17 polarising cytokines within the artery wall, namely IL-6, suggests an inability of naïve T-cells to differentiate into Tregs but rather the environment favours differentiation towards a Th17 phenotype, resulting in a Th17/Treg imbalance, which is commonly seen in autoimmune diseases, such as rheumatoid arthritis (Wang *et al*., 2012). In comparison to healthy controls, numbers of Treg cells in patients with GCA are decreased within the circulation (Samson *et al*., 2012) suggesting a defect in their regulation in these individuals and an imbalance in T-cell homeostasis, which contributes to autoimmunity and the prolonged inflammatory response seen in GCA patients.

1.3.1.2.5 Th9 cells

Recently it has been reported that the newly identified Th9 subset is found within temporal artery biopsies from GCA patients (Ciccia *et al*., 2015). Due to their novelty, the functions of Th9 cells are largely unknown, but they have been observed in a number of inflammatory diseases and have been found to secrete Th2 associated cytokines, including IL-4, albeit in low amounts. Th9 cells require TGF-β and IL-4 for their differentiation but a lineage-specific transcription factor

has yet to be elucidated (Veldhoen *et al.*, 2008) therefore it has been suggested that Th9 cells represent a specialised Th2 subset rather than a separate T-cell lineage. Their associated cytokine, IL-9, has been found to be increased in the circulation of GCA patients, with the expression of IL-9 in arteries found to be accompanied with overexpression of Th9 polarising cytokines IL-4 and TGF-β, at the protein level. This suggests differentiation of Th9 cells occurs within the artery wall. Additionally, numbers of Th9 cells have been found to only slightly decrease in response to glucocorticoids, suggesting a role in the chronic pro-inflammatory response (Ciccia *et al*., 2015). Ciccia *et al*. (2015) also observed that varying ratios of Th17 and Th9 cells were associated with different histological patterns of inflammation within the temporal artery, therefore differently polarised T-cells may drive different inflammatory pathways within GCA. In a mouse model of colitis, Th9 cells were found to produce IL-17 (Dardalhon *et al.*, 2008), whilst in an adoptive transfer model of experimental autoimmune encephalomyelitis (EAE), Th9 cells were found to produce IFNγ (Jäger *et al*., 2009), suggesting they have the ability to switch their phenotype depending on the microenvironment they encounter. Furthermore, Th1, Th9 and Th17 cells in EAE were found to induce different disease phenotypes, identifying different roles in disease progression (Jäger *et al*., 2009). The separate transfer of *in vitro* differentiated T-cells, however, provides only a simplistic view of the complex conditions found *in vivo.* Evidence from these disease models suggests differences in the microenvironment between GCA patients could be a contributing factor to the switching of Th9 cells into different T-cell subsets, and subsequently, the heterogeneity that is seen in the patterns of inflammation between patients.

1.3.1.2.6 T follicular helper cells

Increased amounts of IL-21 producing T-cells have been identified both in the circulation and temporal arteries of GCA patients, with levels of IL-21 correlating with disease activity (Terrier *et al*., 2012). IL-21 is produced almost exclusively by follicular T-cells (Tfh) but can also be secreted by Th17 cells. Recent observation of artery tertiary lymphoid organs (ATLOs) within the media of biopsy positive arteries and accompanied protein expression of the Tfh molecule Chemokine (C-X-C) receptor (CXCR)5 (Ciccia *et al*., 2017), may suggest Tfh cells are derived from these structures and play a role in B-cell development. The findings by Ciccia *et al.* (2017) have not been corroborated by other groups (van der Geest *et al*., 2014; Cid *et al.*, 1989; Graver *et al*., 2018), as B-cells have only been found within the adventitia and not the media. Tfh cells are also found to secrete IL-4 (Ma *et al.*, 2009) and therefore may be the cell type which secretes

IL-4 within the artery wall. An important role of IL-21 in GCA, along with IL-6, has been identified as causing a decrease in Treg activation with preferential stimulation of naïve CD4+ T-cells towards Th1 and Th17 subsets. Blocking of IL-21 receptors resulted in decreased numbers of both these T-cell phenotypes (Terrier *et al.*, 2012).

1.3.1.3 CD8+ T-cells

CD8+ T-cells are also known as cytotoxic T-cells and are activated when presented with antigen to their MHC class I receptor, along with exposure to IL-12 (Pearce and Shen, 2007). They play a role in the removal of pathogens through their release of pro-inflammatory cytokines IFNγ and tumour necrosis factor (TNF), their secretion of cytotoxic granules causing cell membrane rupture of infected cells (de Saint Basile *et al.*, 2010; Kagi *et al*., 1994) as well as their ability to induce apoptosis via their expression of FasL (Kagi *et al*., 1994). Furthermore, they are also thought to provide a regulatory role, secreting the immunosuppressive cytokine IL-10, to prevent tissue destruction (Palmer *et al*., 2010).

Studies into CD8+ T-cells in GCA have long suggested their numbers are decreased in the circulation compared to healthy controls (Macchioni *et al*., 1993; Dasgupta *et al.*, 1989; Benlahrache *et al*., 1983). A more recent study carried out by Samson *et al*. (2016) however, found that CXCR3 expressing CD8+ T-cells are increased in number in GCA patients. When compared to controls, however, overall numbers of CD8+ T-cells were still found to be much lower in GCA arteries compared to CD4+ T-cells. The role of CD8+ T-cells in GCA has been linked to remodelling of the vascular wall and recruitment of further CD4+ and CD8+ T-cells, due to their secretion of IFNγ and IL-17, along with CXCL9, CXCL10 and CXCL11. Furthermore, increased amounts of these CD8+ cells have been associated with an increase in disease severity due to a correlation with increased visual loss (Samson *et al.,* 2016). It was also found that Th1 cells recruit CD8+ T-cells in GCA via their secretion of CXCR3 ligands CXCL9, CXCL10 and CXCL11 (Samson *et al.*, 2016). Additionally, IL-21, secreted by Th17 cells, has been linked to increased differentiation of CD8+ T-cells (Sutherland *et al.*, 2013). Furthermore, like CD4+ T-cells, CD8+ T-cells have been found to be oligoclonal in GCA arteries (Martinez-Taboada *et al.*, 1996) which may be due to their protective role against viral infections. The concept of age-induced oligoclonality, however, cannot be ignored as CD8+ T-cells have been found to be persistently clonally expanded against cytomegalovirus (CMV) in the elderly (Vescovini *et al.*, 2004; Almanzar *et al*., 2005). A study into CMV

transcript levels in artery biopsies, using a clinically validated qPCR method, detected CMV in 1 out of 35 cases (Cankovic and Zarbo, 2006). This provides evidence to suggest that recruitment of CD8+ T-cells to the artery wall is not induced by CMV, however, other pathogens, yet to be detected, could cause this oligoclonality. The treatment of patients with glucocorticoids was found to significantly decrease CD8+ T-cell numbers within the circulation and in temporal artery biopsies when compared to untreated patients (Samson *et al*., 2016).

1.3.1.4 Multinucleated Giant cells

Multinucleated giant cells (MGCs) are formed through the fusion of neighbouring macrophages, forming large, highly activated, cells. Although GCA is named after MGCs, MGCs are not always found in GCA cases (Rodriguez-Pla *et al.*, 2005), yet despite this, the presence of giant cells has been associated with clinical features such as jaw claudication and intimal thickening (Ting *et al*., 2016). MGCs are found spread along the IEL, at sites of fragmentation, and have been observed to produce tissue damaging mediators, such as metalloproteinases, which are thought to contribute to the destruction of the media and IEL (Rodriguez-Pla *et al*., 2005). MGCs are the main producer of vascular endothelial growth factor (VEGF) in GCA, promoting further infiltration of recruited immune cells into the artery wall, as well as secreting the growth factor, platelet derived growth factor (PDGF), which has been associated with intimal hyperplasia (Rittner *et al.*, 1999b). It could be implied, due to their location at sites of fragmented IEL, that MGCs promote the migration of inflammatory and stromal cells towards the intima, supporting intimal hyperplasia. The contributing factors that lead to the formation of MGCs in GCA are still to be determined; the ability of MGCs to release a variety of cytokines within the artery allude to MGCs which may be polarised into different phenotypes, similarly to macrophages, and may be a result of different macrophage phenotypes fusing together.

1.3.1.5 Other immune cells

Neutrophils have been identified within GCA positive temporal artery biopsies which display a pattern of inflammatory pan-arteritis (Ciccia *et al.*, 2015), with this increased number of neutrophils found to be linked to the expression of IL-8 (Ciccia *et al*., 2015; Nadkarni *et al*., 2014). Th17 specific recruitment of neutrophils via secretion of IL-17 (Camporeale and Poli, 2012) and vDCs recruitment of CCR6+ Th17 cells results in a transmural-specific pattern of inflammation (Deng *et al.*, 2009). This provides further evidence to suggest that different patterns of inflammation in GCA occur in response to different immune

response mechanisms and their associated downstream signalling pathways. Neutrophils have also been implicated in relapse. Nadkarni *et al.* (2014) identified changes in neutrophil phenotypes, isolated from blood samples of GCA patients over a 24-week longitudinal study. Circulating neutrophils were observed to express a suppressive phenotype in response to high-dose glucocorticoid treatment, yet, in response to glucocorticoid tapering, neutrophils were observed to show an inability to suppress T-cell proliferation and displayed a more activated phenotype. A significant increase in neutrophil count was also observed between GCA patients and healthy controls.

Very small numbers of other cells, including B-cells, mast cells, natural killer (NK) cells and eosinophils are found in temporal artery biopsies in GCA. Within GCA artery biopsies, B-cells have been found in very low quantities within the adventitia (Lavignac *et al*., 1996), with a more recent study identifying IL-6 secreting B-cells within the artery wall and within the circulation (van der Geest *et al*., 2014). The recent observation of ALTOs in the media of GCA positive artery biopsies, along with the expression of B-cell differentiation and proliferating factors, such as B-cell activating factor (BAFF) (Ciccia *et al.*, 2017) and a role in IL-6 secretion, suggests B-cells may play a more important role in the development of GCA than previously thought. Furthermore, treatment with the B-cell depleting anti-CD20 monoclonal antibody, Rituximab, has been found to resolve GCA symptoms in 2 patients (Bhatia *et al*., 2005; Mayrbaeurl *et al*., 2007). However, as explained in Section 1.3.1.2.6, no other studies have corroborated the findings of Ciccia *et al.* (2017). Furthermore, B-cell numbers have been inversely correlated with markers of systemic inflammation, C-reactive protein and erythrocyte sedimentation rate (van der Geest *et al*., 2014).

Mast cells are found in low amounts in biopsies from GCA patients (Cavazza *et al*., 2014; Banks *et al.*, 1983). An immunohistological study of 18 biopsies identified mast cells to express tryptase, cathepsin and VEGF in GCA biopsies and were associated with neovessels within the intima, suggesting mast cells may contribute to angiogenesis (Mayranpaa *et al.*, 2008); this confirms findings in a GWAS study which associated *VEGF* with neoangiogenesis (Carmona *et al*., 2017). The low number of biopsies examined however limits the power of this study. Additionally, mast cells are thought to interact with other immune and vessel wall cells, helping to control their functions. Very low numbers of NK cells have also been found in GCA artery lesions (Cid *et al*., 1989).

The most abundant cell types within GCA lesions are the innate immune cell, macrophages which carry out fundamental roles in the pathogenesis of the disease. The biology of macrophages is described in detail in Section 1.7.

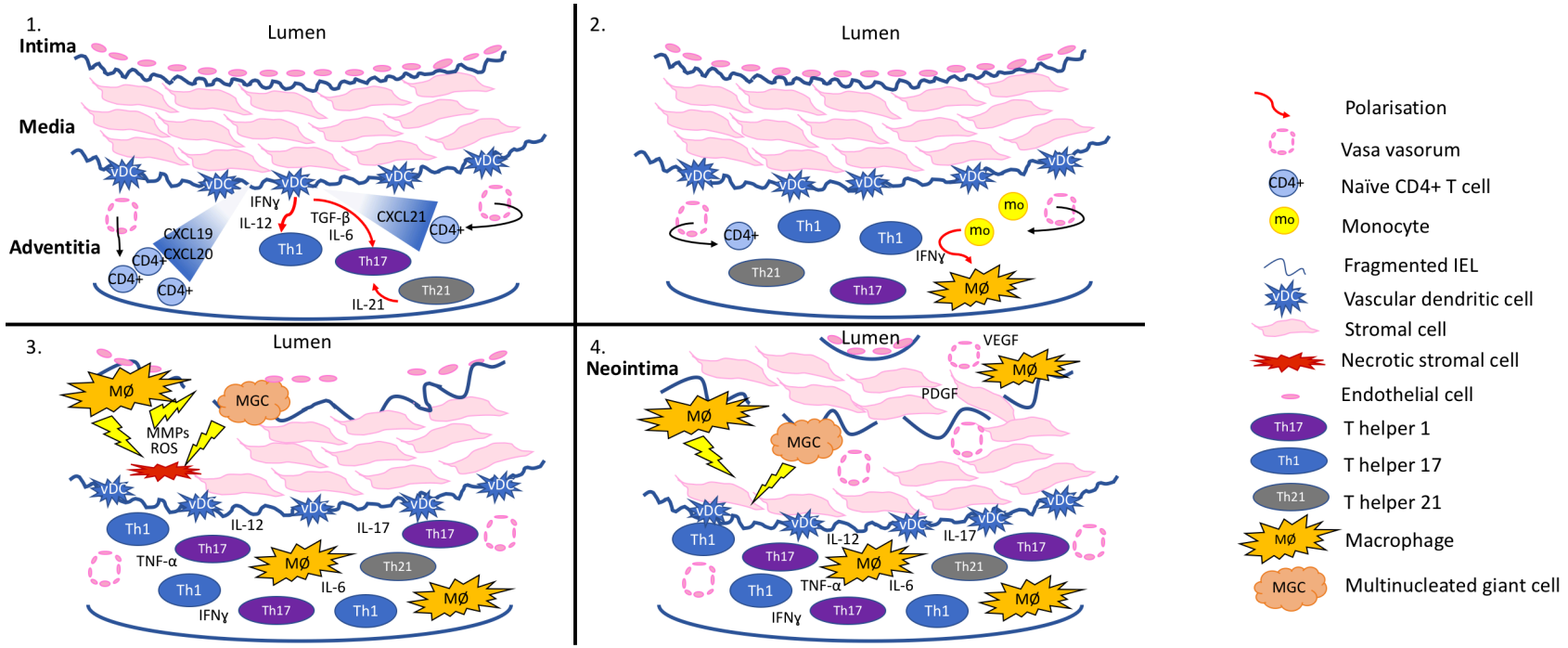


Figure 1.1: Schematic to show the hypothesised phases of GCA development

Stage 1: Activated vDCs at the adventitia-media border recruit naïve CD4+ T-cells via the adventitial vasa vasorum through secretion of chemokines CXCL19, -20 and -21, inducing their differentiation into Th1 cells via IFNγ and IL-12 or Th17 cells via TGF-β and IL-6. Th21 secrete IL-21, contributing to Th17 polarisation. **Stage 2**: Th1 and Th17 cells recruit further naïve T-cells and monocytes. Pro-inflammatory macrophages (M1) are polarised from monocytes via stimulation with IFNγ released from Th1 cells. **Stage 3**: The adventitia is highly inflammatory with high concentrations of IL-12, IL-6, IL-17, TNF-α and IFNγ. Macrophages migrate into the media where multinucleated giant cells are formed through macrophage fusion, both promoting stromal cell damage, media thinning, as well as internal elastic lamina fragmentation, through release of destructive molecules metalloproteinases and reactive oxygen species. **Stage 4**: Anti-inflammatory macrophages (M2) move into the intima, promoting vascularisation, stromal cell migration and proliferation, via release of vascular endothelial growth factor and platelet-derived growth factor, resulting in luminal occlusion. CXCL, chemokine (C-X-C) ligand; IFNγ, interferon gamma; IL, interleukin; TGF-β, transforming growth factor-beta; TNF-α, tumour necrosis factor-alpha.

1.3.2 Vascular pathology

The interactions between immune cells and stroma, such as immune cell activation of stromal cells and stromal cell polarisation of immune cells, are fundamental to the progression of GCA. Understanding how immune and vessel wall cells regulate each other and how this contributes to disease pathogenesis is crucial to identifying alternative drug targets.

1.3.2.1 Endothelial cells

Endothelial cells within the artery wall behave differently in relation to their location. An unknown number of endothelial cell types can be found within the vessel wall, for example, lining the lumen of the temporal artery, lining the vasa vasorum within the adventitia, and lining the neovessels within the media and intima (Watanabe *et al.*, 2017). Endothelial cells therefore contribute to the supply of oxygen and nutrients within the different artery layers via their contribution to their formation into vasa vasorum. They also contribute to the promotion of inflammation through the recruitment of inflammatory cells via upregulation of inducible endothelial adhesion molecules, such as E-selectin and vascular endothelial adhesion molecule (VCAM)-1, both of which have been correlated with systemic inflammation (Cid *et al*., 2000). Endothelial cells are activated by IL-6, and the expression of Endothelin (ET)-1 in GCA arteries implies endothelial cells are in an activated state (Dimitrijevic *et al*., 2010). Furthermore, endothelial cells lining neovessels have been found to secrete M2 macrophage polarising cytokine IL-33 and the pro-inflammatory cytokine IL-32 (Ciccia *et al.*, 2011; Ciccia *et al*., 2013).

Like other autoimmune and vasculitic diseases, studies into autoantibodies in GCA have identified anti-endothelial cell antibodies (AECA) within patient serum (Régent *et al*., 2011; Navarro *et al*., 1997). These antibodies belong to a family of autoantibodies which detect different antigens in different diseases, resulting in different functional outcomes (Bordron *et al*., 2001). AECA have been observed to induce pro-inflammatory molecules, such as secretion of IL-6 and upregulation of adhesion molecules E-selectin and VCAM-1 on endothelial cells in systemic lupus erythematosus (SLE) (Holmen *et al.*, 2007; Del Papa *et al*., 1999). This suggests that AECA found in GCA may also induce a pro-inflammatory phenotype in endothelial cells which promote inflammation. Furthermore, AECA may also induce apoptosis of stromal cells, as observed in SLE (van Paassen *et al*., 2007).

1.3.2.2 Vascular smooth muscle cells

Autoantibodies directed at antigens expressed by vascular smooth muscle cells (VSMCs) have also been detected in the serum of GCA patients (Régent *et al.*, 2011), however this is the only study to investigate anti-VSMC in GCA. Both AECA and anti-VSMC antibodies were found to recognise Grb2 (Régent *et al,* 2011), a protein associated with VSMC proliferation and the development of the neointima in response to injury, in a mouse model (Zhang *et al*., 2003). Grb2 has been suggested to be overexpressed by VSMC and endothelial cells in the wall of GCA arteries (Régent *et al.,* 2011).

VSMCs in GCA are thought to be activated after stimulation with ET-1 secreted by T-cells and macrophages, initiating their migration into the intima of the artery wall. Activation also results in metalloproteinase (MMP)-2 secretion by VSMCs, which may cause destruction of the IEL enabling migration into the intima (Planas-Rigol *et al*., 2017).

The growth factor, neurotrophin (NT), has also been shown to play a role in VSMC migration within GCA artery biopsies. Along with PDGF and TGF-β, the identification of NT in VSMC migration suggests the artery wall microenvironment provides a multitude of ways in which VSMC can contribute to the hyperplasia of the intima (Ly *et al*., 2014). The small number of cases examined in this study (n=22), limits the findings of this study.

New insights into immune dysfunction in GCA have identified dysfunctional NOTCH and PD-1 signalling pathways in endothelial cells and VSMCs within the inflamed artery wall (Zhang *et al*., 2017; Piggott *et al*., 2011; Wen *et al.*, 2017). This has implicated both endothelial and VSMCs in the breaking of immune-privilege of the artery wall. The role of these stromal cells and signalling pathways in GCA are described in more detail in Section 1.3.3.

1.3.2.2.1 Myofibroblasts

Myofibroblasts within the intima are thought to derive from VSMCs after exposure to growth factors secreted by mononuclear cells and injured VSMCs. Differentiation of VSMCs into myofibroblasts was also found to be induced via ET-1 (Planas-Rigol *et al*., 2017) while the blockade of the ET-1 receptor was found to inhibit the proliferation of VSMCs (Régent *et al.*, 2017). Secretion of PDGF by macrophages promotes VSMC proliferation while ET-1 induces their migration towards the intima (Planas-Rigol *et al.*, 2017). This proliferation results in an enlarged intima which secretes a range of extracellular matrix proteins, causing remodelling of the vessel wall, resulting in hyperplasia and luminal

occlusion (Lozano *et al*., 2008). At the mRNA level, these cells have been found to express pro-inflammatory chemokines, such as CXCL13 and CCL21, which are upregulated through stimulation with pro-inflammatory cytokines including IL-17 and IL-6. These chemokines are involved in lymphoneogenesis and are also chemoattractants for B-cells, suggesting interaction of B-cells and myofibroblasts within the artery wall (Ciccia *et al*., 2017).

1.3.3 Immune dysfunction

Dysfunction of the immune system has been associated with age-related changes to the immune response (as explained in more detail in Section 1.2.1). Immune checkpoint molecules are required to control immune cell activation, autoimmunity, and tissue destruction via co-stimulatory or co-inhibitory signals to T-cells (Watanabe *et al.,* 2017). In GCA, dysregulation of immune checkpoints, an antigen-nonspecific immune regulatory mechanism, has recently been described, resulting in immune cell migration into the immune-privileged site and an over-reactive immune response. The dysregulated immune checkpoints are described in more detail below.

1.3.3.1 NOTCH pathway

The NOTCH pathway is an antigen-nonspecific mechanism that has been associated with stromal-stromal and stromal-immune cell interactions, via upregulation of NOTCH receptors and ligands. The pathway has been implicated in VSMC differentiation as well as endothelial cell activation and function, including the formation of neovessels (Boucher *et al.*, 2012). Furthermore, NOTCH is implicated in the differentiation of T-cells into various subsets, including Th1, Th12, Th17 and Th9 cells (Amsen *et al*., 2015).

The NOTCH ligand, Jagged1, has been found to be expressed in GCA patients by endothelial cells lining the vasa vasorum within the adventitia but found to be absent in healthy controls when investigated in an immunohistochemistry study (Wen *et al.,* 2017). Furthermore Wen *et al.* (2017) identified that over-expression of circulating VEGF induced expressed of Jagged1 on endothelial cells in an *in vitro* model while in an *in vivo* human artery-SCID chimera model, VEGF was found to exacerbate disease in GCA diseased arteries (Wen *et al.,* 2017). Previous studies have identified a 20-fold increase in $CD4^+$ T-cell expression of the NOTCH receptor, Notch1, in GCA patients compared to controls (Piggott *et al.*, 2011). Wen *et al.* (2017) using GCA serum in an *in vitro* endothelial monolayer-$CD4^+$ T-cell co-culture system, identified Notch1 on $CD4^+$ T-cells was stimulated by endothelial cell expression of Jagged1. This stimulation resulted in

the activation of lineage specific Th1 and T17 transcription factors, T-bet and RORγt. Piggott *et al.* (2011) also identified RNA and protein expression of NOTCH ligands, Jagged1 and Delta1, on vDCs, VSMCs and endothelial cells in GCA patients. Their investigation into blocking NOTCH signalling in a humanised mouse model carrying human arteries identified inhibition of $CD4^+$ T-cell activation, with a bias towards suppression of IL-17.

NOTCH receptor and ligand expression may therefore provide a mechanism through which selective pro-inflammatory T-cell subsets enter the immune privileged site, which is discrete from an antigen-specific response, and could provide multiple targets for immunotherapies.

1.3.3.2 PD-1

The programmed death-1 (PD-1) receptor/programmed death ligand-1 (PD-L1) immune checkpoint provides another antigen-nonspecific immunosuppressive mechanism to inhibit the actions of activated T-cells via TCR signal transduction through ligation of their PD-1 receptor (Chemnitz *et al*., 2004). The ligand PD-L1, is expressed by a number of cell types including antigen-presenting cells and stromal cells, allowing them to protect tissue from an unwanted immune response.

A very recent study into this immune checkpoint in GCA by Zhang *et al.* (2017) used a SCID mouse engrafted with a human artery and transplanted with human T-cells and monocytes from GCA patients to induce vasculitis. The group identified reduced transcripts of PD-L1 in GCA arteries compared to healthy controls. Furthermore, a defect in the ability of DCs within the artery wall of GCA patients to upregulate PD-L1 was identified. Consequently, DCs were unable to provide pro-inhibitory signals to activated PD-1 expressing T-cells to induce their apoptosis and prevent an unwanted inflammatory reaction within the artery wall. Transcript analysis in explanted inflamed human arteries in which PD-1 signalling was blocked were found to upregulate lineages-specific transcription factors *Tbet* and *RORγT* as well as *IFNγ, IL-17* and *IL-21,* suggesting selection for pro-inflammatory Th1 and Th17 cells. The use of transcripts within the artery wall limits this study as this may not fully translate to protein, an occurrence which has been observed in previous experiments into IL-4 expression in the artery wall (Ciccia *et al.,* 2015). The greater the number of PD-1 expressing T-cells found within the artery wall, the greater the induction of endothelial cell activation, T-cell cytokine secretion, intimal hyperplasia and neovascularisation that was observed.

1.3.3.3 CTLA-4

Checkpoint molecule cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) is another T-cell suppressing immune mechanism, however, unlike PD-1, CTLA-4 enables the suppression of T-cells during their activation, rather than after activation (Watanabe *et al.,* 2017). T-cells require two co-stimulatory signals from the antigen-presenting cell to induce activation: firstly, antigen presentation to their TCR and secondly, CD80/86 binding to their CD28 receptor. However, to control for self-reactive T-cell activation, T-cell expression of CTLA-4 and binding to CD80/86 antagonises CD28, inducing inhibitory signals which results in the suppression of the signalling cascade preventing T-cell activation after, for example, recognition of self-antigens (Koster and Warrington, 2017). The use of the CTLA-4 immunotherapy, Ipilimumab, to block CTLA-4 immunosuppression in cancer patients has been found to induce GCA in these patients (Goldstein *et al*., 2014). The mechanisms for which CTLA-4 induces suppression of T-cells is highly controversial (Walker and Sansom, 2015) which makes understanding how GCA is induced in Ipilimumab-treated cancer patients difficult. However, the effects of this monoclonal therapy support the concept of further checkpoint dysfunction within the arteries of GCA patients.

Coupled with T-cell entry and activation via NOTCH into an immune privileged site, along with a lack of downregulation of highly active T-cells within the artery wall, identifies a multi-step process which contributes to inflammation of the artery wall involving various dysfunctional immune mechanisms which can be seen in Figure 1.2. The disruption of these immune checkpoints also points to a lack of exogenous antigen involvement in the initiation of the disease, but rather an inability of APCs to downregulate T-cells, perhaps in response to an autoantigen.

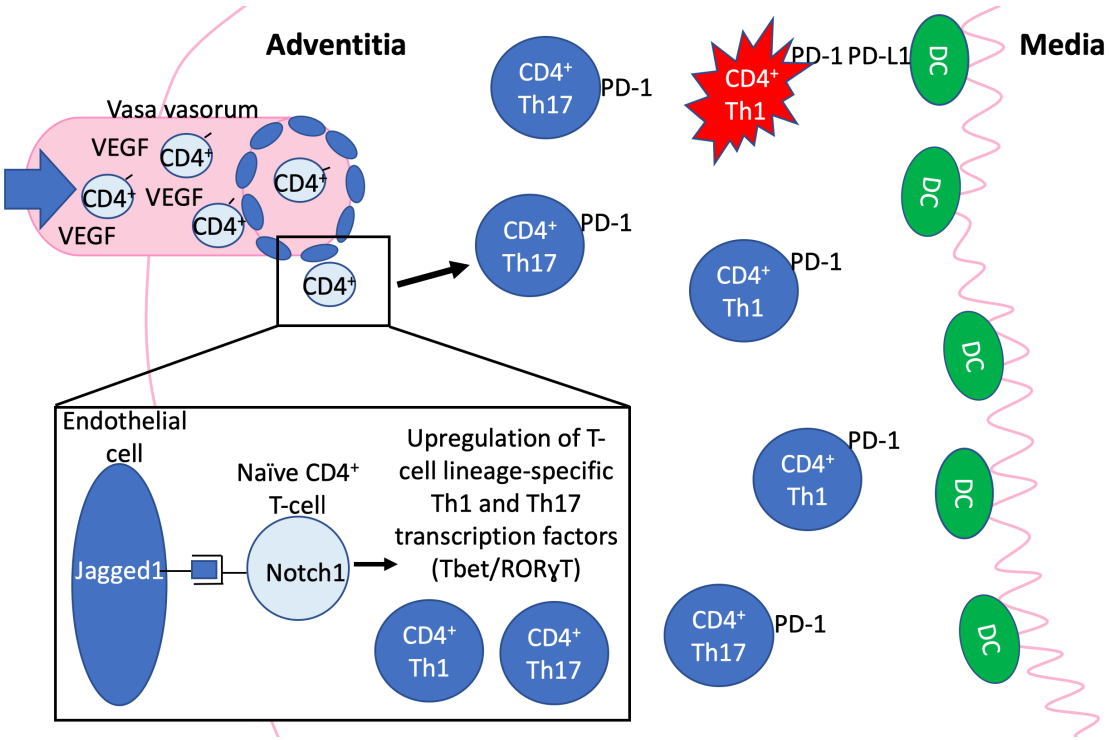


Figure 1.2: Schematic to show NOTCH and programmed death-1 checkpoint dysregulation in GCA arteries.

Naïve $CD4^+$ T-cells overexpressing Notch1 receptor enter the adventitia of the GCA diseased artery via the vasa vasorum network. Increased circulating levels of vascular endothelial growth factor induce expression of Notch1 ligand, Jagged1, on endothelial cells that line the vasa vasorum. Interaction of Jagged1 on endothelial cells and Notch1 on naïve $CD4^+$ T-cells results in the upregulation of T-cell lineage specific transcription factors, *Tbet* and *RORγT*, responsible for differentiation of $CD4^+$ T-cells into Th1 and Th17 cells respectively. Vascular DCs at the adventitia-media border lack expression of programmed death-1 receptor to prevent unwanted T-cell activation within the artery wall, therefore apoptosis of activated T-cells via the programmed death-1 checkpoint is insufficient to remove activated T-cells. DC, dendritic cell; PD-1, programmed-death 1 receptor; PD-L1, programmed-death ligand 1; VEGF, vascular endothelial growth factor.

1.4 Systemic inflammatory response

In addition to the inflammatory response within the arterial wall, systemic inflammation is another component of the disease, resulting in malaise, fever, night sweats and weight loss in patients. Within the circulation of patients with GCA, a number of cytokines and other inflammatory-related factors are elevated. Patients present with varying degrees of systemic inflammation.

1.4.1 Acute-phase proteins

The release of cytokines into the blood stream results in an increase in acute phase proteins, including serum amyloid A (SAA) and C-reactive protein (CRP), which is thought to be due to cytokine stimulated release of acute phase proteins from macrophages. The production of these proteins from hepatocytes within the liver, driven by IL-6 (Roche *et al.*, 1993), are associated with a higher inflammatory reaction (Kermani *et al*., 2012; Salvarani and Hunder, 2001) and are thought to contribute to the pathogenesis of GCA. Furthermore, levels of IL-6 correlate with the systemic response as well as CRP levels in GCA patients (Emilie *et al*., 1994; Roche *et al.*, 1993). Erythrocyte sedimentation rate (ESR), a measure of inflammation and one of the ACR classification criteria for GCA (Hunder *et al.,* 1990), is routinely measured in laboratories, alongside CRP, to identify systemic inflammation in GCA patients (Kermani *et al*., 2012). Recently, O'Neill *et al.* (2015) found SAA induced angiogenesis and hyperplasia within the artery wall, through its respective induction of activated MMP-9 and pro-inflammatory cytokines IL-6 and IL-8, and its promotion of myofibroblast outgrowth, in temporal artery explants. The artery explant method used in this study does not allow for the identification of the cell types which secrete these pro-inflammatory cytokines.

Like circulating cytokines, acute phase proteins are found to decrease in concentration in response to glucocorticoid treatment (Pountain *et al*., 1994; Andersson *et al*., 1986), however, levels can begin to increase after tapering increasing the risk of relapse.

1.4.2 Cytokines

Expansion of T-cell subsets, Th1 and Th17, have been observed within the circulation of patients with GCA, along with activated macrophages. Levels of Th1 and Th17 polarising cytokines, IL-12, and IL-1β, IL-6 and IL-23, respectively, are elevated in the circulation of untreated, GCA patients (Deng *et al.,* 2010). Additionally, TNFα has also been observed within the circulation of GCA patients

(Hernandez-Rodriguez *et al.,* 2004). As discussed in Section 1.3.1.2.3 glucocorticoids have been shown to ablate Th17 polarising cytokines IL-6, IL-1β, and IL-23, yet have no effect on Th1 polarising cytokine IL-12 (Deng *et al.,* 2010). IL-6 is thought to be a key cytokine that maintains the disease activity of GCA (Samson *et al.,* 2012).

IL-6 is a complex pleiotropic cytokine expressed by a wide range of cell types in response to infection and injury. IL-6 has been reported to induce both pro- and anti-inflammatory functions, resulting in numerous effects on the immune response (Tanaka *et al*., 2014), some of which have been described in this Chapter. IL-6 can bind both the membrane bound IL-6 receptor (IL-6R) and the soluble IL-6 receptor (sIL-6R), which comprises a downstream signalling chain, glycoprotein (gp)130. Soluble IL-6 receptors allow cells that do not express IL-6R to induce downstream signalling, described as trans-signalling, and is thought to promote inflammation (Rabe *et al*., 2008). Activation of gp130 induces activation of major signalling pathways, Janus Kinase (JAK)-signal transducer and activator of transcription (STAT)3 along with the mitogen-activated protein kinase (MAPK), which are implicated in T-cell and macrophage differentiation (Seif *et al*., 2017; Zhou *et al*., 2014). Furthermore, induction of STAT3 also results in upregulation of suppressor of cytokine signalling (SOCS)3 which can inhibit the JAK/STAT pathway, regulating IL-6 signalling (Babon *et al*., 2014).

Patients presenting with more severe systemic inflammation display a higher concentration of circulating pro-inflammatory cytokines, IL-6, IL-1β, and TNFα (Samson *et al.*, 2012; Roche *et al*., 1993) and exhibit a higher degree of resistance to glucocorticoid treatment (Hernandez-Rodriguez *et al.*, 2004). Furthermore, those with higher circulating TNF and IL-6 levels have also been found to require a longer treatment regimen of glucocorticoids (Hernandez-Rodriguez et al., 2004b; Hernandez-Rodriguez et al., 2002), however, it must be noted that TNFα levels are difficult to measure within the circulation and could impact results.

The observation of higher systemic levels of IL-6 has conversely also been related to patients exhibiting a lack of vessel occlusion (Hernandez-Rodriguez *et al*., 2003). This may be a protective mechanism, as greater neoangiogenesis has been correlated with a strong systemic reaction (Cid *et al*., 2002). Patients found to have high levels of TNFα and IL-6 after long-term follow-up however have been found to be more prone to relapse when glucocorticoids are tapered (Hernandez-Rodriguez *et al*., 2004). These cytokines could therefore potentially be used as biomarkers for identifying patients most likely to relapse. Further

studies are required to confirm these findings, as well as investigations into the levels of cytokines that allow identification of relapsing patients from non-relapsing patients.

Cytokines have been used as targets for therapeutics in a number of diseases, for example the use of the anti-TNF therapy, Infliximab, for treatment of patients with rheumatoid arthritis. More recent studies into the targeting of cytokines inducing their inhibition in GCA have revealed their importance in the contribution to disease progression, including the anti-IL6R drug, tocilizumab (Stone et al., 2017).

1.5 Histological features

Understanding the histological features of GCA affected arteries is critical due to the use of artery biopsies as the “gold standard” method for GCA diagnosis. Medium and large arteries are composed of three distinct layers: the outermost layer, the adventitia (which contains a network of small vessels called vasa vasorum), the media and the innermost layer, the intima, with external and internal elastic laminae separating these three layers (Figure 1.3a). Multiple histological features, such as an inflammatory infiltrate, giant cells, hyperplasia within the intima and luminal occlusion, are seen within the artery wall and a positive diagnosis requires the observation of a number of these histological features in combination with clinical examination criterion tests (Hunder *et al.,* 1990). The common histological features found in GCA positive arteries are discussed in more detail below. In most GCA cases, the characteristic disease features are not always found; pathological features can vary between patients, such as the presence of giant cells (Wang *et al*., 2017).

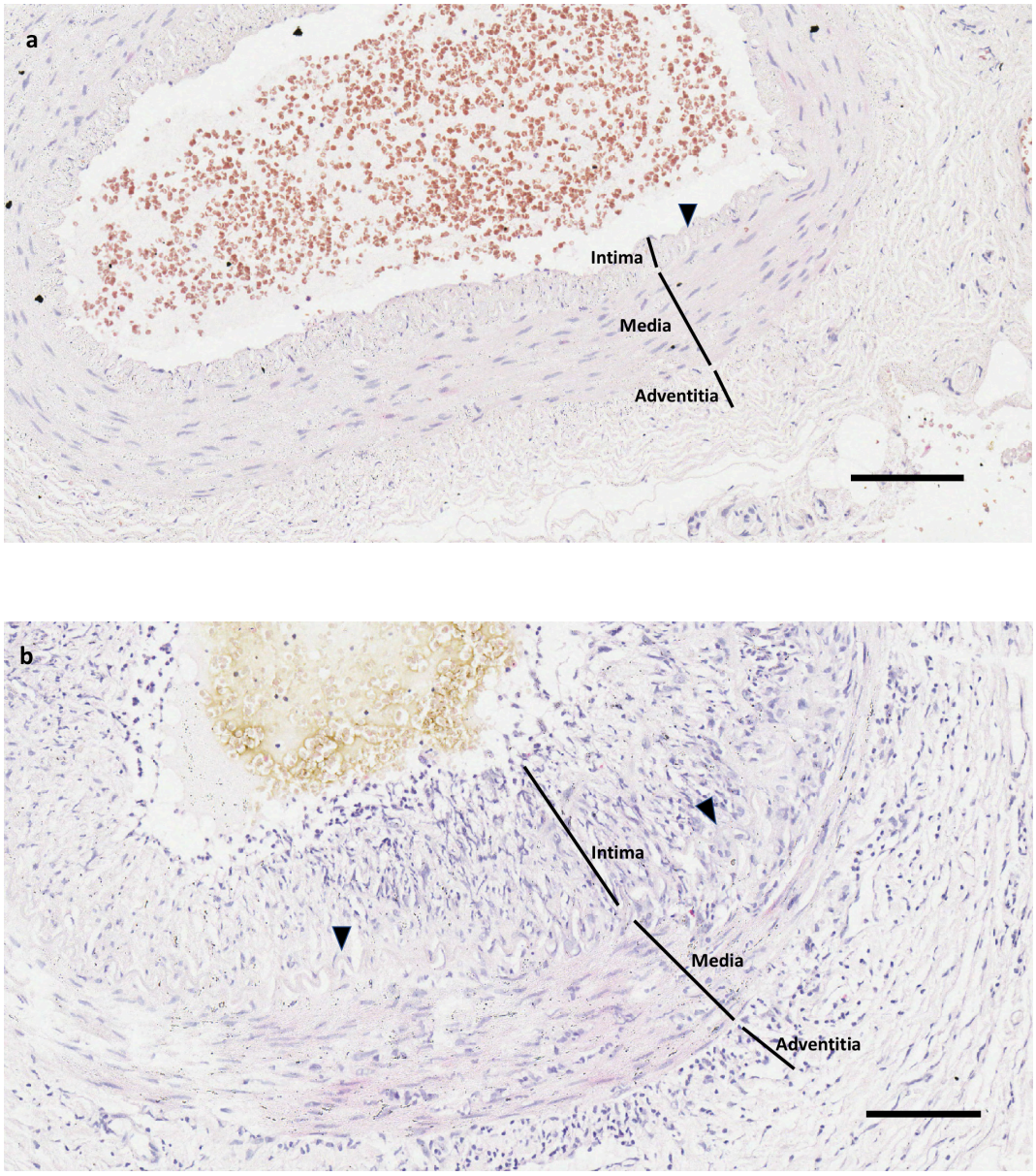


Figure 1.3: Images of healthy and GCA positive artery biopsies.

Artery sections were stained with haematoxylin and eosin to show differences in the artery structure between **a)** a healthy artery and **b)** a GCA-positive artery with a fragmented internal elastic lamina. The layers of the artery wall have been annotated to show the differences in size between the two arteries, arrows indicate internal elastic lamina. Images were taken at x150 magnification. Scale bar is 100μm. Ethical approval and patient consent was given (see Section 2.2).

1.5.1 Inflammatory infiltrate

An influx of immune cells can be seen within the artery of a GCA patient in Figure 1.3b. This influx mainly consists of dendritic cells (DCs), T-cells (CD3+) and macrophages (CD68+) that are thought to infiltrate the artery wall through the vasa vasorum vascular network and migrate through the different layers towards the intima (Segarra *et al*., 2007; Cid *et al*., 2000). This infiltrate, however, is found to occur in different regions along the length of the artery, described as skip lesions, and explains why a positive diagnosis of GCA can be made in the presence of a negative biopsy. Multinucleated giant cells are also not a universal histological feature of GCA, as giant cells are only observed in 50% of GCA cases (Wang *et al*., 2017; Restuccia *et al*., 2012). When giant cells are identified, they are commonly found at the intima-media border, close to fragmented internal elastic lamina and are described histologically as part of typical GCA.

1.5.1.1 Patterns of inflammation

The influx of immune cells through the vessel wall can occur in many different patterns (Cavazza et al., 2014). Those with a smaller inflammatory infiltrate, confined to the adventitia are described as showing atypical GCA (Restuccia et al., 2012; Cavazza et al., 2014), whilst those with an infiltrate throughout all layers of the vessel wall (termed pan-arteritis) are described as having typical GCA. Characterising GCA using these patterns may be too simplistic. More recently, other groups have identified further patterns of inflammation (Wang et al., 2017; Hernandez-Rodriguez et al., 2016). Hernandez-Rodriguez identified 4 different patterns of inflammation: adventitial pattern, adventitial invasive pattern (inflammation of the adventitia and local inflammation of the media), concentric bilayer pattern (in which inflammation was observed within the adventitia and intima with no media involvement) and a pan-arteritic pattern, (where inflammation is observed throughout all artery layers). These patterns can be seen in Figure 1.4. They observation of these different patterns within the same artery led them to suggest these patterns represent phases of GCA progression, with the first two phases occurring during early GCA development and the last two phases during the later phases of GCA development. The degree to which inflammatory cells infiltrate the artery has been associated with increased incidence of cranial clinical manifestations including jaw claudication and scalp tenderness (Hernandez-Rodriguez et al., 2016; Cavazza et al., 2014) therefore these different patterns may be of clinical significance.

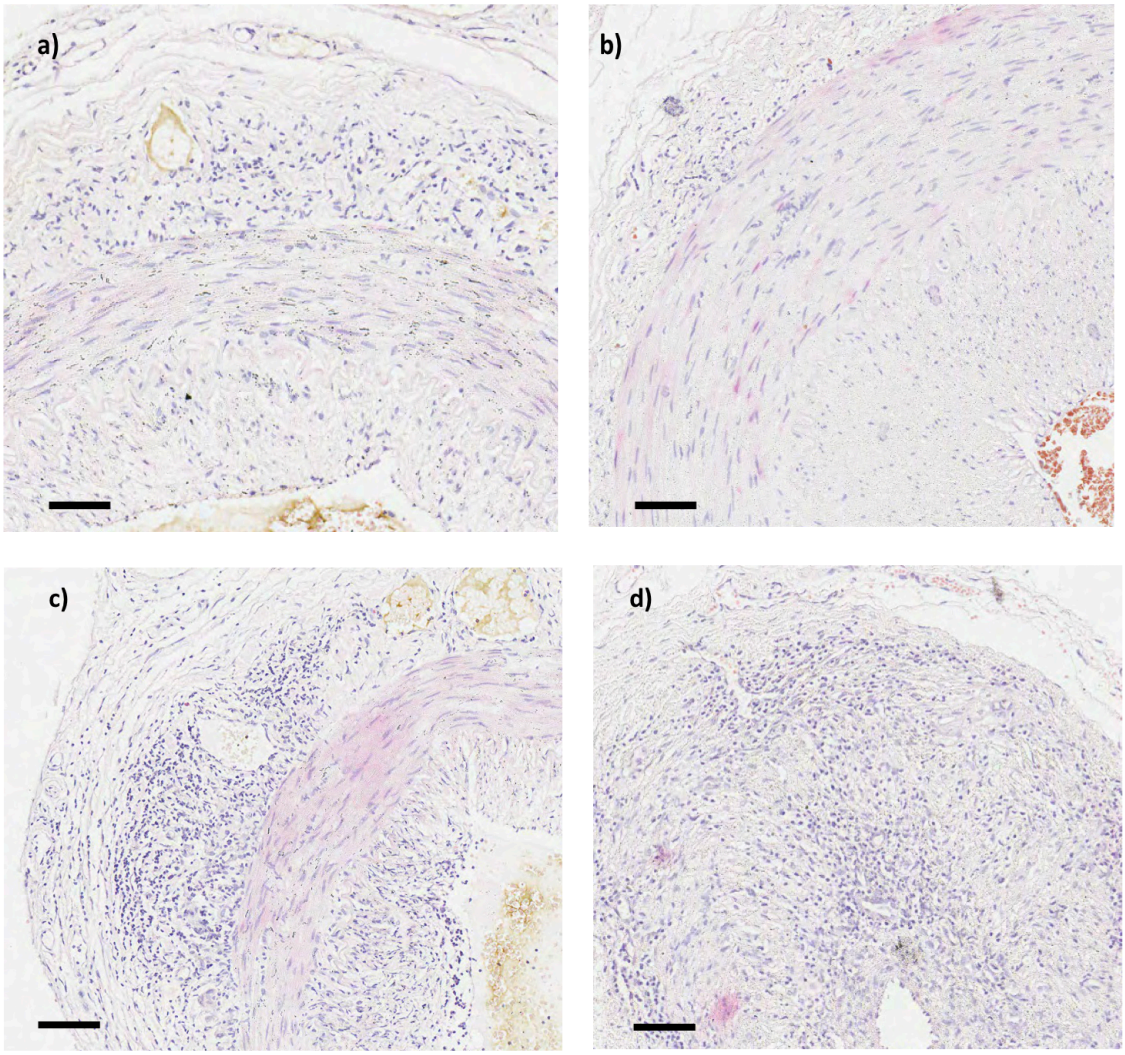


Figure 1.4: Patterns of infiltrating inflammatory cells in the wall of GCA affected arteries.

The four patterns of infiltrating inflammatory cells described by Hernandez-Rodrigeuz *et al*. (2016) are shown in haematoxylin and eosin stained artery sections. **a)** adventitial-restricted inflammation, **b)** adventitial-invasive inflammation, **c)** concentric bilayer inflammation and **d)** pan-arteritic inflammation. Images were taken at a x100 magnification. Scale bars represent 100μm.

1.5.2 Intimal thickening and occlusion

The extent of intimal thickening leading to vessel occlusion has been correlated with the amount of inflammation within the artery wall, as well as the degree of internal elastic lamina (IEL) fragmentation as indicated in Figure 1.3b (Hernandez-Rodriguez *et al.*, 2016; Segarra *et al*., 2007; Nordborg and Petursdottir, 2000). This IEL fragmentation is thought to be a wound healing process, instigated by macrophages and giant cells exposed to IFNγ, promoting migration and proliferation of stromal cells, as well as tissue fibrosis through production of pro-fibrotic mediators and extracellular matrix components, such as fibronectin, collagen, and elastic fibres (Kaiser *et al*., 1998).

1.5.3 Angiogenesis

The development of neovessels is an important aspect of GCA which occurs in all vessel wall layers, however, like giant cells, not all patients have signs of new vessel development. Angiogenesis is thought to be promoted by macrophages and is required to provide the artery wall with further oxygen and nutrients to supply a high energy environment, as well as providing another way in which inflammatory cells can reach the artery wall (Rittner *et al.*, 1999b).

1.6 Treatments

Glucocorticoids are the “gold standard” treatment for GCA yet their wide array of side effects and their inability to fully treat the condition warrants the need for new treatments which can target multiple pathways, leading ultimately to a disease regression. A number of new treatment options have been investigated for GCA, such as the anti-TNF agent, Infliximab, yet their lack of efficacy (Yates et al., 2014; Hoffman et al., 2007) or an inability to treat the Th1 arm of the disease, such as NOTCH pathway blockade (Piggott *et al.*, 2011) has hindered their progression in drug repurposing trials. The anti-IL-6R therapy Tocilizumab, however, has just been licensed as an adjunctive therapy.

1.6.1 Glucocorticoids

Glucocorticoids have been the mainstay of treatment for GCA patients over the past 60 years and are prescribed immediately at high doses to prevent vision loss and stroke. The optimum treatment regimen has not been defined in clinical trials, however, British society for rheumatology (BSR) guidelines (Dasgupta *et al.*,

2010) suggest prednisolone is given orally at doses of 40 to 60mg/d to GCA patients over 4 to 6 weeks before tapering. Those showing symptoms of vision loss are given high-dose pulsed intravenous (IV) methylprednisolone of up to 1g per day over 3 days, before oral dosages of 40mg/d.

1.6.1.1 Mechanisms of glucocorticoids

Glucocorticoids have a wide range of effects due to the pleiotropic nature of its receptor, the glucocorticoid receptor (GR), which can induce resolution of inflammation but when used over a prolonged period and at high doses, also results in numerous side effects (Huscher *et al*., 2009). Furthermore, the effects of glucocorticoids vary between different cell and tissue types. The GR has the ability to inhibit a number of different signalling pathways involved in inflammation making glucocorticoids an effective treatment option for a lot of different diseases.

Glucocorticoid binding of the GR induces GR to act as a transcription factor, translocating to the nucleus and altering gene expression. This can occur through transcriptional activation by binding to the glucocorticoid response element (GRE) inducing expression of genes such as IL-10, or transcriptional repression by either binding to the negative GRE (nGRE) or interacting with other transcription factors, such as nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) and PD-1 (Ramamoorthy and Cidlowski, 2013). Interaction with other transcription factors has been found to inhibit a large range of pro-inflammatory cytokines, such as IL-6 and TNFα (Almawi and Melemedjian, 2002).

1.6.1.2 Effect of glucocorticoids in GCA

The response of systemic symptoms to glucocorticoids is quick, with malaise and fever, as well as vasculitic symptoms such as headaches, improving within days. Vascular inflammation, however, decreases slowly over time due to the persisting Th1 inflammatory arm resulting in chronic inflammation that is not completely resolved (Maleszewski *et al*., 2017). Glucocorticoids are given over a long duration and although tapered, half of patients relapse after glucocorticoid withdrawal (Andersson *et al*., 2009) therefore requiring glucocorticoid treatment over a period of many years. The nature of glucocorticoids, especially in dosages given to GCA patients results in a wide range of side effects, such as bone loss and diabetes, with a dosage of more than 10mg/d found to cause serious adverse events (Strehl *et al*., 2016; Chandran *et al.*, 2015). The requirement of new GC-

sparing treatments with increased efficacy, especially the ability to target the Th1 disease arm, and a lower number of side effects, are therefore greatly needed.

1.6.2 Methotrexate

Methotrexate, an immunosuppressive agent of which its mechanisms of action are poorly understood, is used for the treatment of rheumatoid arthritis. Studies into its use in GCA have shown conflicting results. Two studies identified no effect on adverse events when used as an adjuvant with glucocorticoids (Hoffman *et al*., 2002; Spiera *et al*., 2001), whilst Jover *et al*. (2001) described a decrease in relapse and cumulative dose of glucocorticoids. Meta-analysis of these three methotrexate studies in GCA reported a decrease in cumulative glucocorticoid dose and a decrease in probability of relapse after glucocorticoid withdrawal (Mahr *et al.*, 2007). The low number of patients used to investigate methotrexate as an adjuvant therapy limits all three of these studies.

1.6.3 Tocilizumab (anti-IL-6R)

The newly licenced drug for GCA, Tocilizumab, is now used as an adjunctive therapy targeting the Th17 arm of the disease by competitively binding membrane and soluble receptors for the cytokine IL-6. This blockade may enable the rebalancing of the Th17:Treg ratio as shown in rheumatoid arthritis (Samson *et al*., 2012), whereas glucocorticoids only partially rebalance this defect. The Giant-Cell Arteritis Actemra (GiACTA) trial, carried out by Stone *et al*. (2017) into the use of tocilizumab as an adjuvant therapy found it decreased the overall duration and dosage of glucocorticoids given to patients, increased the number of patients achieving remission without relapse, and increased the duration of remission, confirming other findings of efficacy (Vitiello *et al*., 2018; Villiger *et al.*, 2016). Consequently, tocilizumab has been approved as an adjuvant therapy prescribed weekly for the treatment of GCA. Nevertheless, the longer-term effects of tocilizumab need to be investigated further. Additionally, the cost implications of adding a monoclonal antibody treatment are large and therefore the advantages of its use must be weighed against the cost and side-effects.

1.7 Macrophages

1.7.1 Background

Macrophages are important innate immune cells, their name ‘big eater’ refers to their primary homeostatic role in the removal of dead cells and debris via efferocytosis, however, they perform a myriad of functions including the removal of pathogens via phagocytosis, promoting and resolving inflammation and the repairing of damaged tissue in response to environmental and physiological changes.

Macrophages originate from myeloid progenitor cells and are present throughout the different organ systems of the body. They belong to a family of professional phagocytic cells that also include monocytes, dendritic cells and osteoclasts that carry out specialised functions according to differentiation status (Gordon and Plüddemann, 2017). Macrophages are described as ‘professional’ phagocytes as they have the ability to recognise pathogens via their expression of pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) which identify pathogen associated molecular patterns (PAMPs), such as lipopolysaccharides (LPS), as well as danger associated molecule patterns (DAMPs) (Ren *et al*., 2017). These molecules are expressed on the surface of many pathogens and results in their phagocytosis. Phagocytosis and subsequent degradation of these pathogens into antigens allows macrophages to present antigens to other immune cells via their MHC II molecules, promoting an inflammatory response (Watts, 1997)

The ability of macrophages to perform a multitude of different functions has drawn attention to their role within disease, especially within GCA. It is thought that an imbalance of macrophages with opposing functions may impact disease onset, severity and progression. For example, in GCA macrophages observed within different layers of the artery wall have been described to secrete different cytokines and mediators and are thought to contribute to the varied histological changes that occur within the vessel wall (Wagner *et al.*, 1996).

In GCA, macrophages can be found within all three layers of the arterial wall of GCA temporal arteries (Wang *et al*., 2017) and have been described as producing a range of different factors in these different layers (Rittner *et al.,*1999b; Weyand *et al.,* 1996). This has led to the hypothesis that different macrophage phenotypes contribute to different aspects of GCA pathogenesis, such as those that promote inflammation and those that support wound healing. The role of macrophages in GCA is discussed in more detail in Section 1.8.

1.7.2 Polarisation

Macrophages are highly plastic cells that respond to environmental cues by altering their phenotype, and as a result express different markers of polarisation (Xue *et al*., 2014; Stout *et al*., 2005), enabling them to reshape inflammatory responses (Guiducci *et al*., 2005). Originally it was proposed that *in vitro* polarisation with the Th1 cytokine IFNγ resulted in a pro-inflammatory macrophage phenotype, termed classically activated macrophages (Pace *et al*., 1983) whilst macrophages polarised with the Th2 cytokines, IL-4 or IL-13, were found to induce a differently activated phenotype and were termed alternatively activated macrophages (Martinez *et al*., 2013; Stein *et al*., 1992). Crudely, polarised macrophages have long since been described in this binary fashion, with classical or pro-inflammatory macrophages, termed M1, at one end of the scale and alternative or anti-inflammatory macrophages, termed M2, at the other (Mills *et al.*, 2000). This simplified concept of macrophage polarisation has been and still is, the basis for *in vitro* studies into macrophage phenotypes and is explained in detail below.

1.7.3 Pro-inflammatory macrophages

Pro-inflammatory M1 macrophages are polarised *in vitro* through stimulation with IFNγ and the TLR4 ligand, LPS. Additional molecules, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and the less commonly used TNF, when used alone or in combination with the agents above, are also known to induce a M1 phenotype (Mantovani *et al.*, 2005).

IFNγ is secreted by a variety of innate and adaptive immune cell types including NK cells, however, M1 polarisation is associated with an adaptive immune cell response and the efficient production of IFNγ by Th1 polarised T-cells to maintain this macrophage activation state.

Functionally, M1 macrophages exhibit anti-microbial and anti-tumour properties and have the ability to mount an inflammatory response due to their potent production of pro-inflammatory cytokines (Table 1.1). The production of IL-12 induces a feedforward loop, further stimulating Th1 polarisation whilst the release of IL-1β, IL-6 and IL-23 promotes Th17 differentiation (Arnold *et al*., 2015), both boosting recruitment of specific T-cell subsets to the area of inflammation. As well as cytokines, M1 macrophages release toxic molecules, reactive oxygen species (ROS) and nitric oxide (NO) which contribute to the destruction of invading intracellular pathogens (Jung *et al.*, 2013). M1 macrophages also have

the ability to present antigen to T-cells (Arnold *et al*., 2015) as well as exhibit an increased phagocytic ability (Lam *et al*., 2016).

The strong pro-inflammatory properties of M1 macrophages highlight their importance in the innate immune response but also emphasises the need for their functions to be controlled since dysregulation can ultimately lead to chronic inflammation, tissue damage and autoimmune disease such as rheumatoid arthritis (Brennan *et al*., 1989).

The release of high amounts of IFNγ by Th1 cells within the adventitia of inflamed GCA arteries is thought to promote macrophage polarisation towards an M1 phenotype. The release of Th1 and Th17 polarising cytokines from M1 cells supports the amplification of these two T-cell subsets, driving inflammation and tissue damage within the artery wall (Deng *et al.,* 2010).

1.7.4 Anti-inflammatory macrophages

Anti-inflammatory macrophages were initially termed M2 due to the ability of the Th2 cytokines, IL-4 and IL-13, to induce the polarisation of macrophages into an anti-inflammatory phenotype (Martinez *et al.*, 2013). The recognition of further molecules that induced an M2-like phenotype *in vitro*, such as IL-10 and glucocorticoids, resulted in the division of M2 macrophages into three subtypes, M2a, M2b and M2c. Each of these subclasses are differentiated using various molecules and carry out different anti-inflammatory functions (Table 1.1) but still gives a simplistic view of macrophage polarisation. The main functions of M2 macrophages are thought to be tissue repair exemplified by wound healing. Interestingly it has been speculated that during different phases of the wound healing process M2 macrophages are found with different wound healing functions (Lucas *et al*., 2010). Furthermore, the addition of M-CSF has also been identified as a M2 polarising factor and is commonly used in *in vitro* human macrophage polarisation protocols (Martinez *et al.*, 2006).

1.7.4.1 M2a macrophages

M2a macrophages are polarised through treatment with IL-4 and/or IL-13 and exhibit wound healing functions such as inducing cellular proliferation, supporting the formation of neovessels, and promotion of fibroblasts into myofibroblasts which aid in wound closure (Wynn and Vannella, 2016). Activation of IL-4 induces STAT6 which promotes downstream upregulation and subsequent secretion of extracellular matrix components supporting tissue repair and regeneration (Yan *et al.*, 2015). M2a macrophages are also associated with the removal of parasites

and helminths during invasion depending on the microenvironment and cell types they encounter (Martinez *et al.*, 2009). Similarly to M1 macrophages, the functional processes carried out by M2a macrophages need to be tightly controlled as excessive wound healing can lead to hyperproliferation and fibrosis (Duffield *et al.*, 2005). In GCA, M2a macrophages are thought to play a part in promoting intimal hyperplasia and neovascularisation due to their ability to secrete mediators that promote wound healing (Hernandez-Rodriguez *et al*., 2016; Kaiser *et al.,* 1999).

1.7.4.2 M2b macrophages

M2b macrophages are induced through stimulation with immune complexes in combination with IL-1β or LPS, and are termed immunoregulatory macrophages as M2b are thought to recruit Tregs through their expression of the CCR8 ligand CCL1 (Mantovani *et al*., 2004). Interestingly, although these cells are the opposite of M1 cells in terms of their cytokine secretion (high IL-10, low IL-12) they are not classed as anti-inflammatory cells due to their ability to secrete pro-inflammatory cytokines IL-6 and IL-1β (Mantovani *et al*., 2004). Rather they have the ability to induce a type II adaptive immune response, stimulating the release of IgG1 antibodies from B-cells. M2b cells also express high levels of the co-stimulatory molecules CD80 and CD86 which allows these cells to activate T-cells through antigen presentation. M2b cells have not been described within the artery wall of GCA patients, however, the identification of ATLOs within the artery wall (Ciccia *et al.,* 2017) may suggest a role for M2b cells promoting B-cells antibody production against endothelial and VSMCs within the artery wall (Régent *et al*., 2011).

1.7.4.3 M2c macrophages

M2c macrophages can be generated through treatment of unpolarised macrophages with IL-10, glucocorticoids, or TGF-β and are described as deactivated macrophages in light of their ability to switch from a M1 phenotype to a M2 phenotype (Porcheray *et al*., 2005). Furthermore, M2c macrophages are involved in immunosuppression through secretion of cytokines including TGF-β and IL-10 (Yoshimura and Muto, 2011; Martinez *et al*., 2009; Taylor *et al*., 2006). IL-10 mediates the inhibition of M1 activating signalling pathways induced by LPS via STAT3 (Mantovani *et al.* 2004), therefore M2c macrophages are able to prevent the secretion of a range of pro-inflammatory cytokines such as IL-6, IL-12 and TNFα, downregulate the MHC class II receptor preventing antigen presentation to T-cells, and suppress reactive oxygen and nitrogen species

(Maynard and Weaver, 2008). M2c cells may also have wound healing properties due to the secretion of TGF-β which has been shown to promote fibroblast proliferation and differentiation (Evans *et al*., 2003) as well as the upregulation of a number of genes involved in neovascularisation and matrix remodelling, such as *VCAM* and *MMP8,* respectively (Lurier *et al*., 2017).

Due to the high dosages and prolonged use of glucocorticoids for treatment of GCA it could be assumed that macrophages within the artery wall could be polarised towards an M2c phenotype. These cells may promote the resolution of the disease by suppressing the inflammatory response.

Table 1.1: Description of M1 and M2 polarisation stimuli and their functions.

Macrophage phenotype	Polarisation stimuli	Cytokines secreted	Chemokines expressed	Other factors released	Functions
M1	**IFNγ**, **TNFα**, LPS (TLR4 ligand), GM-CSF	**IFNγ**, **IL-1β**, **TNFα**, **IL-12**, **IL-23** and **IL-6**	**CXCL10**, **CXCL13**, CXCL15, CXCL19, CXCL20	**iNOS**, **ROS**, RNS, **MMPs-2** and **-9**	Pro-inflammatory, anti-microbial, anti-tumour.
M2a	**IL-4***, IL-13	IL-4*	CCL13, CCL14, CCL17, CCL18, CCL22, CCL23	**MMP12**, **MMP9**, **TIMP1**.	Resolution of allergy; parasite recognition; endocytosis; antagonise Th1/M1 functions; pro-tumour
M2b	Immune complexes, IL-1Ra or LPS	**IL-6**, **IL-1β**, **IL-10**, and, **TNFα**	CCL1	**iNOS**	Immunoregulatory; antigen presentation; pro-tumour; recruit Tregs and eosinophils
M2c	**IL-10**, **glucocorticoids** (in treated patients), **TGF-β**	**IL-10**, **TGF-β**	CCL16, CCL18	**VEGF**, **PDGF**, MMP8	Deactivated; wound healing; tissue remodelling; pro-tumour, endocytose; antagonise M1 functions; suppress inflammatory cells; activate Th2 and Tregs

Those in red are known to be upregulated in GCA. Those with an astrix (*) have been described to be upregulated in GCA but there is disagreement in the literature. (Ciccia *et al.,* 2017; Ciccia *et al.,* 2015; Murray *et al.,* 2014; Martinez *et al.,* 2013; Mantovani *et al.,* 2009; Rodríguez-Pla *et al.,* 2009; Martinez *et al.,* 2008; Segarra *et al.,* 2007; Martinez *et al.,* 2006; Mantovani *et al.,* 2004; Kaiser *et al.,* 1998; Kaiser *et al.,* 1999; Weyand *et al.,* 1996). IFN, interferon; TNF, tumour necrosis factor; LPS, lipopolysaccharide; TLR, toll-like receptor; GM-CSF, Granulocyte-macrophage colony-stimulating factor; IL, interleukin; CXCL, chemokine (C-X-C) ligand; CCL, chemokine ligand; iNOS, inducible nitric oxide species; ROS, reactive oxygen species; RNS, reactive nitrogen species; MMP, metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; TGF, transforming growth factor; VEGF, vascular endothelial growth factor; PDGF, platelet-derived growth factor.

1.7.5 Mechanisms of macrophage polarisation

Numerous mechanisms have been identified that result in transcriptional changes in macrophages causing polarisation into various phenotypes, however, the process of polarisation is highly complex. Exposure to different stimuli induces a wide range of downstream signalling pathways and epigenetic changes, regulating transcription factors such as STAT, interferon-regulatory factor (IRF), NF-κB and peroxisome proliferator-activated receptor (PPAR), which together bring about upregulation and downregulation of a diverse range of proteins, cytokines and receptors (Czimmerer *et al.*, 2018; Glass and Natoli, 2015). These mechanisms also allow switching between polarisation states.

An imbalance towards M1 polarising transcription factors STAT1/SOCS3 or M2 polarising transcription factors STAT3/SOCS1, mediated by microRNAs, has been observed to be one mechanism which results in the polarisation of macrophages, with this imbalance having the ability to effect a number of downstream signalling pathways (Beldi-Ferchiou *et al.*, 2017; Ma *et al.*, 2016). Polarisation into M1 cells is driven by activation of STAT1 and NFκB via IFNγ, TLR4-ligand and IL-6 stimulation with the negative feedback loop of SOCS3 preventing M2 polarisation. Increased expression of SOCS3 inhibits STAT3 activation, enabling TLR4 signalling induced by LPS, as well as inhibiting IL-6 induced IL-10 expression. Without SOCS3 expression, polarisation towards an M1 phenotype cannot occur and instead results in the upregulation of M2a markers, such as CD206 and SOCS1 (Zhou *et al.*, 2017; Arnold *et al*., 2014; Liu *et al*., 2008). In contrast, induction of SOCS1 via IL-4 signalling and STAT3 activation, results in the blockade of IFNγ and LPS induced effects, enabling downstream IL-4 signalling through phosphatidylinositide 3-kinase (PI3K) and STAT6 activation and the subsequent transcription of M2 genes (Maruoka *et al*., 2017; Strebovsky *et al.*, 2010; Nakagawa *et al.*, 2002). This balance of signalling pathways exposes a way in which the ability of macrophages to alter their phenotype could be manipulated to redirect polarisation (Malyshev and Malyshev, 2015; Zhou *et al.*, 2014).

1.7.6 Phenotypic markers

Markers have long been used to characterise cellular phenotypes, with T-cell populations being the exemplar of this method. Similarly, to T-cell immunology, this concept has been used to try to characterise M1 and M2 macrophages in tissues helping to identify their roles in disease. Markers which are used most in macrophage biology studies to define macrophage phenotypes can be found in Table 1.2. The difference in macrophage biology to that of T-cell biology is the lack of specific markers that have been identified for each phenotype resulting in the problem of defining phenotypes (Geissmann *et al*., 2010; Fujiwara *et al.*, 2016). This is in part due to the use of the binary model for *in vitro* macrophage polarisation when in reality, macrophage polarisation is not an end-point event. Additionally, macrophages *in vivo* encounter an array of cytokines and molecules and are known to be able to express a multitude of markers that are associated with more than one phenotype, blending their polarisation states (Vogel *et al.*, 2013a). Moreover, the absence of clear macrophage polarisation protocols, with differences in polarising agents, their concentrations and time scales of exposure, as well as variable markers used for characterising phenotypes results in variation between studies (Murray *et al*., 2014; Arnold *et al.*, 2015).

Table 1.2: Common markers for identification of differently polarised macrophages used in macrophage studies.

Macrophage phenotype	Marker (gene)	Marker (protein)
M1	*IRF5* (Krausgruber *et al.*, 2011), *STAT1*, *SOCS3* (Whyte *et al*., 2011; Arnold *et al*., 2015)	CD80, CD64 (Ambarus *et al.,* 2012)
M2a	*TGM2* (Arnold *et al*., 2015; Martinez *et al*., 2013) *IRF4* (Satoh *et al*., 2010), *PPARγ* (Bouhlel *et al*., 2007), *STAT3, SOCS1* (Martinez *et al*., 2013; Whyte *et al*., 2011)	CD163, MRC1 (Arnold *et al*., 2015; Arnold *et al*., 2014; Ambarus *et al*., 2012), CD200R (Ambarus *et al.,* 2012)
M2b	CD36, MRC1 (Mantovani et al., 2004)	MRC1 (Mantovani *et al.,* 2004)
M2c	*CD163, MRC1* (Ehrchen *et al*., 2007), *MARCO* (Park-Min *et al*., 2005)	CD163 (Ehrchen *et al*., 2007; Ambarus *et al*., 2012), CD32, CD16 (Ambarus *et al.,* 2012)

CD, cluster of differentiation; IRF, interferon-regulatory factor; MARCO, macrophage receptor with collagenous structure; MRC, mannose receptor C-type; PPAR, peroxisome proliferator-activated receptor; SOCS, suppressor of cytokine signalling; STAT, signal and transducer of transcription; TGM2, transglutaminase.

As macrophage biology is highly conserved between mice and humans, murine macrophages and models are commonly used as substitutes due to their ease of use (Ingersoll *et al.*, 2010). Consequently, macrophage literature has become difficult to interpret due to studies which assumed parallels between mice and human macrophage gene and protein expression that further studies found were restricted to mice (Arnold *et al*., 2015; Vogel *et al*., 2014; Ambarus *et al.*, 2012). The use of high throughput RNA methods to identify upregulated and downregulated genes has provided a huge number of markers for a wide range of differently treated macrophages. This has resulted in a large variety of markers, some much more specific than others to identify macrophage phenotypes (Fujiwara *et al*., 2016; Becker *et al*., 2015; Xue *et al*., 2014; Beyer *et al.*, 2012; Martinez *et al*., 2006). In GCA, analysis of these markers using an end-point based approach to RNA and protein expression would help provide a way in which to specifically characterise macrophage subsets within the artery wall using immunohistochemistry.

1.7.7 Polarisation as a spectrum

The linear model of macrophage polarisation has made *in vitro* macrophage polarisation and defining macrophage populations easier, but this oversimplified view, although useful *in vitro*, has prevented gaining a true picture of macrophage phenotypes and their functions since it does not mimic all the conditions seen *in vivo,* where macrophages can encounter numerous molecules simultaneously. The recognition of additional polarising agents, such as LPS, and the use of highly sensitive transcriptome profiling is slowly altering this linear macrophage model (Xue *et al*., 2014; Martinez *et al*., 2006; Martinez *et al.*, 2013; Sudan *et al*., 2015) and bringing about a change in how we conceptualise macrophage polarisation. Macrophages are highly plastic cells and have the ability to switch their phenotype at any stage due to changing environmental conditions, including physiological changes such as pH and hypoxia (Chen *et al*., 2018; Raggi *et al*., 2017; Guiducci *et al*., 2005). Macrophages therefore have the ability to express a mixture of differently associated phenotypic markers. More recently macrophage polarisation *in vivo* has been described as a spectrum (Figure 1.5), with no phenotypic extremes, but rather overlapping populations of polarised macrophages which exhibit overlapping phenotypic markers and functions (Xue *et al.*, 2014; Martinez and Gordon, 2014; Mosser and Edwards, 2008). Indeed, this concept in part agrees with the phenotypic state of adipose tissue macrophages, (macrophages treated with glucose, insulin and free fatty acids), which secrete pro-inflammatory cytokines yet express M2 associated markers and are instead termed ‘metabolically active’ macrophages (Kratz *et al*., 2014). Adipose tissue macrophages therefore conform to the spectral model but macrophages identified in atherosclerosis, described as Mhem, M(Hb) and M4 (Gleissner *et al*., 2010; De Paoli *et al.*, 2014; Boyle *et al*., 2009) are described separately from M1 and M2 macrophages and therefore do not match this M1-M2 spectral model, suggesting that many other macrophage phenotypes are yet to be identified.

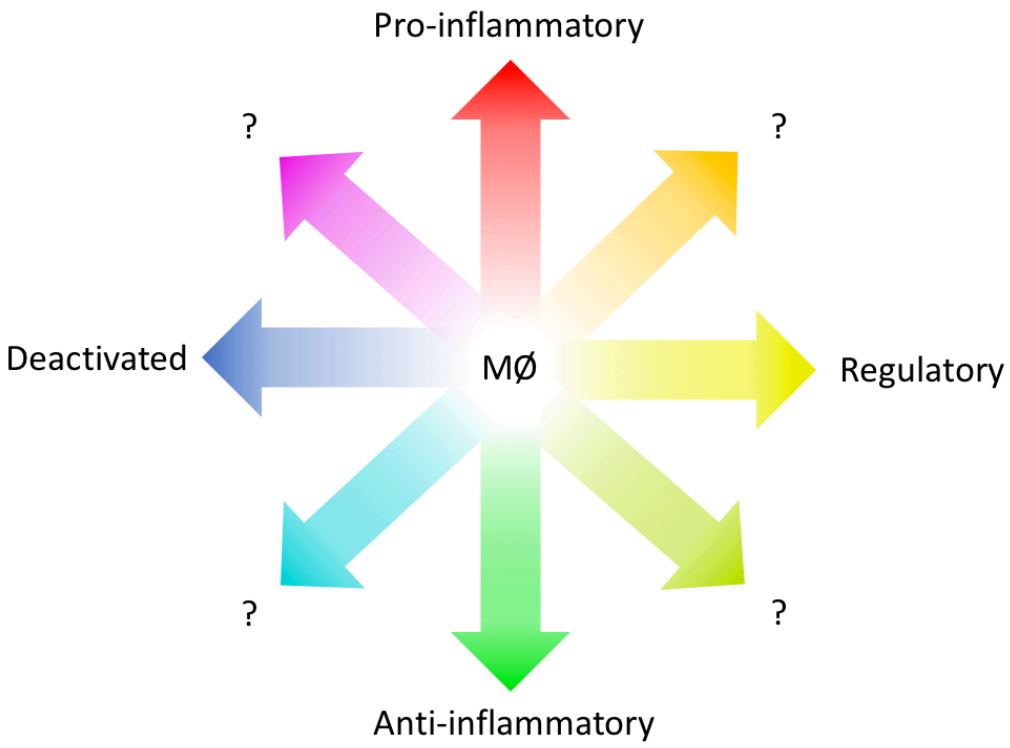


Figure 1.5: A representation of the spectrum model of macrophage polarisation.

The spectrum model proposes that macrophages can fall anywhere within the continuum, therefore they have the ability to display a mixture of phenotypes.

This concept, however makes defining macrophages and understanding their role in diseases difficult. Until cutting edge techniques such as single cell analysis are made cheaper and more accessible, the linear model for macrophage polarisation, along with defining macrophage marker specificity, will still be used to molecularly and biologically understand the role of macrophages in disease. Currently, techniques such as RNA-sequencing are now used more frequently and are helping to bridge the gap between broad M1 and M2 characterisation (Xue *et al.*, 2014) but macrophage polarisation and their functions in disease remain poorly understood.

1.7.8 Polarisation states

A change in macrophage nomenclature has been suggested by Murray *et al.* (2014) in light of: the increasing number of' "artificial" polarisation states, such as M1 and M2, the increasing range of polarising signatures that can result in the same phenotype, and the lack of specific markers. Murray *et al.* (2014) have proposed macrophages should instead be defined by their polarisation (or activation) stimuli, rather than the "artificial" M1/M2 definition. Consequently, as recommended by Kratz *et al.* (2014) markers used to identify macrophage polarisation states would therefore be based on stimuli-induced-signalling pathways, rather than related to artificial subsets, making the identification of different macrophage phenotypic states and interpreting macrophage studies, easier.

Macrophages polarised in this study will be defined by their polarising stimuli, for example M(LPS, IFNγ) rather than as M1, as suggested by Murray *et al.* (2014) and used by a number of groups (Spiller *et al*., 2016; Becker *et al.*, 2015; Sudan *et al*., 2015). To make it less confusing, due to most previous studies using the old concept to describe macrophage phenotypic subsets, M1, M2a, M2b, M2c nomenclature will be used when generalising and when referring to other studies. For ease of interpretation of the results in this study to those from previous studies using the old nomenclature, Table 1.3 shows how the macrophages produced and defined in this study relate to the old concept of macrophage phenotypic subsets.

Table 1.3: Macrophage phenotypes commonly described in the literature and their relation to the differently polarised macrophages in this study.

Macrophage phenotypic subset	Macrophage states defined in this study by their polarising stimuli
M1	M(LPS,IFNγ)
M2a	M(IL-4)
M2c	M(IL-10), M(GC)

GC, glucocorticoid; IFNγ, interferon gamma; IL, interleukin; LPS, lipopolysaccharide;

1.7.9 Macrophage cell lines

In vitro investigations into human macrophage polarisation and function are commonly carried out using primary, peripheral blood mononuclear cell (PBMC) derived macrophages, also known as monocyte-derived macrophages (MDMs), however, their impracticality and heterogeneity has resulted in the use of 31 monocyte-like cell lines (Drexler, 2002) which allow for an indefinite supply of homogenous monocytic cells. The most commonly used monocyte cells lines for investigations into macrophage biology are discussed in more detail.

1.7.9.1 THP-1 cell line

The THP-1 cell line, a promonocytic cell line derived from the blood of a patient with acute monocytic leukaemia, is one of the most commonly used myeloid cell lines displaying monocyte-like properties that are used to study macrophages (Drexler, 2002; Qin, 2012). Maturation of THP-1 monocytes towards macrophages requires pre-treatment with a protein kinase C (PKC) activator which include 1,25-dihydroxyviamin D_3 (VD_3), retinoic acids or phorbol 12-myristate 13-acetate (PMA), the latter being the most common agent used for THP-1 maturation (Chanput *et al.*, 2014; Chanput *et al*., 2013; Shiratori *et al.*, 2017; Daigneault *et al.*, 2010; Park *et al.*, 2007; Genin *et al*., 2015). This is due to the ability of PMA to produce THP-1 derived macrophages that more closely resemble primary macrophages as a result of activation of different signalling pathways such as upregulation of anti-apoptotic molecules which VD_3 was not found to upregulate (Schwende *et al*., 1996; Daigneault *et al*., 2010). Pre-treatment with PMA results in a resting macrophage phenotype, M(0), the precursor to polarised macrophages. Cells adhere to cell culture plastic and exhibit an increase in cell size and no proliferative ability. The use of high concentrations of PMA, however, has been suggested to result in a bias towards a M1 phenotype prior to the addition of polarising cytokines (Maess *et al*., 2014), which can make comparing studies difficult, especially due to the lack of consistency in concentration and exposure time to PMA (Lund *et al.*, 2016). This is especially important when polarisation towards an M2 phenotype is carried out and highlights the need for a standardised THP-1 model for macrophage studies.

THP-1 monocytes, matured towards a M(0) cell, can be polarised towards an M1 or M2 phenotype that express known M1 and M2 markers (Chanput *et al.*, 2013), secrete phenotype-specific cytokines and carry out relevant functions, using the same polarising stimuli as described previously for human MDMs, verifying their use as a model to study human macrophages (Shiratori *et al.*, 2017). Although the same method for polarisation is used, Shiratori *et al.* (2017) compared

polarised MDMs with polarised THP-1 derived macrophages and identified a number of markers that differed in their expression levels, mainly in M2 polarised macrophages, and suggested a restricted ability of THP-1 cells to polarise towards a M2 phenotype compared to MDMs. This could be due to the use of 50ng/mL of PMA over 48 hours to mature THP-1 monocytes into macrophages in this study, which has been shown to bias polarisation towards an M1 phenotype (Park *et al.,* 2007).

Importantly, THP-1 cells, due to their cancerous origin, contain genetic defects which are continuously altered with increasing passages. Partial deletion of the phosphatase and tensin homolog (*PTEN*) gene in cancerous cell lines, including THP-1 cells, has been described in a number of studies (Dahia *et al*., 1999) and is thought to be a major contributor to the characteristics of the THP-1 cell line such as immortalisation (Adati *et al*., 2009). Considerable differences remain between THP-1 cells and monocytes (Daigneault *et al*., 2010), for example THP-1 monocytes express low levels of CD14 unlike primary monocytes and therefore are less responsive to LPS (Bosshart and Heinzelmann, 2004). Despite this difference in CD14 expression, microarray data comparing responses of THP-1 and human monocytes to LPS, showed considerable similarities in expression profiles at the RNA level using real time PCR (Sharif *et al*., 2007). At the protein level, however, THP-1 cells have been found to secrete lower levels of IL-18 and do not secrete cytokines IL-6 and IL-10 compared to human monocytes in response to LPS (Schildberger *et al.,* 2013). Treatment with PMA, however, does induce the expression of CD14 as well as CD11b on matured THP-1 derived macrophages (Aldo et al., 2013; Schwende et al., 1996) suggesting THP-1 derived macrophages may be more representative of human MDMs compared to THP-1 monocytes and human monocytes. Overall, their similarities to primary macrophages allow them to be used to understand a wide range of macrophage functions including polarisation, antigen presentation, cytokine secretion, cell interactions, and phagocytosis. The advantages and disadvantages of using primary cells and THP-1 cells can be found in Table 1.4.

1.7.9.2 U937 cell line

The oldest myeloid cell line, U937, is another frequently used cell line derived from a histiocytic lymphoma of a patient with myeloid leukaemia (Drexler, 2002). U937 cells are a more mature form of monocytes compared to THP-1 cells, having been derived from tissue, yet U937 cells also require a PKC activator for monocyte maturation, of which PMA is again most commonly used. Although very similar cell lines, considerable differences in cytokine and chemokine

expression in result of treatment with LPS have been identified compared to that of THP-1 and MDMs, where only 7 out of 34 genes analysed using real time PCR were found to show similar expression levels (Sharif *et al*., 2007). Contamination of LPS, however, could have affected activation of TLR2, inducing activation of other signalling pathways (Hirschfeld *et al.*, 2000). U937, like THP-1 cells, have mutations that allow these cells to continually proliferate which may affect downstream signalling cascades. The PU.1 transcription factor and its activation of the oncogene MEIS1 have been implicated in the ability of U937 cells to proliferate (Zhou *et al*., 2015; Kumar *et al*., 2009). U937 cells isolation from tissue and their expression of different cytokines and chemokines could suggest that they may represent a model for tissue resident macrophages rather than monocyte-derived macrophages. It is therefore important to consider the biological question when choosing a cell line.

1.7.9.3 Mono Mac 6 cell line

The Mono Mac 6 cell line is another monocytic cell line derived from the blood of a patient with monoblastic leukaemia which expresses CD14, unlike THP-1 cells. Importantly, however, this cell line is not able to produce IFN, a critical cytokine secreted by M1 polarised macrophages (Neustock *et al*., 1993).

Table 1.4: Advantages and disadvantages of cell lines versus primary macrophages

	Advantages	Disadvantages
General cell line	Lack of variation between cells; Immortalised cell line, therefore unlimited supply; Doubling time is high; Safe to use; Can be kept in liquid nitrogen and removed without affecting cell viability and function; Culture time to macrophages is shorter than primary cells.	Derived from cancerous cells; Requires artificial stimulation to induce maturation towards macrophages; Differences in amount of gene expression and cytokine expression; Differences may be genotype dependent; Alterations in cell cycling pathway; Mutations are more likely to form due to continuous passaging.
THP-1	Shown to highly mimic primary macrophages; Able to polarise towards a M1 and M2 phenotype.	No standardised protocol for maturation into macrophages; Varied use of VD_3 and PMA for maturation.
U937	May be a model for tissue macrophages; Can be differentiated towards macrophages.	Lack of response to LPS compared to THP-1 and primary cells; M1 polarisation via LPS will be different to primary and THP-1 cells.
Mono mac 6	Expresses mature monocyte markers (M42).	Form giant cells; Used in monocyte biology not macrophage.
Primary cells	Do not require treatment for maturation to occur; Are a more reliable method to understand human macrophages.	Donor to donor variation; Limited supply; Low yield; Monocyte to macrophage maturation is time consuming; Limited use when studying interaction with other cell types; Contamination with other cells types during isolation.

Overall, each cell line has properties that are dissimilar to that of primary macrophages, however, THP-1 derived macrophages have been shown to more closely resemble monocyte-derived macrophages when compared to other monocyte-like cell lines, such as U937 and Mono Mac 6 cells. THP-1 cells are able to upregulate a greater number of monocyte and macrophage-associated genes and proteins which are found in primary monocytes and macrophages, such as lipoprotein lipase (LPL) (Auwerx, 1991). The expression of these markers has resulted in THP-1 cells being extensively use in vascular diseases, such as atherosclerosis (Qin, 2012; Auwerx, 1991). Furthermore, the ability to differentiate THP-1 monocytes towards different macrophage subsets using similar stimulation protocols to primary cells, makes these cells extremely useful for studying macrophage biology in different disease settings (Li *et al*., 2016; Genin *et al.*, 2015).

1.7.10 Macrophages as therapeutic targets

The idea that an imbalance of macrophage subsets results in the progression of different diseases, such as GCA, cancer and atherosclerosis (Ohradanova-Repic *et al*., 2018; Mei *et al.*, 2016; Fadini *et al*., 2014), and the knowledge that macrophages perform a range of pro- and anti-inflammatory functions, suggests macrophages could be good targets for cell-specific therapeutics to restore homeostasis.

Work into the targeting of macrophages in different diseases, especially cancer, has focused on a number of mechanisms, mainly through the use of murine models. One mechanism is the inhibition of monocyte recruitment to diseased sites through the suppression of chemokines (DeNardo *et al*., 2011). The suppression of CCL2/CCR2 to inhibit recruitment of monocytes has been investigated in atherosclerosis, resulting in improvement of the disease (Winter *et al.*, 2018) as well as improvement in a number of cancers, where a phase I clinical trial into Carlumab, a monoclonal antibody to CCL2, showed CCL2 suppression in solid cancers (Sandhu *et al*., 2013). A phase II clinical trial however found no suppression of CCL2/CCR2 signalling in metastatic castration-resistant prostate cancer (Pienta *et al*., 2013). Differences in the suppressive effect of Carlumab may be due to the focus on a more specific form of cancer in the Phase II clinical trial. Another mechanism is the reduction of macrophage numbers in sites of inflammation termed “macrophage depletion” (Ponzoni *et al.*, 2018). Studies into the depletion of macrophages have been carried out using chlodronate, a bisphosphonate which induces apoptosis in monocytes and macrophages through the inhibition of NFκB binding to DNA (Piaggio *et al.*, 2016;

Ries *et al.*, 2014; Barrera *et al*., 2000). The use of chlodronate has been found to be beneficial for the resolution of rheumatoid arthritis (Barrera *et al*., 2000). Furthermore, utilising macrophage plasticity, strategies into macrophage re-education by manipulating polarising pathways have been investigated to alter macrophage phenotypes (Malyshev and Malyshev, 2015; Klug *et al.*, 2013; Hagemann *et al.*, 2008). The re-polarisation of M2 pro-tumour macrophages to M1 anti-tumour macrophages to promote tumour regression has been studied. Blocking of the colony stimulating factor (CSF-1), a differentiating molecule, was found to decrease M2-like macrophages markers and prevented tumour growth in combination with cytokines released by the tumour into the microenvironment, such as IFNγ (Pyonteck *et al.*, 2013).

The phagocytic function of macrophages has been exploited to study delivery systems which, rather than effect a range of cell types, can deliver disease- and cell-specific therapeutics (Singh *et al*., 2017). One such method studied, using a mouse model of myocardial infarction, was targeting of the IRF5 transcription factor involved in macrophage differentiation, through the delivery of nanoparticles containing *Irf5* siRNA. Macrophage-specific knockdown of *Irf5* was observed with a decrease in M1 markers IL-1β and MMP9 whilst cardiac wound healing was increased (Courties *et al*., 2014). Other methods include the delivery of liposomes containing Clodronate which are phagocytosed by macrophages (Piaggio *et al*., 2016) as well as delivery of IL-10 encoded plasmid DNA via nanoparticles to macrophages inducing re-polarisation of M1 towards an M2 phenotype in experimental arthritis (Jain *et al*., 2015).

In inflammatory diseases, which require macrophages for both the inflammatory response as well as the resolution of disease, reprogramming macrophages is seen as a more useful therapeutic method for disease resolution, targeting cytokine-specific signalling pathways by delivering therapeutics directly to macrophages. The deletion of subset-specific macrophages is also seen as an important avenue for therapeutic targeting. The challenge, however, is characterising macrophage subsets in disease, identifying those which drive disease progression and therefore targeting these subsets specifically. Safety and efficacy of these drug delivery therapies is yet to be determined but provides an opportunity to develop new therapeutics which can be personalised for each individual.

1.8 Macrophages in GCA

1.8.1 Circulating vs resident macrophages

Macrophages are derived from monocytes which originate from myeloid progenitor cells, and are released from the bone marrow, circulating throughout the body until they are attracted to inflamed or damaged tissue, receiving cytokines and other factors that result in their maturation into macrophages. Circulating monocytes are required to replenish macrophages within areas of inflammation or wound healing.

In addition to circulating monocytes, individual tissues and organs, specifically at barrier-tissue sites, have specifically adapted, resident (or tissue) macrophages, which are thought to be derived from the embryonic yolk sac, and from birth remain within the different organ tissues (Ginhoux *et al.*, 2010; Epelman *et al.*, 2014). Barrier-tissue sites are constantly exposed to exogenous threats therefore they require resident cells, including macrophages, to maintain homeostasis, barrier integrity and prevent unwanted inflammatory responses (Mowat *et al.,* 2017). Resident macrophages are thought to replenish themselves without the need for the recruitment of circulating monocytes and are distinct from human monocyte derived macrophages (hMDM) (Ajami *et al.*, 2007; Hashimoto *et al*., 2013). Their adaptation to different tissues and different sites within the same tissue, for example within the intestine (Asano *et al.,* 2015) and the skin (Mowat *et al.,* 2017), suggests resident macrophages carry out specific tissue- and organ-related functions. This adaptation is thought to be induced by the surrounding microenvironment.

In a model of cardiac injury, resident macrophages have been described as displaying an anti-inflammatory phenotype, preventing inflammatory responses during inflammation and contributing to wound healing (Lavine *et al.,* 2014), whereas recruited hMDMs to areas of inflammation are found to be pro-inflammatory (Lavine *et al*., 2014). The shortcomings of these studies are their sole use of murine models and therefore results may not fully transfer to human biology.

Studies into macrophages in GCA have focussed on the concept of infiltrating macrophages and their polarisation within the artery wall rather than gaining an understanding of both infiltrating and resident macrophages. It may be important to consider the artery as a barrier-site, like the skin and the lungs, and consider the functions of macrophages in maintaining this site. It would also be important to consider whether resident macrophages were found within the different artery

wall layers and whether these cells had layer-specific functions, such as maintaining barrier integrity. Evidence to suggest that resident and recruited macrophages respond differently to an inflammatory response also highlights the importance in designing macrophage-specific therapies. The lack of studies into resident macrophages in GCA leaves a large gap in the understanding of macrophage biology in GCA as well as the pathogenesis of the disease and its treatment.

1.8.2 Identification of macrophage phenotypes

Macrophage behaviour with regards to GCA pathophysiology is largely unexplored, as explained by Weyand *et al*. (2012) “it is unknown whether macrophages in the GCA lesions differentiate *in situ*, which signals regulate their functional commitment, how long they survive, and how they contribute to the distinct phases of the disease”.

Various markers have been used to identify macrophages and their phenotypes in GCA positive arteries and commonly CD68 is used to identify all macrophages within the artery (Rodriguez-Pla *et al*., 2005; Rittner *et al*., 1999a; Rittner *et al*., 1999b; Kaiser *et al*., 1998; Weyand *et al*., 1996). The use of markers for the identification of specific macrophage polarisation states within the artery wall is very limited.

From immunohistochemistry studies, we know that macrophages are key players in the pathogenesis of disease. They have been observed to display specific functional phenotypes which correlate to the immunological processes occurring at that location. For example, an initial immunohistochemistry study by Weyand *et al.* (1996) into macrophages in GCA temporal artery biopsies identified macrophages within the adventitia which displayed a pro-inflammatory $CD68^{+}TGF\text{-}\beta1^{+}IL\text{-}1\beta^{+}IL\text{-}6^{+}iNOS^{-}$ phenotype. At the intima-media border they exhibited a tissue destructive $CD68^{+}MMP\text{-}2^{+}$ phenotype, thought to cause fragmentation of elastin and destroy the IEL. Within the intima they expressed $CD68^{+}iNOS^{+}TGF\text{-}\beta^{-}$, another macrophage-destructive phenotype, whereas at the base of the neointima they are found to be $CD68^{+}TGF^{-}\beta1^{+}$ which promote wound healing. Additional studies have identified further molecules expressed by location-specific macrophages which relate to the histological change. Kaiser *et al.* (1998) identified macrophages within the media and at the media-intima border which secreted PDGF whilst corroborating findings by Weyand *et al.* (1996) observing $CD68^{+}MMP\text{-}2^{+}PDGF^{+}$ macrophages at the media-intima border. PDGF expression correlated with migration of cells from the media

towards the intima, contributing to intimal hyperplasia. $CD68^{+}MMP2^{+}$ medial macrophages were also confirmed by Rittner *et al*. (1999b), however, ROS-induced damage was only identified within the media and correlated with $CD68^{+}MMP2^{+}$ macrophages, contradicting the observation by Weyand *et al.* (1996) of CD68+iNOS+ macrophages within the intima. This contradiction may be in result of differences in defining areas of the artery wall of which there is no specific guidelines, definitions of artery layers in this study are described in Section 2.7.5. Kaiser *et al.* (1999) described media-intima residing macrophage found close to neovessels also secreted the growth factor, VEGF, involved in neovascularisation. Additional publications went on to identify MMP2+ macrophages at the media-intima border also expressed MMP9 which they found was associated with IEL destruction, intimal hyperplasia and occlusion, suggesting MMP9 rather than MMP2 contributes to disease progression (Rodriguez-Pla *et al.,* 2005).

Only more recent publications have attempted to characterise subset-specific macrophage in GCA artery biopsies. Ciccia *et al.* (2013), using 20 patients who had artery biopsies prior to glucocorticoid treatment, defined M1 macrophages as $iNOS^{+}$ and found they correlated with IFNγ levels and defined M2 macrophages as $CD163^{+}$ which correlated with levels of IL-33, a cytokine associated with both pro- and anti-inflammatory properties (Pastorelli *et al*., 2013). The sole use of iNOS however has been described as unsuitable to define M1 macrophages (Barros *et al.,* 2013) while the use of CD163 as a M2-specific marker is controversial as it has been suggested to be used as a pan-macrophage marker (Bertani et al., 2017; Cai et al., 2014; Barros et al., 2013; Barros et al., 2012), therefore M1 macrophages may have been wrongly described as a M2 phenotype in the Ciccia *et al.* (2013) study. Additionally, many biopsies are taken after the beginning of glucocorticoid treatment, and therefore the effects of glucocorticoids may result in phenotypic skewing towards an M2c phenotype and therefore inducing CD163 expression. Furthermore, the reliance on CD68 as a marker for macrophages in all these studies may have resulted in mischaracterisation of cell types as CD68 has also been observed to be expressed on DCs (Vakkila *et al.*, 2005). A schematic to show these different macrophage functions can be seen in Figure 1.6.

Whether macrophages in the artery wall are recruited from circulating monocytes or whether they originate from resident macrophages is unknown. Additionally, no defined M1 or M2 macrophage phenotypes have been described within the

artery wall using appropriate subset-specific markers (Ciccia *et al.,* 2013). Furthermore, the age of some of the studies reference above has implications on their research as well as future GCA studies. The use of old methods of immunohistochemistry means specificity of antibodies may be lower, along with lower sensitivity, compared to the use of polymer-based detection systems, therefore older studies may wrongly identify epitopes and may not identify low expressing protein markers (Ramos-Vara and Miller, 2006). It also highlights the lack of studies into macrophages in this disease area and therefore, unlike in cancer research, the application of new research findings into macrophage biology is not applied as quickly in GCA. Furthermore, the lack of studies results in the reliance of interpreting macrophage biology in other disease settings such as atherosclerosis and rheumatoid arthritis.

Overall, interpretation of these findings suggests macrophages found at different locations within the artery wall can be associated with either a pro-inflammatory M1 macrophage phenotype or an anti-inflammatory, tissue reparative M2 phenotype and may be characterised using different markers specific to their subset. The different roles of macrophages in the pathophysiology of GCA are described in more detail.

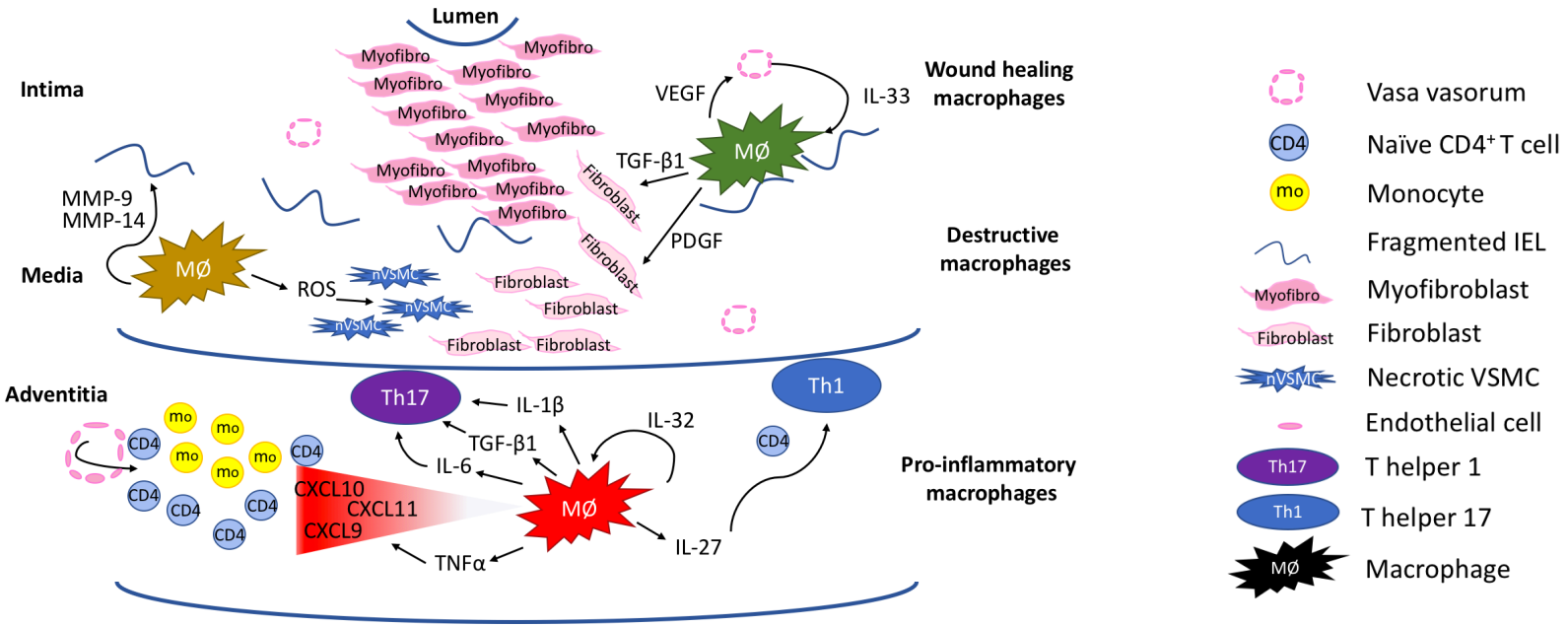


Figure 1.6: Schematic showing the different functions macrophages are thought to perform within the different areas of the artery wall in GCA.

Adventitial macrophages recruit monocytes and naïve T-cells via secretion of chemokines CXCL9, -10 and -11, inducing polarisation of naïve T-cells into Th1 and Th17 cells via IL-27 and IL-1β, TGF-β1 and IL-6, respectively. Macrophages release TNFα promoting leukocyte recruitment while IL-32 promotes further release of pro-inflammatory cytokines IL-1β, TNFα, TGF-β1 and IL-6 by macrophages. In the media, macrophages produce destructive mediators metalloproteinase -9 and -14 which degrade the internal elastic lamina, and reactive oxygen species which causes necrosis of vascular smooth muscle cell. Macrophages close to the intima-media border are found to release vascular endothelial growth factor, promoting neovascularisation, as well as growth factors platelet-derived growth factor and TGF-β1, inducing fibroblast migration from the media towards the intima, their differentiation into myofibroblasts and subsequent proliferation. Endothelial cells release IL-33 in response to stress, inducing M2 polarisation. Due to the different mediators and cytokines secreted by macrophages in the different layers of the artery wall, macrophages have been suggested to perform pro-inflammatory, destructive or wound healing functions. CXCL, chemokine (C-X-C) ligand; IL, interleukin; MMP, metalloproteinase; PDGF, platelet-derived growth factor; ROS, reactive oxygen species; TGF-β, transforming growth factor beta; VEGF, vascular endothelial growth factor; VSMC, vascular smooth muscle cell.

1.8.3 Cytokine and chemokine secretion

The release of cytokines IL-1β, IL-6, and TNFα by macrophages in GCA, observed using immunohistochemistry, is mainly localised to granulomas at the intima-media border and within the adventitia, in which a high degree of pro-inflammatory cells can be seen (Hernandez-Rodriguez *et al.,* 2004).

As explained earlier, cytokines released by M1 macrophages are potent pro-inflammatory cytokines (Section 1.7.3). IL-6 is pleiotropic and has been implicated in promoting systemic inflammation through inducing acute-phase protein release as well as the recruitment of further T-cells and contributing to T-cell differentiation and the imbalance of Th17 and Treg cells (Roche *et al*., 1993; Gauldie *et al*., 1987; Bettelli *et al.*, 2006). In GCA, a greater level of IL-6 and TNFα protein expression was found to significantly correlate with a greater acute-phase response and a stronger systemic reaction in patients. IL-1β was also found to correlate but was not found to be significant (Hernandez-Rodriguez *et al.,* 2004). Furthermore, the greater the transcript level of TNFα, the longer patients took to respond to glucocorticoid therapy (Hernandez-Rodriguez *et al.,* 2004) IL-6 stimulation of SAA secretion from macrophages has been associated with vascular remodelling in the intima due to SAA induction of VEGF and MMP-9 release from VSMCs as well as promoting myofibroblast migration and proliferation. Furthermore, IL-6 has been shown to promote M2 polarisation, with upregulation of STAT3 and subsequent IL-10 gene expression, as described previously (Braune *et al*., 2017).

Pro-inflammatory cytokines IL-32 and IL-27 have been implicated in a number of diseases including rheumatoid arthritis (Joosten *et al.*, 2006) and Crohn's disease (Netea *et al.*, 2005) and have more recently been observed by Ciccia *et al.* (2011) within the inflamed arteries of GCA patients at the RNA and protein level and are thought to be secreted by macrophages. High IL-32 expression was identified in both macrophages and giant cells within GCA arteries and studies have identified IL-32 as inducing the formation of osteoclasts, a giant multinucleated cell (Kim *et al*., 2010). Additionally, they found IL-32 correlated with high IL-27 expression, a cytokine which has been implicated in the polarisation of naïve T-cells towards a Th1 phenotype, prior to IL-12 polarisation (Owaki *et al*., 2005). Together these two cytokines induce pro-inflammatory cytokine production, including IL-6, IL-1β and TNF (Netea *et al.*, 2005; Kim *et al.*, 2005) and therefore IL-32 and IL-27 could act as a feedforward mechanism in macrophages to promote inflammation within the artery wall.

Macrophages within the adventitia have been shown to produce chemokines CXCL9, CXCL10 and CXCL11 (Corbera-Bellalta *et al*., 2016), stimulating the recruitment of naïve T-cells and monocytes into the vascular wall, promoting further inflammation.

1.8.3.1 IL-33

IL-33, a cytokine described as a possible inducer of M2 polarisation has been observed within the artery wall of GCA patients (Ciccia *et al.,* 2013). Within GCA arteries, Th2 cells have not been identified, however, their associated cytokine IL-4, although not detected at the mRNA level, has been observed to be overexpressed within the artery wall using immunohistochemistry (Ciccia *et al.,* 2015). It is unknown which cell types are the main producers of these two cytokines.

IL-33 has been implicated in a number of inflammatory diseases including rheumatoid arthritis and is thought to contribute to inflammation (Pastorelli *et al*., 2013; Duan *et al*., 2013). IL-33 has been shown to be secreted by damaged endothelial cells as well as activated DCs in GCA (Ciccia *et al.,* 2017). IL-33 has been found to induce the polarisation of naïve macrophages into a M1 phenotype but it also enhances a M2 macrophage phenotype (Joshi *et al*., 2010). In addition IL-33 promotes a Th2 response by polarising naïve T-cells into a Th2 phenotype, inducing the release of IL-5, IL-4 and IL-25 (Furukawa *et al*., 2017).

The role of IL-33 in macrophage polarisation remains controversial as it has been suggested IL-33 can affect macrophages polarisation in different ways. For example, IL-33 has been shown to polarise splenic and lymph node murine macrophages towards a MRC1 expressing M2 phenotype, in an *in vivo* murine model of EAE (Jiang *et al*., 2012). M2 polarisation however did not occur in an *in vitro* cell culture model using murine bone marrow-derived macrophages (BMDM) (Kurowska-Stolarska *et al.*, 2009) but expression of chemokines was only used to define macrophage phenotypes in this study. Furthermore, use of murine macrophages from different lineages, along with investigations in diseased and healthy murine macrophages may impact differences in findings of these two studies.

In contrast to murine macrophages, IL-33 was found to polarise human M0 BMDMs towards a M1 phenotype but could not induce M2 polarisation, however IL-33 was found to re-polarise M1 macrophages towards an M2 phenotype (Joshi *et al*., 2010). The addition of M2 polarising stimuli, along with IL-33, promoted enhanced M2 polarisation in both murine and human BMDMs and human MDMs

(Joshi *et al*., 2010; Kurowska-Stolarska *et al.*, 2009). IL-33 has also been found to promote both pro- and anti-inflammatory responses in different murine models of inflammation (Palmer *et al*., 2009; Miller *et al*., 2008), therefore the microenvironment may be fundamental to the effects of IL-33. The lack of studies in humans and the inconsistencies found between murine models and humans makes it hard to draw conclusions.

In GCA temporal artery biopsies Ciccia *et al.* (2013) found increased expression of IL-33 and its receptor, suppression of tumorigenicity (ST2), localised to endothelial cells of neovessels. Increased IL-33 expression in addition correlated to an increase in inflammatory infiltrates within the vessel wall. Furthermore, IL-33 mRNA levels were associated with a significant increase in $CD163^{+}$ M2 macrophages, with levels of IL-33 protein returning to control levels after glucocorticoid treatment. Interestingly, however, no Th2 related cytokines such as IL-5 and IL-4 were identified at the RNA level, confirming studies in which gene expression of IL-4 is consistently absent in GCA (Watanabe *et al.,* 2017; Ciccia *et al.,* 2015; Ciccia *et al.,* 2013; Terrier *et al*., 2012). IL-4 however was found to be overexpressed at the protein level in GCA arteries (Ciccia *et al.,* 2015). CD163 may not identify M2-specific macrophages in GCA, however and therefore IL-33 may induce M1 and M2 phenotypes separately or M1 and M2 macrophage phenotypes concurrently within the artery wall.

1.8.4 Contribution to structural changes of the vessel wall

The ability of macrophages to carry out pro-inflammatory and tissue remodelling functions means they can be both destructive and reparative. As explained previously, macrophages most likely carry out both these functions within different areas of the artery wall in GCA, resulting in distinct structural changes.

1.8.4.1 Tissue destruction

Macrophages are known to release a wide range of different mediators which when uncontrolled, can result in tissue destruction. In GCA, macrophages have been implicated in the destruction of the media and the IEL, through the release of MMPs, reactive oxygen intermediates, and chemokines, thus enabling migration of fibroblasts and inflammatory cells into the intima from the media (Rittner *et al.,* 1999b).

MMPs belong to a family of proteases that are secreted as a proenzyme that require cleavage for their activation. Together this family allows the breakdown of all extracellular matrix components in order to allow for tissue remodelling,

however, dysregulation of their release ultimately results in tissue destruction. Macrophages have been shown to release a number of MMPs, which are controlled through exposure to a range of factors including cytokines, growth factors and interactions with other cell types. In addition to these controlling factors, MMP's activities can be prevented through their binding to tissue inhibitors of MMPs (TIMPs).

In GCA, a number of MMPs have been identified that have been suggested to contribute to the degradation of the IEL via destruction of elastin as well as intimal hyperplasia and luminal occlusion. MMP-12 has been identified as being highly upregulated in GCA artery biopsies, along with upregulation of MMP-9 (Rodríguez-Pla *et al.*, 2009), MMP-2 (Rittner *et al.,* 1999b; Weyand *et al*., 1996) and MMP-14 . Using gelatin zymography, Segarra *et al.* (2007) were able to show MMP-2, MMP-9 and MMP-14 are found in their active form within highly inflamed pan-arteritic artery biopsies. Furthermore, using immunohistochemistry Segarra *et al.* (2007) also observed expression of MMP-2, MMP-9 and MMP-14 by leukocytes and described greater destruction of extracellular matrix in areas where all three MMPs were found, due to their coordinated regulation which is described in Figure 1.7.

Protein expression of MMP-9 and MMP-14 has additionally been associated with the breakdown of elastin due to their expression surrounding granulomas and the destructive macrophages at the IEL border (Segarra *et al.*, 2007; Nikkari *et al.*, 1996; Sorbi *et al*., 1996). However, a later microarray study using 19 GCA positive biopsies suggested only *MMP-9* and *MMP-12* are overexpressed within the artery wall (Rodríguez-Pla *et al*., 2009). Furthermore, there is controversy around the contribution of MMP-2 by macrophages in light of the lack of mRNA expression difference between GCA and healthy artery tissue (Rodríguez-Pla *et al*., 2009; Rodriguez-Pla *et al*., 2005). However, these studies failed to investigate both expression at the protein level and activation within the artery wall unlike Segarra *et al.* (2007). Furthermore, this lack of difference in mRNA expression may be expected as cleavage is the critical mechanism that allow MMPs to carry out their remodelling functions and therefore regulation at the RNA level is not essential. Segarra *et al.* (2007) were able to show that unlike in healthy controls, MMP-2, MMP-9 and MMP-14 are found to be in their active form in GCA patients. It could therefore be assumed that MMP-12 may not be functionally active in GCA lesions. Expression of TIMP1 and TIMP2 to inhibit MMP-9 and MMP-2, respectively have been found in GCA positive arteries yet both ratios of TIMPs to MMPS is lower compared to healthy controls, suggesting a lack of MMP regulation in GCA positive arteries (Segarra *et al*., 2007; Tomita and Imakawa,

1998), supporting the association between elevated levels of MMPs and destruction of artery wall structures.

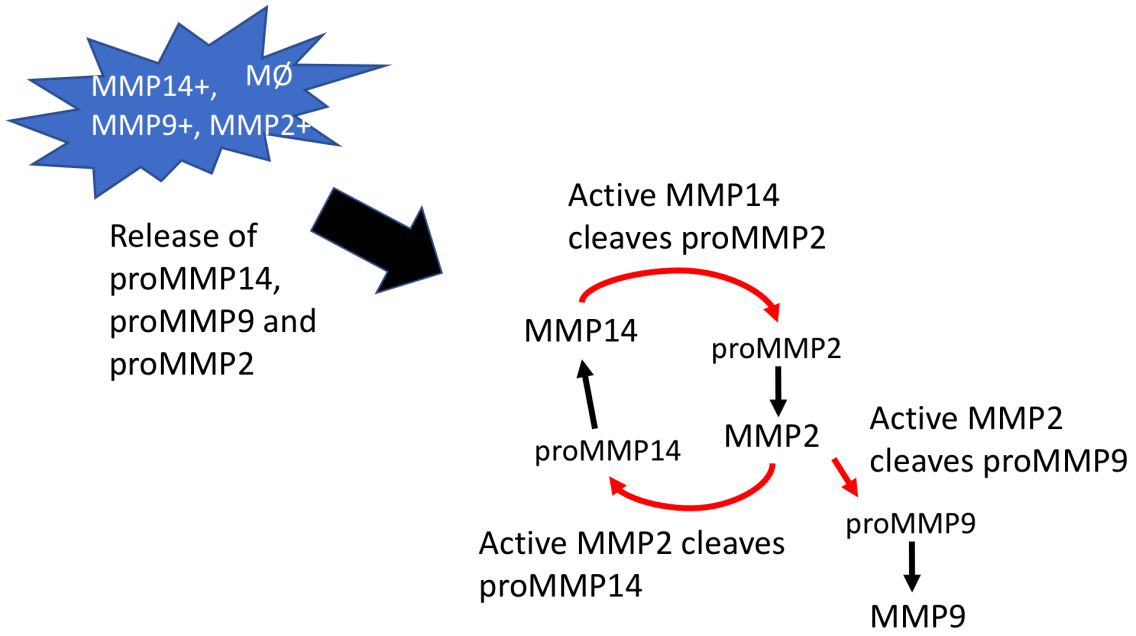


Figure 1.7: The process of metalloproteinase self-activation and regulation.

Cleavage of inactive pro-metalloproteinases to their active form is co-ordinated by other active metalloproteinases. MMP, metalloproteinase.

In addition to MMPs secreted by macrophages within the media and at the IEL border, ROS and reactive nitrogen intermediates (RNIs) have also been shown to be co-expressed by macrophages at these sites as well by MGCs (Rittner *et al.,* 1999b). It is thought that macrophages contribute to further tissue destruction both directly and indirectly through oxidative stress-induced apoptosis via lipid peroxidation and cleavage of MMP proenzymes, respectively (Rittner *et al*., 1999b; Wagner *et al*., 1996). Production of ROS, specifically iNOS through the arginine metabolism pathway, has been associated with a pro-inflammatory, M1 phenotype and contributes to the elimination of invading pathogens (Mantovani *et al*., 2004). Rittner *et al.* (1999b) identified a subset of highly destructive $CD68^{+}$ macrophages, expressing MMP-2 along with high levels of mitochondrial activity with additional peroxidised lipids on their surface membranes using immunofluorescence, suggestive of ROS production from macrophages in GCA positive arteries. Using immunohistochemistry these macrophages were found within areas of high tissue destruction, including the media, EEL and IEL, co-localising to areas with ROS-damaged cells, whereas ROS-damaged cells were not found within the adventitia or the intima. In addition to this, Ciccia *et al*. (2013)

utilised iNOS for the identification of M1 macrophages in GCA positive arteries. Similarly to MMPs, a mechanism of regulation of oxidative stress caused by ROS has been identified in GCA arteries to prevent tissue injury which has been attributed to macrophages, T-cells and VSMCs and their release of aldose reductase (Rittner *et al*., 1999a). Using a human temporal artery-SCID mouse chimera Rittner *et al.,* (1999a) found inhibition of aldose reductase resulted in increased downstream products of lipid peroxidase and an increase in apoptotic cells.

Release of MMPs and ROS, along with a lack of regulation of both these mechanisms, can therefore combine to result in the destruction of the IEL, of which can be seen in Figure 1.8.

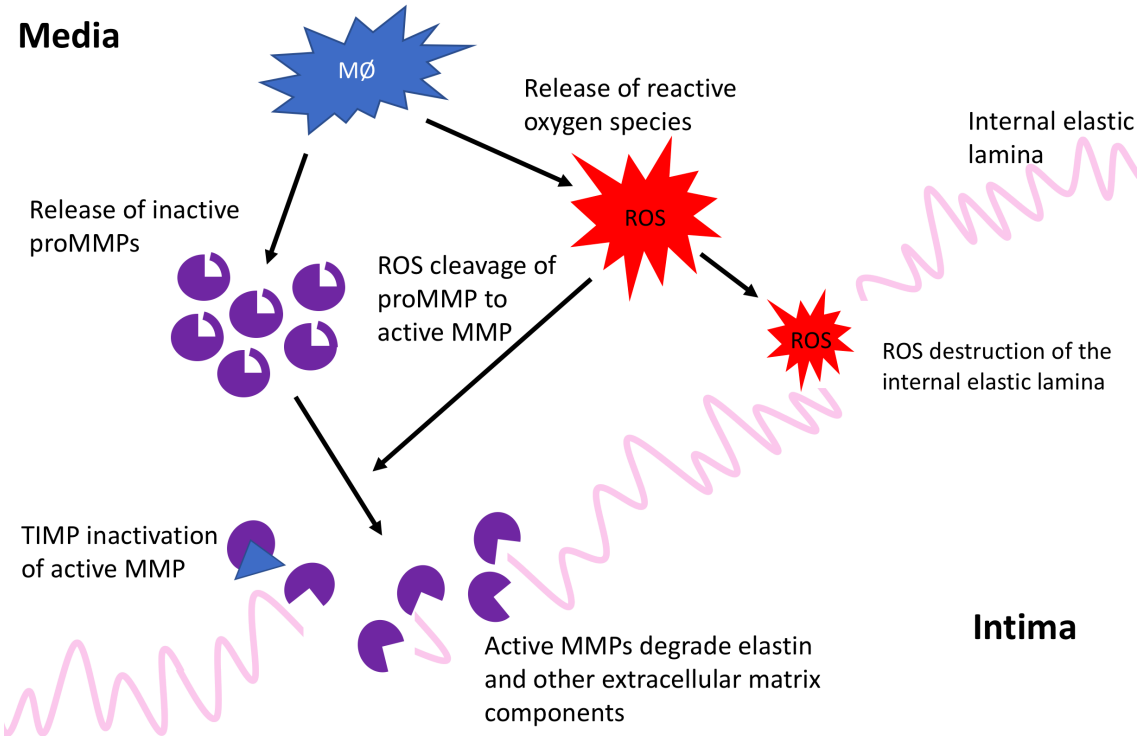


Figure 1.8: The relationship of metalloproteinases and reactive oxygen species in the destruction of the internal elastic lamina.

Macrophages release metalloproteinases in the form of inactive proenzymes. Cleavage of the proenzyme to its active form occurs through the action of other activated metalloproteinases and the release of reactive oxygen species by macrophages. Secretion of reactive oxygen species and the activation of metalloproteinases, coupled with the low ratio of tissue inhibitors of metalloproteinases to metalloproteinases, results in a system that destroys the extracellular matrix components of the media and the internal elastic lamina, allowing the migration of cells towards the intima and development of the neointima. MØ, macrophage; MMP, metalloproteinase; ROS, reactive oxygen species; TIMP, tissue inhibitor of metalloproteinases.

1.8.4.2 Tissue repair

A number of growth factors have been detected within arteries of GCA patients including VEGF, PDGF and TGF-β which contribute to the 'repair' of tissue within the artery.

Macrophages and MGCs found on the media-intima border have been shown to be the main producers of VEGF via observation of co-expression of CD68 and VEGF using immunohistochemistry (Rittner *et al*., 1999b). VEGF is known to induce angiogenesis and has been associated with the abnormal formation of neovessels within the media and intima of GCA positive arteries, which normally form exclusively in the adventitia of healthy arteries. The formation of additional neovessels within the media and intima is thought to contribute to the recruitment of further immune cells to promote both the pro-inflammatory and anti-inflammatory response within the artery wall.

In addition to VEGF, macrophages found at the IEL and the media-intima border are also the main produces of PDGF identified using immunohistochemistry and dual staining of CD68 and PDGF on temporal artery biopsies (Kaiser *et al*., 1998). Therefore, they may contribute to hyperplasia within the intima and subsequent luminal occlusion due to the effect of PDGF on the migration of VSMCs from the media into the intima and subsequent effects on their proliferation. The degree of PDGF secretion has been shown to correlate with the level of hyperplasia and luminal occlusion that occur within the artery (Kaiser *et al*., 1998). Furthermore, increased acute phase protein, SAA, has been found to induce VEGF secretion as well as VSMC proliferation and migration in a GCA model (O'Neill *et al*., 2015).

Macrophages within the adventitia have been identified to secrete TGF-β1 and it has been postulated that TGF-β1 contributes to the differentiation of adventitial fibroblasts to myofibroblasts and promotes their migration towards the intima (Wagner *et al*., 1996; Shi *et al*., 1996). This concept is still debated as the origin of intimal myofibroblasts is still unknown and multiple hypotheses exist, including VSMC dedifferentiation via PDGF (Owens *et al*., 2004). Additionally, macrophages found at the base of the neointima have also been identified to secrete TGF-β1 and are thought to contribute the differentiation and migration of myofibroblasts within the intima, adding to the degree of hyperplasia (Wagner *et al*., 1996). The study by Weyand *et al*. (1996) relies heavily on interpretation however due to the inability of antibodies to identify the active form of TGF-β1 from the inactive form. Moreover, overexpression of TGF-β1 has been associated with fibrosis when secreted by macrophages, with hepatic studies showing significant decrease in fibrosis when TGF-β1 macrophages were depleted.

1.9 Summary and hypothesis

GCA is a highly heterogeneous disease whereby patients present with varying clinical symptoms and histological features and respond differently to glucocorticoid treatment. To date, no study has investigated macrophage polarisation with validated phenotype-specific macrophage markers. Consequently, there is a limited understanding of the importance of M1 and M2 macrophage phenotypes within the inflamed arterial wall of GCA patients and the contribution of macrophages to histological changes and different disease manifestations.

I hypothesise that the heterogeneity of histological and clinical manifestations seen between individuals with GCA is in part due to the phenotypic heterogeneity of macrophages found within the artery wall of different patients. This phenotypic heterogeneity is hypothesised to be in result of:

1. The stage at which a patient's disease is identified (i.e. inflammatory response or healing phase).
2. Functional differences of M1 and M2 polarised macrophages which may correlate with destructive or tissue reparative histological features, respectively due to the stage of disease.
3. Differences in the pattern (numbers and location) of infiltrating macrophages across the arterial wall layers.
4. An imbalance of macrophage polarisation towards greater M1 or M2 subsets resulting in arteries with greater artery destruction or greater tissue repair.

To investigate these hypotheses, the aims of this study were:

- To identify and validate "on" and "off" markers that distinguish between macrophage polarisation states which are suitable for immunohistochemistry.
- To characterise macrophage polarisation states in GCA temporal artery biopsies using these novel macrophages markers.
- To determine the relationship of macrophage phenotypes with histological features of artery destruction and repair.
- To identify stages of disease and their relationship with macrophage phenotypes.
- To determine the relationship of macrophage phenotypes with clinical manifestations.

Therefore, the objectives of this study were:

- To develop a cell line model to produce M1 and M2 macrophages to enable studies into macrophage marker expression in different polarisation states.
- To validate markers at the RNA and protein level using end-point PCR and western blotting to confirm their specificity for macrophage subsets, their similarity to human MDMs, as well as their use in immunohistochemistry studies.
- To utilise temporal artery biopsies and clinical data from patients recruited to the UKGCA consortium study to:
    - i) Perform immunohistochemical studies on artery biopsies using identified antibodies for macrophage phenotypic markers and other histological features.
    - ii) Use histological features to understand the phase of disease.
    - iii) Use clinical and laboratory data to understand the relationship of macrophage phenotypes with clinical manifestations including ischemic features such as permanent vision loss, jaw claudication, systemic features including weight loss, night sweats or fever, as well as systemic inflammatory markers CRP and ESR.
    - iv) To develop a scoring system of macrophage marker expression to characterise macrophage phenotypes and their location and distribution of staining within the adventitia, media and intima, as well as identifying their relationship with histological manifestations including media destruction, neovascularisation and luminal occlusion.

Chapter 2. Materials and Methods

2.1 Patients and cohorts

The UK GCA Consortium study, a multicentre observational study, was initiated in 2005 and is still ongoing. In this study, patients with a diagnosis of GCA confirmed by a specialist were recruited following the provision of informed consent. Only one single study visit was required, at which clinical details were collected on a structured case report form by a combination of medical notes review and interview with a trained researcher. Clinical details included presence of jaw claudication, permanent visual features, fever, night sweats, weight loss, systemic inflammatory markers (CRP and ESR), duration of prednisolone, time to reach 5mg of prednisolone and time to stop prednisolone. Table 2.1 provides details of the definitions of these clinical features and the method by which data on these clinical features was collected. Patient cohort outcomes described in Table 2.1 are associated with caveats as they are dependent not only on the severity of GCA, but multiple factors including presentation, professional opinion and treatment regimen.

As an integral part of the study patients consented to use of their archived, formalin-fixed, paraffin-embedded temporal artery biopsy specimen if this had previously been taken for clinical diagnostic purposes. Patients also gave permission for later access to their medical records so that the time taken to stop steroids permanently could be recorded. The clinical data relating to the patients selected for the current study have been published (Mackie *et al*., 2011). Patients used in this study were recruited between 2005 and 2009 and recruited patients were diagnosed with GCA between 1995 and 2009.

Table 2.1: Definitions of the clinical features (systemic and vascular inflammatory manifestations) that were used in this study.

Clinical feature	Definition	Method of collection
Jaw claudication	Masticatory muscle pain when chewing. Resolved with glucocorticoid treatment.	Medical notes
Permanent visual features	Visual blurring and vision loss associated with GCA activity which did not completely resolve once glucocorticoids treatment was initiated.	Medical notes
Fever, night sweats and weight loss	History of weight loss (>2kg or clothes becoming looser), night sweats or fever associated with GCA activity.	Medical notes review, and patients interview at study visit
Systemic inflammatory markers	Laboratory markers for systemic inflammation, CRP and ESR, performed using routine protocols by hospital laboratory.	Medical notes
Time to reach prednisolone dose of: 5mg and zero	Time taken (in months) for patient to become stable on a daily dose of 5mg pf prednisolone, or to permanently stop prednisolone treatment. These doses were chosen from studies of steroid toxicity in GCA (Proven *et al*., 2003).	Medical notes

CRP, c-reactive protein; ESR, erythrocyte sedimentation rate.

2.2 Ethics

Patient data and archived temporal artery biopsy specimens were originally collected within the ongoing UK GCA Consortium study, the full title of which is *Clinical and immunogenetic characterisation of giant cell arteritis (GCA) and polymyalgia rheumatica (PMR)* (Leeds East Research Ethics Committee (REC) reference number 05/Q1108/28). The current work falls within the scope of the study protocol approved by the ethics committee.

Blood samples from healthy volunteers were collected following written informed consent, obtained as part of another ongoing ethically-approved study *(Functional characterization of the genes and proteins involved in the development and severity of autoimmune and (auto)inflammatory diseases* (REC reference number 04/Q1206/107)).

2.3 Cell culture

2.3.1 Human monocyte-derived macrophage cell culture

Whole blood (15ml) was taken from consenting healthy volunteers. Blood was diluted 1:1 with Dulbecco's Phosphate Buffered Saline (DPBS) (Sigma-Aldrich, Missouri, USA). To a SepMate™tube (STEMCELL technologies, Vancouver, Canada), 15ml Lymphoprep™ (Axis-Shield, Oslo, Norway) was added and 30ml diluted blood was carefully layered on top. The tube was centrifuged at 1200xg for 10 minutes with the brake off. The top layer of peripheral blood mononuclear cells (PBMCs) was pipetted into a fresh Falcon tube, careful not to disturb the lower red blood cell layer. DPBS was added to the PBMCs to make the volume up to 40ml and the tube was spun at 300xg for 10 minutes to wash and remove platelets. Supernatant was removed, the pellet was re-suspended in 10ml PBS and centrifuged at 300xg for 10 minutes. Cells were re-suspended in 50ml Roswell Park Memorial Institute medium (RPMI-1640, Sigma-Aldrich, Missouri, USA) supplemented with L-glutamine, $NaHCO_3$ and 10%v/v fetal bovine serum (FBS) (Sigma-Aldrich, Missouri, USA) (subsequently referred to as media). PBMCs were plated into uncoated T75 flasks (Corning, New York, USA) in 15ml of media at a density of approximately 6×10^5 cells/ml. Cells were left for 24 hours at 37°C, 5% CO2 before media containing unattached cells was removed by pipetting and replaced with fresh media. After 4 days, cells were washed and 250ng/ml LPS plus 20ng/ml IFN-γ, 30ng/ml IL-4 or 20ng/ml IL-10 (R&D systems, Minneapolis, USA) was added to polarise attached macrophages towards an M(LPS, IFNγ), M(IL-4) or M(IL-10) phenotype, respectively. Cells were left for a further 2 days to differentiate before they were lysed for either RNA or protein isolation, described in Section 2.5.1 and Section 2.6.1 respectively.

2.3.2 THP-1 cell culture system

2.3.2.1 Phorbol 12-myristate 13-acetate titration

THP-1 cells were seeded in wells of a 6-well tissue culture plate (Corning, New York, USA) at 3.3×10^5 cell/ml in 3ml of media. Phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich, Missouri, USA) was added to each well at a concentration of 2.5 ng/ml, 5 ng/ml, 10 ng/ml, 20 ng/ml, 40 ng/ml, 80 ng/ml or 160ng/ml and cells were incubated at 37°C, in 5% CO_2 for 24 hours prior to cell viability experiments and RNA analysis.

2.3.2.2 Cell viability

Media was carefully removed from each well and 2ml of cold (3°C) PBS was replaced. Cells were placed in the cold room (3-5°C) for 20 minutes to promote cell detachment. Cells were moved back to the cell culture hood and were thoroughly scraped using a cell scraper to detach cells from the culture surface. Cells were then pipetted 10 times to ensure cells were fully in suspension.

To enable staining of dead cells, cell suspension (10µl) was added to 10µl of trypan blue stain (0.4%) (ThermoFisher Scientific, Massachusetts, USA) and pipetted up and down several times to ensure thorough mixing. The trypan blue-cell suspension mixture (10µl) was pipetted onto a Countess™ counting chamber slide (ThermoFisher Scientific, Massachusetts, USA), making sure to cover all of the counting area. The Countess™ counting chamber slide was inserted into the Countess™ machine (ThermoFisher Scientific, Massachusetts, USA), the image was adjusted using the Focus Knob to allow for optimised readings (live cells show a bright centre and dark edges; dead cells are dark blue in colour). The Focus Knob was locked for all subsequent counts. Cells were then counted and the percentage viability along with the total number of live cells per ml was noted. Cells were counted on the Countess™ within 3 minutes of mixing the cell suspension with trypan blue to prevent toxicity to cells.

2.3.2.3 Rest period time course

Maturation of THP-1 cells was carried out using the titrated concentrations of PMA, as described previously in Section 2.3.2.1. THP-1 cells were seeded ($3.3x10^5$ cell/ml) in three, 6-well tissue culture plates, with wells labelled according to the PMA concentration, in duplicate. THP-1 cells were treated with the relevant PMA concentration for 24 hours, as described above. Media was removed from the cells and cells were washed 3 times with 2ml of DPBS. Fresh media (3ml) was added to each well and the cells were incubated at 37°C, 5% CO_2 for either 1, 2, 3, 4 or 5 days before it was used for RNA extraction.

2.3.2.4 IL-4 titration and time course

THP-1 cells were seeded in 12, T25 culture flasks at a seeding density of approximately $2.5x10^6$ cells/ml in 5ml of media. Flasks were labelled either M(IL-4) 24 hours (20ng/ml, 25ng/ml, or 30ng/ml), M(IL-4) 48 hours (20ng/ml, 25ng/ml, or 30ng/ml) or M(IL-4) 72 hours (20ng/ml, 25ng/ml, or 30ng/ml). Additionally, flasks were labelled M(0) 24h, M(0) 48h and M(0) 72h. To all flasks, 5ng/ml of PMA was added to the THP-1 cells and they were incubated at 37°C, at 5% CO_2 for 24 hours to allow maturation into macrophage-like cells M(0). After 24 hours

of maturation, media was removed from the cells and they were washed 3 times with 2.5ml of DPBS. The flasks were then rested for the optimum 72 hours at 37°C, at 5% CO_2. Next, IL-4 was added to the M(IL-4) labelled wells at the correct concentration of either 20ng/ml, 25ng/ml or 30ng/ml. Control M(0) wells were not treated with cytokine. Cells were returned to the incubator and left at 37°C, 5% CO_2 for either 24, 48 or 72 hours prior to use for RNA analysis.

2.3.2.5 Optimised THP-1 polarisation protocol

THP-1 cells were seeded at a density of $5x10^6$ cells/ml in three, T75 flasks with 15ml of media which were labelled M(0), M(LPS, IFNγ) or M(IL-4). PMA (5ng/ml) was added to each flask and the cells were placed in an incubator at 37°C, 5% CO_2 for 24 hours. Media was removed, and cells were washed with 5mL of DPBS three times. Fresh media was replaced (15ml) and cells were placed back into the incubator at 37°C, 5% CO_2 for 72 hours. Matured cells were treated with the relevant cytokines: M(LPS, IFNγ), LPS (250ng/ml) and IFNγ (20ng/ml); M(IL-4), IL-4 (30ng/ml); and M(0) received no cytokine. Cells were then placed in the incubator at 37°C, 5% CO_2 for a further 48 hours to differentiate. For protein analysis, cells were treated with cytokine over 48 and 72 hours.

2.3.2.5.1 IL-10 treatment

THP-1 cells were cultured following the optimised THP-1 polarisation protocol described in Section 2.3.2.5. Briefly, THP-1 cells were seeded at a density of $5x10^6$cells/ml in T75 flasks labelled M(0) or M(IL-10). Cells were treated with PMA for 24 hours before being washed three times with DPBS and then replaced with fresh media. Cells were rested for three days and then treated with 20ng/ml of IL-10 (ThermoFisher Scientific, Massachusetts, USA) cytokine to the same media before returning cells to the incubator for a further 48 hours to differentiate. For protein analysis, cells were treated with cytokine over 48 and 72 hours.

2.3.2.5.2 IL-33 treatment

THP-1 cells were cultured following the optimised THP-1 polarisation protocol described in Section 2.3.2.5. Briefly, THP-1 cells were seeded at a density of $1x10^6$cells/ml in 6-well plates. Plates were labelled either ‘without IL-33’ or ‘with IL-33’ and wells were divided into M(0), M(LPS, IFNγ), M(IL-4) or M(IL-10). Cells were treated with PMA for 24 hours before being washed three times with DPBS. Fresh media was added, and cells were rested for three days. The relevant cytokines were added at the concentrations stated in Section 2.3.2.5 for M(0), M(LPS, IFNγ), M(IL-4) and Section 2.3.2.5.1 for M(IL-10). Cells were returned to the incubator for 24 hours before the addition of IL-33 (Peprotech, New Jersey,

USA) to all polarised cell types in the ‘with IL-33’ labelled plate, resulting in M(IL-33), M(LPS, IFNγ, IL-33), M(IL-4, IL-33) and M(IL-10, IL-33). All plates were returned to the incubator for a further 24 hours prior to use for RNA analysis.

2.3.2.5.3 Glucocorticoid treatment

THP-1 cells were cultured following the optimised THP-1 polarisation protocol described in Section 2.3.2.5. Briefly, THP-1 cells were seeded at a density of $5x10^6$cells/ml in T75 flasks and labelled M(0), M(LPS, IFNγ), M(IL-4) or M(IL-10) in triplicate. Each cell type was additionally labelled no glucocorticoids (GC), +Dexamethasone or +Fluticasone Propionate. This was carried out in duplicate with one experiment adding glucocorticoids during polarisation and the other experiment adding glucocorticoids post polarisation. Both glucocorticoids were used at a concentration of 100nM (39ng/ml Dexamethasone or 50ng/ml Fluticasone propionate).

Cells were treated with PMA for 24 hours before being rested for 72 hours. Cells were then stimulated with the relevant cytokines. The relevant glucocorticoid was added to “during polarisation” labelled flasks for 48 or 72 hours. To the “post polarisation” labelled flasks, glucocorticoids were added after 48 hours of cytokine stimulation to the same media and cells were incubated for a further 48 or 72 hours before being used for protein expression analysis.

2.3.2.6 Cellular morphology

To investigate the polarisation of THP-1 cells into an M(LPS, IFNγ) or M(IL-4) macrophage phenotype, cells were visualised using bright field microscopy at a magnification of x20 and x40 using an EVOS XL cell imaging system (ThermoFisher Scientific, Massachusetts, USA).

2.4 Immunofluorescent staining

Staining of polarised macrophage actin filaments was carried out by culturing THP-1 cells, using the final protocol described previously (Section 2.3.2.5), on glass coverslips. Media was removed, and coverslips were washed 3 times with PBS before incubation in 1ml of paraformaldehyde fixing solution for 5 minutes. The solution was replaced with 500µl of 0.5% Triton X-100-PBS solution and incubated at room temperature for 5 minutes to enable intracellular staining of cells. A PBS staining solution containing phalloidin (ThermoFisher Scientific, Massachusetts, USA) at 1:1000 weight/volume (w/v) concentration and DAPI (ThermoFisher Scientific, Massachusetts, USA) at 1:1500w/v was pipetted into a humidified chamber and glass coverslips were placed on top of the PBS staining solution, cell side down, and left in the dark for 1 hour at room temperature. Coverslips were washed with PBS, the excess removed and a drop of mounting medium (Abcam, Cambridge, UK) was placed onto the coverslips before they were mounted onto slides. Cells were imaged using the Eclipse Ti-E, widefield fluorescent microscope (Nikon, Tokyo, Japan).

2.5 RNA expression analysis

2.5.1 Preparation of cell lysates

For cell lysis, cell culture media was removed and 2ml of DPBS was added to wash cells. To each well, 350µl of RNeasy plus RLT lysis buffer (Qiagen, Hilden, Germany), a high-salt buffer containing guanidinium isothiocyanate, was added. Cells were scraped using a cell scraper and the lysate was pipetted into a microcentrifuge tube. To ensure no clumping of cells, and to homogenise the cell lysate, lysates were pipetted up and down carefully but with vigour. Lysates suspended within the RNeasy plus RLT lysis buffer were stored at -20°C until needed.

2.5.2 RNA isolation

Following the manufacturers' instructions, RNA was extracted from cell lysates using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). Briefly, homogenised cell lysate samples suspended in RNeasy plus RLT lysis buffer were added to a gDNA Eliminator spin column and centrifuged at 10,000 rpm for 30 seconds. RNA was passed through the silica-based membrane within the spin column into the flow through, with the high salt-buffer selectively leaving DNA bound to the silica membrane. To the flow through, 1 volume (350µl) of 70% ethanol was added and thoroughly mixed via pipetting. Up to 700µl of the ethanol-flow through sample was transferred to a RNeasy spin column and centrifuged at 10,000 rpm for 15 seconds. The ethanol and high salt buffer allowed RNA to bind to the silica membrane. The flow through containing contaminants was discarded. To remove further impurities, RW1 buffer (700µl) was added to the RNeasy spin column and centrifuged for 15 seconds at 10,000 rpm. The flow-through was discarded. The RNeasy spin column was washed with the addition of RPE buffer (500µl) and centrifuged for 15 seconds at 10,000 rpm. The flow-through was discarded. The RNeasy spin column was washed again with 500µl RPE buffer and centrifuged for 2 minutes at 10,000 rpm. The collection tube was discarded with the flow through inside and the RNeasy spin column was placed in a new collection tube. The column was centrifuged at full speed for 1 minute to prevent carryover of RPE buffer and to dry residual ethanol. The RNeasy spin column was then placed in a 1.5ml Eppendorf tube, 30µl of RNase-free water was pipetted directly onto the column membrane and the column was centrifuged at 10,000 rpm for 1 minute to elute the RNA from the silica-based membrane. The concentration of the eluted RNA was measured using a spectrophotometer (NanoDrop,

ThermoFisher Scientific, Massachusetts, USA). RNA samples were stored in RNase-free water at -20°C until use.

2.5.3 cDNA synthesis

Reverse transcription of RNA products was performed with the addition of the following (all from Invitrogen, California, USA): 1µl of dNTP (10mM), 1µl of Oligo(dT)$_{12-18}$, 20ng total RNA heated to 65°C for 5 minutes. Next 4µl of reverse transcription buffer (Appendix 1), 2µl of dithiothreitol (DTT) was added and incubated for 2 minutes at 42°C. Finally, 1µl of SuperScript II Reverse Transcriptase was added and sterile, distilled water to make the volume up to 20µl, this was incubated at 42°C for 50 minutes. The reaction was inactivated by heating samples to 70°C for 15 minutes. cDNA samples were stored at -20°C in the cDNA synthesis buffer at a concentration of 1ug/ml.

2.5.4 Designing of primers

Primers were designed for polymerase chain reaction (PCR) spanning approximately 20 base pairs (bp), amplifying a cDNA sequence between an estimated 100 to 300bp. Primers were designed to anneal at approximately 60°C and span an intron-exon boundary of larger than 500bp.

2.5.5 End-point PCR amplification

End-point polymerase chain reaction (PCR) amplification was performed. Expression was determined using primers (Integrated DNA technologies, Leuven, Belgium) the sequences of which can be found in Table 2.2 The cycling parameters were as follows: 94°C for 30 seconds, varying temperature for 30 seconds and 72°C for 30 seconds for a total of 25 to 30 cycles. M1 and M2a polarised monocyte-derived macrophages were assayed along with the experimental THP-1 polarised cells as a positive control for M(LPS,IFNγ) or M(IL-4) marker expression. Loading control was determined using the house keeping gene, RPL37A which has been validated and recommended by Maess *et al.* (2010) for normalising RNA expression in THP-1 cells differentiated towards macrophages using PMA.

2.5.6 Agarose gel electrophoresis

Agarose gels at 3% w/v were prepared using 1 x Tris Acetate EDTA (TAE) buffer (appendix 1). Each PCR product (10µL) was added to loading dye (2µL) containing bromophenol blue and glycerol. To the gel, 10µL of the PCR and loading dye solution was pipetted into each well. The gel was run at 115 volts for 60 minutes and was visualised using the Gel Doc™ XR+ Documentation system (Bio-Rad, California, USA).

Table 2.2: Table of primer targets, forward and reverse primer sequences and their respective sequence length with annealing temperatures used.

	Primer target	Forward primer sequence	Reverse primer sequence	cDNA sequence length (bp)	Genomic sequence length (bp)	Annealing temperature (ºC)	Cycles
Housekeeping gene	RPL37A	5'-dGTACCACTTGCTCTTTCTGTGGC-3'	5'-dCTGCATGAAGACAGTGGCTG-3'	89	639	56.3	25
M1 primers	ANKRD22	5'-dGAAGGACCAGCATGGGAATC-3'	5'-dGAAGACAGCAGCTATGCCAAC-3'	110	19670	58	27
	CXCL9	5'-dGCTGGTTCTGATTGGAGTGC-3'	5'-dCAATTTGCCCCAAGCCCTTC-3'	129	1240	56.9	25
	CXCL10	5'-dCCTTATCTTTCTGACTCTAAGTGG-3'	5'-dCCTGTTAATACAAGGTCTTTA G-3'	107	660	51.7	25
	GBP5	5'-dGACCAAGAACCCACCAATTCC-3'	5'-dCTCCGCTGCATACAAATCAGG-3'	112	1701	56.2	25
	HLADRB1	5'-dCACTCTGGACTTCAGCCAAGAG-3'	5'-dCCTCCTGGCTGTTATTCTTCCAC-3'	121	909	57.5	30
	IL-6	5'-dCCAGCTATGAACTCCTTCTCC-3'	5'-dCCCCAGGAGAAGATTCCAAAG-3'	118	280	58.4	30
	INHBA	5'-dGATCATCACGTTTGCCGAGTC-3'	5'-dCTGGCTCTTCCTAAAAGTCCC-3'	120	9564	56.2	30
	IRF1	5'-dGGAAGGGAAATTACCTGAGG-3'	5'-dCCTACTCAATGAACCTGGAG-3'	101	1278	52.4	3
	STAT1	5'-dCAATGCTTGCTTGGATCAGC-3'	5'-dCCTACGAACATGACCCTATCAC-3'	129	2765	54.8	35
	SERPING1	5'-dGATGTCCAAGTTCCAGCCCAC-3'	5'-dGACCTTAACCTGTGTGGGCTG-3'	130	2521	63	25
	TNFAIP6	5'-dGGGATGCCTATTGCTACAACC-3'	5'-dCAGGCTTCCCAAATGAGTACG-3'	99	3901	59	25
	TSC22D1	5'-dCTCTGGTGCAAGTGTGGTA-3'	5'-dGGTCGGAGAAGAAGTGGAGG-3'	98	1258	63	30
M2a primers	ALOX15	5'-dCAGATACTCCGGTACTTCCACC-3'	5'-dCTGCACCTGGTTGTTGGAAC-3'	122	2007	56.7	30
	CCL17	5'-dACTGAAGATGCTGGCCCTGGTCA-3'	5'-dGCTGAAGACGTGGTACCAGACATCT-3'	154	1232	61.5	30
	CCL22	5'-dCCAACATGGAAGACAGCGTCTGCT-3'	5'-dCCTACTCTGATGACCGTGGCCTT-3'	224	3164	61	30
	CCL23	5'-dCGTGTTCACTCCTGGAGAGTTAC-3'	5'-dGTTTCTGTGCCAACCCCAGTG-3'	105	540	61	25
	CCL26	5'-dGAAGGGCCTGATTTGCAGCATC-3'	5'-dGGAGTGACATATCCAAGACCTG-3'	120	220	56.9	25
	CD200R1	5'-dCTGTACATAGAGCTACTTCCTGTTCC-3'	5'-dCCAGTTGTTGAGGAGGATGAAATGC-3'	187	5048	57.2	30
	CD206	5'-dCGAGGAAGAGGTTCGGTTCACC-3'	5'-dGCCATGAGAACCGGGATTGC-3'	84	2963	59.4	30
	HOMER2	5'-dCCTCAGCTCATGTCAGAGTGC-3'	5'-dCCTGAAGGTGGAGTTGAAGAGC-3'	153	1400	57.7	30
	PPARγ	5'-dCCATTCACAAGAACAGATCCAGTGGTTG-3'	5'-dCCAGAAGCTTTATCTCCACAGACACG-3'	200	1590	65	30
	TGM2	5'-dGCAGTGACTTTGACGTCTTTGCCC-3'	5'-dCGTGGAGCCAGTTATCAACAGCTAC-3'	269	5881	60.4	30
M2c primers	AMAC	5'-dGACTTTCTCCTCTCCTCCTG-3'	5'-dGTTCTTCCCTGGAACTCTCC-3'	102	102	56.4	30
	C1QA	5'-dGAGCATCCAGTTGGAGTTGAC-3'	5'-dTCTATGGTGACCGAGGACTTG-3'	115	1022	54.5	30
	CCL16	5'-dCTGTCTCTCCTTGTCCTCATC-3'	5'-dCCACCTGCTGCCTGAAGTATT-3'	103	3184	61.4	30
	CCL18	5'-dCCTTGTCCTCGTCTGCACCATG-3'	5'-dCTATACCTCCTGGCAGATTCC-3'	96	6133	57.3	30
	CD163	5'-dGAGGAGACCTGGATCACATGTGA-3'	5'-dGTGTGTCAACAACTTGGCTGTGGTC-3'	166	1586	59.4	27
	CXCL13	5'-dAAATCTTGCCCCGTGGGAATG-3'	5'-dCCCTCAAGCTGAATGGAATAC-3'	99	2877	54.3	30
	CXCL15	5'-dGTCTCTGAACCCAGGGAAGAC-3'	5'-dAGGAAAACCTATGTTTGCCGC-3'	127	127	59.5	30
	FOLR2	5'-dCTGAGGACAAGCTGCATGAC-3'	5'-dCGCCTGTACAACTTTAACTGGG-3'	114	2249	56	30
	IL2RA	5'-dGTTCCTTCCTTGTAGGCCATG-3'	5'-dCCATGATGAACGTGAGCAGTC-3'	109	36171	54.4	30
	MARCO	5'-dGACGAGCTCTTGAGTGAGAC-3'	5'-dGGTGAACTTCTCCCTAGCTG-3'	115	26877	54.8	30
	merTK	5'-dGGATCGCCATAGAAAGTCTTGCAGAC-3'	5'-dCCGAAGACTGCCTGGATGAACTGT-3'	192	6824	57	30
	TMIGD3	5'-dGCATACTGATCACGGGTTTGG-3'	5'-dCACTTTGAAGCCCTTCTCGCG-3'	109	2901	61.2	30
	SEPP1	5'-dCCTAACTTTCCCATATGTAGAAGAAGCC-3'	5'-dGAAACTCCATCGCCTCATTACCAT C-3'	161	3485	59.5	25
	SLC16A	5'-dCTGTGGCTTGATTGCAGCTTC-3'	5'-dCCAGCTCTGACCATGATTGGC-3'	118	4063	57.7	30

2.6 Protein expression

2.6.1 Sample preparation

Media was removed from the cell culture and cells were washed with PBS. Cells were gently scraped with a cell scraper to remove all adherent cells from the cell culture surface. PBS was pipetted 10 times, up and down, quickly, to dislodge and wash cells off the culture surface. The cell suspension was then transferred to an Eppendorf and spun at 1500 rpm for 5 minutes. The supernatant was aspirated, and the cell pellet was re-suspended in RIPA (appendix 1) lysis buffer (200µl for 6-well plate, 500µl for T75 flask) with the addition of 100x Halt Protease Inhibitor cocktail (ThermoFisher, Massachusetts, USA) and kept agitated for 30 minutes. The Eppendorfs were then centrifuged at 16000 rpm for 10 minutes to remove debris from the lysate and the supernatant was collected in a fresh Eppendorf and stored at -20°C until needed.

2.6.2 Bicinchoninic acid protein assay

The protein concentration of sample lysates was determined through the use of the Pierce bicinchoninic acid (BCA) Protein Assay Kit (ThermoFisher Scientific, Massachusetts, USA). The assay was carried out as described in the manufacturer's instructions. Briefly, a set of protein standards were prepared with concentrations ranging from 0-2000ug/ml. To each well of a 96 flat-bottomed plate, 25µl of the standards and the samples were added in duplicate.

The BCA working reagent (WR) was prepared with a ratio of reagent A to reagent B at 50:1. To the standards and samples, 200µl of WR was added. The plate was covered, shaken for 30 seconds and then incubated at 37°C for 30 minutes. The plate was then read at 562nm using a VarioSkan (ThermoFisher Scientific, Massachusetts, USA) and absorbance readings were calculated into concentration values (µg/ml) using the standard curve. Total protein concentrations were then normalised across samples to 500µg/ml, diluted using RIPA buffer.

2.6.3 Western blot

Protein samples (5µl) were loaded into 8-tube PCR strips with an equal volume of 2x Laemmli sample buffer (Appendix 1) and were heated at 95°C for 5 minutes to allow denaturing of proteins. A 12% Mini-PROTEAN TGX^{TM} precast protein gel (Bio-Rad, California, USA) was placed in a Mini-Protean Tetra cell (Bio-Rad),

taking care to remove the green strips and 1x sodium dodecyl sulphate (SDS)-running buffer (1L) (Appendix 1) was added, making sure to cover the wells of the gel. To the first well, 7.5µl of Precision Plus Protein™ Dual Xtra Prestained Protein Standards (Bio-Rad, California, USA) was pipetted and denatured protein samples (10µl) were pipetted into the remaining wells. The gel was run at 150 volts for approximately 45 minutes at room temperature or until the proteins had reached the black line.

A transfer sandwich was prepared at 3°C. Polyvinylidene difluoride (PVDF) membrane and blotting paper (Amersham, Buckinghamshire, UK) were cut to the same size as the gel. The membrane was pre-wet in methanol for 20 seconds, placed in distilled water for 5 minutes and then moved to cold transfer buffer (appendix 1) and incubated for at least 15 minutes. Sponges and blotting paper were pre-wet in cold transfer buffer in the gel tank for at least 15 minutes. Once the gel had run, the transfer sandwich was constructed beginning at the anode with three sponges, three pieces of blotting paper, the gel (turned 180°), PVDF membrane (making sure not to touch the membrane), three further blotting papers and three sponges, topped with the cathode. The sandwich was placed into the gel tank and topped with transfer buffer. The clamp was closed multiple times to squeeze air bubbles out of the sandwich before the transfer was run at 25 volts for 1 hour at 3°C.

The membrane was removed from the transfer sandwich and placed into a Falcon Tube (Corning, New York, USA) with Tris-Buffered saline with 0.1% Tween- 7% Bovine Serum Albumin (TBS-T-BSA) (Appendix 1) blocking solution. The membrane was left to block at room temperature for 1 hour on a roller. After blocking, antibody was added to the TBS-T-BSA solution (10ml) (details of antibodies used, and their dilutions can be found in Table 2.3) and left to incubate at 3°C, overnight. The antibody solution was removed, the membrane was washed with 10ml Tris-buffered saline with 0.1% Tween (TBS-T) (Appendix 1) for 3 x 5 minutes. TBS-T (10ml) was added with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody at a 1:10000 dilution (Agilent, California, U.S.A) and incubated at room temperature for 1 hour. The membrane was then washed with 10ml TBS-T for 5 x 5 minutes. Membrane was developed using Amersham ECL Western Blotting Detection Reagent (Amersham, Buckinghamshire, UK) by mixing reagent A with reagent B (1:1 solution) and applying to the membrane. Protein expression was then visualised using the Gel Doc™ XR+ Gel Documentation system (BioRad).

Table 2.3:Details of antibodies used for western blotting.

Antibody	Host	Working dilution	Manufacturer
ANKRD22	Rabbit polyclonal	1:5000	Atlas antibodies (Abcam, Cambridge, UK)
GBP5	Rabbit polyclonal	1:5000	ThermoFisher
TNFAIP6	Rabbit polyclonal	1:1000	Novus biologicals
SERPING1	Rabbit polyclonal	1:2000	Atlas antibodies
ALOX15 (Clone OTI3G8)	Mouse monoclonal	1:2000	Abcam
CD200R1	Rabbit polyclonal	1:2000	Abcam
MRC1/CD206	Rabbit polyclonal	1:2000	Abcam
TGM2 (Clone CUB 7402)	Mouse monoclonal	1:5000	ThermoFisher
CD163 (Clone EDHu-1)	Mouse monoclonal	1:2000	Bio-Rad
Beta-actin (Clone BA3R)	Mouse monoclonal	1:10000	ThermoFisher

2.7 Histology

2.7.1 Preparation of cell pellets for positive cell staining

THP-1 cells were polarised to M(0), M(LPS,IFNγ), M(IL-4) and M(IL-10) following the optimised THP-1 cell model protocol (Section 2.3.2.5), cells were plated in T75 flasks in replicates of 3 to ensure adequate number of cells to form each cell pellet. Once polarised, media was removed from the flasks and replaced with 10ml of cold DPBS. Flasks were moved to the cold room (3-5°C) and incubated for 20 minutes to help detach cells from the culture surface. Cells were then scraped using a cell scraper to remove all other attached cells. The cell suspension was spun at 1200rpm for 5 minutes to form a cell pellet before the supernatant was removed and cells were re-suspended in 500µl of DPBS. Cell suspension was moved to a 1.5ml Eppendorf and spun at 10,000rpm for 5 minutes to form a cell pellet. The supernatant was removed and replaced with enough 4% paraformaldehyde to cover the cell pellet. The cell pellet was left overnight at room temperature before the paraformaldehyde was removed and replaced with enough 70% ethanol to cover the cell pellet. The cell pellet was then embedded in paraffin wax before being sectioned.

2.7.2 Sections

Sections (5µm) were cut sequentially from formalin-fixed paraffin embedded (FPPE) tissue blocks. Tissue sections were mounted onto Superfrost™ Plus microscope slides (ThermoFisher Scientific, Massachusetts, USA) and dried at 37°C overnight. Slides were then stored at 4°C for up to three weeks prior to staining. Slides were placed on a hotplate set to 70°C for 30 minutes to allow sections to stick to the slide before they were dewaxed in Xylene four times, each time for 3 minutes. Slides were rehydrated in 100% alcohol twice for 2 minutes, 90% alcohol once for 2 minutes, and 70% alcohol once for 2 minutes and then immersed quickly in PBS to wash.

2.7.3 Haematoxylin and Eosin staining

Tissue sections were prepared as described in Section 2.7.2.

Excess PBS was removed from the slides by blotting on paper and were then placed in filtered Mayer's haematoxylin for 30 seconds, rinsed in PBS for 1 minute, placed in Scott's tap water (Appendix 1) for a further minute and then washed again in PBS for 1 minute. Slides were then placed in eosin for 2.5 minutes, washed in PBS for 1 minute and then immersed quickly and sequentially

in two containers of PBS to ensure slides were fully washed. Slides were blotted and then dehydrated three times for 1 minute in 100% alcohol, air dried for 5 minutes and cleaned with paper. Slides were then cleared in Xylene, four times for 1 minute before mounting carefully under glass coverslips using distyrene/plasticiser/xylene (DPX, Sigma Aldrich, Missouri, USA).

2.7.4 Immunohistochemistry

Tissue sections were prepared as described in Section 2.7.2.

Antigen retrieval was performed by pre-heating Vector Antigen Unmasking Solution, a pH 6.0 citric acid solution (Vector Labs, Peterborough, UK), to 95-100°C in a steamer. Slides were immersed in the pre-heated Unmasking Solution for 30 minutes. For GBP5, antigen retrieval was performed using a pressure cooker, incubating slides in pH 6.0 citric acid solution at 120°C for 2 minutes. Following antigen retrieval, slides were removed and left to cool for 20 minutes prior to rinsing with TBS-T for 5 minutes. Vector BLOXALL blocking solution (Vector, Peterborough, UK) was added to the slides (100µl) and left to incubate for 15 minutes at room temperature before washing with TBS-T for 5 minutes. To reduce non-specific binding of the primary antibody, protein blocking was carried out by incubating the slides in 100µl of 1x casein solution (Vector, Peterborough, UK) diluted in Antibody Diluent (ThermoFisher Scientific, Massachusetts, USA) for 20 minutes. Blocking solution was removed from the slides and 100µl of primary antibody diluted in Antibody Diluent (details of antibodies and their dilutions are given in Table 2.4) was added to each slide and incubated overnight at 4°C. Slides were washed in TBS-T once for 4 minutes and again for 5 minutes. Secondary antibodies, either ImmPRESS HRP reagent kit anti-mouse (100µl) or ImmPRESS HRP reagent kit anti-rabbit (100µl) (both Vector, Peterborough, UK) were added to the correct slides and incubated for 30 minutes at room temperature. Slides were washed in TBS once for 4 minutes and again for 5 minutes. ImmPACT diaminobenzidine (DAB) peroxidase (HRP) substrate-Chromogen solution (1:20 solution) (Vector, Peterborough, UK) was added to the slides (100µl), making sure to cover the whole tissue section, for 5 minutes at room temperature to enable visualisation of antibody binding. Excess solution was removed, slides were placed in PBS to rinse for 1 minute and immersed quickly and sequentially in 2 containers of PBS to ensure thorough washing of slides. Slides were then counterstained using Mayer's haematoxylin for 30 seconds, washed with PBS for 1 minute, moving quickly and sequentially to 2 other containers of PBS. Slides were blued in Scott's tap water solution for 1 minute, rinsed with PBS as described previously. Slides were then dehydrated in

100% ethanol and cleared in xylene as described previously (Section 2.7.3) and mounted under glass coverslips using DPX. Slides were left to dry before examining under a microscope. Staining of positive and negative controls was routinely carried out. Cell pellets (Section 2.7.1) were used as a positive control and negative controls were performed by carrying out the procedure without the addition of the primary antibody.

Optimisation of staining was performed using sections taken from PFFE temporal artery biopsies which had severe inflammatory infiltrate. Parameters that were changed were the method of antigen retrieval (including microwave, pressure cooker and steamer), duration of antigen retrieval and the concentration of antibody. The parameters used for each antibody can be seen in Table 2.4.

Table 2.4: Details of antibodies used for immunohistochemistry and their working dilutions.

Antibody	Host	Working dilution	Manufacturer	Antigen retrieval method
CD68 (Clone PG-M1)	Mouse monoclonal	1:200	DAKO	Steamer, 30 minutes
ANKRD22	Rabbit polyclonal	1:200	Atlas antibodies	Steamer, 30 minutes
GBP5	Rabbit polyclonal	1:600	ThermoFisher	Pressure cooker, 2 minutes
MRC1/CD206	Rabbit polyclonal	1:600	Abcam	Steamer, 30 minutes
TGM2 (Clone CUB 7402)	Mouse monoclonal	1:500	ThermoFisher	Steamer, 30 minutes
CD163 (Clone EDHu-1)	Mouse monoclonal	1:400	Bio-Rad	Steamer, 30 minutes
CD31	Rabbit polyclonal	1:100	Abcam	Steamer, 30 minutes
Alpha Smooth muscle actin (SMA)	Rabbit polyclonal	1:400	Abcam	Steamer, 30 minutes

2.7.5 Analysis of staining

Slides were digitised up to a maximum magnification of x400 (x40 objective) by the virtual pathology team at the University of Leeds. A scoring system was established with the help of Dr Aruna Chakrabarty and can be seen in Table 2.5. A staining atlas was then produced (Appendix 2) providing images of each type of staining score for each antibody that was used in this study. This atlas presented examples of the different types of staining to allow for comparison between artery biopsies sections, allowing for a more consistent semi-quantitative scoring approach.

Staining of macrophage marker expression in artery biopsy sections was scored in a semi-quantitative manner. For each artery layer (adventitia, media and intima) the locality of staining was scored from 0-3 and the intensity of staining was scored from 0-3, the values of which were added together to give a total staining score for each layer between 0 and 6. The total staining scores for each artery layer were also totalled to provide a total artery score, of which could be between 0 and 18. The different artery layers were visualised using H&E staining of artery sections and compared to arteries stained with other antibodies (Figure 2.1). No data was entered if sections were damaged or if staining failed (no staining of any artery section). To better describe the different patterns of macrophage infiltration across the artery layers, the numerical scoring system for staining locality and staining intensity were interchangeably adapted into a character scoring system as shown in Table 2.5.

SMA staining of fibroblasts and myofibroblasts within the intima were used to assess the degree of luminal occlusion within each artery section.

CD31 was used to identify neovessels within the media and intima layers of the artery wall. Absence of neovessels was scored as 0, a small number of neovessels within the media was scored a 1, a large number of neovessels within the media and small number of neovessels within the intima was scored as 2, and a larger number of neovessels within the media and intima was scored as 3.

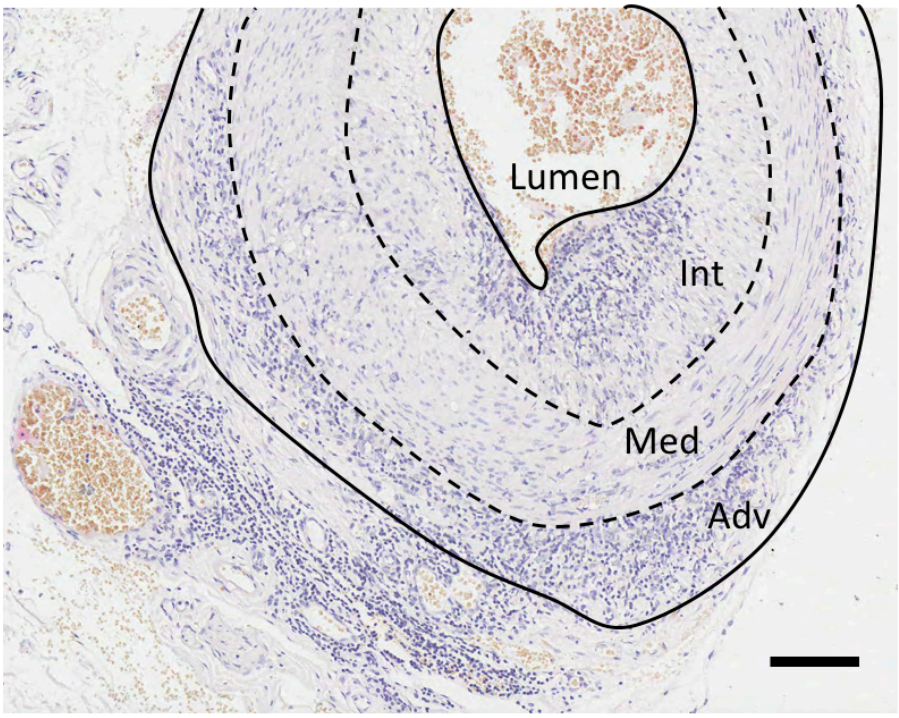
SMA staining of the VSMCs within the media was used to determine the degree of VSMC loss within the media. An intact media (i.e. no media destruction) was scored a 0, partial destruction in isolated areas was scored as 1, complete loss of VSMCs within one section of the media was scored as 2 and complete loss of VSMCs from more than one section of the media with additional partial destruction of other areas of the media was scored as 3.

Table 2.5: Semi-quantitative scoring system used for analysing staining of macrophage markers

Variable	Numerical scoring system	Character scoring system for use in Section 5.9	Description of score
Locality of macrophage marker staining	0	N	No staining
	1	F	Focal staining
	2	M	Multifocal staining
	3	D	Diffuse staining
Intensity of macrophage marker staining	0	N	No staining
	1	m	Mild staining
	2	M	Moderate staining
	3	S	Strong staining
Luminal occlusion (SMA)	0		0%
	1		0-25%
	2		25-50%
	3		50-75%
	4		>75%
Neovascularisation (CD31)	0		No vascularisation
	1		Mild
	2		Moderate
	3		Severe
Media destruction (SMA)	0		No destruction
	1		Partial destruction in isolated area
	2		Complete loss of VSMCs from one area of the media
	3		Complete loss of VSMCS from more than one area of the media.

SMA, smooth muscle actin.

a)



b)

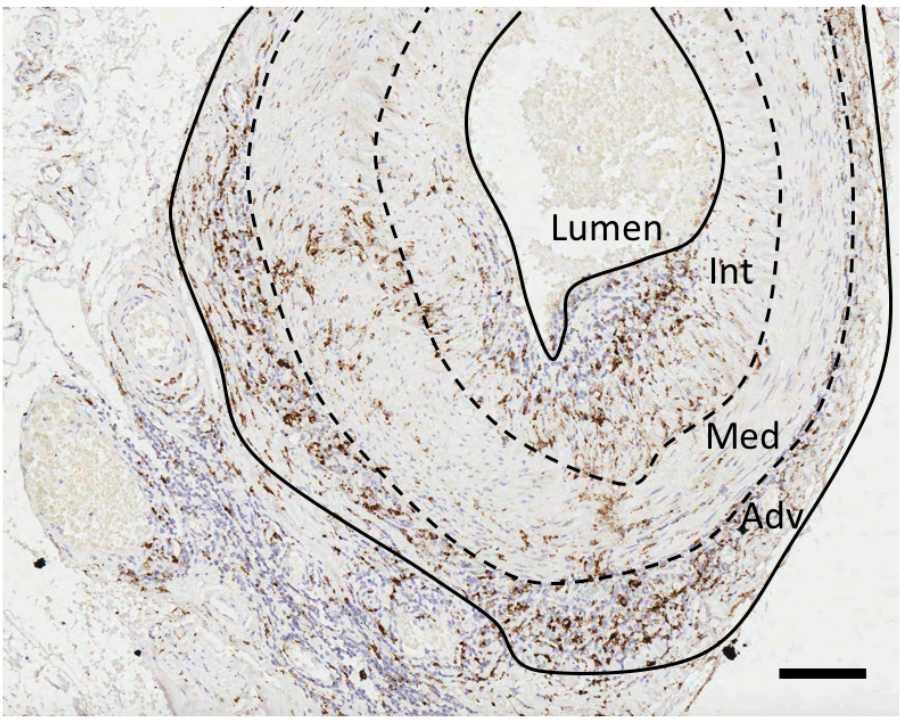


Figure 2.1: Layers of the same artery stained by haematoxylin and eosin and CD68.

Different layers of the artery wall can be seen in sections of the same artery stained with **a)** haematoxylin and eosin and **b)** CD68 staining of macrophages. The external elastic lamina (separating the adventitia from the media) and the internal elastic lamina (separating the media from the intima) are shown by dotted lines. The border of the adventitia and the lumen are visualised by the solid line. Images were taken at a magnification of x50. Scale bar represents 100μm. Adv, adventitia; Med, media; Int, intima.

2.8 Statistical analysis

Analysis was performed using SPSS version 24 (Chicago, IL, USA). Descriptive statistics including means, medians and interquartile range and standard deviations were reported in this study. Non-parametric statistics were generally used because the variables were not normally distributed. Spearman rank was run using a two-tailed test and used to test for correlation between two continuous variables. Mann-Whitney U was used to compare values of continuous variables in two categories. According to convention, a threshold of $p<0.05$ was required for a result to be considered statistically significant. Correction for multiple comparisons was not made

Chapter 3. Development of a THP-1 cell model system for the study of macrophage phenotype marker expression

3.1 Introduction

The literature on macrophages is highly convoluted: polarisation protocols for both primary cells and cell lines is highly variable, the binary method for describing macrophage phenotypes is too simplistic; new stimuli and polarisation states are being continually identified, much of the macrophage literature is derived from mice studies, and there is no consensus on subset-specific markers for these differently polarised macrophages. Together, this makes understanding and studying macrophages in humans difficult, therefore tackling these problems requires standardisation of protocols and nomenclature and the need for new, phenotype-specific macrophage markers.

The THP-1 monocytic cell line has been used extensively as a model to study human monocytes and macrophages (Tsuchiya *et al.*, 1982) and provides a less variable experimental method compared to primary cells, however, similarly to primary cells, no standardised protocol has been established for the maturation of THP-1 cells into macrophages and subsequent polarisation into different macrophage phenotypes. In addition to a lack of standardisation, no method of achieving an M2a-like macrophage phenotype from THP-1 cells, which closely resembles that of primary M2a-like macrophages, has been validated (Shiratori *et al*., 2017). Furthermore, for macrophages in general, there is no consensus on the most appropriate markers to allow for the characterisation of M1 and M2a macrophages at both the transcript and protein level (Fujiwara *et al.*, 2016), this makes producing cell lines to mimic primary macrophage phenotypes difficult. The use of transcripts can be very useful in characterising macrophages, yet transcripts do not necessarily translate into protein, due to modifications in translation. This is further complicated by the plasticity of immune cells (Ecker *et al.*, 2017). A lot of the macrophage literature has focused on mice models, and although murine and human monocytes and macrophages have been found to be very conserved, differences have been identified (Arnold *et al*., 2015; Jablonski *et al*., 2015; Vogel *et al*., 2014; Ambarus *et al*., 2012). Furthermore, different methods to produce macrophage phenotypes from THP-1 and primary cells are frequently used, due in part to different experimental needs. Differences in the stimulants used to polarise macrophages are therefore very common, leading to upregulation and downregulation of different signalling pathways and

therefore, different marker expression (Xue *et al.,* 2014; Martinez *et al.,* 2006). A lack of thorough detailing of methods makes comparing studies difficult and the lack of clear descriptions regarding the polarising cytokines used results in confusion and a poor understanding of which markers are the most useful for certain macrophage phenotypes (Xue *et al.,* 2014; Murray *et al.,* 2014). As described previously, the recommendations outlined by Murray *et al*. (2014), to describe macrophages in terms of their polarising stimuli will be followed when referring to the macrophage phenotypes produced in this study. For example, macrophages treated with IL-4 will be described as M(IL-4) rather than the broad M2a definition. M1 and M2 nomenclature will be used when generalising markers and when referring to previous studies. Table 1.3 describes the nomenclature used for the macrophage polarised in this study and how they relate to M1 and M2 macrophage phenotypes that are commonly described in the literature.

3.2 Aims

Markers which were specific for differently polarised macrophage phenotypes were required to enable macrophage phenotype characterisation at the tissue level in artery biopsies to ultimately help ascertain macrophage phenotypes within GCA inflamed arteries. Therefore, my aims were:

1. To identify phenotype-specific markers which were membrane or intracellularly expressed, and therefore suitable for immunohistochemistry studies, from publicly available RNA-Seq datasets and published literature.
2. To confirm polarisation of macrophages into specific states and to analyse the specificity of selected markers for their macrophage phenotype.

To achieve these aims, my objectives were:

- To optimise a THP-1 cell model protocol that allowed for the polarisation of THP-1 cells into different macrophage phenotypes, closely resembling polarised human primary macrophages, using previously published protocols as the foundation for the development of the THP-1 cell model system, as well as known subset-specific macrophage markers.
- To optimise and carry out end-point PCR experiments to analyse subset-specific marker expression at the transcript level at different phases of the THP-1 culture protocol to understand optimum conditions for the different phases of macrophage maturation, as well as confirming polarisation of macrophages into different phenotypes.

3.3 M1 and M2a macrophage marker selection

To ensure confidence in knowing the THP-1 cells had been polarised towards either an M1 or M2a phenotype, commonly used transcript and protein markers in macrophage phenotyping studies were identified via a literature search. Journal database Web of Science was searched using search terms including macrophage(s), activation, differentiation, polarisation, transcriptional, transcript, proteomics, protein and markers, sorting results by times cited. The inclusion criteria specified articles which defined human macrophages, of which were polarised using IFNγ and LPS to induce an M1 macrophage phenotype, or with IL-4, with or without IL-13, for M2a polarisation. Markers that had been previously identified as being highly upregulated at the transcript or protein level in M1 or M2a phenotypes, with additional confirmation and experimental validation as to their use as specific M1 or M2a markers in other papers, were defined as commonly used markers and were selected to produce an initial panel of markers. This literature search was carried out before November 2015.

A parallel project (carried out by Nikki Re), cross-examined the initial panel of putative phenotypic transcript markers taken from the literature search, with a primary human macrophage RNA-Seq dataset (Beyer *et al*., 2012). Cross-examining produced a select group of commonly used transcript markers, identified as also being highly upregulated in the Beyer dataset (within the top 40 M1 or M2a upregulated genes) and were subsequently used to ensure the THP-1 cells had been polarised towards either an M1 or M2a phenotype.

This method of marker selection provided further evidence for their phenotype-specific expression. The marker panel shown in Table 3.1 was then compiled using both published literature and “RNA-Seq” markers. This panel would enable the optimisation of a cell culture model system to differentiate THP-1 cells into M1- or M2a-like macrophages that closely resembled primary human macrophages. Further details of M1 markers can be seen in Table 3.2 and details of M2a markers can be seen in Table 3.3.

Table 3.1: Panel of M1 and M2a markers used to optimise the THP-1 cell model system to produce M1 and M2a macrophage phenotypes.

MØ subset	Marker	Source	Studies
M1	Chemokine (C-X-C motif) ligand (CXCL)9	Literature and RNA-Seq	(Martinez *et al*., 2006)
	Chemokine (C-X-C motif) ligand (CXCL)10	Literature and RNA-Seq	(Martinez *et al.,* 2006)
	Human leukocyte antigen (HLA)-DRB1	Literature and RNA-Seq	(Helm *et al*., 2014)
	Interleukin (IL)-6	Literature and RNA-Seq	(Martinez *et al.,* 2006)
	Interferon regulatory factor (IRF)1	Literature	(Martinez *et al.,* 2006)
	Signal transducer and activator of transcription (STAT)1	Literature and RNA-Seq	(Lawrence and Natoli, 2011)
M2a	Chemokine (C-C motif) ligand (CCL)17	Literature and RNA-Seq	(Martinez *et al.,* 2006)
	Chemokine (C-C motif) ligand (CCL)22	Literature and RNA-Seq	(Martinez *et al*., 2009; Mantovani *et al*., 2004)
	Chemokine (C-C motif) ligand (CCL)23	Literature and RNA-Seq	(Novak *et al*., 2007; Martinez *et al*., 2006)
	Chemokine (C-C motif) ligand (CCL)26	Literature and RNA-Seq	(Stubbs *et al*., 2010; Martinez *et al*., 2006)
	Mannose receptor C-Type (MRC)1/CD206	Literature and RNA-Seq	(Sica and Mantovani, 2012; Martinez and Gordon, 2014)
	CD200R1	Literature and RNA-Seq	(Koning *et al*., 2010)
	Peroxisome proliferator-activated receptor (PPAR)γ	Published literature	(Bouhlel *et al*., 2007)
	Transglutaminase (TGM)2	Published literature	(Martinez *et al*., 2013)

Table 3.2: Description of M1 marker cellular expression, location and function.

MØ subset	Gene	Cellular location	Cellular expression	Function in macrophages
M1	*CXCL9*	Secreted	Mononuclear cells	Recruits CXCR3 expressing cells: (CD4+ (Th1) and CD8+ T cells) (Park *et al.,* 2002; Loetscher *et al*., 1996).
	CXCL10	Secreted	Mononuclear cells (DCs, B cells, monocytes, macrophages), endothelial cells.	Recruits CXCR3 expressing cells and NK cells (Park *et al*., 2002; Loetscher *et al.,* 1996).
	HLA-DRB1	Membrane	Antigen presenting cells: Macrophages, B cells, naïve DCs.	Belongs to HLA class II, presenting peptides to T cells (Sturniolo *et al.*, 1999).
	IL6	Secreted	Fibroblasts, endothelial cells. Innate immune cells (monocytes, macrophages, DCs, mast cells, B cells, activated T cells).	Promotes Th17 T cell and B differentiation. Inhibits Treg differentiation. Induces pro-inflammatory genes. Involved in angiogenesis (Van Snick, 1990; Bettelli *et al*., 2006).
	IRF1	Intracellular	Macrophages, DCs, NK cells, CD4+ (Th1 and Th2) and CD8+ T cells, epithelial cells, endothelial cells	Transcription factor involved in activation of pro-inflammatory response and cytokine production induced by IFNγ (Langlais *et al*., 2016).
	STAT1	Intracellular	Macrophages, DCs, T cells, endothelial cells, B cells	Transcription factor regulating differentiation of macrophages. Involved in pro-inflammatory responses and cell survival. Requires phosphorylation for its activation. Induced by pro-inflammatory cytokines (Varinou *et al*., 2003).

Table 3.3: Description of M2a marker cellular expression, location and function.

MØ subset	Gene	Cellular location	Cellular expression	Function in macrophages
M2	*CCL17*	Secreted	Macrophages, epithelial cells, DCs	Recruits CCR4$^+$ cells (Macrophages and Th2 cells) (Sallusto *et al.*, 1999; Mantovani *et al*., 2004).
	CCL22	Secreted	Macrophages, DCs	Induces trafficking and migration of CCR4$^+$ cells (Andrew *et al*., 1998), monocytes, DCs, NKs (Godiska *et al*., 1997) and Tregs (Iellem *et al*., 2001).
	CCL23	Secreted	Macrophages, DCs	Recruits CCR1$^+$ cells (DCs, monocytes, lymphocytes, endothelial cells). Differentiates endothelial cells; promotes angiogenesis (Novak *et al*., 2007; Mantovani *et al.,* 2004; Hwang *et al.*, 2005).
	CCL26	Secreted	Monocytes, macrophages, epithelial and endothelial cells	Recruits CCR3$^+$ cells (Eosinophils, basophils, CD4$^+$ Th2 T cells). Blocks recruitment of CCR1-, 2- and 5- expressing cells (Monocytes, CD4+ Th1 cells) (Stubbs *et al.,* 2010; Mantovani *et al*., 2004; Petkovic *et al.*, 2004).
	CD200R1	Membrane	Macrophages, CD4$^+$ T cells, DCs, B cells	Inhibits cell activation; decreases cytokine secretion (Rijkers *et al.*, 2008; Jenmalm *et al.*, 2005)
	MRC1	Membrane, intracellular	Macrophages, DCs, nonvascular endothelium	Scavenger receptor, role in endocytosis and phagocytosis (Gordon, 2003). Recognises bacteria with mannan-coated cell walls (Taylor *et al.*, 2005; Azad *et al.*, 2014)
	PPARG	Intracellular	Macrophages, DCs, CD4+ T cells, B cells, endothelial cells, fibroblasts	Transcription factor controlling anti-inflammatory macrophage activation, gene and cytokine expression (Odegaard *et al*., 2007; Bouhlel *et al*., 2007).
	TGM2	Membrane, cytoplasmic, secreted, nucleus	Macrophages, DCs, B cells, CD8+ T cells VSMCs, endothelial cells, neutrophils.	Controls integrin functions in macrophages, affecting phagocytosis (Toth *et al.*, 2009). Involved in clearance of apoptotic cells (Roszer *et al.*, 2011; Nadella *et al.*, 2015) Crosslink extracellular matrix proteins (Chen *et al.,* 2013). Regulate PDGF and VEGF signalling (Dardik and Inbal, 2006; Heldin and Westermark, 1999).

M2a transcripts *PPARG* and *TGM2* were not identified within the top 40 genes within the RNA-Seq dataset, however were identified within published literature as subset-specific M2a macrophage transcripts.

Martinez *et al.* (2013) have described greater sensitivity of M2a macrophages to culture conditions when compared to other macrophage phenotypes. The discrepancies in marker expression may therefore be a result of different conditions used in their cell culture protocols to mature and differentiate macrophages into M2a macrophages. Examples of these protocols from RNA-Seq and microarray studies can be found in Table 3.4.

Beyer *et al*. (2012) used one protocol for M2a polarisation of human monocyte-derived macrophages (hMDMs) whereas both Xue *et al.* (2014) and Martinez *et al.* (2013) used multiple methods for culturing hMDMs towards an M2a phenotype which varied in maturation and polarising conditions, the most similar M2a polarisation protocol used by both groups compared to that used by Beyer *et al.* (2012) are described in Table 3.4. Interestingly Martinez *et al.* (2013) pooled the polarisation results to identify commonly upregulated markers specific for M2a at both the transcript and protein level from the different polarising methods whereas Xue *et al*. (2014) analysed data separately from the different polarising methods.

Table 3.4: Protocols carried out by different groups to produce macrophage phenotypes for RNA-Seq and microarray analysis.

Study	Monocyte isolation	Macrophage maturation	M1 Polarisation	M2a polarisation
(Xue *et al.*, 2014)	PBMC isolation from human donors. CD14+ monocyte isolation with MACS beads	50ng/ml GM-CSF for 72 hours	10ng/ml IFNγ, + 10ng/ml LPS for 72 hours	30ng/ml IL-4 for 72 hours
(Martinez *et al.*, 2013)	PBMC isolation from human donors. Cd14+ monocyte isolation with MACS beads	100ng/ml M-CSF for 7 days	N/A	20ng/ml IL-4 for 18 hours
(Beyer *et al.*, 2012)	PBMC isolation from human donors. CD14+ monocyte isolation with MACS beads	30ng/ml GM-CSF for 72 hours	10ng/ml IFNγ for 72 hours	30ng/ml IL-4 for 72 hours

3.4 THP-1 cell model system optimisation

3.4.1 Literature search of protocols for THP-1 polarisation

Comparisons into THP-1 cell line polarisation studies, exposed a wide variation in cell culture procedures that resulted in the production of M1 and M2a macrophages. These different polarisation protocols can be seen in Table 3.5. Most protocols comprised a number of sequential steps to induce THP-1 differentiation into different macrophage subsets: PMA treatment for the maturation of THP-1 cells into M(0) cells, polarisation of matured THP-1 cells towards an M1 phenotype with IFNγ and LPS, and polarisation with IL-4 to induce an M2a phenotype. However, the precise protocols, timing and concentrations of stimuli used were found to vary considerably between studies. Additionally, some protocols included a PMA removal step after THP-1 maturation for a duration of time before treatment with polarising cytokines.

Treatment of THP-1 cells with PMA to produce M(0) cells included differences in both the concentration of PMA used to induce maturation and the amount of time THP-1 cells were exposed to PMA. Concentrations of PMA were found to range from 2.5 to 160ng/ml, with duration of exposure ranging from 6 hours to 3 days.

Timing of the rest period differed between studies, with durations of PMA withdrawal lasting up to 5 days (Daigneault *et al*., 2010) and other groups omitting the rest period altogether (Zhong and Yi, 2016).

Table 3.5: Variation of THP-1 maturation and macrophage polarisation into M1 and M2 macrophages.

Study	PMA concentration and duration	Rest period	M1 polarisation and duration	M2 polarisation and duration
(Shiratori *et al.*, 2017)	50ng/ml for 48hrs	72hr rest	50ng/ml LPS + 20mg/ml IFNγ for 6, 24 and 48hrs	20mg/ml IL-4+ 20mg/ml IL-10 for 6, 24 and 48hrs
(Zhong and Yi, 2016)	92.5ng/ml (or 150nM) for 24hrs	No rest	10pg/ml LPS + 20ng/ml IFNγ for 24hrs	20ng/ml IL-4 + 20ng/ml IL-13 for 24hrs
(Buckley *et al.*, 2016)	61.7ng/ml (or 100nM) for 6hrs	No rest	100ng/ml LPS + 20ng/ml IFNγ for 72hrs	20ng/ml IL-4 + 20ng/ml IL-13 for 72hrs.
(Li *et al.*, 2016)	Varied for M1 or M2 polarisation	No rest	Initial 200ng/ml PMA for 6hrs then 200ng/ml PMA + 100ng/ml LPS + 20ng/ml IFNγ for 66hrs	200ng/ml PMA + 20ng/ml IL-4, + 20ng/ml IL-13 for 66hrs
(Genin *et al.*, 2015)	92.5ng/ml (or 150nM) for 24hrs	24hr rest	10pg/ml LPS + 20ng/ml IFNγ, duration not stated	20ng/ml IL-4 + 20ng/ml IL-13, duration not stated
(Wang *et al.*, 2014)	197.4ng/ml (or 320nM) for 48hrs	No rest	5ng/ml IFNγ for 48hrs	100ng/ml M-CSF for 48hrs
(Chanput *et al.*, 2013)	100ng/ml for 48hrs	24hr rest	1ug/ml LPS + 20ng/ml IFNγ for 6hrs	20ng/ml IL-4 for 24hrs
(Freytes *et al.*, 2013)	50ng/ml for 48hrs	No rest	240ng/ml LPS + 20ng/ml IFNγ for 48hrs	20ng/ml IL-4 + 20ng/ml IL-13 for 48hrs

3.4.2 Titration of PMA for THP-1 monocyte maturation

To identify the minimum dose of PMA required to induce maturation of THP-1 cells towards M(0) cells, without activating other intracellular signalling pathways, a PMA titration was carried out and the effect of increasing PMA concentrations on transcriptional markers was analysed using end-point PCR. The use of hMDMs, isolated from human donors and polarised into different macrophage phenotypes were used as positive controls to confirm the upregulation of phenotype-specific transcripts in their respective macrophage phenotypes. In addition to this, no-reverse transcriptase and no-template controls were run as negative controls to confirm a lack of DNA or RNA contamination in both the cDNA and the RNA template. These controls were used for all end-point PCR experiments carried out in this study.

A series of PMA concentrations were chosen in result of the literature search. PMA concentrations 0ng/ml, 2.5ng/ml, 5ng/ml, 10ng/ml, 20ng/ml, 40ng/ml, 80ng/ml, and 160ng/ml were used to identify the optimum concentration of PMA for the maturation of monocytic THP-1 cells into M(0) cells. Concentrations higher than 100ng/ml were included due to the minimum concentration for THP-1 maturation being suggested as 100ng/ml after 48 hours treatment (Chanput *et al*., 2014). Higher concentrations of PMA were excluded from the titration due to described induction of protein kinase C (PKC) (Schwende *et al.*, 1996) and subsequent NF-κB pathway activation (Chanput *et al*., 2014; Bomsztyk *et al*., 1991).

NF-κB, a transcription factor induced by various inflammatory stimuli such as LPS, is known to direct M1 differentiation and activate downstream regulators of pro-inflammatory cytokines, chemokines and other mediators of inflammation in M1 macrophages (Sica and Mantovani, 2012). It is therefore important to limit NF-κB expression when maturing THP-1 cells towards macrophages to avoid unwanted polarisation resulting in skewing towards an M1 phenotype.

3.4.2.1 M1 marker expression

Expression of M1 transcripts induced in M(0) macrophages through treatment with PMA and without the addition of M1 polarising cytokines (non-specific marker expression) was investigated using end-point PCR (Figure 3.1a). It was found that expression of *STAT1* and *IRF1* transcripts were highly upregulated at 24 hours by all concentrations of PMA that were investigated. Additionally, transcripts *CXCL10*, *HLA-DRB1* and *IL6* also underwent upregulation, however, expression of these transcripts was found to be lower, with expression of *IL6* seen very faintly at 20 and 40ng/ml only. Unexpectedly, as PMA concentration was increased, expression of all the non-specifically induced transcripts (*STAT1*, *IRF1*, *CXCL10* and *HLA-DRB1*) decreased, with 160ng/ml of PMA showing the lowest amount of expression.

CXCL9 was not upregulated by PMA.

3.4.2.2 M2a marker expression

Expression of M2a transcripts induced non-specifically by PMA was also investigated using end-point PCR (Figure 3.1b). After the addition of PMA for 24 hours, *PPARG* expression was found to be highly upregulated for all concentrations of PMA, yet no expression could be seen for the M2a polarised hMDMs used as a positive control. Further experiments using this hMDM positive control for *PPARG* expression confirmed a lack of upregulation therefore, due to this repeated lack of expression in hMDMs, *PPARG* was not used for the optimisation of M2a macrophages and was removed from the marker panel. Expression of chemokines transcripts *CCL22* and *CCL26* was also induced in both the THP-1 cells and the primary M2a macrophages, however, expression was found to be much lower than *PPARG*. Interestingly, as seen with M1 transcripts, the higher PMA concentrations resulted in the lowest upregulation of M2a transcripts. This can be seen clearly for the expression of *TGM2*, which has the strongest expression after 2.5ng/ml of PMA with a gradual reduction in expression as the concentration of PMA was increased.

Transcriptional markers *CCL17, CCL23, CD200R1, MRC1* were not induced by the addition of PMA.

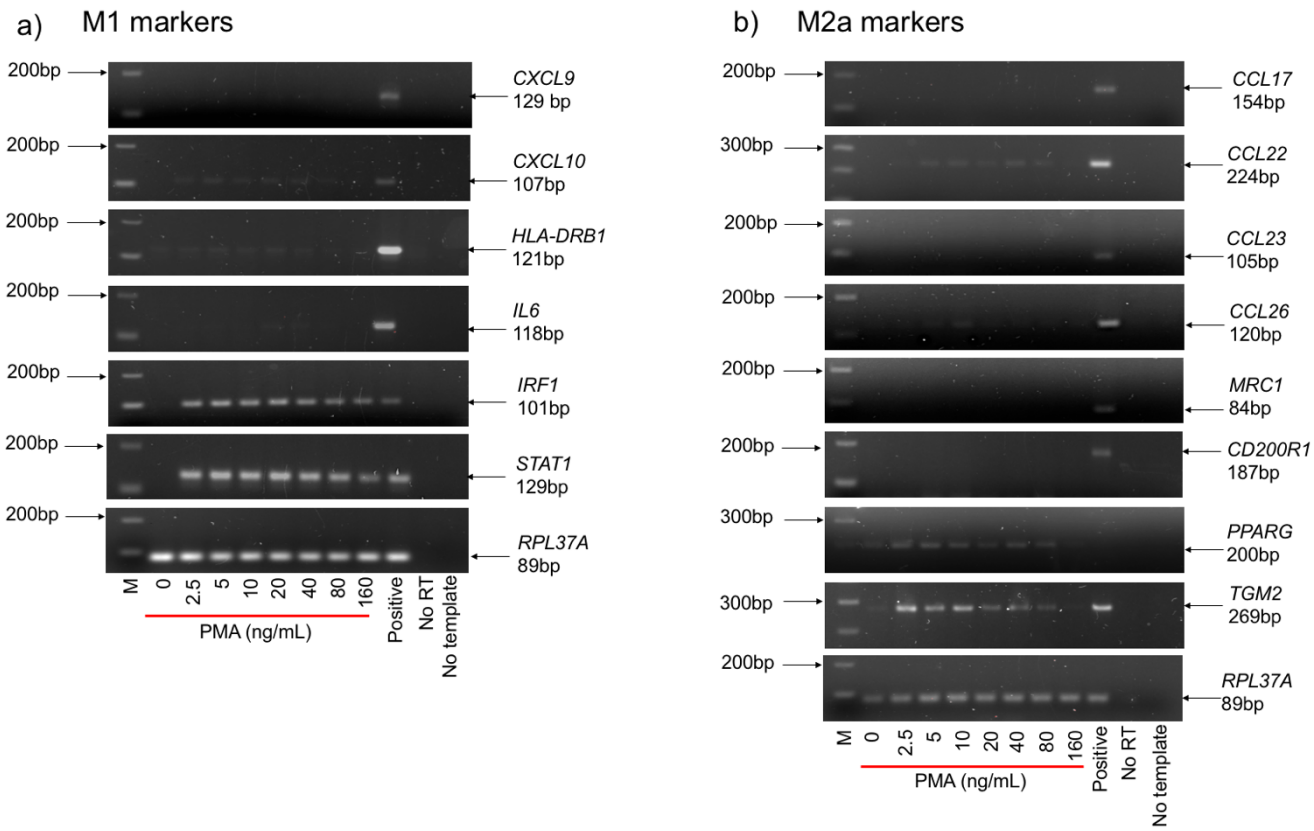


Figure 3.1: Expression of markers in THP-1 cells treated with a PMA titration.

THP-1 cells were treated with a range of PMA concentrations (2.5, 5, 10, 20, 40, 80 and 160ng/ml over 24 hours before cell lysis. Expression of markers **a)** M1 and **b)** M2a were then analysed using end-point PCR. hMDMs polarised using LPS and IFNγ or IL-4 were used as a positive control for M1 and M2a markers, respectively. RPL37A was used as a loading control. The experiment was perfomed once. IFNγ, interferon gamma; IL, interleukin; LPS, lipopolysaccharide; hMDM, human monocyte-derived macrophage; M, molecular ladder; PMA, phorbol myristate acetate; PCR, polymerase chain reaction; RT, reverse transcriptase.

3.4.3 Morphology of PMA-treated THP-1 cells

Changes to the morphology of THP-1 cells, after the addition of varying concentrations of PMA over 24 hours, were investigated (Figure 3.2). As the concentration of PMA was increased, an increasing proportion of cells could be seen to adhere to cell culture plates and expand in size, indicating maturation towards a M(0) macrophage-like cell type. It was noticed that some cells exhibiting a spindle-like structure with filopodia, a morphology associated with M1 polarised cells, could be seen from the lowest concentration of 2.5ng/ml PMA (Figure 3.3) and the number of cells with this morphology increased as the concentration of PMA was increased to 160ng/ml. This may imply non-specific cellular polarisation towards an M1 phenotype induced through PMA treatment. Interestingly, cellular adherence was visibly lower after 24 hours exposure to the highest concentration of PMA (160ng/ml) compared to 80ng/ml which suggests an increased amount of cell death when high concentrations of PMA are used.

3.4.4 Relationship of PMA concentration and cell viability

To understand whether the lowered cellular adhesion was due to a decrease in cell viability because of high PMA concentrations, the number of viable cells was analysed using an automated cell counter, viability was determined with the use of trypan blue. No difference in the viability of the PMA-treated THP-1 cells across all the concentrations of PMA that were used was found, with viability of cells not falling lower than 60% (Figure 3.4).

Cells treated with 2.5ng/ml of PMA showed the highest viability of 78% and cells treated with 10ng/ml of PMA showed the lowest viability at 61%. From this data, it can be inferred that higher concentrations of PMA are not toxic to THP-1 cells and do not contribute to the lowered adhesion of THP-1 cells at concentrations of 160ng/ml.

This data provides evidence to show that, even at concentrations as low as 2.5ng/ml of PMA, it is essential to remove the non-specific marker expression induced by this treatment prior to polarisation of matured THP-1 cells into M1 and M2a macrophage.

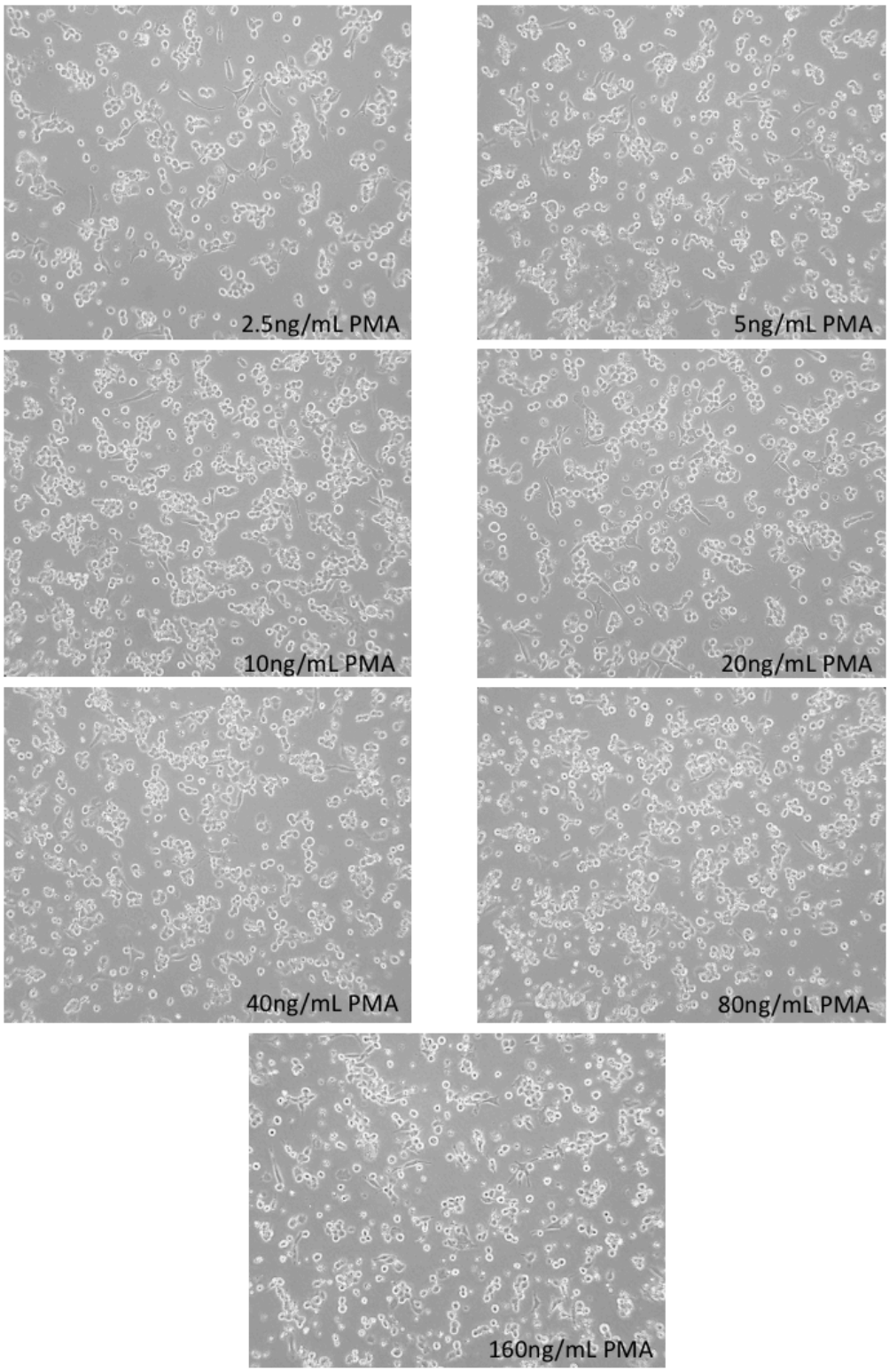


Figure 3.2: Effect of increasing PMA concentrations on THP-1 cell morphology.

THP-1 cells were exposed to 2.5, 5, 10, 20, 40, 80 or 160ng/ml PMA for 24 hours before cells were imaged. Morphological changes of cells after varying concentrations of PMA treatment was examined. Images of cells were taken at x200 magnification. PMA, phorbol myristate acetate.

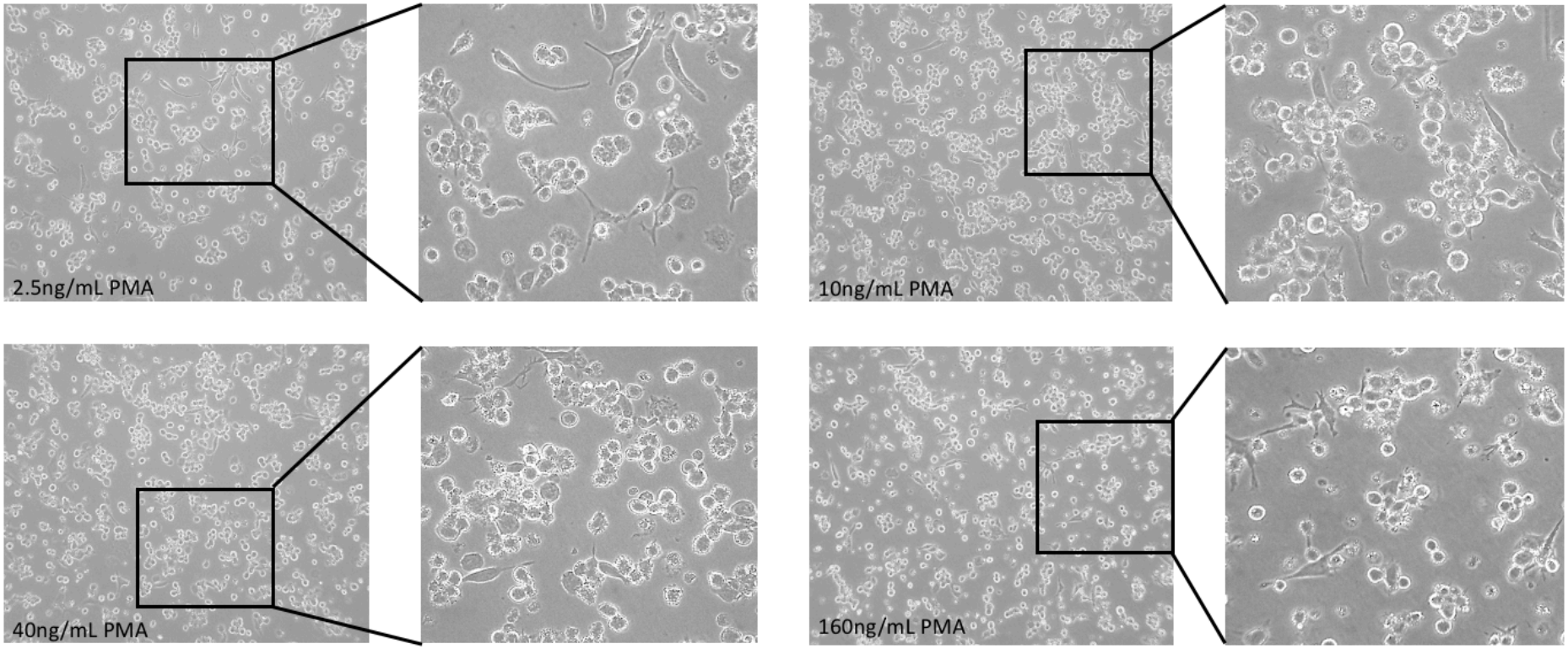


Figure 3.3: Increased magnification of THP-1 cells treated with increasing concentrations (2.5, 10, 40 and 160ng/ml) of PMA.

THP-1 cells were treated with 2.5, 10, 40 and 160ng/ml of PMA over a 24 hour period. Cells were imaged at x200 and x400 magnification to visualise the changes in morphological appearance of THP-1 cells treated with increasing concentrations of PMA in more detail. PMA, phorbol myristate acetate.

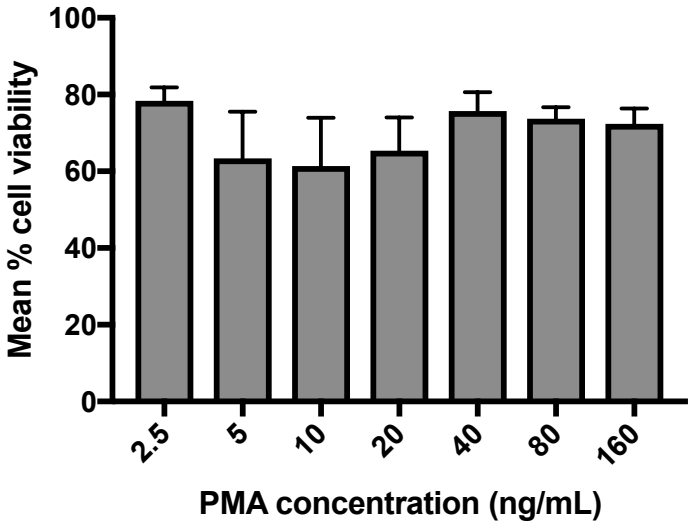


Figure 3.4: Viability of THP-1 cells after exposure to increasing PMA concentrations.

THP-1 cells were exposed to PMA concentrations of 2.5, 5, 10, 20, 40, 80 and 160ng/ml for 24 hours before cell viability was visualised by staining cells with trypan blue. Cell viability was determined using 3 biological replicates and expressed as mean ± S.D. PMA, phorbol myristate acetate.

3.4.5 Effect of rest period on THP-1 cell maturation.

The removal of PMA from the THP-1 cell culture over different durations was investigated after the initial 24 hour PMA treatment, to understand whether non-specific marker expression could be reduced or eliminated, and the bias towards a M1 phenotype could be abolished. The results from the literature search led to the investigation of 0, 1, 2, 3, 4 and 5 days of PMA withdrawal (Daigneault *et al*., 2010; Lund *et al*., 2016; Shiratori *et al*., 2017; Genin *et al*., 2015).

3.4.5.1 Non-specific marker expression

End-point PCR was used to analyse the expression of the markers that were previously found to be induced non-specifically by PMA, after rest periods of 1, 2, 3, 4, and 5 days. Results of M1 transcript markers *CXCL10, HLA-DRB1, IL6, IRF1* and *STAT1* can be found in Figure 3.5, with M2a transcript expression of *CCL22, CCL26* and *TGM2* after different rest periods can be seen in Figure 3.6. Gene transcription of markers *CXCL10*, *HLA-DRB1* and *IL6*, and *CCL26* were found to be absent after 24 hours of rest and remained absent throughout all rest periods tested. It was found that the more strongly a gene was upregulated through PMA stimulation, the longer the rest period required to remove non-specific expression. This can be seen for *TGM2*, which showed high levels of transcription at all concentrations after 24 hours of rest, however, as the rest period increased, the intensity of expression was found to decrease until base line expression levels were detected up to a concentration of 10ng/ml PMA. The higher the concentration of PMA that was used, however, the longer the rest period was required to remove non-specific upregulation. A 5 day rest period was found to be too short to remove non-specific *TGM2* expression in cells treated with PMA at a concentration of 20ng/ml and higher. The same response to the rest period could be seen for *CCL22*, where an increasing amount of rest resulted in lower expression of the gene, interestingly however, the lower the amount of PMA used to induce a mature phenotype, the stronger the upregulation of *CCL22*. For PMA concentrations between 40ng/ml and 160ng/ml, expression of *CCL22* was found to decrease as the rest period was increased until it was absent by day 5. *STAT1* and *IRF1* transcripts were seen to be ubiquitously expressed at all PMA concentrations over the 5 day rest period. As previously mentioned, *STAT1* and *IRF1* have been implicated in the polarisation of cells towards an M1 phenotype and both induce the expression of *CXCL10*. The lack of *CXCL10* expression after 24 hours of rest suggests a lack of further cell signalling via these two pathways and therefore a return to a non-polarised state.

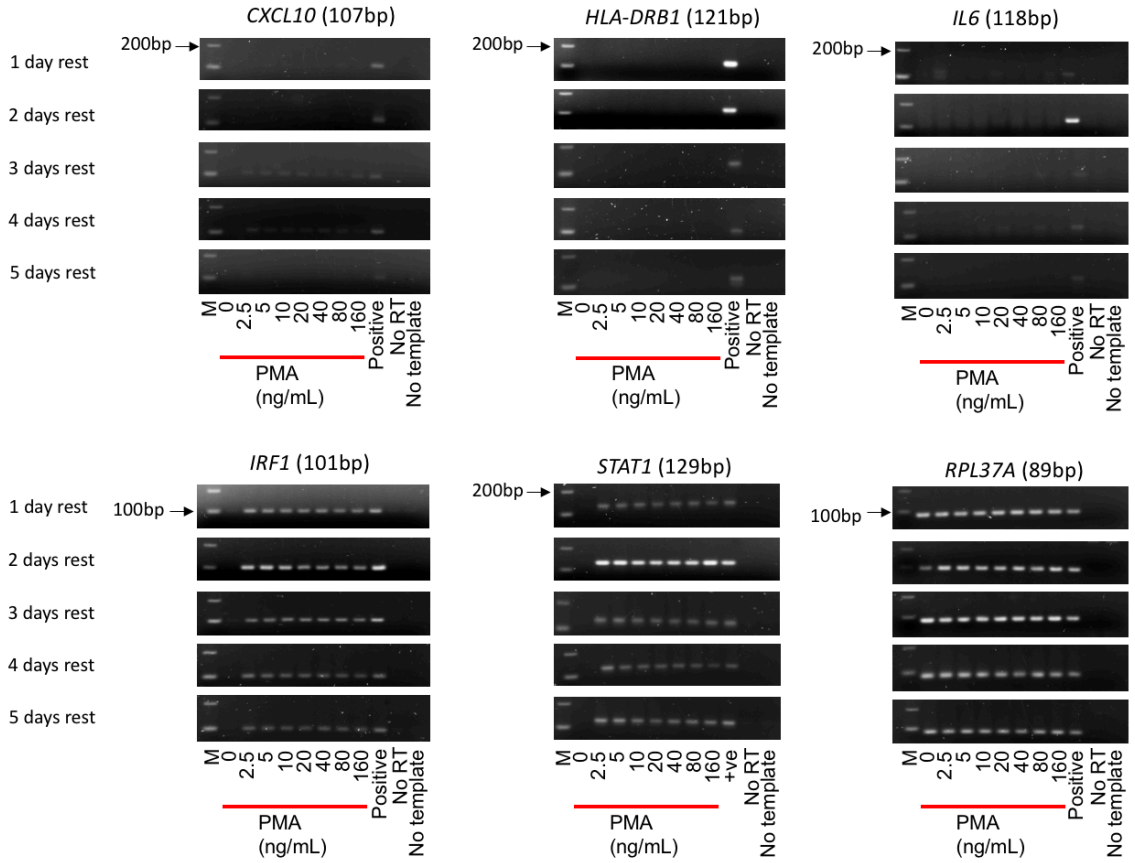


Figure 3.5: Expression of non-specifically induced M1 transcript markers in THP-1 cells at various durations of PMA withdrawal.

THP-1 cells matured with PMA for 24 hours were rested in PMA-free media for 1 to 5 days. Expression of non-specifically upregulated M1 markers *CXCL10*, *HLA-DRB1*, *IL6*, *IRF1* and *STAT1* were analysed at these different time points. hMDMs polarised using LPS and IFNγ were used as positive controls for M1 markers. *RPL37A* was used as a loading control. The experiment was performed once. IFNγ, interferon gamma; LPS, lipopolysaccharide; hMDM, human monocyte-derived macrophage; M, molecular ladder; PCR, polymerase chain reaction; PMA, phorbol myristate acetate; RT, reverse transcriptase.

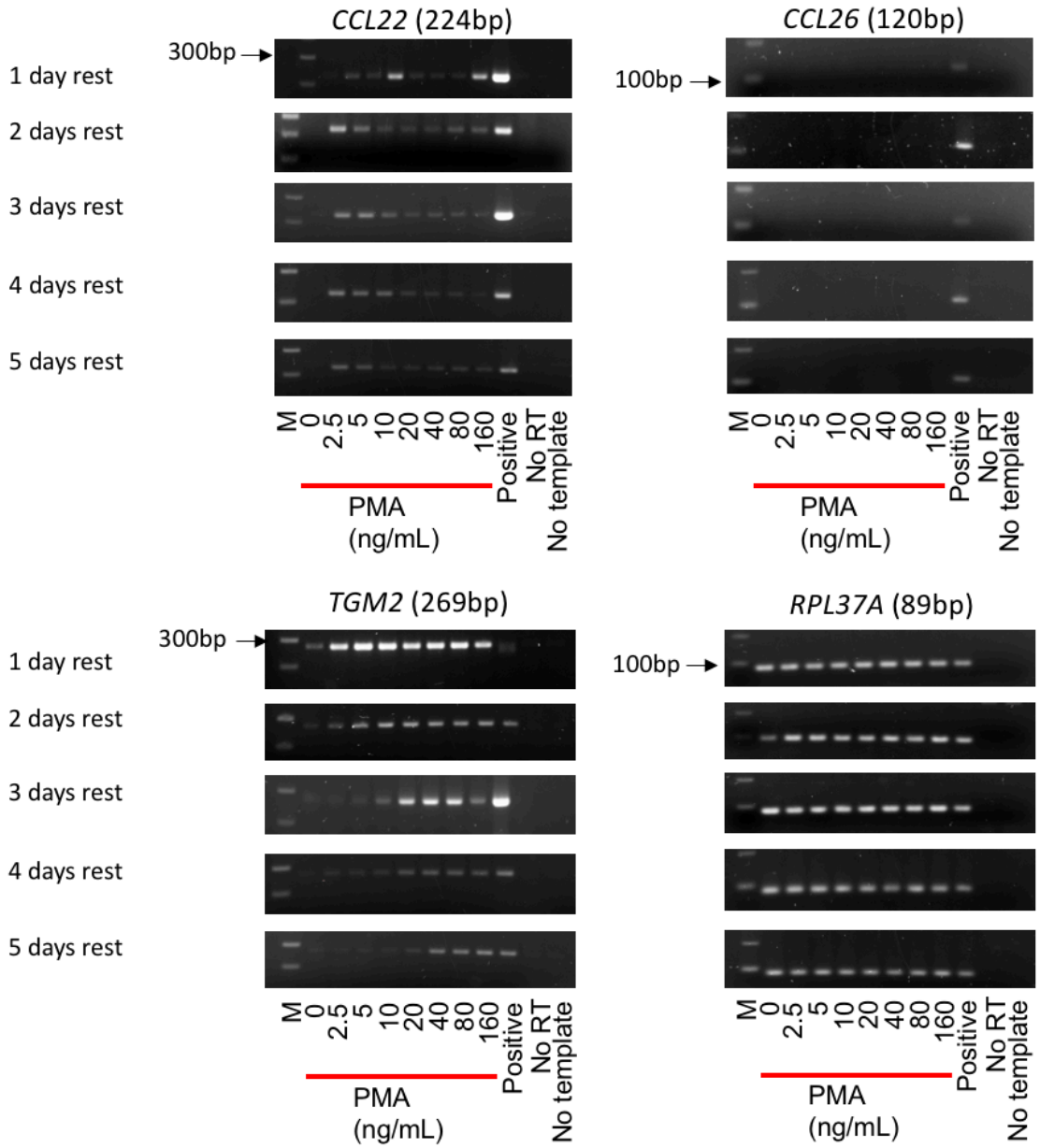


Figure 3.6: Expression of non-specifically induced M2a transcript markers in THP-1 cells at various durations of PMA withdrawal.

THP-1 cells matured with PMA for 24 hours were rested in PMA-free media for 1 to 5 days. Expression of non-specifically upregulated M2a markers *CCL22*, *CCL26* and *TGM2* were analysed at these different time points. hMDMs polarised using Il-4 were used as positive controls for M2a markers. *RPL37A* was used as a loading control. The experiment was performed once. IL, interleukin; hMDM, human monocyte-derived macrophage; M, molecular ladder; PCR, polymerase chain reaction; PMA, phorbol myristate acetate; RT, reverse transcriptase

Marker panels were altered by removing M1 markers *STAT1* and *IRF1* and M2a marker *CCL22*, which showed non-specific gene upregulation in M(0) controls after 5 days of PMA withdrawal (Figure 3.7). The remaining markers were used to optimise the polarisation of matured THP-1 cells into polarised M1 or M2 macrophages.

Carrying on from these experiments it was decided that a 5ng/ml concentration of PMA together with a 3-day rest period in PMA-free media was optimum for the maturation of THP-1 cells towards a macrophage-like cell. Using these conditions, matured THP-1 cells showed a lack of non-specific upregulation of the M2a marker *TGM2*, where higher concentrations of PMA that were used induced *TGM2* expression. Additionally, cells showed an adhesive morphology with cell spreading whilst exhibiting a loss of spindle-like cells. A lower concentration of 2.5ng/ml PMA was not chosen due to the increased number of detachable cells, a problem also encountered by Park *et al*. (2007); Takashiba *et al*. (1999).

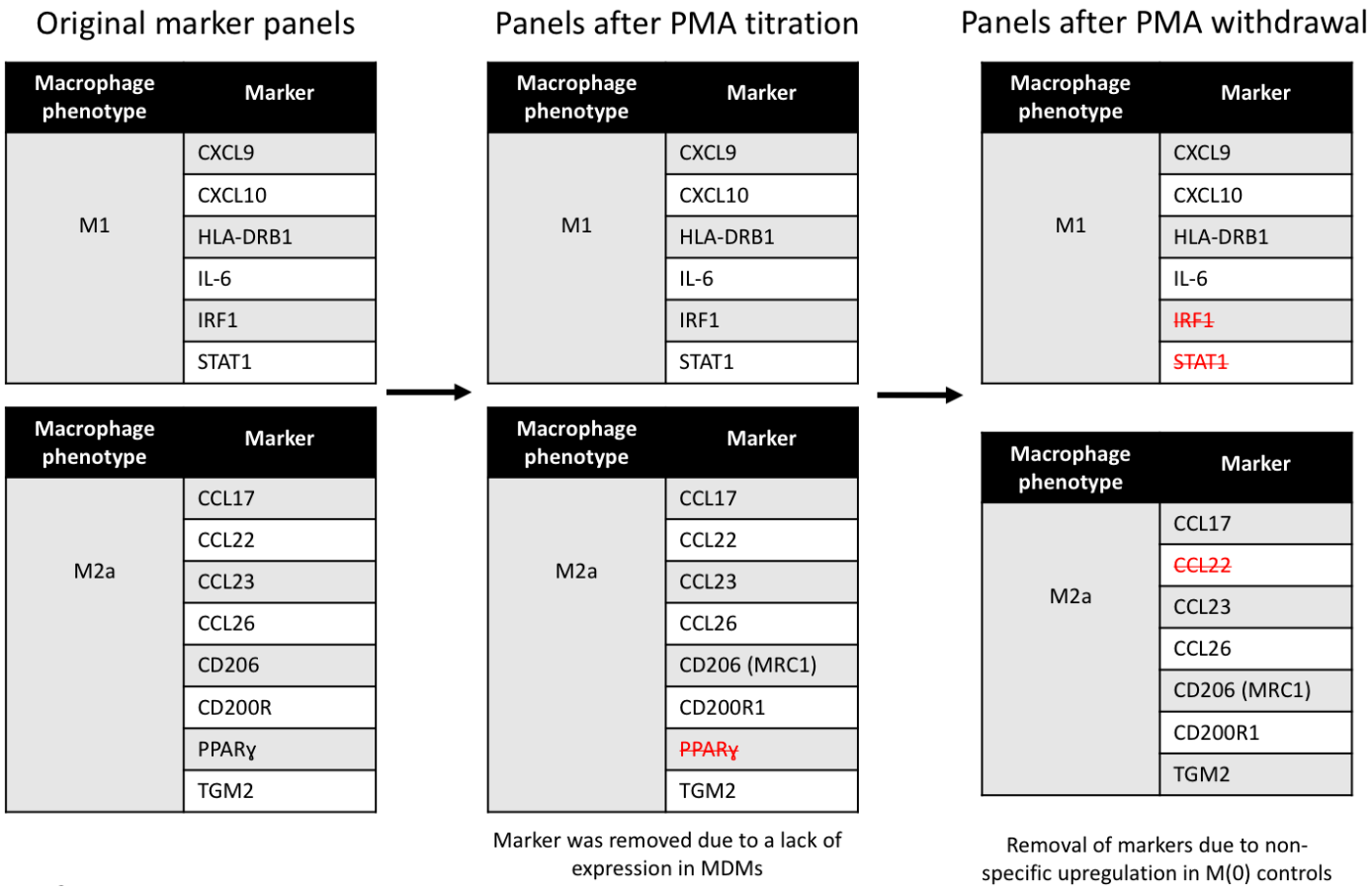


Figure 3.7: Updated marker panels to show process of removing phenotypic markers after PMA withdrawal experiments.

IL, interleukin; MDM, monocyte-dervied macrophage; PMA, phorbol 12-myristate 13-acetate.

3.4.6 Polarisation of macrophage-like cells

Macrophages can be polarised into different phenotypes using various combinations of cytokines. In this study, macrophages were polarised using the most commonly used cytokines M(LPS, IFNγ) for pro-inflammatory macrophages and M(IL-4) for anti-inflammatory macrophages for optimisation of the THP-1 cell model.

3.4.6.1 Polarisation of macrophage-like cells towards a M(LPS, IFNγ) phenotype

Experiments were carried out in a parallel study by Nikki Re to determine the optimum concentration of polarising cytokines, LPS and IFNγ, for differentiation of THP-1 derived M(0) cells into an M(LPS, IFNγ) phenotype. It was found that culturing THP-1 derived M(0) cells in 250ng/ml LPS and 20ng/ml of IFNγ over 48 hours, resulted in cells which expressed the most markers taken from the updated M1 marker panel in Figure 3.7 therefore more closely resembling M1 hMDMs.

3.4.6.2 Polarisation of macrophage-like cells towards a M(IL-4) phenotype

An anti-inflammatory macrophage phenotype can be induced through IL-4 stimulation. Variations in concentration and duration of IL-4 treatment was found throughout the literature, both using hMDMs and THP-1 cells. These parameters were optimised for differentiation of the matured THP-1 M(0) cells.

3.4.6.3 IL-4 concentration titration

Following the maturation of THP-1 cells towards a M(0) cell type via PMA treatment (5ng/ml) for 24 hours and a 72 hour rest, a 24 hour, IL-4 titration was carried out using either 20ng/ml, 25ng/ml or 30ng/ml of cytokine; expression of M2a markers were analysed in these cells as well as in M(0) control cells using end-point PCR (Figure 3.8a).

Expression of *CCL17* and *TGM2* were found to be induced at 24 hours at the same intensity across all the different concentrations of IL-4 analysed. Expression of *TGM2* was also seen in M(0) cells. Additionally, *CCL26* was expressed at all concentrations after 24 hours of cytokine stimulation however, 20ng/ml was found to produce the highest amount of gene expression with 25ng/ml showing the lowest expression. Importantly, no expression of M2a markers *CCL23*, *CD200R1* and *MRC1* at any IL-4 concentration by 24 hours could be seen.

3.4.6.4 IL-4 exposure time course

Increasing the length of IL-4 exposure to 48 and 72 hours was carried out due to the lack of expression found for some markers after 24 hours of cytokine stimulation. Cells were again treated with the same concentrations of IL-4 and expression was analysed using end-point PCR (Figure 3.8b).

Expression of M2a markers *CCL17*, *CCL26*, *MRC1*, *CD200R1* and *TGM2* could be seen by 48 hours, with titration of the cytokine not having a noticeable effect on expression levels. Expression of the chemokine *CCL23* was not induced by 72 hours of IL-4 stimulation and therefore would not be used in further experiments as a M2a marker. Interestingly, expression of all markers, apart from *CD200R1*, showed no difference in expression levels between 48 and 72 hours. Markers that were found to be upregulated at 24 hours, such as *CCL17*, *TGM2* and *CCL26*, showed a gradual decrease in their expression over 48 and 72 hours of IL-4 exposure.

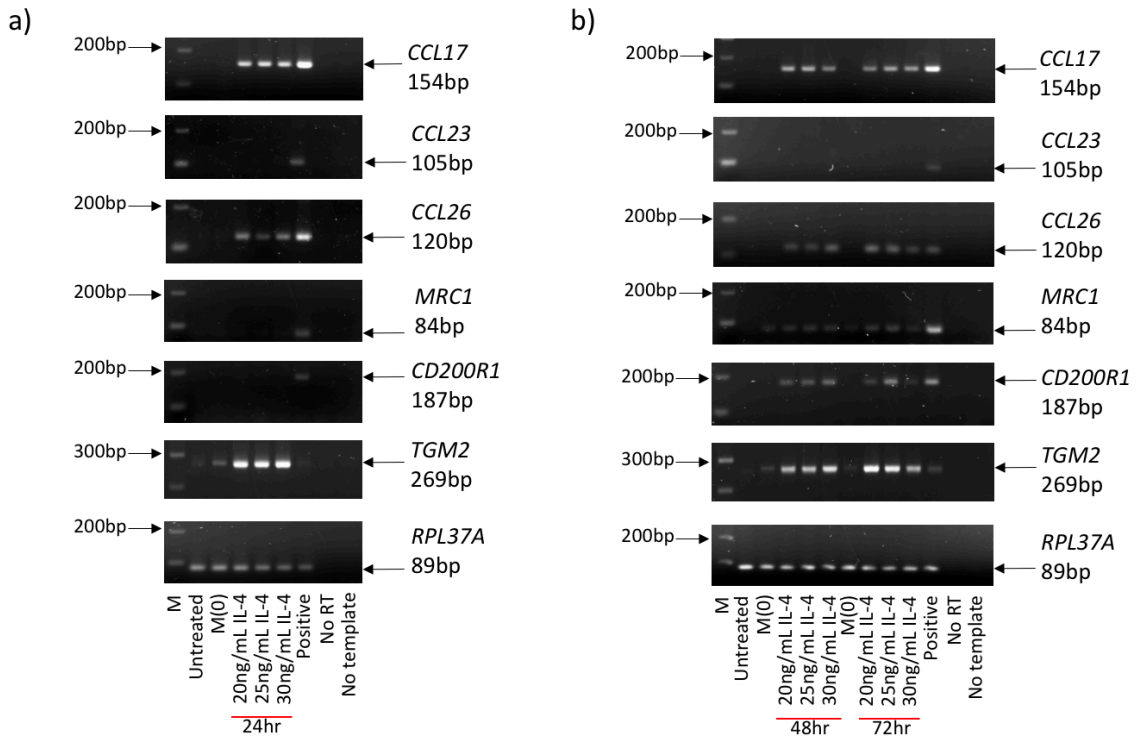


Figure 3.8: Expression of M2a markers in response to a titration of IL-4 over varying durations of cytokine exposure.

THP-1 cells were treated with 5ng/ml PMA over 24 hours and were rested for 72 hours to induce a M(0) macrophage phenotype. M(0) cells were treated with 20ng/ml, 25ng/ml or 30ng/ml of IL-4 for either **a)** 24 hours or **b)** 48 and 72 hours and expression of M2a markers was analysed using end-point PCR. hMDMs polarised using IL-4 were used as a positive control. *RPL37A* was used as a loading control. The experiment was perfomed once. IL, interleukin; hMDM, human monocyte-derived macrophage; PCR, polymerase chain reaction; RT, reverse transcriptase.

Combining the results of M(LPS, IFNγ) and M(IL-4) polarisation optimisation steps, it was found that the highest number of transcripts that were upregulated at any one time was after 48 hours of cytokine stimulation. To obtain polarised cells that closely resembled that of a hMDMs in terms of marker expression, naive THP-1 macrophage-like cells, M(0), were treated using 250ng/ml of LPS and 20ng/ml of IFN-γ to obtain M(LPS, IFNγ) macrophages. For polarisation of THP-1 cells towards an M(IL-4) macrophage, M(0) cells were treated with 30ng/ml of IL-4.

The above results led to the final panels of markers used for the optimisation of the THP-1 optimisation protocol which can be seen in Figure 3.9.

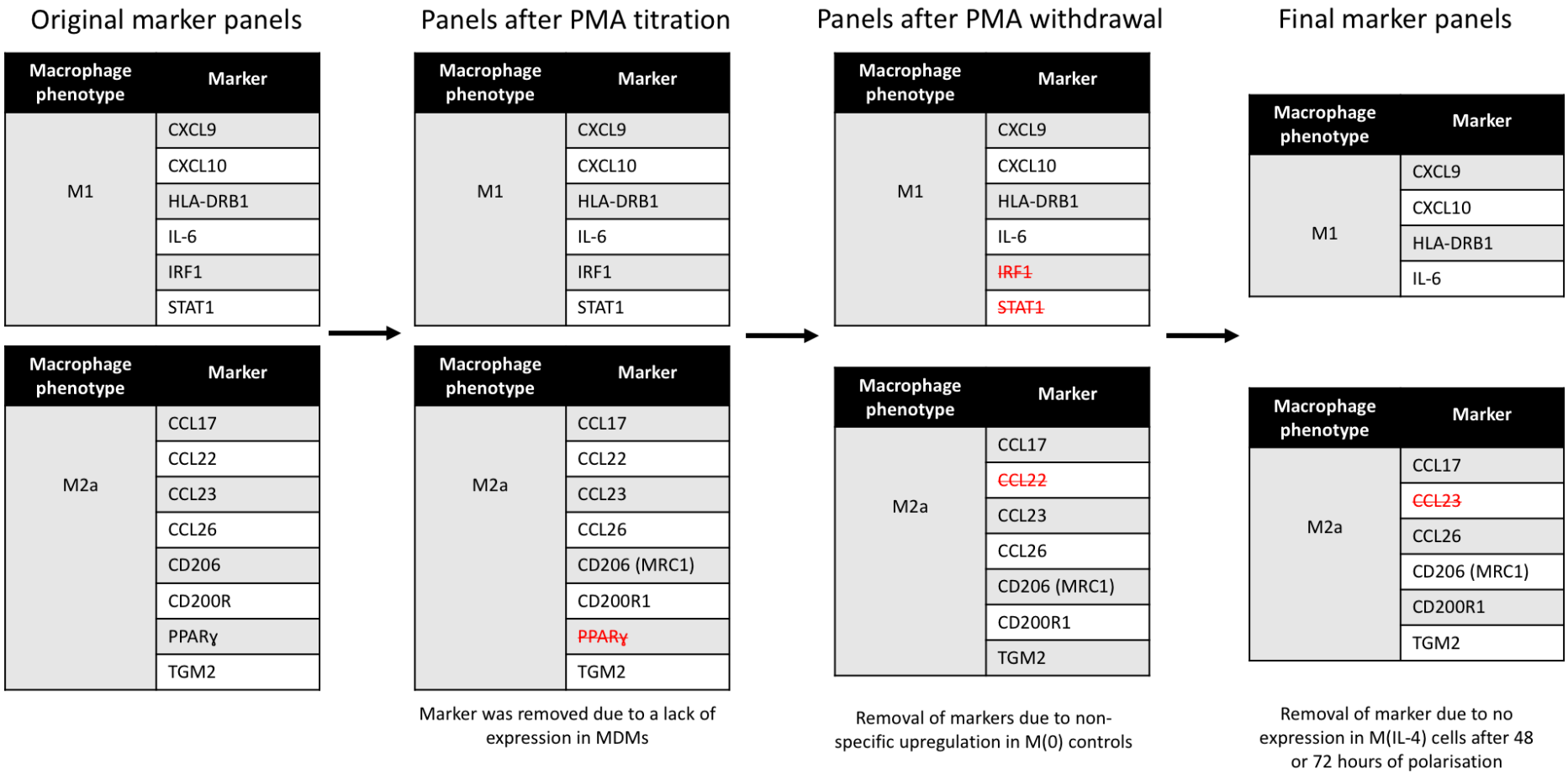


Figure 3.9: Process of producing the final marker panels used for optimising the THP-1 cell model.

IL, interleukin; MDM, monocyte-derived macrophage; PMA, phorbol 12-myristate 13-acetate.

3.5 Final THP-1 cell model protocol.

To investigate the robustness of the THP-1 cell model, the morphological appearance and viability of M(LPS, IFNγ) and M(IL-4) macrophages, cultured using the final protocol, was examined. M1 and M2a markers from the optimisation panel were also analysed and the specificity of these markers for their corresponding macrophage phenotype was determined to identify their suitability for further studies. The final protocol can be seen in Figure 3.10.

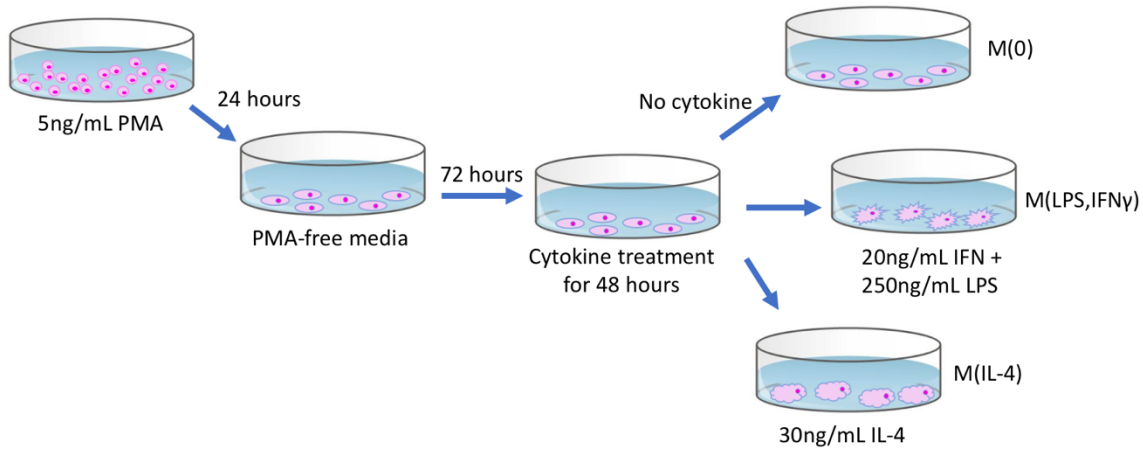


Figure 3.10: Schematic of the final THP-1 cell model protocol for producing M(0), M(LPS, IFNγ) and M(IL-4) macrophages.

THP-1 cells were seeded in media containing 5ng/mL PMA for 24 hours to enable THP-1 macrophage maturation before THP-1-derived macrophages were rested for 72 hours in fresh, non-PMA containing media. Cells were then either left for a further 48 hours or were exposed to 250ng/ml LPS and 20ng/ml IFNγ or 30ng/ml IL-4 for 48 hours to polarise THP-1-derived macrophages into M(LPS, IFNγ) and M(IL-4) cells, respectively. IFNγ, interferon gamma; IL, interleukin; LPS, lipopolysaccharide; PMA, phorbol myristate acetate.

3.5.1 Morphology of polarised macrophage phenotypes

Monocytes undergo substantial changes in regard to their morphological appearance once differentiation towards a mature macrophage begins and once again following differentiation with various cytokines. The differences in cellular form between M1 and M2a-polarised cells are well publicised (Waldo *et al.*, 2008; Cassol *et al.*, 2009; Bertani *et al.*, 2017) and provide a quick and easy way in which to indicate polarisation has occurred.

Morphological differences between M(0), M(LPS, IFNγ) and M(IL-4) polarised macrophages were visualised using immunofluorescence by staining the actin cytoskeleton of the differently polarised cells (Figure 3.11). Considerable differences in morphology could be seen between the three different phenotypes, where Figure 3.11a shows M(0) macrophages and their smaller morphological appearance, M(LPS, IFNγ) macrophages (Figure 3.11b) displayed a distinctive spindle-like structure and M(IL-4) polarised cells (Figure 3.11c) showed a rounded phenotype, described in the literature as ‘fried-egg’ shaped. It is important to note that not all the macrophages, from both phenotypes, show such a pronounced morphology and this may be due to differences in the amount of polarisation of individual cells.

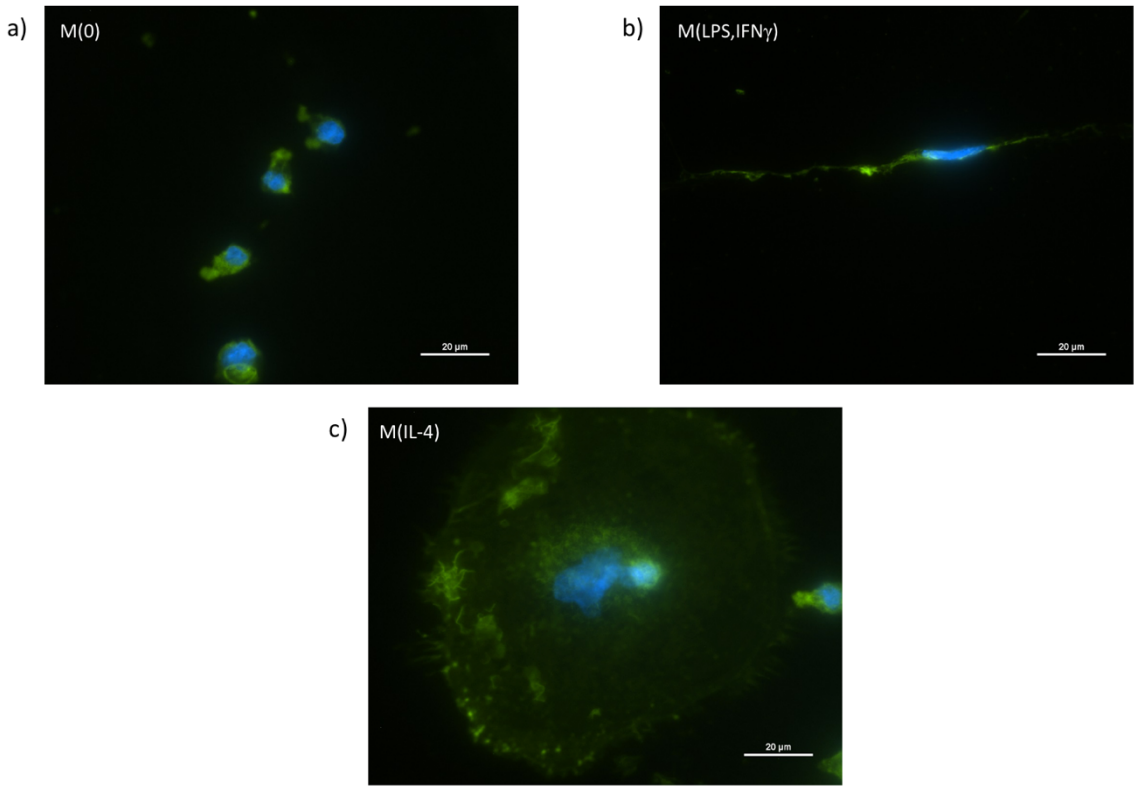


Figure 3.11: Immunofluorescent images of differently polarised macrophages stained to visualise cell morphology.

THP-1-derived macrophages were polarised into **a)** M(0), **b)** M(LPS, IFNγ) and **c)** M(IL-4) phenotypes after 48 hours exposure to cytokine before fixation. Cells were stained with DAPI (blue) and phalloidin (green) to allow visualisation of the nuclei and cytoskeleton, respectively. Cells were imaged at a magnification of x600. IFNγ, interferon gamma; IL, interleukin; LPS, lipopolysaccharide.

3.5.2 Viability

Viability of cells after polarisation for 48 hours towards M(0), M(LPS, IFNγ) or M(IL-4) macrophages was examined using an automated cell counter. The percentage of viable cells from the approximate $5x10^6$ cells originally plated that became and remained attached to the culture plate after THP-1 maturation and polarisation over 6 days, can be seen in Figure 3.12a for M(0), M(LPS, IFNγ) and M(IL-4) cells respectively. On average, for all phenotypes, less than 50% of cells attached to the plate, differentiated and remained attached for 6 days before their use in subsequent experiments. M(LPS, IFNγ) exhibited some degree of variation in the amount of cells that attached and remained so after 6 days. As expected, of those cells remaining attached to the plate for each phenotype, a high percentage of cells remained viable after 6 days of cell culture (Figure 3.12b) as non-viable cells would eventually become detached.

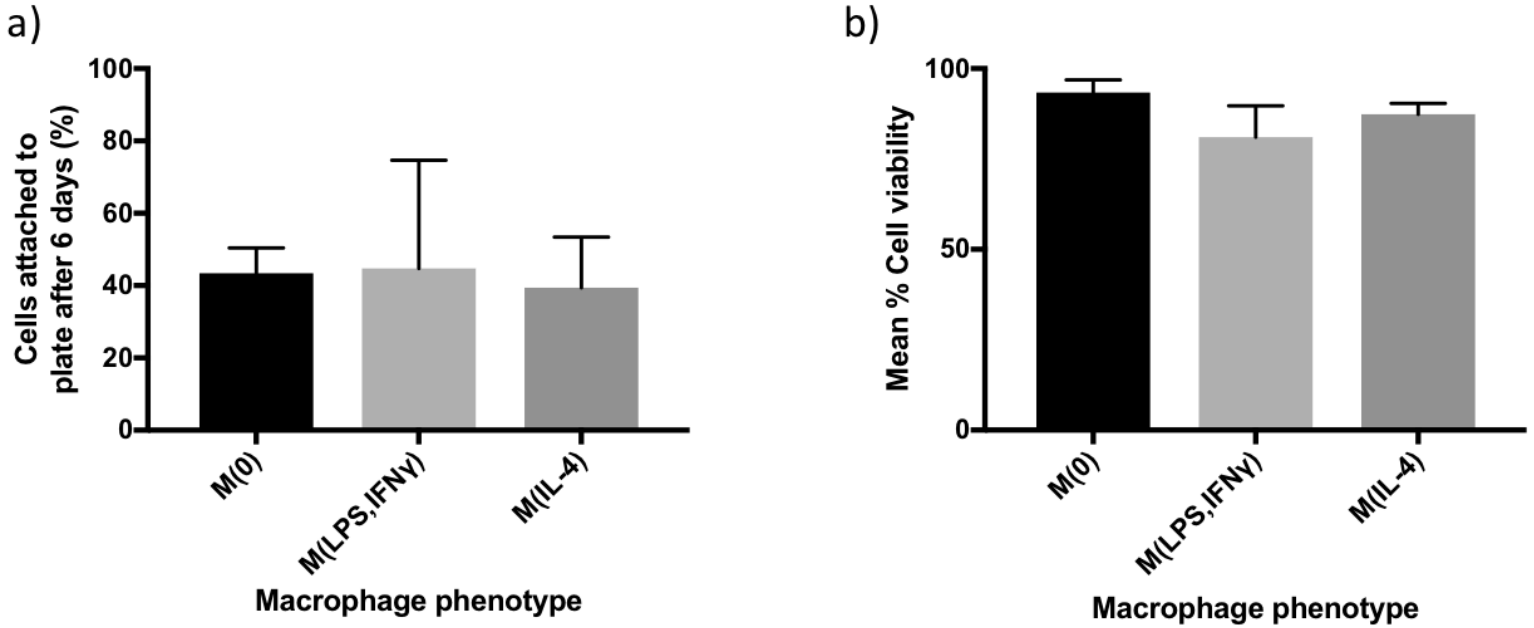


Figure 3.12: Analysis of cellular adhesion and viability after polarisation of THP-1-derived macrophages.

THP-1-derived macrophages were polarised into M(0), M(LPS, IFNγ) and M(IL-4) phenotypes after 48 hours cytokine exposure. Cells were detached from the culture surface using cold PBS, cells were stained with trypan blue. **a)** The mean percent of viable polarised macrophages (M(0), M(LPS, IFNγ) or M(IL-4)) which attached to the plate after 6 days of cell culture from the approximate $5x10^6$ cells originally plated and remained attached. **b)** The mean percent viability of attached THP-1 derived macrophages polarised towards M(0), M(LPS, IFNγ) and M(IL-4) after 6 days of cell culture. Values were determined using 3 biological replicates for each phenotype and are expressed as mean ± S.D. IFNγ, interferon gamma; IL, interleukin; LPS, lipopolysaccharide.

3.5.3 M1 marker expression and specificity

After analysing the panel of M1 markers in M(0), M(LPS, IFNγ) and M(IL-4) macrophages (Figure 3.13a), the chemokine, *CXCL9*, was found to be highly specific for M(LPS, IFNγ) polarised THP-1 cells with no expression seen for the untreated, M(0) or M(IL-4) phenotypes, although in comparison to the primary M(LPS, IFNγ) macrophages, expression of *CXCL9* in THP-1 polarised cells was much higher. Additionally, the transcriptional expression of the chemokine *CXCL10* and the cytokine *IL6* was also very specific for M(LPS, IFNγ) polarised THP-1 cells with expression also seen in primary cells. The transcriptional expression of *HLA-DRB1*, a surface marker, was also found to be specific to M(LPS, IFNγ) THP-1 cells, however, expression of this gene in M(LPS,IFNγ) polarised THP-1 cells was extremely low. None of the M1 transcript markers analysed were found to be expressed in THP-1 derived M(IL-4) macrophages. The expression of chemokines and cytokines associated with M(LPS, IFNγ) macrophages seem to be a reliable tool to define M(LPS, IFNγ) macrophages due to their high specificity for these polarised cells. The expression of these transcript markers has helped to prove the robustness of the THP-1 model, however, due to their secreted nature, chemokine and cytokine protein markers are not suitable for identifying macrophages using IHC and therefore would not be used for these studies.

3.5.4 M2a marker expression and specificity

Similar to chemokines *CXCL9* and *CXCL10* in M(LPS, IFNγ) macrophages, the chemokines *CCL17* and *CCL26* were highly specific for M(IL-4) polarised macrophages (Figure 3.13b). Additionally, the surface markers *MRC1* and *CD200R1* were also found to be highly specific for the M(IL-4) phenotype at the transcript level, furthermore, these four markers were found to be more highly expressed in the hMDMs positive control than in THP-1 polarised cells. *TGM2*, a marker which was not expressed in M2a macrophages in the RNA-Seq and microarray data, but was cited in published literature as a specific M(IL-4) marker (Martinez *et al.*, 2013) showed very high transcriptional expression in THP-1 cells polarised towards M(0), M(LPS, IFNγ) and M(IL-4) macrophages, however, expression in the primary MDMs positive control was much lower. *TGM2* expression has been described as being induced through a number of substances, including phorbol esters, of which PMA is one (Eckert *et al*., 2014), which may account for the increased expression in M(IL-4) cells and the non-specific upregulation in other macrophage phenotypes.

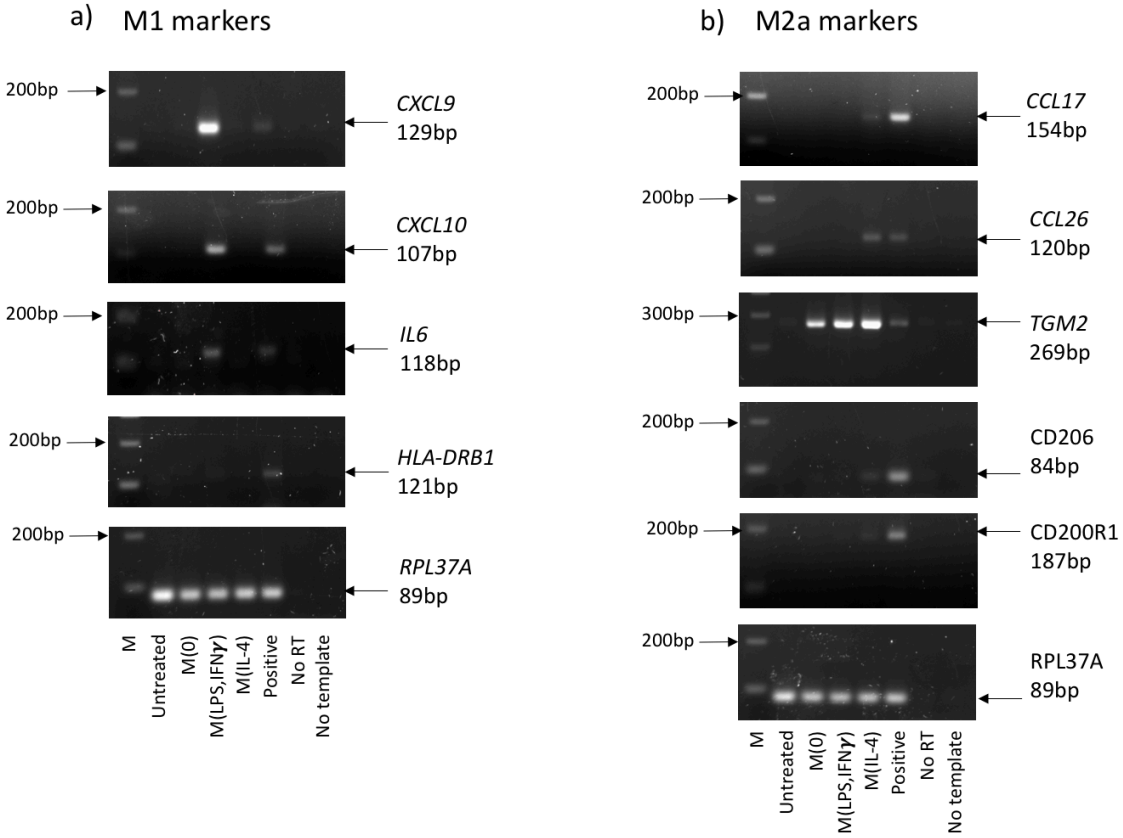


Figure 3.13: Specificity of markers for their respective macrophage phenotype.

Expression of **a)** M1 transcript markers, and **b)** M2a transcript markers, was analysed in THP-1-derived macrophages polarised into M(0), M(LPS, IFNγ) and M(IL-4) phenotypes. hMDMs polarised into either M(LPS, IFNγ) or M(IL-4) were used as a positive control for M1 and M2a markers respectively. *RPL37A* was used as a loading control. The experiment was performed once. IFNγ, interferon gamma; IL, interleukin; LPS, lipopolysaccharide; hMDM, human monocyte-derived macrophage; PCR, polymerase chain reaction; RT, reverse transcriptase.

3.6 Other macrophage phenotypes

Additional phenotypes which included IL-10-treated cells M(IL-10) and IL-33-treated cells M(IL-33) were investigated to determine upregulation of specific genes. M2c cells can be polarised in response to IL-10 as well as exposure to glucocorticoids (GCs), an important point to consider as most GCA patients receive high-dose GCs prior to their biopsy. Furthermore, previous studies have found affected arteries of GCA patients to overexpress IL-33 (Ciccia *et al*., 2013), a cytokine which has been associated with inducing various macrophage phenotypes depending on their polarised state. In mice, IL-33 has been shown to induce an M2a macrophage phenotype from naïve macrophages (Yang *et al.*, 2013) as well as amplifying the M2 phenotype resulting in improved wound healing (He *et al*., 2017). In hMDMs however, treatment of naïve macrophages with IL-33 resulted in cells that expressed M1 chemokine markers whilst treatment of IL-33 to M1 macrophages was found to switch M1 macrophages to an M2a phenotype (Joshi *et al*., 2010).

3.6.1 Treatment with IL-10

Due to the lack of transcript and protein markers described in the literature for M(GC) macrophages, IL-10, a more widely used cytokine to induce a M2c phenotype was used to analyse commonly used M2c transcript markers. The induction of genes in macrophages through treatment with IL-10, identified via literature searches (Table 3.6) were examined in IL-10 treated THP-1 cells using end-point PCR. Specificity of these markers for this macrophage phenotype was also analysed (Figure 3.14).

Table 3.6: M2c markers described in the literature and their location of cellular expression.

M2c marker	studies	Cellular location
CCL18	(Park-Min *et al*., 2005)	Secreted
CD163	(Olmes *et al*., 2016)	Membrane
CXCL13	(Park-Min *et al*., 2005)	Secreted
IL-2Rα	(Park-Min *et al*., 2005)	Membrane
MARCO	(Park-Min *et al*., 2005)	Membrane
MerTK	(Zizzo *et al*., 2012)	Membrane

CCL18 and *MARCO* was shown by Park-Min *et al*. (2005) to be expressed in hMDMs after 48 hours of IL-10 exposure, however, our data shows a lack of expression in both THP-1 and primary M(IL-10) cells as well as for M(0), M(LPS,IFNγ), M(IL-4) polarised THP-1 cells. Expression of *CXCL13* was found in all phenotypes, but strongest in M(LPS, IFNγ) polarised cells. Expression of *CD163* and *MERTK* was also found in all phenotypes, however, strongest expression for both of these markers was induced in THP-1 cells stimulated with IL-10. Interestingly, *CD163* expression was not apparent in hMDMs after both 2 and 7 day exposures to IL-10 (Park-Min *et al.*, 2005), yet CD163 has been commonly used as a marker for M2 macrophages (M2a and M2c), including characterising M2 macrophages in GCA arteries (Ciccia *et al*., 2013). More recent data, however, has suggested CD163 should be used as a pan-macrophage marker rather than a marker for a M2 phenotype (Barros *et al.*, 2013) due to its expression in all macrophage phenotypes, which the RNA-Seq data analysis suggested. The expression of *MERTK* has been associated with M2c macrophages with its expression being suppressed in M1 and M2a macrophage phenotypes (Zizzo *et al*., 2012). Interestingly, however, our data suggests that *MERTK* is not a specific marker for M2c macrophages as high expression could be seen in M(0) and M(LPS, IFNγ) cells. Furthermore, Zizzo *et al*. (2012) described the lack of induction of *MERTK* in response to IL-10 alone, with the

need of M-CSF or glucocorticoids for its upregulation, yet out data contradicts this, with expression of *MERTK* being apparent in both IL-10 treated THP-1-derived macrophages and hMDMs. Overall, at the transcript level, no marker was specific for IL-10 treated THP-1 cells.

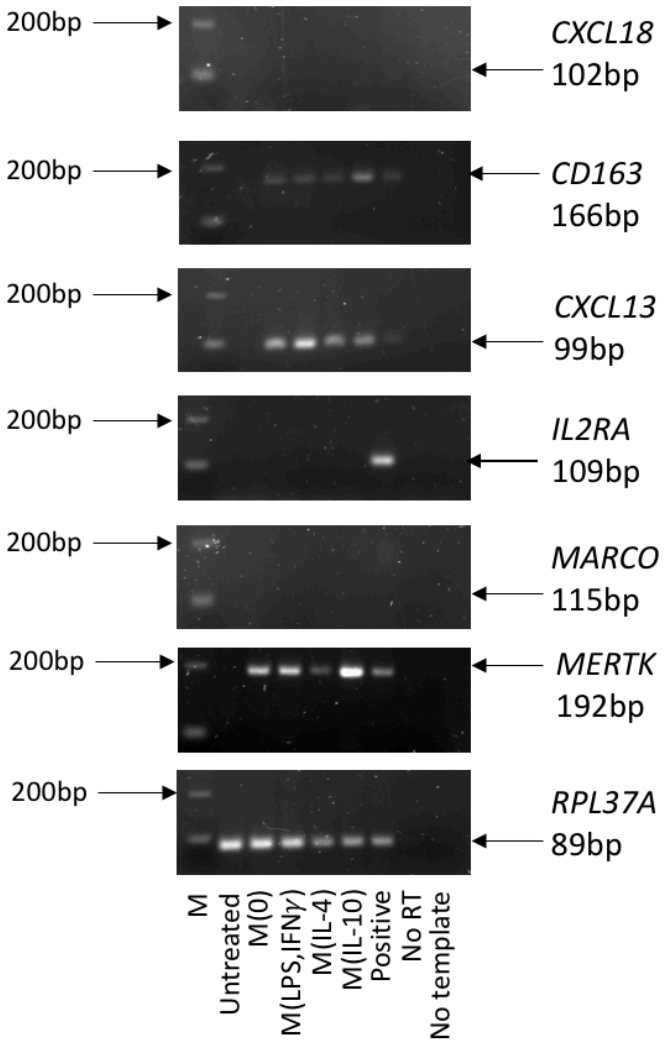


Figure 3.14: End-point PCR showing specificity of M2c markers identified from the literature.

THP-1 derived macrophages were polarised towards a M(0), M(LPS, IFNγ), M(IL-4) and M(IL-10) phenotype and were lysed after 48 hours exposure to cytokine. M2c markers were examined using endpoint PCR in these phenotypes. hMDMs polarised into an M(IL-10) phenotype were used as a positive control. *RPL37A* was used as a loading control. The experiment was performed once. IFNγ, interferon gamma; IL, interleukin; LPS, lipopolysaccharide; hMDM, human monocyte-derived macrophage; PCR, polymerase chain reaction; RT, reverse transcriptase.

3.6.2 Treatment with IL-33

Expression of M1 and M2a markers were analysed using end-point PCR in THP-1 cells which had been polarised to M(LPS, IFNγ), M(IL-4) and M(IL-10) cells using the THP-1 cell model protocol prior to the addition of IL-33 for an additional 24 hours, to confirm amplification of a M2a-like macrophage phenotype.

It was found that the addition of IL-33 did not result in the induction of any M1, M2a or M2c transcript markers in any macrophage phenotypes derived from THP-1 cells, with treatment of IL-33 alone to M(0) cells also showing no induction of any transcriptional expression (Figure 3.15). In contrast, the addition of IL-33 alone to primary macrophages, hMDM(IL-33), resulted in the induction of pro-inflammatory M1 markers *CXCL9*, *CXCL10* and *IL6* (Figure 3.15a), however, no M2a and M2c transcripts were induced by IL-33 (Figure 3.15b and c).

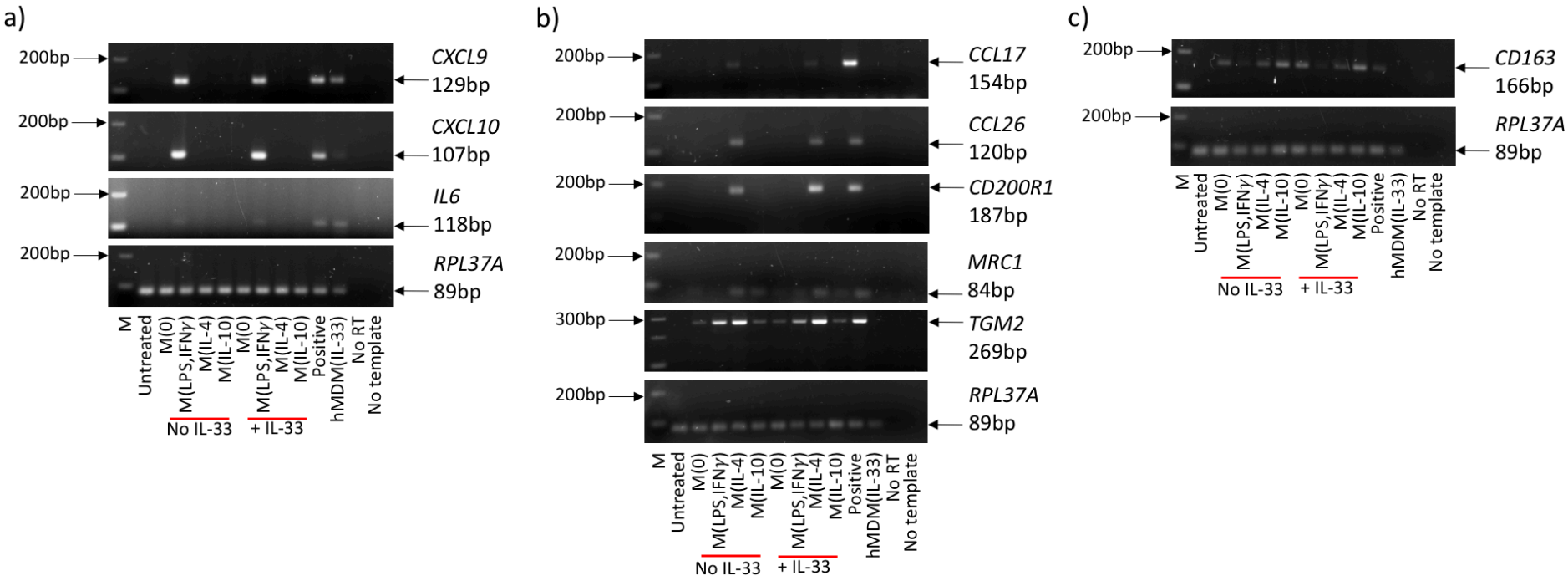


Figure 3.15: The effect of IL-33 on the expression of M1, M2a and M2c markers.

THP-1 derived macrophages were polarised into a M(0), M(LPS, IFNγ), M(IL4) or M(IL10) phenotype. After 24 hours of polarisation, cells were treated with or without IL-33 and left for an additional 24 hours before cell lysis. **a)** M1 markers, **b)** M2a markers and **c)** M2c marker were analysed at the transcript level using end point PCR. hMDMs polarised into M(LPS, IFNγ), M(IL4) or M(IL10) were used as a positive control for M1, M2a and M2c markers respectively. *RPL37A* was used as a loading control. The experiment was performed once. IFNγ, interferon gamma; IL, interleukin; LPS, lipopolysaccharide; hMDM, human monocyte-derived macrophage; PCR, polymerase chain reaction; RT, reverse transcriptase.

3.7 Conclusions

In this study, I developed an optimised method to polarise THP-1 cells towards different macrophage phenotypes, which resemble human primary polarised macrophages in their marker expression.

Expression of transcripts to determine polarisation into specific phenotypes was carried out using end-point PCR due to the requirement of markers to either be “switched-on” or “switched-off” in different phenotypes.

The analysis of gene expression after PMA addition confirmed the upregulation of pro-inflammatory M1 transcripts described by other groups (Park *et al.*, 2007; Maess *et al*., 2014; Genin *et al*., 2015), as well as the M2a marker *PPARG*, in a concentration-dependent manner. These genes (*STAT1, IRF1, CXCL10, HLA-DRB1, IL6, PPARG* and *CCL26*) are implicated with the PKC pathway, the pathway upregulated by PMA, and its downstream transcription factors, such as NF-κB (Salonen *et al*., 2006; Giroux *et al*., 2003; Kiriyama *et al.*, 2001). Additionally, *STAT1* and *IRF1* are involved in the polarisation of macrophages towards an M1 phenotype (Xie *et al.*, 2016; Varinou *et al*., 2003) whilst *IL6* is a downstream gene of NF-κB, and *CXCL10* is a downstream gene of both NF-κB and STAT-dependent pathways, including *STAT1* (Clarke *et al*., 2010; Devaraj *et al.*, 2005). Upregulation of M2a markers was also seen, of which *PPARG* is also a downstream gene of the PKC pathway and has previously been shown to be induced in THP-1 cells through treatment with PMA (Ricote *et a*l., 1998). Upregulation of *CCL26* can be linked downstream of *PPARG* activation, induced by PMA (Ueki *et al*., 2006; Nakahigashi *et al.*, 2012).

It was found that the use of PMA at concentrations of 2.5ng/ml and lower was inadequate for the maturation of THP-1 cells due to the low number of cells that matured and adhere to the cell culture surface, resulting in low RNA concentrations. Interestingly, however, non-specific upregulation of M1 and M2a markers induced by PMA, was observed in matured cells when using a concentration of PMA as low as 2.5ng/ml, highlighting the strong effect of this stimulant on downstream signalling pathways in THP-1 cells. Thus, in agreement with Daigneault *et al.* (2010) a rest period was included to remove the polarisation bias induced by PMA. Cells were consequently treated with an optimum PMA concentration of 5ng/ml for 24 hours followed by a 3-day rest. This resulted in a matured macrophage phenotype that exhibited the least polarisation bias and allowed for sufficient RNA concentrations.

Interestingly, all PMA concentrations after 24 hours were found to induce the same level of expression of M1 transcription factors STAT1 and IRF1, proteins involved in M1 polarisation and the downstream regulation of pro-inflammatory mediators. Both transcription factors were found to be constitutively expressed once induced by PMA, with a 5 day rest period showing no effect on the levels of their expression. Furthermore, the expression of STAT1-associated-IFNγ-inducible genes, *CXCL10* and *IL6*, induced after 24 hours of PMA stimulation, suggests *STAT1* activation via phosphorylation. This however, cannot be confirmed solely from *STAT1* expression at the transcript level and would require further analysis at the protein level. The absence of *CXCL10* and *IL6* after 24 hours of PMA removal may indicate a loss of *STAT1* and *IRF1* downstream signalling, and consequently, their activation, but not their expression.

Following maturation, the generation of M(LPS, IFNγ) cells was found to be optimal when treated with 250ng/ml LPS and 20ng/ml IFNγ and M(IL-4) macrophages with 30ng/ml IL-4 over 48 hours. It was found that all phenotype-specific transcript markers from the updated marker panel (Figure 3.9) were induced at these concentrations over this time period, with some markers only appearing after 48 hours of cytokine stimulation. This highlights the variability in transcript marker expression in response to the duration of cytokine stimulation which has been described recently by Shiratori *et al*. (2017) and therefore the importance in understanding the expression changes of transcripts being used.

The final THP-1 polarisation protocol resulted in macrophages that were found to be morphologically similar to that described in polarised primary hMDM. Additionally, THP-1-derived macrophages were found to express phenotype-specific macrophage transcripts which could be compared to their expression in hMDM cells. Furthermore, expression of *CD163* was found to be expressed in all phenotypes of THP-1-derived macrophages, a finding that has been described in hMDMs (Barros *et al*., 2013). Our model contradicts the results produced by Shiratori *et al*. (2017) who described an inability to polarise THP-1 cells into an M2a macrophage phenotype. Their lack of M2a surface marker expression may be due to their decision to mature THP-1 cells over 48 hours in a concentration of PMA 10 times higher than that used in our model. Additionally, cells were only rested for 3 days in PMA-free media which may not allow enough time for the removal of the M1 bias induced by PMA, therefore preventing the upregulation of M2a transcript *MRC1* and *CD200R1* in their M2a polarised THP-1 cells. Additionally, the use of lower concentrations of PMA may not be as important when the aim is to induce M1 polarisation of THP-1 cells.

From the transcript markers that were used to optimise THP-1 polarisation, cytokines and chemokines were found to be the most reliable to distinguish M(LPS, IFNγ) from M(IL-4) macrophages due to their high specificity for their respective macrophage phenotype. Due to their inability to distinguish specific macrophages when used in immunohistochemistry however, these markers were not carried forwards for use in characterising macrophages in GCA biopsies and instead, attention would be made in identifying suitable surface and intracellular protein markers. From the optimisation marker panel, candidate markers for use in immunohistochemistry experiments which were carried forward included M2a markers CD200R1, MRC1 and TGM2, however no suitable protein markers for M1 were found. Furthermore, the expression of these candidate markers at the transcript level does not necessarily translate into their expression at the protein level.

The lack of specific transcript markers for M2c macrophages does not suggest that THP-1 cells cannot be polarised towards this phenotype but rather it could be inferred that these markers used to characterise M2c macrophages are not useful and therefore more work needs to be carried out to identify M2c-specific markers.

The upregulation of genes *CXCL9* and *CXCL10* after the treatment of hMDMs with IL-33 confirms the observation that IL-33 induces the expression of M1 chemokines in untreated hMDM(0) described by Joshi *et al.* (2010), albeit in hMDMs rather than bone marrow derived human macrophages. This data also suggests IL-33 can upregulate the expression of M1 cytokines due to the induction of *IL6*. THP-1 derived macrophages did not show upregulation of any M1 markers in M(0) cells. This could suggest a lack of ST2 receptor expression and therefore THP-1 cells may not be a useful cell line to understand the effect of IL-33 on macrophage polarisation.

Analysis of markers at the transcript level provided evidence that the THP-1 cell line had been polarised into different subsets of macrophages closely resembling that of polarised hMDMs. Chemokines and cytokines were very useful markers to determine polarisation due to their high specificity for different subsets yet their use in identifying macrophages at the tissue level, due to their secreted nature, prevents them from being candidates for IHC studies. In result of this, the reduced number of markers for each phenotype makes it hard to be able to identify macrophage subsets and therefore new markers need to be identified. Additionally, specificity of markers for IL-10 treated cells was poor and therefore to be able to characterise these cells, novel markers must be analysed to identify

suitable and specific markers, with a focus on cell surface and intracellularly expressed markers.

The use of end-point PCR to determine transcript expression of the chosen macrophage markers was adequate for identifying “on” and “off” expression and provided a quick method for understanding which markers were specific and which markers were not specific for their respective macrophage phenotypes. Some transcripts were found to be faint for their respective macrophage subset, making it hard to decipher expression, and comparing expression between different markers was difficult, due to it being a semi-quantitative method. The use of other methods of PCR, such as quantitative-PCR, would provide a quantitative value for the amount of expression, allowing a better comparison of markers to determine which are more robust for charactering macrophage phenotypes and more reproducible results as numbers can be compared, rather than images. Using quantitative-PCR, more PCRs can be performed, running all replicates at the same time, in the same conditions and decreases the chances of contamination. This method also enables expression changes to be detected at a much lower level

Chapter 4. Subset-specific macrophage marker identification and validation

4.1 Introduction

The objective of developing the THP-1 cell model system was to understand better the different macrophage phenotypes in GCA, through the identification and use of subset-specific markers to characterise macrophage polarisation states in affected arteries. Work carried out in the previous Chapter identified commonly used markers within the marker panel, of which some were suitable for immunohistochemistry. Some of these markers, however, lacked specificity, especially for the M1 phenotype. There is a lack of suitable markers for characterising macrophage phenotypes in tissue.

Another important aspect of any potential marker in partially treated inflammatory diseases is its response to glucocorticoid therapy. As mentioned previously, patients with GCA are prescribed high doses of glucocorticoids days or weeks prior to biopsy, resulting in a change to transcription in cells, especially macrophages. For this reason, in addition to identifying signature-specific macrophage markers suitable for immunohistochemistry, it is also important to identify subset-specific markers that do not alter in response to glucocorticoid treatment, allowing pre- and post-treatment polarising signals to be explored.

4.2 Aims

Subset-specific macrophage markers which were suitable for immunohistochemistry (i.e. not secreted) as well as remaining specific when exposed to glucocorticoids were needed for further experiments. Therefore, my aims were:

1. To confirm the expression of novel markers, which are suitable for immunohistochemistry studies (identified through the mining of publicly-available RNA-Seq datasets of hMDMs in a parallel project carried out by Nikki Re) for their respective macrophage phenotype in THP-1 cells polarised towards an M1, M2a or M2c phenotype. Additionally, determining the degree of specificity of these novel markers for their respective phenotypes in both THP-1-derived macrophages and hMDMs.
2. To help identify markers suitable for identifying macrophages in GCA biopsy tissue, confirming marker expression and specificity at the protein level and understanding the effects of glucocorticoids on the chosen markers to further establish their use as subset-specific markers in GCA artery biopsies.

To address these aims, my objectives were:

- To develop western blotting methods to identify protein expression of selected markers specific for differently polarised macrophages.
- To modify the THP-1 cell model system to help understand the effect of glucocorticoids on cellular signalling and protein expression at different phases of macrophage polarisation.

4.3 Marker selection from the optimisation marker panel

The marker panels used for the optimisation of THP-1 cell polarisation towards an M1 and M2a macrophage from Chapter 3 were refined as follows: only markers shown in previous studies to be expressed as non-secreted protein were kept as suitable markers to characterise macrophages using immunohistochemistry in GCA temporal artery biopsies. After the removal of unsuitable markers, only M2a markers MRC1 (mannose receptor C-type 1, also known as CD206), CD200R1 and TGM2 were found to be adequate. No plasma membrane or intracellularly expressed markers specific for M1 macrophages were identified in the previous experiment (Section 3.5.3).

4.4 M1 and M2 marker selection

4.4.1 Identification of M1 and M2 novel markers

Owing to the absence of M1- and M2c-specific markers from the literature and the low number of M2a markers which were identified, novel markers identified from RNA-Seq datasets as subset-specific (within the top 40 markers) for M1, M2a or M2c macrophages as well as being membrane-bound or intracellularly expressed, were analysed in macrophages cultured using the THP-1 model system. The identified novel markers can be found in Table 4.1 and Table 4.2.

Table 4.1: Novel M1 markers identified from the RNA-Seq dataset and their cellular location and function.

MØ subset	Marker	Cellular location	Cellular function
M1	Ankyrin Repeat Domain (ANKRD)22	Nucleus, cytoplasm.	Specific function unknown. Found to be induced by interferons and functions to protect against viral infection (Bin *et al*., 2016)
	Guanylate Binding Protein (GBP)5	Cytoplasm, membrane, endoplasmic reticulum.	Involved in the inflammatory response and the formation of the inflammasome (Fujiwara *et al*., 2016; Shenoy *et al*., 2012)
	Inhibin Beta A Subunit (INHBA)	Cytoplasm	Thought to promote pro-inflammatory macrophages by altering polarisation outcome (Sierra-Filardi *et al*., 2011)
	Serpin Family G Member (SERPING)1	Cytoplasm, membrane	Involved in the complement cascade, regulating its activation by inhibiting associated proteins (Wagenaar-Bos and Hack, 2006)
	TNF Alpha Induced Protein (TNFAIP)6	Cytoplasm, membrane. Also suggested to be secreted.	Involved in a negative feedback loop in macrophages in response to pro-inflammatory mediators (Mittal *et al.*, 2016; Milner and Day, 2003)
	TSC22 Domain Family Member (TSC22D)1	Nucleus, cytoplasm.	Functions as a repressive transcription factor (Kester *et al*., 1999)

Table 4.2: Novel M2a and M2c markers identified from the RNA-Seq dataset and their cellular location and function.

MØ subset	Marker	Cellular location	Cellular function
M2a	Arachidonate 15-Lipoxygenase (ALOX15)	Cytoplasm, membrane	Can promote both pro- and anti-inflammatory functions in macrophages (Uderhardt et al., 2012)
	Homer Scaffolding Protein (HOMER)2	Cytoplasm, membrane	Involved in cytoskeleton rearrangement, suppresses filopodia-like structures (Shiraishi et al., 1999). Regulates Ca2+ signalling (Yang et al., 2014)
M2c	Complement C1q A Chain (C1QA)	Cytoplasm, membrane	Promotes macrophage clearance of apoptotic cells (Galvan et al., 2012). Promotes an anti-inflammatory macrophage phenotype (Ho et al., 2016)
	Selenoprotein P (SELENOP /SEPP1)	Cytoplasm, membrane	Induces an anti-inflammatory macrophage phenotype (Barrett et al., 2015)
	Solute Carrier Family 16 Member 1 (SLC16A)1	Cytoplasm, membrane	Transports molecules involved in metabolism and maintains pH homeostasis (Adijanto and Philp, 2012)
	Transmembrane and Immunoglobulin Domain Containing (TMIGD)3	Membrane	Suppressor of NF-κB pathway (Iyer et al., 2016)

4.4.2 Transcriptional expression and subset-specificity of selected markers

To ascertain the specificity of these newly identified transcriptional macrophage markers, expression of M1 and M2a markers was analysed using end-point PCR in THP-1 monocytes and THP-1-derived macrophages polarised into M(0), M(LPS,IFNγ) and M(IL-4) phenotypes, with M2c markers also analysed in M(IL-10) polarised macrophages. The requirement of the selected markers to detect macrophages states when carrying out immunohistochemistry on tissue sections, justified the use of end-point PCR for identifying “on/off” macrophage markers. hMDMs from three healthy donors were polarised using LPS and IFNγ, IL-4 or IL-10 to induce hMDM(LPS,IFNγ), hMDM(IL-4) or hMDM(IL-10) phenotypes and were used as a positive control. No reverse transcriptase (No RT) and no template samples were used as negative controls to confirm no contamination of both RNA and cDNA samples.

M1 transcript markers *ANKRD22, GBP5, SERPING1* and *TNFAIP6* were found to show specificity for M(LPS,IFNγ) polarised macrophages with expression of these markers seen in both THP-1-derived macrophages and hMDM M(LPS,IFNγ) cells, without expression in untreated, M(0) and M(IL-4) cells (Figure 4.1a). M1 transcripts *INHBA* and *TSC22D1,* however, were found to lack specificity for M(LPS,IFNγ) macrophages. *INHBA*, although showing the highest level of expression for THP-1-derived M(LPS,IFNγ) polarised macrophages was also expressed in M(0) and M(IL-4) cells at similar levels of intensity to hMDM M(LPS,IFNγ) positive control cells. *TSC22D1* was found to be similarly expressed in M(0) and THP-1-derived M(LPS,IFNγ) macrophages. *TSC22D1* was also seen in hMDM M(LPS,IFNγ) control cells albeit at a lower intensity, with even lower expression seen in M(IL-4) polarised cells. Figure 4.1b shows the novel M2a marker *ALOX15* has specificity for M(IL-4) polarised macrophages, with expression seen only in THP-1-derived and hMDM M(IL-4) control cells. For *HOMER2,* similar levels of expression were seen in M(0) and THP-1-derived M(IL-4) macrophages, however, lower expression was also seen (at comparable intensities) in M(LPS,IFNγ) and the positive hMDM M(IL-4) control cells.

M2c markers *C1QA, SELENOP, SLC16A* and *TMIGD3* were analysed in M(0), M(LPS,IFNγ), M(IL-4) and M(IL-10) polarised macrophages (Figure 4.1c) however, no marker was found to be specific to M(IL-10) cells. *C1QA* was found to show the highest expression, and with similar levels of intensity, in both THP-1-derived macrophages and in the hMDM M(IL-10) positive control, however, expression could also be seen in M(LPS,IFNγ) THP-1-derived macrophages and slight expression in M(0) cells. *TMIGD3* interestingly was not expressed in the positive hMDM-derived M(IL-10) control cells and additionally showed a lack of specificity for THP-1-derived M(IL-10) macrophages, with expression found in all other analysed macrophage phenotypes, apart from untreated THP-1 monocytes. *SCL16A* was found to be expressed at the same level in all macrophage phenotypes, without expression in untreated THP-1 monocytes. Finally, *SELENOP* was also found to show be expressed in all macrophages, with the lowest levels seen in THP-1-derived and hMDM M(IL-10) phenotypes. No expression of this marker was found in untreated THP-1 monocytes. C1QA may be a candidate marker for M2c macrophages but due to a lack of complete specificity at the RNA level, this marker was not carried forward for further analysis at the protein level, due to time constraints.

A summary of the RNA expression of these novel markers can be seen in Table 4.3.

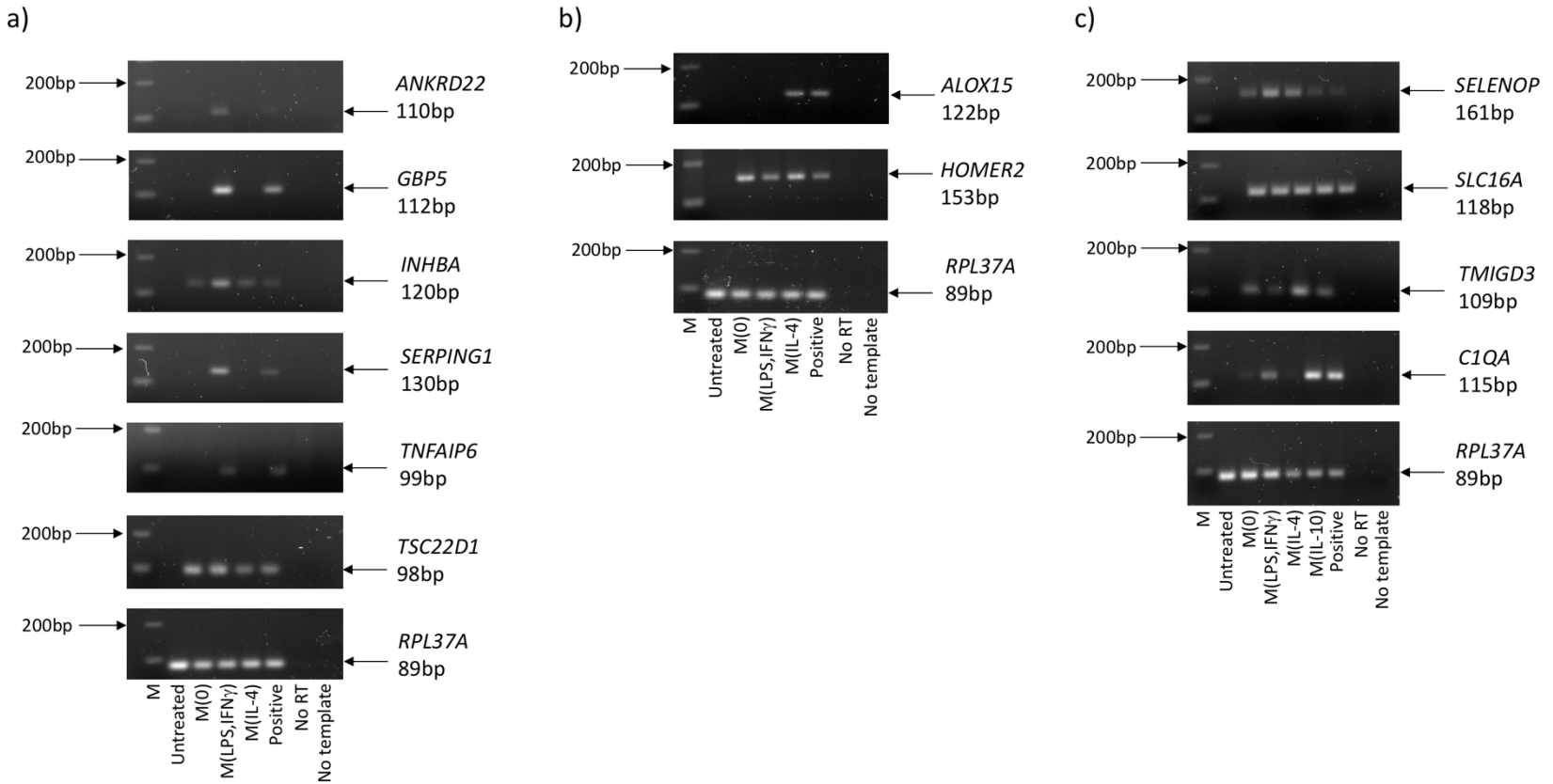
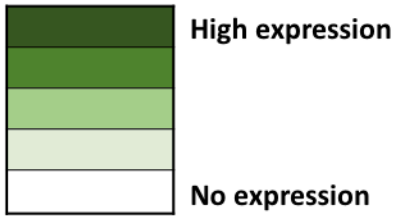


Figure 4.1: Novel marker expression and specificity for their respective phenotype.

THP-1-derived macrophages were polarised into M(0), M(LPS,IFNγ), M(IL-4) or M(IL-10) phenotypes for 48 hours before cell lysis. **a)** novel M1, **b)** M2a and **c)** M2c markers were analysed using end point PCR in these phenotypes, as well as in untreated THP-1 monocytes. hMDMs polarised into their respective phenotype were used as a positive control. *RPL37A* was used as a loading control. The experiment was carried out once. bp, basepair; IFNγ, interferon gamma; IL, interleukin; LPS, lipopolysaccharide; M, marker; hMDM, human monocyte-derived macrophage; PCR, polymerase chain reaction; RT, reverse transcriptase.

Table 4.3: Summary of novel marker expression and specificity in THP-1 cells after 48 hours polarisation.

Macrophage phenotype	Marker	RNA at 48 hours				
		Untreated	M(0)	M(LPS, IFNγ)	M(IL-4)	M(IL-10)
M1	ANKRD22					
	GBP5					
	INHBA					
	SERPING1					
	TNFAIP6					
	TSC22D1					
M2a	ALOX15					
	HOMER2					
M2c	SELENOP					
	SLC16A					
	TMIGD3					
	C1QA					



The amount of RNA expression of novel markers in M(0), M(LPS,IFNγ), M(IL-4), and M(IL-10) phenotypes from Figure 4.1 was summarised in a semi-quantitative manner in the form of varying colour intensities. Marker expression not examined in the M(IL-10) phenotype is shaded in black. IFNγ, interferon gamma; IL, interleukin; LPS, lipopolysaccharide.

4.4.3 Further testing of selected markers

Markers found to be specific for either M(LPS,IFNγ) and M(IL-4) macrophages were analysed in additional phenotypes to better understand their specificity for their respective macrophage phenotypes. Due to the lack of specific markers for the M2c phenotype, shown previously, the commonly used M2c marker *CD163* (Ambarus *et al*., 2012; Buechler *et al*., 2000) was used in further analysis to determine its specificity. Markers were analysed at the transcript level in THP-1-derived macrophages polarised towards 5 different phenotypes: M(0), M(LPS,IFNγ), M(IL-4), M(IL-10) and M(IL-33), as well as in untreated THP-1 monocytes.

M1 markers (Figure 4.2a) *ANKRD22* and *TNFAIP6* showed expression in cells treated with IL-10, however, markers *GBP5* and *SERPING1* were only expressed in THP-1-derived macrophages and the positive control hMDMs treated with LPS and IFNγ.

M2a markers *ALOX15, CD200R1* and *MRC1* were again specific for M(IL-4) macrophages (Figure 4.2b) with expression only seen in IL-4 treated THP-1 derived macrophages and the IL-4 treated hMDMs, used as the positive control. *TGM2,* however, was highly expressed in M(0), M(LPS,IFNγ), M(IL-4), M(IL-10) and M(IL-33) as well as the positive control. Out of the 3 markers specific for M(IL-4), *CD200R1* showed the highest amount of expression for both THP-1-derived and the positive control primary macrophages.

For the M2c marker *CD163* (Figure 4.2c), expression was not specific to IL-10 treated cells as expression was also induced in IL-4 and IL-33 treated macrophages at similar levels of intensity. CD163, is thus a pan M2 marker and does not distinguish between the different subsets.

A summary of the RNA expression of these markers can be seen in Figure 4.4.

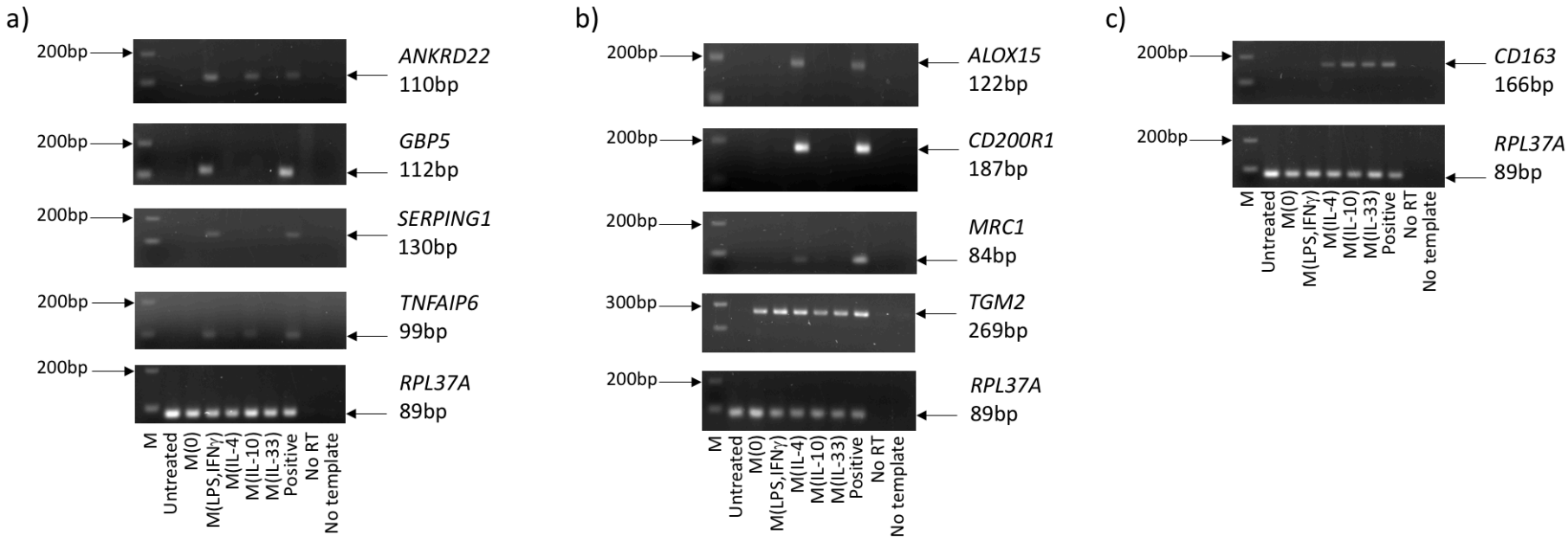
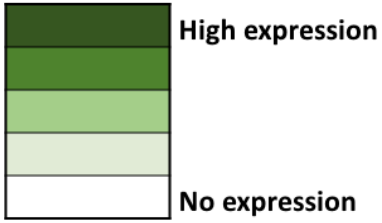


Figure 4.2: Expression of selected markers in a wider range of macrophage phenotypes.

THP-1-derived macrophages were polarised into an M(0), M(LPS,IFNγ), M(IL4), M(IL-10) or M(IL-33) phenotype as described previously before cell lysis. Selected **a)** M1, **b)** M2a and **c)** M2c markers were analysed using end-point PCR in these phenotypes, as well as in untreated THP-1 monocytes. hMDMs polarised into their respective phenotype were used as a positive control. *RPL37A* was used as a loading control. The experiment was carried out once. bp, basepair; IFNγ, interferon gamma; IL, interleukin; LPS, lipopolysaccharide; M, marker; hMDMs, human monocyte-derived macrophages; PCR, polymerase chain reaction; RT, reverse transcriptase.

Table 4.4: Summary of marker RNA expression in THP-1 cells after 48 hours of polarisation.

Macrophage phenotype	Marker	RNA at 48 hours					
		Untreated	M(0)	M(LPS,IFNγ)	M(IL-4)	M(IL-10)	M(IL-33)
M1	*ANKD22*						
	GBP5						
	SERPING1						
	TNAFIP6						
M2a	*ALOX15*						
	CD200R1						
	MRC1						
	TGM2						
M2c	*CD163*						



The amount of RNA expression of markers in M(0), M(LPS,IFNγ), M(IL-4), M(IL-10) and M(IL-33) phenotypes from Figure 4.2 was summarised in a semi-quantitative manner in the form of varying colour intensities. IFNγ, interferon gamma; IL, interleukin; LPS, lipopolysaccharide.

4.5 Validation of markers at the protein level

Due to the known discrepancies between RNA and protein expression in cells, the sole use of transcript expression cannot be used to assume expression at the protein level. To verify marker expression and to provide further evidence of THP-1 polarisation towards M1, M2a and M2c phenotypes, selected markers were then analysed at the protein level using western blotting. The positive controls taken from polarised hMDMs were run as technical replicates unless otherwise stated.

4.5.1 Expression at 48 hours

Protein expression of selected markers was first analysed after 48 hours of exposure to LPS and IFNγ, IL-4, IL-10, IL-33 or were not stimulated.

4.5.1.1 M1 marker expression

Figure 4.3 shows a representative example of two biological replicates (whole experiment repeated). Novel markers ANKRD22 and GBP5 showed specificity at the protein level for THP-1-derived macrophages polarised towards M(LPS,IFNγ) and specificity was consistent between the two biological replicates. When comparing expression of GBP5 and ANKRD22 in differently polarised primary hMDMs, however, overlap was observed between the different macrophage phenotypes and additionally, there was variation in the technical replicates. ANKRD22 expression showed specificity for M(LPS,IFNγ) hMDMs however the second replicate showed expression of ANKRD22 in M(0), M(LPS,IFNγ), M(IL-10) and M(IL-33) macrophages. GBP5 was shown to be expressed strongly in M(LPS,IFNγ) and M(IL-33) hMDMs with lower expression in M(IL-4) and M(IL-10) macrophages, whilst the second replicate showed similar levels of expression in M(LPS,IFNγ), M(IL-4) and M(IL-33) cells. No expression of M1 markers TNFAIP6 and SERPING1 could be seen at the protein level and therefore no replicate western blots were carried out at 48 hours.

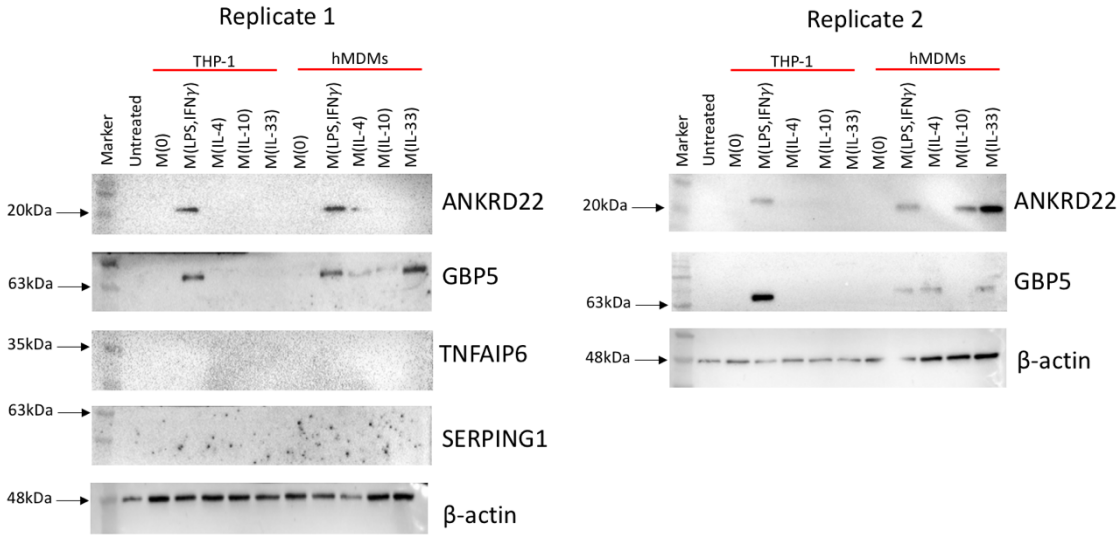


Figure 4.3: Protein expression of M1 markers in THP-1 and hMDMs after 48 hours of cytokine exposure.

THP-1-derived macrophages and hMDMs were polarised into an M(0), M(LPS,IFNγ), M(IL4), M(IL-10) or M(IL-33) phenotype as described previously before lysis after 48 hours. Selected M1 markers were analysed using western blotting in these phenotypes, as well as in untreated THP-1 monocytes. β-actin was used as a loading control. THP-1 derived macrophages were run using biological replicates, hMDMs were run using technical replicates, unless no expression was seen and were only run once (TNFAIP6 and SERPING1). IFNγ, interferon gamma; IL, interleukin; LPS, lipopolysaccharide; hMDMs, human monocyte-derived macrophages.

4.5.1.2 M2 marker expression

When determining protein expression of M2a markers at 48 hours (Figure 4.4) expression of MRC1 in both replicates was not found in any phenotype polarised from THP-1 cells, yet in primary hMDMs, expression could be seen in M(0), M(IL-4), M(IL-10) and M(IL-33), but no band was present for M(LPS,IFNγ) cells. TGM2 was expressed non-specifically in both THP-1-dervied macrophages and in hMDM cells, however, a large amount of expression was evident for THP-1 derived M(IL-4) cells and the expression pattern in hMDMs looked similar to that of MRC1. Expression of markers ALOX15 and CD200R1 was not seen at the protein level after 48 hours of cytokine stimulation and therefore only one replicate was carried out.

The expression pattern of the M2c marker CD163 was found to be different in THP-1 derived macrophages and hMDMs as seen for most M1 markers and all M2a markers (Figure 4.5). CD163 was found to be expressed in M(0) and M(IL-4) THP-1 derived macrophages, whereas in hMDMs, CD163 expression was seen in M(0) and M(IL-33) cells. It is interesting to note that the M(IL-10) macrophage phenotype, which has been described to express this marker, did not show any evidence of the protein in either the primary or secondary cell lines. This may suggest expression of CD163 is controlled post-translationally as protein expression is seen in THP-1-derived M(0) and M(IL-4) cells, whereas RNA expression of CD163 is seen in M(IL-4), M(IL-10) and M(IL-33) THP-1-derived macrophages.

An overall summary of the protein expression data produced in Figure 4.3, Figure 4.4 and Figure 4.5 and can be seen summarised in Table 4.5. It is important to consider the variation in protein expression over time and the time required for RNA to be translated into protein. The expression of M2a markers in THP-1 derived macrophages for example, has been described to take longer than in M1 polarised THP-1 macrophages and hMDMs (Shiratori *et al.*, 2017; Genin *et al*., 2015). The expression of markers seen at the RNA level, 48 hours post cytokine treatment therefore, may not yet be expressed at the protein level, and so protein expression would also be analysed after 72 hours of cytokine exposure.

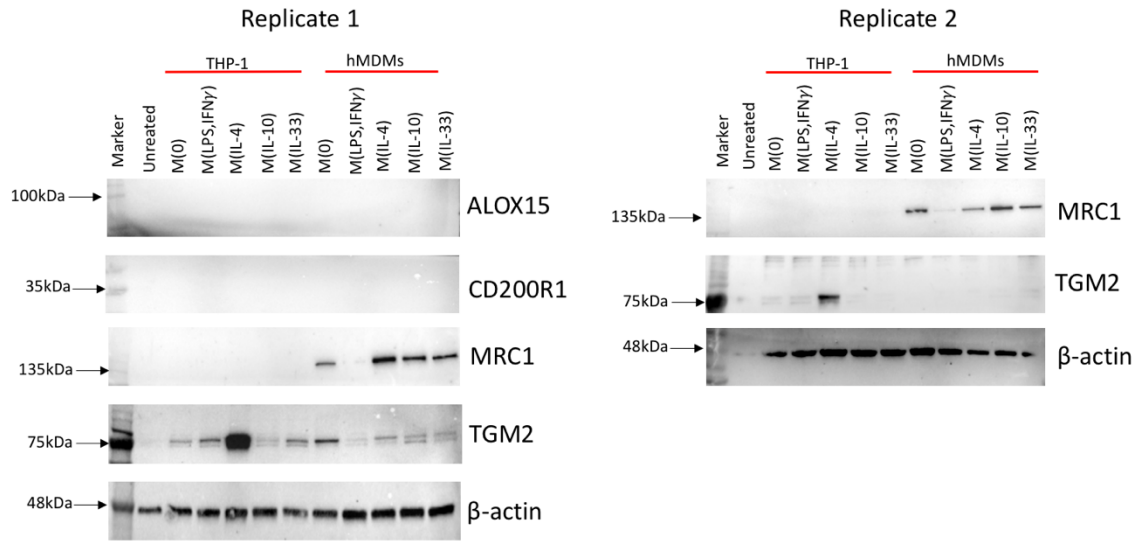


Figure 4.4: Protein expression of M2a markers in THP-1 and hMDMs after 48 hours of cytokine exposure.

THP-1-derived macrophages and hMDMs were polarised into an M(0), M(LPS,IFNγ), M(IL4), M(IL-10) or M(IL-33) phenotype as described previously before lysis after 48 hours. Selected M2a markers were analysed using western blotting in these phenotypes, as well as in untreated THP-1 monocytes. β-actin was used as a loading control. THP-1 derived macrophages were run in 2 biological replicates, hMDMs were run in 2 technical replicates, unless no expression was seen and were only run once (ALOX15 and CD200R1). IFNγ, interferon gamma; IL, interleukin; LPS, lipopolysaccharide; hMDMs, human monocyte-derived macrophages.

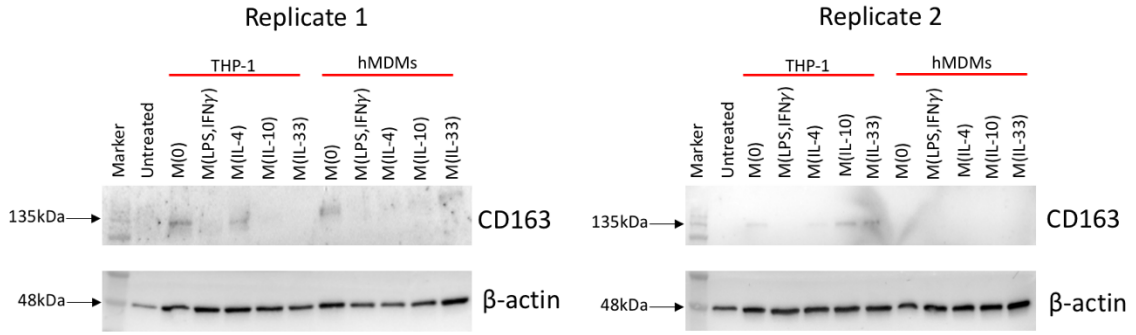
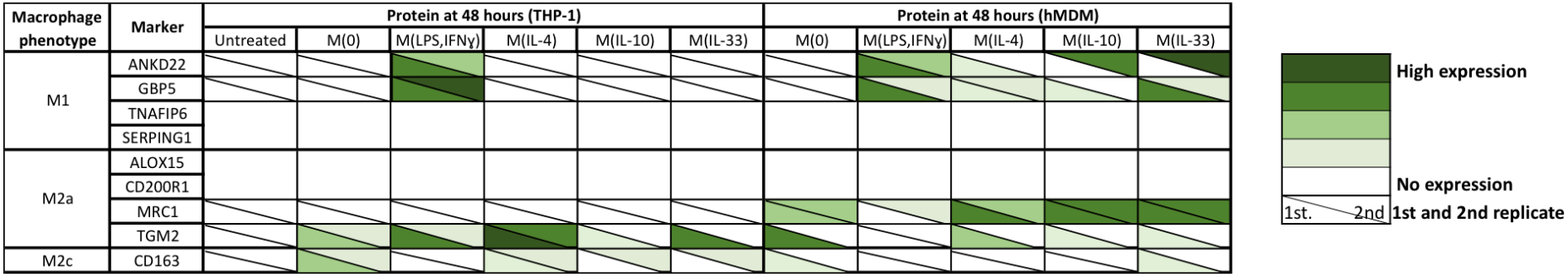


Figure 4.5: Protein expression of the M2c marker CD163 in THP-1 and hMDMs after 48 hours of cytokine exposure.

THP-1-derived macrophages and hMDMs were polarised into an M(0), M(LPS,IFNγ), M(IL4), M(IL-10) or M(IL-33) phenotype as described previously before lysis after 48 hours. Selected M2c marker CD163 was analysed using western blotting in these phenotypes, as well as in untreated THP-1 monocytes. β-actin was used as a loading control. THP-1 derived macrophages were run in 2 biological replicates, hMDMs were run in 2 technical replicates. IFNγ, interferon gamma; IL, interleukin; LPS, lipopolysaccharide; hMDMs, human monocyte-derived macrophages.

Table 4.5: Summary of marker protein expression in THP-1 cells and hMDMs after 48 hours of polarisation



The degree of protein expression of markers in M(0), M(LPS,IFNγ), M(IL-4), M(IL-10) and M(IL-33) phenotypes after 48 hours of polarisation Differences in protein expression taken from the data in Figure 4.3, Figure 4.4 and Figure 4.5 has been summarised as a range of colours in a semi-quantitative manner. Cells have been split to show differences in expression between the two THP-1 biological replicates and the two hMDM technical replicates. IFNγ, interferon gamma; IL, interleukin; LPS, lipopolysaccharide; hMDM, human monocyte-derived macrophage.

4.5.2 Expression at 72 hours

Due to a lack of expression of some M1 and M2a markers after 48 hours, cells were exposed to cytokine for 72 hours. Protein expression was analysed using western blotting to investigate whether there were differences in protein translation time and therefore resulted in a lack of expression of proteins after 48 hours of polarisation.

4.5.2.1 Expression of M1 markers

M1 markers ANKRD22 and GBP5 (Figure 4.6) were specific for THP-1-derived M(LPS,IFNγ) macrophages after 72 hours of cytokine exposure in replicate 1, and this specificity remained consistent for ANKRD22 in the second biological replicate (different sample taken from the THP-1 cell model system). Interestingly, no expression of GBP5 was noted in any THP-1-derived phenotype in the second biological replicate. In polarised hMDMs, however, ANKRD22 was highly expressed in M(LPS, IFNγ) cells but again, faint protein expression in M(IL-4) hMDMs was also observed, similarly to that seen after 48 hours of polarisation. Additionally, differences between the hMDM technical replicate was once again seen, with ANKRD22 expression occurring in M(0), M(LPS,IFNγ), M(IL-4) and M(IL-33) hMDMs in the second replicate. GBP5 was found to be upregulated in an LPS,IFNγ-independent manner in hMDMs cells at 72 hours, a pattern of expression that was not seen at 48 hours. Similarly to ANKRD22, differences in the hMDM technical replicate were also noticed for GBP5 in the second replicate, with very faint expression apparent in M(0), M(LPS,IFNγ) and M(IL-33) hMDMs. Furthermore, the additional 24 hours of polarisation did not result in the upregulation of M1 markers TNFAIP6 and SERPING1 in any macrophage phenotype derived from either THP-1 cells or hMDMs.

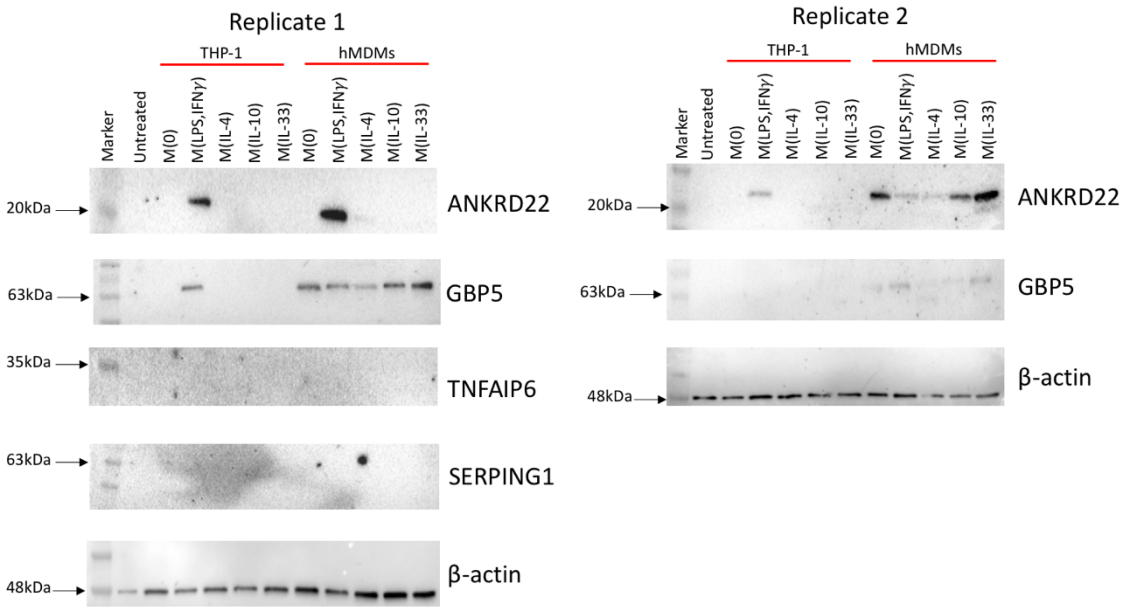


Figure 4.6: Protein expression of M1 markers in THP-1 and hMDMs after 72 hours of cytokine exposure.

THP-1-derived macrophages and hMDMs were polarised into an M(0), M(LPS,IFNγ), M(IL4), M(IL-10) or M(IL-33) phenotype as described previously before lysis after 72 hours of polarisation. Selected M1 markers were analysed using western blotting in these phenotypes, as well as in untreated THP-1 monocytes. β-actin was used as a loading control. THP-1 derived macrophages were run in 2 biological replicates, hMDMs were run in 2 technical replicates, unless no expression was seen and were only run once. IFNγ, interferon gamma; IL, interleukin; LPS, lipopolysaccharide; hMDMs, human monocyte-derived macrophages.

4.5.2.2 Expression of M2 markers

Figure 4.7 shows that expression of M2a markers after 72 hours of cytokine treatment was found to mimic that seen at 48 hours, with MRC1 showing no expression in any THP-1-derived macrophage phenotype, yet showed expression in M(0), M(IL-4), M(IL-10) and M(IL-33) hMDMs, which was also confirmed in the second replicate. Comparably to expression at 48 hours, TGM2 showed a lack of subset specificity, however, expression was very strong in M(IL-4) polarised THP-1 derived macrophages with expression also strongest for M(IL-4) cells in hMDMs. No second replicate was carried out for TGM2. Additionally, ALOX15 and CD200R1 expression was not seen in any macrophage phenotype derived from THP-1 cells or hMDMs at 72 hours.

Interestingly expression of M2c marker CD163 was seen in M(IL-4), M(IL-10) and M(IL-33) polarised THP-1 derived macrophages with no expression in hMDM polarised cells a different pattern to that seen after 48 hours of cytokine stimulation (Figure 4.8). No second replicate was performed for CD163. Table 4.6 summarises the western blotting results presented in Figure 4.6, Figure 4.7 and Figure 4.8.

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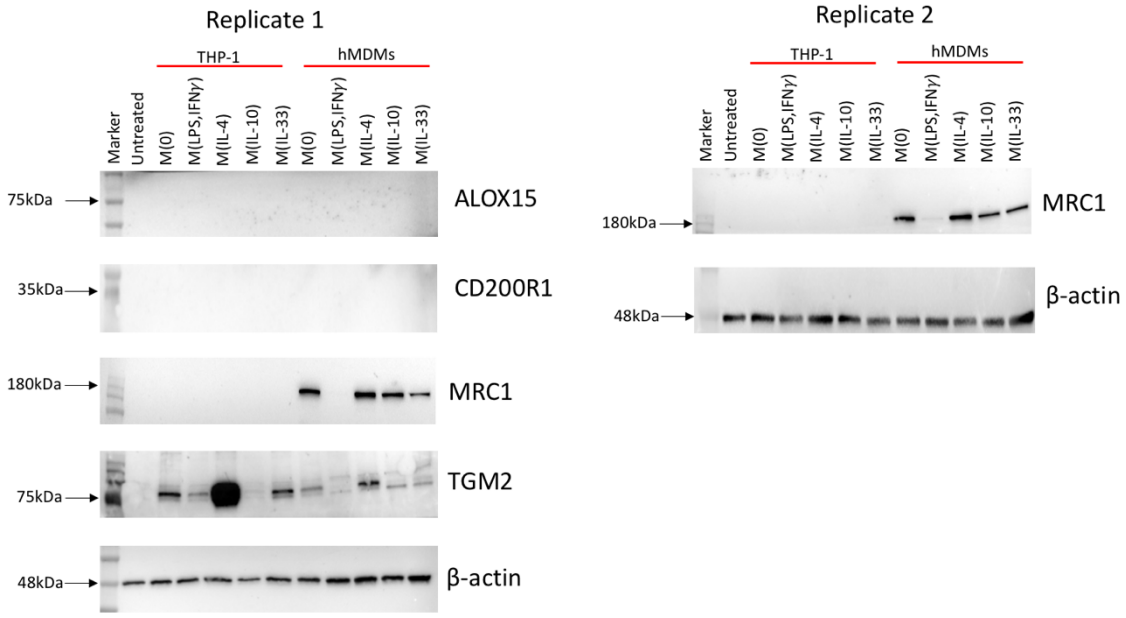


Figure 4.7: Protein expression of M2a markers in THP-1 and hMDMs after 72 hours of cytokine exposure.

THP-1-derived macrophages and hMDMs were polarised into an M(0), M(LPS,IFNγ), M(IL4), M(IL-10) or M(IL-33) phenotype as described previously before lysis after 72 hours of polarisation. Selected M2a markers were analysed using western blotting in these phenotypes, as well as in untreated THP-1 monocytes. β-actin was used as a loading control. THP-1 derived macrophages were run in 2 biological replicates, hMDMs were run in 2 technical replicates, unless no expression was seen and were only run once. IFNγ, interferon gamma; IL, interleukin; LPS, lipopolysaccharide; hMDMs, human monocyte-derived macrophages.

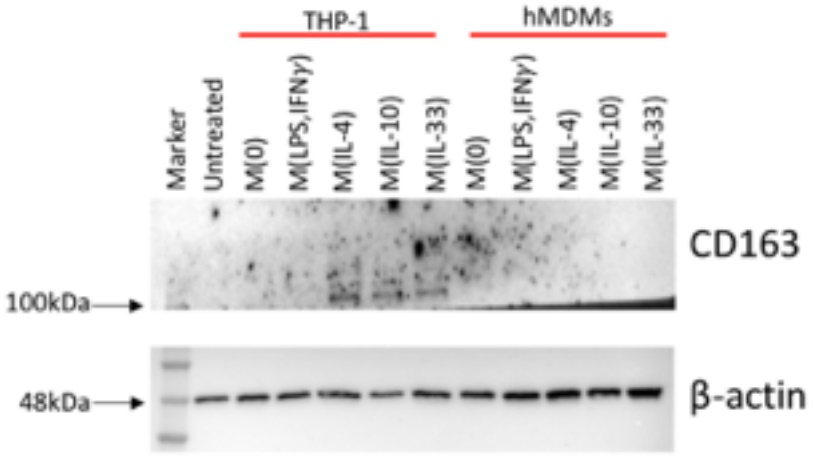
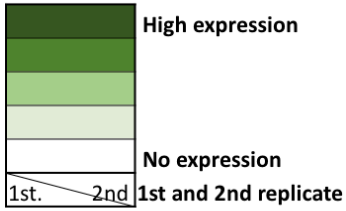


Figure 4.8: Protein expression of the M2c marker CD163 in THP-1 and hMDMs after 72 hours of cytokine exposure.

THP-1-derived macrophages and hMDMs were polarised into an M(0), M(LPS,IFNγ), M(IL4), M(IL-10) or M(IL-33) phenotype as described previously before lysis after 72 hours of polarisation. Selected M2c marker CD163 was analysed using western blotting in these phenotypes, as well as in untreated THP-1 monocytes. β-actin was used as a loading control. The experinemt was only performed once. IFNγ, interferon gamma; IL, interleukin; LPS, lipopolysaccharide; hMDM, human monocyte-derived macrophage.

Table 4.6: Summary of marker protein expression in THP-1 cells and hMDMs after 72 hours of polarisation

Macrophage phenotype	Marker	Protein at 72 hours (THP-1)						Protein at 72 hours (hMDM)				
		Untreated	M(0)	M(LPS,IFNγ)	M(IL-4)	M(IL-10)	M(IL-33)	M(0)	M(LPS,IFNγ)	M(IL-4)	M(IL-10)	M(IL-33)
M1	ANKD22											
	GBP5											
	TNAFIP6											
	SERPING1											
M2a	ALOX15											
	CD200R1											
	MRC1											
	TGM2											
M2c	CD163											



The amount of protein expression of markers in M(0), M(LPS,IFNγ), M(IL-4), M(IL-10) and M(IL-33) phenotypes after 72 hours of polarisation. Differences in protein expression taken from the data Figure 4.6, Figure 4.7 and Figure 4.8 has been summarised as a range of colours in a semi-quantitative manner. Cells have been split to show differences in expression between the two THP-1 biological replicates and the two hMDM technical replicates. IFNγ, interferon gamma; IL, interleukin; LPS, lipopolysaccharide; hMDM, human monocyte-derived macrophage.

The above data illustrates that M1 marker protein expression in THP-1 derived macrophages is more specific to M1 polarised THP-1 cells when compared to M1 marker expression in hMDMs. It also highlights differences in the ability of cell lines and primary cells to upregulate certain proteins, which may be due to primary cells being in an inactive state in healthy controls. The data also demonstrates the amount of variation in protein expression that can occur between biological replicates. This is most apparent for ANKRD22 protein expression which was found to be specific for THP-1-derived M1 macrophages in both of the 72 hour biological replicates and one of the 48 hour replicates, however, expression in the second 48 hour biological replicate was not specific, with expression seen in M(0), M(LPS,IFNγ), M(IL-10) and M(IL-33) phenotypes. Full loading controls show adequate loading of samples and expression in multiple phenotypes albeit in M(IL-4) cells, the cell type most opposing of M(LPS,IFNγ) phenotype, indicate biological variation rather than an experimental error, however human error in loading the correct samples into the correct wells cannot be ruled out as well as overflow of samples into neighbouring wells. Passaging of cells can also result in accumulation of mutations resulting in changes to protein expression.

Differences in the expression of markers between technical primary cell replicates that was seen may be in result of freeze-thawing, with some proteins more susceptible to these conditions than others.

Comparing RNA to protein expression, it is clear that RNA expression does not translate to that seen at the protein level, as described by other groups (Tuomisto *et al*., 2005; Wu *et al*., 2013). This variability may be due to a number of factors including differences in time needed for RNA and protein to be expressed, as well as variants in transcripts and post-transcriptional modifications.

To gain a better picture of variation between biological replicates of macrophage phenotypes derived from hMDMs, it was decided that marker expression would be analysed in a further two hMDM biological replicates to firstly understand marker specificity in primary cells and secondly, the amount of variation that occurs between different donors.

4.5.3 Analysis of primary hMDM marker expression

In light of the results from protein expression analysis of the marker panel in THP-1 derived macrophages and hMDMs, expression of M1 markers ANKRD22 and GBP5, and M2a markers MRC1 and TGM2 was further analysed in biological replicates (from different donors) of differently polarised hMDMs after 48 hours of cytokine polarisation. As this study would ultimately use these markers to identify primary human macrophages, understanding the expression of these selected M1, M2a and M2c markers in hMDM M(0), M(LPS,IFNγ), M)IL-4), M(IL-10) and M(IL-33) phenotypes, and determining how specific these markers were for their respective polarised macrophages was critical.

Surprisingly, in both biological replicates M1 marker ANKRD22 (Figure 4.9a) was not expressed in any macrophage phenotype contradicting the results seen in Figure 4.3 and Figure 4.6 however, this may be the result of human error, with a lack of antibody being added. Expression of GBP5 was found to be non-specific for M(LPS,IFNγ) cells as seen previously, but the pattern of expression was different for both replicates. Replicate 1 showed expression of GBP5 in all phenotypes, albeit with highest expression in M(0) and M(LPS,IFNγ) polarised cells whereas replicate 2 showed highest expression in M(IL-4) macrophages with expression also evident in M(LPS,IFNγ) and M(IL-10) cells.

Confirming the results in Figure 4.4 and Figure 4.7 protein expression of the M2a marker MRC1 was not specific for the hMDM derived M(IL-4) (Figure 4.9b), however, expression patterns in hMDM cells were unlike that seen in Figure 4.4 and Figure 4.7. Replicate 1 revealed highest expression of MRC1 in M(0), M(IL-10) and M(IL-33) cells, lower expression was seen in M(IL-4) cells, with the lowest expression exhibited in M(LPS,IFNγ) cells. In replicate 2, MRC1 was found to be more specific to IL-4 treated cells with highest expression evident for M(IL-4) and M(0) phenotypes. M(LPS,IFNγ) cells exhibited the lowest MRC1 protein expression whilst M(IL-10) and M(IL-33) showed similar levels of MRC1 expression. This suggests MRC1 expression is upregulated in both M1- and M2-like macrophages, however, is more highly upregulated in M2-like phenotypes and lowly expressed in M1-like macrophages and therefore can be assumed to be associated with a M2-like macrophage. TGM2 showed the most specific expression in polarised hMDMs with replicate 2 showing expression in the M(IL-4) phenotype and very low expression in M(0) cells with no bands detected for M(LPS,IFNγ), M(IL-10) and M(IL-33) cells, a more specific pattern of TGM2 expression than seen previously in hMDMs. Replicate 1, however, showed very low levels of TGM2 expression in all phenotypes that were analysed. The M2c

marker CD163 had a similar pattern of expression in both biological replicates (Figure 4.9c), but a different pattern to that seen in Figure 4.5 and Figure 4.8 with expression evident in M(0), M(IL-10) and M(IL-33) cells. In replicate 1, there was no expression seen in M(LPS,IFNγ) and M(IL-4) cells and similarly for replicate 2 there was no expression in M(LPS,IFNγ) cells, however, there was evidence of a band for M(IL-4) cells which may suggest CD163 is expressed in M(IL-4) phenotypes and may be a general M2 marker. β-actin loading control shows samples were loaded at similar protein concentrations.

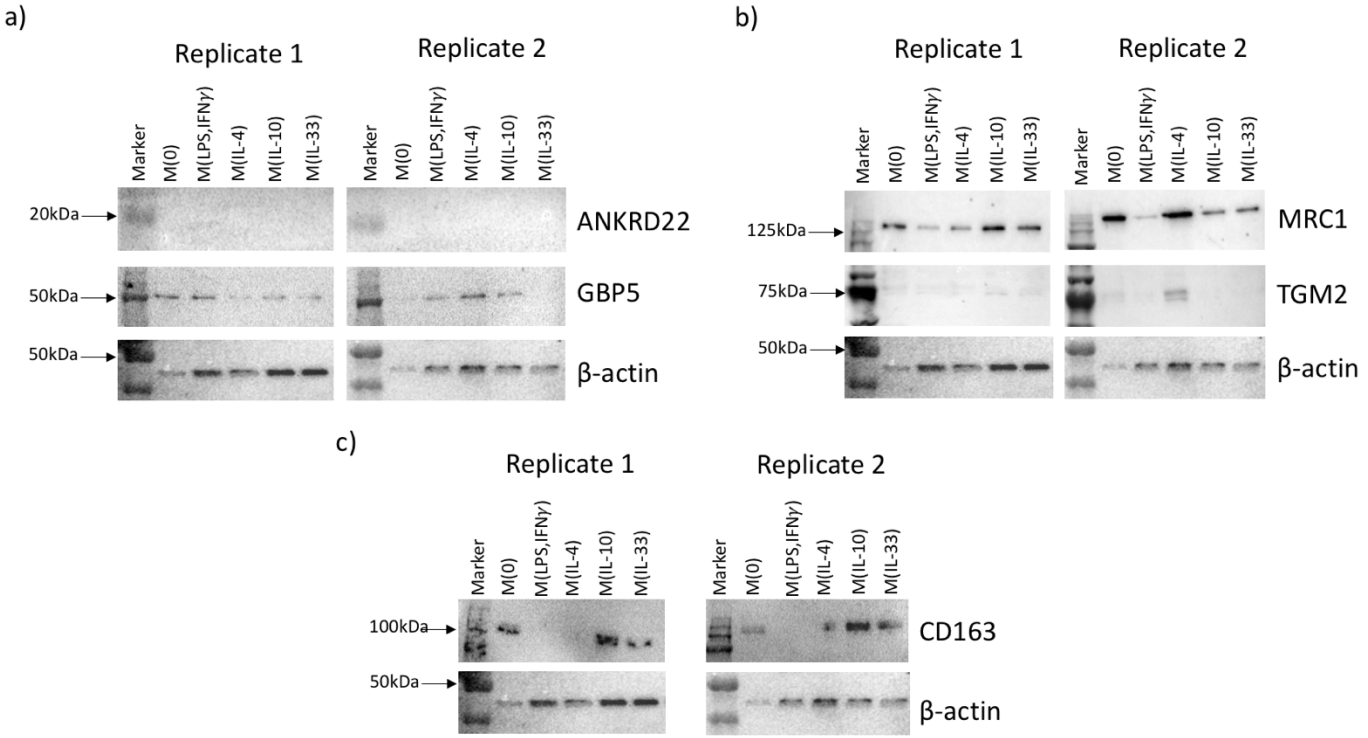


Figure 4.9: Expression of selected markers in two biological replicates of primary hMDM cells, polarised into various macrophage phenotypes after 48 hours of cytokine exposure.

Primary hMDMs were polarised into M(0), M(LPS,IFNγ), M(IL-4), M(IL-10), and M(IL-33) phenotypes after a 48 hour cytokine treatment before cell lysis, the experiment was run using 2 biological replicates (1 and 2). These different phenotypes in the 2 biological replicates were analysed for the expression of **a)** M1, **b)** M2a and **c)** M2c markers using western blotting. β-actin was used as a loading control. IFNγ, interferon gamma; IL, interleukin; LPS, lipopolysaccharide; hMDM, human monocyte-derived macrophage.

4.6 Effect of glucocorticoid treatment on marker expression

Considering the high doses of glucocorticoids administered and the long-term use of the drug in patients with GCA to help treat the condition, it was essential to understand the effect of glucocorticoids on the expression of selected markers ANKRD22, GBP5, TGM2 and MRC1 in M(0), M(LPS,IFNγ), and M(IL-4) phenotypes at the protein level. It is known that the treatment of glucocorticoids in macrophages induces an M2c phenotype, therefore it was important to determine whether expression of markers was lost in their specific macrophage phenotype after treatment with glucocorticoids as well as whether expression was induced in other phenotypes. Both types of changes would affect the results of immunohistochemical staining of macrophages in arterial biopsies taken from GCA patients, therefore, it was important to know the effects of glucocorticoids on these markers in order to interpret staining on temporal artery biopsies. Due to the more common use of glucocorticoids dexamethasone and fluticasone propionate compared to prednisolone when performing macrophage cell culture, these two glucocorticoids were chosen to examine the effects on marker expression, due to a better understanding of appropriate concentrations to use. The use of both glucocorticoids would give an insight into the effects of glucocorticoids with a higher potency and therefore anti-inflammatory effect higher than that of prednisolone (Johnson, 1998; Smith and Kreutner, 1998). Furthermore, a higher glucocorticoid potency would mimic the high doses of glucocorticoids given to patients presenting with visual manifestations of GCA, who are given up to 1g of methylprednisolone iv.

Since it is unknown whether macrophages identified at the site of arterial inflammation are derived from circulating monocytes polarised *in situ* or from polarised resident macrophages, THP-1 derived macrophages were treated with glucocorticoids either during polarisation to mimic circulating monocytes or after polarisation to mimic resident macrophages. A schematic to visualise the protocol that was carried out to generate these macrophages and their subsequent treatment with glucocorticoids can be seen in Figure 4.10.

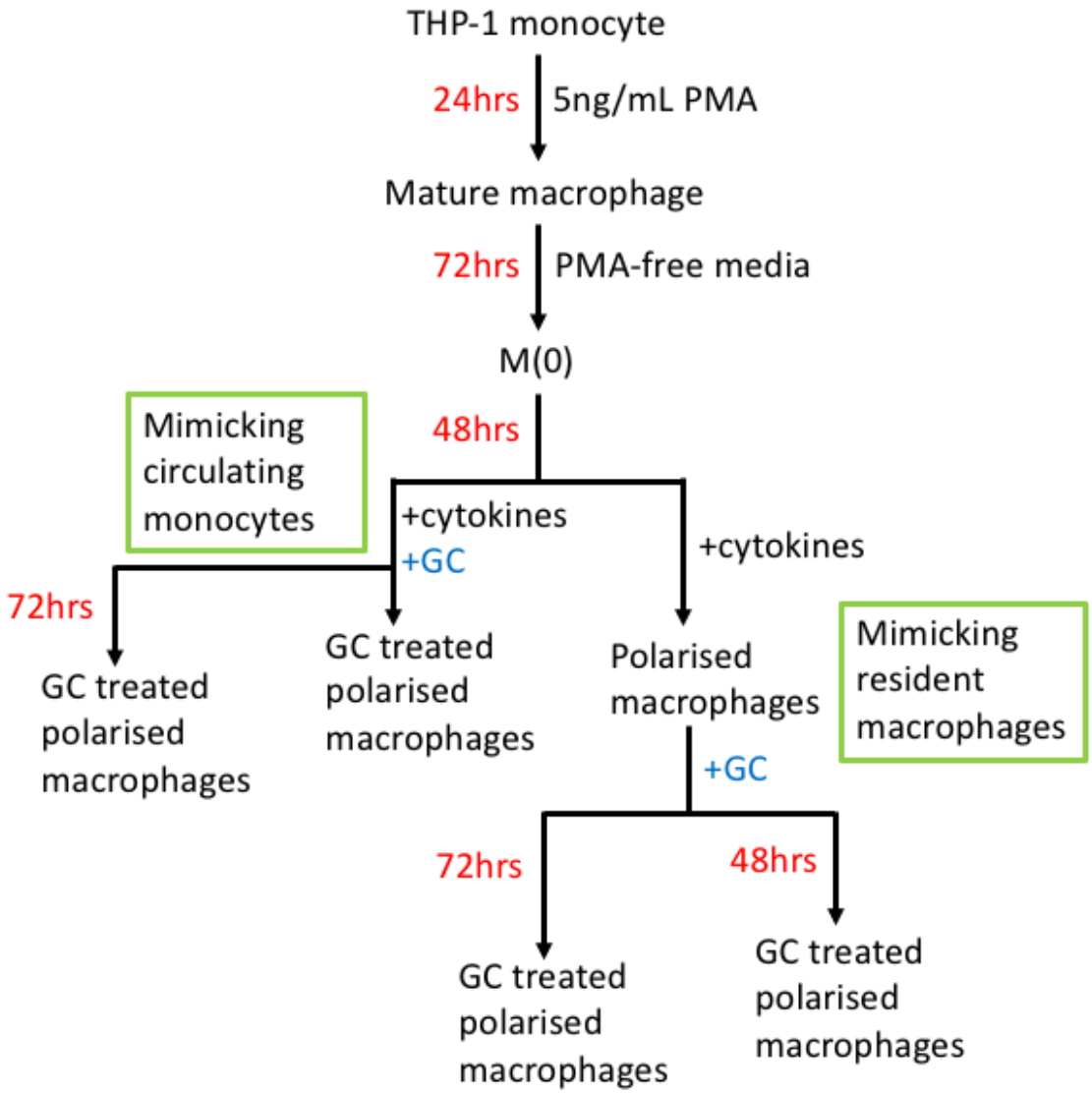


Figure 4.10: Process of generating macrophages which mimic circulating monocytes and resident macrophages and exposing to glucocorticoids.

THP-1 cells were polarised into M(0) macrophages using our THP-1 protocol. These M(0) were then treated in various ways to mimic circulating monocytes or resident macrophages and subsequently exposed to either the glucocorticoid dexamethasone or fluticasone propionate. Polarised macrophages included M(0), M(LPS,IFNγ) and M(IL-4) phenotypes. Cell were lysed for use in western blotting studies once treatment was finished. GC, glucocorticoids; IFNγ, interferon gamma; IL, interleukin; LPS, lipopolysaccharide; PMA, phorbol myristate acetate.

4.6.1 Addition of glucocorticoids during macrophage polarisation

To understand the effect of glucocorticoids on the expression of selected markers in THP-1 derived macrophages which mimicked circulating monocytes, dexamethasone and fluticasone propionate were added during 48 or 72 hours of polarisation of macrophages into M(0), M(LPS,IFNγ) and M(IL-4) phenotypes. The effect of adding these glucocorticoids was examined through western blotting to determine protein expression of selected markers in the different phenotypes.

4.6.1.1 Protein expression after 48 hours polarisation with glucocorticoids

The expression of markers after 48 hours of polarisation with glucocorticoids can be seen in Figure 4.11a. The M1 marker ANKRD22 was found to be further induced in M(LPS,IFNγ) cells after treatment with either dexamethasone and fluticasone propionate when compared to M(LPS,IFNγ) cells treated without glucocorticoids. Additionally no expression of ANKRD22 could be seen in M(0) and M(IL-4) phenotypes when treated with either dexamethasone or fluticasone propionate. A similar pattern could be seen for the M1 marker GBP5 after 48 hours of polarisation with glucocorticoids. In comparison to non-glucocorticoid treated M(LPS,IFNγ) cells, the addition of the glucocorticoid fluticasone propionate resulted in an increased upregulation of GBP5 whilst the addition of dexamethasone resulted in an even greater upregulation. Expression of GBP5 remained absent in M(0) and M(IL-4) cells after treatment by both glucocorticoids. Two bands show similar sizes for the M2 marker TGM2, however, the top band is specific for TGM2 protein expression. It is clear that TGM2 is highly upregulated in non-glucocorticoid treated M(IL-4) cells but low expression can be seen in M(0) and M(LPS,IFNγ) cells. The addition of dexamethasone during polarisation results in TGM2 expression increasing in M(LPS,IFNγ) cells whilst decreasing in M(IL-4) and M(0) cells. In M(IL-4) and M(0) cells treated with fluticasone propionate, TGM2 expression is absent with M(LPS,IFNγ) phenotype showing low expression of the protein. The M2 marker MRC1 is expressed in M(0) and M(IL-4) phenotypes at similar levels when treated without glucocorticoids, no MRC1 can be seen in M(LPS,IFNγ) cells. The addition of dexamethasone increased MRC1 expression slightly in the M(0) phenotype and highly upregulated expression in M(IL-4) cells without inducing any expression in the M(LPS,IFNγ) phenotype. Similarly for fluticasone propionate, the treatment of this glucocorticoid resulted in MRC1 expression increasing in M(0) cells with an even larger upregulation of expression in M(IL-4) cells when compared to non-

glucocorticoid treated macrophages. M(LPS,IFNγ) cells did not show any MRC1 expression after fluticasone propionate treatment.

4.6.1.2 Protein expression after 72 hours polarisation with glucocorticoids

Figure 4.11b shows expression after 72 hours of cytokine stimulation with or without glucocorticoid treatment. The amount of ANKRD22 expression after 72 hours of polarisation was found to decrease in M(LPS,IFNγ) cells treated with dexamethasone when compared to non-glucocorticoid treated M(LPS,IFNγ) cells. Yet, similar levels of expression could be seen between M(LPS,IFNγ) cells treated with fluticasone propionate and M(LPS,IFNγ) cells polarised without glucocorticoids. Expression of ANKRD22 was not induced in any other phenotype. No positive control was evident for this western blot, the reason for which is unknown. The expression of GBP5 after 72 hours of polarisation was found to decrease in M(LPS,IFNγ) cells treated with dexamethasone and fluticasone propionate compared to cells polarised without glucocorticoids, with dexamethasone inducing a greater down regulation of the protein. Importantly, however, dexamethasone and fluticasone propionate did not induce GBP5 expression in either M(0) or M(IL-4) phenotypes. The treatment of M(IL-4) phenotypes with both dexamethasone and fluticasone propionate resulted in the elimination of TGM2 protein expression. Additionally, expression in the M(LPS,IFNγ) phenotype was also decreased when polarised with dexamethasone and removed completely when polarised with fluticasone propionate. Changes to MRC1 expression after 72 hours was similar to that seen after 48 hours of polarisation. Both M(0) and M(IL-4) phenotypes showed increased MRC1 expression when polarised with dexamethasone and fluticasone propionate treatment compared to those phenotypes polarised without glucocorticoids.

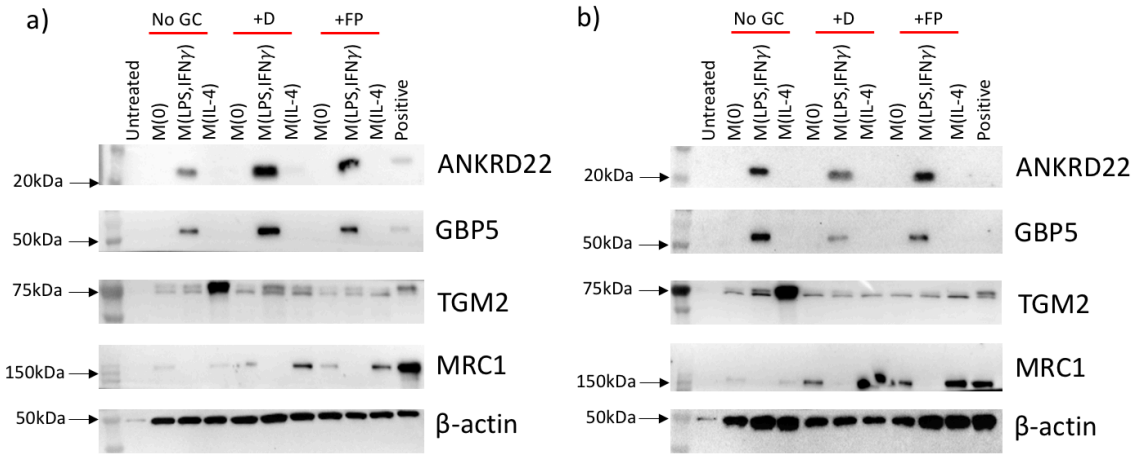


Figure 4.11: The effect of treating macrophages with glucocorticoids over a period of 48 or 72 hours whilst polarising towards various phenotypes.

THP-1 cells were treated with PMA (5ng/mL) to induce a M(0) phenotype before treatment with polarising cytokines LPS and IFNγ or IL-4 alone (No GC) or with the addition of the glucocorticoid Dexamethasone (+D) or Fluticasone Propionate (+FP) for either **a)** 48 hours or **b)** 72 hours. The expression of markers was analysed using western blotting, β-actin was used as a loading control and hMDMs polarised into their respective phenotype were used as a positive control. No replicates were performed for these experiments. D, Dexamethasone; FP, Fluticasone propionate; GC, glucocorticoid; IFNγ, interferon gamma; IL, interleukin; LPS, lipopolysaccharide; hMDM, human monocyte-derived macrophage.

4.6.2 Addition of glucocorticoids after macrophage polarisation

To understand the effect of glucocorticoids on already polarised macrophages, glucocorticoids dexamethasone and fluticasone propionate were administered to THP-1-derived macrophages for 48 or 72 hours after their initial 48 hours of polarisation towards M(0), M(LPS,IFNγ) and M(IL-4) phenotypes. This was to understand the effect of glucocorticoids on polarised resident macrophages which reside within the arterial wall.

4.6.2.1 Protein expression 48 hours after glucocorticoid addition

Figure 4.12a shows the expression of selected markers after 48 hours of glucocorticoid treatment in polarised macrophages. The M1 marker ANKRD22 could be seen to remain specific for M(LPS,IFNγ) polarised macrophages after both dexamethasone and fluticasone propionate treatment. No expression was seen in M(0) or M(IL-4) polarised cells after stimulation with either glucocorticoid. GBP5 also showed specificity for the M(LPS,IFNγ) phenotype after treatment with either dexamethasone or fluticasone propionate. Both types of glucocorticoids were found to decrease the expression of GBP5 in M(LPS,IFNγ) cells in comparison to non-glucocorticoid treated M(LPS,IFNγ) cells. The M2a marker TGM2 was seen to remain highly specific for the M(IL-4) phenotype after treatment with either glucocorticoid and no decrease in expression levels could be seen in these cells in comparison to non-glucocorticoid treated cells. The marker MRC1 was seen to be further upregulated in M(0) and M(IL-4) phenotypes after dexamethasone treatment, with M(IL-4) showing highest upregulation. Interestingly the addition of fluticasone propionate caused a decrease in the expression of MRC1 in M(IL-4) cells as well as M(0) when compared to the non-glucocorticoid cells. No expression was induced in M(LPS,IFNγ) cells after treatment with either glucocorticoid.

4.6.2.2 Protein expression 72 hours after glucocorticoid addition

The expression of markers in cells treated with glucocorticoids for 72 hours after polarisation can be seen in Figure 4.12b. ANKRD22 expression in M(LPS,IFNγ) cells was not altered by the addition of either dexamethasone or fluticasone propionate when compared to non-glucocorticoid treated M(LPS,IFNγ) cells and remained specific for this phenotype after treatment with both glucocorticoids. Expression of GBP5 showed a similar pattern to ANKRD22 with specificity of the marker remaining for the M(LPS,IFNγ) phenotype after treatment with either glucocorticoid and additionally, no change to the intensity of expression in comparison to the non-glucocorticoid M(LPS,IFNγ) treated cells was seen.

Expression of TGM2 after 72 hours of glucocorticoid was seen to be very different to that after 48 hours where high expression of TGM2 was seen in non-glucocorticoid treated M(IL-4) cells with some expression also seen in M(LPS,IFNγ) cells. When treated with dexamethasone for 72 hours after polarisation TGM2 expression was induced in M(0) and M(LPS,IFNγ) cells to similar levels with expression in M(IL-4) remaining higher. Addition of fluticasone propionate to polarised M(0) and M(LPS,IFNγ) cells also induced expression of TGM2 with further upregulation of the protein seen in the M(IL-4) phenotype. Further replicates would be needed to understand whether these differences are due to additional 24 hours of glucocorticoid exposure, or variation in cell expression. No positive control could be seen for TGM2 in this western blot, however, due to the loading control being present and at a similar intensity, it could be assumed that the wrong sample was added to this well or due to trimming of the membrane, the positive control could have been accidently cut off. Protein expression of MRC1 could not be seen in any non-glucocorticoid treated cells 72 hours after polarisation yet treatment with dexamethasone could be seen to induce expression in M(IL-4) cells only. Conversely treatment with fluticasone propionate did not induce MRC1 in either M(0), M(LPS,IFNγ) or M(IL-4) phenotype.

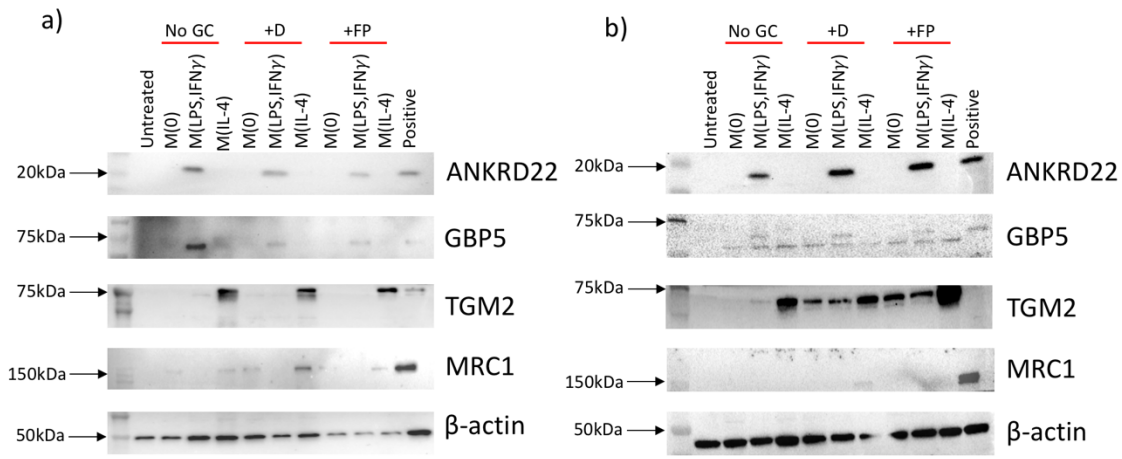


Figure 4.12: The effect of treating macrophages with glucocorticoids over a period of 48 or 72 hours after polarisation towards various phenotypes for 48 hours.

THP-1 cells were treated with PMA (5ng/mL) to induce a M(0) phenotype before treatment with polarising cytokines to induce a M(LPS,IFNγ) or M(IL-4) phenotype over 48 hours. After polarisation, glucocorticoid Dexamethasone (+D) or Fluticasone Propionate (+FP) was added for a further **a)** 48 hours or **b)** 72 hours. The expression of markers was analysed using western blotting, β-actin was used as a loading control and hMDMs polarised into their respective phenotype were used as a positive control. No replicates were performed for these experiments. D, Dexamethasone; FP, Fluticasone propionate; GC, glucocorticoid; IFNγ, interferon gamma; IL, interleukin; LPS, lipopolysaccharide; hMDM, human monocyte-derived macrophage.

Overall the expression of M1 markers ANKRD22 and GBP5 remained specific for M(LPS,IFNγ) cells after treatment with glucocorticoids dexamethasone and fluticasone propionate both during and post-polarisation, although decreases in marker expression was apparent after some glucocorticoid treatments. These results, however, suggested that these proteins could be used as markers of M1-like macrophages in GCA temporal artery biopsies.

Conversely M2a markers MRC1 and TGM2 show variation in their expression across the different phenotypes and a lack of specificity. MRC1 can be seen to be upregulated by glucocorticoids in M(IL-4) cells during and after polarisation, however, expression in M(0) seems to mimic that seen in M(IL-4) cells when cells are treated with glucocorticoids during polarisation. This suggests that the phenotype induced by glucocorticoids, described as M2c, expressed the M2a marker MRC1. Treatment of cells with glucocorticoids post-polarisation does not seem to induce MRC1 expression in M(0) cells and overall has less of an effect on MRC1 expression. TGM2 looks to be more susceptible to changes in

expression after glucocorticoid treatment, however, changes were found to be extremely variable, with no same general pattern of expression seen in any glucocorticoid treatment protocol. Table 4.7 summarises the effects of different glucocorticoid treatment on macrophage phenotypes taken from Figure 4.11 and Figure 4.12.

The reason for the variability in TGM2 expression may be due to a number of factors, both biological and technical. These include expression of different transcript variants in different cells, of which may behave differently in result of glucocorticoid treatment. TGM2 protein within cells can exist in different forms, such as membrane bound as well as being secreted as an enzyme (Tovar-Vidales *et al*., 2008) and these different forms of TGM2 may be induced in different quantities between experiments and may respond differently to different stimuli, such as glucocorticoids. Western blots of TGM2 showed the highest intensity of protein expression, and those with very high intensity bands showed the most variation in expression and suggests that if a lower concentration of primary antibody was used, lower variation may have been seen. Additionally, two bands of very similar size can be seen for TGM2, with the above band being specific to TGM2 expression as seen by the positive control. The lower band makes it difficult to determine whether TGM2 is expressed in these cells or not. Human error may have also caused variability seen in these results. To understand this variation further, replicates of these experiments would need to be performed, however, due to time constraints this was not achievable in this study. This highlights the difficulty in identifying M2a macrophages in tissue, especially in those patients taking glucocorticoids, which appear to have a larger impact on M2a marker expression than on the M1 markers ANKRD22 and GBP5.

Table 4.7: Summary of marker protein expression in differently polarised THP-1 derived macrophages after different treatment conditions with glucocorticoids.

a)

	Macrophage phenotype	Marker	Macrophage phenotype								
			No GC			+ Dexamethasone			+ Fluticasone propionate		
			M(0)	M(LPS,IFNɣ)	M(IL-4)	M(0)	M(LPS,IFNɣ)	M(IL-4)	M(0)	M(LPS,IFNɣ)	M(IL-4)
48 hours of GC treatment during polarisation	M1	ANKRD22									
		GBP5									
	M2a	MRC1									
		TGM2									
72 hours of GC treatment during polarisation	M1	ANKRD22									
		GBP5									
	M2a	MRC1									
		TGM2									

High expression

No expression

b)

	Macrophage phenotype	Marker	Macrophage phenotype								
			No GC			+ Dexamethasone			+ Fluticasone propionate		
			M(0)	M(LPS,IFNɣ)	M(IL-4)	M(0)	M(LPS,IFNɣ)	M(IL-4)	M(0)	M(LPS,IFNɣ)	M(IL-4)
48 hours of GC treatment post polarisation	M1	ANKRD22									
		GBP5									
	M2a	MRC1									
		TGM2									
72 hours of GC treatment post polarisation	M1	ANKRD22									
		GBP5									
	M2a	MRC1									
		TGM2									

The degree of protein expression of markers in M(0), M(LPS,IFNγ), and M(IL-4) phenotypes which mimic **a)** circulating monocytes polarised over a 48 or 72 hours period with glucocorticoids or **b)** resident macrophages polarised for 48 hours and treated with glucocorticoids for a further 48 or 72 hours. Expression has been summarised as colours in a semi-quantitative manner from the data in Figure 4.11 and Figure 4.12. GC, glucocorticoid; IFNγ, interferon gamma; IL, interleukin; LPS, lipopolysaccharide.

4.7 Conclusions

Six new markers were identified from the RNA-Seq dataset as being M1 subset-specific and were subsequently analysed at the RNA and protein level to validate their subset-specificity. Out of the six M1 markers, ANKRD22 and GBP5 were confirmed to be specific for THP-1 derived M(LPS,IFNγ) cells at the protein level. *GBP5* also showed specificity for M(LPS,IFNγ) cells at the RNA level yet transcriptional analysis of *ANKRD22* revealed upregulation in M(IL-10) treated THP-1-derived cells, demonstrating the variability in the translation of RNA to protein.

The marker SERPING1 also displayed specificity for M1 macrophages at the RNA level in THP-1 derived macrophages, however, this could not be replicated in western blot analysis with no bands visible for any THP-1-derived macrophage or hMDM phenotype. Similarly marker TNFAIP6 showed the same pattern of expression to ANKRD22 when analysed at the RNA level in THP-1 derived cells with expression apparent for M(LPS,IFNγ) and M(IL-10) cells, yet at the protein level, no bands were detected for any THP-1 derived macrophage or hMDM phenotype. Further analysis would need to be carried out for both SERPING1 and TNFAIP6 to investigate their expression at the protein level, for example, the use of alternative antibodies which identify different epitopes for western blot analysis as it seemed there was a problem with the antibodies used in this study. Other methods of marker analysis such as flow cytometry could also be performed as transcripts were seen in THP-1 and hMDM M1 cells, therefore both genes should not yet be ruled out as protein markers for M1 macrophages.

Two new markers were identified from the RNA-Seq dataset as being M2a subset-specific, which included ALOX15 and HOMER2. Out of these M2a markers, *ALOX15* showed specificity for THP-1 derived M(IL-4) cells, however, this could not be replicated at the protein level using western blotting as no bands were evident for any macrophage phenotype, suggesting a problem with the antibody identifying the epitope, therefore different antibodies should be tested in future western blotting experiments for ALOX15. The second marker HOMER2 was found to lack specificity at the RNA level and was subsequently removed from the panel of markers.

The M2a markers MRC1 and CD200R1 used in Chapter 3 for the development of the THP-1 model system using RNA expression data were found to display high specificity for THP-1 derived M(IL-4) cells at the RNA level, however, similarly to the M1 markers SERPING1 and TNFAIP6, CD200R1 was not found

to be expressed in any phenotype at the protein level in both THP-1 derived macrophages and hMDMs, again this suggests a problem with the antibodies used and repeat western blots should be carried out using different antibodies that identify alternative epitopes to determine protein expression. No protein expression of MRC1 was evident in any THP-1 derived macrophage phenotype, however, expression was seen in hMDM phenotypes, albeit not specifically.

No M2c markers selected from the RNA-Seq dataset were found to be specific for the THP-1 derived M(IL-10) phenotype at the RNA level. This absence of marker specificity for THP-1 derived M(IL-10) macrophages was also observed by Shiratori *et al*. (2017) who found a lack of M2c marker upregulation in THP-1 derived macrophages in response to IL-10 treatment. It was therefore decided that the M2c markers from the RNA-Seq dataset would not be investigated further. The most commonly used M2c markers, CD163, was found to be expressed in the M2-like phenotypic cells as well as induced by IL-33. Further protein analysis showed discrepancies in phenotypic expression between western blots, however, analysis in hMDMs provided evidence that expression was not induced in M(LPS,IFNγ) cells but upregulated in M(0), M(IL-10), M(IL-33) and one blot showed expression in M(IL-4) cells. This confirmed the pattern of RNA expression seen in THP-1-derived macrophages.

It was important to understand the expression of chosen markers in hMDMs as they would be used to characterise human macrophages in GCA positive patient tissue. Cell lines are useful as a snapshot to understand marker expression, however it was clear from the results that there were differences in specificity of markers between polarised THP-1 derived macrophages and polarised hMDMs. The lack of similarity between protein expression of markers in THP-1-derived macrophages and hMDMs was evident in Figure 4.3 and Figure 4.6. These figures showed markers ANKRD22, GBP5, MRC1, TGM2 and CD163 lacked subset-specificity in hMDMs at 48 and 72 hours of polarisation, although a pattern of upregulation for their respective phenotype and downregulation for non-specific phenotypes was apparent. Further analysis of markers in more donors emphasised the variability that is apparent in macrophage phenotypes and their marker expression between different subjects. Markers GBP5 and MRC1 displayed a lack of specificity for their respective phenotypes, as well as variation in expression between donors. No bands were observed for ANKRD22 in either donor whilst CD163 was the only marker which showed the same pattern of expression between donors. TGM2, however, showed more specificity for M(IL-4) hMDM phenotype compared to THP-1 derived macrophage phenotypes, this may be due to a much higher expression of TGM2 in THP-1-derived

macrophages. Differences between the cell line and the primary controls may be due to up-regulated cancerous pathways in THP-1 cells and a lack of inactive pathways in healthy control primary cells, making it difficult to identify markers upregulated in both healthy and diseased patients.

The cytokine IL-33 was found to induce the M2a marker TGM2 and the M2c marker CD163 in THP-1 derived cells at the protein level which was consistent with the data presented in Chapter 3. However in hMDMs, IL-33 was found to upregulate MRC1 and CD163 at the protein level which was not evident at the RNA level. Additionally, IL-33 induced the expression of the M1 marker GBP5 in hMDMs. The suggestion that IL-33 induces a M1 macrophage phenotype when added to M(0) cells (Joshi *et al.*, 2010) is contradicted at the protein level in hMDMs with the induction of both M2a and M2c markers.

Treatment of selected markers with glucocorticoids provided further evidence for their potential usefulness in identifying macrophage states in GCA artery biopsies. Treatment of macrophages with glucocorticoids during and after polarisation was found to have little effect on selected M1 macrophage markers with expression of ANKRD22 and GBP5 remaining specific for the M(LPS,IFNγ) phenotype. Expression of M2a marker MRC1 was further upregulated in M(0) and M(IL-4) macrophages after treatment with both dexamethasone and fluticasone propionate when added during polarisation over 48 and 72 hours.

TGM2 was found to be more susceptible to glucocorticoid treatment when added during macrophage polarisation as expression in M(IL-4) cells was downregulated after the addition of both dexamethasone and fluticasone propionate after 48 and 72 hours of polarisation. Treatment after polarisation however, did not seem to effect TGM2 expression in M(IL-4) cells. After 48 hours of glucocorticoid treatment to already polarised macrophages, TGM2 was specific for M(IL-4) macrophages only and the amount of expression in glucocorticoid treated M(IL-4) cells was no different to cells not treated with glucocorticoids. At 72 hours however, non-specific upregulation seemed to be induced in M(LPS,IFNγ) cells when macrophages were treated with glucocorticoids, however expression of TGM2 increased in both M(IL-4) phenotypes treated with glucocorticoids, with fluticasone propionate, the most potent glucocorticoid, causing the greatest expression increase. As glucocorticoids are given to patients after the development of GCA, polarised macrophages would encounter glucocorticoids *in situ* and therefore the results of glucocorticoid treatment after polarisation may be more useful in understanding their effect on marker expression in GCA.

Chapter 5. Macrophage phenotypes in giant cell arteritis

5.1 Introduction

The role of macrophages in the pathogenesis of GCA is still to be elucidated. The ability of macrophages to polarise into an array of phenotypes, as well as having the ability to switch between phenotypes during different phases of disease progression (Porcheray *et al.*, 2005) has made defining macrophage phenotypes in GCA difficult.

An understanding of the pathogenesis of GCA is still largely based on observations from immunohistological and molecular studies of temporal artery biopsies combining functional knowledge of molecules. These findings are then correlated with different clinical presentations of the disease. The adventitia is dominated by a Th1 and Th17 response, with Th1 cells releasing high levels of IFNγ. IFNγ is thought to promote M1 polarisation within the adventitia and subsequent release of pro-inflammatory cytokines, such as IL-6 and TNFα, into the adventitia and the circulation, cytokines which correlate with disease duration and relapse (Hernandez-Rodriguez *et al.*, 2004a). In different patients the media of GCA temporal arteries is found in different states of destruction. External and internal elastic lamina, which respectively border the adventitia-media and media-intima and are thought to provide immune privilege, are also found in different states of fragmentation. IFNγ-stimulated M1 macrophages are thought to release destructive ROS molecules into the media, resulting in VSMC apoptosis and media destruction (Ciccia *et al.,* 2013; Rittner *et al.,* 1999b). A number of elastolytic MMPs are also produced by macrophages found within the media and close to the adventitia-media and media-intima borders (Segarra *et al.,* 2007; Rittner *et al.,* 1999b). These molecules are thought to contribute to the destruction of media and fragmentation of the external and internal elastic lamina (Segarra *et al.,* 20007) allowing migration of cells from the media into the intima. The intima is an area in which tissue remodelling occurs and where a number of growth factors are found. Migration of fibroblasts from the media into the intima and their subsequent proliferation is thought to be induced by the release of PDGF from macrophages and giant cells at the media-intima border (Lozano *et al.*, 2008; Kaiser *et al.*, 1998). Proliferating fibroblasts are thought to result in hyperplasia of the intima causing intimal thickening and luminal occlusion (Lozano *et al.,* 2008). Furthermore VEGF, an angiogenic factor, is found at higher levels in the circulation of GCA patients compared to healthy controls

(Goodfellow *et al*., 2017). VEGF is thought to be released by macrophages and giant cells within the media and intima, resulting in neoangiogenesis within these two layers, promoting recruitment of further immune cells to the artery wall (Kaiser *et al.,* 1999). Studies have associated a greater inflammatory infiltrate, identified using H&E staining, with increased intimal hyperplasia (Hernandez-Rodriguez *et al.*, 2016; Nordborg and Petursdottir, 2000). Neoangiogenesis is also thought to act as a compensatory mechanism for the increased cellular proliferation occurring within the intima (Cid *et al.*, 2002).

These heterogeneous populations of macrophages, however, were identified within the different layers of the artery wall two decades ago (Kaiser *et al*., 1998; Rittner *et al*., 1999b; Wagner *et al*., 1994). The lack of more recent macrophage studies in GCA means newer ideas and theories regarding macrophage biology and GCA pathogenesis have not been investigated. The concept of macrophage subsets means these different populations of macrophages may be characterised into M1 and M2 phenotypes using the markers identified in the previous Chapters. The different immunological processes described above and the association of different macrophage phenotypes with these different processes may give insights into dominant cytokine profiles in the different parts of the vessel wall. This may reveal which macrophage phenotypes should be therapeutically targeted. More recent studies that have attempted to characterise macrophages within GCA temporal artery biopsies have not adequately answered the questions posed here. This is due to the use of markers that lack specificity for macrophage subsets as well as markers which are affected by glucocorticoid treatment. Studies into macrophage subsets in GCA have used CD163 as a marker of the M2 macrophage phenotype (Ciccia *et al*., 2013; Shirai *et al*., 2015), a marker which has been described as specific to M2 macrophages and used as an M2 marker in a number of studies (Hu *et al*., 2017; Weber *et al*., 2015; Wehrhan *et al*., 2014; Stöger *et al*., 2012). CD163, however, is also thought to be expressed specifically on the M2c subset (Ehrchen *et al*., 2007; Tran *et a*l., 2015). Furthermore, it is known that M2c macrophages are polarised in response to glucocorticoids and the marker CD163 is upregulated in response to glucocorticoid treatment (Ritter *et al*., 1999; van den Heuvel *et al*., 1999). The results from this study identified CD163 expression in primary human M(0), M(IL-4) and M(IL-10) macrophages but not in M(LPS, IFNγ) macrophages (Section 4.5.3) suggesting CD163 should be used as a marker to define M2a and M2c macrophage subsets. In some previous studies, CD163 had been suggested to be used as a pan-macrophage marker, as a greater number of macrophages stained with CD163 in comparison to the commonly used pan-macrophage

marker, CD68 (Klein *et al*., 2014; Barros *et al*., 2013; Barros *et al*., 2012; Lau *et al.*, 2004).

The notion that macrophages within GCA artery biopsies perform different functions and processes could help to explain the large clinical heterogeneity that is observed between patients (Janssen *et al.*, 2008).

5.2 Aims

It is hypothesised that macrophages in GCA artery biopsies can be characterised into different phenotypes, and these phenotypes may infiltrate arteries in different amounts depending on the patient, stage of disease and length of glucocorticoid treatment. As a consequence, different macrophage phenotypes correlate to areas of differing immunological processes including media destruction, neovascularisation and luminal occlusion, and also that macrophage phenotypic heterogeneity in biopsies could explain some of the clinical heterogeneity of the disease. This relationship of macrophage phenotype with clinical features could be of potential value in predicting treatment durations.

To test this hypothesis macrophages in GCA temporal artery biopsies from patients with GCA were phenotyped using macrophage markers identified in Chapter 4.

The aims of this study therefore were:

1. To characterise macrophage phenotypes within temporal artery biopsies to determine their location and the extent of their infiltration within the arterial wall.
2. To identify the association of macrophage phenotypic markers with clinical manifestations such as vision loss or jaw claudication and the different histological features that are found in GCA arteries, such as media destruction, angiogenesis and luminal occlusion.
3. To understand the relationship between macrophage phenotypic marker expression and duration of glucocorticoid treatment.

To meet these aims, the objectives were:

- To develop immunohistochemistry protocols and to optimise antigen retrieval.
- To perform immunohistochemistry on archived formalin-fixed paraffin embedded (FFPE) temporal artery biopsies from GCA patients.
    - Using markers identified in Chapter 4 to identify their use in characterising macrophage subsets in GCA artery biopsies.
    - Using αSMA and CD31 respectively for scoring media destruction and luminal occlusion, and neovascularisation.
- To score the staining of different macrophage markers as well as the different histological changes observed within the artery wall by:

    - Developing a scoring system for the locality and intensity of antibody staining as well as a scoring system for the degree of histological change seen within the artery for neovascularisation, media destruction and luminal occlusion.
    - Produce a staining atlas of these antibodies and their scores to allow for consistent scoring of arteries.
- To compare macrophage populations with clinical and histological features using previously obtained clinical data on patients to understand the relationship between clinical outcomes with macrophage phenotypes.

5.3 Marker expression in THP-1 derived macrophages

Markers ANKRD22 and GBP5 were selected for characterising M1 macrophages and markers MRC1 and TGM2 were chosen for identifying M2a macrophages in GCA temporal artery biopsies due to their specificity for their respective macrophage subset and their lack of expression change in response to glucocorticoids (Section 4.6.1). Due to studies showing that CD163 may be a more useful pan macrophage marker compared to C68 in different tissues and diseases (Barros *et al.,* 2013), the pan macrophage marker CD68 and M2c marker CD163 were chosen to help identify overall macrophage numbers as they are commonly reported within the literature and are used in routine laboratories, making them a suitable benchmark against which novel markers could be compared.

To understand the use of CD68 and CD163 as pan macrophage markers, ANKRD22 and GBP5 in staining an M1 macrophage phenotype, and CD163 and MRC1 in staining an M2 macrophage phenotype at the tissue level, THP-1 cells were polarised into M(0), M(IFNγ, LPS), M(IL-4) and M(IL-10) phenotypes following the THP-1 model. Polarised cells were then pelleted and embedded in paraffin to allow for sectioning and staining with the above macrophage markers. Their staining intensity was then semi-quantitatively scored from 0 to 3 based on the intensity of staining of macrophages seen in the staining atlas in Appendix 2. The results of this can be seen in Table 5.1.

Table 5.1: Semi-quantitative scoring of THP-1 derived macrophage phenotype pellet sections stained with selected markers.

Macrophage phenotype	Marker					
	CD68	ANKRD22	GBP5	CD163	MRC1	TGM2
M(0)	(3,2,1) 2	(1,2,3) 2	(0,0,0) 0	(2,2,2) 2	(3,3,3) 3	(2,2,3) 2
M(LPS, IFNɣ)	(3,1,2) 2	(3,2,0) 2	(0,0,0) 0	(0,1,1) 1	(1,1,0) 1	(1,1,3) 1
M(IL-4)	(1,1,2) 1	(0,1,1) 1	(0,0,0) 0	(2,2,2) 2	(3,3,3) 3	(3,2,2,) 2
M(IL-10)	(3,1,2) 2	(3,0,0) 0	(0,0,0) 0	(2,2,2) 2	(3,2,3) 3	(2,2,2) 2

THP-1 cells were polarised into M(0), M(LPS, IFNγ), M(IL-4) and M(IL-10) phenotypes after 48 hours of polarising stimulation. Cells were pelleted and embedded in paraffin before sectioning. FFPE sections were stained with pan-macrophage marker CD68, M1 markers ANKRD22 and GBP5, M2a markers MRC1 and TGM2 and M2c marker CD163 and were then scored from 0 – 3 based on the intensity of staining of cell pellet sections. Results show the score of each replicate (replicate 1, replicate 2, replicate 3) and the median of these scores. Replicates were all cultured and pelleted at the same time. FFPE, Formalin-fixed paraffin-embedded; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide.

CD68, a pan-macrophage antibody, was found to stain the cell surface and cytoplasm of differently polarised macrophages with a similar intensity of 2, however staining of CD68 was found to be lower for M(IL-4), therefore staining of CD68 may have a bias towards M1 macrophage phenotypes. When looking at the scores for the different replicates, however, there was a great degree of variation in staining intensity for M(0), M(LPS, IFNγ) and M(IL-10) macrophage pellets. The M1 marker ANKRD22 was found to stain M(LPS, IFNγ) cells with the highest intensity whilst staining M(IL-4) at the lowest intensity. Additionally M(0) cells were also stained with a median intensity of 2, with M(IL-10) staining with ANKRD22 at an intensity of 0. This is a different pattern of staining in comparison to THP-1 cells polarised for 48 hours and analysed using western blotting, where ANKRD22 was highly specific for M(LPS, IFNγ) cells (Figure 4.3). Like CD68, there was variation across the three replicates. The other M1 marker, GBP5, was found not to stain any macrophage phenotype in any of the replicates. The reason for this may be due to expression that is too low to be picked up by the antibody at the tissue level, as western blot samples were pooled and lysed but not

subjected to paraffin fixation. The protocol was not optimised for this experiment due to shortage of time, therefore antigen retrieval methods and concentrations of antibodies would need to be optimised in subsequent experiments with the use of human liver cancer tissue as a positive control.

Expression of the M2a marker, MRC1, a scavenger receptor, was found to show a similar pattern of expression in the different polarised subsets to protein expression observed in polarised hMDMs when analysed using western blotting in Section 4.5.3. Western blotting showed M(0), M(IL-4) and M(IL-10) polarised macrophages had a higher expression of MRC1 than M(LPS, IFNγ) cells. Immunohistochemistry staining of MRC1 showed this marker was highly expressed on the cell surface as well as in the cytoplasm of M(0) and M(IL-4) phenotypes with a median intensity scores of 3, expression of MRC1 has also been observed in these phenotypes in other studies (Raggi *et al*., 2017; Tran *et al*., 2015). Expression was also highly expressed in M(IL-10) phenotype, with an intensity score of 3, which confirms observations seen in other studies (Tran *et al*., 2015; Svensson-Arvelund *et al.*, 2015). MRC1 expression in M(LPS, IFNγ) macrophages was lower, with a median intensity score of 1, showing its upregulation for unpolarised, M(0) macrophages, and M2-like polarised macrophages.

TGM2, another M2 antibody, was also found to stain M(0), M(IL-4) and M(IL-10) phenotypes with the highest intensity, with a median score of 2, whereas M(LPS, IFNγ) phenotypes stained with the lowest intensity, with a score of 1. This is a similar pattern of TGM2 protein expression that was seen when analysed using western blotting where TGM2 was found to be expressed highly in M(IL-4) macrophages but showed protein expression in all other phenotypes too, albeit at a lower expression.

The M2c antibody CD163 stained M(0), M(IL-4) and M(IL-10) polarised macrophages at the same intensity with a score of 2, whilst staining of M(LPS, IFNγ) cells was lower, with a median intensity score of 1, a pattern which is similar to MRC1. Therefore, in contrast to CD68, CD163 may have a bias towards macrophages which have a M2 phenotype. For all three M2 markers, there was less variation seen across the three replicates compared to CD68 and ANKRD22 markers. The pattern of expression seen for CD163 in this study is different to other studies into CD163 expression in macrophages (Barros *et al.,* 2013). The difference in expression seen in the Barros *et al.* study, however, may be due to staining of polarised hMDM cells taken from 6 different tissue types in a range of disease settings, with differing immune responses. This included tissue from

Crohn's disease patients, a Th1 dominant disease, and skin biopsies showing wound healing, a Th2 immune response. Although a snapshot, this provides a more accurate representation of macrophages and their constitutive expression of markers across a variety of different disease microenvironments in which different cytokines are produced.

If not optimised properly for each antibody in each tissue, immunohistochemistry may not be as useful in recognising specific epitopes when compared to western blotting. This may account for the differences in expression of markers when comparing immunohistochemistry results to western blot results. This is due to formalin fixation and paraffin embedding of tissue resulting in changes to molecules within the tissue, termed "antigen masking" which can affect the way in which the tissue is stained using immunohistochemistry. Optimised heat-induced antigen retrieval with optimal pH buffers can alter these modifications, enabling tissue molecules to revert to a closer natural conformation (Scalia *et al*., 2017; Dapson, 2007). Furthermore, the age of stored paraffin sections may have a detrimental effect on the intensity of immunohistochemical staining of epitopes (Ramos-Vara *et al.*, 2013).

5.4 CD68 as a pan macrophage marker in GCA

Carrying on from the staining of differently polarised THP-1 derived macrophages, selected markers were used to stain FFPE temporal artery biopsies (n=59), which were consecutively sectioned. Characteristics of patients used in this study can be seen in Table 5.2. In total, 154 sections were stained due to one or more artery sections per FFPE block. The process of artery sectioning is shown below in Figure 5.1.

CD68 is commonly used as a pan-macrophage marker and in GCA studies is the marker used to identify infiltrating macrophages (Sultan *et al.*, 2018; van Sleen *et al.*, 2017; Zhou *et al.*, 2009) however, Ciccia *et al.,* (2013) used iNOS and CD163 respectively to identify M1 and M2 macrophages in GCA biopsies. There is contradicting evidence regarding the use of CD68 to characterise macrophages as CD68 has been observed to stain dendritic cells (Vakkila *et al*., 2005) as well as underestimating macrophage numbers in both Th1- and Th2-related diseases (Barros *et al.*, 2013). In contrast, in other diseases, CD163 has been shown to be more specific for macrophages and does not appear to stain other immune cell populations (Lau *et al*., 2004) as well as staining a larger number of macrophages in comparison to CD68 (Barros *et al*., 2013). This might suggest that CD163 could be a more useful pan macrophage marker in the immunohistochemistry of GCA biopsies and may have implications on the use of CD163 as an M2 marker in the Ciccia *et al.* (2013) study.

Table 5.2: Characteristics of the patients included in the biopsy study

Variable	Patients (n=59)
Sex, Male:Female	20:39 (34%:66%)
Age at GCA onset	72 (68-77) n=57
Length of diagnosis prior to recruitment (months)	24 (5-56) n=56
Duration of glucocorticoids prior to biopsy (days)	2 (0-5) n=58
Range in date of recruitment	August 2005 – May 2009

Continuous variables are given as a median (interquartile range). Data was not available for a small number of cases (medical notes lost or inaccessible).

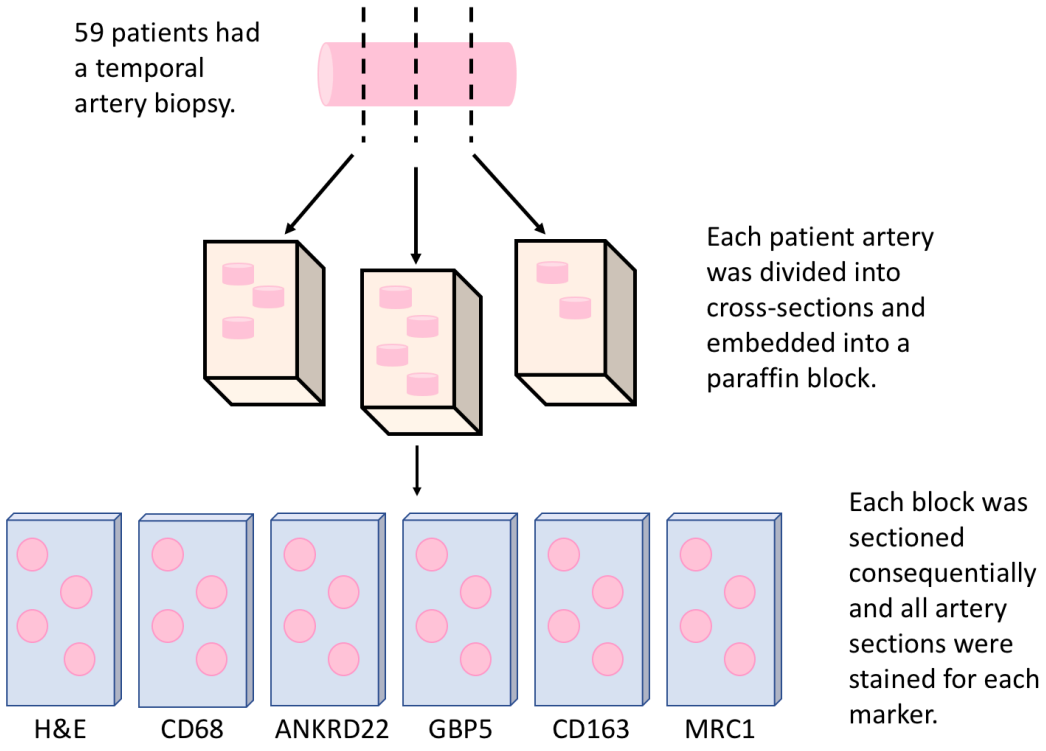


Figure 5.1: Process of artery sectioning.

Each patient had a temporal artery biopsy and each patient artery was divided into a number of cross-sections (depending on the length of the artery biopsy). Each cross-section was formalin-fixed and embedded into a paraffin block. Paraffin blocks were sectioned consecutively, and each artery section was stained for each marker. H&E, haematoxylin and Eosin.

5.4.1 CD68 vs CD163 macrophage expression

To compare the use of CD68 and CD163 as pan-macrophage markers in GCA, all GCA temporal artery biopsy sections were stained (n=154) with CD68 and CD163 and their locality and intensity of staining was semi-quantitatively examined. The locality and the intensity of the staining in the adventitia, media and intima layers of the artery wall were scored from 0 to 3, and the values from both scores were added together to give a total score for each artery layer, the maximum of which was a score of 6. Details of the scoring system can be found in Section 2.7.5.

It was found that, compared to CD163, CD68 had a greater total staining score across the whole artery as well as in each layer of the artery, as shown in Table 5.3. Furthermore, the mean CD68 scores for locality and intensity within each artery layer were also greater than the mean CD163 scores. This suggests that CD68 stains a greater number of macrophages in GCA artery biopsy tissue than CD163. This does not agree with the results of Barros *et al.* who observed a lower number of macrophages stained by CD68 compared to CD163 in cases of classical Hodgkin lymphoma (Barros *et al*., 2012) and observed the same pattern in both Th1 and Th2 diseases (Barros *et al.,* 2013). Barros *et al.* (2013) also identified the ability of CD163 to be co-expressed with M1 transcription factor, pSTAT1 or RBP-J and therefore suggested CD163 is not specific to the M2 phenotype. The differences observed, however, may be due to differences in disease state as well as differences in the location of the disease, of which both can impact macrophage polarisation. This emphasise the importance in optimising antibodies for different disease states and for seeing which antibodies provide the most relevant diagnostic or prognostic information.

Table 5.3: Mean total staining score for CD68 and CD163 in each layer of the artery wall.

Marker	(Locality, intensity) Adventitia	(Locality, intensity) Media	(Locality, intensity) Intima	(Locality, intensity) Whole artery
CD68 n=148	(1.79, 1.62) 3.41	(1.39, 1.33) 2.71	(1.57, 1.51) 3.05	(1.58, 1.49) 3.06
CD163 n=140	(1.56, 1.45) 2.94	(1.05, 0.89) 1.88	(1.13, 1.10) 2.23	(1.25, 1.15) 2.35

Temporal artery biopsies were stained with CD68 and CD163 and the locality and intensity of staining were both semi-quantitatively scored from 0 to 3, the values of which were summed giving a total staining score for each artery layer. The mean values for locality, intensity and total area score for each artery layer and mean values for locality, intensity and total area score for the whole artery are shown in the table.

Fewer macrophages were observed within the media, and those macrophages within the media were found to express both CD68 and CD163 markers at a lower intensity in comparison to both the adventitia and the intima (Table 5.3). Macrophages, regardless of their marker expression, were most commonly found along the adventitia-media or media-intima border. Those macrophages which were observed within the media were found in areas in which the media had been destroyed and could be found to be infiltrating the intima at the same site, as shown in Figure 5.2.

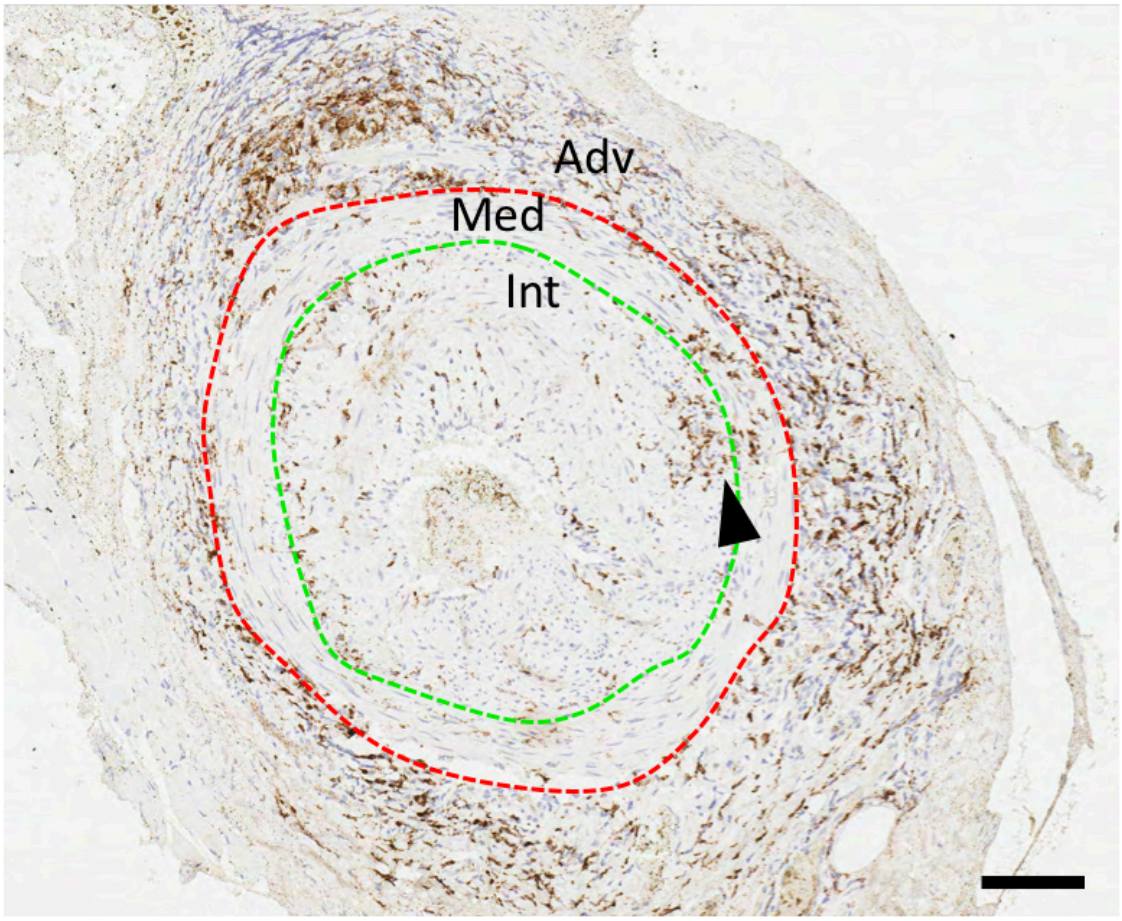


Figure 5.2: Pattern of overall macrophage staining across the whole artery using CD68 pan macrophage marker.

Macrophages can be found to localise to the boundary of the media-intima border (green dotted line) and to a lesser extent, the adventitia-media border (red dotted line). Macrophages can be seen to be infiltrating the intima where a greater number of macrophages are observed within the media, as indicated by the arrow. Image was taken at a magnification of x50, scale bar indicates 100μm. Adv, adventitia; Med, media; Int, intima.

In this study, the lower scoring values for CD163 does suggest that CD163 may be staining a different subset of macrophages, potentially the M2 subset, as described by other groups (Barbosa *et al.*, 2015). In Figure 5.3 areas of cells within the adventitia of a GCA diseased artery which show a morphology of macrophages (large cytoplasm with indented or irregular, pale nuclei (Cline, 1994)) can be seen to be stained by CD68 antibodies, as indicated by the arrows. Areas of cells which show a similar morphology to macrophages but no CD68 staining, as indicated within the circled areas, can also be seen in Figure 5.3. Without dual or multiple staining using multiple cellular markers to define macrophages and other cell types, such as DCs, it cannot be concluded that these are CD68- macrophages. Further research would need to be carried out to

determine whether CD68- macrophages exist within the adventitia of GCA affected arteries, as described by Barros *et al.* (2012) in multiple disease settings.

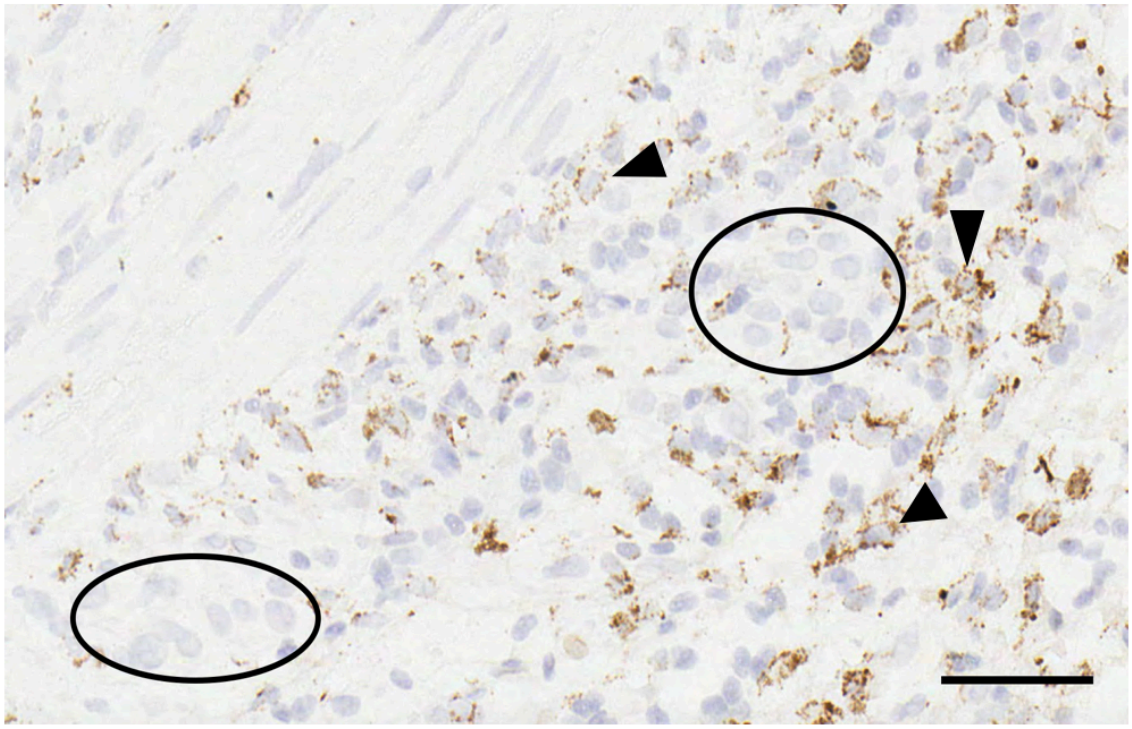


Figure 5.3: Staining of macrophages by CD68.

GCA positive temporal artery biopsies were stained with the pan-macrophage marker, CD68. Cells with a macrophage morphology not stained by CD68 can be seen within the circles, cells with a macrophage morphology stained by CD68 are indicated by arrows. Image was taken at a magnification of x400, with a scale bar of 50μm.

5.5 Within-case variation

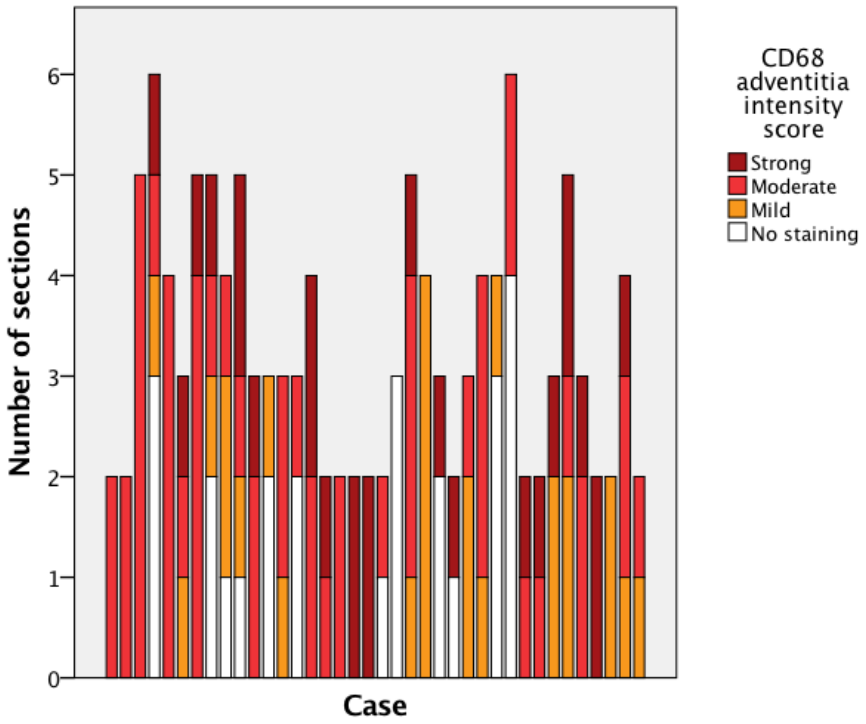
Out of all the artery biopsies used in this study, 38 artery biopsies had more than one artery section that was used for analysis of marker expression. The variation of staining in the different sections of each artery biopsy was analysed. Examples of the variation in adventitial CD68 staining in biopsies with more than one artery section can be seen in Figure 5.4. The adventitia was chosen as the more suitable layer as monocytes are thought to infiltrate through vasa vasorum into the adventitia, and therefore it is expected that most sections would have macrophages in this layer.

The percentage of artery biopsies which showed variation in CD68 adventitial intensity scores across the sections was 71% (27/38) (Figure 5.4a), with variation increasing to 76% (29/38) when locality and intensity scores were summed to give a total adventitia score (Figure 5.4b). For all other markers, similar variation was seen, with variation occurring in 63% (22/35) of artery biopsies for ANKRD22, 49% (17/35) for GBP5, 83% (29/35) for CD163 and 82% (28/34) for MRC1. Due to the amount of variability of staining seen in the different sections of each artery biopsy for all the macrophage markers, I decided that CD68 would be taken as a reference antibody which would be used to identify the section of each artery biopsy with the greatest number of macrophages of both M1 and M2 phenotypes. This would firstly ensure that a degree of macrophage infiltration would be present within the artery wall for each case, providing internal validity for this study and secondly, it would prevent the inclusion of skip lesions, where parts of the artery are healthy and show no signs of vasculitis. Therefore, the section with the highest overall score of CD68 marker staining for each artery biopsy was used for further analysis. The sum of the locality and intensity CD68 score was calculated to give an overall artery CD68 staining score, producing 59 sections from 59 artery biopsies. Therefore, from now on sections will be referred to as arteries.

Looking at the data from the cell pellet staining (Table 5.1), selecting for sections with the greatest CD68 score may mean that this is likely to select for M1-dominant sections of the artery, given that CD68 was found to stain M(IL-4) macrophages at a lower intensity. However, if we were to select sections with the greatest CD163 score, the data suggests these sections would be preferentially M2-dominant. Nevertheless, most studies into macrophages in GCA have used CD68 as a pan-macrophage marker and therefore using CD68 as our reference antibody would help to compare results with other studies. Furthermore, CD68 is also the NHS gold standard antibody for identifying macrophages in histology,

therefore it is essential to benchmark new antibodies to those used routinely by pathologists.

a)



b)

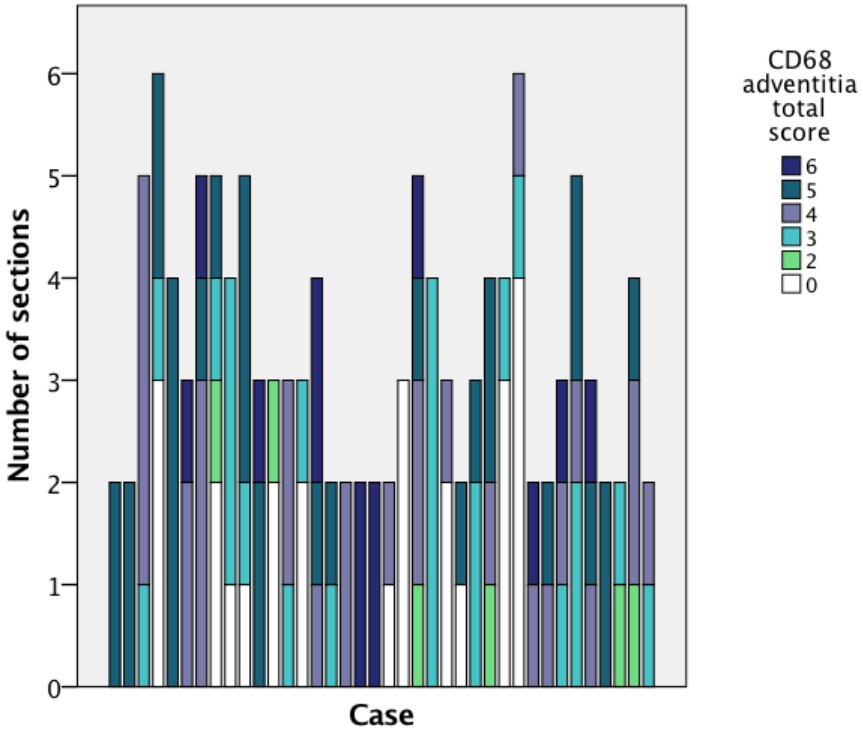


Figure 5.4: Variability of adventitial CD68 scores of different sections within cases.

The number of sections per artery biopsy was plotted to determine **a)** variability of CD68 intensity scores of the adventitia and **b)** variability of CD68 adventitia total score (intensity + locality).

5.5.1 Analysis of clinical phenotype of GCA and heterogeneity of macrophage expression

Taking the results from Figure 5.4a into consideration, it was important to understand whether severe clinical phenotypes were associated with consistently greater macrophage-specific marker intensity staining of the artery wall.

Patients which more than one cross-section taken from their artery were included in this analysis. Firstly, the intensity score of marker staining for the adventitia, media and intima for each macrophage marker was summed for every cross-section, giving total intensity. The mean total intensity was then calculated using all the cross-sections that were stained for each patient.

The mean total intensity for each macrophage marker was correlated using Spearman rank with different clinical outcomes (permanent visual features, temporary jaw claudication, fever, night sweats or weight loss, CRP level and ESR level) to determine if a consistently greater staining intensity correlated with a more severe clinical outcome. It was found that consistently greater CD68 intensity staining correlated with greater ESR levels (r_s = 0.602, n = 24, *p*=0.002**) suggesting an association with systemic inflammation, however no other clinical outcomes were found to be associated with any macrophage markers.

5.5.2 Glucocorticoid effect on marker expression of different macrophage markers

GCA is treated with high-dose glucocorticoids, usually preceding biopsy by a variable number of days, therefore the biopsy is done in the context of systemic exposure to high-dose glucocorticoids and this could be a potential complicating factor in interpreting these results. Due to this, it was important to determine whether expression of markers selected for macrophage phenotype characterisation was dampened or ablated following a typical duration of glucocorticoid treatment prior to biopsy. If so, a cut-off period to remove patients from data analysis after this duration of glucocorticoid treatment would need to be implemented.

Previous experiments using PCR and western blotting techniques were performed to understand the effect of glucocorticoids on the RNA expression and protein expression of different macrophage markers selected for characterising macrophage phenotypes in artery biopsies. Using patient data on prednisolone

use prior to artery biopsy, the effect of prednisolone treatment duration on macrophage marker expression within the inflammatory infiltrate was analysed using immunohistochemistry, to determine whether glucocorticoids ablated marker expression and if so, after what duration of prednisolone treatment. In light of the changes in expression of markers after different durations of glucocorticoids as described in Section 4.6, cases were grouped into different periods of glucocorticoid duration prior to biopsy, which were 0 days, 1-3 days, 4-7 days, 8-14 days, and more than 15 days. This also allows for a similar distribution of cases per group, apart for the 8-14 and >15 days groups, which occur less frequently. Differences in marker total score (locality score + intensity score) across the adventitia, media and intima were analysed (Figure 5.5, Figure 5.6 and Figure 5.7, respectively), where a maximum score of 6 could be given. The number of arteries within the 8-14 days group (n=4) and the >15 days group (n=2 or 3) were very low and therefore the data interpreted from these are less likely to represent what occurs within the population.

5.5.2.1 Macrophage marker expression in the different artery layers

All markers showed a degree of adventitial staining after 15 days (Figure 5.5). For all the artery layers, CD68 expression (indicating the majority of macrophages) appeared to be similar for the first week on prednisolone, however a decrease in expression was observed after the second week and reduced further in subsequent days after this. This is in agreement with Deng *et al.* (2010) who observed a decrease in the expression of macrophage-derived cytokine transcripts in a human artery-SCID mouse chimera model, suggesting a decrease in activated macrophages within the artery wall.

However, in the adventitia of the same arteries, a slight decrease in GBP5 staining and an increase in the M2 markers CD163 and MRC1 was observed in the 4-7 days category. Unexpectedly an increase in adventitial ANKRD22 staining in the same category was found which paralleled M2 marker expression compared to GBP5 expression. Like the staining in the adventitia, staining within the media (Figure 5.6) and the intima (Figure 5.7) for all antibodies did not decrease in relation to an increased duration of prednisolone treatment. The effect on the different macrophage markers expression to glucocorticoids in these two layers seem to also be similar to the adventitia.

The media was found to have lower total scores for all markers. The low scores for arteries which had not been treated with prednisolone suggests that the low scores are not due to prednisolone treatment but may be due to a lower degree of macrophage infiltrate within the media. This would support the idea that

inflammatory cells infiltrate the media to a greater degree during the later phases of inflammatory cell infiltration as described by Hernandez-Rodriguez *et al.* (2016). A greater percentage of arteries would therefore be expected to have a smaller number of macrophages in the media compared to the adventitia and the intima.

Overall, there was no evidence that duration of glucocorticoid treatment before biopsy had any effect on expression of the novel markers. The similar response of the M1 marker ANKRD22 after 4-7 days of glucocorticoid treatment to the response of both M2 markers, in which an upregulation of the marker was seen, suggests ANKRD22 may be an important marker of the individual susceptibility to the effects of glucocorticoids.

For GBP5 expression in the different layers, the data is harder to interpret due to number of scores of zeros that were given for each layer.

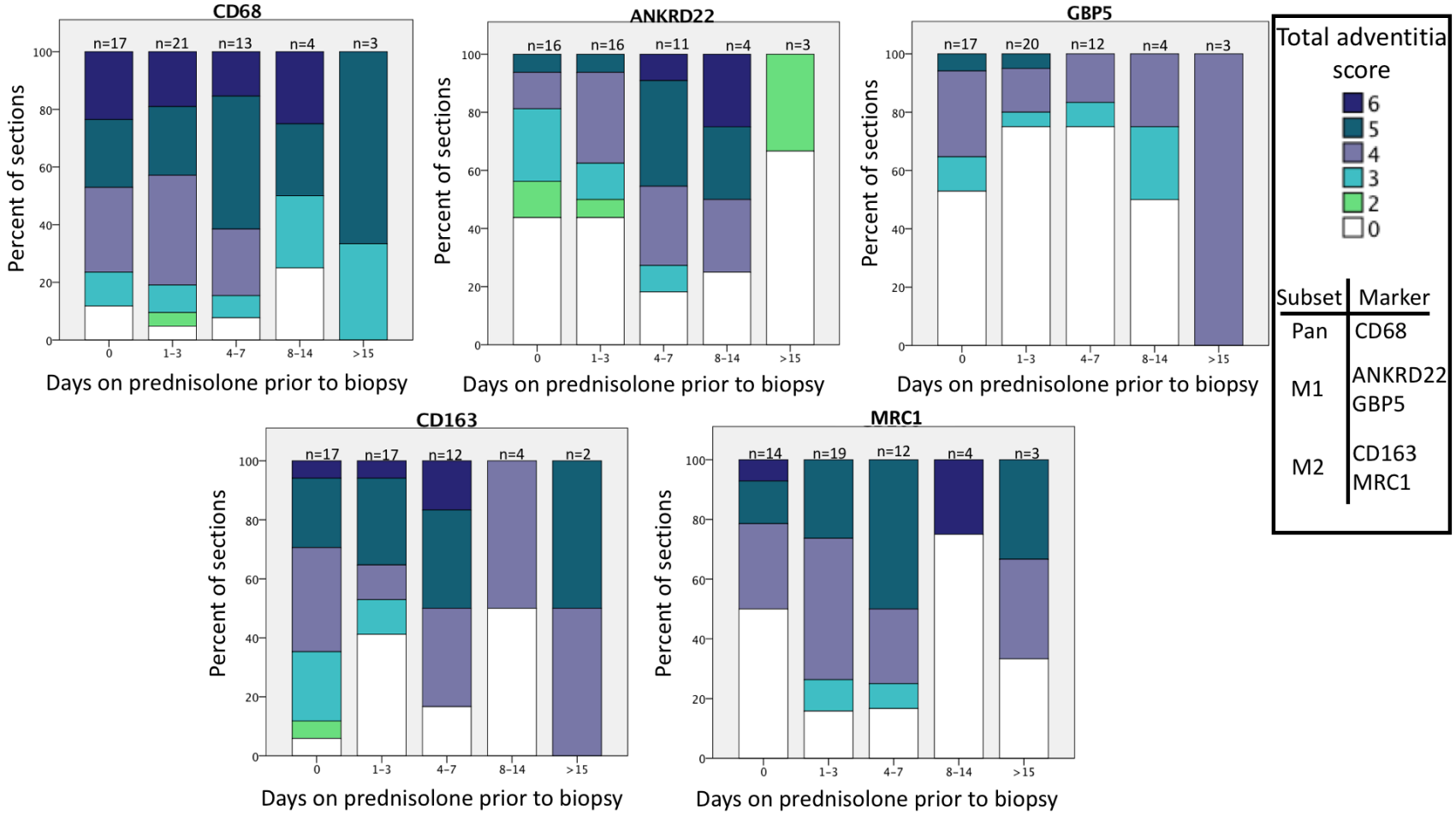


Figure 5.5: Total adventitia score for all macrophage markers after varying days of prednisolone use prior to biopsy.

The percentage of arteries with an adventitia total score after various days of prednisolone treatment prior to biopsy for markers CD68, ANKRD22, GBP5, CD163 and MRC1. A total score includes the sum of the locality and intensity score, of which the maximum score is 6.

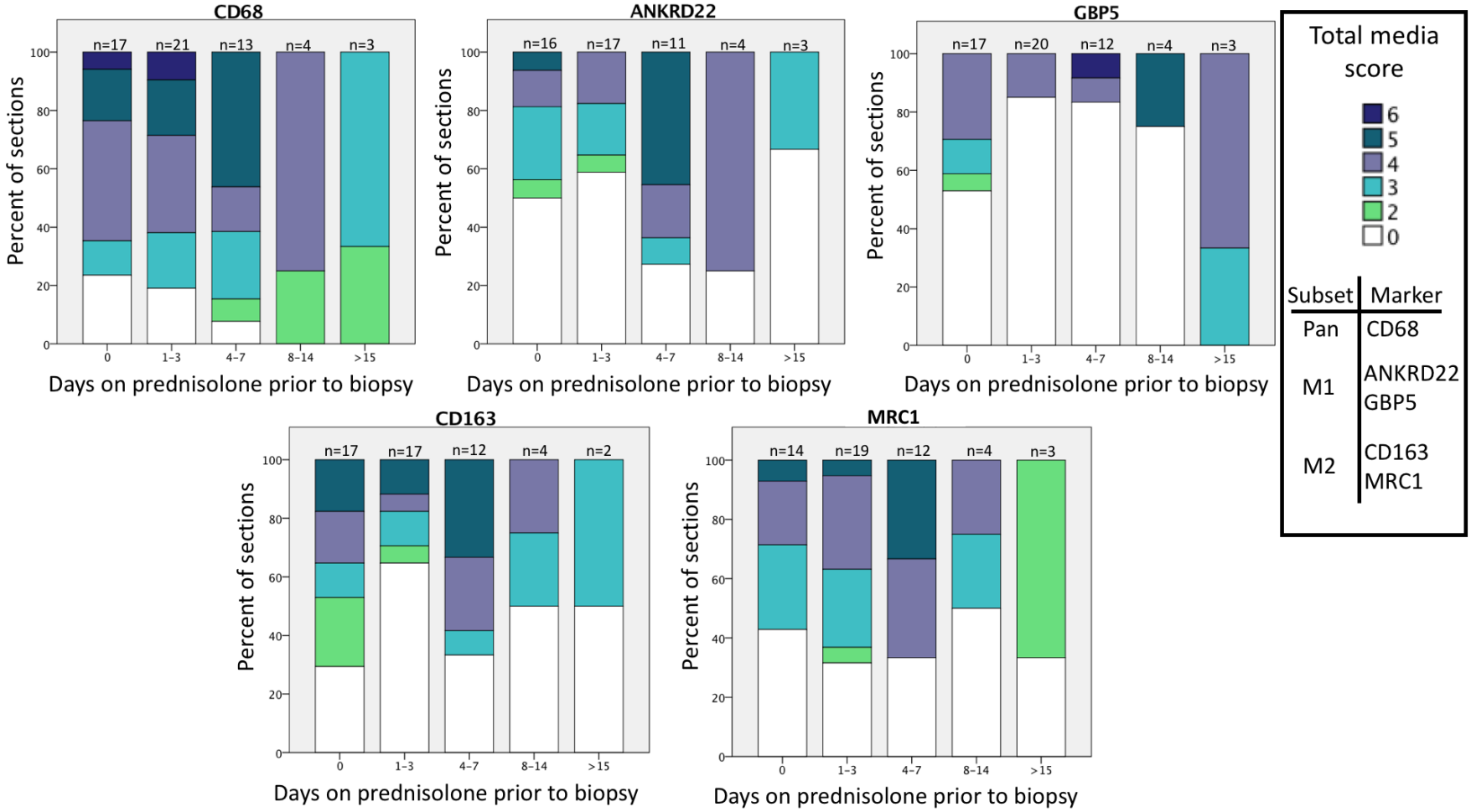


Figure 5.6: Total media score for all macrophage markers after varying days of prednisolone use prior to biopsy.

The percentage of arteries with a media total score after various days of prednisolone treatment prior to biopsy for macrophage markers CD68, ANKRD22, GBP5, CD163 and MRC1. A total score includes the sum of the locality and intensity score, of which the maximum score is 6.

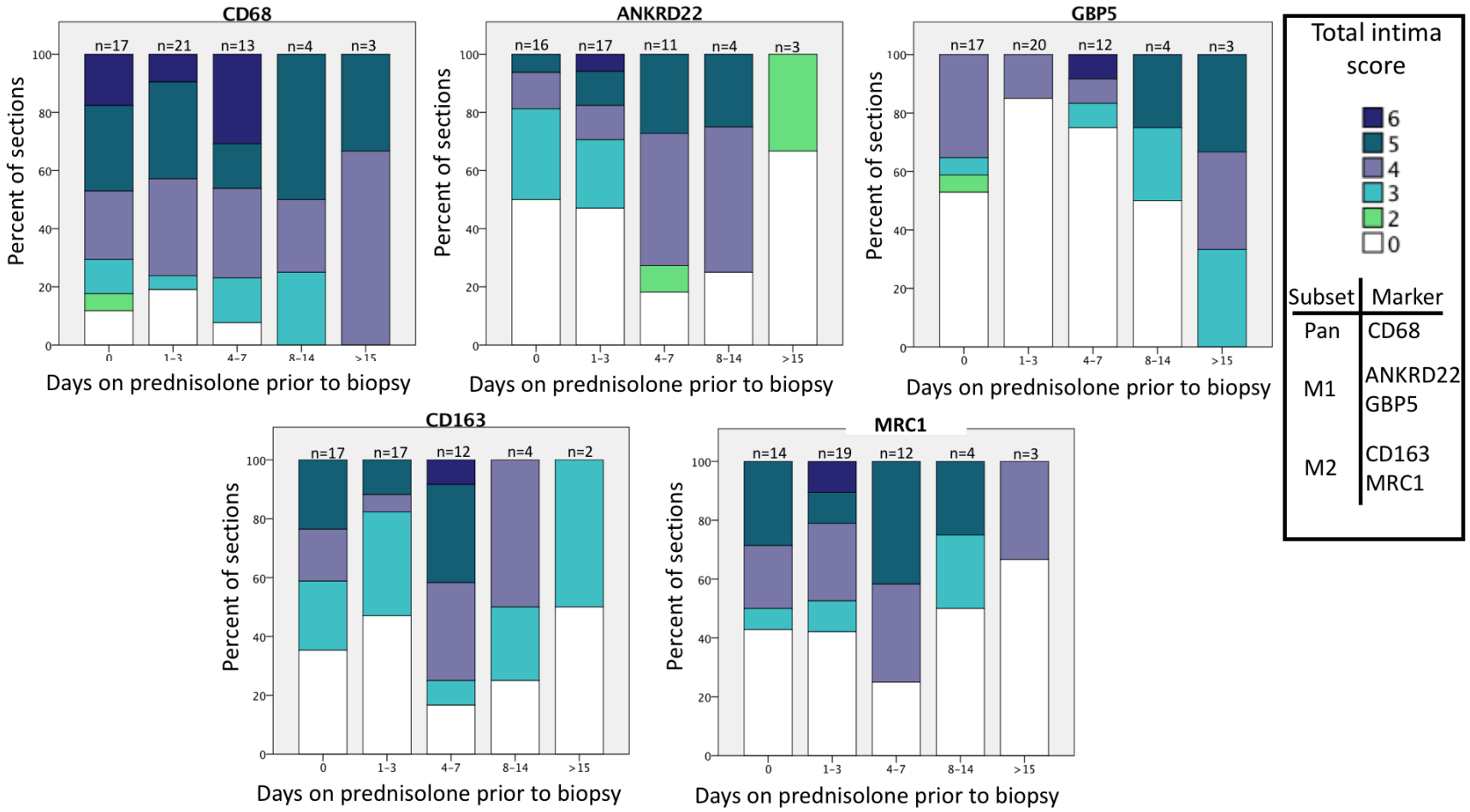


Figure 5.7: Total intima score for all macrophage markers after varying days of prednisolone use prior to biopsy.

The percentage of arteries with a media total score after various days of prednisolone treatment prior to biopsy for macrophage markers CD68, ANKRD22, GBP5, CD163 and MRC1. A total score includes the sum of the locality and intensity score, of which the maximum score is 6.

Overall, no markers were found to decrease in total staining score across all layers of the artery wall and therefore no cut-off for prednisolone treatment needed to be included in further analysis of marker scores, such as correlation with histological and histological features. Furthermore, some markers were found to increase in their total scores up to 4 to 7 days of prednisolone treatment. The lack of patients treated with prednisolone for longer than this means it is difficult to fully assess the effect of glucocorticoids on marker expression after 7 days of treatment. The wide range of total scores for each marker across all treatment groups and within treatment groups exemplifies the heterogeneity of macrophage infiltration, phenotypes and functions in GCA.

5.6 Macrophage phenotype heterogeneity in GCA arteries

As explained previously, GCA has been described as a heterogeneous disease, with patients presenting with different clinical manifestations, such as headache and visual loss (Koster *et al*., 2018; Janssen *et al*., 2008). In addition to clinical manifestations, there is wide variation in histological changes observed within the diseased artery (Hernandez-Rodriguez *et al*., 2016). The heterogeneity seen between patients may be due to variation in many immunological processes (Ciccia *et al*., 2015), but could also represent different stages of the disease process (Hernandez-Rodriguez *et al*., 2016). However, histological changes have been associated with clinical manifestations, such as a greater degree of intimal hyperplasia (of which tissue remodelling macrophages are thought to contribute to) and permanent visual features, such as vision loss (Makkuni *et al*., 2008). Therefore, to understand whether macrophage phenotypes may play a role in the heterogeneity that is found between patients in their histological appearance and therefore clinical manifestations, heterogeneity within the macrophage phenotype populations in GCA patients was investigated.

5.6.1 Novel macrophage marker staining in GCA artery biopsies

Firstly, macrophage markers were examined to determine the ability of the novel markers identified as disease-suitable (not altered by glucocorticoids) and subset-specific at the protein level in Section 4.6, to characterise macrophage populations in GCA diseased tissue. Serial sections were taken from arteries embedded in FFPE and were stained with markers in the order: CD68, ANKRD22, GBP5, CD163, MRC1 and TGM2 allowing M1 markers and M2 markers to be grouped together.

5.6.1.1 Marker specificity for macrophage phenotypes

Although it was found that the M2 marker TGM2 stained macrophages in GCA temporal artery biopsies, a high degree of non-specific staining of a range of different cell types, including strong staining of smooth muscle cells across all layers of the artery wall, made it difficult to identify macrophages from other staining across the different layers. It was decided, therefore, that TGM2 would not be used to characterise M2 macrophages in GCA artery biopsies. Novel M1 marker, ANKRD22, was also found to non-specifically stain endothelial cells and dendritic cells and thought to also stain monocytes.

5.6.1.2 Macrophage characterisation

Markers were found to stain the same artery at different locations and intensities, producing different overall patterns. Figure 5.8 shows serial sections cut from the same artery and markers were stained in the order: CD68, ANKRD22, GBP5, CD163 and MRC1. The different staining patterns found for each marker suggest that they may be able to identify different macrophage populations within the artery wall. Yet, markers which identify the same phenotypes were found to display different patterns of staining, both in terms of their staining intensity and locality, such as M1 markers ANKRD22 and GBP5, and M2 markers CD163 and MRC1. The reason for this difference in staining may be due to the ability of one marker to be more specific to their respective phenotype than another. In previous experiments, RNA and protein expression of GBP5 was found to be more specific to THP-1 derived M(LPS, IFNγ) macrophages compared to the expression of ANKRD22, which was also expressed in M(IL-10) THP-1 derived macrophages. Some markers were found to stain more macrophages and with greater intensity (Figure 5.8) such as macrophages stained in the adventitia by the M2 marker MRC1 compared to the pan-macrophage marker CD68, suggesting M2 macrophages may be increased in the adventitia. In comparison, CD163 and CD68 showed similar number and intensity of macrophage staining in the adventitia; however, CD163 staining intensity within the media and intima was much lower compared to CD68. Taking the results from the cell pellet staining in Table 5.1 into consideration, in which M(IL-4) showed decreased expression of CD68 and M(LPS, IFNγ) showed decreased expression of CD163, there may be less M2 macrophages and more M1 macrophages within the intima compared to the adventitia. Furthermore, metalloproteinases within the media and intima may cleave CD163 from the surface of macrophages, reducing the intensity of CD163 staining. In addition, markers for different phenotypes were observed to have a similar pattern of staining, such as the M1 marker ANKRD22 and the M2 marker MRC1. Staining of serial sections will also have an effect as more or less macrophages may appear as the sections are cut through the artery wall.

Overall these results point towards a trend for M1 macrophages locating to the media and intima and for M2 macrophages locating to the adventitia. Combining the markers of the different subsets and the same subsets seems to both show staining of different macrophage populations within the different layers. This confirms the spectral model of macrophage polarisation and may demonstrate different cytokine environments within the vessel wall. Dual staining of these markers would be useful to confirm these findings.

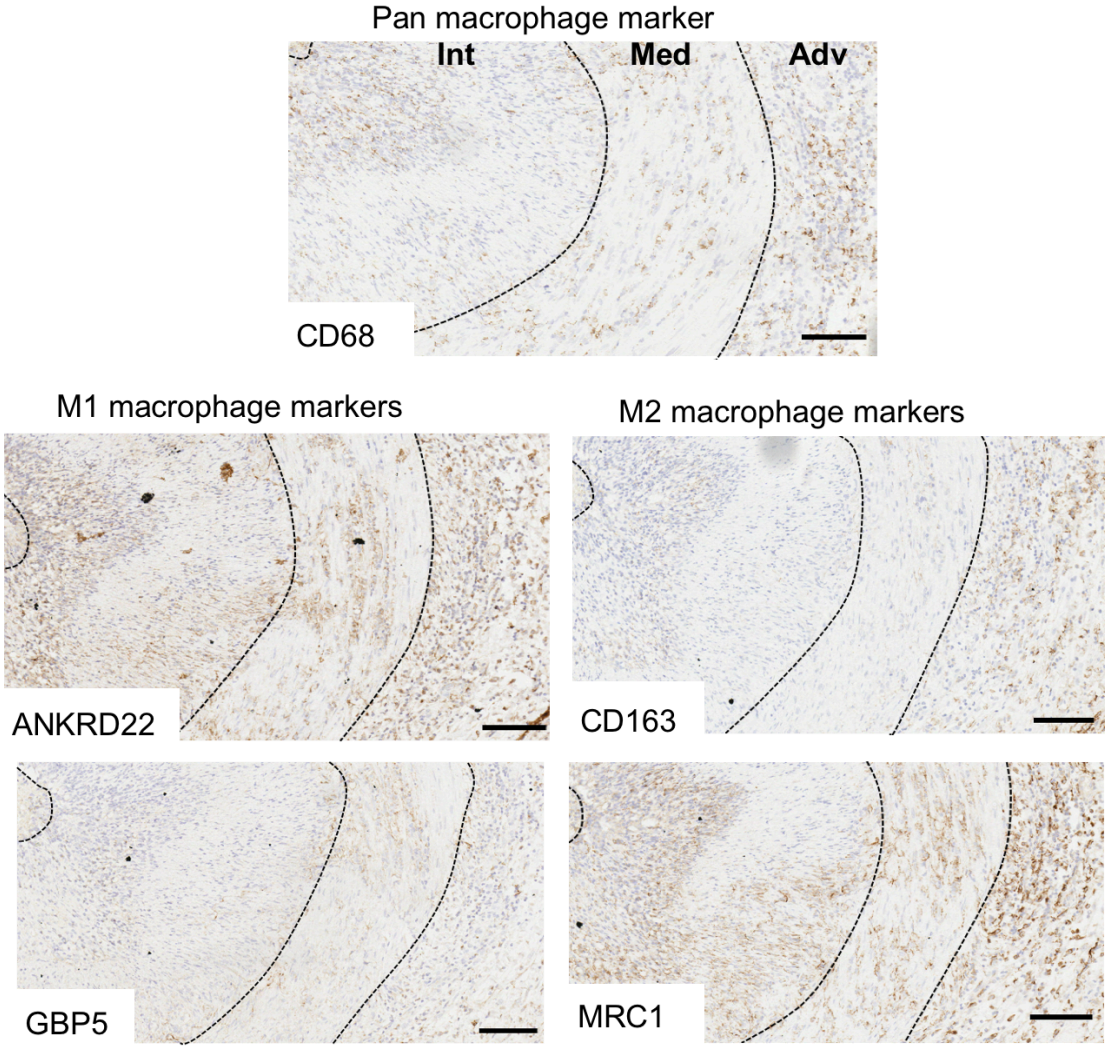


Figure 5.8: Macrophage marker staining of the different artery layers.

Serial sections of the same artery were stained with different macrophages markers to understand the locality and intensity of their staining, to help identify the location of macrophage phenotypes within the artery wall. Serial sections were cut and stained in the order: Pan macrophage marker CD68, M1 markers ANKRD22 and GBP5, and M2 markers CD163 and MRC1. Dotted lines provide an approximate guide to help distinguish the adventitia, media, intima and the lumen. Images were taken at a magnification of x200, scale bars indicate 50μm. Adv, adventitia; Med, media; Int, intima.

5.7 Antibody staining of giant cells

Giant cells were observed to express both M1 and M2 macrophage markers (Figure 5.9), however, staining intensities differed between these different macrophage markers. The M1 marker, GBP5 (Figure 5.9b), was found to stain giant cells at a greater intensity compared to CD68 and MRC1, whilst the M2 marker, CD163 (Figure 5.9c), was not expressed by the same giant cells. Different giant cells within the same artery section could be found to express markers at different intensities as shown for CD68 in Figure 5.9a, CD163 in Figure 5.9c and MRC1 in Figure 5.9d. This provides evidence that giant cells express the same markers as macrophages in GCA and that these cells can exhibit a mixed phenotype, an observation which has been made previously in biomaterial-induced giant cells which were found to express both pro- and anti-inflammatory macrophage markers (Barbeck *et al*., 2015) but has not been observed in GCA to my knowledge. Across the different cases, giant cells were found to express different macrophage markers, suggesting different giant cell populations occur in GCA arteries.

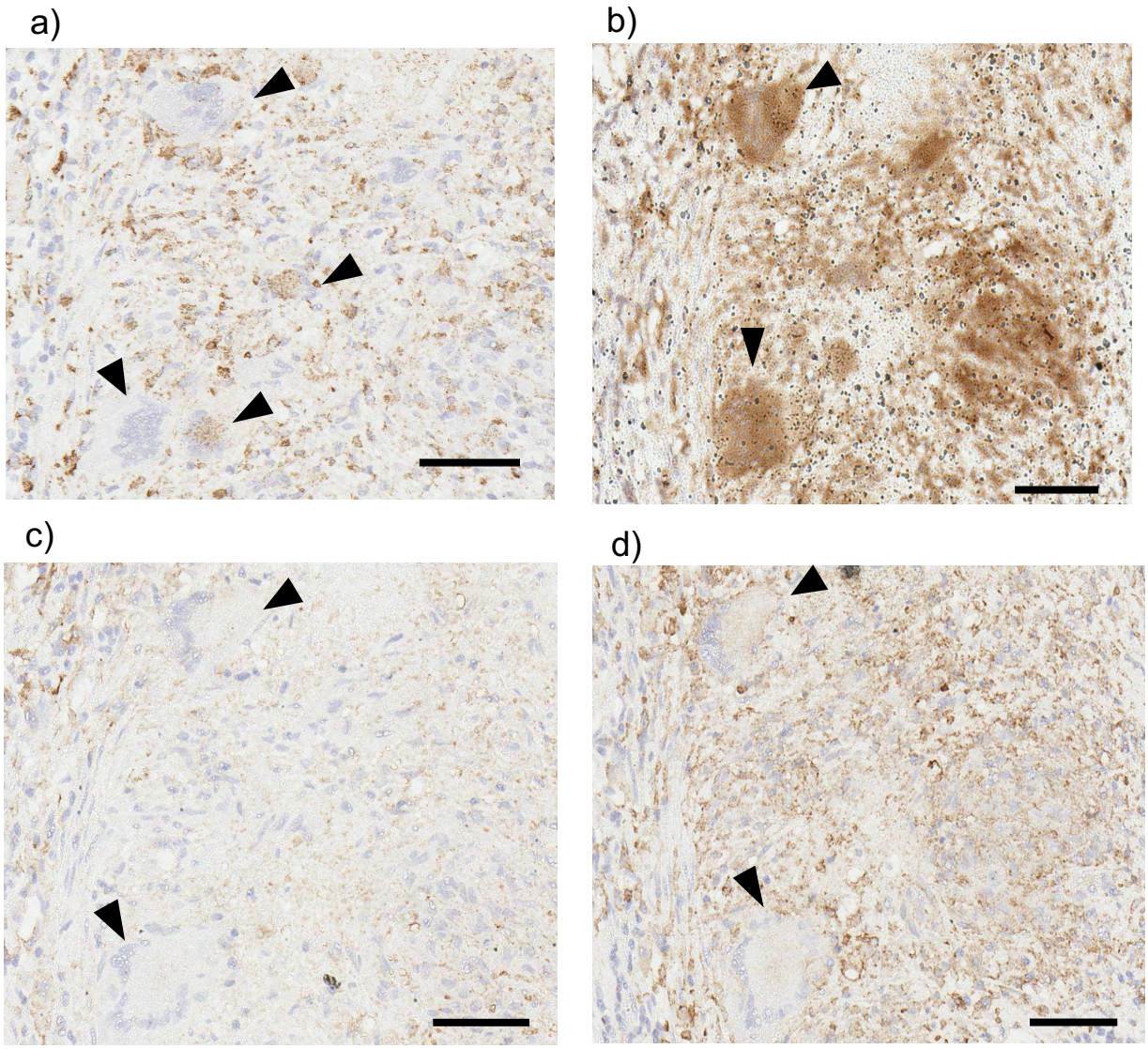


Figure 5.9: Giant cells stained with different macrophage markers.

Sections from the same GCA artery biopsy were stained with different macrophage markers, a) CD68, b) GBP5, c) CD163 and d) MRC1, to identify differences in their expression in giant cells, of which are indicated by arrows. Images were taken at a magnification of x200, scale bars represent 50μm.

5.8 Macrophage phenotypes within the artery layers

Carrying on from macrophage phenotypic characterisation, the heterogeneity of macrophage marker expression within the different layers of the artery wall was investigated due to the opposing inflammatory functions which occur within the adventitia and the intima. It is therefore hypothesised that a greater amount of inflammatory, M1 macrophages would be observed within the adventitia whilst a greater amount of wound healing, M2 macrophages would be found within the intima. Therefore, it was important to determine whether M1 and M2 macrophages were observed within these specific areas of the artery wall which are thought to correspond respectively with areas of inflammation and tissue repair. The location of each macrophage phenotype within each artery layer was investigated to identify the layer in which these different phenotypes were most likely to be found.

5.8.1 Number of macrophages and location of macrophage phenotypes

To identify the location in which most markers were found to be expressed, the number of macrophages expressing the different macrophages markers within the different areas of the artery wall was determined. As the total number of macrophages expressing CD68, ANKRD22, GBP5, CD163 and MRC1 was not analysed quantitatively, locality scores were used to provide an estimate of the number of macrophages within each layer. This was because it could be assumed an adventitia with a focal macrophage staining score would have less macrophages throughout the layer than an adventitia with a diffuse macrophage staining score. Calculation of the mean locality score for each marker in each artery layer was performed to give an indication of the number of macrophages expressing each marker in each artery layer.

Using all cases, the number of arteries with different locality scores (score from 0 to 3) for each artery wall layer, for each marker, were multiplied by their respective score, totalled and divided by the total number of arteries (59), giving a mean locality score for each artery layer, the maximum of which is 3. An example of this calculation using CD68 locality staining of the adventitia can be seen in Table 5.4. The mean locality scores for each marker for each layer of the artery wall, calculated using the process detailed in Table 5.4 can be seen in Table 5.5.

Table 5.4: Example of the mean locality score calculation using CD68 locality scores for the adventitia of all 59 arteries that were stained,

Locality score of adventitia	CD68 stained arteries (n,59)	CD68 stained arteries multiplied by locality score	
No staining (0)	5	0	
Focal staining (1)	4	4	
Multifocal staining (2)	29	58	
Diffuse staining (3)	21	63	
Total	59	125	125/59 = 2.11

The number of arteries with a locality score of no staining (0), focal staining (1), multifocal staining (2) and diffuse staining (3) in the adventitia was determined. The number of arteries was multiplied by their respective locality score and totalled. This was then divided by the total number of arteries to give the mean locality score for CD68 expressing macrophages within the adventitia.

Table 5.5: Mean locality score for all three artery layers, calculated using locality scores for all 59 arteries stained representing the number of macrophages expressing each marker within each artery layer.

Artery layer	CD68	ANKRD22	GBP5	CD163	MRC1
Adventitia	2.11	1.27	0.77	1.87	1.62
Media	1.38	1.04	0.60	1.23	1.23
Intima	1.95	1.25	0.68	1.30	1.23

The mean locality score for each marker within each artery layer was calculated to represent the number of macrophages expressing each marker within each layer.

As expected, CD68 showed the highest number of macrophages in all layers of the artery wall, providing further evidence of its use as a pan-macrophage marker in GCA temporal artery biopsies compared to CD163. Macrophages expressing either CD163 and MRC1 showed similar numbers within the media, whereas a higher number of CD163 expressing macrophages were found within the intima and an even greater number within the adventitia. When compared to M1 markers, a higher number of macrophages expressing MRC1 was seen for the adventitia in comparison to both ANKRD22 and GBP5, however, within the intima, there was a lower number of MRC1 expressing cells compared to ANKRD22 expressing cells. This refutes the hypothesis that a greater amount of M1 macrophages would be observed within the adventitia whilst a greater amount of M2 macrophages would be found within the intima. The number of GBP5 expressing macrophages was found to be lowest throughout all layers of the artery wall compared to all other markers, however, analysis of marker staining found a greater number of arteries in which GBP5 had not stained any macrophages. This may be due to GBP5 having greater specificity for its specific macrophage phenotype, but this lack of staining could be a disease-specific phenomenon due to the particular combination of cytokines and pathways which contribute to GCA.

Within the media, lower numbers of macrophages were found to express the different macrophage markers compared to both the adventitia and the intima. This would corroborate the suggestion by Hernandez-Rodriguez *et al.* (2016) in which greater infiltration of inflammatory cells is observed in the adventitia and intima prior to infiltration of the media, which is thought to occur during the later stages of the disease.

5.9 Patient heterogeneity in patterns of macrophage phenotype infiltration

In light of the variation between cases in terms of macrophage marker staining across the different artery layers, I decided that the pattern of locality and intensity staining of each macrophage marker for each artery layer would be evaluated, producing an overall locality and intensity pattern score.

5.9.1 Patterns of locality staining across the artery

The different locality staining scores for the three layers of the artery wall were individually analysed and collated, producing an overall locality pattern score for each case. The scoring system used in this chapter was adapted from the numerical score (0-3) to a character score as described in Table 2.5. The initial numerical score given to each layer of the artery wall was exchanged for an individual letter: no staining, 0 (N), focal staining, 1 (F), multifocal staining, 2 (M) and diffuse staining, 3 (D). The combination of these letters gave an overall locality pattern score, for example, an artery which was observed to have a focal score within the adventitia, a multifocal score within the media and a diffuse score within the intima, would have an overall locality staining pattern of FMD. The percentage of all patterns that were observed in the GCA cases for each marker were calculated.

It was found that, across all cases, a multifocal staining pattern [MMM] was the most common overall locality staining pattern for all markers: CD68 (19%), ANKRD22 (21%), GBP5 (19%), CD163 (19%) and MRC1 (21%) (discounting those arteries with no staining throughout the three layers (Figure 5.10)) yet great variation was seen in patterns observed across the cases of GCA. CD163 had the greatest degree of variation in locality patterns, with 21 different patterns of staining across the three different layers, whereas only 7 different patterns of staining for MRC1 across the different layers were observed. The range in the degree of staining locality in these patterns was also large, for example in a percentage of cases, a diffuse pattern of staining was seen across all layers of the artery [DDD] for markers CD68 (12%), ANKRD22 (4%), CD163 (9%) and MRC1(9%), whilst in other cases, staining of the adventitia only was observed, for example multifocal staining of the adventitia, but no staining of the media and the intima [MNN] was seen for markers CD68 (3%), GBP5 (4%), CD163 (8%) and MRC1 (11%).

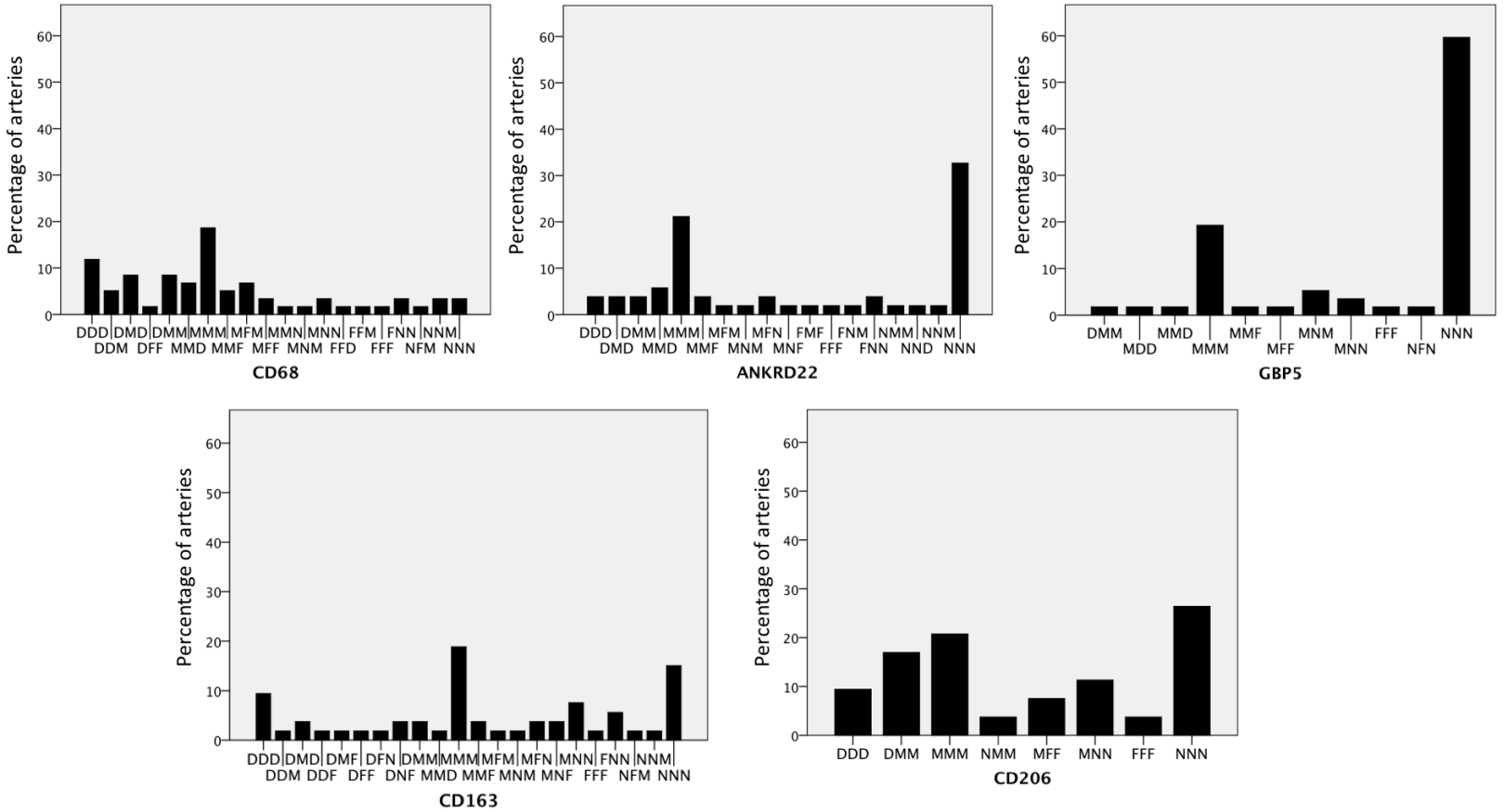


Figure 5.10: Patterns of locality staining across all three layers of the artery wall for different macrophages markers.

The range of different patterns of locality staining and the number of arteries observed with this type of locality pattern was analysed. Patterns were ordered from greatest inflammatory infiltrate to those with the least inflammatory infiltrate across the whole artery. Each locality score for the three artery layers is denoted by a letter: D, diffuse staining; M, multifocal staining; F, focal staining; N, no staining. Together this provided an overall locality staining pattern for each artery.

5.9.2 Patterns of intensity staining across the artery

In addition to the overall locality pattern scores, each intensity score was analysed individually for each layer of the artery wall to produce an overall intensity staining pattern score for each artery. As explained previously, the scoring system used in this chapter was adapted from the numerical score (0-3) to a character score as described in Table 2.5. The initial numerical score given to each layer of the artery wall was exchanged for an individual letter: no staining, 0 (N), mild staining, 1 (m), moderate staining, 2 (M) and strong staining, 3 (S). The combination of these letters gave an overall locality pattern score, for example, an artery with an intensity score of mild within the adventitia, moderate within the media and strong within the intima, would have an overall intensity pattern score of mMS.

The percentage of all patterns that were observed in the GCA cases for each marker were calculated. The most commonly occurring overall intensity staining pattern score across the GCA cases was moderate for all markers, across all artery layers [MMM]: CD68 (28%), ANKRD22 (21%), GBP5 (21%), CD163 (19%) and MRC1 (26%) (Figure 5.11) (discounting those arteries where no staining was observed across the whole artery). Despite the common overall intensity staining pattern for each marker, across the GCA cases, a wide variation of overall locality patterns was seen. CD163 and MRC1 were both found to have the greatest variation in overall intensity staining patterns, with 19 different patterns observed across the GCA cases. The marker with the smallest variation was GBP5, where only 10 different intensity patterns were observed across the GCA cases.

Together with the degree of pattern variation, the range of intensity staining across the artery layers was large, with a small percentage of arteries stained for CD163 (2%) observed to have strong staining within the adventitia and no staining within the media or the intima [SNN] whilst 2% of arteries had no staining of CD163 within the adventitia but was observed to have mild staining within the media and the intima (Nmm).

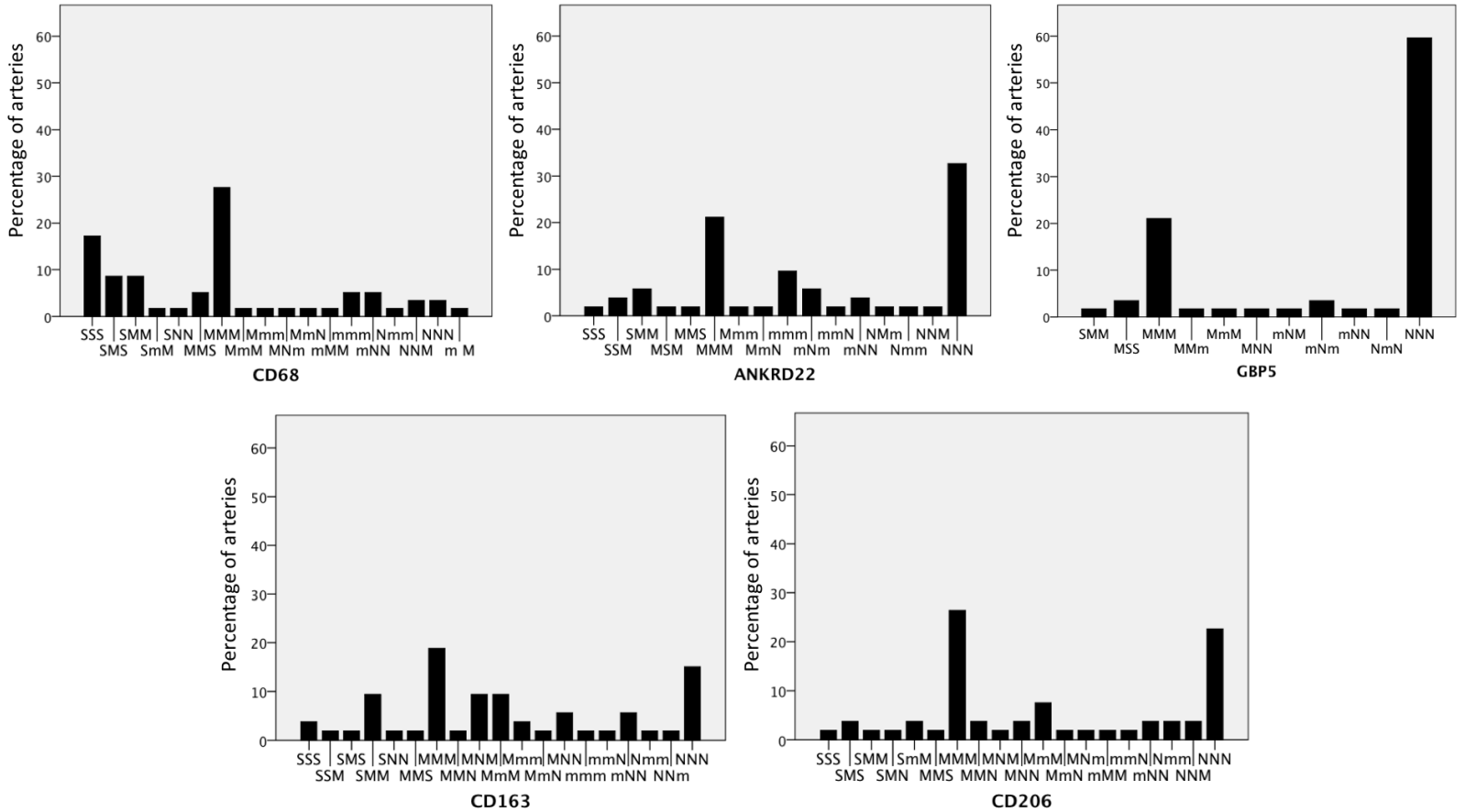


Figure 5.11: Patterns of intensity staining throughout all layers of the artery wall for the different macrophage markers.

The range of different patterns of intensity staining and the frequency of arteries observed with this type of locality pattern was analysed. Patterns were ordered from greatest intensity to those with the smallest intensity across the whole artery. Each intensity score for the three artery layers is denoted by a letter: S, strong staining; M, moderate staining; m, mild staining; N, no staining. Together this provided an overall intensity staining pattern for each artery.

The types of macrophage infiltration patterns observed across the artery and the degree of variation seen between patients supports the concept that different patterns of inflammation exist in GCA, and that there is great heterogeneity in inflammatory responses between patients. Different patterns of inflammation occurring in GCA diseased arteries have been described by Cavazza *et al.* with subsequent groups also confirming the finding of different patterns of inflammation across the artery wall in different patients (Hernandez-Rodriguez *et al*., 2016; Ciccia *et al*., 2015; Cavazza *et al*., 2014). These different patterns have been described to range from inflammation localised to the adventitia to pan-arteritic inflammation, which Hernandez-Rodriguez *et al.* (2016) have suggested to represent different phases of the disease response, rather than differences in inflammatory response to disease. The results above provide evidence for the occurrence of these patterns for the different populations of macrophages. Furthermore, the degree of intensity and severity of these patterns may vary between patients, as differences in locality and intensity of marker staining were observed in these different patterns of infiltration, from strong pan-arteritic staining to mild, localised staining.

5.10 Macrophages and histological features

An increased inflammatory infiltrate between patients has been associated with the severity of histological changes and clinical manifestations that are presented by patients with GCA (Hernandez-Rodriguez *et al*., 2016; Segarra *et al*., 2007; Nordborg and Petursdottir, 2000). These histological features include artery destruction such as the extent of media destruction as well as artery remodelling, such as the degree of intimal hyperplasia and therefore luminal occlusion as well as the degree of neovascularisation. Due to the heterogeneity found in the staining of M1 and M2 markers between patients, I analysed the staining of the different macrophage phenotypic markers to determine their association with different histological features: media destruction, neovascularisation and luminal occlusion. These features were scored for each artery in this study.

Spearman rank correlation was carried out for each marker, using the combination of their locality and intensity scores for each artery layer. Scores were combined, producing a composite score, as each score provided different aspects of the overall degree of macrophage infiltrate within the artery wall. This would therefore help understand whether macrophage phenotypes are associated with the occurrence of different histological features that are common in patients with GCA.

5.10.1 Macrophages and artery destruction

Smooth muscle cells which form the media are thought to contribute to intimal hyperplasia through their differentiation into migratory and proliferating cells which move towards the intima, resulting in the media thinning and its destruction and subsequent thickening of the intima. Smooth muscle actin-alpha (αSMA) was used to identify smooth muscle cells within the artery wall, which form the media layer, a standard NHS practice in some centres. Media destruction was scored in this study from 0 to 3, where 0 signifies an intact media, 1 signifies partial destruction of areas of the media, 2 signifies complete destruction of an area of the media layer and 3 signifies where the media shows complete destruction in more than one area. Examples of staining scores can be seen in Appendix 2. The relationship of media destruction to macrophage marker expression in the different artery layers is shown in Table 5.6. Spearman rank was used to identify which marker in which layer showed the strongest relationship with destruction of the media. Destruction of the media was not significantly associated with CD68 expression in any layer of the artery but was significantly associated with

expression of two macrophage markers in the media. (GBP5, n = 47, p = 0.038; CD163, n = 44, p = 0.048).

Table 5.6: Association of the different macrophage markers within the different layers of the artery wall and across the whole artery with the occurrence of media destruction.

Artery layer	Marker	n	r_s	Correlation of locality + intensity score (degree media destruction)
Adventitia	CD68	47	0.135	p=0.366
	ANKRD22	43	0.065	p=0.680
	GBP5	46	0.258	p=0.083
	CD163	44	0.125	p=0.419
	MRC1	43	-0.122	p=0.434
Media	CD68	47	0.249	p=0.091
	ANKRD22	43	0.136	p=0.383
	GBP5	46	0.307	p=0.038*
	CD163	44	0.300	p=0.048*
	MRC1	43	0.068	p=0.666
Intima	CD68	47	0.118	p=0.430
	ANKRD22	43	0.027	p=0.865
	GBP5	46	0.269	p=0.070
	CD163	44	0.157	p=0.310
	MRC1	43	-0.094	p=0.550

Spearman rank was performed, * denotes $p < 0.05$.

The association of medial GBP5 and CD163 expression, but lack of association of CD68 media expression, with media destruction, implies that the activation state of macrophages is important in the destruction of this layer rather than simply the presence of macrophages within the media. This would support the concept that M2 macrophages are thought to be involved in the destruction of the media via their secretion of matrix degrading enzymes, MMPs, as well as growth factors, such as PDGF, to initiate the migration of smooth muscle cells, which form the media, towards the intima, contributing to the process of luminal occlusion. Whereas M1 macrophages are thought to contribute to the destruction of the media via their secretion of ROS, which results in cellular apoptosis as well as activation of MMP enzymes. It may also indicate that macrophage marker expression in the media is potentially important for telling us about structural change of the artery, an important part of the biology of the disease.

The use of multiple significance testing in Table 5.6 means that there is a potential to generate spurious (false-positive) associations, but it provides a way to explore the data and quickly identify potential associations which require further investigation (Bender and Lange, 2001).

5.10.2 Macrophages and artery remodelling

Previous studies have associated a greater inflammatory infiltrate, identified using H&E staining, with increased intimal hyperplasia. Furthermore, the observation of neovessels in the media and the expanding intima of patients with GCA has been associated with macrophages due to their expression and secretion of the neovascular factor, VEGF. To further understand the role of macrophages in the process of tissue remodelling in GCA, the relationship between macrophage marker expression and the degree of luminal occlusion as well as the degree of neovascularisation was investigated. Expression of markers, using combined locality and intensity scores, in different artery layers and across the whole artery was used to determine whether different macrophage phenotypes were associated with these disease process.

The degree of luminal occlusion was scored using αSMA to identify fibroblasts within the intima from 0 to 4. The scoring system was adapted from (Makkuni *et al*., 2008) and used to score each artery in this study. No luminal occlusion was scored as 0, 1% to 25% occlusion of the lumen was scored as 1, 25% to 50% occlusion was scored as 2, a score of 3 identified those with 50% to 75% of occlusion and those arteries with 75% to 100% occlusion were given a score of 4. Examples can be seen in the staining atlas in Appendix 2. Spearman rank was

used to identify which marker in which layer showed the strongest relationship with luminal occlusion.

For neovascularisation, neovessels within the media and the intima were identified using CD31 and arteries were scored between 0 and 3, where no neovascularisation was scored as 0, mild neovascularisation was scored as 1, moderate neovascularisation was scored as 2, and strong neovascularisation was scored as 3.

No macrophage marker in any layer or across the whole artery was found to be significantly associated with luminal occlusion or with neovascularisation in patients with GCA.

5.11 Macrophage markers and progression of vascular inflammation

The concept that patterns of inflammation within the artery of GCA patients relate to different phases of GCA progression, identifies the degree of medial layer involvement in temporal arteries as an important indicator of disease severity and vascular inflammation. Hernandez-Rodriguez *et al.* (2016) categorised different inflammatory infiltrates observed in 285 H&E stained GCA artery biopsies into four different patterns of inflammation. The first two phases had little involvement of the media and therefore a lack of media destruction. In the last two phases, a high degree of inflammatory infiltration of the media and the intima was observed, where media destruction was found to occur. Media destruction is therefore hypothesised to be a key event in the pathogenesis of GCA allowing further spread and persistence of the inflammatory infiltrate. Macrophages are believed to play a key role in this via production of mediators, such as MMPs and ROS.

Correlation analysis of macrophage marker expression with histological manifestations in this study identified a relationship with media destruction (Section 5.10.1), but not with intimal hyperplasia or neovascularisation (Section 5.10.2), which are phenomena within the intima. The correlation of medial macrophages with media destruction, along with the finding that the degree of media destruction may indicate stages in the progression of inflammation, suggests an important role of medial macrophages in vascular inflammation and disease progression. However, the work done by Hernandez-Rodriguez *et al*. (2016) was limited to haematoxylin and eosin stained sections and did not specifically assess the role of macrophages. In the present study it was hypothesised that the different distributions of macrophages observed within the media (focal, multifocal, diffuse) might reflect different functional roles of macrophages and be reflected in differing patterns of marker expression across all layers of the artery.

The pattern of macrophage infiltration of the media, using CD68 medial locality scores identified in this study, was mapped onto the different stages of GCA described by Hernandez-Rodriguez *et al.* (2016). CD68 was chosen as this antibody was found to label the greatest number of macrophages in GCA temporal arteries in this study as seen in Section 5.4.1 and Section 5.8.1 and therefore macrophage subsets could be compared to the majority of macrophages observed within the artery wall. Table 5.7 describes the stages of disease progression detailed by Hernandez-Rodriguez *et al.* (2016) as well as their description of the involvement of the media in each stage. The different

locality scores given to CD68 expressing macrophages in the media in this study are also given to show how they might broadly relate to the different stages of disease progression.

Table 5.7: Stages of GCA progression within the artery wall described by Hernandez-Rodriguez *et al*. (2016), associating patterns of macrophage infiltration within the media (as assessed by CD68 locality score) with stages of GCA inflammation.

Stage	Pattern of inflammation described by Hernandez-Rodriguez *et al.* (2016)	Inflammation of the media described by Hernandez-Rodriguez *et al.* (2016)	CD68 locality staining of the media described in this study
1	Adventitial infiltration	Media is spared from infiltration	No staining
2	Early stages of invasion	Invasion of the media	Focal
3	Early stage of pan-arteritis	Macrophages activated in media and intima, small degree of media destruction	Multifocal
4	Late stage of pan-arteritis	Media destruction with widespread, persistent inflammation across all three vessel layers	Diffuse

Using CD68 locality staining scores given to macrophages in the media, each artery was categorised into either no staining, focal, multifocal or diffuse, serving as an indicator for the stage of vascular inflammation. The locality scores of CD68 expressing macrophages cannot be mapped exactly onto the stages described on H&E staining by Hernandez-Rodriguez *et al.* (2016) but they are an alternative way to capture the functional role of macrophages in the media. Macrophage polarisation within the whole artery was then assessed by identifying the greatest staining intensity observed in any layer of the artery wall for each marker. The same arteries were therefore categorised into none, mild, moderate or strong staining for each macrophage marker. The number of arteries with different intensities of staining for each macrophage marker was then compared to CD68 locality staining of the media. M1 markers ANKRD22 and GBP5 can be seen in Figure 5.12 and M2 markers CD163 and MRC1 can be seen in Figure 5.13.

As the media locality score increased it was found that the percentage of arteries with a strong ANKRD22 and GBP5 staining intensity also increased. This may suggest that the M1 macrophage subset is associated with the later stages of disease in which there is a greater degree of artery destruction. This would corroborate earlier findings in which GBP5 medial staining correlated with media destruction. Both M2 markers were observed to have strong staining intensity for all types of CD68 media locality staining, suggesting that M2 markers may not be associated with specific stages of the disease. However, the interpretation is limited by small numbers of cases with either no CD68 staining or diffuse CD68 staining within the media.

a)

CD68 media locality	ANKRD22 no staining in any layer, n/N (%)	ANKRD22 mild staining, n/N (%)	ANKRD22 moderate staining, n/N (%)	ANKRD22 strong staining, n/N (%)
None (N=7)	4 (57)	1 (14)	2 (29)	0 (0.0)
Focal (N=10)	5 (50)	2 (20)	2 (20)	1 (10)
Multifocal (N=26)	5 (19)	7 (27)	9 (35)	5 (19)
Diffuse (N=8)	3 (38)	2 (25)	1 (13)	2 (25)

b)

CD68 media locality	GBP5 no staining in any layer, n/N (%)	GBP5 mild staining, n/N (%)	GBP5 moderate staining, n/N (%)	GBP5 strong staining, n/N (%)
None (N=9)	6 (67)	0 (0)	3 (33)	0 (0)
Focal (N=9)	3 (33)	1 (11)	5 (56)	0 (0)
Multifocal (N=30)	19 (64)	3 (10)	7 (23)	1 (3)
Diffuse (N=9)	6 (67)	0 (0)	1 (11)	2 (22)

Figure 5.12: Comparison of highest intensity staining of M1 macrophage markers ANKRD22 and GBP5 to the different stages of GCA inflammation.

The highest marker staining intensity for **a)** ANKRD22 and **b)** GBP5 in each artery was compared to the medial locality score for the same artery to determine the relationship of the M1 macrophage phenotype with stages of vascular inflammation.

a)

CD68 media locality	CD163 no staining in any layer, n/N (%)	CD163 mild staining, n/N (%)	CD163 moderate staining, n/N (%)	CD163 strong staining, n/N (%)
None (N=8)	2 (25)	3 (38)	2 (25)	1 (13)
Focal (N=10)	1 (10)	0 (0)	6 (60)	3 (30)
Multifocal (N=26)	4 (15)	3 (12)	14 (54)	5 (19)
Diffuse (N=9)	1 (11)	1 (11)	5 (56)	2 (22)

b)

CD68 media locality	MRC1 no staining in any layer, n/N (%)	MRC1 mild staining, n/N (%)	MRC1 moderate staining, n/N (%)	MRC1 strong staining, n/N (%)
None (N=8)	3 (38)	2 (25)	3 (38)	0 (0)
Focal (N=10)	2 (20)	0 (0)	6 (60)	2 (20)
Multifocal (N=26)	5 (19)	3 (12)	12 (46)	6 (23)
Diffuse (N=9)	2 (22)	0 (0)	7 (78)	0 (0)

Figure 5.13: Comparison of highest intensity staining of M2 macrophage markers CD163 and MRC1 to the different stages of GCA inflammation.

The highest marker staining intensity for **a)** CD163 and **b)** MRC1 in each artery was compared to the medial locality score for the same artery to determine the relationship of the M2 macrophage phenotype with stages of vascular inflammation.

Due to the low numbers of arteries with different patterns of media locality staining, it was decided that arteries with none and focal CD68 media staining would be combined into a category in which there was no pan-arteritis, therefore relating to early stages of GCA development and inflammation in which macrophages are likely to be less activated. Arteries with multifocal and diffuse CD68 media staining would also be in combined into a category which pan-arteritis is observed with a high degree of inflammation, relating to late stages of GCA development in which macrophages are more likely to be activated. Using the Mann-Whitney U test, these categories were then compared to determine whether the different macrophage markers were more likely to be observed at early or late stages of GCA development. The results can be seen in Table 5.8.

The results showed that greater staining intensity of ANKRD22 was found in arteries in which pan-arteritis was observed ($p = 0.044$), and therefore it may be hypothesised that macrophages are being polarised towards a more destructive, M1 phenotype in the later stages of GCA. This also corroborates the finding that the M1 marker, GBP5 is associated with media destruction. The high number of arteries which showed negative staining for GBP5 may have reduced the statistical power in this study.

Table 5.8: Mann-Whitney U test to identify the relationship of macrophage marker expression with the different stages of GCA.

Macrophage marker	n	*p*
ANKRD22	51	0.044*
GBP5	57	0.388
CD163	53	0.799
MRC1	53	0.400

Mann-Whitney U test was performed, * denotes $p < 0.05$.

Overall, these results may suggest that ANKRD22 could be potentially useful in phenotyping macrophages which are associated with media destruction and the pan-arteritis pattern of inflammation. Therefore, the marker ANKRD22 was taken forward to examine its association with clinical features of GCA, such as permanent visual features and jaw claudication.

5.12 ANKRD22 and clinical features

Carrying on from the results of analysis into macrophage marker intensity in arteries at different stages of GCA, it was important to consider the clinical features associated with the M1 marker ANKRD22 in GCA patients. It has been suggested that a larger inflammatory infiltrate is associated with higher circulating levels of the pro-inflammatory cytokine IL-6 (thought to be secreted by M1 macrophages), and longer glucocorticoid treatment (Hernandez-Rodriguez *et al*., 2003; Hernandez-Rodriguez *et al.*, 2004a; Hernandez-Rodriguez *et al.*, 2002). Furthermore, higher levels of circulating IL-6 has been associated with a lower risk of ischaemic events, such as vision loss (Hernandez-Rodriguez *et a*l., 2003). There are however conflicting studies with regards to the association of the degree of inflammatory infiltration and ischemic events, such as vision loss (Hernandez-Rodriguez *et al.,* 2016; Breuer *et al*., 2013).

It was therefore important to understand whether the M1 marker, ANKRD22, thought to be associated with later stages of GCA (in which there is a greater inflammatory infiltrate that contributes to artery destruction), could be correlated to systemic and vascular inflammation.

5.12.1 Vascular inflammation

Total artery staining of ANKRD22, combining intensity and locality scores for each artery layer was correlated using Spearman rank with vision loss, jaw claudication, and the combination of fever, night sweats or weight loss. Analysis of these clinical features of vascular inflammation (Table 5.9) identified a negative association of ANKRD22 total artery staining with permanent visual features (n = 51, r_s = -0.312, P = 0.026). However, of these 51 cases, only 7 patients had permanent visual features, therefore there is a greater chance of producing spurious results and so these results should be interpreted with caution. No correlation was found between ANKRD22 and combined fever, night sweats or weight loss. There was also no correlation of ANKRD22 with jaw claudication.

Table 5.9: Correlation of the M1 marker ANKRD22 staining of arteries as a whole, with different vascular inflammatory manifestations

Vascular inflammatory manifestations	N	r_s	*p*
Permanent visual features	56	-0.312	0.026*
Temporary jaw claudication	51	-0.107	0.484
Fever, night sweats or weight loss	49	-0.107	0.463

Spearman rank, * denotes $p < 0.05$,

5.12.2 Systemic inflammation

The association of ANKRD22 with the later stages of GCA, in which there is a greater inflammatory infiltrate and the negative association of ANKRD22 with permanent visual features suggests ANKRD22 may be correlated with systemic inflammation. In this study, data on patient circulating levels of IL-6 were not available however clinically, systemic inflammation is determined by levels of circulating CRP and ESR (Watanabe *et al.,* 2016). Furthermore, the secretion of acute phase proteins such as CRP from the liver is induced by IL-6 (Watanabe *et al.,* 2016), therefore circulating levels of CRP are useful to correlate levels of circulating IL-6.

Spearman rank was used to correlate the total artery staining score of ANKRD22 with circulating levels of CRP and ESR. No associations however were found for ANKRD22 with these systemic inflammatory markers (Table 5.10).

Table 5.10: Correlation of the M1 marker ANKRD22 staining of arteries as a whole, with different markers of systemic inflammation

Systemic inflammatory marker	N	r_s	*p*
CRP	43	0.084	0.594
ESR	51	0.279	0.135

Spearman rank, * denotes $p < 0.05$

5.13 Prognostic value of markers

It has been proposed that the severity of the inflammatory response and the degree of systemic inflammation, and histological change within the artery of a patient, could help determine the duration of glucocorticoid treatment (ter Borg *et al.*, 2007). Macrophages ability to secrete a variety of cytokines, including IL-6, which has been implicated with a more severe inflammatory response and lower risk of ischemic complications, suggests macrophage markers could be prognostic. Subsequently, increased CD68 expression within the artery has been identified as a prognostic marker for the response to glucocorticoid treatment (Braun *et al.*, 2009). Furthermore, the evidence above to suggest the M1 macrophage marker, ANKRD22, is negatively correlated with visual features implies that the ANKRD22 marker could also have a prognostic value for those patients which are on prednisolone longer.

5.13.1 Time taken to reach 5mg of prednisolone

Locality and intensity scores were totalled for each layer to give an overall macrophage marker artery staining score which was used in the analysis. Spearman rank association tests between the macrophages markers and time to reach 5mg of prednisolone were carried out. The results can be seen in Table 5.11.

A negative association was found for the total MRC1 artery staining score (p = 0.015), therefore greater MRC1 staining associated with a shorter time for patients to reach 5mg of prednisolone. No association was found for any other macrophage marker. Due to increased IL-6 levels being associated with a longer glucocorticoid duration and increased inflammation, analysis of the total MRC1 artery staining score with the same vascular inflammatory manifestations and systemic inflammatory markers tested in Section 5.12 was performed using Spearman rank. This identified a negative association with fever, night sweats or weight loss (n = 50, r_s = -0.302, P = 0.033) however no association was found for any systemic inflammatory markers. Caution must be taken with the results seen here as the time to reduction of prednisolone to 5mg is not only dependent on severity of disease but on multiple factors such as treatment regimen and disease presentation.

Table 5.11: Association of macrophages markers with the time patients took to reach 5mg of prednisolone

Marker	n	r_s	*p*
CD68	40	-0.289	0.071
ANKRD22	35	-0.251	0.146
GBP5	38	0.049	0.770
CD163	34	-0.267	0.128
MRC1	34	-0.414	0.015*

Spearman rank, * denotes p <0.05

5.13.2 Time taken to stop prednisolone treatment

To understand the use of macrophage markers to identify patients who are more likely to stop the use of prednisolone, Spearman rank tests between total macrophage marker artery staining scores and the time it took patients to stop prednisolone was analysed (Table 5.12). No associations were found for any macrophage markers. This identifies that although MRC1 might be a useful marker to identify patients who will respond better to prednisolone treatment and be able to reach a low dose (5mg) sooner, it does not appear to be so useful for identifying those patients who will completely stop prednisolone treatment sooner.

Table 5.12: Association of macrophages markers with the time patients took to stop prednisolone treatment.

Marker	n	r_s	*p*
CD68	44	0.028	0.859
ANKRD22	39	-0.114	0.491
GBP5	42	-0.081	0.609
CD163	40	0.125	0.443
MRC1	39	0.101	0.542

Spearman rank, * denotes p <0.05

5.14 Conclusions

Analysis of macrophage marker staining identified CD68 as a marker that allowed the identification of more macrophages in GCA compared to CD163, a finding which corroborates a study into CD68 staining in 48 GCA arteries which observed CD68 was useful in identifying macrophages within the artery lesion (Wang *et al.*, 2017). Furthermore, CD68 staining was also found to be useful in distinguishing a positive diagnosis in indeterminate cases (Zhou *et al.,* 2009) and identifying patients more likely to respond better to prednisolone (Braun *et al.*, 2009). A number of groups had suggested CD163 is a more reliable and specific pan-macrophage marker than CD68 in other diseases and tissues, for example for tumour associated macrophages in classical Hodgkin lymphoma (Barros *et al.,* 2013; Vakkila *et al*., 2005; Lau *et al.*, 2004). This suggests that different tissues and diseases require the use of different pan-macrophage markers to identify general macrophage populations.

In contrast to Wang *et al.* (2017) who identified the greatest amount of CD68 staining within the adventitia and the media, the results from this study found macrophages expressing CD68 were more likely to be found within the adventitia and the intima, with fewer $CD68^{+}$ macrophages found within the media. In addition to this, macrophage phenotypic markers ANKRD22, GBP5, CD163 and MRC1 were found to be expressed in greater abundance within the adventitia and lowest within the media. The number of macrophages, however, was not determined quantifiably, due to the extent of macrophage infiltration throughout the artery, therefore abundance was estimated using mean locality scores. As a result of this, an underestimate in the degree of infiltrating macrophages within the different artery layers may have been made.

The variation in macrophage marker staining that was seen between arteries within the same case, highlights the heterogeneity that is seen in the inflammatory infiltrate of the artery wall at different sites, an observation also made by Hernandez-Rodriguez *et al*. (2016). A lack of marker expression within arteries may be a result of skip lesions as well as sections cut near to skip lesions which may have lower inflammatory infiltration. This highlights the importance that cases with only one section may not give a full representation of what is occurring within the artery wall and therefore more than one section should be used to determine the degree of inflammatory infiltrate. In this study, the section with the greatest CD68 total staining score in cases with more than one section was used, maximising the number of macrophages available for study within the section. This also reduces the possibility of missing macrophage-rich sections

due to skip lesions, although not all cases were found to have more than one section and so it was not possible in all cases to do this. The disadvantage of selecting sections with the greatest number of macrophages is the possibility of missing out on information about earlier stages of the disease that may have fewer macrophages. Although the artery with the greatest degree of CD68 staining was used for each case, it was found that other macrophage markers were not necessarily expressed in the same artery, therefore using CD68 as the deciding marker for the presence of macrophages does not necessarily mean that there will be M1 and M2 macrophages present within the same artery. This difference could be in result of lower concentrations of polarising cytokines required to upregulate these macrophage markers within the artery wall and also suggests that the markers used in this study do not stain all macrophage populations.

Overall, the use of macrophages markers CD68 and CD163 in the NHS pathology laboratories suggest that both markers might be useful in identifying total numbers of macrophages in GCA biopsies, however, the results from this study allude to a lack of prognostic information when used on their own. However, the use of CD68 for dual staining with the novel marker ANKRD22 may provide a way to improve the characterisation of the destructive, M1 macrophage subset, thought to contribute to artery wall damage, as ANKRD22 was also observed to stain cells other than macrophages in GCA artery biopsies.

The observation that M1 and M2 macrophage markers were expressed in all layers of the artery wall, rather than localised respectively to the adventitia and intima suggests macrophages are more likely to be a mixture of phenotypes rather than pure-M1 or pure-M2 macrophage populations. Macrophages which expressed M2 markers MRC1 and CD163 were found with macrophages which expressed M1 markers ANKRD22 and GBP5 within the adventitia, an area of pro-inflammatory cytokines such as IL-6 and IFNγ (Weyand *et al.,* 1996). These two M2 markers are induced respectively via cytokines IL-4 and IL-13, and IL-10 *in vitro*, yet within GCA arteries, these cytokines have been shown to be absent (Weyand *et al.,* 1997; Weyand *et al.,* 1994). Upregulation of CD163 and MRC1 in the adventitia along with M1 markers such as GBP5, may be due to polarisation of macrophages with IL-33. IL-33, a cytokine observed in the arteries of GCA patients (Ciccia *et al.,* 2013), was found to upregulate CD163, MRC1 and GBP5 in hMDMs in Chapter 4, as shown in Figure 4.9. This may be a reason why M1 and M2 macrophage markers are upregulated in the same arterial layers and dual staining studies would be useful to confirm whether GBP5 was co-expressed with CD163 and MRC1. Furthermore, studies into pulmonary hypertension

(another disease that occurs within the artery wall) using both *in vivo* and *in vitro* models, have suggested fibroblasts within the adventitia promote the polarisation of macrophages into a novel chronic, pro-fibrotic phenotype in an IL-4/IL-13 independent manner within this layer. This has been suggested to occur via secretion of IL-6 and subsequent activation of chronic inflammation and tissue remodelling-associated transcription factors, such as STAT3, resulting in the expression of the STAT3-associated marker MRC1, as well as CD163 (El Kasmi *et al*., 2014). Although the study performed by El Kasmi *et al.* is from a different disease setting, the greater abundance and stronger expression of CD163 and MRC1 markers within the adventitia of arteries supports the concept that M2 macrophages can be polarised within the adventitia without the requirement of the conventional IL-4/IL-13 cytokines in GCA. More recent evidence suggests that IL-4 is overexpressed at the protein level in inflamed GCA artery biopsies but is absent at the RNA level (Ciccia *et al*., 2015), although this work has not yet been reproduced, and the cells which produce the cytokine have not been identified. Furthermore, a subpopulation of CD163 expressing macrophage in psoriasis, a chronic Th1 and Th17 driven disease, have been identified as pro-inflammatory cells, releasing inflammatory molecules, IL-12, TNF and iNOS when exposed to an IFNγ microenvironment (Fuentes-Duculan *et al*., 2010).

The observation that giant cells within the artery wall co-express M1 and M2 macrophage markers, a finding also described by Barbeck *et al*. (2015), albeit in biomaterial-induced giant cells, suggests that these cells may perform both pro- and anti-inflammatory functions within the artery wall. It was found that GBP5 stained giant cells at a higher intensity than routinely used markers CD68 and CD163, making them easier to identify and therefore the use of GBP5 may help in making a diagnosis of GCA in those patients who have giant cells present. Furthermore, different giant cells within the same location were found to express these macrophage markers at different intensities, suggesting giant cells can specifically up-regulate and down-regulate macrophage markers and exist as different phenotypes, as suggested by Barbeck *et al.,* (2015) and Miron and Bosshardt (2017). This is supported by the ability of giant cells to secrete M1 associated, tissue destructive ROS (Rittner *et al.,* 1999b) and M2-associated mediators, such as MMPs (Rodríguez-Pla *et al*., 2005) along with the suggestion that they break down the internal elastic lamina (Cameselle-Teijeiro *et al*., 2013). Giant cells are also thought to be implicated in the development of the neointima, as they are able to secrete PDGF, a growth factor involved in the migration and proliferation of fibroblasts (Kaiser *et al*, 1999). Whether the mixed phenotype displayed by giant cells is in result of the macrophages engulfing other

macrophage phenotypes, and whether their ability to release a multitude of mediators is due to their mixed phenotype, is unknown.

This study supports the finding that arteries with different patterns of inflammation exist between patients with GCA, from layer-specific infiltration to pan-arteritis (Cavazza *et al*., 2014; Hernandez-Rodriguez *et al*., 2016). This study, however, identified a much greater variation in infiltrating macrophage patterns, albeit in low frequencies, which may be due to the small number of cases that were used. For example, not all arteries observed to have no CD68 macrophage staining of the media (and therefore assumed no involvement of the media) had staining limited to the adventitia. As shown in Figure 5.10 multifocal staining of CD68 was observed in the intima and adventitial layers of an artery in which there was no CD68 staining of the media, giving a locality pattern of MNM. The increased number of different patterns of infiltration may also be due to a pattern categorisation system used by Hernandez-Rodriguez *et al.* (2016) which is too simplistic and may incorporate or miss other types of infiltrating patterns. A greater number of cases would help to identify those patterns of macrophage infiltration which are more common and these different patterns identified in this study should be explored in future studies to understand how they fit into the patterns of GCA development described by Hernandez-Rodriguez *et al*. Differences in patterns of inflammation, both within cases and between cases, have been suggested to represent disease progression (Hernandez-Rodriguez *et al*., 2016), due to evidence of artery sections with healed arteritis and sections of the same artery with high degrees of inflammatory infiltrate. Initiation of GCA at different parts of the artery are unlikely to occur at exactly the same time and progress over the time period which strengthens the hypothesis that different phases of the disease can be seen in different sections of the artery. Furthermore, in atherosclerosis, the number of macrophages and their phenotype has been found to change during the different phases of disease progression and remission (Tabas and Bornfeldt, 2016).

These patterns of inflammatory infiltrate may also represent differences in the inflammatory response between patients and could be a result of the dysfunction in the regulation of the immune response which has been described in GCA patients (Wen *et al*., 2017) along with genetic factors which skew T helper cell polarisation as well as environmental factors. The finding that the intensity of M1 marker ANKRD22 staining is associated with arteries in which a pan-arteritis pattern is observed, as assessed by multifocal or diffuse patterns of CD68 staining of the media, suggests ANKRD22 is expressed at higher levels in arteries which have a greater inflammatory infiltrate. This suggests that macrophages are

being polarised towards a more destructive, M1 phenotype as GCA progresses and is suggestive of a bias towards M1 polarisation compared to M2 polarisation of macrophages in the later stages of GCA, where M1 macrophages are becoming more activated and contributing to the later phases of GCA, especially media destruction. Due to its novelty, there is little information on the function of ANKRD22 (Yin *et al*., 2017). *ANKRD22* has been found to be upregulated in IFNγ treated macrophages yet it's role in macrophage function has not been elucidated (Court *et al.*, 2017; Venner *et al*., 2014; Pereira *et al*., 2010). ANKRD22 has been found to upregulate E2F1 (Yin *et al.*, 2017), a transcription factor which regulates NFκB genes associated with a LPS-TLR4 pro-inflammatory response in macrophages (Lim *et al.*, 2007). The transcription factor has also been shown to suppress VEGF expression in a murine cardiac and murine skin model, preventing wound healing (Wang *et al*., 2016; Wu *et al.*, 2014). Furthermore, E2F1 has also been associated with upregulation of TLR3, a receptor which recognises viral RNA, corroborating findings in which ANKRD22 on PBMCs protects against RNA viruses via release of IFNα and IFNβ (Bin *et al*., 2016). These cytokines, however, have not been identified within the artery wall. Further studies into the role of ANKRD22 in IFNγ polarised macrophages, such as siRNA knockdown, would be important to understand the significance of this protein and the role it may play in host defence and wound healing.

T-cell subsets have been observed to be selectively controlled by NOTCH signalling pathways to enter the vessel wall (Wen *et al*., 2017; Piggott *et al*., 2011). Whether this selectivity of T-cell subsets causes downstream macrophage polarisation bias within the artery is unknown, but the entry of certain T-cell subsets which secrete specific cytokines that result in macrophage polarisation, could suggest a role of immune checkpoint dysfunction in macrophage polarisation and the bias towards an M1 phenotype in the later stages of the disease. The NOTCH signalling pathway has also been implicated in selectively promoting polarisation of human M1 macrophages while preventing polarisation towards an M2 phenotype (Pagie *et al.*, 2018) therefore the dysfunction of immune checkpoints in GCA may also promote M1 polarisation directly. Yet, not all arteries with diffuse CD68 staining within the media were found to have strong intensity staining of ANKRD22 and therefore this cannot be assumed for all patients. This further highlights the heterogeneity of the disease and the different processes and pathways involved in the pathogenesis of GCA.

Furthermore, the idea that glucocorticoid dosage and duration has an effect on the histological appearance of the artery, such as the loss of giant cells (Maleszewski *et al.*, 2017), may have implications on the patterns that are

observed between patients. In this study, CD68 staining was found to decrease in all layers of the artery wall over a period of 14 days suggesting the number of macrophages within the artery wall decreases in response to glucocorticoids (Section 5.5.2.1). It was also found however that the subset-specific macrophage markers ANKRD22, CD163 and MRC1 increased in response to glucocorticoids (Section 5.5.2.1), therefore glucocorticoids also alter the phenotype of macrophages within the artery wall and could therefore have implications on histological changes of the artery wall. Glucocorticoids have been associated with artery biopsies that display a “healed” histology after long-term exposure to treatment where changes to histology are apparent, such as fibrosis (Fauchald *et al*., 1972; McDonnell *et al*., 1986). Controversy exists regarding glucocorticoid effects on histology, as some studies show high dose glucocorticoids, over a period of a month, does not alter morphology or the inflammatory infiltrate (Hernandez-Rodriguez *et al*., 2016; Narvaez *et al*., 2007). The differences in the duration of glucocorticoids taken in these studies, however, may have implications in their observations. McDonnell *et al*. (1986) identified histological changes in arteries in which glucocorticoids were taken seven or more weeks prior to biopsy, while Hernandez-Rodriguez *et al.* (2016) only investigated histology of arteries in which patients had received glucocorticoids only four weeks prior to biopsy.

The association of ANKRD22 with a greater degree of inflammatory infiltrate along with the negative association of ANKRD22 with permanent visual features is consistent with the findings of a number of groups in which increased inflammation is associated with a lower risk of ischemic events, such as vision loss and stroke, in GCA patients (Cid *et al*., 2004; González-Gay *et al*., 2000). ANKRD22, however, was not found to correlate with markers of systemic inflammation indicative of IL-6 levels in GCA patients. IL-6, secreted by M1 macrophages, has been shown to be associated with the degree of the inflammatory response and patient resistance to glucocorticoid treatment (Hernandez-Rodriguez *et al*., 2002). Data on the levels of circulating IL-6 in patients was not available in this study, therefore CRP and ESR were used as related variables. A lack of association with systemic inflammation may be due to the low n numbers used in the test. A future study with a larger sample size and data on circulating IL-6 levels in GCA patients would be useful to determine the association of ANKRD22 and levels of IL-6.

The heterogeneity in presenting symptoms may have an impact on the statistical power to detect associations with other clinical features. The results should also be interpreted with great caution due to multiple significance testing increasing

the risk of spurious associations being identified. They however hint at ANKRD22 being more useful as a macrophage marker for the degree of inflammation in GCA arteries and may help predict downstream implications of this increased inflammation on clinical outcomes, such as increased risk of relapse (Hernandez-Rodriguez *et al*., 2002).

The results here did not identify an association with the extent of M2 macrophage marker staining and the degree of luminal occlusion and neovascularisation, processes thought to be promoted by tissue reparative M2 cells. Currently there is no standard approach to scoring of GCA artery biopsies (Bharadwaj *et al*., 2005). Differences in the way the scoring of artery staining is carried out may have implications on the outcome of data analysis. This may be why some differences in the relationship between inflammation and histological features was observed in this study. The staining atlas produced in this study (Appendix 2) for the scoring of the different antibodies to keep scoring consistent between arteries would allow others to replicate this work. Spearman rank tests were carried out by combining locality and intensity staining scores to produce a composite score. The method of scoring in this study therefore has weaknesses and without validation, can only be viewed as a relatively crude indicator of the amount of marker expression within an area of the artery wall. This produces imprecision in the measurement method, due to the limitations of immunohistochemistry as well as the semi-quantitative scoring system. This may partly explain the lack of significant associations found. Input from a biostatistician would have been instructive for developing a more informative and reliable scoring system.

The finding that patients with greater artery staining of the M2 macrophage marker, MRC1, were more likely to reach 5mg of prednisolone sooner suggests this marker is useful for predicting which patients will respond better to glucocorticoids. Together with the association of increased MRC1 artery staining with decreased fever, night sweats or weight loss, this also alludes to MRC1 being an indicator of a decreased inflammatory response. This is due to an increased frequency of fever being associated with an increased inflammatory response (Gonzalez-Gay *et al*., 2004) and less severe ischemic manifestations (Sun *et al*., 2016). Glucocorticoids were found to promote further upregulation of MRC1 on M(IL-4) polarised THP-1 cells as shown in Section 4.6.1 and MRC1 staining of the different artery layers was found to increase with an increased duration of prednisolone treatment (Section 5.5.1). This suggests that patients with greater MRC1 staining are more susceptible to glucocorticoid treatment. This may result in a quicker and therefore greater frequency of macrophages altering

their phenotype, upregulating MRC1 and switching towards a more anti-inflammatory, tissue remodelling M2c macrophage phenotype compared to patients less susceptible to the effects of glucocorticoids.

The lack of any macrophage markers showing an association with the time taken to stop prednisolone suggests that other factors are likely to be involved in the ability of those patients to stop prednisolone treatment sooner. For example, there may be variation in susceptibility of other immune cells to the effects of glucocorticoids, for example neutrophils, which have been shown to become more activated during glucocorticoid tapering and have been implicated with an increased incidence of relapse (Nadkarni *et al*., 2014). A future study with greater numbers of cases would allow to time-to-event analysis to be performed using Cox regression. As explained previously, patient outcomes such as permanent visual manifestations, jaw claudication and time taken to reach 5mg prednisolone and to cease prednisolone treatment are dependent on multiple factors not just severity of GCA which can have implications on the results from this study.

Overall, the results here suggest M1 macrophages are more highly implicated in the progress of GCA in the later stages of the disease and the histological changes that occur during this phase of the disease. It also highlights that phenotyping M1 and M2 macrophages is important for understanding the different phases of GCA and ANKRD22 may be a suitable marker for identifying M1 macrophages. Furthermore, the findings of this study suggest MRC1 might be a potential marker to identify those patients with a decreased inflammatory response and further studies are required to confirm or refute the finding that MRC1 expression is associated with a good prognosis as measured by reaching 5mg of prednisolone in a shorter time.

Chapter 6. Discussion

6.1 Summary of results

A THP-1 macrophage model system was developed which allowed for the polarisation of macrophages into subsets that closely resembled human M1 and M2a macrophages. End-point PCRs showed their upregulation of published subset-specific RNA transcripts. Differences in cell culture procedure, such as PMA concentration and duration of cytokine exposure, were found to have a large impact on the expression of macrophage markers. *In vitro* experiments performed using the THP-1 cell model verified the expression of both published markers and novel markers identified from RNA-Seq datasets, in their respective macrophage phenotypes at the RNA level. Corroboration of expression of novel markers in polarised hMDMs identified large variation in marker expression between human donors compared to that seen in THP-1-derived macrophage subsets, with markers showing greater specificity for THP-1-dervied macrophage subsets compared to hMDMs. Verification of RNA expression at the protein level however identified candidate markers. Analysis of candidate marker expression in different macrophage phenotypes in response to glucocorticoids was required prior to their use for characterising macrophages in GCA temporal artery biopsies. Expression of M1 macrophage markers were found be unaffected by glucocorticoids, remaining specific for THP-1 derived M(LPS,IFNγ) macrophages. The M2a marker MRC1 was found to be upregulated in M(0) macrophages and further upregulated in M(IL-4) whilst TGM2 was upregulated in M(LPS, IFNγ) macrophages on glucocorticoid exposure, thus becoming non-specific.

Macrophages in temporal artery biopsies were characterised into M1 macrophages using markers ANKRD22 and GBP5 and M2 macrophages were characterised using CD163 and MRC1 markers. CD68 and CD163 are both used in routine histopathology laboratories, particularly CD68, which was used as the reference marker in my analysis. CD68 was the most useful marker for identifying the majority of macrophages in GCA and was therefore used as a pan-macrophage marker. Expression of all markers was observed within each artery layer. The number of macrophages expressing each marker was found to differ between the adventitia, media and intima and heterogeneity was also seen in the locality and intensity of expression between the two M1 phenotype-specific macrophage markers and between the two M2 phenotype-specific macrophage markers. In general, there was a greater number of M2 marker-expressing

macrophages than M1 marker-expressing macrophages within all layers of the artery wall. The most common pattern of macrophage infiltration was multifocal with moderate intensity staining and this was observed for each marker in all three artery layers, however great inter-individual heterogeneity in the patterns of macrophage infiltration was observed. Although each layer had a greater amount of M2 macrophages than M1 macrophages, a higher intensity of ANKRD22 macrophage staining was observed in arteries with a greater inflammatory infiltrate suggestive of an imbalance in macrophage activation in the artery wall biasing towards increased activation of M1 macrophages but was not observed for M1 marker GBP5. Furthermore, GBP5 macrophage staining was significantly associated with media destruction. Associations of marker staining with the time it took patients to reach 5mg of glucocorticoids and to stop glucocorticoids identified the M2 marker, MRC1, as an indicator of patients who might respond better to initial glucocorticoid treatment as indicated by reaching a dose of 5mg prednisolone earlier. Further analysis negatively associated MRC1 with occurrence of fever and therefore this may be a potential marker of a lower inflammatory response. No markers were found be associated with patients most likely to stop glucocorticoids sooner.

6.2 THP-1 cell line as a model for macrophages

THP-1 cells are a primary cell line used to study human macrophages and were used in this study as they were considered to represent human MDMs more closely than any other monocytic cell line (Maess *et al*., 2014; Chanput *et al*., 2014) and provide a less variable, longer-lasting tool for studies into macrophage biology. The caveat to using immortalised cell lines like the THP-1 cells is their cancerous origin and therefore their genetic defects that for example, allow them to continuously replicate and which may implicate downstream signalling pathways.

6.2.1 Mimicking of polarised human MDMs

Macrophages are highly plastic cells which enables them to switch between phenotypes (Mantovani *et al*., 2004; Stout and Suttles, 2004; Stout *et al.*, 2005). This is an important issue when considering the use of PMA and its ability to induce an M1 phenotype prior to the addition of any M1 polarising cytokines. The inclusion of a rest after PMA treatment allows those non-specifically polarised macrophages to revert back to a resting macrophage-like cell (M(0)), which more closely resembles a hMDM prior to polarisation (Daigneault *et al.,* 2010). It is important to include a rest period of sufficient length to remove the bias of PMA, a stimulant which induces downstream PKC signalling and may therefore upregulate IFNγ-associated M1 genes (Radzioch and Varesio, 1988). Furthermore this “resetting” of macrophage cells enables more defined polarisation to be obtained after cytokine treatment, as recently described by Lund *et al.* (2016). Their investigation into the effect of rest on THP-1 polarisation using LPS identified that a rest period of over 24 hours was required, and indeed optimal, in producing macrophages exhibiting minimal pro-inflammatory characteristics induced by PMA. Yet, the effect of a rest period has also been suggested to revert macrophage-induced THP-1 cells towards their initial proliferative, monocytic phenotype when using PMA at a concentration between 6 (3.7ng/mL) to 30nM (18.5ng/mL) (Spano *et al*., 2013).

The findings of this study show each step of the THP-1 cell culture protocol has implications on the polarisation of THP-1 derived macrophages and the upregulation of markers. This impacts the ability to compare results from different THP-1 macrophage model studies which use different protocols for the maturation of macrophages from THP-1 cells. Inconsistences include PMA concentration (Shiratoi *et al.,* 2017; Buckley *et al.,* 2016; Genin *et al.,* 2015) and duration (Shiratori *et al.,* 2017; Zhong and Yi, 2016; Buckley *et al.,* 2016) as well

as use of rest periods between PMA treatment and cytokine exposure (Shiratori *et al.,* 2017; Buckley *et al.,* 2016; Genin *et al.,* 2015).

THP-1 derived macrophages, although optimised to resemble hMDMs as closely as possible, were found to upregulate some markers at the transcript and protein level differently to hMDMs in this study. This implies that THP-1 cells, although they have the ability to be differentiated into different phenotypes, cannot be used as a complete substitute for hMDMs when used for studies into macrophage biology and should therefore always be compared to hMDMs. A similar approach to using THP-1 cells as a cell model has also been described by Tedesco *et al*. (2018). THP-1 derived macrophages have previously been shown to respond to stimuli less effectively than hMDMs (Tedesco *et al.*, 2018; Maess *et al*., 2014). The reasons for such differences between THP-1 derived macrophage and hMDMs could be a result of variations in signalling pathways between the cell line and primary cells, especially as THP-1 cells are a cancerous cell line. Levels of activation may also vary in hMDMs due to genetics, environmental factors and infections. A major advantage of the THP-1 cell line is that it can be used to provide an unlimited number of cells for experimental controls which can be challenging when using primary cells and due to less variability between cells, cell lines also enable greater comparison between studies.

Overall, this model can be used confidently to produce different macrophage phenotypes which mimic human MDMs closely, and they can provide a tool to understand basic macrophage biology, such as responses to different stimuli and identifying markers that different phenotypes express. This model enabled *in vitro* studies into macrophage polarisation and phenotypic marker expression that could be transferred to *in situ* studies, allowing the identification of macrophage subsets in GCA artery biopsies.

6.3 Macrophage heterogeneity

6.3.1 The microenvironment in GCA arteries

The abundance of a wide range of different inflammatory mediators in GCA, and the clear topographical arrangement of these mediators, as well as different cell types within the layers of the artery wall, points to a complex microenvironment that may lead to different macrophage phenotypes found within these three layers. My study hypothesised, in light of the different mediators and stimuli, as well as the different histological changes which occur within the different layers of the artery wall, that macrophages could be characterised into destructive, M1 and tissue reparative, M2 macrophages within the different artery layers. Furthermore, it was also hypothesised that these changes in histology could be explained by a disparity in the ratio of these macrophage phenotypes, which is seen in a number of diseases including rheumatoid arthritis (Fukui *et al*., 2017), atherosclerosis (Feig *et al*., 2012) and chronic obstructive pulmonary disease (Shaykhiev *et al.*, 2009). In my study it was found that both M1 and M2 macrophage markers were observed across all layers of the artery with a greater number of M2 marker expressing macrophages than M1 expressing macrophages in each artery layer. The intensity of destructive, M1 macrophage marker expression however, was found to be associated with a greater inflammatory infiltrate in which greater media destruction is observed (Hernandez-Rodriguez *et al*., 2016). Although ANKRD22 did not correlate with media destruction, expression of the M1 marker GBP5 within the media was found be significantly associated with destruction of the media. Performing dual staining of ANKRD22 and GBP5 would be useful to understand the co-expression of these two M1 markers on M1 macrophages and how they may differ in their expression on different macrophage populations. Together this suggests a bias towards greater M1 activation in the advanced stages of inflammatory infiltration of the artery wall, and therefore it could be hypothesised that like other diseases, increased tissue damage may be attributed to a bias towards greater M1 activation

Additionally, identifying macrophage markers which do not alter their expression in response to glucocorticoids was an important aspect of this study. These markers provided a more robust approach to characterising macrophages in GCA arteries which almost uniformly will have been treated with glucocorticoids prior to their biopsy.

6.3.2 Spectrum of polarisation

The plastic nature of macrophages (Stout *et al.*, 2005), and the idea that macrophage polarisation is a spectrum (Sudan *et al.*, 2015; Xue *et al.*, 2014; Mosser and Edwards, 2008), makes identifying M1 and M2 macrophages difficult. It is now thought macrophages can adopt a mixed phenotype in response to different stimuli (Bystrom *et al*., 2008) and in response to different disease settings, for example in atherosclerosis (Finn *et al*., 2012) multiple sclerosis (Vogel *et al.*, 2013b) and cancer (Pettersen *et al*., 2011). The microenvironment within GCA artery biopsies is composed of an array of cytokines, including IFNγ, TGFβ, IL-1β and IL-6 (Weyand *et al.,* 1997; Weyand *et al.,* 1996), all of which have been associated with the polarisation of macrophages into different phenotypes (El Kasmi *et al.*, 2014; Martinez *et al.,* 2008). The concept of M1 and M2 macrophages polarised *in vitro* are now thought of as extremes of this spectrum, suggesting that macrophages which only express either M1 or M2 markers are unlikely to be found within the artery wall.

It could be assumed therefore that due to the range of polarising cytokines found within the artery wall of GCA patients, mixed phenotype macrophages, co-expressing different markers, are highly likely to be found. The overexpression of IL-33 and the finding that it upregulates both M1 and M2 markers *in vitro* in hMDMs (Figure 4.9) supports the concept of mixed phenotypes. The likelihood that both M1 and M2 markers are found in all artery layers therefore is high, as evident from the immunohistochemistry results. Similarly, opposing processes in atherosclerosis were initially thought to be in result of M1 and M2 polarised macrophages (Moore *et al*., 2013; Chinetti-Gbaguidi *et al.*, 2011). Investigations into macrophage biology in this disease setting, through cell culture of human monocytes, and qPCR and immunohistochemistry of human atherosclerotic plaques have identified novel haemoglobin-stimulated M(Hb) macrophage phenotypes, distinct from M(IL-4) macrophages, in areas of haemorrhage and angiogenesis, specific to atherosclerotic lesions which express CD163 and MRC1 and contribute to the progression of atherosclerosis (Finn *et al*., 2012). The increased neovascularisation of GCA arteries and the identification of CD163 and MRC1 expression in GCA arteries suggests further experiments should be performed to determine whether M(Hb) macrophages are observed within the artery wall of GCA patients. The co-localisation of different phenotype-specific markers and the concept that different disease patterns of GCA are due to differences in the phase of the disease process (Hernandez-Rodriguez *et al*., 2016) suggests macrophages within the different artery layers could be switching between phenotypes. Therefore macrophages may co-express different

phenotypic markers and functions in response to a changing environment as observed by macrophages, for example during the process of tissue repair (Lucas *et al.*, 2010; Novak and Koh, 2013; Kussell and Leibler, 2005; Martins *et al*., 2017). Furthermore, the observation that different giant cells were found to express different mixtures of M1 and M2 macrophage markers supports the concept that these markers can be expressed on the same cell. Giant cells may therefore be able to perform different functions depending on the markers they express. Although experiments were carried out to identify markers unaffected by glucocorticoids to limit the effect of glucocorticoids on the identification of macrophage subsets in GCA arteries, the different glucocorticoid dosages and durations may have implications on the frequency of macrophages switching towards a more M2c macrophage phenotype. This does not mean the use of the markers chosen in this study are not useful, but suggests that the more markers that can be used simultaneously to identify macrophages, the more information that can be obtained to understand their phenotype, function and role.

In general, it is difficult to gain a true picture of macrophage phenotypes due to their highly plastic nature and their ability to express a multitude of markers which belong to a range of phenotypes. The markers identified in this study in combination with other macrophages markers, both published and novel, will help to expand our knowledge about these important and highly diverse immune cells. Furthermore, the use of new technologies, such as single cell analysis and multiplex immunohistochemistry staining techniques will provide a more detailed understanding of macrophages in GCA arteries.

6.3.3 Heterogeneity between individuals

The variation in the patterns of macrophage marker staining observed between macrophages within the artery wall as well as between individuals with GCA identified in this study suggests there are differences in the way patients respond to the different stimuli within the different disease environments. This is further supported by the finding that artery expression of MRC1 may be useful to determine those patients who are more likely to respond better to initial glucocorticoid treatment.

A genetic element to differences in the way macrophages react to stimuli have been found in inbred mouse strains, with differences in the way transcription factors result in downstream signalling and activation of macrophage genes, through genetic variation of enhancers and other genomic regulatory elements (Heinz *et al*., 2013). GWAS and Immunochip studies of GCA patients have

implicated major pathways in the predisposition to GCA (Carmona *et al.,* 2017; Carmona *et al.* 2015). One pathway included the hypoxia-inducible factor-1 (HIF-1) (Carmona *et al.,* 2017) and its associated genes, including *VEGFA, MMP9* and *IL6*, previously identified in candidate gene studies (Carmona *et al.*, 2014) and are known to be expressed by macrophages in GCA. Furthermore, *in vitro* experiments using monocytes from different atherosclerosis patients identified individual variability in the way they responded to different stimuli (Orekhov *et al*., 2015). This was a similar finding to the results in this study, where upregulation of markers at the protein level was found to vary considerably between the macrophages isolated from different human donors in response to the same stimuli.

Inter-individual variability may therefore be found in the ability of macrophages to polarise into different phenotypes, and as such, may be the reason different diseases occur in different individuals, the way the disease progresses and the way in which patients respond to glucocorticoid treatment. As discussed in Chapter 5, high intensity ANKRD22 staining is not always observed in arteries with pan-arteritis, emphasising the heterogeneity in macrophage polarisation between patients. A lack of information about this novel marker makes it difficult to know whether this difference in expression is due to functional genetic variants of ANKRD22. Additionally, identification of greater arterial MRC1 expression in those patients who initially respond better to glucocorticoid treatment could be due to heterogeneity between patients in their response to disease, heterogeneity between patients in their sensitivity to glucocorticoids, heterogeneity in the ability of patients' macrophages to respond to glucocorticoids or a combination of these factors. This points to differences in the response of patients to processes in GCA, as well as their response to treatment, which may be in result of macrophage genetics and subsequent variations in signalling pathways. Further studies to replicate findings into ANKRD22 and MRC1 expression in arteries are required to determine firstly, whether MRC1 could be a useful predictive biomarker and secondly, whether ANKRD22 and MRC1 could be useful stratification markers to identify groups of patients where different immunological pathways were upregulated. Drug repurposing studies would be useful to identify whether these groups of patients respond differently to different drugs for the treatment of GCA.

The combination of macrophage plasticity, their ability to polarise into a spectrum of different phenotypes, along with genetic variability of macrophage polarisation between individuals, highlights the complexity of macrophage biology. This emphasises potential for the application of personalised medicine in GCA rather

than the conventional blanket approach, where all patients receive the same therapy. Analysis of macrophage biology, therefore, needs to be studied on an individual patient level; single-cell analysis could be crucial to identify inter-individual differences in genetics, cellular phenotypes and signalling pathways which could be utilised for personalised drug development.

6.4 Limitations

As described previously, the use of a cell line to investigate macrophage biology has its limitations, largely due to its cancerous origin, and the investigation of cellular processes in a dissimilar environment, therefore, this method may not fully mimic human MDMs both *in vitro* and *in vivo* (Tedesco *et al.,* 2018).

The reliance on the binary M1 and M2 macrophage model, and the idea that markers could be “switched on” in one phenotype and “switched off” in another, although initially very useful in optimising the THP-1 cell model, may be too simplistic for the characterisation of macrophages in GCA artery biopsies. Use of the binary model relied heavily on LPS and IFNγ as stimulants for M1 polarisation, whilst polarisation of M2 cells requires stimulation with IL-4 in *in vitro* studies, resulting in macrophages polarised to the extremes of the polarisation spectrum. Additionally, as many different cytokines, growth factors and other molecules have been observed within the arteries of GCA patients, the use of M(LPS, IFNγ) and M(IL-4) polarised macrophages and their associated markers to characterise these macrophages within artery biopsies is likely to disregard many other macrophage phenotypes within the artery. Transcription factors may play a role in guiding polarisation into one phenotype or another (Porta *et al*., 2009; Bystrom *et al.*, 2008) and the continuous exposure to multitudes of different polarising signals may induce expression of both M1 and M2 markers via different downstream signalling pathways. This method, therefore, does not mimic *in vivo* conditions found within the inflamed artery wall of GCA patients, where a multitude of mediators and cytokines have been described (Weyand *et al*., 1997).

The ability to characterise different macrophage subsets in this study depended highly upon the stimuli investigated in the RNA-Seq studies for macrophage polarisation, and the usefulness of the markers identified for immunohistochemistry. This therefore limited the phenotypes that could be characterised, and the markers used to characterise them, within the GCA lesions. Yet, due to the unknown mechanisms that induce M2-like macrophages within GCA lesions, and the limitations of *in vitro* cell culture, the binary method is currently the most useful method to identify subset markers to characterise macrophages in GCA.

In this study, M2 macrophage markers, such as the widely-used CD163 were found to be expressed on overlapping M2 phenotypes, such as M2a and M2c cells at the protein level. Furthermore, MRC1 was found to be upregulated by glucocorticoids at the protein level in M(0) macrophages, but was not upregulated by IL-10, which are both M2c polarising molecules. This suggests that MRC1 can

be upregulated on both M2a and M2c polarised cells and highlights the difficulty in identifying M2 macrophage markers specific for their M2a, M2b or M2c subset, where more overlap is seen compared to M1 macrophages. This may have implications on the results which were obtained in this study from the immunohistochemistry staining, however, the idea that macrophages co-express markers may be the cause of this inability to identify M2a-specific markers.

A major limitation throughout this study is the ability of the methods used to only take a ‘snapshot’ of the processes that are occurring, which is especially important if macrophages are able to switch between phenotypes. Optimisation of the THP-1 cell model identified differences in marker expression as a result of variations in cytokine exposure time and highlights the variability of marker expression. This has implications for the RNA-Seq data that was used, the protocol used for the study of THP-1-derived macrophage marker expression, as well as the characterisation of macrophages in GCA artery biopsies. An understanding of the processes of both macrophage polarisation and their role in the changes that occur within the artery wall, is therefore incomplete.

The opinion that macrophage polarisation exists as an overlapping spectrum of subsets means the use of single markers to stain separate sections of GCA artery biopsy tissue is a limitation to this study. The use of dual-staining has the ability to determine whether macrophages in GCA exist as separate M1 and M2 marker expressing cells or whether macrophages co-express M1 and M2 markers. This would help to gain a better insight into the polarisation spectrum of macrophages within the artery layers.

Furthermore, the low number of stained artery biopsies in this study limits the power of the statistical tests that were performed and therefore future studies would require a greater number of artery biopsies to identify differences in inflammatory patterns as well as macrophage marker staining.

6.5 Future work

6.5.1 Multiplex staining of GCA artery biopsies

To confirm the idea of mixed macrophage phenotypes in GCA artery biopsies, multiple staining immunohistochemical methods would need to be performed to determine whether macrophages simultaneously expressed M1 and M2 macrophages markers. The number of suitable genes identified at the RNA level that required further analysis at the protein level limited the time available for full optimisation of all antibodies for analysis by western blotting. Therefore markers that were identified as being highly specific for M(LPS,IFNγ) or M(IL-4) macrophages at the transcript level, such as SERPING1 and ALOX15 and CD200R1, respectively were not found to be expressed at the protein level. Future experiments would benefit from further optimisation of antibodies for SERPING1, ALOX15 and CD200R1. This may identify further subset-specific markers for each phenotype that could be used for subsequent experiments exploring M(LPS,IFNγ) and M(IL-4) macrophages.

The utilisation of methods which would allow staining of more than two markers at once on paraffin embedded tissue would also provide much more information on cellular processes, such as macrophage interactions with other cell types in GCA. One possibility is the use of the Perkin Elmer Vectra Automated Quantitative Pathology Imaging System. This is a multiplex system allowing multicolour immunohistochemistry (Stack *et al.*, 2014), an avenue of research that was proposed in my study, however, due to time constraints, was not used. Marker panels identified for macrophage characterisation in this study, along with panels for other interesting cell types, such as T-cell subsets and stroma, could be used simultaneously on whole FFPE tissue. This would help identify the variety of macrophage phenotypes within the artery wall and understand cell-cell interactions with other cell types, helping to understand the mechanisms of the disease. The advantage of the Vectra system over immunofluorescence staining is the ability to observe the histological arrangement of the whole section using H&E, along with the location of particular stained cells. Furthermore, the image analysis software enables identification and quantification of the different cell phenotypes that express different cell markers (Parra *et al.*, 2017; Hu *et al.*, 2011).

6.5.2 Macrophages as targets in GCA

The breadth of immunological processes which involve macrophages in GCA makes these cells an ideal target for future treatments, however understanding their phenotypes is crucial to enabling cell-specific targeting. It may be possible, with further research into macrophage biology, to target specific macrophages and switch their function so they contribute to the resolution of GCA. Their plasticity makes them a very attractive target in the resolution of disease (Stout *et al.*, 2005), as their function could be switched to help promote anti-inflammatory functions, which has been carried out in cancer biology (Klug *et al*., 2013; Pyonteck *et al*., 2013). In GCA, complete switching of macrophages from pro-inflammatory phenotype to anti-inflammatory macrophages may have implications on increasing severity of ischemic symptoms due to increased tissue remodelling and therefore intimal hyperplasia. A more targeted approach to specific pathways involved in different macrophage functions in GCA may be more useful to treating the disease. For example, the identification of ANKRD22 expressing macrophages as an indicator of greater media destruction in GCA highlights a potential cellular candidate for drug targeting to help in the resolution of the GCA.

Furthermore, a better understanding of macrophage phenotypes found in GCA may ultimately help to repurpose drugs to inhibit certain arms of the disease. It may also identify areas of the immune response which are dysregulated. Additionally, identifying and understanding patterns of certain macrophage phenotypes may help to recognise patients most likely to respond best to certain treatments as well as those patients more likely to relapse after treatment withdrawal. This is particularly important due to the high doses of glucocorticoids prescribed for GCA and the wide range of side effects patients experience.

6.5.3 Phenotyping of giant cells

The analysis of giant cells in artery biopsies in this study identified individual giant cells expressed variable intensities for each of the markers, such as CD163, which was found to be absent in one giant cell, but a giant cell in close vicinity expressed CD163 at low levels (Figure 5.9). Furthermore, this study also identified giant cells that co-expressed different M1 and M2 markers. Only 11 out of the 59 arteries analysed in this study were found to have giant cells present within the artery wall. This prevented further analysis of the association between giant cells expressing different markers to different histological and clinical manifestations. Additional work to include a greater number of cases with giant

cells would allow further investigations into the different giant cell phenotypes and their association with histological and clinical features, such as media destruction, luminal occlusion and ischemic manifestations.

6.5.4 Macrophage markers and other diseases

The lack of reliable markers to identify macrophage phenotypes has hindered investigations into macrophage biology in different diseases. The novel markers identified in this study may therefore be useful in identifying macrophage phenotypes within diseases which have a strong macrophage component to their progression. This includes, but is not limited to, atherosclerosis, cancer and psoriasis.

Markers identified in this study could provide a multi-marker approach which would allow detection of various macrophage phenotypes at the tissue level. Analysis of gene expression within tissue has proven unreliable, for example in GCA where IL-4 mRNA expression is absent in artery biopsies, yet, has been observed to be overexpressed at the protein level (Ciccia *et al.,* 2015). This may be due to the use of primers that do not identify the correct IL-4 transcript found in GCA arteries. Furthermore, the cell type which produces IL-4 is unknown and therefore, the number of cells may be too low to allow for detection of IL-4 at the RNA level. Furthermore, studies into macrophage behaviour within different diseases have used simplistic methods to identify different macrophage phenotypes, similarly to the studies into macrophages in GCA. For example, M1 and M2 macrophages in an atherosclerosis study, were identified solely by ‘on’ or ‘off’ expression of MRC1 by Chinetti-Gbaguidi *et al.* (2011) and these macrophages were attributed to different processes. Additionally, a lot of the studies into macrophages are performed using apoE-/- mice models of atherosclerosis or *in vitro* cell culture models (Gui *et al*., 2012). Multiple staining of the novel macrophage markers identified in my study, using multiplexing techniques, could provide more valuable information into the different macrophage phenotypes within different disease settings, along with the functions they carry out.

6.6 Summary

To summarise, through the development of a THP-1 cell culture protocol to produce M1 and M2 polarised macrophages I have identified phenotypic-specific macrophage markers suitable for immunohistochemistry. Their use in immunohistochemistry of GCA temporal artery biopsies found large heterogeneity between patients in the infiltration of macrophages throughout the artery wall. The association of the M1 marker GBP5 with media destruction was found to be statistically significant, supporting the concept that M1 macrophages contribute to tissue damage. Furthermore, the association of the M1 marker ANKRD22 with a greater inflammatory infiltrate and the association of the M2 marker MRC1 with a greater response to glucocorticoids suggests these markers could be used together to identify groups of patients who might require different treatment regimens of glucocorticoids.

Appendix 1. Reagents, solutions and manufacturers

Reagents

RNeasy Plus mini kit (Qiagen), containing:

RNeasy Plus RLT lysis buffer

gDNA eliminator spin columns

RNeasy spin columns

RW1 buffer

RPE buffer

RNase-free water

Pierce™ BCA Protein Assay kit (ThermoFisher Scientific), containing:

BCA reagent A

BCA reagent B

Albumin standard ampules (2mg/mL)

Superscript II reverse transcriptase, containing:

5x first strand buffer

0.1M DTT

Superscript™ II RT

Antigen Unmasking solution (Vector): contains citrate, pH 6.0.

BLOXALL endogenous peroxidase and alkaline phosphatase blocking solution (Vector)

10x casein solution (Vector)

Ready-too-use antibody diluent with BSA (ThermoFisher Scientific)

ImmPRESS™ (Peroxidase) polymer horse anti-mouse IgG reagent (Vector)

ImmPRESS™ (Peroxidase) polymer horse anti-rabbit IgG reagent (Vector)

ImmPACT™ diaminobenzidine (DAB) peroxidase (HRP) substrate (Vector)

Solutions

For immunohistochemistry, solutions were kept at room temperature and made fresh every 2-3 weeks.

For western blotting, solutions were kept at room temperature and were made up fresh every 2-3 weeks (unless otherwise stated).

Reverse transcription buffer (no $MgCl_2$) (10x)

100mL 1MKCl

20mL 1M Tris-Cl (pH 8.3)

2mL Triton X-100

Make up to 200mL with dH_2O

Tris acetate EDTA (TAE) for electrophoresis (50x)

242g Tris base

57.1mL Acetate

18.61g Disodium EDTA

Make up to 1L with dH_2O

Tris-buffered saline (TBS) (10x)

24.2g Tris base

80g NaCl

pH to 7.6 with HCl and make up to 1L with distilled water (dH_2O)

Tris-buffered saline, 0.1% Tween 20 (TBS-T) (1x)

100mL TBS 10x (see above)

1mL Tween 20

Make up to 1L with dH_2O

Scotts tap water (1L)

2g Sodium bicarbonate

10g $MgSO_4$

dH_2O 1L

RIPA buffer

30mL 5M NaCl

5mL 1M Tris (pH 8.0)

5mL 20% (w/v) Triton X-100

5mL 10% (w/v) sodium deoxycholate

0.5mL 20% SDS

Make up to 50mL with dH_2O

Laemmli sample buffer (2x)

4mL 10% (w/v) SDS

2ml Glycerol

1.2mL 1M Tris-Cl (pH 6.8)

500uL β-mercaptoethanol

2.3mL dH_2O

Add bromophenol blue to a final concentration of 0.02%

SDS-running buffer (10x)

10.08g SDS

30.3g Tris

144g Glycine

Transfer buffer (10x)

30.3g Tris

144g Glycine

Transfer buffer with methanol (1x) (made up fresh)

100mL 10X transfer buffer (see above)

200mL methanol

Made up to 2L with dH_2O

TBS-T-BSA (0.1% Tween 20, 7% BSA) blocking solution

0.7g BSA

10mL TBS-T (see above)

PBS for immunofluorescence

Five 1g PBS tables were dissolved in 500mL dH_2O

This provides a solution containing:

0.14M NaCl

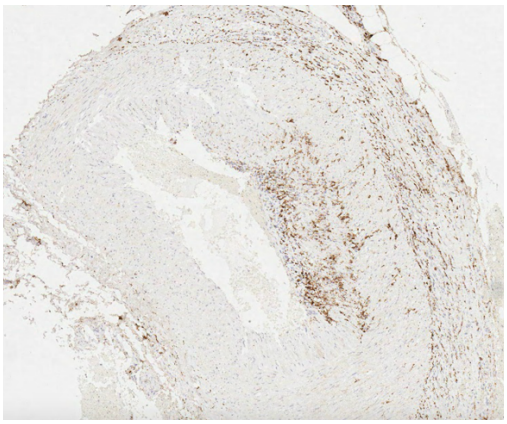
0.01M phosphate

0.003M KCl

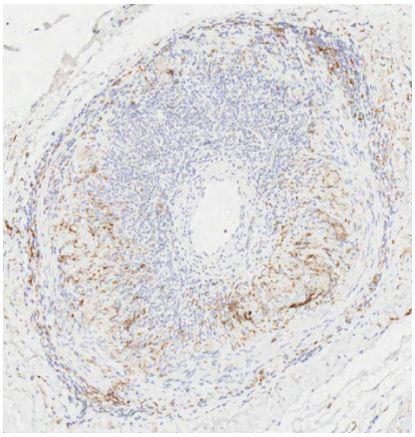
Appendix 2. Staining Atlas

Locality of staining (using CD68 as an example): No staining (0), focal staining (1), multifocal staining (2), diffuse staining (3)

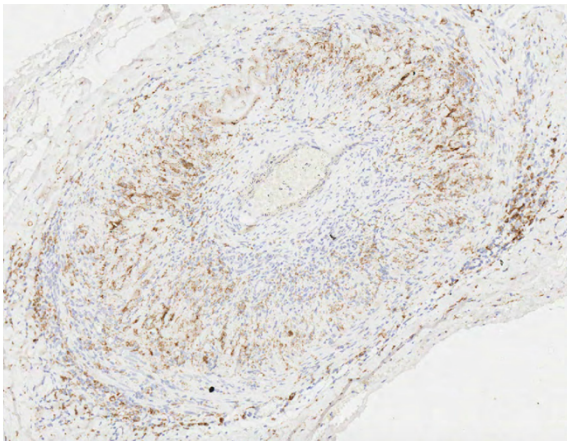
Score 1: Focal staining - Focal staining of adventitia, media and intima



Score 2: Multifocal staining - Multifocal staining of intima and media. Diffuse staining of adventitia.



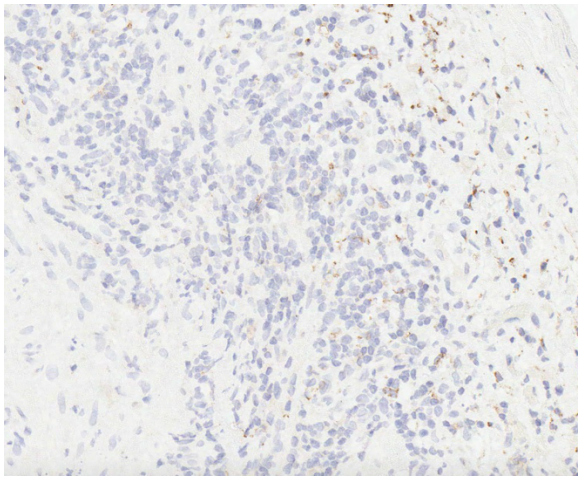
Score 3: Diffuse staining - Diffuse staining of all layers.



Intensity of staining: No staining (0), mild staining (1), moderate staining (2), strong staining (3)

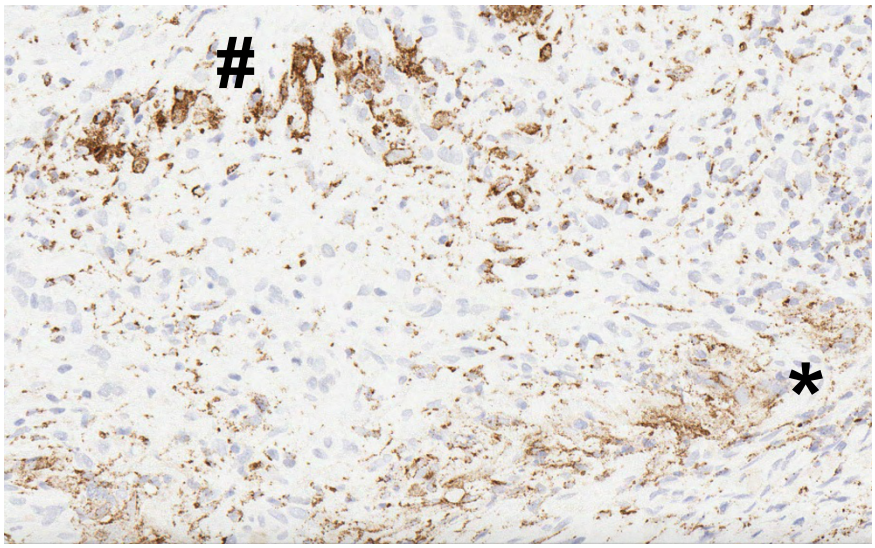
CD68

Score 1: Mild staining: Mild staining of adventitia, no staining of media or intima



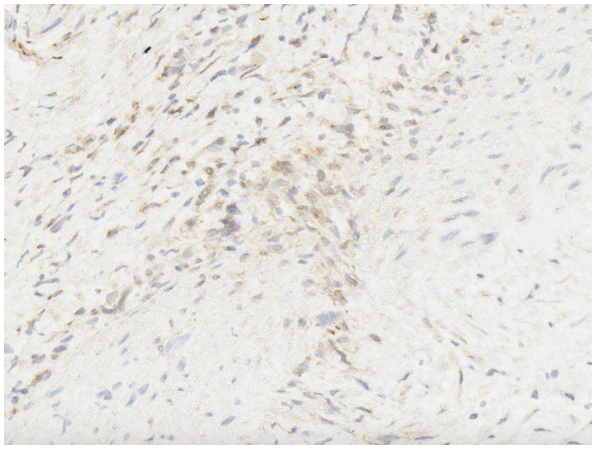
Score 2: Moderate staining – Moderate staining of the adventitia (*)

Score 3: Strong staining - strong staining of intima (#)

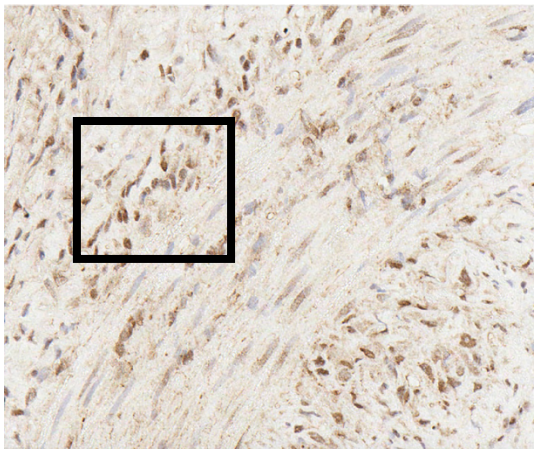


ANKRD22

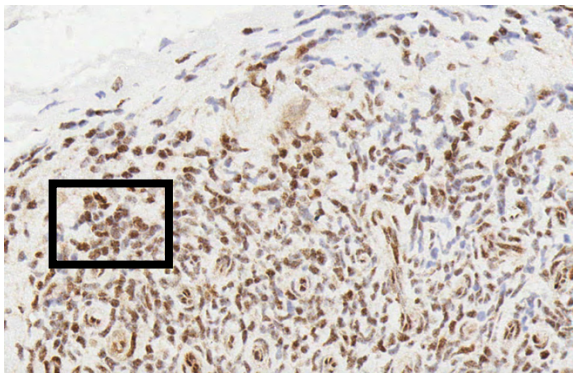
Score 1: Mild staining - Mild staining of all layers (non-specific staining of other cell types)



Score 3: Moderate staining - Moderate staining of all layers (Macrophage shown in box) (clear non-specific staining of other cell types)

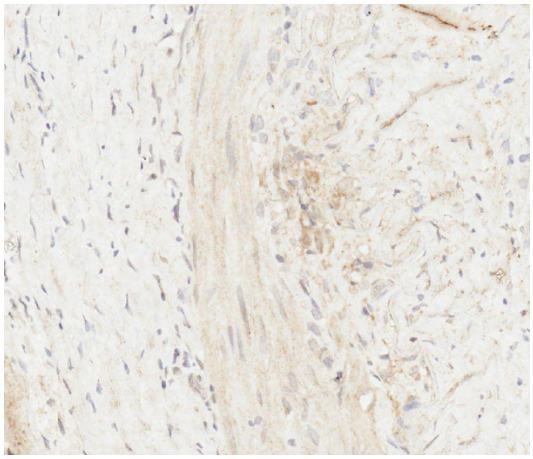


Score 3: Strong staining - Strong staining of all layers (Macrophages shown in box) (very clear non-specific straining of other cell types).

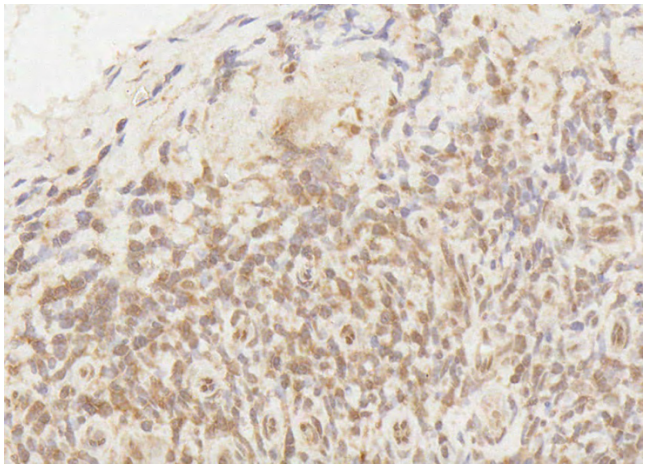


GBP5

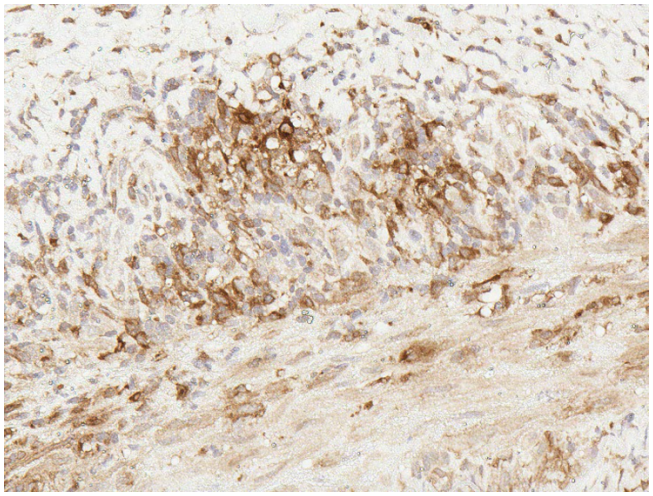
Score 1: Mild staining - Mild staining of the intima, no staining of adventitia (non-specific staining of media)



Score 2: Moderate staining - Moderate staining of all layers (non-specific staining of other cell types).

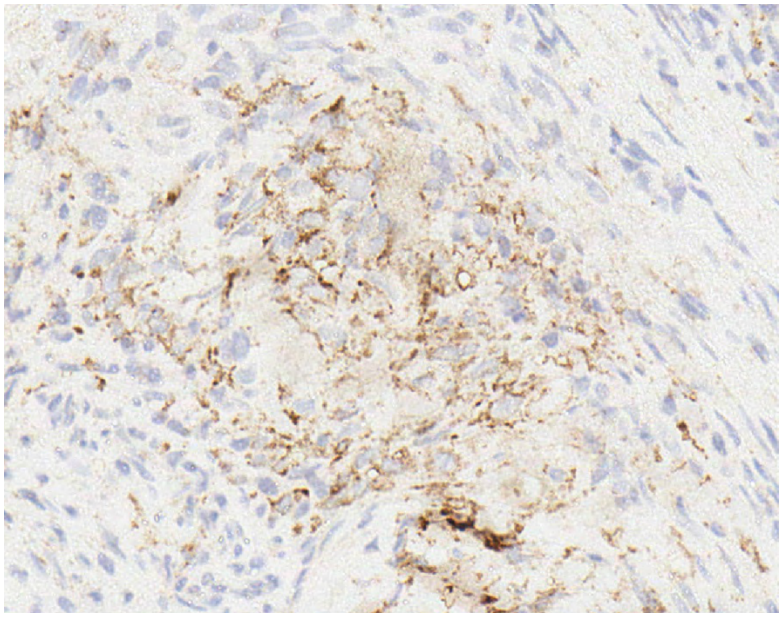


Score 3: Strong staining - Strong staining of the adventitia



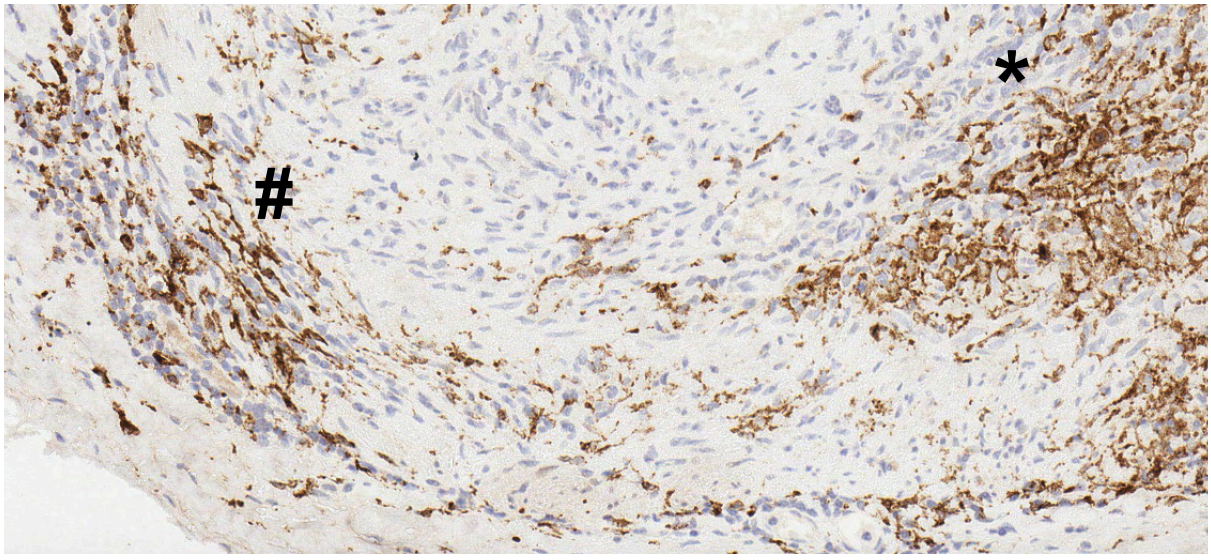
CD163

Score 1: Mild staining - Mild staining of the intima



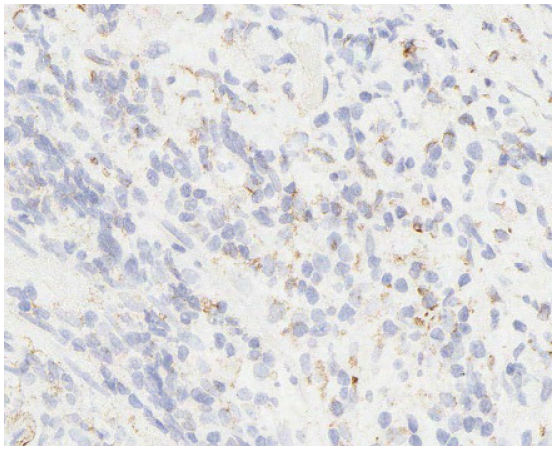
Score 2: Moderate – Moderate staining of the media and intima (*)

Score 3: Strong staining- Strong staining of the adventitia (#)

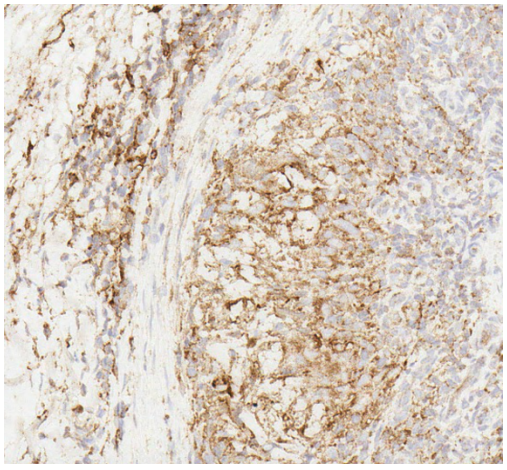


MRC1

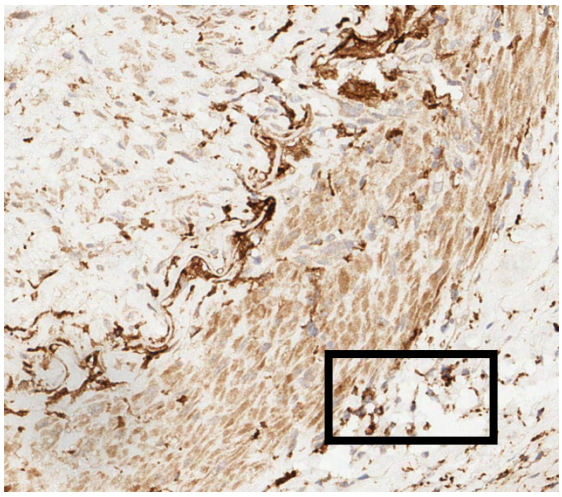
Score 1: Mild staining - Mild staining adventitia and media, no staining of intima.



Score 2: Moderate staining - Moderate staining of all layers

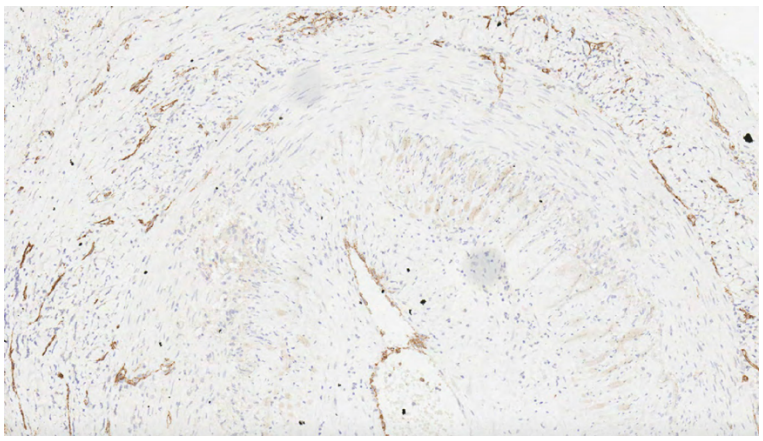


Strong staining (3): Strong staining of all layers (macrophages shown in box) (non-specific staining of VSMCs in media)

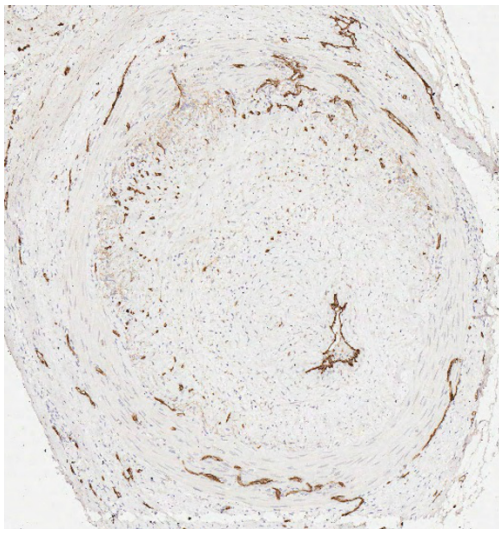


CD31 (neovascularisation) [Excludes any vascularisation within the adventitia]

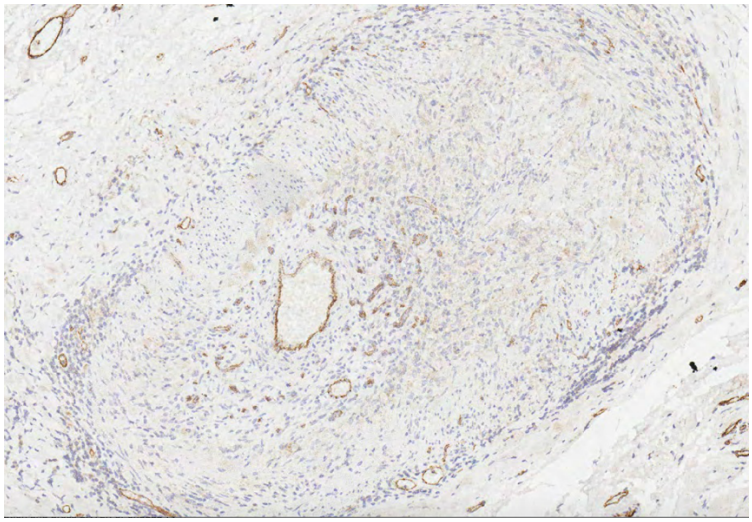
Score 1: Mild neovascularisation:



Score 2: Moderate neovascularisation:



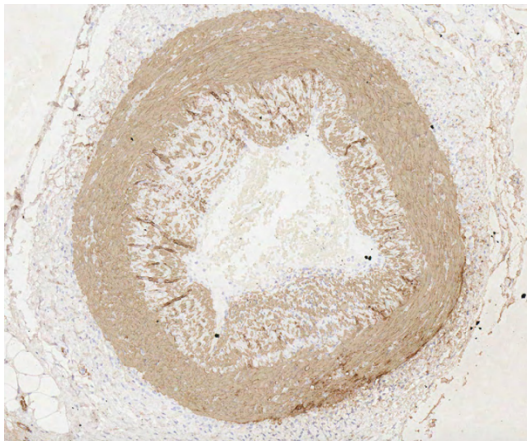
Score 3: Strong neovascularisation:



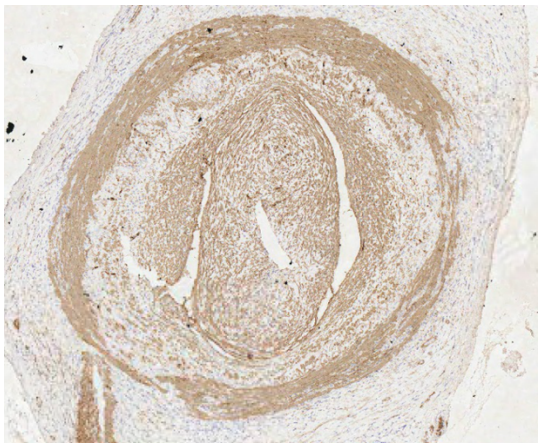
SMA

Degree of media destruction

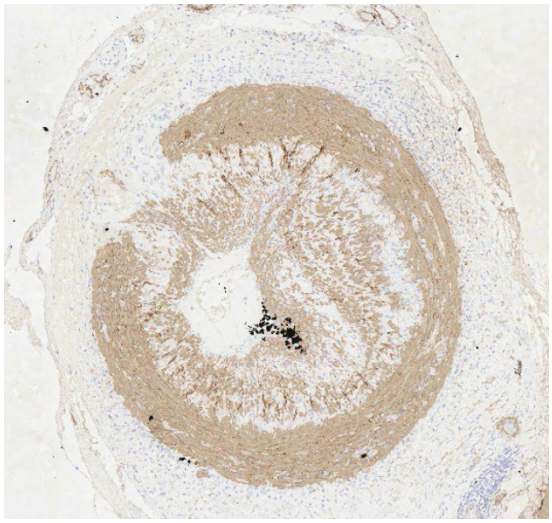
Score 0: No media destruction - Intact media



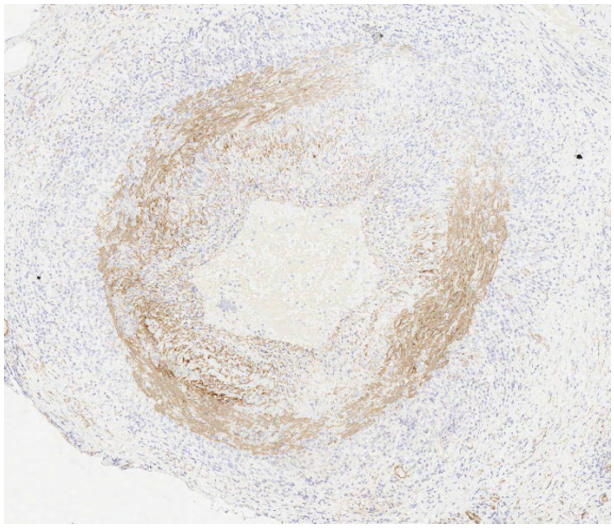
Score 1: Mild media destruction - Partial destruction in localised areas



Score 2: Moderate media destruction - Complete destruction of an area of the medial layer



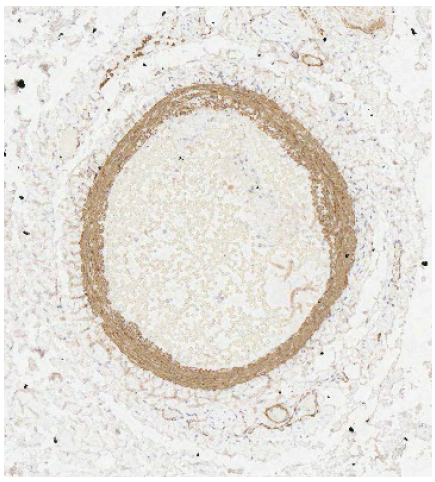
Score 3: Severe media destruction - Complete destruction of areas of the medial layer plus partial destruction of most of the media.



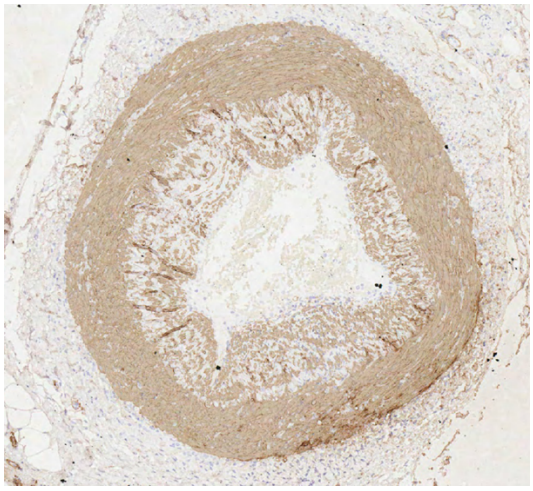
SMA

Degree of occlusion

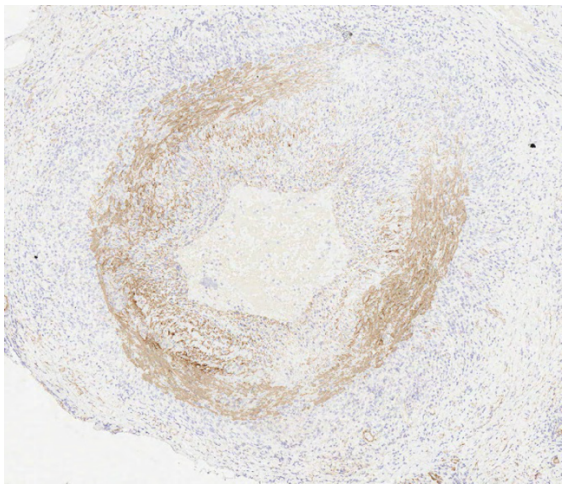
Score 0: No occlusion of the intima (0%)



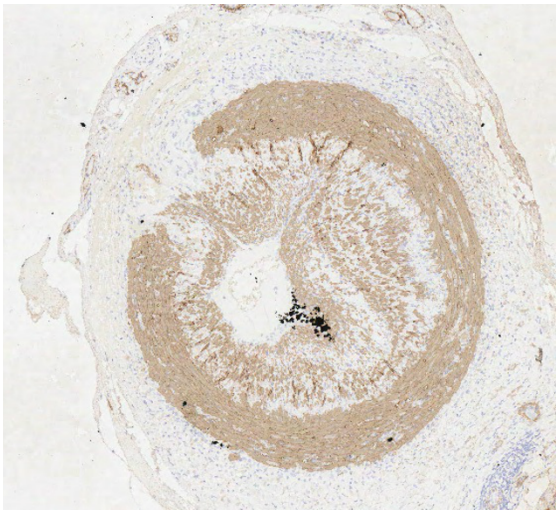
Score 1: 1%-25% occlusion



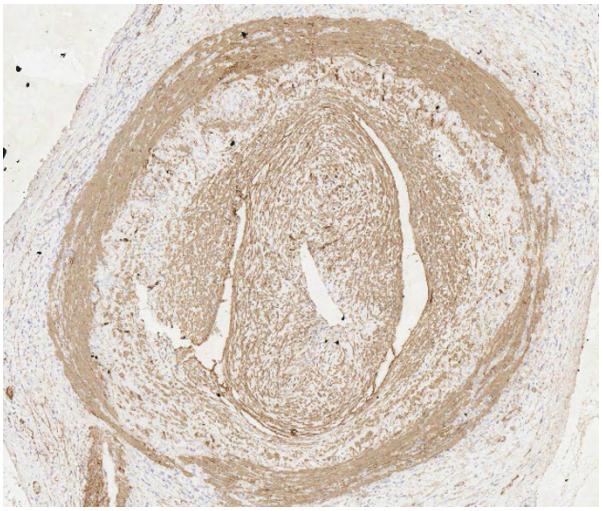
Score 2: 25%-50% occlusion



Score 3: 50-75% occlusion



Score 4: 75%+ occlusion



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