The University Of Sheffield.
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The molecular mechanisms driving chronic macrophage dysfunction in ageing

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Declaration

I, Charlotte Moss, confirm that the work submitted in this thesis is my own. I confirm that appropriate credit has been given within the thesis where reference has been made to the work of others. I am aware of the University's Guidance on the Use of Unfair Means (www.sheffield.ac.uk/ssid/unfair-means). No part of this thesis has been previously presented for an award at this, or any other, university.

This thesis contains two manuscripts that were collaborative efforts with multiple authors. I am the primary contributor and listed as first or co-first author for both. My detailed contribution and that of co-authors are outlined at the start of these chapters. In Chapter 3, all authors designed and planned the manuscript. Myself and Hew Phipps performed the search, screening and data extraction. Data analysis and writing of the manuscript was performed by myself with support from all authors. In Chapter 5, I performed all experiments with the assistance of Martha Clements and Simon Johnston, apart from the western blot which was performed and analysed by Joshua Kimble. I performed all data analysis with the exception of the eQTL data presented in Figure 5.3 and Supplementary table 8.4.2-8.4.3, performed by Veryan Codd and Stephen Hemby. Writing of the manuscript was done by myself, with all authors contributing to the development and editing of the manuscript. All authors reviewed the manuscript.

All remaining work presented in this thesis is my own and has been written by myself, without contribution from others.

Charlotte Moss, March 2024

Associated publications

Conference papers

Published abstracts

Moss CE, Clements M, Wilson HL, Kiss-Toth E. BS45 Transcription factors MYC and USF1 drive chronic macrophage dysfunction in ageing. Heart. 2023; 109:A278.

Manuscripts

Moss CE, Phipps H, Wilson HL and Kiss-Toth E. Markers of the ageing macrophage: a systematic review and meta-analysis. Front. Immunol. 2023; 14:1222308. doi: 10.3389/fimmu.2023.1222308

Moss CE, Johnston SA, Kimble JV, Clements M, Codd V, Hamby S, Goodall AH, Deshmukh S, Sudbery I, Coca D, Wilson HL, Kiss-Toth E. Ageing-related defects in macrophage function are driven by *MYC* and *USF1* transcriptional programmes. Cell Reports. 2024; 43(4):114073. doi: 10.1016/j.celrep.2024.114073

Other contributions

Martinez-Campesino L, Kocsy K, Cañedo J, Johnston JM, **Moss CE**, Johnston SA, Hamby S, Goodall AH, Redgrave J, Francis SE, Kiss-Toth E and Wilson HL. Tribbles 3 deficiency promotes atherosclerotic fibrous cap thickening and macrophage-mediated extracellular matrix remodelling. Front. Cardiovasc. Med. 2022; 9:948461. doi: 10.3389/fcvm.2022.948461

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List of Abbreviations

AD – Alzheimer's disease

APC – Antigen presenting cell

ARD – Age-related disease

ATP8B1 – ATPase phospholipid transporting 8B1

BMDM – Bone marrow-derived macrophage

BSA – Bovine serum albumin

CALHM6 – Calcium homeostasis modulator family member 6

COPD – Chronic obstructive pulmonary disease

Ct – Cycle threshold

CVD – Cardiovascular disease

DEG – Differentially expressed genes

DMSO – Dimethyl sulfoxide

eQTL – Expression quantitative trait loci

FBS – Foetal bovine serum

FC – Fold change

FDR – False discovery rate

GEO – Gene expression omnibus

GOI – Gene of interest

GSEA – Gene set enrichment analysis

GWAS – Genome wide association study

HSC – Haematopoietic stem cell

iBMDM – Immortalised bone marrow-derived macrophage

IFN – Interferon

IL – Interleukin

LDL – Low density lipoprotein

LPS – Lipopolysaccharide

MAIC – Meta-analysis by information content

MCP-1 – Monocyte chemoattractant protein 1

M-CSF – Macrophage colony stimulating factor

MDM – Monocyte-derived macrophage

MHC – Major histocompatibility complex

MMP – Matrix metalloproteinase

mTOR – Mammalian target of Rapamycin

NFKB – Nuclear factor kappaB

NO – Nitric oxide

NOS – Nitric oxide synthase

oxLDL – Oxidised low density lipoprotein

PBMC – Peripheral blood mononuclear cell

PBS – Phosphate-buffered saline

PCA – Principal component analysis

PCSK9 – Proprotein convertase subtilisin/kexin type 9

PFA – Paraformaldehyde

PRR – Pathogen recognition receptor

RBC – Red blood cell

ROI – Region of interest

ROS – Reactive oxygen species

RT-qPCR – Real-time quantitative polymerase chain reaction

SASP – Senescence associated secretory phenotype

siRNA – Short interfering RNA

SLR – Systematic literature review

T2DM – Type 2 diabetes mellitus

TAM – Tumour-associated macrophage

TGF – Transforming growth

TNF – Tumour necrosis factor

TLR – Toll-like receptor

USF1 – Upstream stimulatory factor 1

Thesis Summary

Macrophages are key to initiating the early immune response. They have a diverse set of roles in homeostasis that are known to become dysregulated with age. Additionally, macrophages have been implicated in the progression of several age-related diseases. However, the majority of work on macrophages to date has been carried out in mouse models and the underlying molecular mechanisms resulting in their age-related dysregulation are not yet known.

The work presented in this thesis aimed to first comprehensively and critically review the existing literature in the field of macrophage ageing, with the idea of taking this forward into assessing the functioning of human cells. We characterised the functional changes with age in human primary macrophages before setting out to uncover transcriptional networks underpinning these changes. We identified multiple transcription factors potentially driving age-related transcriptional changes and narrowed these down to *MYC* and *USF1* that have key roles in macrophage function, including phagocytosis and chemotaxis, and drive their deregulation with age. We further assessed the contribution of MYC and USF1 to the ageing macrophage phenotype by assessing their expression in human monocytes, finding that *MYC* but not *USF1* was also dysregulated in these precursor cells, that also have reduced phagocytosis and chemotaxis with age.

Lastly we assessed whether macrophage function could be restored in older individuals through modulating expression of key genes and through intervention with senolytic treatments. We found that phagocytosis was restorable in monocyte-derived macrophages isolated from older human donors following modulation of USF1 targets and that MYC expression was restored in bone marrow-derived macrophages isolated from older mice following senolytic treatment.

Overall, this thesis highlights functional and mechanistic changes occurring in human macrophages with age and provides evidence that therapeutically targeting these cells could improve the overall immune response and limit progression of age-related diseases.

Chapter 1. General Introduction

The introduction to my thesis covers the broad concepts relating to macrophages and ageing that are key in understanding my work. This highlights the knowledge gaps and relevance of this thesis and is followed by the hypothesis and aims of my research. A detailed systematic review of macrophages and ageing is covered as a published manuscript in Chapter 3.

Declaration of Contribution

The chapter was written by myself, without contribution from others.

In the developed world, people are living longer, with those aged over 65 years accounting for 18% of the population of Great Britain in 2018, compared with 10.8% in 1950. This is projected to increase still further to 25% by 2050 [1] and is due mainly to improvements in healthcare, such as the expansion of antibiotics and development of many vaccinations and therapeutics against previously fatal diseases, as well as improvements in living standards [2,3]. However, the number of years an individual lives in good health are not increasing proportionally. Ageing is one of the key drivers of multimorbidity: the presence of two or more long-term health conditions. Understanding the mechanisms that contribute to age-related diseases is therefore crucial to improving quality of life in this population, as well as the humanistic, societal and economic burden of disease in older populations.

Age-related dysfunction is multi-faceted, spanning neurodegenerative disorders, cardiovascular disease (CVD), cancers and metabolic diseases; it is an intricate process involving deterioration at multiple levels. However, this dysfunction seemingly converges on inflammation [4], a process in which macrophages are intimately involved. Macrophages, are a set of diverse innate immune cells of myeloid origin that are functional in every physiological system across all vertebrate species [5–8]. They have been implicated in the development and progression of many age-related and inflammatory diseases, including being fundamental to the pathogenesis of atherosclerosis [9,10]. While the role of macrophages in individual disease pathogenesis has been much studied, the molecular mechanisms driving their shift from being functionally protective to being drivers of chronic dysfunction and disease progression in ageing, are not known. Research into understanding the role of macrophages in age-related decline and multimorbidity could aid in the development of therapeutics as well as preventative measures for age-related diseases, ultimately leading to a better correlation between the increasing lifespan across the developed world and “healthspan”: the healthy life expectancy of the population [11].

1.1 Macrophages are essential components of the innate and adaptive immune systems

Macrophages, first discovered in the 19th century by Metchnikoff [5,6], are a diverse set of innate immune cells. They were classified almost 50 years ago as part of the mononuclear phagocyte system, highlighting their perceived main functional role [5]. Since then, this heterogenous cell population has received a large amount of interest, and their importance in proper immune functioning has been confirmed. Alongside phenotypes able to clear foreign material or apoptotic cells through phagocytosis, it is known that macrophages have key roles in mediating the innate and adaptive immune responses, not limited to antigen presentation and cytokine production [12,13]. They have functions crucial to many aspects of immunity, interacting with many cell types in order to initiate defence against foreign infiltrates, and control tissue homeostasis and repair. To properly maintain this dynamic array of functions, macrophages have plasticity and are uniquely able to switch phenotype; this is something that likely becomes defective in the shift from health to disease. To better understand the contribution of macrophages to age-related disease progression, it is crucial to first understand normal macrophage function.

1.1.1 Macrophage ontogeny

Macrophages reside in all tissues of the body and their development has been well investigated in mice through fate mapping [7,14,15]. It was initially thought that all macrophages were derived from bone marrow progenitors such as monocytes. However, it is now clear that this is an inadequate description of most tissue populations. While some tissues rely on infiltration of circulating monocytes derived from haematopoietic stem cells (HSCs) in the bone marrow, which differentiate to supply macrophages to maintain the tissue cell population, other tissue resident macrophage populations are entirely reliant on primitive macrophages produced in the foetal liver and yolk sac during development, before the initiation of haematopoiesis [16]. These macrophages have been found to be genetically distinct from those that derive from bone marrow, including the requirement of

transcription factor MYB activity in the development of macrophages derived from HSCs but not the yolk sac [15] (Figure 1.1).

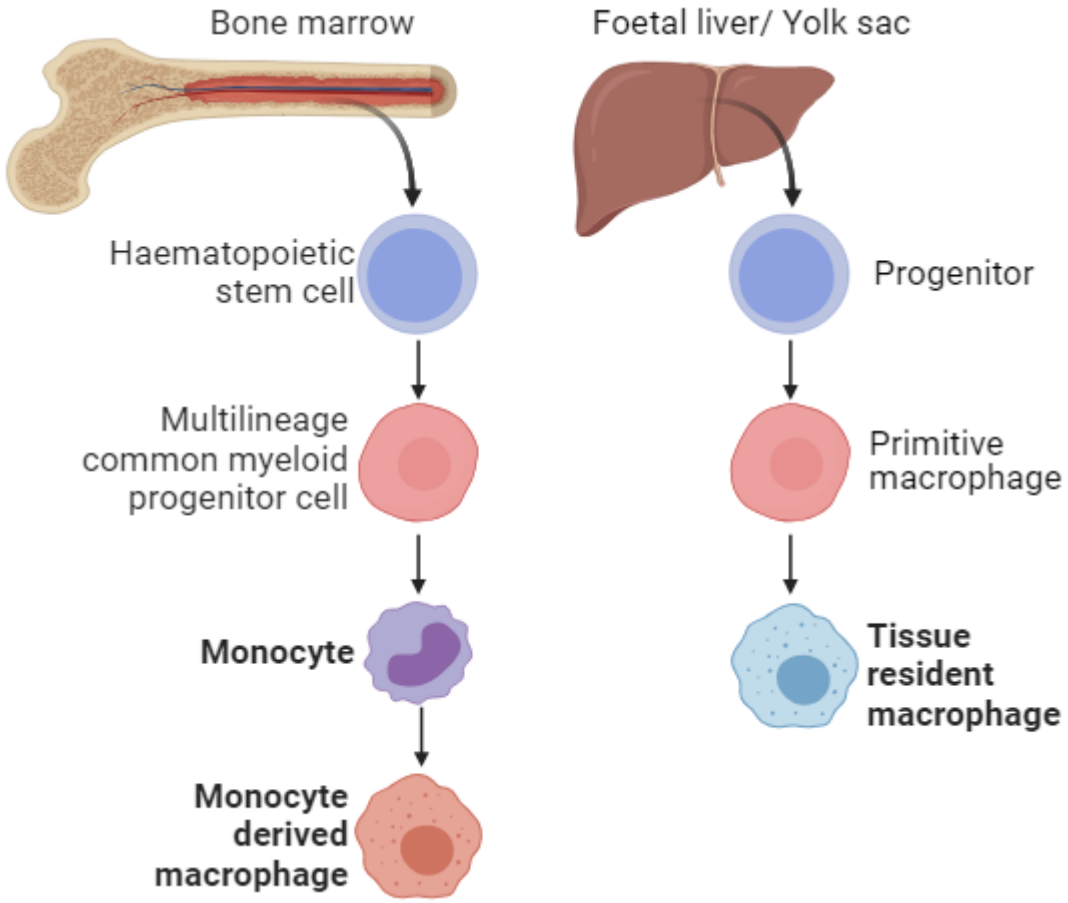

Figure 1.1 Macrophage Ontogeny

Fate mapping of macrophages and precursor cells has shown that tissue macrophages are derived from two separate lineages [14,16]. Macrophages are either produced throughout adulthood from monocyte precursors, in turn derived from haematopoietic stem cells in the bone marrow, or from the foetal liver and yolk sac during development and self-maintained throughout life. Created in Biorender.

Monocyte-derived macrophages (MDMs) are produced throughout life in order to replenish tissue populations and this population expands extensively in response to an infectious stimuli, injury or other stress. This differs from tissue resident macrophages derived from the foetal liver or yolk sac, that are produced during embryonic development and are self-maintained [17,18]. The macrophages of the skin and gut are continually replaced with MDMs from adult HSCs due to their constant exposure to potential threats, making this the primary macrophage population in these tissues [17]. In contrast,

microglia (macrophages of the central nervous system and brain) develop and migrate from the yolk sac during embryogenesis and remain a self-maintained population [19]. Other tissue macrophage populations, such as the Kupfer cells of the liver, alveolar macrophages of the lung and macrophages of the peritoneum, have both yolk sac-derived and HSC-derived populations [6,20]. Finally, tissue populations such as that of the heart are self-maintained apart from during an inflammatory response, which is governed by MDM expansion [16] (Figure 1.2). The reason for embryonic population displacement is not entirely understood; studies have revealed that there is a gender-specific change in certain tissues, not just replacement of proliferative exhausted cells, highlighting the role of local environment over tissue origin in the need for monocyte replenishment [20].

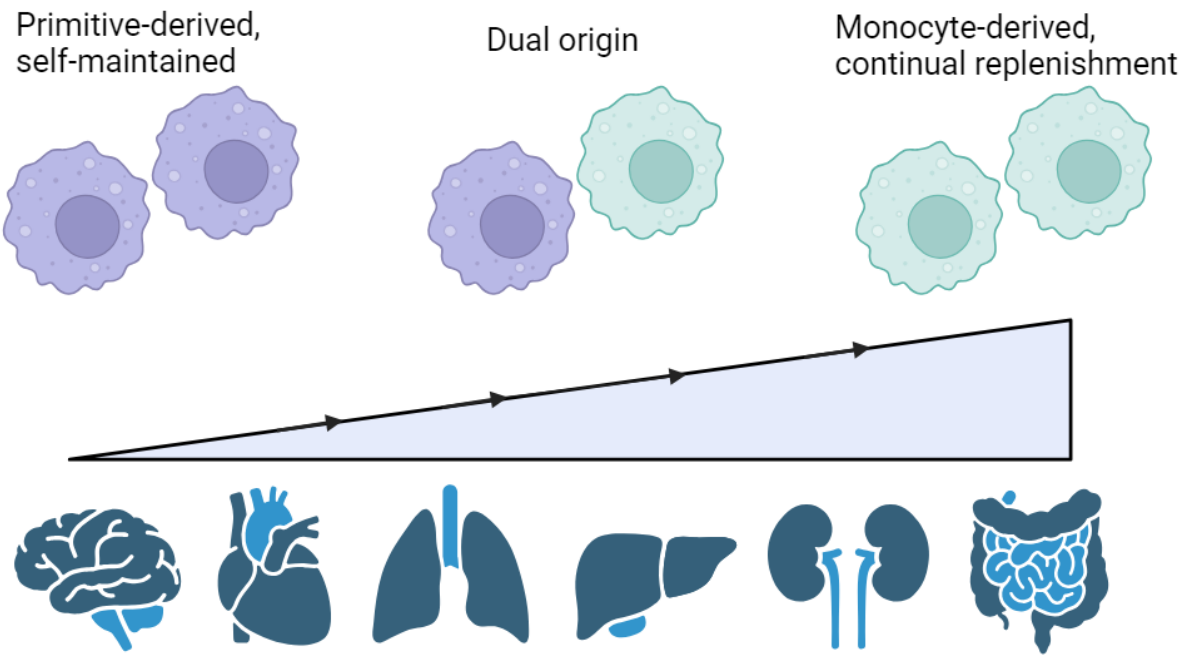

Figure 1.2 Monocyte-derived macrophage replenishment in different tissues

Tissue macrophages are either derived entirely from the foetal liver and yolk sac during development and self-maintained, such as the microglia in the brain, continually replenished by recruited monocytes, such as in the skin and gut, or have both primitive and monocyte-derived macrophages making up the population [17,19,20]. Created in Biorender.

The tissue environment plays a major role in programming the functional specificity of its resident macrophages. As each tissue has a unique environment, and many tissues have primarily self-maintained macrophage pools, macrophage populations are largely distinct from one another. These

distinctions include the transcriptional makeup of each tissue-resident population and although each population has a role in early tissue development and homeostasis [21], this creates the organ-specific functional and phenotypic niches required, making different tissue macrophage populations readily distinguishable from one another [22]. For example, a population of F4/80^{dim}, CR3^{dim} and CD206^{+} alveolar macrophages isolated from mouse lung function to clear particles and metabolise surfactants, whereas F4/80^{+} and CR3^{+} microglia isolated from mouse brain interact with live and dead neurons and are involved in development and repair [22,23]. The transcriptional regulatory pathways that drive development of individual populations have not yet been determined [24]. However, the Immunological Genome Project set out to characterise many adult populations by producing a complete microarray dissection of gene expression in the immune system of the mouse [25]. This has led to the characterisation of many tissue-resident macrophage populations such as peritoneal [26], lung [27], microglia [28], red pulp [29] and bone marrow-derived macrophages (BMDMs) [30].

1.1.2 Macrophage phenotype and function

Macrophages shift to different phenotypes in order to perform specific functions as a consequence of stimuli in the local environment. Much published research has attempted to better understand the spectrum of macrophage phenotypes that exist. However, the multitude of potential stimuli in the different tissue environments makes classifying them a challenge. Historically, and particularly for *in vitro* modelling, macrophages have largely been classified as resting, denoted M0, or have been associated with two main types: classically activated/pro-inflammatory or alternatively activated/anti-inflammatory, denoted an M1 or M2 phenotype, respectively. The M1 phenotype is most commonly produced by stimulation with pro-inflammatory cytokines interferon (IFN)-γ or tumour necrosis factor (TNF) and toll-like receptor (TLR) ligands such as Gram-negative bacterial cell wall component lipopolysaccharide (LPS). These stimuli cause an increase in expression of genes such as inducible nitric oxide synthase (*iNOS*) and production of pro-inflammatory cytokines such as interleukin (IL-6), TNF and IL-1β. The M2 phenotype encompasses many anti-inflammatory subtypes; anti-inflammatory cytokines such as IL-4, immune complexes and IL-10 can be used to stimulate

macrophages *in vitro* towards M2a, M2b and M2c phenotypes, respectively. This leads to the upregulation of genes such as Arginase-1 or mannose receptor C1, leading to upregulation of key anti-inflammatory cytokines such as transforming growth factor (TGF)-β and IL-10 [31,32] (Figure 1.3A). The expected *in vivo* function of this *in vitro* model, in response to a pathogen, includes pro-inflammatory M1 macrophages initiating the immune response, producing pro-inflammatory cytokines and phagocytosing foreign bodies to bring in more immune cells and activate the adaptive immune response. Once under control in healthy tissue, cells switch from the pro-inflammatory M1 population to an anti-inflammatory M2 population, that function to repair the tissue by secreting cytokines such as growth factors and clearing apoptotic cells and cellular debris (Figure 1.3B).

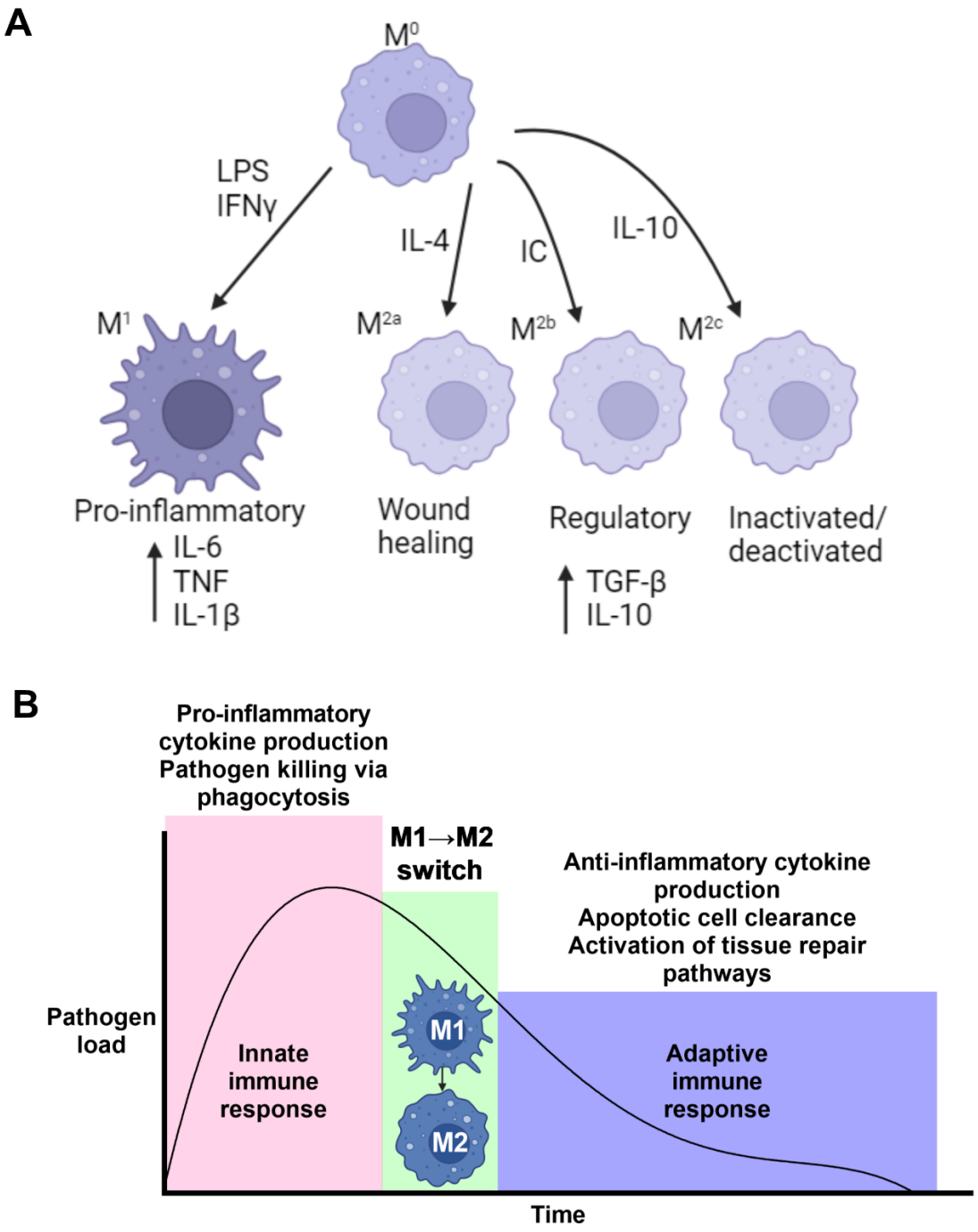

Figure 1.3 Common macrophage phenotypes produced *in vitro* and their roles *in vivo*

A – *In vitro*, unstimulated macrophages are referred to as M^0. Pro-inflammatory M1 macrophages are most commonly produced as a result of inflammatory stimuli, such as lipopolysaccharide (LPS) and interferon (IFN)-γ. M1 macrophages produce pro-inflammatory cytokines including interleukin (IL)-6, tumour necrosis factor (TNF) and IL-1β. Anti-inflammatory macrophages are most commonly produced by stimulation with anti-inflammatory cytokines such as IL-4, denoted M2a, immune complexes (IC), denoted M2b, or IL-10, denoted M2c. These aid in wound healing, immunoregulation and tissue remodelling by producing anti-inflammatory cytokines such as transforming growth factor (TGF)-β and IL-10.

B – *In vivo*, different macrophage phenotypes are produced in response to pathogens for effective clearance and repair. There is an initial pro-inflammatory response that includes production of cytokines and phagocytosis of the pathogen. Once the pathogen is under control there is a switch to an anti-inflammatory response, requiring anti-inflammatory cytokine production, apoptotic cell clearance and activation of tissue repair pathways. Created in Biorender.

The ability of macrophages to shift phenotype is still not completely understood. It was initially thought that the M1 and M2 functions were produced by two separate macrophage populations [31]. However, it is now apparent that individual macrophages can undergo phenotypic switching so that the same macrophages that initiate the response are then able to shift towards tissue repair phenotypes [33]. This has been attributed to transcription factor Maf, which negatively regulates downstream targets of Nrf2 to remain pro-inflammatory, and is then suppressed to allow the phenotypic change [33]. Of course, this M1/M2 paradigm is a very simplistic idea of macrophage roles. In reality, a plethora or spectrum of phenotypes exist in the microenvironment to respond to various stimuli, with many shared characteristics; macrophages are able to shift in accordance with environmental cues. Existing literature has since adapted the way in which macrophages are classified. One suggestion is the multidimensional model of activation. This provides stimulus-specific nomenclature that aid in experimental correlations between stimulus and gene expression readouts [34,35]. The macrophage subtypes depicted in Figure 1.3 would therefore be denoted $M^{LPS+IFN\gamma}$, $M^{IL\text{-}4}$, M^{IC} and $M^{IL\text{-}10}$ from left to right. Throughout this thesis, stimulus-specific nomenclature will therefore be used to aid in repeatability and comparison with published literature.

In recent years, the development of single-cell sequencing has allowed us to further distinguish the different macrophage phenotypes at play in different physiological contexts. One such example is the discovery that it is M2-like macrophages rather than inflammatory macrophages that become foam cells in atherosclerosis. These cells are initially beneficial in preventing atherosclerosis as they detoxify the blood with respect to oxidised low-density lipoprotein (oxLDL) and other modified lipids. However, as the disease progresses and the macrophages become lipid-laden and accumulate in arterial walls, these cells begin to fail and form the necrotic core of the plaque [36]. This was contrary to our previous understanding as this was thought to be an inflammatory process. Further, a single cell study looking at inflammation in the lungs uncovered five distinct subtypes of macrophages that were present during inflammation and that were unique in both transcriptional profile and function. Out of these subtypes, three were present only during inflammation. However, two were also seen

during homeostasis, highlighting the presence of a spectrum of macrophage phenotypes contributing to both health and disease [37].

In summary, macrophages are involved in a plethora of immune functions, as well as disease states, with their full spectrum of functions remaining largely unknown. Understanding the molecular mechanisms governing their unique phenotypes and plastic nature forms a large focus of current macrophage research. The use of novel techniques, such as single-cell sequencing, and the move to more accurate nomenclature will bring a better understanding of the phenotypical changes associated with homeostasis or macrophage dysfunction.

1.1.3 Macrophages in homeostasis

During an immune response, macrophages are one of the first effector cells to respond. Their primary roles include pathogen recognition and killing, and secretion of pro-inflammatory and antimicrobial mediators into the local environment [12,38]. Further, macrophages are involved in the initiation of the adaptive immune response, as they break down the ingested pathogen and present associated molecular patterns on their surface [39]. Finally, once the pathogen is under control, macrophages have a key role in resolution, through secretion of anti-inflammatory mediators, initiation of tissue repair and clearance of dying cells [40,41].

As mentioned previously, plasticity allows macrophages to tailor responses to the local environmental stimuli, enabling their involvement in a plethora of functions that require cooperation across the whole immune system. During homeostasis, the main phenotype of macrophages in tissues appears to be more M2-like, a sustainable energy phenotype that can survey the environment and quickly switch depending on the environmental needs [42]. Macrophages recognise pathogens through specific pathogen recognition receptors such as TLRs, whereby a signalling cascade initiates an inflammatory response. At the macrophage level, this involves an energetically costly M2-to-M1 switch of the tissue population and infiltration of MDMs due to release of pro-inflammatory cytokines and chemokines [42]. These MDMs cross the blood vessel wall as monocytes

and then differentiate and migrate through the tissue to the site of inflammation. The pro-inflammatory signalling also enables infiltration and activation of other immune cells such as neutrophils, dendritic cells and helper T cells that aid in pathogen killing [38]. While both macrophages and neutrophils engulf and degrade pathogen via phagocytosis and production of reactive oxygen species (ROS) and nitric oxide (NO), dendritic cells primarily act as antigen presentation cells (APCs), that work closely with macrophages to take up degraded pathogen and quickly migrate to the thymus to activate the T cells, ensuring a targeted immune response against the specific pathogen [39]. Macrophages can also work as APCs at the site of infection and so the pathogen is degraded and the antigen is presented on the cell surface. Depending on the type of pathogen, antigen presentation involves major histocompatibility complex (MHC) class I or II on the macrophage interacting with CD8+ or CD4+ T cells, respectively. This bridges the innate and adaptive immune responses. Once the pathogen is under control, it is then crucial that macrophages are able to resolve the immune response to prevent excess tissue damage [43]. This resolution response varies depending on the tissue, but it is thought that macrophages do this by undergoing normalisation of phenotype back to an M2-like state. This allows for expansion of the tissue population, orchestrating a dampening of the immune system that includes suppressing inflammation and promoting tissue repair through production of anti-inflammatory cytokines and growth factors and by clearing debris [40,41].

Macrophages clear cellular debris and cells that have undergone apoptosis through recognition by surface receptors such as scavenger receptors, integrins and complement receptors. Through different signalling pathways, this phagocytic role can occur independently from other cells, indicating that the macrophage remains unstimulated, either by not inducing inflammatory signals or by producing inhibitory signals, allowing the tissue to be cleared of debris without inducing inflammation [32]. Macrophages also have the ability to clear senescent cells and perform autophagy, a mechanism for recycling unneeded cellular components such as damaged organelles, misfolded proteins and ATP to increase energy efficiency for activation [44,45]. This can also affect the function of

the macrophage; for example, macrophages can specifically degrade nuclear factor kappaB (NFKB) by autophagy to maintain an M2-like phenotype and suppress the immune response [46].

1.2 Defining ageing as a disease

The biology of ageing, comprising inflammation, cellular senescence, oxidative stress or impaired mitochondrial function among others, and the mechanisms of age-related diseases; collectively referred to as "geroscience" are an area of active research [11,47], with the hope of better understanding the age-related dependency of many chronic diseases. Ageing is an evolutionary process as the body adapts to changes in the environment over time [48]. It affects each physiological system and cell type, often in different ways, but the process is largely thought to have an inflammatory contribution, as increased pro-inflammatory signals and chronic inflammation are considered to be key markers of ageing [4,49]. Ageing can be defined as the gradual deterioration of the physiological functions that enable both survival and fertility [50]. As opposed to age-related diseases, all individuals are affected by these mechanisms of ageing, although there is also some disparity between men and women. Simply, women live longer than men, but men remain fitter for longer, with a longer period of fertility and reporting of fewer illnesses [51]. Many hypotheses have been suggested to explain this, including improved immune functioning, protection from oestrogen or reduction in growth hormone all seen in women, however no conclusion yet fully supports these phenomena [52]. There are a number of risk factors associated with the burden of ageing, these include: injury, development of non-communicable diseases, poverty, social isolation, and maltreatment. Addressing these risk factors may reduce the progression of ageing [53]. Although it is not the mission of geroscience to find a "cure" for ageing, treating ageing as a disease and maintaining good health for longer in old age is one of the main aims. In order to do this, a much greater understanding of the underpinning cellular and molecular mechanisms of ageing and age-related disease is needed [54].

It is thought that the immune system is a central player in contributing to the ageing phenotype. "Inflammaging", a term coined to describe the chronic, low-grade and sterile inflammation associated with ageing, is believed to be a significant risk factor to the elderly population's increased susceptibility to infection and inflammatory disease [55]. Similarly, immunosenescence, described as the gradual decline in immune function seen in aged individuals, is likely correlated with persistent infections and a reduction in the elderly population's ability to respond to both new and previously encountered infections [56]. As both inflammaging and immunosenescence increase with age, these are likely linked to increased susceptibility to infection and disease and reduced ability to manage diseases and infections encountered in aged individuals [57,58].

The remodelling of the immune system and increase in pro-inflammatory signals described is considered to be a "hallmark" of ageing, as it is common to the ageing phenotype. The hallmarks of ageing were proposed to describe the consistent features associated with normal mammalian ageing, at both the cellular and molecular level, that appear to both accelerate the ageing process when experimentally activated and slow ageing when ameliorated [59]. These hallmarks form a highly interconnected process of ageing but have been assigned three separate categories: primary, antagonistic and integrative (Table 1.1). Although not all of these hallmarks are yet fully supported, it is thought that they largely determine age-related outcomes in practice, making them crucially important to understand when researching ageing and human health.

Table 1.1 The hallmarks of ageing

The table shows descriptions of the hallmarks of ageing as well as the main examples, taken from [59].

Hallmark Category	Description	Examples
Primary	Hallmarks are undoubtedly negative and likely the initiating triggers of the dysfunction associated with ageing that progressively accumulates over time	Genomic instability and DNA damage Telomere loss Epigenetic drift Defective proteostasis
Antagonistic	Hallmarks have opposite effects depending on their intensity: at low intensity they are beneficial, at higher intensity they become harmful	Senescence ROS-mediated cell signalling and survival Nutrient sensing and anabolism
Integrative	Hallmarks arise when the accumulative damage from primary and antagonistic hallmarks cannot be compensated for	Stem cell exhaustion Altered intracellular communication

ROS: reactive oxygen species

Many theories exist to describe the process of ageing, in an attempt to try to pinpoint a single overarching age-related change. The Hayflick limit describes the number of divisions a cell can go through, that accounts for telomere shortening. This would make ageing a regulated and unavoidable process [60]. Alternatively the stochastic theory attributes ageing to random chance mutations that lead to damage accumulation and reduced capacity for repair [60]. Additionally, accumulation of oxidative damage from increased ROS production could be the main driver in reducing cell function, referred to as the oxidative stress theory of ageing [61,62]. Immune system functional decline with age is also widely described [48,55]. Overall, it is clear that ageing is a multifaceted process with much work still required to understand the contribution of a multitude of factors rather than any singular change, as described by the increasing hallmarks of ageing (Table 1.1).

1.2.1 Cellular responses in ageing

As previously described, ageing is associated with an increase in inflammation and a decline in immune function and together these changes can be detrimental to many processes, such as immunity to infection and vaccine responses [63–65]. Most vaccines are less effective in the elderly population due to immune system remodelling, described as a relative decline and shift in adaptive immune cell populations and a functional decline in innate immune cells [64,65]. The T, B and dendritic

cells represent the adaptive immune system, and demonstrate a shift towards memory cell populations and away from naïve cells as the bone marrow and thymus become involuted, producing fewer B and T cell progenitors [66–68]. A reduction in repertoire also manifests in the decreased production of antibodies by B cells, overall making the adaptive immune system less able to respond to both new and previously encountered pathogens [64,65]. On the other hand, the innate immune cells including macrophages, neutrophils, natural killer cells, basophils and eosinophils are less able to perform functions such as phagocytosis and granulation [69–71], while producing more pro-inflammatory stimuli [72], meaning they too can less easily respond to new and previously encountered pathogens through chronic activation and reduced function (Figure 1.4) [64,65].

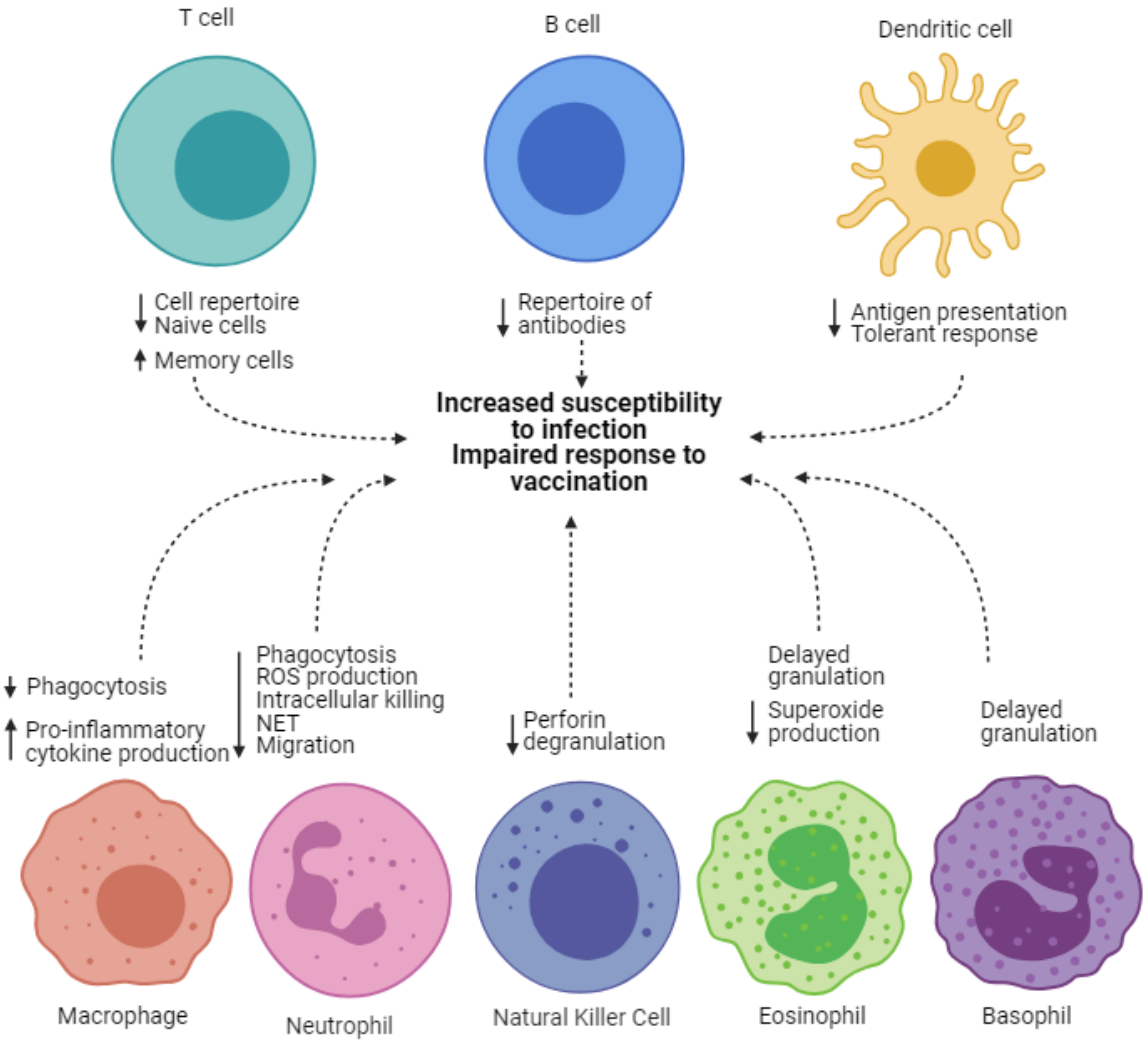

Figure 1.4 Adaptive and innate immune cells contribute to age-related dysfunction

Functional decline of immune cells of the adaptive (top) and innate (bottom) immune system has been linked to increased susceptibility to infection and reduced vaccine response. Created in Biorender.

NET: neutrophil extracellular trap

1.2.1.1 Cellular senescence

Continual exposure to pathogens, changes in nutrition and metabolism or tissue damage all result in increased cell death [64,73]. There are many different mechanisms of cell death, from regulated and programmed cell death resulting from the activation of such signals, to cellular senescence involving an irreversible loss of proliferative signals and the release of the senescence-associated secretory phenotype (SASP) [74]. Cellular senescence is thought to be a major contributor to the ageing phenotype and in recent years this has been widely researched. It is broadly described as the

irreversible arrest of the cell, without it then undergoing apoptosis. This can be a useful anti-tumorigenic response but in ageing is often caused by stress leading to a DNA damage response [75]. The senescent state is still not fully characterised and lacks universal markers due to highly heterogenous senescent programmes in different tissues and cell types. Senescent cells are currently measured by combining a number of different hallmarks that would be individually non-specific, including robust increase in expression of cyclin D-dependent kinase inhibitor p16 and lysosomal senescence-associated (SA)-β-Galactosidase [76]. Removal of senescent cells has been shown to delay age-related disease in the mouse, particularly those removed from adipose tissue, skeletal muscle and the eye, in which senescent cells have previously been found to contribute to pathology [77].

Senescence is one of the main contributors to inflammaging through immune cell recognition of SASP components as well as pro-inflammatory cytokine and chemokine production in an attempt to clear these senescent cells from the body [78,79]. This rise in circulating pro-inflammatory molecules such as IL-6, TNF and C-reactive protein has been shown to be a strong predictor of age-related morbidity and mortality, as well as cognitive decline in the elderly population [58,80,81]. In addition to this increase in senescent cell accumulation seen with age, mechanisms to remove these cells, as well as unneeded cell components also become dysregulated [82].

1.2.1.2 Autophagy

Autophagy involves the degradation and recycling of cellular components through delivery of cargo to the lysosome, allowing the maintenance of homeostasis [83]. It can both remove damaged organelles and degrade proteins into metabolites to aid in cell survival [84]. There is a growing body of evidence to suggest that autophagy declines with age [85,86]. In fact, autophagy has been linked to longevity in a mouse model overexpressing autophagy gene *Atg5* [87], through maintenance of the lysosomal receptor for chaperone-mediated autophagy [88], and in human centenarians who have been found to have increased levels of beclin-1, a biomarker for autophagy activity [89]. There is a strong association between decreased autophagy and increased senescence, including cell cycle

arrest and increased SA-β-Gal, indicating that autophagy is protective against the senescence phenotype [90].

The age-related decline in autophagic processes and increase in senescence has been associated with many diseases. However, age-related disease progression is still poorly defined and needs further investigation, particularly the role of the immune system and macrophages in these processes.

1.3 Macrophages in ageing

There is already a plethora of published literature addressing age-related changes that occur in macrophages, with focus on key macrophage functions such as phagocytosis and secretion of cytokines relating to polarisation. So far, the majority of this work has been done in tissue populations, predominantly from model organisms. However, with such variation between tissue populations, microenvironment or *in vitro* stimulation, there are many differing conclusions being drawn, and an overall lack of collective evidence. We therefore addressed this systematically in a review publication presented in Chapter 3; here we assessed the published literature and used a meta-analysis to identify overarching changes in macrophages with age.

Macrophages have already been implicated as key players in the initiation of inflammaging. Because of this, a subset of inflammaging termed "macrophaging" has been proposed to incorporate the chronic activation of macrophages in ageing [55]. Damaged cells and cellular debris, that increase with age, are recognised by macrophages through pattern recognition receptors (PRRs), whereby inflammation is promoted. Continuation of this inflammatory phenotype drives intestinal permeability, allowing pathogens into the circulation that further stimulate macrophage PRRs to produce more inflammatory mediators. In addition, this inflammaging phenotype causes a phenotypic switch of resting tissue macrophages to inflammatory-primed M1-like cells, that further secrete these pro-inflammatory mediators into the tissue, as found in mouse cardiac tissue macrophage populations [91,92]. Additionally, a population of M2-like macrophages with a pro-

inflammatory phenotype has been identified that accumulates in some tissues with age, such as human skeletal muscle. Like M1 macrophages, they show an increase in pro-inflammatory cytokine production [93,94].

This consistent basal inflammatory environment is unsustainable, mainly due to the large amount of energy required to maintain macrophage activation [42], and this leads to diminished or hyperactive inflammatory responses, resulting in decreased bacterial clearance, excess tissue damage and progression of age-related disease [92]. Although this is now a well-established phenomenon, we are still unaware of the underpinning molecular mechanisms that are changing with age in the macrophage to cause this ageing phenotype.

The ability of macrophages to undergo senescence is also beginning to be researched. It was previously thought that the lack of proliferative capacity of terminally differentiated macrophages meant that they could not undergo senescence [95,96]. However, macrophages do share some commonality with senescent cells, particularly in that they produce many of the same mediators of the SASP cocktail [97]. They are also largely involved in senescent cell clearance [98,99]. Senescence-associated macrophages have previously been reported, a subclass of macrophages with increased p16 and SA-β-Gal. These cells appear to accumulate in response to senescent cells or an unresolved innate immune response, further contributing to inflammaging [100], although senescent cells may not be required to produce this macrophage phenotype and further work is needed to confirm this [101,102]. Further, p16-high macrophages have been observed in the mouse liver that show phenotypic similarities to senescent cells, but these too have not yet been fully defined [103].

1.4 Macrophages in age-related diseases

While the systematic review of the literature in Chapter 3 focuses only on published data pertaining to healthy ageing in an attempt to understand how age alone influences macrophage function, this is not necessarily a true perspective of human ageing, as age-related disease and multimorbidity are commonplace in our ageing population. Age is a major risk factor for CVD, neurodegeneration

leading to Alzheimer's Disease (AD), Type-2 diabetes mellitus (T2DM), osteoarthritis, chronic obstructive pulmonary disease (COPD) and many cancers [104,105]. It has been established that having one age-related disease can make someone more susceptible to developing another [106,107], so tackling the problem at the initial stage would be far more beneficial, both in terms of improving quality-of-life of our ageing population and reducing the burden of disease on humanistic, societal and economic pressures. However, to date much more research has been done looking into individual age-related diseases.

A commonality between many of these diseases is dysregulation in the immune system and inflammation [4]. Therefore, of course macrophages, neutrophils and other cells that govern inflammation have been assessed for their roles in these diseases. Particularly, macrophage dysfunction has been found to be common in age-related disease, but it has not been established whether this is present in healthy ageing and exacerbated to cause age-related disease or occurs through an alternative mechanism of disease pathology. One example is M2-like macrophage dysfunction that is common in many cancers. These are referred to as tumour-associated macrophages (TAMs) and as they dampen the immune response and prevent inflammation, TAMs are strong influencers of tumour initiation, able to subvert immune cells to hide the cancer [108,109]. There is much research looking into targeting TAMs to eliminate cancer through removal, depletion, reprogramming or suppression of these cells [110]. Additionally, macrophages have been attributed to progression and pathology of other age-related diseases including AD [111] and T2DM [112].

1.4.1 Macrophages in atherosclerosis

According to the World Health Organisation, CVD is the number one cause of death globally, particularly heart attack and stroke which contribute to four out of five CVD deaths, including many that occur prematurely in people under the age of 70 years [113]. A major risk factor of heart attack and stroke is atherosclerosis. This is a highly researched disease associated with ageing that characteristically sees a build-up of fatty plaques in arterial walls. The progression of the disease, as

well as the role of macrophages in its progression, is well understood, partly due to its long-standing prevalence in society [114,115].

Despite our current knowledge, atherosclerosis remains a major problem around the world, which may be in part because the transcriptional profiles associated with the macrophage phenotypes within the atherosclerotic regions, and the way these change with age and disease progression, have not been elucidated. This knowledge may be crucial to better predicting lesion development and plaque stability and could therefore improve therapeutics and disease outcomes.

Atherosclerotic plaques generally occur in medium or large arteries, typically in arterial bifurcations where there are low or oscillatory shear stress patterns [116,117]. The makeup of the plaque has been well characterised as it progresses, as shown in Figure 1.5, with cholesterol, lipoproteins, inflammatory cells such as macrophages, foam cells and T lymphocytes, inflammatory cytokines, smooth muscle cells, collagen and elastin from the membrane of the blood vessel making up the plaque core [118].

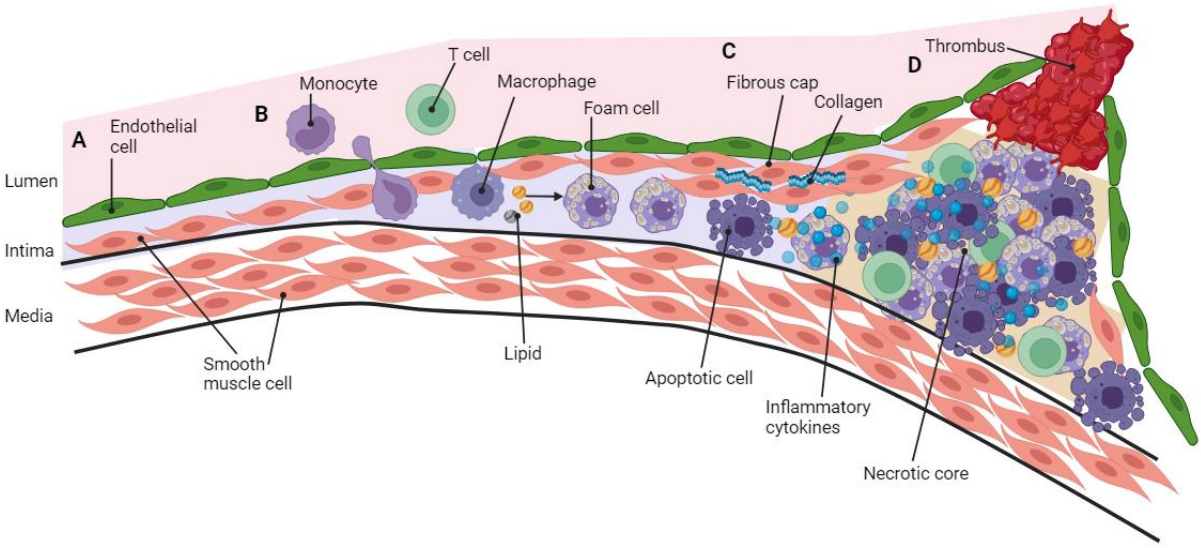

Figure 1.5 The progression of arterial atherosclerotic plaque

A – Healthy human artery with smooth muscle cells in the intima.

B – Early atherosclerosis characterised by increase in intima layer, monocyte and T cell infiltration and ingestion of lipids by macrophages to form foam cells.

C – Fibrous cap formation with smooth muscle cells and collagen, arterial wall bulging, lipid-laden and apoptotic cell accumulation.

D – Necrotic core formation and erosion of endothelium causing thrombus development.

Created in Biorender.

Atherosclerosis formation is thought to initiate from endothelial damage and build-up of LDL cholesterol and lipoproteins, that are retained in the arterial bifurcations and modified in the oxidant-rich environment to oxidised LDL [116,119,120]. Macrophages and smooth muscle cells recognise oxLDL via scavenger receptors and this stimulates an inflammatory response [121]. During the early stages of disease progression, oxLDL accumulation in both macrophages and smooth muscle cells can lead to apoptosis of the cell, which is then effectively cleared via macrophage efferocytosis [122]. However, there is no regulation in the amount of oxLDL that the arterial macrophages can internalise, meaning that in the later stages of the disease, macrophages accumulate more lipids and cholesterol ester and transform into foam cells [121,123]. This can lead to endoplasmic reticulum stress and therefore necroptosis of the cell [124]; efferocytosis then becomes defective. This leads to the release of inflammatory mediators, proteases and coagulation factors, each contributing to the progression of plaque vulnerability [125]. The chronic inflammation within the plaque also means that

the phagocytic cells become defective and inflammation is unresolved [126]. Plaque necrosis follows, making the plaque unstable, and as the disease progresses further, risk of plaque rupture, thrombosis, stroke or other cardiovascular event increases [127].

Through mouse models, it has been established that knocking out macrophages diminishes the pathology of atherosclerosis, suggesting their essential role in its progression [128]. Macrophage heterogeneity significantly impacts atherosclerosis with many macrophage phenotypes being characterised in atherosclerotic lesions. Inflammatory macrophage polarisation occurs via endogenous TLR and scavenger receptor ligands, such as oxLDL activating these receptors leading to stimulation of the NFKB pathway, and activation of the NLRP3 inflammasome [129]. Their roles in atherosclerosis include aggravating formation of the atherosclerotic plaque and expansion of the necrotic core through pro-inflammatory cytokine production [130]. In contrast, M2-like macrophages are stimulated by the presence of certain lipid products and immune complexes, as well as Th2 cytokines produced by engulfing apoptotic cells. Their polarisation is dependent on STAT6 and these macrophages exhibit increased phagocytosis and efferocytosis and reduced foam cell formation leading to reduced inflammation and plaque regression [131].

A plethora of additional macrophage phenotypes have been identified in atherosclerotic lesions [9]. M4 macrophages are a subset of pro-inflammatory macrophages induced by the chemokine CXCL4 that upregulate expression of IL-6, TNF and matrix metalloproteinase (MMP)-7 [132]. They also reduce phagocytosis by downregulating the scavenger receptor CD163 and contribute to plaque instability. Two additional subtypes have been characterized as anti-inflammatory, denoted M(Hb) and Mhem, that are induced by haemoglobin and heme, respectively [133]. M(Hb) macrophages upregulate IL-10 causing a decrease in iron and ROS production and increase in CD163 and cholesterol efflux [134]. Similarly, Mhem macrophages upregulate IL-10 and HMOX-1, leading to plaque stabilisation and tissue regeneration, as well as decreasing foam cell formation [135]. Finally, an antioxidant macrophage, Mox, has been established that is largely induced by oxidised phospholipids. Mox macrophages

decrease oxidative stress and do not readily perform phagocytosis. The characterisation of each of these subsets highlights the role of the macrophage as both athero-protective and atherogenic in disease progression [136]. Fundamentally, macrophages are involved in oxLDL clearance, efferocytosis, plaque stabilisation, foam cell formation and necrotic core formation, and so understanding the mechanisms and alterations in gene expression driving the phenotypic shifts and functions of macrophages in atherosclerotic plaques and in ageing is of key importance in better preventing and treating the disease.

1.5 Transcriptional networks in macrophages and/or ageing

As previously mentioned, the mechanisms underpinning macrophage function are not fully understood, involving a highly coordinated and complex cascade of signalling pathways [137]. However, transcription factors are beginning to be attributed to more functions, such as that of Maf that has been associated with phenotypic switching of the macrophage during the inflammatory response [33]. Transcription factors are proteins able to modulate gene expression, through binding enhancers and promoters of the genes they regulate, enabling or preventing binding of RNA polymerase and allowing transcription to occur or not [138,139]. This then influences various cellular processes and functions, such as the response to environmental stimuli [138,139]. A number of other transcription factors have been well associated with macrophage responses, such as PU.1 that has a long-standing association with macrophage differentiation and proliferation [140,141], NFKB in the activation of macrophages following inflammatory stimulation, allowing phagocytosis and bacterial killing to occur [142,143], interferon regulatory factor (IRF) transcription factors involved in macrophage response to interferons and regulation of phenotype heterogeneity [144] and hypoxia-induced factor (HIF) transcription factors which have numerous roles in macrophage biology, including promotion of M1 or M2 polarisation through maintaining nitric oxide homeostasis [145] and inducing chemokines and glucose metabolism to modulate migration [146–148].

Dysregulation of transcription factor activity can have various consequences, including many age-related diseases such as cancer and cardiovascular disease. For example, dysregulation of HIF-1α signalling in macrophages has been associated with necrotic core formation in atherosclerosis [149], while dysregulation of Forkhead box O (FOXO) transcription factors is well associated with ageing, as they regulate genes involved in the stress response, DNA repair, apoptosis and cellular metabolism [150]. It is likely that dysregulation in transcription factor activity is contributing to many of the negative effects of ageing, including age-related macrophage dysfunction. Therefore, understanding these mechanisms could aid in the development of better targeted therapies to promote healthy ageing and prevent age-related diseases.

1.5.1 MYC

MYC is a proto-oncogene and transcription factor that has three conserved box domains at the N terminus and a basic helix-loop-helix (bHLH) leucine-zipper domain at the C terminus that forms a zipper structure with MAX to exert its transcriptional regulation effects [151]. MYC has an extremely large gene network, potentially including 15% of all human genes, making it a global regulator of transcription [152]. It mediates a vast range of biological events including proliferation, differentiation, cell survival, programmed cell death, metabolism, protein synthesis and immune regulation [153]. Under normal conditions, MYC expression is tightly regulated [151]. It increases rapidly at the initiation of the G1 phase of the cell cycle and then rapidly decreases again [154]. It is also known that expression of MYC decreases in response to DNA damage leading to apoptosis of the cell via p53 pathways [155–158]. However, dysregulation in expression of MYC is well known to contribute to cellular dysfunction and tumorigenesis [159]. This dysregulation in expression is not thought to be as a result of mutations to the DNA, rather it has been proposed that epigenetics have a role in MYC overexpression [160].

MYC-driven cancers may also be able to subvert targeting by the immune system through the ability of MYC to push macrophages towards an M2 phenotype, dampening inflammation and suppressing immune cell infiltration at the tumour microenvironment, while increasing tumour cell proliferation

[161,162]. These anti-inflammatory macrophages are often referred to as TAMs and have been shown to play a pivotal role in the progression of many cancers [163]. MYC is a known marker of M2 macrophage polarisation, both in murine and human populations [164–166]. However, its role in the macrophage is still not completely understood [167].

MYC also has a complex association with senescence, whereby MYC downregulation can lead to senescence phenotypes [168] and MYC activation can dampen down p53-p21 and p16INK4a pathways to suppress senescence, exploited in many cancers [169]. When overexpressed, MYC was the first oncogene to be shown to induce senescence, acting as a tumour suppressor [158,170]. However, MYC overexpression is also required for this mechanism to be bypassed in cancer [171]. Additionally, mice with deletion of one copy of Myc from the genome show increased lifespan alongside a reduction in cancer and several age-associated pathologies [172], while Myc inactivation in juvenile mice has been shown to give rise to many features of premature ageing and has been proposed as a model for studying ageing that is better recreated prematurely than existing models [173]. Overall, this further highlights the complexity and pleiotropy of MYC biology; much like the macrophage it has an extremely diverse role in health and disease, including ageing, that is not yet fully understood.

1.5.2 Upstream stimulatory factor

Upstream stimulatory factor (USF) 1 is also a bHLH leucine zipper transcription factor that binds E-box elements in the DNA sequences of target genes [174,175]. It forms a heterodimer with USF2 and has a ubiquitous role in regulation of transcription in a variety of cellular processes that underpin energy homeostasis, lipid metabolism and cholesterol, triglyceride and glucose level maintenance [176–178], regulating key genes such as apolipoproteins in response to metabolic cues [179]. It also has established roles in cell proliferation and differentiation [180] and is a common marker of macrophages and other myeloid cells [181]. USF1 has been attributed to the development of many diseases. However, the transcriptional regulation of USF1 itself is not yet understood [179].

USF1 has been implicated in metabolic disorders such as metabolic syndrome, where patients have high blood cholesterol and triglyceride levels attributed to perturbed expression of USF1 [178], and T2DM where glucose and lipid metabolism are also dysregulated [182]. USF1 has also been assessed in atherosclerosis [183], its deficiency has been associated with improved cardiometabolic health [184]. Genetic variations in USF1 have been linked to familial combined hyperlipidaemia [185], increased risk of CVD [186] and incidence of T2DM [182]. In addition, USF1 is associated with pathogenesis of several cancers, including liver, oral and gastric cancers [187–189], as well as poor prognosis of glioma, as it promotes glioma cell proliferation and stemness, and the immunosuppressive nature of TAMs in the tumour microenvironment [190]. Finally, USF1 has been assessed in macrophages in the context of atherosclerosis, where it has been found that silencing USF1 increased cholesterol removal from macrophages. Additionally, these macrophages had a reduced inflammatory burden, indicating that USF1 plays a role in pro-inflammatory cytokine secretion by macrophages [191].

1.5.3 Epigenetics

The role of epigenetics in ageing is becoming better understood, with epigenetic drift being a cellular hallmark of ageing [59]. Epigenetics encompasses changes to gene expression that are not as a result of alterations to the DNA sequence itself, rather chemical alterations to DNA or associated proteins that are thought to be reversible [192]. Epigenetic modifications play a role in regulating many biological processes in homeostasis and disease [193], the main types being DNA methylation that influences gene expression through modifying the accessibility of DNA to regulatory proteins such as transcription factors [194], and histone modifications including acetylation, methylation, phosphorylation and ubiquitination that influence the accessibility of chromatin to affect gene expression [195–198]. Epigenetic drift refers to the changes in epigenetic marks such as DNA methylation patterns, the most common and well researched being 5mC [199], that occur over time in cells and tissues [200]. These age-associated changes have been found both in specific individual gene loci [201,202] and globally/genome wide, where hypomethylation occurs with increasing age [203]. It is also well established that low density CpG regions tend to start out as methylated and therefore undergo

hypomethylation with age, whereas high density CpG regions undergo an age-associated hypermethylation [204]. In human whole blood, these sites of age-associated hypermethylation have been found to preferentially occur at the promoters of developmental genes such as in embryonic stem cells, including those known to be tumour suppressors or transcription factors necessary for differentiation [204–207]. Age-associated changes in DNA methylation occur in many different assessed tissues [206,208,209], and have also been validated in bone marrow-derived mesenchymal stem cells [205] and haematopoietic progenitor cells [210] that remain in differentiated cell populations and could therefore be the cause of similar age-associated changes in methylation patterns seen across tissues [200]. It has also been found that these age-associated patterns of DNA methylation are similar to what has also been observed in cancer [205]. Overall, it is thought that the machinery responsible for maintaining normal DNA methylation patterns becomes dysregulated with age, leading to this epigenetic drift. However, the functional consequences and effect on gene expression are also not yet well understood [200].

As epigenetic changes are considered reversible, there is a drive to find therapeutics able to restore the epigenome, particularly in the context of cancers [211,212]. Small molecule drugs targeting enzymes involved in the epigenome, including DNA methyl transferases needed for DNA methylation [213], and histone deacetylases needed for histone acetylation [214], already exist to disrupt transcription, so far mainly focusing on tumour suppressor and DNA repair pathway genes [215,216]. Such treatments are yet to be investigated in the context of ageing and epigenetic drift.

1.6 Therapeutic interventions in ageing

There are many therapeutics able to treat the symptoms of individual age-related diseases, often with numerous side effects that can even necessitate additional treatment. Geroscience research is therefore trying to identify treatments against the burden of ageing as a whole, able to slow the progression of multimorbidity before it occurs. This includes attempting to both repurpose existing therapeutics and find new compounds with the same potential. This has led to the development of

DrugAge, a database that has collated therapeutics with anti-ageing properties, such as lifespan extension in model organisms, that may be beneficial and require further study [217].

There are many different compounds or druggable targets for anti-ageing with local or systemic effects. One class of anti-ageing drugs is senolytics, which work to remove senescent cells from the body by inducing cell death leading to reduced inflammation and increased health. Senolytics currently under investigation include BCL-2 inhibitors such as Fisetin, FOXO4-related peptides and GLS1 inhibitors [218–220]. Additionally, senomorphic agents act to suppress the negative effects of senescence through reducing SASP. This class of drugs includes Rapamycin and other rapalogs [221,222]. Rapamycin is a currently approved treatment for organ transplant rejection that is now also being investigated for treatment of ageing and age-related disease as it extends the lifespan of many model organisms [223,224]. It works to inhibit mammalian target of rapamycin (mTOR), a major regulator of cell signalling, growth, cytoskeleton organisation and metabolism, that has been implicated in many diseases and is important in longevity regulation. It has been suggested that rapamycin slows the effects of ageing directly rather than acting on lethal diseases [225,226]. The effect of rapamycin on macrophages is less well documented. Rapamycin has been found to restrict M1 macrophage activation by increasing autophagy, reducing production of mitochondrial ROS through NRF2 signalling and reducing pro-inflammatory cytokine production [227]. Contrastingly, it has also been found to skew polarisation of human monocyte-derived macrophages towards an M1 phenotype, which may be beneficial in diseases with an M2 macrophage dominance [228]. However, it is not fully understood whether rapamycin exerts its effects directly on macrophages to improve their function.

Metformin has also been assessed for its potential to be repurposed as an anti-ageing therapeutic. It is a widely used drug for the treatment of T2DM that was also found in clinical trials to have anti-ageing effects, including reduced morbidity and delayed mortality compared with controls [229]. Although much work is still needed to properly understand its effects, studies have already looked at the potential effects of metformin on macrophages. Initial findings indicate that it causes both a

reduction in M1-like macrophages to reduce production of pro-inflammatory cytokines and ROS, and an increase in M2-like macrophages that contribute to tissue repair and accelerated wound healing [230–232]. In studies of T2DM, it is already known that metformin acts to modulate inflammatory gene expression [233]. In osteoarthritis, metformin therapy has been shown to suppress macrophage infiltration and differentiation towards a pro-inflammatory phenotype [234]. In the context of atherosclerosis, metformin has been found to reduce monocyte differentiation into macrophages, foam cell formation, inflammation and apoptosis and increase macrophage polarisation towards an anti-inflammatory phenotype [230].

There are many other potential therapeutics on the DrugAge database [217] including Zoledronate, a bisphosphonate used to treat bone diseases, and Fisetin, a flavonoid found in many plants that acts as an anti-oxidant, that are discussed in Chapter 6. Although many of these appear promising, much more research is still needed to understand their full effects and mechanisms of action that contribute to improved lifespan. For example, it has not yet been established whether they exert positive effects on macrophages, or indeed if macrophage function can be improved in older individuals, despite the extensive evidence suggesting their dysfunction with age.

1.7 Summary

In summary, it is clear that macrophages have a significant impact on every physiological system, residing in each tissue as well as the bone marrow, where macrophages are derived from monocyte precursors. While much is known about how they function within the immune system during homeostasis, including pathogen clearance, initiating the adaptive immune response, inflammation resolution and efferocytosis of dying cells, there is a lack of robust evidence as to how age-related alterations in macrophages arise. Clearly, macrophages are key instigators of inflammaging and many age-related diseases such as atherosclerosis, they undergo functional decline including reduced pathogen killing, tissue repair and removal of senescent cells. However, current knowledge focuses on single disease systems, tissues and mouse models; little work has gone into addressing how

ageing alone affects macrophage function and particularly human primary macrophages that are expanded in response to inflammation; the molecular mechanisms driving age-related changes and their contribution to the overall ageing phenotype is largely unknown. In addition, anti-ageing therapeutics are beginning to be assessed in combination with current treatments for age-related diseases. Understanding how these treatments effect macrophages may shed more light onto the molecular mechanisms underpinning their dysfunction and improve their potential as therapeutic targets.

The true contribution of macrophage dysfunction to unhealthy ageing has not yet been fully elucidated, as well as whether macrophage age-related dysfunction can be reversed, either through transcriptional manipulation or therapeutic targeting. Gaining this knowledge could be key in further understanding the ageing phenotype that is becoming commonplace in our ageing population.

1.8 Hypothesis and Aims

Although macrophage ageing has been well characterised in mouse models or tissue macrophage populations, there is still a lack of consensus as to the mechanisms underpinning functional changes. Furthermore, there is a lack of published research addressing human macrophage ageing and the corresponding underlying mechanisms.

We hypothesise that human macrophages have an attenuated ability to function with age, and that there are specific dysregulated transcriptional networks driving these changes. The aims of our research are as follows:

Chapter 3. Identify what is known already about macrophage ageing

Chapter 4. Using published datasets, identify age-related dysregulation in transcription factor expression in macrophages and optimise techniques for assessment of human primary macrophage ageing

Chapter 5. Identify functional changes with age in human primary macrophages and hone in on the transcription factors regulating this dysfunction

Chapter 6. Assess whether macrophage function in ageing can be restored through modulation of gene expression and senolytic treatments

Chapter 2. General Materials and Methods

The methods used throughout my thesis are presented here. Each results chapter will refer back to these methods to avoid duplication and improve pagination, unless methods are unique to the chapter.

Declaration of Contribution

This chapter was written by myself, without contribution from others.

2.1 Ethical considerations

All experiments from human samples were conducted following approval from the University of Sheffield Research Ethics Committee. The project title is "Studies of the function of platelets and leukocytes in blood and plasma from healthy volunteers" and application reference number is 031330. The Research Ethics Committee also approved participant information sheet 1071555 (V1, 06/12/2022; Supplementary figure 8.1.1) and participant consent form 1071553 (V1, 06/12/2022; Supplementary figure 8.1.2). This approval allowed us to take 80 mL fresh whole blood by venepuncture from volunteers over the age of 18 years. Age, sex and current medication were recorded while gaining written, informed consent.

For experiments on mice, femurs and tibias were provided for bone marrow isolation, made available from other studies. All experiments were performed in accordance with UK legislation under the Animals (Scientific Procedures) Act 1986. The University of Sheffield Project Review Committee approved all animal experiments which were carried out under UK Home Office Project Licence P5395C858.

2.2 Dataset identification

The human RNAseq data assessing changes in macrophage transcript expression with loss of *MYC* or *USF1* were deposited in GEO database, accession number GSE240075. The pipeline for analysis of this dataset in R is deposited in github, at:

https://github.com/cemoss1/Moss_et_al/blob/main/RNAseq_pipeline.R

2.3 Cell culture and maintenance

2.3.1 Human monocyte isolation and differentiation to monocyte-derived macrophages

Human blood was collected from gender-balanced healthy volunteers aged 18-30 years (young cohort) or > 50 years (older cohort). Specific age ranges for experiments can be found in results chapters and figure legends. Whole blood was drawn by venepuncture and mixed with 3.8% (w/v) trisodium citrate dehydrate ($Na_3C_6O_7$ X H_20; Sigma). Blood was layered onto Ficoll-Paque PLUS (GE

Healthcare), used to perform gradient centrifugation. The peripheral blood mononuclear cell (PBMC) layer was separated and collected into phosphate-buffered saline (PBS) containing 2 mM EDTA (Fisherbrand). Cells were then incubated with red blood cell (RBC) lysis buffer, a solution of ammonium chloride made up of 155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 M EDTA in H_2O, for 5 minutes. Monocytes were isolated by positive selection using $CD14^+$ magnetic beads (Miltenyi Biotec). This technique allows for isolation of both $CD14^{high}$ and $CD14^{low}$ monocytes to ensure all subsets are selected [235] (Figure 2.1). Monocytes were then differentiated to monocyte-derived macrophages (MDMs) by incubation for 7 days at 37°C and 5% CO_2 in RPMI1640 (Gibco) containing 10% (v/v) heat-inactivated foetal bovine serum (FBS; Pan Biotech), 1% (v/v) L-Glutamine (Lonza Biosciences), 1% (v/v) penicillin-streptomycin (Gibco) and 100 nM macrophage colony stimulating factor (M-CSF; Peprotech).

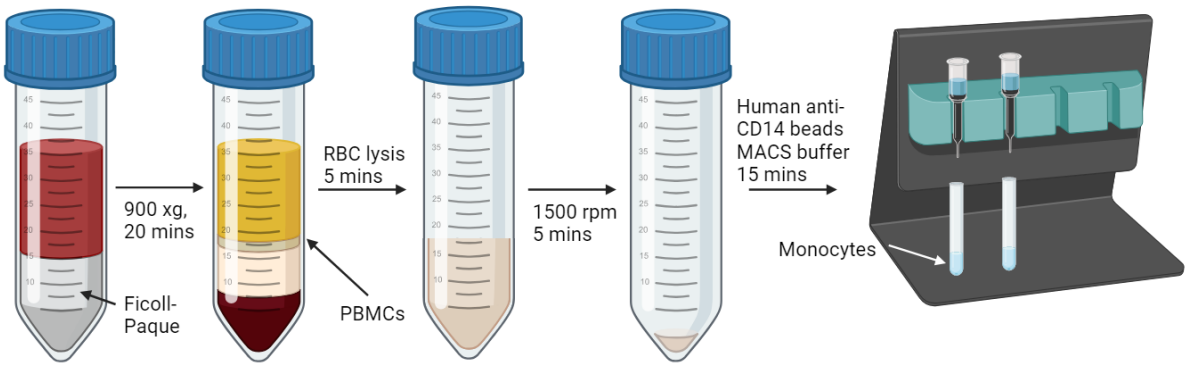

Figure 2.1 Monocyte isolation protocol for monocyte-derived macrophage differentiation

Whole blood was collected from human volunteers and monocytes were isolated through a series of centrifugation steps, lysis of red blood cells and positive selection of CD14+ cells by magnetic cell separation. Following isolation, monocytes were counted and plated with M-CSF for macrophage differentiation. Created in Biorender.

RBC: red blood cell; PBMC: peripheral blood mononuclear cell; M-CSF: macrophage colony stimulating factor

2.3.2 Murine bone marrow isolation and differentiation to bone marrow-derived macrophages

Mouse bone marrow was isolated from femurs and tibias of young (2-5 months) or old (20-22 months) male C57BL/6 mice. Mice were used where available from various other research, with treatments described in Table 2.1.

Table 2.1 Bone marrow-derived macrophage murine origin

Denotation	Mouse age	Treatment	Diet
Young mouse	2-5 months	WT	Standard diet
Old mouse	20-22 months	WT	Standard diet
Zoledronate-treated old mouse	20-22 months	WT	Standard diet with Zoledronate added
Atherosclerotic old mouse	20 months	WT, PCSK9-injected	Western diet
Fisetin-treated atherosclerotic old mouse	20 months	WT, PCSK9-injected	Western diet with 2 week intermittent periods of Fisetin added

PCSK9-injected mice were injected with *iv* rAAV8/mPCSK9 6 x 10^{11} vg total.
PCSK9: proprotein convertase subtilisin/kexin kinase type 9

To isolate bone marrow, under aseptic conditions, both ends of each bone were cut and flushed through using a 2.5 mL syringe fitted with a 25-guage needle into DMEM (Gibco) containing 10% (v/v) heat-inactivated FBS (Pan Biotech). Bone marrow cell suspensions were then passed through a 40 µm cell strainer and centrifuged at 500 *g* for 5 minutes. Cells were resuspended and differentiated into bone marrow-derived macrophages (BMDMs) by incubation for 5 days at 37°C and 5% CO_2 in DMEM (Gibco) containing 10% (v/v) heat-inactivated FBS (Pan Biotech), 1% (v/v) penicillin-streptomycin (Gibco) and 10% (v/v) L929 cell-conditioned medium (taken from growing L929 fibroblasts that secrete M-CSF).

Where cells were not used immediately, following cell straining and centrifugation, they were resuspended in 90% (v/v) heat-inactivated FBS (Pan Biotech) and 10% dimethyl sulfoxide (DMSO) and frozen slowly to -80 °C. They were then stored in liquid nitrogen.

2.3.3 Immortalised bone marrow-derived macrophage culture

Immortalised bone marrow-derived macrophages (iBMDMs) were gifted by David Brough at the University of Manchester, generated from wild-type male mice following a protocol detailed elsewhere [236]. Cells were cultured in DMEM (Gibco) containing 10% (v/v) heat-inactivated FBS (Pan Biotech), 1% (v/v) penicillin-streptomycin (Gibco) and 1% (v/v) sodium pyruvate (Lonza). Cells were passaged every 2 days by cell scraping.

2.3.4 Transient transfection of small interfering RNA constructs *in vitro*

Transient transfection using small interfering RNA (siRNA) was performed in both the iBMDM murine cell line for optimisation of gene silencing and human MDMs. In the iBMDMs, siRNA targeting *Myc* gene (ON-TARGET plus smartpool siRNA #L-040813; Dharmacon) or *Usf1* gene (ON-TARGET plus smartpool siRNA #L-040656; Dharmacon) was mixed with different transfection reagents (Fugene, Dharmafect 3, RNAi Max and Lipofectamine 3000) at different concentrations according to manufacturer recommendations to find optimal conditions. In human MDMs, following 7 days of macrophage differentiation, siRNA targeting *MYC* gene (ON-TARGET plus smartpool siRNA #L-003282-02; Dharmacon), *USF1* gene (ON-TARGET plus smartpool siRNA #L-003617; Dharmacon) or *ATP8B1* gene (ON-TARGET plus smartpool siRNA #L-019995; Dharmacon) was mixed at 12.5 nM with Fugene HD transfection reagent following the manufacturer's instructions. This was incubated with MDMs for 72 hours in Opti-Mem media (Gibco). As a control, non-targeting siRNA (ON-TARGETplus smartpool control non-targeting siRNA #D-001810-10-20; Dharmacon) was used at the same concentration and conditions. Knockdown was confirmed after 72 hours by RT-qPCR for each donor.

2.3.5 Macrophage polarisation *in vitro*

Following macrophage differentiation (and transfection where applicable), cells were either left untreated, denoted M^0, or were further stimulated to $M^{LPS+IFN\gamma}$ or $M^{IL\text{-}4}$ as follows, representing a pro- and anti-inflammatory macrophage phenotype, respectively:

$M^{LPS+IFN\gamma}$ – LPS (100 ng/mL; Enzo) and IFNγ (20 ng/mL; PeproTech) for 24 hours

$M^{IL\text{-}4}$ – IL-4 (20 ng/mL; PeproTech) for 24 hours

After stimulation, functional assays were performed or cell pellets were collected for gene expression profiling.

2.4 Functional assays

Functional assays were performed on human monocytes and MDMs following isolation, differentiation, transient transfection and/or polarisation.

2.4.1 Phagocytosis assay

Human MDMs were cultured as above in 12-well plates in 1 mL of media at a density of 500,000 cells per mL with a 4°C control and 37°C active plate. For monocyte studies, monocytes were isolated and 500 mL of cells were immediately seeded into 24-well plates at a density of 500,000 cells per mL in antibiotic-free RPMI. Fluoresbrite YG microspheres 1.00 µm (Polysciences) were opsonised with heat-inactivated FBS (Pan Biotech) at 37 °C for 30 minutes. Five minutes before the end of this incubation, the control 4 °C cells were placed on ice to get to temperature. Opsonised beads were added to each well at a concentration of 50 µL/mL and cells were incubated at 4 °C or 37 °C for three hours. For phagocytosis of *Staphylococcus aureus* by monocytes, mCherry expressing *S. aureus* was added to the cells at a multiplicity of infection of 10 and cells were again incubated at 4 °C or 37 °C for three hours. Cells were then washed twice with PBS and fixed with 4% paraformaldehyde (Fisher Scientific) for 30 minutes at room temperature. Cells were again washed with PBS three times and stored in PBS at 4 °C until microscopy. Images were taken using ZOE Fluorescent cell imager (BIO-RAD) at 20x zoom. Brightfield (gain: 5, exposure: 340 ms, LED intensity: 59, Contrast: 20), green channel (gain: 40, exposure: 500 ms, LED intensity: 43, contrast: 0) or red channel (gain: 60, exposure: 500, LED intensity: 72, contrast: 0) images were taken for each field of you, with three images being taken per temperature, condition and donor. Images were analysed on ImageJ software v2.9.0/1.53t using the "Analyse particles" function; the same threshold was set for each brightfield image. The image was then converted to mask and regions of interest (ROIs) were created around each cell. ROIs were then overlayed onto the corresponding green or red channel image and mean gray value was calculated for each cell. A geometric mean for each donor condition was then calculated and normalised to the corresponding control 4 °C image. An average was then taken for each donor condition.

2.4.2 Scratch assay

Human MDMs were cultured as above in 12-well plates in 1 mL of media at a density of 500,000 cells per mL. A scratch was drawn through the adherent cells from the top to bottom of each well, cells were washed with PBS and fresh media was added. Using the ZOE fluorescent cell imager (BIO-RAD)

at 20x zoom, brightfield images of the scratch were immediately taken (Gain: 5, Exposure: 340 ms, LED intensity: 59, Contrast: 20) and used as a 0 hour threshold of the number of cells in the scratch. Three images were taken of each scratch, equating to 9 images per condition and donor, and an average number of cells in the field was calculated. Cells were then returned to the incubator for 6 hours, from which point images were taken every 2 hours to the same specification until 14 hours had passed. For analysis of number of cells returning to the scratch, a box was drawn around the scratch at hour 0 and copied over to all other images taken of that scratch. The cells inside each box were then counted and an average was taken from all fields at each timepoint from each donor. This was normalised to hour 0 by subtracting the initial number of cells in the box.

2.4.3 MCP-1 transwell migration assay

Human MDMs were cultured as above in 6-well plates in 2 mL of media at a density of 500,000 cells per mL and then collected using a cell scraper. For monocyte studies, freshly isolated monocytes were counted and resuspended in media at the same density. Cells were centrifuged at 400 *g* for 5 minutes and stained using PKH26 red fluorescent cell linker (Sigma-Aldrich) to better visualise the cell membrane, according to the manufacturer's instructions. Two minutes after dye was added, cells were mixed by pipetting and then an equivalent volume of 1% bovine serum albumin (BSA; Sigma-Aldrich) was added to the cells to remove excess dye. Cells were again centrifuged at 4000 *g* for 5 minutes and resuspended at a density of 1 million cells per mL in 300 µL RPMI 1640 (Gibco) in order to run each sample in triplicate. Using HTS Transwell 96-well permeable support (Fisher Scientific), 100 µL of 1% (v/v) MCP-1 (Peprotech) in RPMI 1640 (Gibco) was added to the bottom well and 100 µL of cell suspension was added to the transwell insert and this was incubated for 3 hours at 37 °C and 5% CO_2. The transwell insert was removed and the bottom plate was imaged using ZOE fluorescent cell imager (BIO-RAD) at 20x zoom including brightfield (gain: 5, exposure: 340 ms, LED intensity: 59, contrast: 20) and red channel (gain: 40, exposure: 500 ms, LED intensity: 50, contrast: 0). Three fields of view were taken per sample. ImageJ v2.9.0/1.53t was then used to analyse each

image by measuring overall mean gray value of the field and an average for each donor and condition was taken.

2.4.4 Actin staining

Human MDMs were cultured as above in 0.5 mL of media as a density of 500,000 cells per mL onto 13 mm cover slips in 24-well plates and fixed with 4% (v/v) formaldehyde (Fisher Scientific) for 10 minutes at room temperature and then kept in PBS at 4 °C. Samples were then blinded so that microscopy and analysis could be conducted without knowledge of condition or donor being assessed. Coverslips were then removed onto parafilm and cells were washed with PBS and quenched with ammonium chloride for 10 minutes before being washed again with PBS. Cells were then permeabilised with 0.1% (v/v) Triton-X diluted in PBS for 4 minutes. Cells were stained with Fluorescein Isothiocyanate labelled Phalloidin (Sigma-Aldrich) for 20 minutes and washed with PBS and then water. Coverslips were mounted onto slides with Mowiol and kept in the dark for at least 24 hours to dry before imaging.

2.4.4.1 Microscopy

Images were taken using a Nikon Ti-E with CFI Plan Apochromat λ 20X, N.A.0.75 objective lens. Four fields of view per condition per donor were taken and analysed using ImageJ software v2.9.0/1.53t. Each cell was manually drawn around and then area, circularity (shape descriptors) and mean gray value were measured. A circularity score of 1.0 indicates a perfect circle whereas values approaching 0.0 indicate an increasingly elongated shape. The median for area and circularity and geometric mean for mean gray value were then calculated for each image and an average was taken. PCA was used to analyse clustering of the cells according to size, circularity and fluorescence.

2.5 Gene expression profiling

2.5.1 RNA isolation

Total RNA isolation was performed using ReliaPrep RNA Cell Miniprep System (Promega) according to the manufacturer's instructions. BL+TG lysis buffer was added to the cell pellet, followed by 100%

isopropanol and cells were vortexed for 5 seconds. Lysate was then transferred to a minicolumn and washed with RNA wash solution. DNase I solution was then added to the column and incubated at room temperature for 15 minutes. The column was then washed again with column wash solution and RNA wash solution and RNA was then eluted from the column in RNase-free water. RNA concentration and purity were measured using NanoDrop™ Spectrophotometer and RNA was then stored at -80 °C. For RNA sequencing, RNA integrity and concentration were also assessed by agarose gel electrophoresis and using Bioanalyzer Agilent 2000.

2.5.2 cDNA synthesis

In order to convert RNA to cDNA, iScript cDNA synthesis kit (BIO-RAD) was used according to manufacturer's instructions. 100 ng total RNA was transcribed to cDNA per reaction using the protocol and cycle conditions in Table 2.2.

Table 2.2 cDNA synthesis protocol

Reaction protocol	
Priming	5 minutes, 25 °C
Reverse transcription	20 minutes, 46 °C
Reverse transcription inactivation	1 minute, 95 °C
Hold/ cooling	Immediate, 4 °C
Storage	4 °C or frozen
Reaction component	**Volume**
5x iScript Reaction Mix	4 µL
iScript Reverse Transcriptase	1 µL
Nuclease-free water	To make up to 20 µL
RNA template (100 fg – 1 µg total RNA)	Variable
Total volume	20 µL

2.5.3 Real-time quantitative PCR

Real time qPCR was performed using Precision PLUS SYBR-green mastermix (Primer Design) and SYBR primers (Sigma-Aldrich) were designed using NCBI BLAST Primer Designing Tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer sequences can be found in Supplementary table 8.1.1 and Supplementary table 8.1.2 for human and mouse transcripts, respectively. The protocol and cycle conditions can be found in Table 2.3. Samples were assayed using CFX384-C1000 Touch Thermal Cycler (BIO-RAD). The specificity of each primer was confirmed

by melt curve analysis prior to overall analysis. Relative expression of the gene of interest (GOI) was measured using the $2^{-\Delta Ct}$ method, where ΔCt is the cycle threshold of the GOI normalised to the housekeeping (HK) gene using the following equation, where the Ct is the average of triplicate values:

$$\Delta Ct_{GOI} = Ct_{GOI} - Ct_{HK}$$

All assays were performed in triplicate and normalised to expression levels of housekeeping genes *PUM1* (human) or *Mau2* (mouse), as these were determined to be the most suitable across young and old samples.

Table 2.3 RT-qPCR cycle conditions (Primer Design)

Process step	**Settings**
Polymerase activation/ denaturing	2 minutes, 95 °C
Amplification	40 amplification cycles: 15 seconds, 95 °C 1 minute, 60 °C
Melting curve analysis	From 60 °C - 95 °C in 0.5 °C increments
Reaction component	**Volume**
SYBR Green Mastermix	5 µL
Forward primer, 10 µM	0.3 µL
Reverse primer, 10 µM	0.3 µL
Diluted cDNA 0.5 ng/µL	5 µL
Total volume	10.6 µL

2.6 RNA sequencing analysis

RNA sequencing was performed on the following samples, from six healthy young donors (22-25 years), deposited under accession number GSE240075. MDMs were differentiated and transfected as described in Section 2.3:

- M^0 control
- si*MYC* M^0
- si*USF1* M^0
- $M^{LPS+IFN\gamma}$ control
- si*USF1* $M^{LPS+IFN\gamma}$

An indexed pair–end sequencing run of 2 × 51 cycles on Illumina HiSeq 2000 was performed on all samples by Novogene. FASTQ files were provided which were mapped onto build 38 of the human genome using STAR [237]. The full pipeline of analysis in R is deposited in GitHub (https://github.com/cemoss1/Moss_et_al/blob/main/RNAseq_pipeline.R). Mapped transcripts were then converted to genes using tximportData in R v1.18.0 [238]. Using edgeR package in R v3.32.1 [239–241], counts per million were computed and used for principle component analysis (PCA). Pairwise differential gene expression analyses for each of the comparisons were performed using DESeq2 in R v1.30.1 [242] with non-targeting siRNA M^0 or $M^{LPS+IFN\gamma}$ control used as the reference for gene knockdown in M^0 or $M^{LPS+IFN\gamma}$, respectively. DEGs were identified using False Discovery Rate (FDR) of < 5%. Gene set enrichment analysis (GSEA) v4.2.3 [243], Enrichr (https://maayanlab.cloud/Enrichr/ accessed on 13.09.23) [244–246] and GOrilla (https://cbl-gorilla.cs.technion.ac.il/ accessed on 03.05.23) [247] analysis were performed on the DEG lists to output associated enriched pathways. The raw data files are deposited on the gene expression omnibus database, accession number GSE240075.

2.7 Microarray analysis

The in-depth pipeline of analysis of the published microarray dataset GSE84901, obtained from Wong *et al*. (2017) can be found in Chapter 4, where the dataset was used extensively to pull out candidate genes in macrophage ageing. This dataset was also used in Chapter 5, where a more concise method of its use can be found.

2.8 Statistical analysis

GraphPad Prism 9 software was used to generate all statistical analysis and graphs. N numbers and statistical analysis are stated for each experiment in the figure legends. Differences were considered to be significant when P values were < 0.05. Donors used in RNA sequencing were blinded prior to analysis. All macrophage samples used for microscopy were also blinded prior to imaging and analysis to prevent bias. Unless otherwise stated, six biological replicates were used for all experiments with each having three technical replicates. N = 6 donors for each condition was used

due to this being previously shown to give statistical power. Where possible, when comparing the same donor under different conditions, paired analysis was performed. ANOVA was used for time course experiments.

Chapter 3. Markers of the ageing macrophage: a systematic review and meta-analysis

This first results chapter is a published systematic literature review and meta-analysis of macrophages and ageing that was researched and written primarily by myself. It is included as a results chapter due to the findings in the meta-analysis.

Declaration of Contribution

Both myself and Hew Phipps did the initial screening of publications for the SLR. The code for the MAIC was run by Hew Phipps, which generated the data in Figure 3.5-3.7. These data were then analysed, interpreted and formulated by myself. All authors conceived the study and supported construction of the manuscript.

Markers of the ageing macrophage: a systematic review and meta-analysis

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Abstract

Introduction: Ageing research is establishing macrophages as key immune system regulators that undergo functional decline. Due to heterogeneity between species and tissue populations, a plethora of data exist and the power of scientific conclusions can vary substantially. This meta-analysis by information content (MAIC) and systematic literature review (SLR) aims to determine overall changes in macrophage gene and protein expression, as well as function, with age.

Methods: PubMed was utilized to collate peer-reviewed literature relating to macrophage ageing. Primary studies comparing macrophages in at least two age groups were included. Data pertaining to gene or protein expression alongside method used were extracted for MAIC analysis. For SLR analysis, data included all macrophage-specific changes with age, as well as species, ontogeny and age of groups assessed.

Results: A total of 240 studies were included; 122 of which qualified for MAIC. The majority of papers focussed on changes in macrophage count/infiltration as a function of age, followed by gene and protein expression. The MAIC found iNOS and TNF to be the most commonly investigated entities, with 328 genes and 175 proteins showing consistent dysregulation with age across the literature. Overall findings indicate that cytokine secretion and phagocytosis are reduced and reactive oxygen species production is increased in the ageing macrophage.

Discussion: Collectively, our analysis identifies critical regulators in macrophage ageing that are consistently dysregulated, highlighting a plethora of targets for further investigation. Consistent

functional changes with age found here can be used to confirm an ageing macrophage phenotype in specific studies and experimental models.

3.1 Introduction

Largely due to improvements in healthcare and living standards, those in the developed world are living longer, with people aged over 65 years making up nearly 20% of the UK population in 2021, compared with less than 11% in 1950 [2,248]. However, this increase in lifespan does not correlate with healthspan, thus much of our elderly population are living with poor quality of life, increasing pressure on societal resources including health and social care. Unhealthy ageing is often described in terms of multimorbidity, the presence of more than one age-related disease (ARD), with each additional ARD increasing the risk of developing another [249]. There is a wide overlap in the causes of developing ARDs, as they occur in no particular order and eventually affect many different organ systems [250]. Thus, geroscience research is steadily gaining ground, with the aim of finding biomarkers and therapeutic interventions to prevent progression of multimorbidity and the associated decrease in quality of life [54].

More recent advances have noted the crucial role of immune system functional decline in the progression of ARDs, in both innate and adaptive immune cells [251]. This is often referred to as immunosenescence [48] and has been linked to a reduced immune response to new and previously encountered infections, as well as vaccines [64]. The functional decline of the adaptive immune system, made up primarily of B-, T- and dendritic cells, is already well characterised [252–256]. The innate immune system is the first responder to pathogens, often starting with recognition by neutrophils and macrophages that go on to initiate the adaptive immune response [257]. Neutrophil phagocytosis and reactive oxygen species (ROS) production has been found to decline with age [258]. Neutrophil extracellular traps have also been shown to be defective with increasing age, both reducing their bactericidal activity [259]. Macrophages also undergo functional decline with age, and this will be discussed in depth throughout this review.

Macrophages are a diverse set of innate immune cells, collectively known for their phagocytic ability [6]. They are uniquely plastic in nature, making them able to shift and reprogram in response to environmental cues [260,261]. This puts them at the forefront of mounting an immune response, enabling them to further inform their surroundings through cytokine and chemokine production [262]. Macrophages also largely govern inflammation, at both the initiation and resolution phase [263]. These macrophage characteristics are largely dependent on stimuli within their microenvironment and different tissue macrophage populations can have widely different functions and appear phenotypically distinct at the transcriptional level [264]. Although the role of macrophages within different tissues is now largely understood, knowledge is lacking in the context of ageing, and there is a pressing need for research data to be collated and consolidated. Our current knowledge of macrophages in ageing tissues centres around inflammaging, a term used to describe the development of a persistent low-grade, sterile inflammatory phenotype that develops with age [55]. One possible cause for this is macrophage chronic activation, leading to production of more inflammatory cytokines that affect neighbouring cells and the tissue environment [265]. Chronic activation of the immune system is energetically costly, and the resultant effect is an inability to mount a full immune response and therefore fight off pathogens, much like that of immunosenescence [48,266].

As macrophages have now been implicated in age-related immune system decline and inflammaging, an increasing body of data assessing macrophage changes with age are being reported. However, data largely remain unstandardised, with the cellular ontogeny and tissue location proving crucial to corresponding function, reaction to stimuli, surface marker expression or cytokine secretion [264,267]. This review therefore aims to consolidate current knowledge on the ageing macrophage, in a way that allows for comparison between macrophage populations from different mammalian species and tissues. Specifically, it aims to highlight where there is consistency in changes in gene and protein expression during ageing and our current knowledge of tissue- and species-specific functional changes in the macrophage with age. We will present and discuss the role of macrophages in ageing

through a narrative review with a systematic approach as well as findings from a meta-analysis by information content (MAIC) in order to provide a comprehensive list of genes and proteins dysregulated with age, a useful tool both for modelling macrophage ageing and uncovering potential therapeutic targets for age-related functional decline.

3.2 Methods

The SLR and MAIC presented here were conducted adhering to PRISMA 2020 guidelines [268].

3.2.1 Search strategy

Two independent reviewers searched PubMed up to March 2022 for MAIC and December 2022 for SLR using the following search term:

> ((((Macrophage[Title/Abstract]) AND ((Ageing[Title/Abstract]) OR (Aging[Title/Abstract]))) OR ((Macrophage[Title/Abstract]) AND (Old[Title/Abstract])) OR ((Macrophage[Title/Abstract]) AND (Longevity[Title/Abstract])) OR ((Macrophage[Title/Abstract]) AND (Age[Title/Abstract])))

Any articles not in English and any duplicates were then removed.

3.2.2 Eligibility and selection process

Studies were included if they met the following criteria:

1. Full-text article available in English
2. Primary research article
3. At least one set of results directly comparing young vs. old samples, with old being defined by the article authors
4. At least one set of results directly assessing isolated macrophages

Additionally, studies were further marked for MAIC if they met the final criteria:

5. Data directly assessing a named gene or protein's expression in young and old macrophages

Where reviewers had differing opinions, studies were discussed in more depth until a consensus decision could be made. Both reviewers' selections were then combined.

3.2.3 Data extraction for systematic literature review

Data extraction for the SLR included the following information:

1. Whether the article fit the SLR, MAIC criteria, or both
2. General article information, such as the title, authors and study design
3. Experimental design including species looked at, type of macrophages assessed, age of groups assessed and experimental techniques used
4. Results pertaining to macrophage ageing

3.2.4 Data extraction for meta-analysis by information content

The MAIC algorithm used was developed by the Baillie Lab [269,270]. The online server and user interface is available at https://baillielab.net/maic (accessed on 01.12.22). Qualifying articles listing gene/protein hits were compiled into a text file to create a database (Figure 3.1). This database was indexed by separating each publication into lists based on the method used to obtain the gene/protein data. If a publication presented experimental results from three methods, the result would be three lists of the respective genes/proteins titled [Xa], [Xb], [Xc] and headed by their respective method.

Separate input files were generated in order to differentiate between genes and proteins, as well as whether expression was up- or downregulated with age, resulting in five separate input files (Supplementary table 8.2.1 – Supplementary table 8.2.6):

1. All entities (genes and proteins)
2. Genes upregulated
3. Genes downregulated
4. Proteins increased
5. Proteins decreased

The MAIC algorithm was executed on each input file using Python and output data were processed in Rstudio.

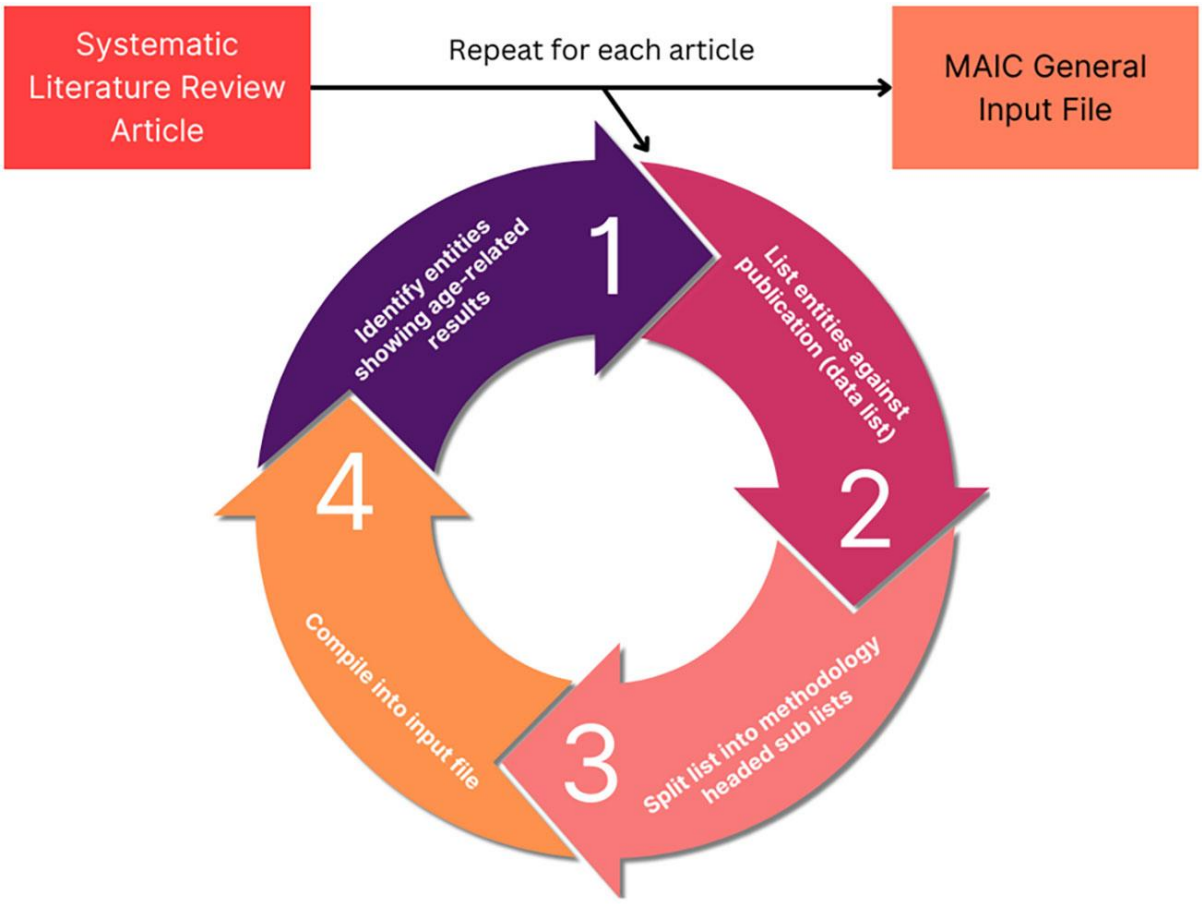


Figure 3.1 Meta-analysis by information content input data processing

The analysis process used to obtain MAIC data. The cycle is repeated for each qualifying study, meeting the inclusion criteria. Each list of data is placed into a tab delimited.txt file stacking into a super list with each data list occupying a row. In the case of the other 4 input files (genes upregulated, genes downregulated, proteins upregulated, proteins downregulated), "entity" is replaced with the respective data element, such as genes upregulated with age.

3.3 Results

3.3.1 Stratifying the literature for MAIC and SLR analysis

An initial search of PubMed identified 4,785 papers, relating to macrophages and ageing, as of December 2022 (Figure 3.2). This was refined to 4,222 full-text publications in English. 1,337 publications were excluded due to not containing primary research or being case reports. A further 2,177 publications were excluded due to lack of comparison between young and aged groups and 458 due to not containing macrophage-specific data on further inspection. This left 250 primary research articles that met all criteria for the systematic literature review. During data extraction, a further 10 papers were excluded due to lack of availability, meaning 240 publications were included

in this SLR. PubMed search was completed in March 2022 for the MAIC, at which time 3,639 articles were identified by our search strategy. 122 of these articles met the more stringent inclusion criteria, containing data pertaining to changes in genes or protein expression in the macrophage with age.

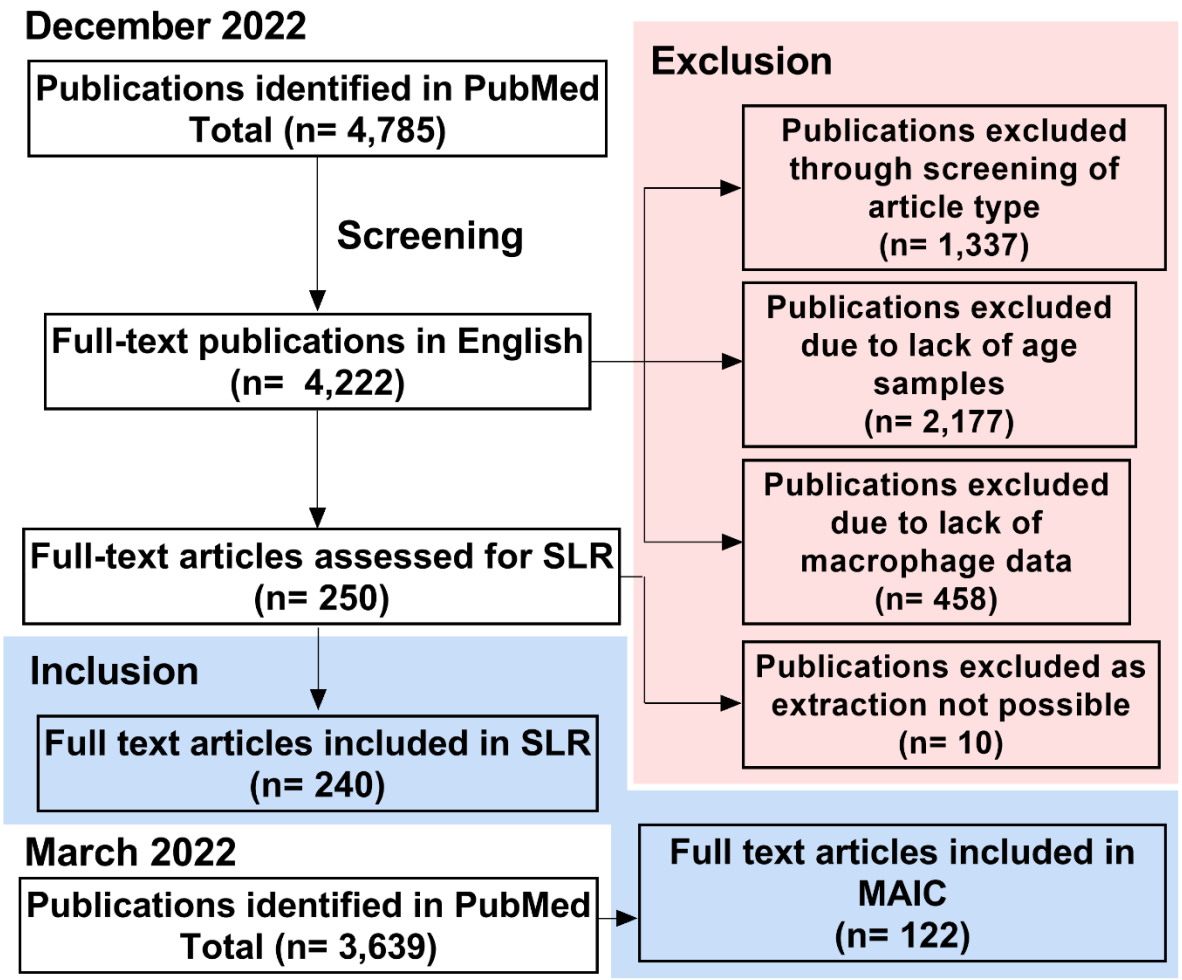


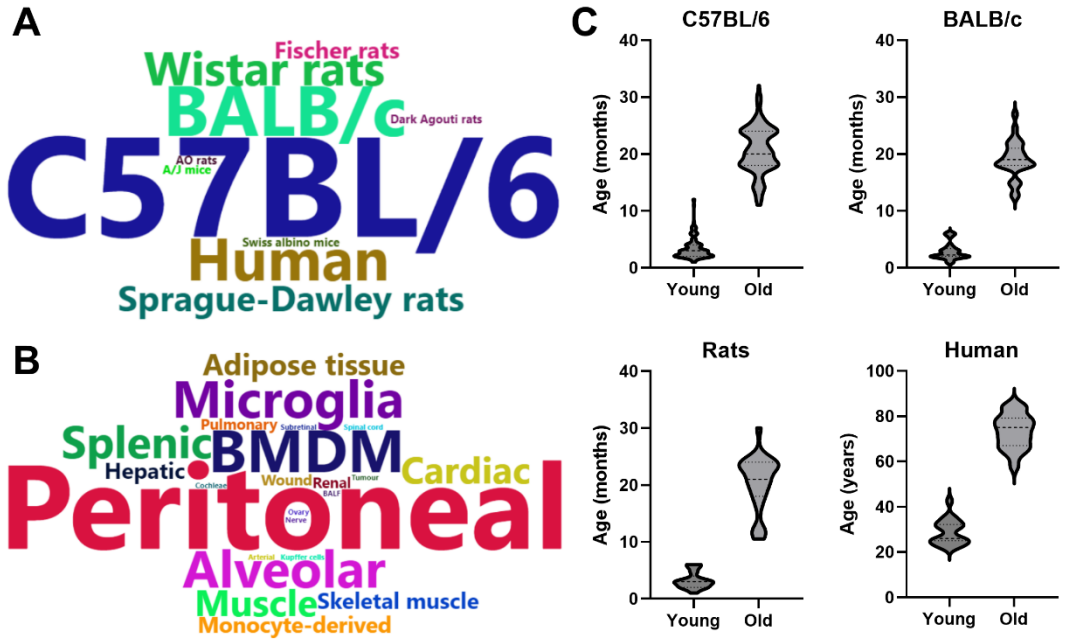
Figure 3.2 Study screening and selection process

Process of study inclusion and exclusion for systematic literature review (SLR) (completed December 2022) and meta-analysis by information content (MAIC) (completed March 2022).

3.3.2 Systematic literature review of the ageing macrophage

A summary of the publications that met inclusion criteria for the SLR can be found in Figure 3.3. Overall, the search strategy identified 240 unique primary research articles for data extraction, as described above. The majority of publications used C57BL/6 mice to study ageing (n = 155), with BALB/c mice (n = 25), humans (n = 17) and Wistar rats (n = 12) being the next most frequently used

species (Figure 3.3A). Publications were further characterised by macrophage population used, whereby peritoneal (n = 82), bone marrow-derived (n = 38), alveolar (n = 17) and microglia (n = 16) were the most common macrophage populations to be assessed (Figure 3.3B). Figure 3.3C highlights the ages most commonly assessed when comparing young and old of the most frequently assessed species. For this, the mean age was used when stated in the publication and when an age range was stated, the median was used. For each species, "young" was more clearly defined, as evidenced by the flatter violins: mean age for C57BL/6 mice was 3.4 months (95% CI: 3.1-3.7), for BALB/c mice was 2.9 months (95% CI: 2.3-3.4), for rats was 3.2 months (95% CI: 2.6-3.8) and for humans was 28.8 years (95% CI: 26.13-31.42). C57BL/6 aged mice were especially varied, with 2 peaks at approximately 18 and 24 months. The mean age here was 20.7 months (95% CI: 20-21.32), for BALB/c mice was 19 months (95% CI: 17.8-20.3), for rats was 20.4 months (95% CI: 18.4-22.5) and for humans was 73.3 years (69.7-76.9). The number of annual publications for the past 10 years that were included in this SLR is shown in Figure 3.3D. Although 2020 saw an increase in publications, this number has remained broadly consistent for the past 10 years. Figure 3.3E and F depicts the overall focus of the publications, with the most common focus of macrophage ageing being changes in overall numbers of resident or infiltrating cells, frequently examined by macrophage surface receptor expression. This was often analysed in conjunction with assessing how aged macrophages differ compared with young in their response to stimuli, such as lipopolysaccharide (LPS) or polarising cytokines. Data from these publications are discussed in more detail below.



D

Year	Count
2022	13
2021	18
2020	28
2019	20
2018	18
2017	16
2016	12
2015	9
2014	14
2013	11

E

Main focus of macrophage ageing	Count
Count/ infiltration	129
Surface receptor expression	89
Gene expression	63
Cytokine expression	44
Polarisation	40
Cytokine secretion	36
ROS production	26
Phagocytosis	20
Transcriptomics	18
Protein expression	16
Macrophage killing	8
Morphology	7
microRNAs	6
Chemotaxis	5
Signalling	5
Senescence	3
Pathogen recognition	2
Adherence	2

F

Additional model assessed	Count
Stimulation	58
Injury	10
Diet	8
Exercise	4
Long-lived	3
Infection	3
Stress	2
Wound	2

Figure 3.3 Systematic literature review summary

A – Word cloud of most commonly assessed species in the context of macrophage ageing. Words are sized based on the frequency that the species is studied in the literature.

B – Word cloud of most commonly assessed macrophage types. Words are sized based on the frequency that the macrophage type is studied in the literature.

C – Violin plots of the ages assessed when comparing young and old macrophages of the four most common species studied in the literature. Either mean age stated in the publication or median of age range was used.

D – Number of included publications produced per year for the past 10 years.

E – Counts of the main focus for results of each included publication.

F – Counts of any disease model assessed in the publication in addition to healthy macrophage ageing.

3.3.2.1 Macrophage infiltration in response to age

Resident macrophage count and infiltration of primary macrophages were by far the most frequent focus when assessing changes with increasing age. For this, most studies analysed surface marker expression by flow cytometry or immunohistochemistry. There are a plethora of different macrophage markers with species- and tissue-specificity, as well as those that indicate the inflammatory phenotype of the cell [264,271]. Data were extracted from publications that assessed these age-related changes and collated by the surface markers assessed. Whether these macrophage populations increased, decreased or underwent no change with age was then collated (Figure 3.4). The species, tissue and macrophage subtype assessed, as well as the publication that the data pertains to, are shown in Supplementary table 8.2.7. According to the literature pool, most commonly tissue macrophage populations were assessed, with pan-macrophage markers making up the bulk of the surface marker expression data. There was no particular trend in whether these cells increased, decreased or stayed the same with age across a number of parameters. Increase in macrophage count or infiltration most commonly occurred in BMDMs (n = 5), peritoneum, adipose and liver (n = 4). Decrease in macrophage count or infiltration most commonly occurred in peritoneal (n = 6), BMDMs and muscle (n = 5), and wounds (n = 2). The proinflammatory (M1) macrophage markers, including CD11c, iNOS, MHC-II and CD80, were more frequently upregulated with age (n = 17), compared with downregulated (n = 3) and no change (n = 2). Increases in M1 macrophage populations were more often seen in wounds (n = 4), adipose tissue (n = 4) and alveoli (n = 3) and were also commonly reported in BMDM populations (n = 3). In contrast, alternatively activated (M2) macrophages were more commonly increased (n = 12) or decreased (n = 11) with age compared with no change (n = 7). The increased M2 population was more frequently seen in peritoneal (n = 3) hepatic (n = 2) or BMDMs (n = 2), whereas a decreased M2 population was most common in the muscles (n = 4) or peritoneum (n = 3). No change in M2 macrophage populations was less common, with two publications finding this in adipose tissue macrophages. We speculate that this lack of overall trend for age-related M2 macrophage counts may reflect the highly diverse phenotypes, with

distinct roles in specific tissues or processes, of alternatively activated macrophages, that are often simplistically grouped together. Finally, IBA-1+ macrophages, IBA-1 being a marker of macrophage activation, were more frequently increased with age, with six out of seven studies, two different species and six different tissue macrophage populations demonstrating this expansion.

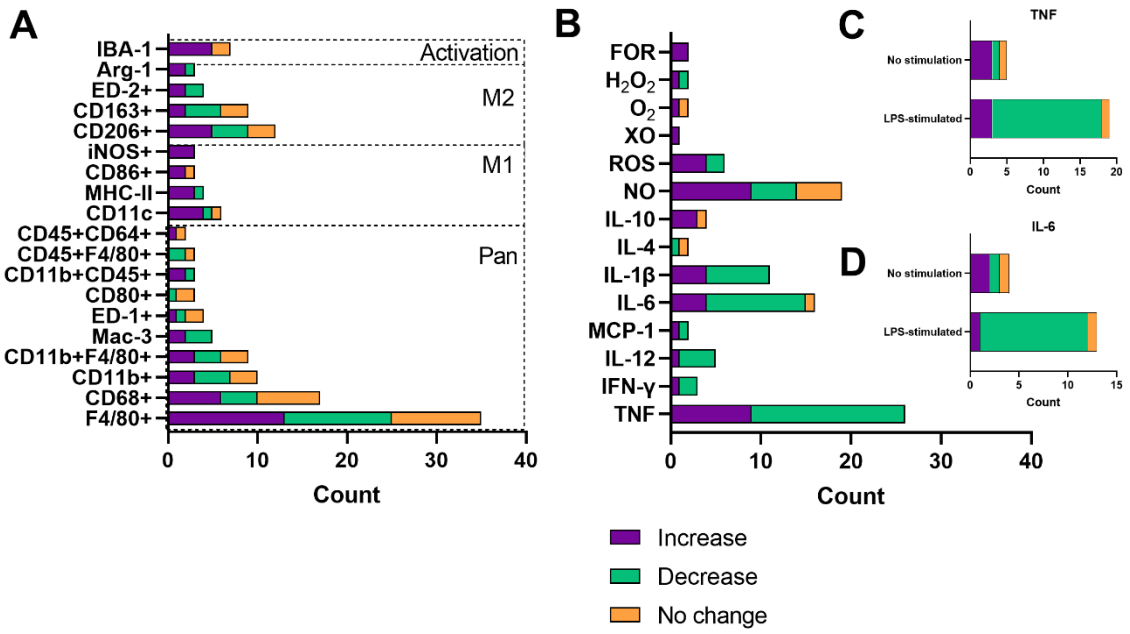


Figure 3.4 Changes with age in macrophage count/ infiltration and soluble mediator release

A – Number of publications assessing changes with age in macrophage infiltration or count by surface marker expression.

B – Number of publications assessing changes with age in soluble mediator release, including cytokines and reactive oxygen species. Data are only included where there were results from more than one publication.

C – Number of publications assessing TNF release as a consequence of age, grouped by whether cells were stimulated with LPS or not stimulated.

D – Number of publications assessing TNF release as a consequence of age, grouped by whether cells were stimulated with LPS or not stimulated.

FOR: free oxygen radicals; XO: xanthine oxidase; ROS: reactive oxygen species; NO: nitric oxide; IL: interleukin; MCP: monocyte chemoattractant protein 1; IFN: interferon; TNF: tumour necrosis factor; LPS: lipopolysaccharide

3.3.2.2 Mediator release

There are a plethora of cytokines, chemokines and other soluble mediators released by macrophages. This is normally dependent on the tissue environment or polarisation state and

governs the inflammatory phenotype of the surroundings [262], as well as the response to insult with ROS having key roles in antimicrobial activity and cellular differentiation [272]. ROS such as superoxide has been linked to ageing, whereby increased catalysis to H2O2 has been associated with extended longevity in Drosophila [273]. Considering the role of macrophages in inflammaging, much of the literature has assessed the effect of age on inflammatory mediator release. Data were extracted from publications that analysed soluble mediator release as a function of age and grouped according to whether an increase, decrease or no change was reported. These findings are summarised in Figure 3.4B as well as Supplementary table 8.2.8, which again highlights the tissue and species of interest, as well as the environment of the cells assessed. TNF was evidently the most commonly assessed cytokine in ageing, most often being associated with reduced production. Most often, this decrease with age was measured after LPS and IFN-γ-stimulation (n = 15, Figure 3.4C), used to model inflammation in vitro, primarily in peritoneal and splenic macrophages. TNF secretion increase with age was most commonly found in BMDMs (n = 4), both in basal or inflammatory conditions. IL-6 and IL-1β were also commonly measured and were most often reported to be decreased with age (69% and 64% of reporting papers, respectively). These were often tested in conjunction with TNF; IL-6 decrease with age was also most commonly found as a response to LPS stimulation (Figure 3.4D). Anti-inflammatory or restorative (M2) macrophages were much less frequently assessed, with IL-10 being the predominantly analysed cytokine that is involved in inflammation resolution. IL-10 was most commonly increased with age, also more often being assessed in an inflammatory setting. ROS production was most commonly increased with age, including nitrogen oxide, free oxygen radicals and hydrogen peroxide.

3.3.2.3 Phagocytosis, chemotaxis and pathogen recognition and killing

Phagocytosis is a core function of the macrophage, critically important in initiating the immune response and rapid pathogen clearance [274]. A total of 20 papers addressed changes in phagocytosis with age; these were collated according to direction of change with age, be that upregulation, downregulation or no change, and then by macrophage type and species. The majority of these

publications analysed peritoneal macrophages and C57BL/6 mice. Overall, 11 of the publications found a reduction in phagocytosis with age, seven found no change and two found phagocytosis to be increased with age (Table 3.1).

Table 3.1 Findings from publications assessing changes in phagocytosis with age, grouped by direction of change

Direction of change with age	Macrophage type	Species	Additional factors	Citation
Upregulated (2)	Peritoneal (1)	BALB/c mice	Measured by phagocytic index, however downregulated compared with young with addition of NPY	[275]
	Pulmonary (1)	C57BL/6 mice	*M. tb* infection of cells	[276]
Downregulated (12)	Alveolar (2)	C57BL/6 mice (2)	Internalisation of *E. coli* particles	[274]
			Looked at percentage of cells phagocytosing latex beads	[277]
	BMDM (1)	C57BL/6 mice	Assessed 4 days after reloading unloaded hindlimbs to assess aged skeletal muscle	[278]
	Peritoneal (8)	C57BL/6 mice (4)	Phagocytic activity of yeast cells	[279]
			Peritoneum-resident M1-like macrophages	[280]
			Fluorescent particle uptake by number of cells and particles	[281]
			Binding of IgG-SRBC and addition of protein diet	[282]
		ICR/CD1 mice (1)	Assessed long-lived mice and found positive correlation between achieved lifespan and phagocytic ability	[283]
		AO rats (1)	Zymosan, stimulation (LPS, GM-CSF, IL-4) enhanced phagocytosis in young but not old	[284]
		Swiss albino mice (1)	Percentage of phagocytes and phagocytic index of yeast cells	[285]
		BALB/c mice (1)	Without stimulation and with addition of NPY	[286]
	Microglia (1)	C57BL/6 mice	Rate of uptake of YG-beads over seven minutes	[287]
No change (7)	BMDM (3)	C57BL/6 mice (2)	Assessed many different treatments e.g., LPS/IFNγ	[288]
			Fluorescent particle uptake at different concentrations by number of cells and particles	[281]
		BALB/c mice (1)	Uptake of *S. pyogenes*	[289]
	Peritoneal (2)	Albino rat (1)	Percentage of phagocytosis in control, TG and TB samples	[290]
		A/J mice (1)	CdCO3 and latex particles	[291]
	MDM (1)	Human	*S. pneumoniae* binding and phagocytosis	[292]
	Hepatic (1)	C57BL/6 mice	FITC-labelled *E. coli* particles assessed in M1 and M2	[293]

In order to successfully clear pathogens from tissues, macrophages also need to be able to migrate towards them, recognise them and kill them, be that via degradation in the phagolysosome or through recruitment of other immune cells [294]. Looking at the chemotactic ability of aged macrophages, it was found, through assessment of macrophages in peritoneal suspension, that long-lived animals had overall better immune function, including phagocytosis and chemotaxis, than those that died at the previous age point studied. The very old and long-lived animals even had an increase in immune function compared with themselves at old age [283]. Additionally, it was found that chemotaxis index increased with age [286], and that chemotaxis towards CCL2 was more robust in old bone marrow-derived macrophages (BMDMs) from C57BL/6 mice, enhanced further by sFASL [295]. In contrast, chemotaxis was found to decrease with age in the final two publications, one assessing white adipose tissue in burns [296], the other looking at exercise, with aged controls having significantly less capacity for chemotaxis than young [297].

Pathogen recognition was a focus for very few papers, likely because of the complexity of the process, involving many different receptors and signalling pathways [298]. However, it was suggested that old peritoneal macrophages had reduced ability to recognise pathogen associated molecular patterns (PAMPs) and signal through toll-like receptors, evidenced by reduced cytokine secretion [280]. Macrophage killing ability was assessed by seven papers, despite this being a less common function of these cells. In two studies, it was found that old BMDMs were less effective at clearing *S. pneumoniae* than young counterparts [299,300]. The same conclusion was drawn for peritoneal macrophages [300], as well as human monocyte derived macrophages (MDMs) [292]. *C. albicans* killing was also found to be less effective with age, this time in peritoneal macrophages isolated from C57BL/6 mice [301]. Conversely, the parasite Leishmania was found to be effectively killed by both young and old BMDMs [302], as well as *C. burnetti* bacteria by peritoneal macrophages [303]. Finally, old peritoneal macrophages were found to be more able to resist HSV-1 than young counterparts [304].

3.3.2.4 Morphology

Macrophage morphology as a function of age was assessed in five publications. Peritoneal macrophages isolated from old C57BL/6 mice were found to be larger and elongated with irregular morphology as compared to those from young mice [305]. Macrophages isolated from the lateral wall of human cochlea from old donors appeared to have larger cytoplasmic volume and fewer projections than those from young donors [306]. In the ageing cochlea of C57BL/6 mice, macrophages were enlarged and grainy with processes projecting towards adjacent macrophages. Giant macrophages with irregularly shaped nuclei were also found in the ageing cochlea but not in the young [307]. Microglia morphology was assessed in two papers: in C57BL/6 mice, old microglia had less convoluted processes and accumulation of lipofuscin when compared with young [308]. Conversely, those isolated from old F433 rats appeared to be more activated with amoeboid-like morphology [309].

3.3.2.5 Gene and protein expression

Together, gene and protein expression have been the main focus in the literature relating to macrophage ageing to date. They are also crucially relevant when looking for biomarkers and potential therapeutic targets and were therefore collated into the MAIC in the following section.

3.3.3 Meta-analysis of macrophage ageing by information content

MAIC is a method of aggregating data from heterogenous sources without reliance on raw values or statistical quality scores. Each entity's information content is quantified by measuring the number of times that it is mentioned across a body of literature. Therefore, the more an entity is mentioned throughout a literature pool, the higher the score is, indicating a strong association with the literature pool. The MAIC algorithm also includes a weighting function, giving a higher score to entities that have been assessed by multiple different methods [269].

We used the MAIC algorithm to assess the genes and proteins most associated with macrophage ageing, compiling 5 separate datafiles (as described in Section 3.2.4) from the same set of 122 articles that had passed the inclusion criteria. These files were as follows:

1. All entities: 127 sets of genes and proteins
2. Upregulated Genes: 29 sets of genes demonstrating macrophage-specific upregulation with age in at least one experimental result
3. Downregulated Genes: 31 sets of genes demonstrating macrophage-specific downregulation with age in at least one experimental result
4. Upregulated Proteins: 50 sets of proteins demonstrating macrophage-specific upregulation with age in at least one experimental result
5. Downregulated Proteins: 53 sets of proteins demonstrating macrophage-specific downregulation with age in at least one experimental result

3.3.3.1 MAIC for all entities

The MAIC for all entities produced an overview of the level of representation of all genes and proteins explored in the literature in relation to ageing macrophages. The higher the MAIC score, the higher the level of representation across varying experimental methods throughout the literature (Figure 3.5). A total of 643 entities were outputted from the MAIC, with scores ranging from 1.0 to 35.5. The level of information content produced by each method is shown in Figure 3.5A corresponding to the magnitude of the respective coloured sectors on the chord diagram. This analysis found qPCR, ELISA, and flow cytometry to be the most frequently used methods for gene and protein expression assessment in this context. The outer circle bars represent the number of entities explored in the respective dataset, clearly showing that the majority of publications only recorded expression data for one or two entities per method used; RNA sequencing yielded the most data per publication. Figure 3.5B shows the calculated MAIC scores for each entity (gene or protein), with a minimum cut-off count of five. Here, TNF and iNOS were the most highly documented and investigated entities, with a score of 35.5 and 28, respectively.

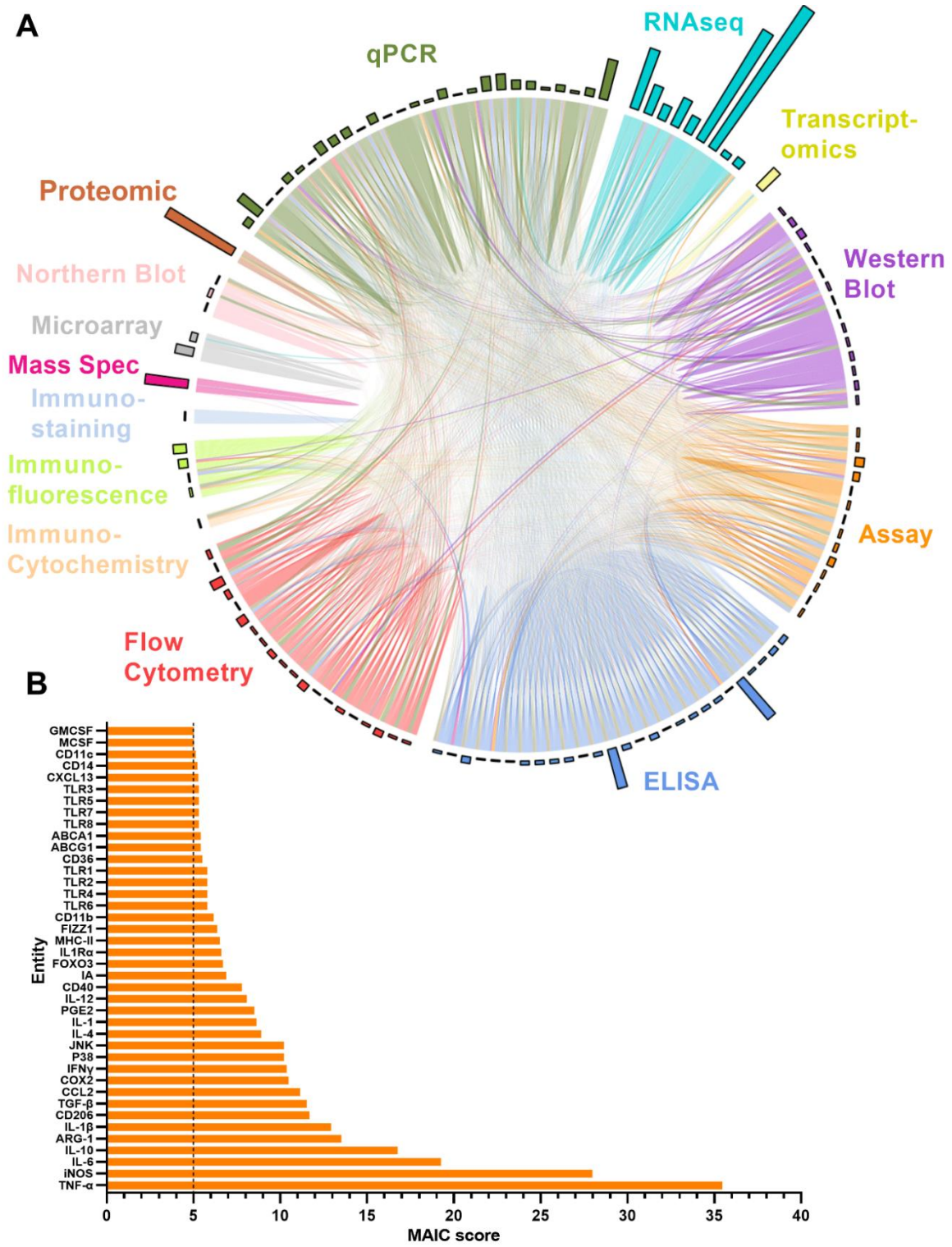


Figure 3.5 Meta-analysis by information content analysis for all entities associated with the ageing macrophage

A – Outputs from the MAIC carried out on data for both genes and proteins across the literature were processed in R to produce a chord diagram where links (chords) between publications (segments) represent the sharing of a gene/protein. Publications are coloured (and grouped into sectors) by the methodology used to explore the entity. Chords stretching between publications differing in methodology are coloured by the more dominant scoring publication. The circle outer bars represent the total entities collected from each dataset/sector. Conducted by myself and Hew Phipps.

B – Top MAIC scoring entities across the literature with a cut-off score of 5.

3.3.3.2 MAIC for gene and protein changes with age

The results of the "all entities MAIC" provides a quantitative evaluation for the level exploration of genes and proteins, as well as a comparative evaluation on the methodologies employed. However, this provided little information on the direction of change for these entities with age. A series of four more MAICs were designed to explore the direction of change for both genes (RNA) and proteins. As described in Section 3.2.4, any genes with results showing upregulation with age were collated into an MAIC input for upregulated genes, and the same applied for downregulated genes and up- and downregulated proteins.

The overall number of genes identified as upregulated and downregulated with age in the literature was highly comparable (Figure 3.6A, Supplementary table 8.2.9), however there is clear dominance of upregulated genes in the MAIC (Figure 3.6B), with the majority of downregulated genes having an MAIC score of only one (appearing in only one publication), putting them below the cut-off threshold. Overall, 159 genes were found to be upregulated with age and 18 were downregulated with age, according to MAIC score. Furthermore, 13 genes had an MAIC score for both upregulation and downregulation with age, indicating variation within the literature, but these scores were also higher for upregulation (Figure 3.6C).

The overall number of proteins identified within the literature was skewed towards upregulation (Figure 3.7A, Supplementary table 8.2.10). However, MAIC scores were more evenly spread across both directions (Figure 3.7B) than that of the gene entities. This was due to the MAIC score for many upregulated proteins falling below the cut-off threshold. Similarly to that of genes, 12 proteins had scores in both directions of change with age, however in this instance scores were higher for downregulation (Figure 3.7C).

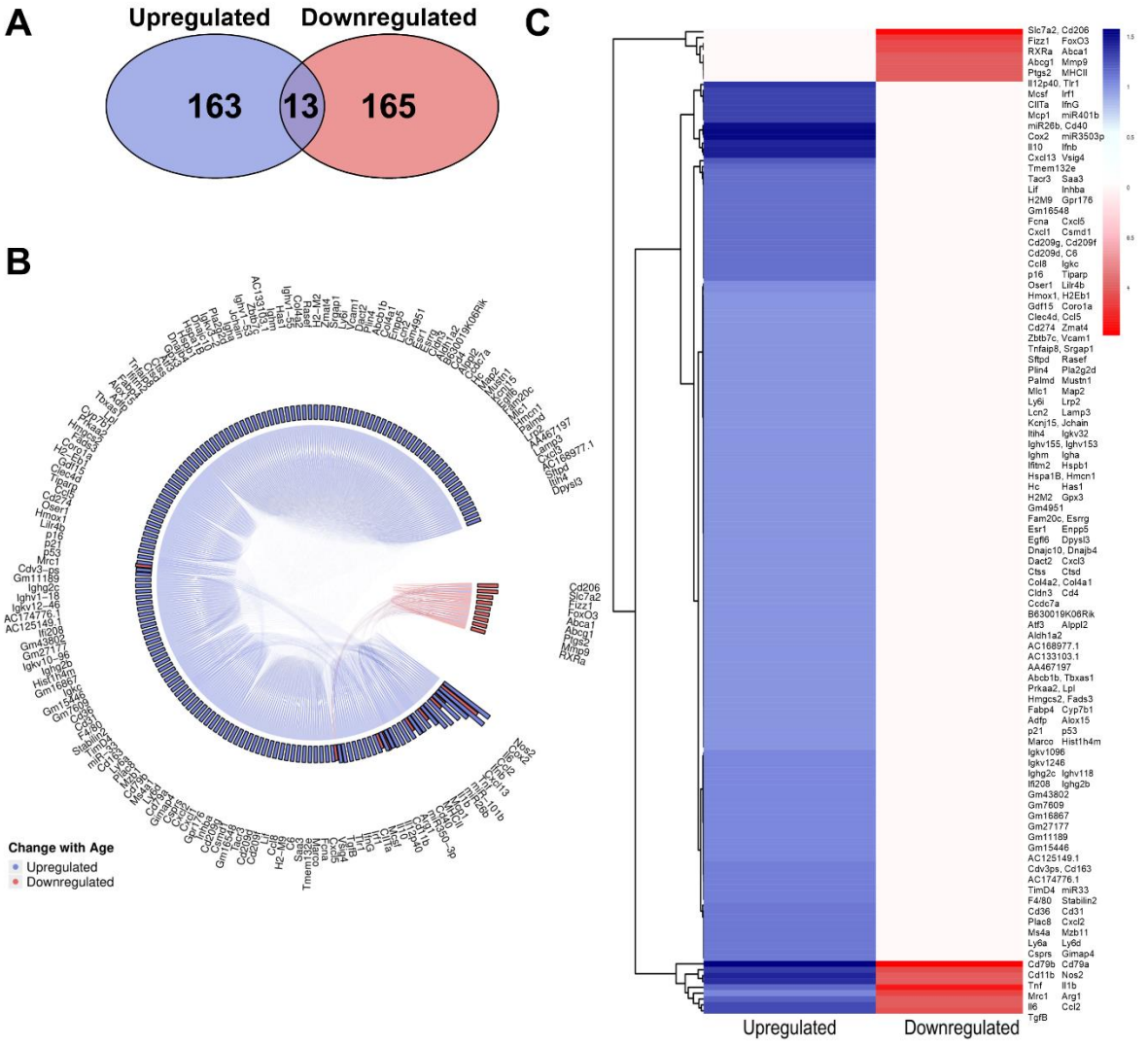


Figure 3.6 Meta-analysis by information content analysis for changes in gene expression associated with macrophage ageing

A – Number of genes observed in the literature according to direction of change with age.

B – Outputs from both the genes downregulated and genes upregulated MAICs were combined and processed with R into a chord diagram where links (chords) between genes (segments) represent cohabitation of the same dataset. Genes are coloured (and grouped into 2 sectors) by the dominant expression change with age. This is determined by whichever regulation shows a higher MAIC score and is portrayed in the outer ring of the circle by bars correlating with MAIC score. Thus, in the case of up- and downregulation observed with age in the literature, both red and blue bars are shown with the larger bar governing the colour of chords stemming from that gene.

C – Mean MAIC scores for all genes, colour coded to match the documented change in expression with age.

Conducted by myself and Hew Phipps.

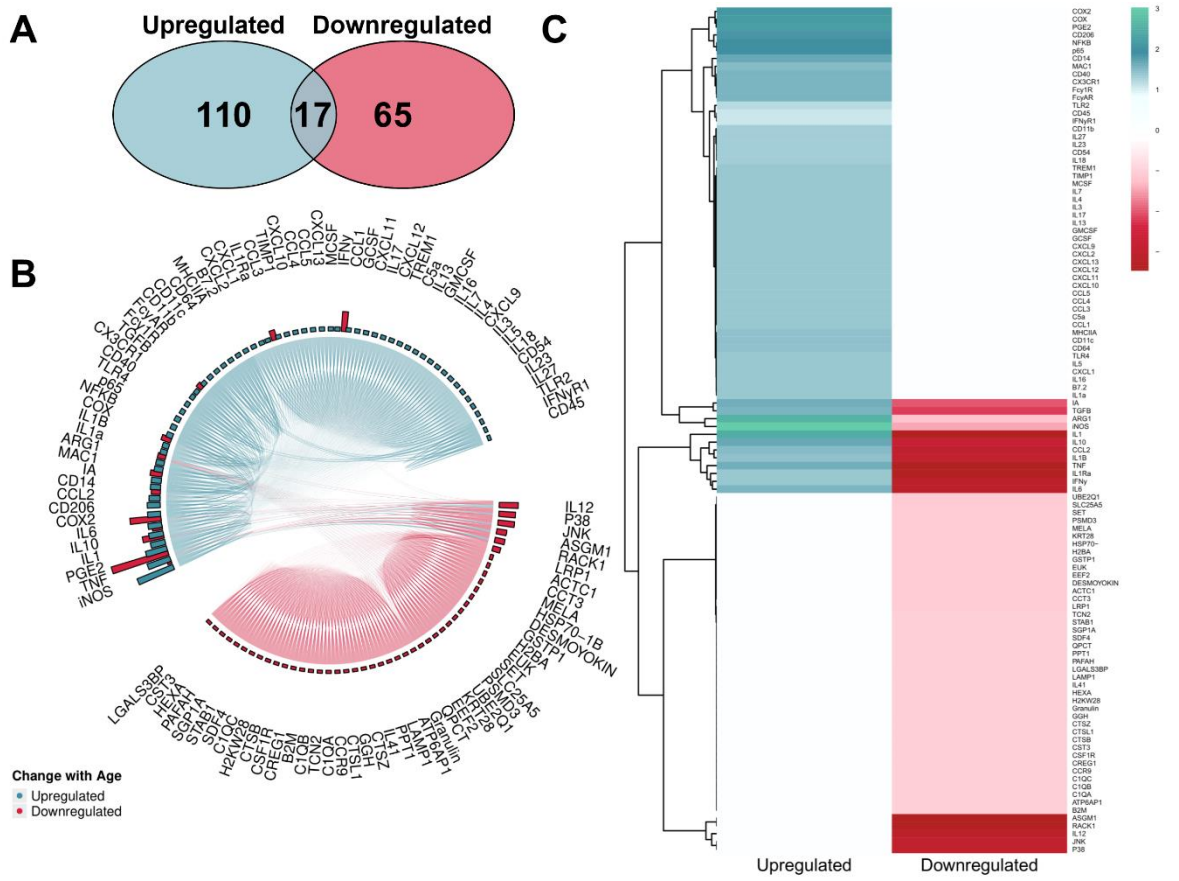


Figure 3.7 Meta-analysis by information content analysis for changes in protein abundance associated with macrophage ageing

A – Number of proteins observed in the literature according to direction of change with age.

B – Outputs from both the proteins downregulated and proteins upregulated MAICs were combined and processed with R into a chord diagram where links (chords) between proteins (segments) represent cohabitation of the same dataset. Proteins are coloured (and grouped into 2 sectors) by the dominant abundance change with age. This is determined by whichever regulation shows a higher MAIC score, shown in the outer ring of the circle by bars correlating with MAIC score. Thus, in the case of up- and downregulation observed with age in the literature, both pink and green bars are shown with the larger bar governing the colour of chords stemming from that protein.

C – Mean MAIC scores for all proteins, colour coded to match the documented change in abundance with age.

Conducted by myself and Hew Phipps.

3.4 Discussion

Macrophages are a diverse and plastic cell type; their phenotypes can be divided into many different subcategories. Tissue populations often have unique ontogenies and differ widely from infiltrating populations. Local tissue environment also has a large impact on the way the macrophage functions [25,27,28,30]. Partly as a consequence of this complexity, the literature has remained fragmented, in most

cases, focussing on single models or tissues when investigating macrophage function. However, the 'spectrum model' is a recent major conceptual advance in our understanding of macrophage biology, highlighting the need to refer to macrophages by the stimulating agent/environmental cues controlling their function [21,35]. This review set out to consolidate published knowledge in the field, and although some conclusions can be drawn, clear gaps in the field have emerged, including the lack of functional work such as phagocytosis, being assessed in aged macrophages from different environmental settings.

Further to the sparsity of publications assessing age-related functional decline in the macrophage, it was surprising to find so few acknowledging the association between macrophages and senescence. Macrophages secrete many factors associated with the senescence-associated secretory phenotype (SASP) [262,310], have been shown to undergo senescence in vitro, in response to ionizing radiation [310] or by high glucose exposure [311], and have a well understood role in clearance of senescent cells from their surroundings [97]. There remains a lack of confidence as to whether non-replication competent cells such as macrophages can be senescent, as well as a lack of association between macrophage age and ability to respond to SASP or to clear senescent cells. One study by Hall *et al*. (2016) has found accumulation of senescent cells with age that appear to be a subpopulation of macrophages due to their removal with clodronate treatment [100]. Two further publications have also found increases in SA-beta-gal positive macrophages with age [305,312]. Finally, Kumar *et al*. (2020) did find that senescence increased in both young and old macrophage populations in the presence of conditioned media from senescent cells, which was rescued when media was switched to that from proliferating cells [305]. A few studies have looked at how macrophages contribute to longevity with interesting results, finding long-lived animals have better overall immune function, including macrophage phagocytosis and chemotaxis, highlighting the need for properly functioning macrophages in healthy ageing [283]. Dimitrivijec *et al*. (2014) also assessed long-lived animals and found long-lived rats had increased IL-10 expression and reduced TNF secretion as compared to old, highlighting the importance of balanced, macrophage-dependent inflammation in healthy ageing [313].

Macrophage cytokine release is a critical component of immunity, directing both initiation and resolution of inflammation. The three most commonly assessed cytokines in the macrophage ageing literature, TNF, IL-6 and IL-1β, are inflammatory cytokines that macrophages release in response to insult in order to recruit other immune cells such as leukocytes, to clear the insult through Th1 responses and induction of B cell proliferation and antibody production [314–316]. Overall, the literature found expression of these cytokines to be decreased in aged compared with young macrophages in a pro-inflammatory setting. If secretion of these cytokines is decreased with age it likely indicates a lack of responsiveness to stimuli [317], so that macrophages are not mounting a full and effective inflammatory response. Although this may also have beneficial effects against some ARDs [318], the many signalling pathways involved have not been fully assessed in macrophages alone and an ageing setting. Frequently, when production of these pro-inflammatory cytokines was found to be increased with age, this was in a resting macrophage state, corresponding to a higher baseline inflammation with age, and indicating presence of low-level inflammation that could be contributing to many ARDs [319]. It should be noted that there were many more data looking at an inflammatory setting than anti-inflammatory/restorative, and this is likely due to the ease of modelling an infection, most commonly stimulating with LPS and IFN-γ [277]. This is compared with the complex nature of tissue restoration, with many different components and macrophage subtypes falling into this category [320,321]. Macrophages can be stimulated with IL-4, to create a wound healing phenotype, immune complexes to create a regulatory phenotype that upregulates IL-10 and many more reviewed elsewhere [31,32,320,321]. Overall, only IL-10 secretion was well characterised across the literature, with an increase with age in an inflammatory setting being the overall conclusion. This could indicate immune system dysfunction, where resolution is occurring during the inflammatory response, with Chelvarajan *et al*. (2005) finding an increase in IL-10 in LPS-stimulated aged macrophages contributing to a decrease in ability to produce proinflammatory cytokines [322]; however, this complex and dynamic system is still not fully understood. Zhao *et al*. (2013) linked an increase in IL-10 to neovascularisation, making age-related macular degeneration more aggressive [295]. Alongside IL-10, overproduction of ROS appeared

commonplace in ageing [323,324], which could lead to excess tissue and DNA damage through oxidative inflammatory stress [323], cytotoxicity and abnormal growth of cancer cells [325].

It is widely thought that overall, tissue macrophage numbers do not change with age [326–328], although expansion of infiltrating macrophages has been reported [329]. Each of the pan macrophage markers and leukocyte specific markers assessed in the literature showed much heterogeneity in expression; F4/80 has a comparable number of papers that find expression increase (n = 13), decrease (n = 10) and no change (n = 10). This may relate to the fact that many different tissue types have been assessed, with decrease in F4/80-expressing macrophages occurring more frequently in the peritoneum [301,312,330] and increase most commonly in infiltrating BMDMs [331,332]. On the other hand, human CD68+ macrophages were more consistently present with age (no change: n = 3, increase: n = 1, decrease: n = 1) and rat ED-1+ macrophages, the homolog of CD68, were more frequent with age (increase n = 6, decrease n = 1, no change n =3). It was more common that macrophages expressing M1 markers were increased with age, while macrophages expressing M2 markers showed a trend towards being decreased with age. This could explain the heterogeneity in pan-macrophage markers as ageing is not leading to an expansion of macrophages so much as a change in marker expression [332–337]. One commonality in the literature was an overall decrease in pan-macrophage marker expression and increase in M1 marker expression in wounds [338–340], supporting the theory that macrophage populations are shifting rather than expanding. This could potentially advance the field in improving recovery time to wounds in older individuals. Another consistency in the literature that could be potentially exploited was that IBA-1+ macrophage populations increased with age [341–344]. This was across a number of different macrophage subtypes assessed, highlighting its homogeneity in the ageing macrophage and thus could be further explored as a potential biomarker or therapeutic target for macrophage ageing.

In terms of functional changes with age, where phagocytosis was assessed, the majority of publications found it to be reduced with age. This likely contributes to reduction in ability to respond

to infection with age, as reduced phagocytosis would reduce pathogen recognition and antigen presentation as well as pathogen clearance, halting the adaptive immune response [345,346]. There were also instances where phagocytosis was upregulated with age, in particular peritoneal macrophages showed more variable phagocytosis with age, as different publications came to different conclusions. In reality, upregulated phagocytosis with age may not correlate with effective processing of phagocytosed contents or presenting antigens to the adaptive immune system, and changes with age in either direction could indicate immune dysfunction [345,346]. Pathogen recognition and antigen presentation are much more difficult to quantify with age; antigen presentation requires more than one cell and at least three different receptor binding events [347]. It has not yet been characterised in ageing more extensively than reduced cytokine secretion after PAMP recognition [280]. Overall chemotaxis was found to be increased in two publications [286,295] and decreased in three with age [283,296,297], although changes with age were found in each instance supporting the notion of immune system dysfunction with age. There is a clear need for more research into macrophage functional decline with age and the mechanisms behind this. Morphological changes were assessed by five papers and commonly, it was found that macrophage size increased with age, as well as projections [305–307]. This suggests an observably distinct phenotype with age compared with healthy young counterparts.

The MAIC was conducted to better define age-related changes of genes and proteins by quantifying the observations in the literature. MAIC is estimative in its statistical power, however the results gained are highly informative if the assumption is made that the literature included is unbiased and the level of investigation of each factor is directly correlated with its association with the ageing macrophage. For this reason the method used is included alongside entity, since RNA sequencing and other high throughput methods have much greater breadth and therefore bias towards no change with age compared with that of high specificity, candidate-based methods such as qPCR or western blot. Therefore, entities sharing the same changes with age across these different methods score much more highly in the MAIC, reflecting the increased likelihood that these results represent a true

change with age. Because of the breadth and lack of specificity in high throughput methods, entities with MAIC scores of 1 were omitted, also indicative that these entities have no shared information content, assessed by only one method.

Cytokines TNF, IL-6, IL-10 and IL-1β were all among the top 10 most assessed entities, as well as macrophage markers iNOS, Arg-1 and CD206. These are, of course, among the most well-known genes and proteins associated with the macrophage, alongside several of the other entities with a score of 5 or higher (Figure 3.5). It is interesting, therefore to recognise that some of the less candidate-based entities may prove to be equally important functionally, and as markers in macrophage ageing. This systematic MAIC provides a new tool to consolidate these findings and identify previously overlooked potential biomarkers or therapeutic targets. ABCA1 and ABCG1 belong to the ATP-binding cassette family and function together as a cholesterol efflux pump for cellular lipid removal [348]. These proteins have been shown to be relatively highly expressed in the macrophage and dysregulation of these genes is known to be associated with dyslipidaemia, which may be relevant in ARDs such as atherosclerosis [349]. FIZZ1, also known as RETNLB or resistin like beta, enables adipose hormone activity [350] and is involved in IL-4 signalling [351]. It has also been implicated in the development of atherosclerosis [352]. Each of these genes could prove interesting to further explore in the ageing macrophage setting.

Overall, 159 genes were found to be upregulated and 18 downregulated with age across at least 2 methods or publications, while 13 genes were found to be both up- and downregulated (Figure 6). These 13 genes constitute many of the entities with the highest overall MAIC scores, probably indicating the amount of data relating to them is dictating the overall findings. It should also be noted that the MAIC presented here does not take into account the tissue environment of the macrophage. Discussed previously in relation to changes in secretion of pro-inflammatory cytokines and expression of different macrophage markers, environmental cues may potentially be causing the changes in regulation reported. Again, it is unsurprising that there are many genes in this list that are

well established in macrophage function, including genes encoding macrophage-specific markers, cytokines and chemokines. For example, MRC1/CD206 is a pattern recognition receptor involved in pathogen sensing that modulates macrophage polarisation [353]. It was found to be both upregulated and downregulated with age, whereas MCP-1 is a monocyte-specific chemokine that was consistently upregulated with age. Of note, CD40 and COX2 were present in the MAIC for genes and had high overall MAIC scores, so will be interesting to research further in the context of macrophage ageing. CD40 is a receptor on antigen presenting cells for binding with T and B cells and bridging the innate and adaptive immune system [354]. As it forms a crucial part of the immune system its dysregulation may very well lead to age-related immune dysfunction. COX2 is a key enzyme in prostaglandin synthesis, with a key role in inflammation [355]. Existing literature has found this to be upregulated with age and this too could be very interesting to further explore in relation to chronic inflammaging.

For the protein MAIC, 62 were upregulated with age and 58 downregulated with a significant MAIC score. 12 proteins were both up- and down-regulated across the literature. This included upregulation of many cytokines and chemokines, as well as CD40 and COX2, consistent with that seen in the gene MAIC. JNK and p38 were both downregulated with age. These are key effectors in MAPK signalling pathways that regulate many cellular processes from proliferation to apoptosis and inflammation [356]. It has been reported that this downregulation seen with age leads to defective TLR-mediated signalling [357], phagocytosis and overall immune function [358]. Receptors CD206 and MAC-1 were upregulated with age. These receptors are involved in pathogen sensing, as well as CD206 being an anti-inflammatory macrophage marker. RACK1 was also found to be downregulated across the literature. This protein has been shown to play a role in M2/M1 macrophage ratio and so its dysregulation could shift macrophages towards an inflammatory phenotype [359]. Overall, there are a number of consistently dysregulated proteins, as well as genes, that could form a panel for assessing biological age of macrophages. An important next step to this review could therefore be to assess

these genes and proteins thoroughly across different species and tissues to validate this list and provide a platform for the discovery of the novel biomarkers in the age-related context.

There is now a plethora of literature relating to macrophage ageing. However, there are still large gaps in the field, which are limited by the lack of functional work that has so far been completed on different macrophage types in an ageing setting. Of the 240 publications included, only 20 investigated phagocytosis, usually the most well-documented function of the macrophage and only 5 assessed chemotaxis, crucially important in infiltration or surveillance of the tissue [360]. Gene and protein expression were much better studied, with as many as 9 separate RNA sequencing datasets, two microarray datasets and a further proteomic dataset contributing to the MAIC, likely accounting for genes and proteins that have been previously under analysed. Data sharing is now crucially important to validate and expand this list of entities critical to the ageing macrophage phenotype; a starting point in biomarker recognition might be macrophage marker of activation IBA-1, for which a clear upregulation with age was seen across the literature. Additionally, many of the genes and proteins found in the MAIC to be consistently dysregulated, such as CD40, COX2, RACK1 and the ABC1 family could be added to produce a panel of entities crucial to the macrophage ageing phenotype.

3.5 Conclusion

By compiling the macrophage ageing literature to date, we can conclude that macrophages undergo functional decline with age, including reduced phagocytosis and chemotaxis, reduction in cytokine release in response to inflammatory stimuli and increased ROS production. Additionally, through our meta-analysis by information content we have found many genes, proteins, and soluble mediators that change with age (Table 3.2). Further assessment of these markers across different macrophage populations and environments could result in the identification of a consistent panel of age-related macrophage markers. Alongside functional decline of macrophages in response to age seen within this review, macrophage biological age could be further established using this panel of genes and

proteins, which may in turn shed light on the pathways resulting in macrophage functional decline. Overall, this panel should serve as a useful resource in designing future studies assessing macrophage ageing.

Table 3.2 Dysregulated genes and proteins with age that have the highest information content from the meta-analysis

Genes		Proteins	
Upregulated	**Downregulated**	**Upregulated**	**Downregulated**
MHC-II	CD206	COX2	ASGM1
MCSF	FIZZ1	PGE2	RACK1
MCP1	ABCA1	CD206	IL12
CD40	ABCG1	NFKB	JNK
COX2	MMP9	CD40	P38
IL-10	FOXO3	MAC1	LRP1

Chapter 4. Using bioinformatic analysis to study primary macrophage ageing

This results chapter identifies age-related changes in macrophages from published datasets and finds reference genes suitable for assessment of primary macrophage ageing: optimisation needed for the following results chapters. It is not intended to be published, but underpins the published findings in Chapter 5.

Declaration of Contribution

These data were analysed, interpreted and formulated by myself. This chapter was written by myself, without contribution from others.

Summary

The study of macrophage ageing is still in its infancy. However, datasets are beginning to be published that have profiled age-related changes in expression of genes in macrophage populations that can be utilised as a starting point for future studies. GSE84901 dataset published by Wong *et al*. (2017), profiled alveolar macrophage ageing. Here, our gene ontology analysis of 335 differentially expressed genes with age (LogFC > 1.5, P value < 0.05) newly identified a number of transcription factors likely governing these changes, including FOXM1, NFYB, MYC and USF1. Additionally, we have uncovered enriched pathways with age, including downregulation in "Regulation of actin skeleton" and "Autophagy". This provides a foundation for assessing changes in gene expression with age in different macrophage populations to better understand the role of transcriptional regulation in macrophage age-related functional decline (Chapter 5).

Housekeeping genes are essential for studying gene expression. To date, few studies have looked at the stability and reliability of housekeeping genes in primary macrophages. Gene expression patterns may be different between macrophage subtype, as a result of *in vitro* stimulation or due to the age of the subjects and should be determined for each set of experimental conditions. Here, we compiled a list of commonly used housekeeping genes that were previously found to be well conserved in different macrophage or ageing studies as well as those found to be well conserved in existing datasets and databases. Using RT-qPCR, we assessed expression of six genes (*PUM1*, *GAPDH*, *CSDE1*, *SF3B1*, *COPG1* and *BECN1*) in human monocyte-derived macrophages (MDMs) and three genes (*Mau2*, *Rpl32* and *Gapdh*) in murine bone marrow-derived macrophages (BMDMs) from young and old subjects that were stimulated *in vitro* to inflammatory or anti-inflammatory conditions. *PUM1* was found to have the most stable expression in human MDMs regardless of age and *in vitro* stimulation, *Mau2* was found to be the most stable gene in mouse BMDMs. These genes are therefore the optimal candidate reference genes for gene expression analysis with age in these cell types and stimulation conditions. The discrepancies in expression of commonly used housekeeping genes such as GAPDH shows the need for verification for each set of experimental conditions.

4.1 Introduction

Bioinformatics is a quickly growing field of biological research that incorporates mathematical, statistical and computational methods in order to analyse raw data [361]. In partnership, the rapid expansion of availability of genomic data has vastly improved bioinformatic software, making it possible for analysis of biological data in new ways, prior to laboratory-based research. This analysis can then inform future research, removing some previously crucial piloting steps and therefore saving time and resources, particularly relevant in animal- and human-based research. High-throughput sequencing techniques such as microarray and next generation sequencing [362], produce vast amounts of data that can be analysed beyond their intended use for publication, identifying key differentially expressed genes and enriched pathways that can influence initial experimental setup.

Macrophages regulate innate and adaptive immune responses, including the inflammatory response through the production of various soluble mediators [33]. Their functional role is largely determined by environmental stimuli that signal changes in gene expression to modulate macrophage function [21,22]. This means that there are large differences between macrophage populations depending on the local environment, exemplified by the resting (M0), inflammatory (M1) and anti-inflammatory (M2) macrophage phenotypes used in *in vitro* systems. M1 macrophages are activated with pro-inflammatory cytokines such as interferon (IFN)-γ and bacterial components such as lipopolysaccharide (LPS). These stimuli cause an increase in pro-inflammatory gene expression to initiate the immune response and allow M1 macrophages to phagocytose pathogens and activate adaptive immune cells. In contrast, M2 macrophages are activated by anti-inflammatory cytokines such as interleukin (IL)-4 and IL-10 and these stimuli lead to an increase in anti-inflammatory cytokine production as well as tissue repair pathways [31,32]. Differences in macrophage phenotype relating to the local environment are also relevant when comparing tissue and primary macrophage populations: tissue populations are affected by previous immune experiences, giving rise to a level of immune memory, such as their ability to de- and re-polarise following an immune response [363]. This gives these cells different phenotypes and functions to primary macrophage populations that are

normally expanded rapidly as monocytes in the bone marrow before migrating and differentiating in the tissue [364]. The differences between tissue and primary cells complicates the studying of macrophage biology, as different macrophage populations can be transcriptionally distinct from one another [21,23]. Additionally, immortalised cell lines, such as human THP-1 cells that are differentiated to macrophages *in vitro* and mouse immortalised bone marrow-derived macrophages (iBMDMs) differ from primary macrophage populations in their phenotype and function due to the immortalisation process [364].

Although resident macrophage responses are now better understood, particularly that of mouse peritoneal macrophages that have been far better studied than other populations [365], more recently it has been made clear that there are discrepancies in macrophage responses with age, including transcriptional programmes [365], polarisation and subsequent cytokine production [280,333,366] and functions such as phagocytosis [63,327,328]. However, the mechanisms underpinning these have not yet been discovered. This includes that of human primary macrophages as it is not yet fully understood whether human macrophages undergo a similar level of dysfunction as mouse counterparts. It has been suggested that murine bone marrow-derived macrophages (BMDMs) bridge the gap between mouse and human macrophages. They have been much better studied, particularly under inflammatory conditions, and it is thought that these cells are a good model population of the human situation [364]. The use of murine cells also poses its own problem as it relies on animals for research. The progression of techniques and increase in data availability in recent years means that scientific research in all forms should be reducing its reliance on model organisms. Rather than starting from scratch for each new study, researchers should be assessing what has already been done and utilising existing literature to aid in hypothesis development and experimental design. This also benefits the 3Rs movement, by reducing the number of model organisms needed in the initial stages of research [367].

In humans, monocyte-derived macrophages (MDMs) are the most exploited macrophage population, due to their relatively easy access and good survival [368]. These cells are differentiated from monocyte precursors isolated from whole blood and are therefore referred to as a primary macrophage population. To date, no datasets exist that directly compare transcriptional changes between MDMs isolated from individuals of different ages. Instead, utilising published datasets assessing age-related changes in gene expression in mouse macrophage populations could form a starting point to assess the human situation. Limited datasets from aged mice macrophages currently exist; one available dataset comes from a study by Wong *et al*. (2017), assessing alveolar macrophages isolated from young (2-4 months) and aged (22-24 months) mice. The authors found that there were widespread transcriptional changes and a reduction in phagocytosis with age in these cells [63]. We propose that consistent dysregulation in gene expression between mouse tissue macrophages such as these alveolar macrophages, mouse BMDMs and human MDMs could highlight key overall drivers of age-related changes in macrophage function.

In order to assess changes in gene expression, such as by real time qPCR (RT-qPCR), housekeeping genes are required. These are non-regulated reference genes that aid in calculating the relative expression of a target gene, resulting in a target/reference ratio that takes into account the changes due to the conditions assessed [369]. Housekeeping genes therefore need to be expressed in a stable manor in the cell of interest as well as between groups to ensure the true change as a result of the experimental condition is calculated [370]. It is known that expression levels of commonly used housekeeping genes can vary between organism, cell type and disease state [371,372], and few studies have already attempted to measure these in different macrophage populations. In addition, experimental conditions can affect gene expression, and so it is advised to undertake housekeeping gene verification before the onset of any study in order to accurately measure the RNA levels of the genes of interest [364,373].

In this study, we searched for published datasets assessing age-related changes in expression profiles of macrophages and located microarray dataset GSE84901 published by Wong *et al*. (2017), that assessed alveolar macrophages from young (2-4 months) and aged (22-24 months) mice [63]. Differentially expressed genes (LogFC > 1.5, P < 0.05) were assessed for enriched pathways and associated transcription factors to be utilised in the study of human MDM ageing. In addition, five published studies and the Housekeeping and reference transcript (HRT) atlas were utilised to collate commonly used housekeeping genes and well-conserved genes in different macrophage populations. This was in order to uncover suitable housekeeping genes for assessment of age-related changes to transcription in human MDMs and mouse BMDMs.

4.1.1 Hypothesis and aims

Data is limited on the study of human macrophage ageing, with only few published high-throughput datasets assessing macrophage ageing in the mouse. There is also a need for reference gene identification in any cell population before the initiation of gene expression studies. We hypothesise that existing datasets assessing resident macrophage ageing can be used as a starting point for assessing primary macrophage ageing by identifying potential regulators as well as reference/ housekeeping genes. In order to test this hypothesis, our aims are as follows:

1. Find key genes up- or downregulated with age in murine alveolar macrophages to take forward as potential regulators of macrophage ageing, acting as a starting point for the study of human MDM ageing and reducing the need for excessive samples for the presented research
2. Identify genes showing stable expression with age and with different *in vitro* stimulations for use as housekeeping controls in gene expression studies of human MDMs and murine BMDMs

4.2 Methods

4.2.1 Microarray analysis

A thorough search of the Gene Expression Omnibus (GEO) database was conducted to find high-throughput sequencing datasets closely related to this research. There were very few datasets relating to macrophage ageing with no human ageing dataset present, highlighting the need for the current research.

The published microarray dataset GSE84901, obtained from Wong *et al*. (2017), was extracted from the GEO database (www.ncbi.nlm.nih.gov/geo/). This dataset used microarray platform GPL21163 and Agilent-074809 SurePrint G3 Mouse GE v2 8x60K microarrays. RNA was extracted from purified alveolar macrophages of C57BL6/J mice at 2-4 months (young, N =6) or 22-24 months (aged, N=6). In total GSE84901 was comprised of alveolar macrophage bulk expression data from 23 samples including six young and six aged, non-infected C57BL6/J mice that were included for analysis. This dataset was analysed in order to find differentially expressed genes as a result of ageing and potential transcription factors driving age-related changes in transcript expression. The full pipeline of analysis in R is deposited in Github (https://github.com/cemoss1/Moss_et_al/blob/main/GSE84901_pipeline.R) and is shown in Figure 4.1, with packages and functions used in Supplementary table 8.1.3.

The "GEOquery" v2.58.0 package [374] was used to load GSE84901 dataset into R. Samples that were infected with purified human influenza virus A/Puerto Rico/8/34 (H1N1) (PR8) were then removed from the dataset analysis and the "boxplot()" function was used to assess the sample distribution. Package "dplyr" v1.0.8 [375] was used to select relevant clinical variables for analysis. A correlation matrix for the samples was created and visualised using "pheatmap" v1.0.12 package [376]. Finally "ggplot2" v3.3.6 [377], "ggrepel" v0.9.1 [378] and "ggfortify" v0.4.16 packages [379,380] and the "prcomp" function were used to generate and plot principal component analysis (PCA), using function

"autoplot()" to show the first two principal components. Expression and feature data were then exported to csv format for visualisation using "readr" v2.1.2 package [381].

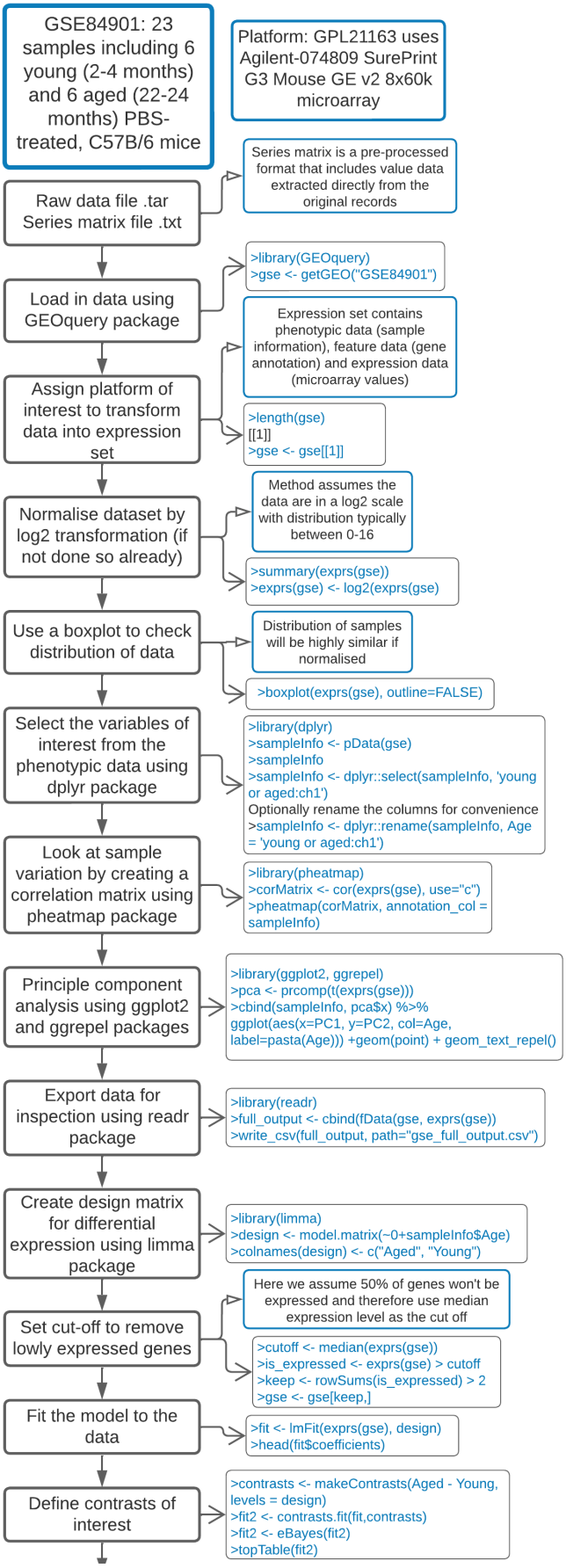
In order to create a design model, "limma" v3.46.0 package [382] was used. Using the assumption that 50% of genes would not actually be expressed, median expression level was used as a cut-off to find genes expressed in each sample. Additionally, genes had to be expressed in at least two different samples. The function "lmFit()" was used to fit the model to the data and contrasts were defined using the "makeContrasts()" function. The Bayes step was applied to pull out differentially expressed genes and relative array weights were calculated to deprioritise outliers in the samples. This means that outliers did not need to be removed, rather they would be down-weighted in analysis. Again using "dplyr" v1.0.8 package [375], features of interest were attached to the data frame. Packages "ggplot2" v3.3.6 and "ggrepel" v0.9.1 [377,378] were utilised to produce a volcano plot using the function "geom_point()" with cut-offs of adjusted P value < 0.05 and absolute LogFC > 1.

Wong *et al*. (2017), produced an overall heatmap of differentially expressed genes with age (adjusted P value < 0.05, LogFC > 1) [63], as well as a heatmap for the most differentially expressed pathways: cell cycle metaphase genes and initiation of mitosis genes. These figures were replicated in R as a training tool using "pheatmap" v1.0.12 [376] and "viridis" v0.6.3 packages [383].

A more stringent cut-off of LogFC > 1.5 and P value < 0.05 was used to define significant differentially expressed genes for this research. This produced a total of 213 upregulated genes and 122 downregulated genes for gene ontology analysis. The "readr" v2.1.2 package [381] was again used to export these data to csv format for further visualisation. Using the "select()" and "filter()" functions in "dplyr" v1.0.8 package [375], a list of gene symbols was prepared for up- and downregulated genes separately to be used as input files for Enrichr. The package "enrichR" v3.2 [244–246,384] was utilised for gene enrichment analysis, where differentially expressed gene lists were compared against KEGG 2019 Mouse, Encode and ChEA consensus TFs from ChIP-X and TargetScan microRNA libraries. These libraries were plotted using the "plotEnrich()" function. Looking specifically at the transcription

factors (TFs) associated with the stringent gene lists, those that were also differentially expressed with age were identified for further analysis.

TFs shown to be differentially expressed with age were assessed through a literature search as of November 2023. This involved a PubMed search of “TF and macrophage” or “TF and ageing”. Information relating to the TF in ageing or macrophages was compiled. Additional criteria for TF selection included a P value of approximately ≤ 0.05 in their association to the up- or downregulated gene lists and the TF being regulated in the same direction as the predicted associated gene list. The number of genes in the up- and downregulated gene lists regulated by the TF were also compiled, reported as a percentage of all differentially expressed genes and as a fraction of the total number of associated genes. Total number of associated genes was taken from CHEA and ENCODE databases and genes not expressed in macrophages were removed. Macrophage-expressed genes were compiled from the GSE84901 dataset, with at least a median level of expression in at least 2 of the 23 samples. In total, 18,349 genes met these criteria to be considered as macrophage-expressed genes.


GSE84901: 23 samples including 6 young (2-4 months) and 6 aged (22-24 months) PBS-treated, C57B/6 mice
Platform: GPL21163 uses Agilent-074809 SurePrint G3 Mouse GE v2 8x60k microarray
Raw data file .tar Series matrix file .txt
Series matrix is a pre-processed format that includes value data extracted directly from the original records
Load in data using GEOquery package
>library(GEOquery)
>gse <- getGEO("GSE84901")
Assign platform of interest to transform data into expression set
Expression set contains phenotypic data (sample information), feature data (gene annotation) and expression data (microarray values)
>length(gse)
[[1]]
>gse <- gse[[1]]
Normalise dataset by log2 transformation (if not done so already)
Method assumes the data are in a log2 scale with distribution typically between 0-16
>summary(exprs(gse))
>exprs(gse) <- log2(exprs(gse)
Use a boxplot to check distribution of data
Distribution of samples will be highly similar if normalised
>boxplot(exprs(gse), outline=FALSE)
Select the variables of interest from the phenotypic data using dplyr package
>library(dplyr)
>sampleInfo <- pData(gse)
>sampleInfo
>sampleInfo <- dplyr::select(sampleInfo, 'young or aged:ch1')
Optionally rename the columns for convenience
>sampleInfo <- dplyr::rename(sampleInfo, Age = 'young or aged:ch1')
Look at sample variation by creating a correlation matrix using pheatmap package
>library(pheatmap)
>corMatrix <- cor(exprs(gse), use="c")
>pheatmap(corMatrix, annotation_col = sampleInfo)
Principle component analysis using ggplot2 and ggrepel packages
>library(ggplot2, ggrepel)
>pca <- prcomp(t(exprs(gse)))
>cbind(sampleInfo, pca$x) %>%
ggplot(aes(x=PC1, y=PC2, col=Age, label=pasta(Age))) +geom(point) + geom_text_repel()
Export data for inspection using readr package
>library(readr)
>full_output <- cbind(fData(gse, exprs(gse))
>write_csv(full_output, path="gse_full_output.csv")
Create design matrix for differential expression using limma package
>library(limma)
>design <- model.matrix(~0+sampleInfo$Age)
>colnames(design) <- c("Aged", "Young")
Set cut-off to remove lowly expressed genes
Here we assume 50% of genes won't be expressed and therefore use median expression level as the cut off
>cutoff <- median(exprs(gse))
>is_expressed <- exprs(gse) > cutoff
>keep <- rowSums(is_expressed) > 2
>gse <- gse[keep,]
Fit the model to the data
>fit <- lmFit(exprs(gse), design)
>head(fit$coefficients)
Define contrasts of interest
>contrasts <- makeContrasts(Aged - Young, levels = design)
>fit2 <- contrasts.fit(fit,contrasts)
>fit2 <- eBayes(fit2)
>topTable(fit2)

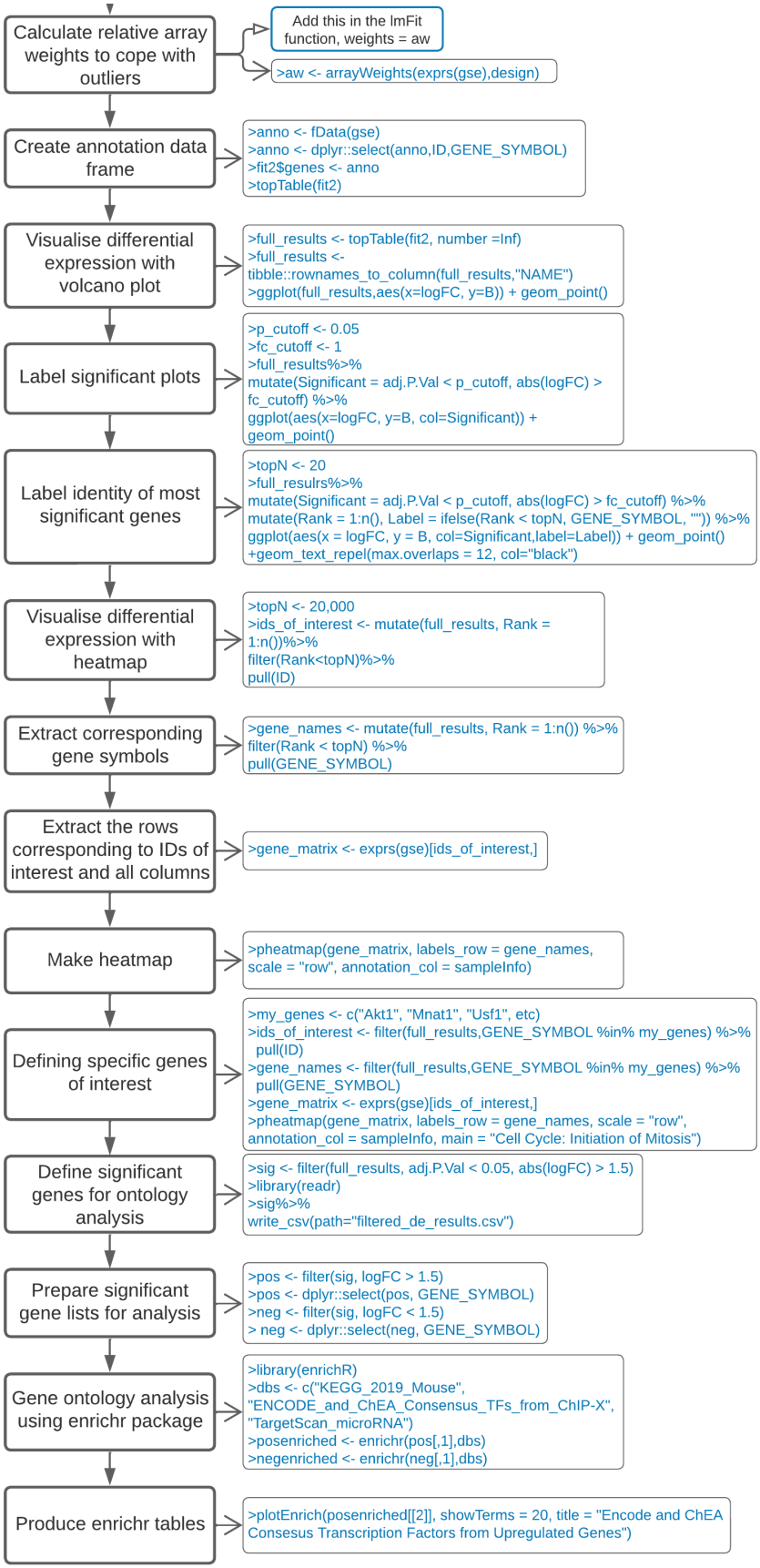


Figure 4.1 Full pipeline used for analysis of GSE84901 microarray dataset in R

Black boxes represent the steps followed, blue writing shows the actual R commands used, blue boxes provide additional information and reasoning.

4.2.2 Cell culture and gene expression analysis

Human monocyte-derived macrophages, murine bone marrow-derived macrophages and immortalised bone marrow-derived macrophages were isolated, differentiated, cultured and polarised as described in Section 2.3. RNA isolation, cDNA synthesis and real-time qPCR were performed as described in Section 2.5.

4.3 Results

4.3.1 Transcriptional profiling of alveolar macrophage ageing: *in silico* analysis

The particular publication chosen for bioinformatic analysis in this research by Wong *et al*. (2017), compared young and aged mice in their responses to influenza infection including alveolar macrophage responses [63]. The main findings of the publication included that aged mice had fewer alveolar macrophages that were critical for influenza-induced mortality compared with young counterparts. The transcriptional profiles of these aged alveolar macrophages were also significantly altered, specifically with downregulated cell cycle pathways and ability to perform phagocytosis. Raw data from this publication were deposited in gene expression omnibus (GEO) as GSE84901, including bulk microarray transcriptomes of six young and six aged healthy/uninfected mouse alveolar macrophages that were controls to this study. This enabled detailed comparisons to be made between the young and aged healthy mice that were not included as part of the publication.

4.3.1.1 Differentially expressed genes in macrophage ageing

GSE84901 dataset was uploaded into R using “GEOquery”, a package that bridges R with the GEO database [374]. A plethora of R packages were then utilised to manipulate the dataset and ascertain differentially expressed genes and associated ontologies. This included “limma” for differential expression analysis, “ggplot2” and “pheatmap” for visualisation and “enrichR” for gene ontology analysis.

In assessing microarray data for differential expression, it was first necessary to assess distribution, correlation and grouping of samples, to ensure that the data were suitable. Samples were Log2

normalised to ascertain expression values and normal distribution of samples was confirmed (Figure 4.2A). Sources of variation between the sample groups can be determined through unsupervised analysis, meaning that samples should be grouped by observational similarity. Correlation between all samples was determined in a pairwise fashion on a scale of 0-1 and this was visualised in a heatmap (Figure 4.2B). This demonstrated that samples were largely clustered in the groups of interest: young and aged, apart from the one young sample "GSM2253767" that correlated more highly with the aged samples. This sample was further tested to confirm whether it was a true outlier. Principal component analysis (PCA) allows visualisation of samples in a 2D, linear plane and shows the principal components that account for variability in the samples [385]. This is an unsupervised analysis meaning groups do not need to be specified, rather the plot will ideally show that the greatest source of variation in the data correspond to the groups of interest. PCA also allows further visualisation of quality control, as outliers can be uncovered at this stage. As shown in Figure 4.2C, young and aged samples were grouped mainly by the first principal component, accounting for 32.65% of the variability between samples, whereas the second principal component accounted for 10.7% of the variability between samples. Through these analyses, distribution of the samples was such that no single sample was an outlier from the others in their specified groups.

Once the dataset had been through these initial checks for suitability and outliers, differential expression of genes between the young and aged groups was assessed. There are multiple different approaches to assessing differential expression; the chosen method used the "limma" R package v3.46.0, due to its extensive use and user-manual [382]. Samples were assigned to the groups of interest (young and aged) in a design matrix and visualised in a volcano plot (Figure 4.2D). Those genes with LogFC > 1 were considered to be significantly upregulated with age ($P < 0.05$), whereas those with a LogFC < -1 were considered to be significantly downregulated with age ($P < 0.05$). The top 20 genes with the greatest fold change were also labelled, with a greater number of these being upregulated than downregulated with age.

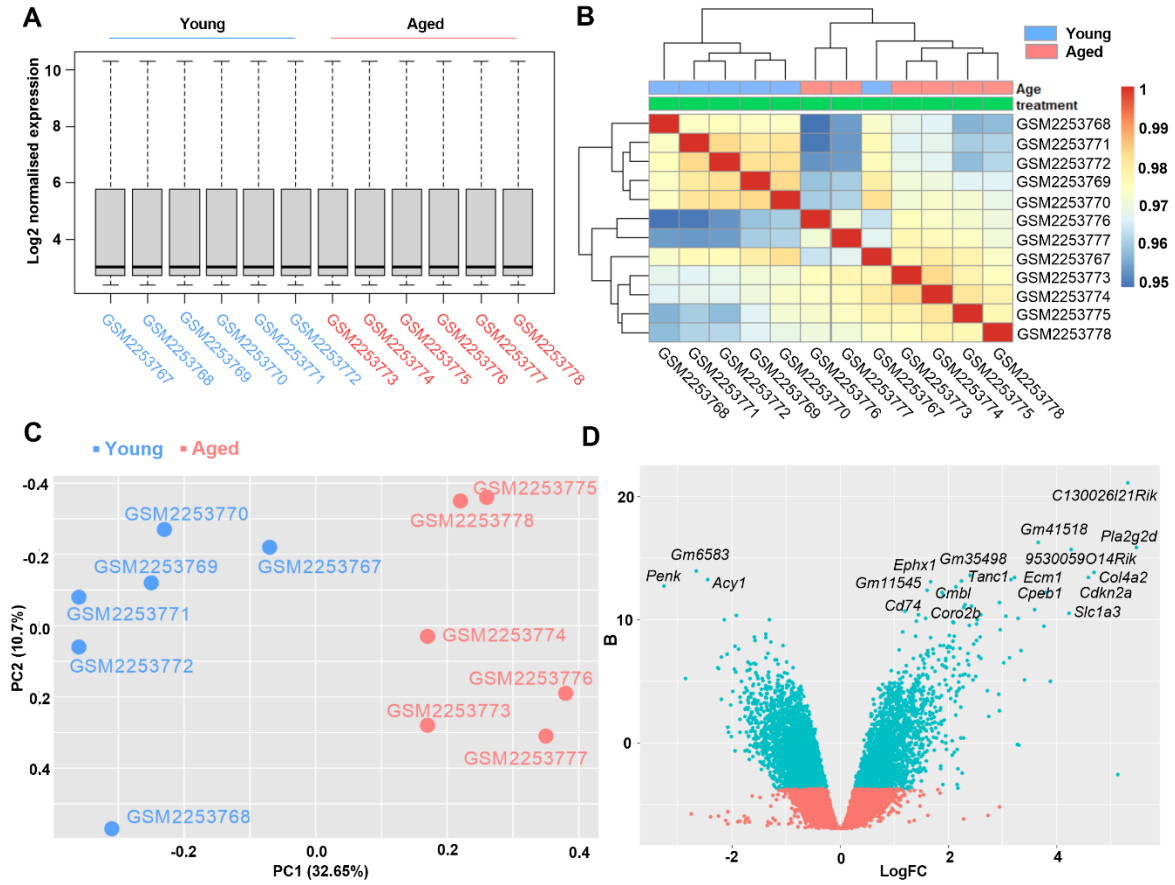


Figure 4.2 Differential expression analysis of untreated young and aged alveolar macrophages

A – Distribution of the young (GSM2253767-72) and aged (GSM2253773-78) untreated samples in the GSE84901 dataset following log2 normalisation using "boxplot()" function in R.

B – Distribution and sample clustering of the young and aged untreated samples using a correlation matrix from the GSE84901 dataset using "pheatmap()" package in R.

C – Grouping and sample clustering of the young and aged untreated samples in the GSE84901 dataset by principal component (PC) analysis using "ggplot2", "ggrepel" and "ggfortify" packages in R.

D – Differentially expressed genes between young and aged mouse alveolar macrophages using "ggplot2" and "ggrepel" packages in R with cut-offs of adjusted P value < 0.05 and absolute Log fold change (FC) > 1. Significant genes are shown in blue and unsignificant in red. The top 20 differentially expressed genes are also labelled with those on the right of 0 being upregulated with age and those on the left downregulated.

Heatmaps were also produced to visualise trends for the whole population of genes that were differentially expressed in the genome. This showed there was a wide-spread difference in the genomes of the alveolar macrophages between young and aged mouse samples. Wong *et al*. (2017), reported a total of 3,545 genes that were significantly different with age (FDR < 0.05). Similarly, for our analysis method the difference extended to 3,437 significantly different genes with age (FDR <

0.05, Figure 4.3A), including many genes associated with cell cycle pathways, that were downregulated with age, concordant with the published findings (Figure 4.3B-C).

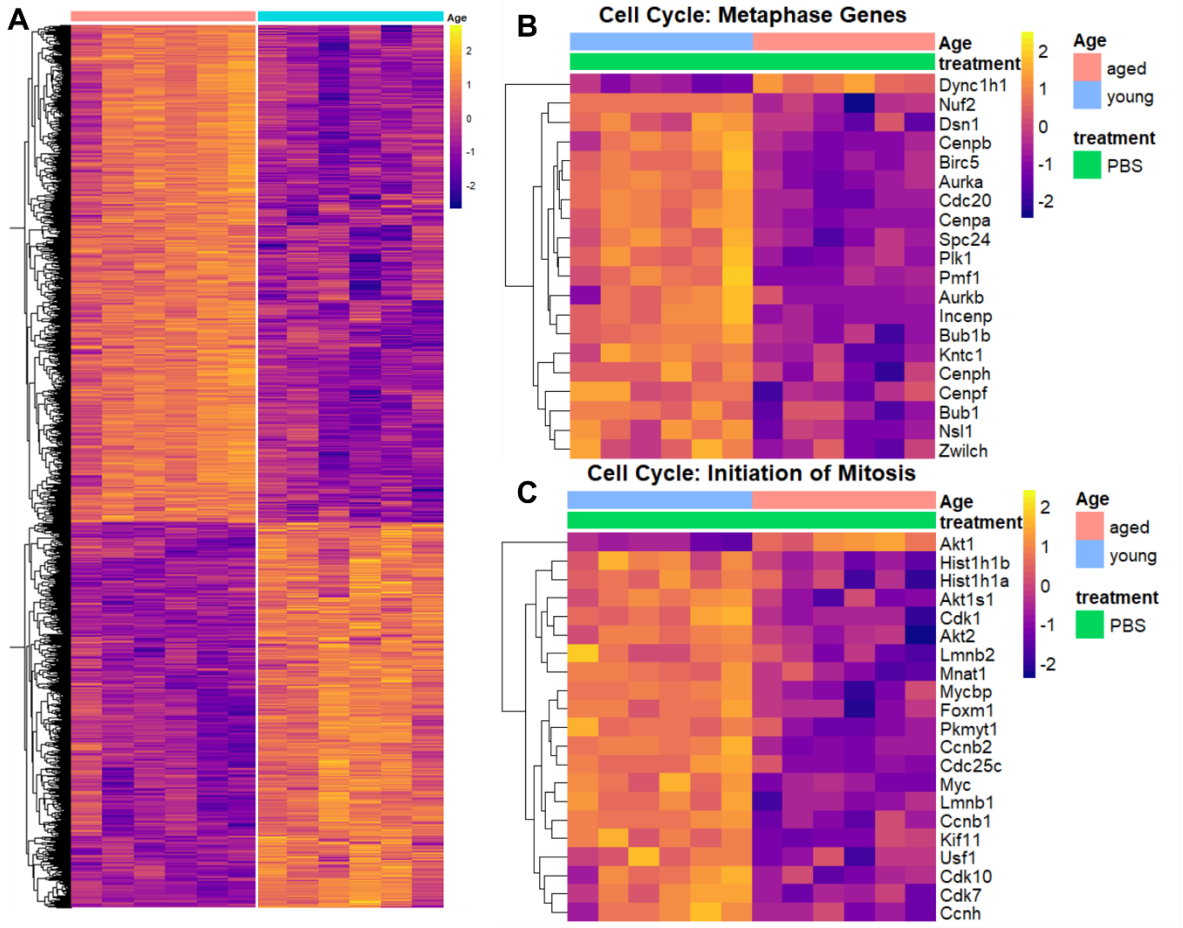


Figure 4.3 Heatmaps of differentially expressed genes between young and aged alveolar macrophages from GSE84901 dataset

A – Heatmap showing overall differentially expressed genes with cut-offs of adjusted P value < 0.05 and absolute LogFC > 1. Upregulated genes with ageing are yellow and downregulated genes are purple.

B – Heatmap showing top differentially expressed pathway of metaphase genes in the cell cycle.

C – Heatmap showing top differentially expressed pathway of initiation of mitosis in the cell cycle.

PBS: phosphate-buffered saline

4.3.1.2 Gene ontology analysis in macrophage ageing

To take the analysis further than that done by Wong *et al*. (2017), gene ontology analysis using the "enrichR" package in R was performed. Enrichr has a plethora of functions and multitude of libraries enabling much gene ontology analysis to be performed [244–246,384]. For this, a more stringent cut-off of

LogFC > 1.5 was used to focus on the genes changing most considerably with age. Within Enrichr, "KEGG 2019 Mouse" library was used to assess enriched biological processes and metabolic pathways, "TargetScan microRNA" library was used to assess the microRNAs (miRNAs) associated with the stringent gene lists and "Encode and ChEA Consensus TFs from ChIP-X" library was used to assess the transcription factors associated with the differentially expressed genes.

In total, 213 genes met the criteria to be considered upregulated with age (LogFC > 1.5, P value < 0.05). Chemokine signalling pathway, Antigen processing and presentation and Morphine addiction were found to be the three most upregulated pathways with age (Figure 4.4A). The miRNAs found to be most associated with the upregulated genes included miR-505, miR-9 and miR-28. Their association with the upregulated genes means these would likely be downregulated with age, as miRNAs are a form of regulation and these genes in the upregulated list were clearly not being targeted for degradation [386] (Figure 4.4B). Finally, the TFs associated with the upregulated gene list included GATA1 (P = 0.0027), NFE2L2 (P = 0.009) and NFIC (P = 0.061) (Figure 4.4C). Because of their association with the upregulated list, they would likely also be upregulated with age, were they actually regulating these genes. These TFs were then assessed for differential expression with age, and those that were differentially expressed are highlighted in green: NFIC, SUZ12, AR, EZH2 and TRIM28 (Figure 4.4D).

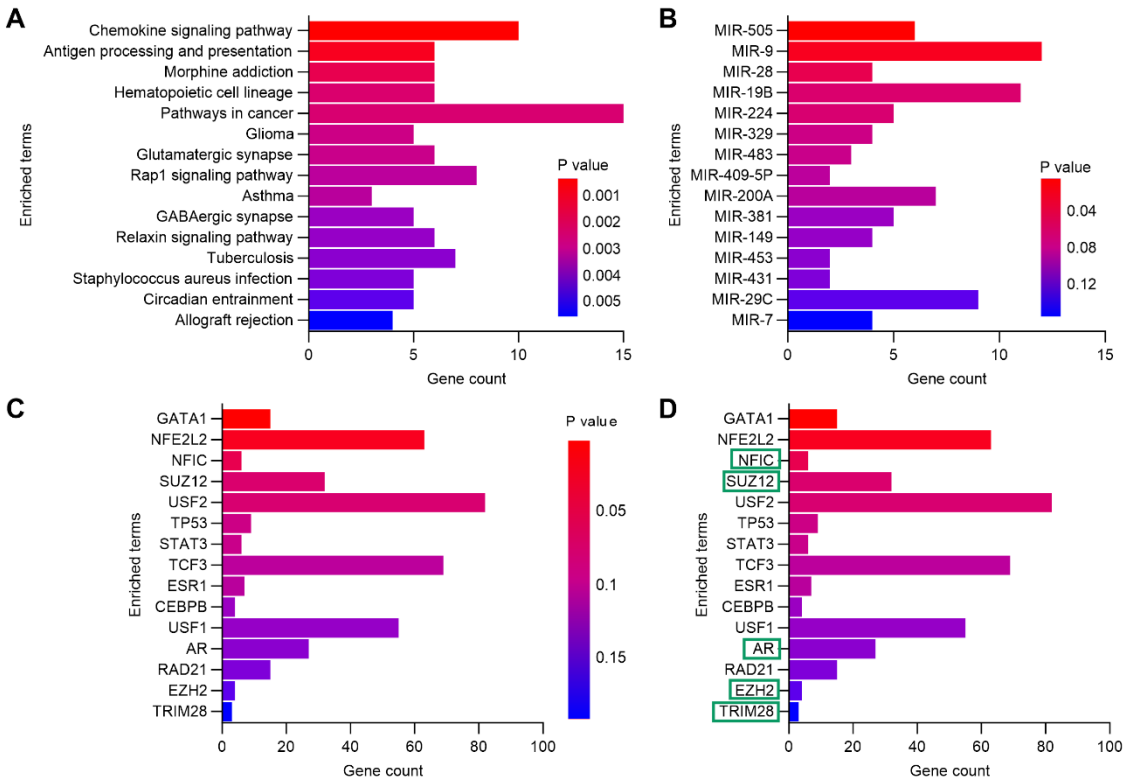


Figure 4.4 Upregulated gene enrichment analysis between young and aged alveolar macrophages from GSE84901 dataset

A – Most upregulated pathways associated with ageing using the KEGG Mouse 2019 database.

B – microRNAs likely targeting the upregulated genes using the TargetScan database.

C – Transcription factors likely regulating the upregulated genes.

D - Transcription factors likely regulating the upregulated genes that are also differentially expressed with age highlighted in green.

Gene enrichment analysis of the upregulated stringent gene list (Adjusted P value < 0.05, LogFC > 1.5) using "enrichR" package in R.

The list of genes downregulated with age was then assessed in the same way. In total 122 genes were downregulated with age (LogFC < -1.5, P value < 0.05). Pathway analysis found that Cell cycle, Oocyte meiosis and Progesterone-mediated oocyte maturation pathways were most associated with genes downregulated with age (Figure 4.5A). The miRNAs associated with the downregulated list included miR-492, miR-449 and miR-216. These would likely themselves be upregulated, and their targeting of the gene for degradation would be the cause of the downregulation [386] (Figure 4.5B). The TFs found to be most associated with the downregulated list included E2F4 (P = 1.28E-13), FOXM1 (P = 3.2E-10) and NFYA (P = 7.21E-9) (Figure 4.5C). As they were associated with the age-related

downregulated genes, downregulation of these TFs would be indicative of a true association with these genes. Again, the TFs associated with the downregulated gene list were assessed for their relative expression between young and aged samples, and those highlighted in red were found to also be differentially expressed themselves with age: FOXM1, NFYB, MYC, ELF1, USF1 and SRF (Figure 4.5D). These TFs, alongside those associated with the age-related upregulated genes that are also differentially expressed, could therefore be governing age-related transcriptional changes in these cells and have the most potential to be master regulators of macrophage ageing, and so these were highlighted for further analysis.

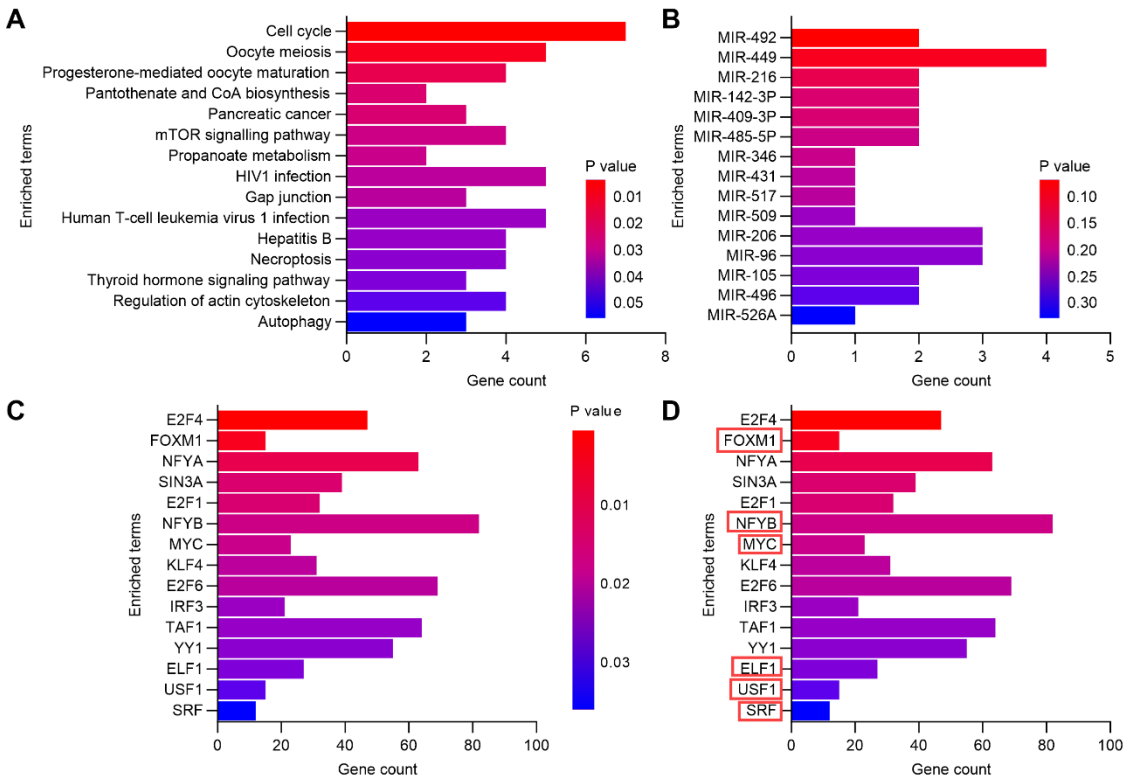


Figure 4.5 Downregulated gene enrichment analysis in young and aged alveolar macrophages from GSE84901 dataset

A – Most downregulated pathways associated with ageing using the KEGG Mouse 2019 database.

B – microRNAs likely targeting the downregulated genes using the TargetScan database.

C – Transcription factors likely regulating the downregulated genes.

D - Transcription factors likely regulating the downregulated genes that are also differentially expressed with age highlighted in red.

Gene enrichment analysis of the downregulated stringent gene list (Adjusted P value < 0.05, LogFC < -1.5) using "enrichR" package in R.

4.3.1.3 Transcriptional control in macrophage ageing

In order to narrow down our list of transcription factors likely regulating macrophage ageing, a literature search was performed as described in Section 4.2.1. The 11 differentially expressed TFs were looked up for their known roles in ageing and macrophage responses. As expected, there was a wide array of information reported for some TFs, such as MYC, AR and SRF, but very limited knowledge on others, such as NFIC and NFYB, with 4 and 3 papers focusing on macrophages, and 4 and 7 papers focusing on ageing, respectively (Table 4.1).

Table 4.1 Papers relating to transcription factors associated with differentially expressed genes in young and aged alveolar macrophages

Number of papers associated with each of the transcription factors that contribute to differential expression of genes with age. Literature searches were done in PubMed up to November 2023, with the transcription factor and ageing or macrophage being present in the title and/or abstract of the paper.

Transcription factor	No. papers for macrophages	No. papers for ageing
NFIC	4	4
NFYB	3	7
SUZ12	4	15
TRIM28	10	17
ELF1	17	6
USF1	21	15
FOXM1	38	101
EZH2	140	198
SRF	60	175
AR	390	1,140
MYC	1,277	1,069

The number of genes regulated by the TF within the list of differentially expressed genes that were macrophage-specific was also calculated to better predict the level of regulation of each transcription factor on macrophage ageing (Table 4.2). NFYB had the highest number of target genes within the differentially expressed genes, equating to nearly 15%, while both NFIC and TRIM28 had only 3 out of the 335 differentially expressed genes in their targets, equating to less than 1%.

Table 4.2 Number of dysregulated genes with age associated with each transcription factor of interest

The number of target genes for each transcription factor was taken from CHEA and ENCODE databases and then only those expressed in the macrophage were used. Dysregulated genes were those with LogFC > 1 and P vale < 0.05. Percentage was then calculated.

Transcription factor	No. target genes	No. genes dysregulated with age	Percentage of target dysregulated genes in stringent gene lists
NFIC	229	3	0.90
NFYB	3004	50	14.93
SUZ12	994	24	7.16
TRIM28	158	3	0.90
ELF1	2012	27	8.06
USF1	1201	15	4.48
FOXM1	83	10	2.99
EZH2	138	4	1.19
SRF	263	5	1.49
AR	801	15	4.48
MYC	1336	17	5.07

The transcription factors selected for further assessment of their contribution to macrophage ageing required P value < 0.05 in their association with the differentially expressed stringent gene list and differential expression of the TF in the same direction as the associated list. Consideration was then made for the number of genes dysregulated with age that were downstream targets of the TF, named in Table 4.3, and from the in-depth literature search of each TF (Figure 4.6).

Table 4.3 Age-related dysregulated genes downstream of key transcription factors associated with murine alveolar macrophage ageing

Transcription factor targets were taken from the ChEA database and looked up within the differentially expressed genes with age in alveolar macrophages isolated from young (2-4 months) and aged (22-24 months) mice, genes in both datasets are presented.

Transcription factor	Target genes dysregulated with age
NFIC	GADD45A, WDFY1, NEAT1
NFYB	PIGS, TOP2A, ROGDI, CDC20, TUBA1C, SGOL1, FLAD1, CHCHD7, BCL7B, NUSAP1, CLCC1, MAP2K1, ATP6AP1, ZFYVE21, CYB561A3, TYK2, CDC25C, MED24, PCCA, RPUSD1, AKT1S1, MCM3, SUCLG2, TRIM59, SEC22A, LRRC14, SLBP, TMEM55B, CDCA8, CENPA, CCNB2, INPP5K, DHX38, MBD3, SLC35A4, CDKN2C, B3GALT6, PYCR2, COQ6, PRC1, CENPK, CDK1, RBM42, PTPN6, SFXN2, NAT9, CENPQ, MXD3, CDKN3, NUP37
SUZ12	CLIC6, CSF1, PRKCB, CDKN2A, CITED4, NDRG1, EPOR, ARHGAP10, CPEB1, ADCY9, MAFB, NR4A3, COL4A2, COL4A1, DNAJA4, TRPV4, ALDH1A2, CLDN23, STAC2, PMP22, KIF1A, S1PR5, HOXB7, ST3GAL1
TRIM28	CDKN2B, COL4A1, MYO5A
ELF1	SLBP, TMEM55B, ARHGAP1, IQGAP3, TUBA1C, FLAD1, CHMP1A, GAR1, INPP5K, DHX38, PLEKHJ1, FBXW9, B3GALT6, CNOT10, PYCR2, COQ9, COASY, GLTSCR2, ZC3HC1, CDK7, RAD51, RPS6KB2, MCM3, ID3, NAT9, CENPQ, NUP37
USF1	TMEM55B, MAP2K1, CLUH, ACY1, ATP6AP1, SLC39A10, B3GALT6, PYCR2, CDK7, SGOL1, FLAD1, AKT1S1, SFXN2, LAMTOR3, MXD3
FOXM1	CDC20, CCNB2, CDKN2C, PRC1, CDK1, TRIM59, CENPA, MXD3, CDKN3, NUP37
EZH2	COL4A2, PDGFB, KIF1A, HOXB7
SRF	FBXW9, RPS6KB2, ARHGAP1, RBM42, MED27
AR	AKNA, MYO5A, UBE2E2, CXCL1, CMBL, IGF1, AOAH, SORBS1, NDRG1, MYO1D, TANC1, MAFB, COL4A1, PPAP2B, POLE2
MYC	MBD3, SLBP, SLC35A4, CLUH, ACY1, PARP1, ARHGAP1, CDCA8, SLC39A10, B3GALT6, TUBA1C, XPO6, AKT1S1, GAR1, MCM3, MXD3, PLEKHJ1

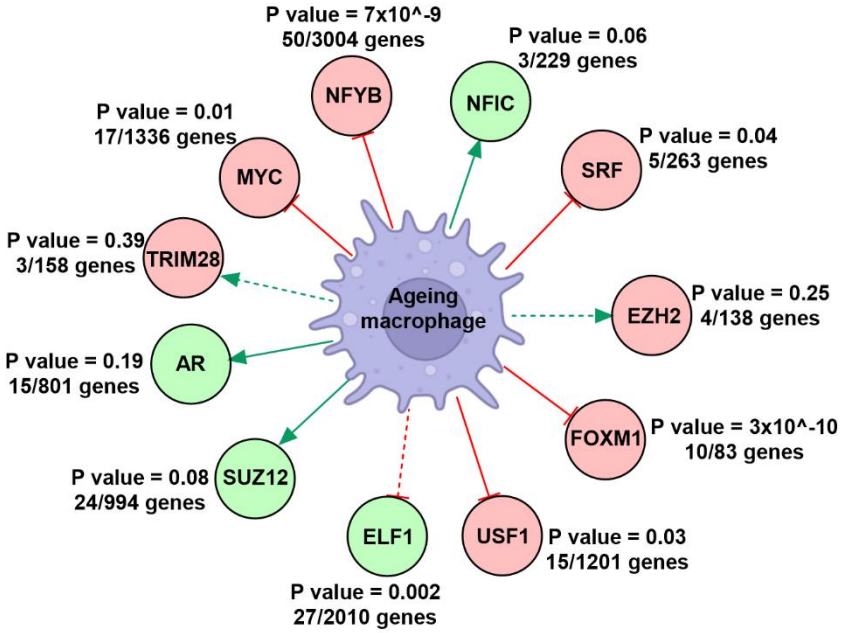


Figure 4.6 Differentially expressed transcription factor analysis in alveolar macrophage ageing

Transcription factor analysis results showing P value in association with age-related differentially expressed genes as well as the number of genes associated with downstream signalling present in the up- or downregulated gene lists. Those in green circles are upregulated with age while those in red circles are downregulated with age. The green arrow corresponds to association with upregulated genes while the red arrow corresponds to association with downregulated genes. Where this is a dashed line the association is opposite to the age-related dysregulation in transcription factor expression.

Six TFs were therefore selected for further analysis: NFYB, SRF, FOXM1, USF1, NFIC and MYC. NFYB, SRF, FOXM1, USF1 and MYC due to their P value < 0.05 in association with the age-related downregulated genes, and downregulation with age, the same differential expression direction as the gene list they are associated with, and NFIC due to being upregulated with age while being associated with the upregulated list and having a P value close to 0.05 in this association. TRIM28, EZH2 and ELF1 were not selected due to their differential expression being opposite to their associated gene list and AR and SUZ12 were not selected due to their P values not reaching significance. The six transcription factors put forward would need to be analysed for changes in gene expression with age in different macrophage populations to understand their overall contribution to macrophage ageing.

4.3.2 Selection of housekeeping genes in macrophage ageing

Housekeeping genes are important in gene expression studies to calibrate or normalise values, enabling direct comparison between samples [387]. Limited data exist assessing potential housekeeping genes with stable expression in macrophages isolated from individuals of different ages. Therefore, we set out to find genes with stable expression between young and older human MDMs or mouse BMDMs. Each ageing process likely needs its own subset of reference genes so it was important to test these genes in our samples to find the most stable [373], including both mouse and human samples separately.

For both human MDMs and mouse BMDMs, existing literature was utilised to compile a list of common reference genes. Five publications were found that assessed housekeeping genes in a number of cell types. A total of 29 genes were found to be most conserved across these publications (Table 4.4). Additionally, the Housekeeping and Reference Transcript (HRT) Atlas [388] was utilised to find further genes with conserved expression. This compiles high-quality RNAseq datasets for different cell types in order to search human and mouse transcripts. For human, 83 high quality macrophage samples were compiled, outputting 30 potential reference transcripts (Table 4.5, Supplementary table 8.3.1). For mouse, 272 macrophage samples were compiled, outputting 896 potential reference transcripts (Supplementary table 8.3.2). In addition to this, bone marrow bulk tissue was used to compile 392 potential reference transcripts from the HRT Atlas (Supplementary table 8.3.3) and those that were present in both macrophage and bone marrow were taken forward (Table 4.6).

Table 4.4 Publications assessing potential housekeeping genes in relevant cells

Cell type	Genes assessed	Most conserved	Reference
Alveolar macrophages from COPD patients	ABL1, ACTB, B2M, GAPDH, GNB2L1, HPRT1, PGBD, RPL32, TBP, TUBB	GNB2L1, HPRT1 and RPL32	[370]
Fibroblast *in vitro* ageing	GAPDH, GUSB, ACTB, HPRT1, PUM1, TBP, OAZ1, TMEM199	GUSB and PUM1	[373]
J774a.1 macrophages with LPS stimulation	B2M, PPIA, GAPDH, HPRT1, RPLP0, TBP, UBC, RPL13A	HPRT1, TBP and UBC	[371]
BMDMs with time course LPS stimulation	GAPDH, ACTB, GUSB, HPRT1, HMBS, B2M, MAU2, RPL13A, HNRNPAB, STX5A, PPIA	HNRNPAB and STX5A	[364]
Human tissues and organs from existing datasets	All genes were assessed using a set of specific parameters to pull out the best fits	RPL41, RPLP0, RPS27, TUBA1B, RPSA, SLC25A3, ACTG1 and EEF1G	[369]

Table 4.5 Most conserved transcripts in human macrophage samples compiled in the HRT Atlas

Rank	Gene symbol	Normalised expression	Standard deviation
1	SF3B1	46.921	0.321
2	CSDE1	90.470	0.363
3	SERINC3	33.897	0.352
4	PRDX5	82.085	0.440
5	STT3B	45.514	0.407
6	CCNI	77.767	0.436
7	PARK7	80.410	0.476
8	ESD	57.143	0.414
9	SND1	51.860	0.428
10	COPG1	49.554	0.445

Table 4.6 Most conserved transcripts in mouse macrophages and bone marrow samples compiled in the HRT Atlas

Gene symbol	Macrophage		Bone marrow	
	Normalised expression	Standard deviation	Normalised expression	Standard deviation
Cpsf3	623.367	0.369	442.895	0.311
Srp72	746.995	0.364	558.121	0.221
Plaa	75.062	0.247	66.322	0.181
Mapre1	451.374	0.318	386.854	0.274
Api5	325.328	0.341	241.373	0.178
Stt3b	422.476	0.375	312.975	0.273
Sec24c	432.656	0.363	354.249	0.158
Canx	486.889	0.408	417.346	0.276
Caprin1	609.657	0.435	572.351	0.298
Bzw1	488.057	0.337	375.018	0.298
Sec31a	554.340	0.455	483.495	0.333
Copb1	355.596	0.368	348.271	0.218
Yeats4	401.171	0.370	255.196	0.223
Psmc6	205.777	0.327	188.887	0.249
Vcp	569.219	0.444	497.455	0.186
Dnajc7	135.544	0.339	113.107	0.219
Arl6ip1	505.361	0.488	518.221	0.372
Psmd1	273.332	0.421	263.961	0.300
Stt3a	503.652	0.448	444.550	0.287
Hnrnpc	397.252	0.379	291.413	0.284
Nrd1	382.992	0.488	335.255	0.300
Hnrnpu	563.036	0.489	293.676	0.304
Ddx1	341.744	0.440	287.168	0.275
Eif3d	319.366	0.426	243.793	0.156
Abcf1	368.542	0.459	315.968	0.269
St13	220.454	0.406	190.310	0.276
Eif3m	135.169	0.375	87.742	0.204
Psmc2	198.492	0.435	213.022	0.154

For human studies, an RNAseq dataset assessing *in vitro* polarised MDMs from 8 human donors was assessed for the most conserved genes between these populations and those genes already identified were also assessed (Table 4.7). The top four genes in Table 4.7 represent those in the RNAseq dataset with the lowest standard deviation and difference (measured as the difference between the maximum and minimum LogFC) between samples, indicating that these genes are the least variable in expression in MDMs following *in vitro* stimulation, while the rest are those found in published literature and on the HRT Atlas. The conserved genes were also searched in the literature for known functions, as it is commonly thought that genes encoding basic cellular processes, such as ribosomal proteins already validated in studies on different cell types [389–391] and those involved in

glycolysis, such as GAPDH, are the most conserved regardless of experimental setup [392]. Overall, six genes were taken forward to assess in human MDMs (Table 4.8). Out of those assessed, *PUM1* was found to be the most conserved between young and older human MDMs regardless of stimulation (Figure 4.7).

Table 4.7 Expression of potential housekeeping genes in *in vitro* stimulated human macrophages

Gene	$M^{LPS+IFN\gamma}$	M^{IL-4}	M^{IL-10}	M^{oxPAPC}	M^{CXCL4}	Average	SD	Difference
BECN1	-0.004	-0.073	0.1056	0.0255	-0.007	0.0090	0.064	0.179
TCEAL4	-0.561	-0.364	-0.439	-0.422	-0.442	-0.446	0.071	0.197
CEP170B	-0.397	-0.602	-0.492	-0.535	-0.416	-0.488	0.084	0.204
NABP2	-0.489	-0.412	-0.470	-0.517	-0.302	-0.438	0.085	0.215
GNB2L1	-0.651	-0.506	-0.504	-0.547	-0.433	-0.528	0.080	0.218
RPL41	-0.317	-0.069	-0.163	-0.380	-0.206	-0.227	0.123	0.311
CSDE1	-0.288	-0.074	-0.079	0.0390	0.0803	-0.064	0.143	0.369
SF3B1	-0.154	-0.185	-0.076	0.2022	0.0653	-0.029	0.161	0.387
PUM1	-0.072	-0.346	0.0388	0.0257	0.0866	-0.053	0.173	0.433
SERINC3	0.2405	-0.172	0.0216	0.3759	0.0386	0.1009	0.212	0.547
PRDX5	0.2347	-0.175	-0.310	-0.202	-0.381	-0.167	0.239	0.616
HPRT1	-0.547	-0.089	-0.362	-0.873	-0.260	-0.426	0.299	0.783
RPS27	0.4554	-0.388	-0.148	0.2210	-0.103	0.0072	0.331	0.844
RPL32	-1.280	-0.197	-0.439	-0.744	-0.352	-0.603	0.428	1.082
RPSA	-1.470	-0.354	-0.821	-1.240	-0.655	-0.908	0.448	1.115
SLC25A3	-1.126	0.0191	-0.504	-0.515	-0.224	-0.470	0.428	1.145
RPLP0	-1.549	-0.297	-0.586	-0.894	-0.473	-0.760	0.491	1.252
GUSB	-1.298	-0.149	-0.280	0.0440	-0.083	-0.353	0.541	1.342
ACTG1	-1.705	0.5503	-0.385	-0.994	-0.230	-0.553	0.847	2.255
TUBA1B	-2.570	0.1704	0.0390	-1.509	-0.239	-0.822	1.182	2.741

Log2 fold change compared to unstimulated MDMs of expressed genes from *in vitro* stimulated MDMs from 8 individuals. Difference was worked out as the difference between the maximum and minimum value.

Table 4.8 Housekeeping genes assessed in human MDMs

Housekeeping genes compiled from the literature and datasets that were taken forward to assess in human MDMs isolated from young and older individuals.

Gene	Reasoning	Known functions	Reference
GAPDH	Current standard housekeeping gene commonly used in the literature	Considered a major housekeeping and multifunctional protein involved in glycolysis, cell metabolism and homeostasis	[393]
PUM1	Found in all assessed published literature to have conserved expression, relatively conserved expression in RNAseq data assessing human MDMs stimulated in vitro	RNA binding protein that may be involved in cell development and differentiation	[394,395]
SF3B1	Conserved expression in the human macrophage samples on the HRT Atlas	Splicing factor 3b subunit 1 of pre-mRNA critical for spliceosome assembly and mRNA splicing	[396]
CSDE1	Conserved expression in the human macrophage samples on the HRT Atlas	RNA binding protein that regulates translation and mRNA stability	[397,398]
COPG1	Conserved expression in the human macrophage samples on the HRT Atlas	Component of COPI protein required for lipid homeostasis	[399,400]
BECN1	Conserved expression in RNAseq data assessing human MDMs stimulated in vitro	Forms an important protein complex in autophagy, including formation of the autophagosome, extension and maturation	[401]

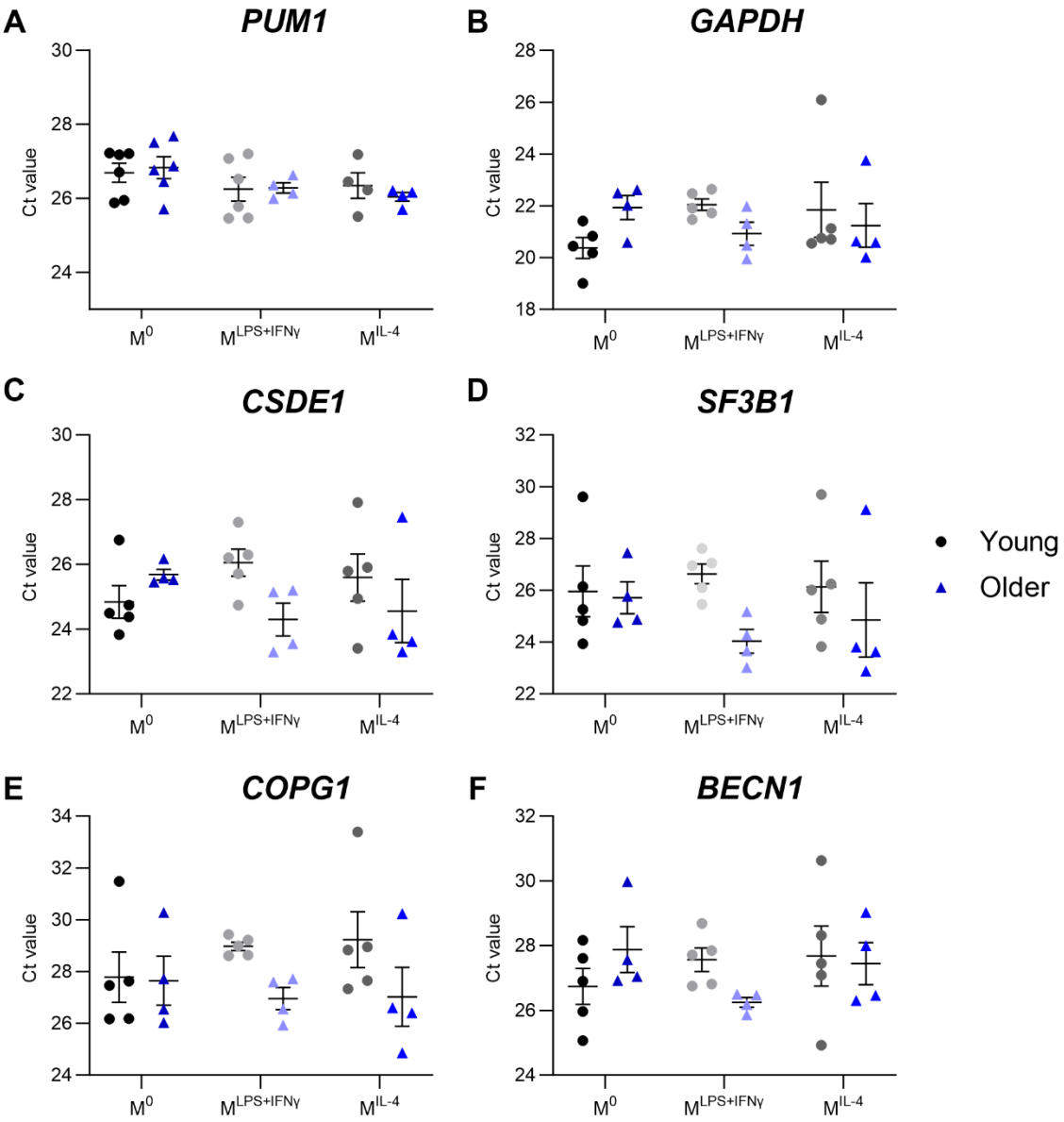


Figure 4.7 RT-qPCR analysis of potential reference genes in young and older human monocyte-derived macrophages

Comparison in expression levels of potential housekeeping genes by RT-qPCR in monocyte-derived macrophages isolated from young (18-30 years) and older (>50 years) human donors. M^0 – cells left unstimulated, $M^{LPS+IFN\gamma}$ – cells stimulated with lipopolysaccharide (LPS) and interferon (IFN)-γ for 24 hours, M^{IL-4} – cells stimulated with interleukin (IL)-4. Circles – young, triangles – older. N = 4-6 biological repeats done in triplicate. Statistical analysis was not performed.

For the mouse studies, the microarray dataset assessing unstimulated alveolar macrophages from young and aged mice was used to look up the compiled gene lists and find further conserved genes. This lead to six genes being assessed in iBMDMs (Table 4.9), used to further narrow down genes before assessing the more valuable BMDM samples (Figure 4.8). These six were further narrowed

down to three genes assessed in mouse BMDMs as these had the most reproducible and conserved expression. *Mau2* was found to be the most conserved between mouse BMDMs of different ages and phenotypes (Figure 4.9).

Table 4.9 Housekeeping genes assessed in mouse iBMDMs

Gene	Reasoning	Known functions	Reference
Gapdh	Current standard housekeeping gene commonly used in the literature, found to be conserved between age groups in alveolar macrophages	Considered a major housekeeping and multifunctional protein involved in glycolysis, cell metabolism and homeostasis	[393]
Actb	Current standard housekeeping gene commonly used in the literature, found to be conserved between age groups in alveolar macrophages	High conserved, ubiquitous cytoskeleton protein required for cell motility, structure and integrity	[402,403]
Mau2	Found to be conserved between age groups in alveolar macrophages and with LPS stimulation in BMDMs	Role in sister-chromatid cohesion. Involved in the cell cycle including loading cohesin onto DNA chromatin during interphase	[404]
Rpl32	Found to be conserved between age groups in alveolar macrophages and in alveolar macrophages from COPD patients	Ribosomal protein that is a component of the 60S subunit	[391]
Psmc2	Conserved expression in mouse macrophages and bone marrow samples on the HRT Atlas	Member of the 19S regulatory subunit of the 26S proteasome and regulates many cellular processes including differentiation, proliferation and energy metabolism	[405,406]
Mapre1	Conserved expression in mouse macrophages and bone marrow samples on the HRT Atlas	Binds to the APC protein and plays a role in microtubule stabilisation	[407]

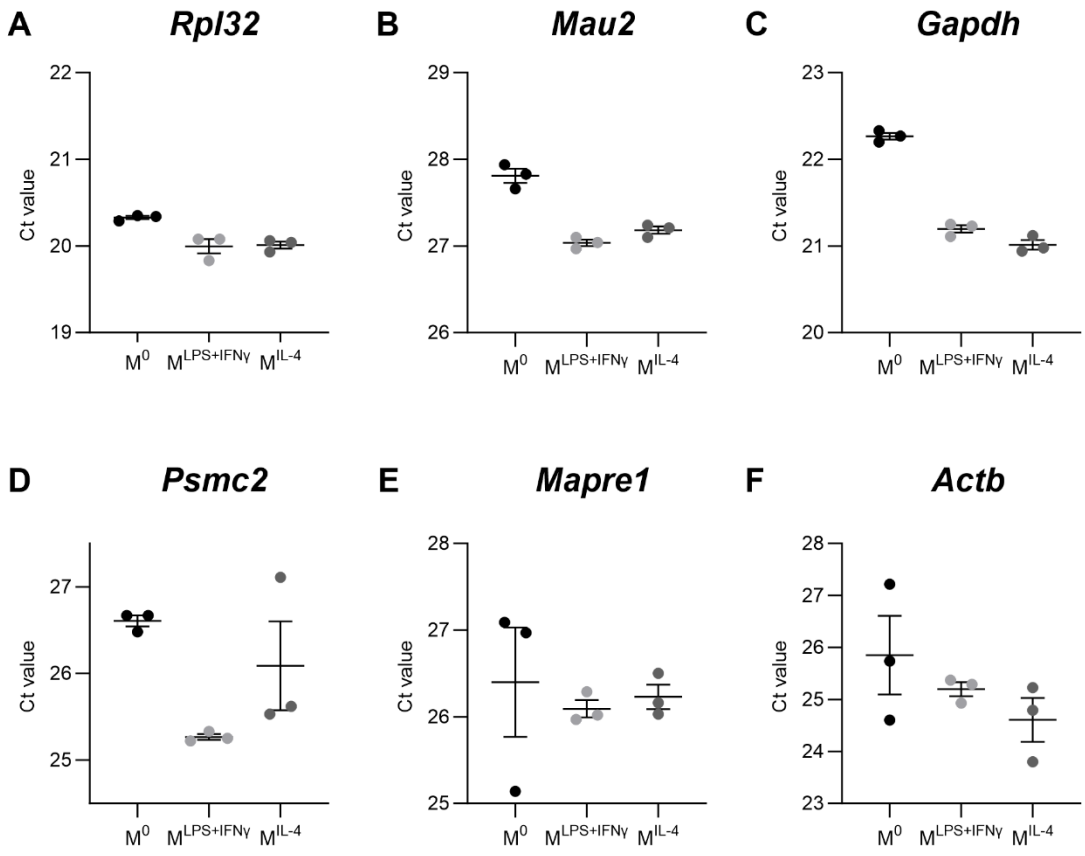


Figure 4.8 RT-qPCR analysis of potential reference genes in immortalised bone marrow-derived macrophages

Comparison in expression levels of potential reference genes by RT-qPCR in immortalised bone marrow-derived macrophages. M^0 – cells left unstimulated, $M^{LPS+IFN\gamma}$ – cells stimulated with lipopolysaccharide (LPS) and interferon (IFN)-γ for 24 hours, M^{IL-4} – cells stimulated with interleukin (IL)-4. N = 1, with 3 technical repeats. Statistical analysis was not performed.

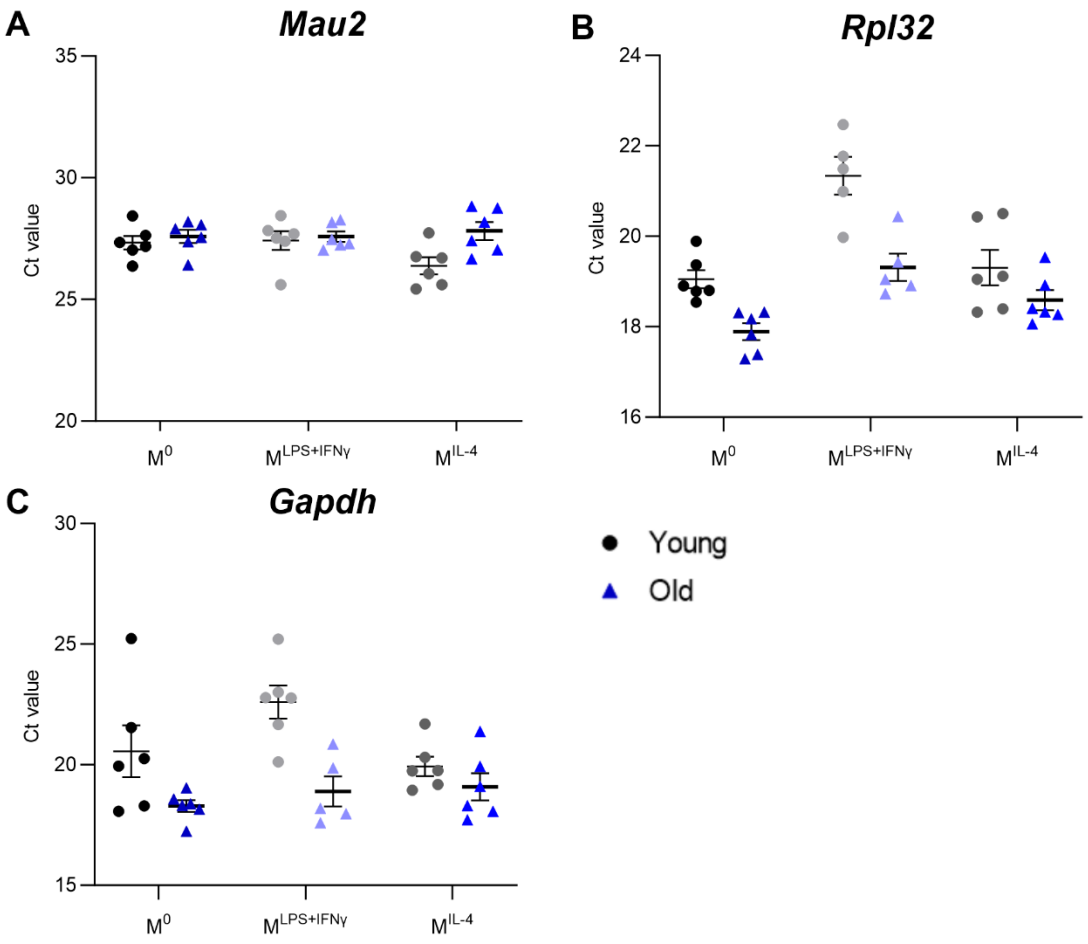


Figure 4.9 RT-qPCR analysis of potential reference genes in bone marrow-derived macrophages isolated from young and old mice

Comparison in expression levels of potential housekeeping genes by RT-qPCR in bone marrow-derived macrophages isolated from young (2-4 months) and old (20-22 months) C57BL/6J mice. M^0 – cells left unstimulated, $M^{LPS+IFN\gamma}$ – cells stimulated with lipopolysaccharide (LPS) and interferon (IFN)-γ for 24 hours, M^{IL-4} – cells stimulated with interleukin (IL)-4. Circles – young, triangles – old. N = 6 biological repeats done in triplicate. Statistical analysis was not performed.

4.4 Discussion

Ageing is a complex physiological process associated with many changes, including regulation of transcription that can cause cellular dysfunction and age-related disease [63]. However, little is known about the effect of ageing on human primary macrophages, such as monocyte-derived macrophages. We set out initially to analyse published datasets assessing macrophages of different ages to find known transcriptional changes that could form a starting point for studying human MDMs, removing

the need for a large-scale systematic approach that would potentially use more resources than required. Analysis of the published dataset GSE84901, that assessed changes in transcriptional profiles between young (2-4 months) and aged (22-24 months) mouse alveolar macrophages, led to the discovery of six transcription factors potentially driving the dysregulation of genes, found through their significant association with age-related transcriptional changes, perpetuated by the transcription factor itself having dysregulation in expression in the same direction. The assumption was made that dysregulation in transcription would be caused, at least in part, by dysregulation in expression of transcription factors. For example, a downregulation in expression of a transcription factor would limit the expression levels of downstream targets. Assessment of the expression of these transcription factors in human MDMs of different ages provides a good starting point for assessing how age affects this cell type, as a similar pattern of regulation would indicate that these transcription factors could be driving functional changes. However, as the study of human MDM ageing is not currently well advanced, no attempt has so far been made to find candidate genes with stable expression during ageing to be used as reference genes in gene expression studies. Additionally, macrophages play a central role in initiating and resolving inflammation and the effect of ageing on these responses is also not yet studied. We therefore set out to find candidate reference genes that were stable both with age and with the addition of inflammatory stimuli such as LPS and IFNγ (to model the initiation of inflammation) or Th2 cytokine IL-4 (to model inflammation resolution).

Through the bioinformatic analysis of published microarray dataset GSE84901, a viable pipeline for sequencing data analysis was produced. This was successfully able to reproduce previously published work by Wong *et al*. (2017), but was also able to newly uncover transcription factors that might be driving dysregulation of genes in alveolar macrophage ageing, as well as the associated enriched pathways and microRNAs. Transcription factors are proteins that regulate the expression of target genes through directly binding to specific DNA sequences such as enhancer elements and modulating the rate of transcription, normally by causing its initiation [138]. Transcription factors are

often cell-specific; the particular set of transcription factors in a cell will govern the transcription of a subset of genes needed for the programming of the cell [139]. If this set of transcription factors were to become dysregulated, this would then have downstream effects and cause cellular dysfunction. Each cell type tends to only have a small number of transcription factors controlling cell type-specific programmes, as evidenced by the ability of three or four transcription factors referred to as Yamanaka factors to convert a number of cells to induced pluripotent stem cells [408]. Many transcription factors have already been implicated in the pathogenesis of disease, particularly many cancers are caused or worsened by amplification of transcription factor and oncogene MYC, leading to elevated expression of target genes, more aggressive cancers and poor outcomes [409,410]. Additionally, mutations in the transcription factor USF1 have been associated with susceptibility to familial combined hyperlipidaemia [185,411] and in AR to susceptibility to prostate cancer [139]. This highlights the crucial role of transcription factors in proper cellular, tissue or organ functioning. Transcription factors can influence the expression levels of a plethora of target genes and form a part of vast gene regulatory networks that enable cell function, particularly those that are cell type-specific, also referred to as master transcription factors [139].

The six transcription factors found to be significantly associated with the ageing alveolar macrophage provide a starting point for discovering mechanisms underpinning the overall ageing macrophage phenotype. The phenomenon of a “master regulator” was first described by Ohno in 1979, in the context of sexual dimorphism [412]. A master regulator was then described as a gene that was not regulated by any other gene, occupying the highest point of the regulatory hierarchy [413]. In the context of gene regulatory networks, master regulators would therefore occupy the very top positions, acting only to drive processes through activation or inhibition of other genes, and not themselves being acted on. Many master regulators have already been proposed, such as MyoD, a myogenic transcription factor essential for the conversion of fibroblasts into myoblasts [414]. But, as each species and cell type express a different array of genes, there is still much to be discerned to fully comprehend gene expression regulation. Should the transcription factors regulating the changes

in gene expression of alveolar macrophages with age also have consistent dysregulation with age in other macrophage populations, the argument for them being master regulators of macrophage ageing could be made.

Alveolar macrophages are derived from both embryonic and blood-monocyte precursors and exist in a unique environment from other macrophage populations [415]. It is yet unknown as to whether these findings would be specific to this population of macrophages only, whether they would be myeloid-specific, ubiquitous to all macrophage phenotypes, or whether these findings could be further translated into a human study. It would therefore be valuable to assess the effects of ageing on these transcription factors in a different population of mouse macrophages to better understand the wider context of these findings.

Alongside transcriptional regulation through transcription factor binding, microRNAs are a form of post-transcriptional control as they bind the 3' untranslated region of genes, causing their degradation or suppression of translation [416,417]. These are small non-coding RNAs with an average of 18-24 nucleotides that have a multitude of biological functions through modulation of gene expression [416,418]. We analysed the GSE84901 dataset to find microRNAs associated with the age-related differentially expressed genes as this could be another potential mechanism of their occurrence; a gene downregulated with age could be caused by an age-related increase in microRNA binding [386]. In looking at the genes upregulated with age in the alveolar macrophages, we found miR-505 to have a significant association. This microRNA has been previously associated with macrophage polarisation towards an M2 phenotype [419], impaired endothelial cell migration and tube formation [420] and is a well-established tumour suppressor in many cancers [421,422]. Its association with the upregulated genes indicates a lack of expression with age and its age-related decline could be a cause of macrophages inability to polarise towards an M2 phenotype and effectively resolve inflammation which has previously been found in ageing [423]. Additionally, miR-9 has been linked to enhancing the inflammatory macrophage response [424] and improving destructive arthritis [425], and

miR-28 has been shown to promote cell proliferation in certain cancers [426,427]. In looking at the downregulated genes with age, miR-492 was significantly associated, which has been shown to promote chemoresistance in gastric cancer [428], cell proliferation and growth and regulation of a number of signalling pathways [429]. It has also been associated with macrophage M2 polarisation alongside SOCS2 when activated [430]. MiR-449 has been shown to inhibit proliferation in a number of cancers [431,432] and improved cardiac function through recruitment of GATA4 in elderly mice [433], and miR-216 has been shown to be involved in macrophage efferocytosis alongside SIRT6, whereby a disturbance in the SIRT6-mir-216/217 axis inhibits efferocytosis [434].

Analysis of the differentially expressed genes also identified key enriched pathways, including an upregulation in pathways associated with cancer, chemokine signalling, asthma, tuberculosis and allograft rejection, all of which could be indicative of a dysregulated immune or inflammatory response. Pathways downregulated with age included cell cycle, mTOR signalling, regulation of actin skeleton and autophagy. Both actin regulation and autophagy are important for macrophage functioning [44,45,435,436]. Specific actin dynamics are required for motility and phagocytosis, functions known to be dysregulated with age in different macrophage populations [274]. Additionally autophagy is required for macrophage activation, as it enables energy to be recycled to increase efficiency [44,45], autophagy and macrophage polarisation are known to decrease with age [85,86]. Overall the transcription factors, microRNAs and enriched pathways found to be associated with the age-related differentially expressed genes in alveolar macrophages need further investigation for their role in the macrophage phenotype and pathological process of ageing.

Assessing how these transcription factors and their targets might change with age in different macrophage populations can be done using RT-qPCR, a method that detects and quantifies the expression of a gene of interest through its amplification in order to uncover the relative changes in RNA levels between experimental conditions [373,437]. RT-qPCR necessitates the use of a housekeeping or reference gene that is stably expressed between experimental conditions, used to normalise the

expression of the gene of interest. Should the reference gene be regulated by age or *in vitro* stimulation, incorrect conclusions would be drawn [364,438]. The current study aimed to find reference genes that would be stably expressed with age and *in vitro* stimulation in human MDMs and mouse BMDMs. The data presented highlight *PUM1* and *Mau2* to be the most stably expressed genes, respectively, while the expression of commonly used housekeeping genes such as *GAPDH* and *ACTB* were much more variable with age and/or *in vitro* stimulation. Moreover, due to the lack of regulation by either experimental condition, these housekeeping genes could be used individually without the need for multiple reference genes.

A review of the published literature identified a number of studies that had previously assessed the stability of housekeeping genes in cell types relevant to macrophages or ageing, leading to the identification of 29 candidate genes to be further prioritised and validated. We also identified additional genes using published datasets assessing human MDMs stimulated *in vitro* and mouse alveolar macrophage ageing, looking for those with the most conserved expression between groups. Finally, the HRT Atlas was used to extract known conserved genes in human macrophages, mouse macrophages and mouse bone marrow. The final list of candidate genes for assessment was compiled by finding the most conserved genes, appearing in multiple outputs or in the experimental conditions most similar to those used in this study. In total, six genes were assessed in human MDMs and six in immortalised BMDMs. The lack of stability between technical replicates for three of these genes meant that this was further narrowed to three genes assessed in mouse BMDMs.

Overall, *PUM1* and *Mau2* were found to be the most stably expressed genes with age in human MDMs and mouse BMDMs, respectively, with or without stimulation by LPS and IFNγ or IL-4. Additionally, in the immortalised BMDMs, *Rpl32* was the most stably expressed gene with or without stimulation. PUM1, also known as Pumillo RNA binding family member 1, is a post-translational repressor that binds the 3' untranslated region of mRNA targets [395]. It is thought to be involved in embryogenesis and cell development and differentiation [394] and was selected as it was previously

found to be well conserved in human fibroblasts aged *in vitro* [373]. Compared with each of the other genes tested in human MDMs, age and *in vitro* stimulation had little effect on its expression, measured by the cycle threshold (Ct) value. This stability across both experimental conditions limits the need for multiple housekeeping genes to be used in assessing human MDM ageing with or without additional *in vitro* stimulation. *Mau2* showed a similar level of stability across both age and *in vitro* stimulation. Mau2 acts as a sister chromatid cohesion factor that loads the cohesion complex onto DNA and also has involvement in the cell cycle [364,439]. Mau2 was assessed due to it being previously found to be well conserved in mouse BMDMs stimulated with LPS [364] and due to its conserved expression with age in the mouse alveolar macrophages.

The use of immortalised BMDMs to test the initial viability of the candidate reference genes highlighted large differences between these and primary mouse BMDMs, not limited to the differing results found with *in vitro* stimulation for the most conserved candidate gene. The current results indicate that *Rpl32* would be the most suitable housekeeping gene in these cells, while *Psmc2*, *Mapre1* and *Actb* showed large variation in expression, even between technical replicates. Rpl32 is a ribosomal protein in the 60S subunit that has previously been shown to be well conserved in human bronchoalveolar cells across a spectrum of lung diseases [391] and alveolar macrophages from COPD patients of varying severity [370]. Psmc2 and Mapre1 were taken forward as they were reported to be well conserved in both mouse macrophages and bone marrow in the HRT Atlas. Similarly in the human MDMs, *SF3B1*, *COPG1* and *BECN1* showed large donor variability alongside the differences between different groups, making these genes particularly unsuitable as reference genes. SF3B1 was assessed due to it being the most conserved transcript in human macrophage samples reported by the HRT Atlas while COPG1 and BECN1 were the most conserved in the different human MDM phenotypes.

Consistent with previous literature, the use of GAPDH as a housekeeping gene in these experimental conditions would not be valid, as GAPDH mRNA levels showed variation between different

stimulation and age groups in all three cell types. This has previously been shown to be the case in human alveolar macrophages isolated from COPD patients of varying severity [370] and mouse BMDMs stimulated with LPS [364].

4.5 Conclusion

Six transcription factors were found to be significantly associated with age-related dysregulation of genes in alveolar macrophages and should form a starting point for future studies of macrophage ageing. It is yet unknown as to whether these findings would be specific only to alveolar macrophages, ubiquitous to all macrophage phenotypes or further translated into human studies. It would therefore be of value to assess the effects of ageing on the expression of these transcription factors in different macrophage populations, including mouse BMDMs and human MDMs. *PUM1*, *Mau2* and *Rpl32* were found to be the most conserved genes with age and *in vitro* stimulation in human MDMs, mouse BMDMs and immortalised BMDMs, respectively. These genes would therefore be ideal reference genes for studying the changes in expression of the transcription factors by RT-qPCR under these experimental conditions. The observed fluctuations in expression of some commonly used housekeeping genes in these cell types, such as GAPDH, and the differing results from the literature that formed the basis of this study highlights the need for validating housekeeping genes for expression studies as part of the study design, ensuring that legitimate changes between groups are uncovered. It is crucial to determine the stability of reference genes under specific experimental conditions employed, and the data presented here highlight a method for collating, prioritising and validating reference genes in this way.

Chapter 5. Ageing-related defects in macrophage function are driven by *MYC* and *USF1* transcriptional programmes

This results chapter assesses the age related changes in gene expression and function in primary human monocyte-derived macrophages.

Declaration of Contribution

This results chapter is a published primary research paper, for which data was obtained, analysed and interpreted, as well as being written up, primarily by myself. The initial processing of RNA sequencing data was performed by Sumeet Deshmukh (all analysis done by myself), eQTL and GWAS database analysis was undertaken by Veryan Codd and Stephen Hamby, the phalloidin staining and microscopy was undertaken by Simon A Johnston (with myself present, image analysis done by myself), Martha Clements assisted with RT-qPCR experiments of downstream targets (analysed by myself) and Joshua V. Kimble performed the western blots. All authors conceived the study and supported construction of the manuscript.

Ageing-related defects in macrophage function are driven by *MYC* and *USF1* transcriptional programmes

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Abstract

Macrophages are central innate immune cells whose function declines with age. The molecular mechanisms underlying age-related immunity changes remain poorly understood, particularly in human macrophages. We report a substantial reduction in phagocytosis, migration and chemotaxis in human monocyte-derived macrophages (MDMs) from older (>50 years) compared with younger (18-30 years) donors, alongside downregulation of transcription factors *MYC* and *USF1* with age. In MDMs from young donors, knockdown of *MYC* or *USF1* decreased phagocytosis and chemotaxis and altered expression of genes associated with these functions, as well as adhesion and extracellular matrix remodelling. A concordant dysregulation of *MYC* and *USF1* target genes was also seen in MDMs from older donors. Furthermore, older age and loss of either *MYC* or *USF1* in MDMs led to an increased cell size, altered morphology and reduced actin content. Together, these results define *MYC* and *USF1* as key drivers of MDM age-related functional decline and identify downstream targets to improve macrophage function in ageing.

5.1 Introduction

Macrophages are a critical component of the cell-mediated immune system and act as both regulatory and effector cells in healthy and disease contexts. Macrophages have diverse functions at the interface of the innate and adaptive immune systems, including chemotaxis and migration through tissues, phagocytosis of pathogens and other foreign material, clearance of dead, dying and senescent cells, antigen presentation, and cytokine production [440]. Many sub-types of macrophages exist, depending on tissue environment, differentiation and activation. The typing of macrophages into pro-inflammatory (M1) and anti-inflammatory (M2) has been invaluable in understanding their biological function *in vitro*. However, much work has also gone into understanding tissue-resident macrophage function, showing that the M1 and M2 classification is much too simplistic for *in vivo* studies. Macrophages exhibit versatility in different settings and nomenclature should focus on stimulating agents or macrophage function [441].

Macrophages have been demonstrated to have a key role in inflammaging, the chronic, sterile inflammatory phenotype that has been intensely studied in ageing research [442], and have been implicated in functional immune decline with age and age-related diseases such as atherosclerosis, diabetes, fibrosis, immunocompromise, autoimmunity and cancer [164]. It is thought that macrophages become chronically activated with age, with their function more closely resembling an inflammatory macrophage phenotype, including increased inflammatory and stress responses [55]. However, most of our current knowledge in this context relies on mouse models, with a notable lack of studies in humans [365]. How the molecular changes that determine the chronic inflammatory phenotype translate to a decline in function is unclear, a critical gap limiting the development of new immunotherapeutics for ageing. Here, we sought to measure ageing-related changes in monocyte-derived macrophage migration and phagocytosis in a human cohort as a phenotypic marker to identify the molecular changes underlying macrophage ageing.

Age-related changes in phagocytosis and chemotaxis have been reported in several contexts in the literature, mainly using murine models, but with differing outcomes. Aged mouse peritoneal macrophages show reduced phagocytosis with diverse phagocytic targets including apoptotic neutrophils [443], fluorescent particles [281], and myelin fragments [444]. In contrast, phagocytosis of zymosan particles by peritoneal macrophages was found to be similar between young and old animals [445], while an earlier study found that phagocytosis activated by adjuvants occurred equally across different age groups [446]. Studies of brain resident macrophages (microglia) revealed that uptake of myelin is reduced with age [444,447], as was uptake of lipid debris [448]. Similarly, alveolar macrophages consistently show a reduction in phagocytic activity with age, including lower phagocytosis of apoptotic neutrophils [63] and *E. coli* [274]. However, aged mouse bone marrow-derived macrophages (BMDM) showed no defect in uptake of zymosan particles [445], fluorescent particles [281], latex particles [278], or *E. coli* [288]. In contrast, decreased phagocytosis with age was seen for uptake of apoptotic Jurkat cells [449], myelin fragments [444] and apoptotic N2A cell debris or *E. coli* bioparticles [450]. To date, no publications have directly assessed the effect of age on phagocytosis in human monocyte-derived macrophages (MDMs).

Mouse peritoneal macrophages and BMDMs showed improved chemotaxis with age, including towards chemoattractants FMLP [286] and CCL2 [295] and in an *in vivo* study measuring the number of antigen presenting cells migrating to the lymph nodes from the site of injection, improvement in chemotactic ability was also seen with age [451]. Contrastingly, aged BMDMs had slower migration towards an implanted polypropylene mesh surface [288]. Therefore, the effects of aging were dependent on the environment, which determines the macrophage phenotype, as well as by the nature of stimulus.

Few publications have studied the effects of ageing on cell migration in humans. Aged human GM-CSF-derived MDMs showed preferential migration towards conditioned media from immune senescent cells [452]. The early macrophage response in human macrophages to pro-inflammatory

stimuli such as lipopolysaccharide (LPS), a bacterial cell wall component, is well understood and utilised in both *in vivo* and *in vitro* models. LPS stimulation has also been well studied in ageing contexts, where macrophages from older animals have tissue-specific alterations in pro-inflammatory responsiveness [453]. Although LPS induces a predominantly M1 phenotype, more varied phenotypes may exist *in vivo* and other phenotypic changes and stimuli have been less studied.

Transcription factor analysis has been key in interpreting macrophage biology and function, but so far has only been used in a small number of studies that focussed on inflammaging. In aged murine macrophages, loss of transcription factor KLF4 has been attributed to loss of diurnal rhythmicity of phagocytosis with age [454]. Transcription factor regulons were used to predict that KLF4 and IRF5 were decreased with age in tissue macrophage populations. This was associated with a trend to a switch from an M1-like to an M2-like macrophage phenotype, known to occur in age-related tumour growth promotion [455]. Older alveolar macrophages have been found to have a "senescent-like" phenotype with reduced proliferation, insensitivity to growth factor stimulation, and increased p16 and senescence-associated secretory phenotype components. Transcription factor CBFβ was found to be lost in these aged cells and preferentially present in proliferating cells, indicating that CBFβ deficiency might push alveolar macrophages towards senescence [456].

Overall, current knowledge indicates differences in dysregulation of macrophage function with age that could be tissue and/or stimulus-specific. It has previously been reported (mostly using murine cells) that macrophages undergo functional decline with age, including a reduction in phagocytosis and chemotaxis [288,450]. However, there is a lack of consensus as to whether, and to what extent this also occurs in primary human macrophages. Additionally, the underlying mechanisms driving these age-related changes are largely unknown. We therefore investigated the impact of ageing on primary human macrophage function, principally in monocyte-derived macrophages (MDMs), to characterise intrinsic, age-related changes to macrophage function and reveal the molecular mechanisms that underpin these changes. We found that ageing significantly reduced macrophage function and

altered morphology. This age-related dysfunction and the morphological changes were replicated with knockdown of either *MYC* or *USF1* (two transcription factors with reduced expression in aged macrophages) in MDMs isolated from young donors. Finally, we identified key transcriptional targets downstream of *MYC* and *USF1* with age-related dysregulated expression that may be driving age-dependent changes in macrophage morphology and function.

5.2 Methods

Human monocyte-derived macrophages and murine bone marrow-derived macrophages were isolated, differentiated, cultured and polarised as described in Section 2.3, that also gives details of transient transfection of differentiated human MDMs with small interfering RNAs targeting *MYC* or *USF1*. For human MDMs, phagocytosis, scratch and chemotactic migration assays, as well as cytoskeletal staining and microscopy, were performed as described in Section 2.4. RNA isolation, cDNA synthesis and real-time qPCR were performed as described in Section 2.5.

Analysis of RNAseq dataset GSE240075 was performed as described in Section 2.6. For the analysis of the published microarray dataset GSE84901, significant differentially expressed genes were extracted using "limma" package in R v3.46.0, defined by adjusted P value < 0.05 and fold change > 1.5. The differentially expressed genes were separated into two lists: genes upregulated and genes downregulated with age and these were used as input files for enrichr analysis (https://maayanlab.cloud/Enrichr/ accessed on 13.09.23) [244–246] with all expressed genes in these samples used as the background list. ENCODE and ChEA Consensus TFs from ChIP-X library was used to establish transcription factors likely governing the dysregulation with age in gene expression. The transcription factors identified as governing differentially expressed genes (DEGs) with age were searched for in the overall DEG lists (FDR < 0.05) and those that were differentially expressed in the same direction as their differentially expressed target genes were taken forward.

5.2.1 Further bioinformatic analysis of identified transcription factors

The published dataset Cardiogenics Consortium transcriptomic study [457–459], comprising MDMs (cultured in vitro in the same way as the M^0 MDMs in this study) and monocyte transcriptional profiles from 596 donors (Illumina's Human Ref-8 Sentrix Bead Chip arrays, Illumina Inc., San Diego, CA), was re-analysed by ranking donors by age and comparing the top and bottom age quartiles (age range of 42-65) to assess age-related differentially expressed genes. It was also used to assess eQTLs present within the dataset associated with MYC and USF1 in MDMs and monocytes. These eQTLs were previously identified and made available, as described in Rotival *et al*. [458]. They were used to identify discrete signals using the locus zoom webserver and looked up in various GWAS catalogues and databases including Open Target (https://genetics.opentargets.org/ accessed on 13.09.23) [460,461] and Phenoscanner (http://www.phenoscanner.medschl.cam.ac.uk/ accessed on 13.09.23) [462,463], both also used to find further signals in published datasets. Further, MYC and USF1 putative targets within the age-related DEGs (FDR < 0.05) in this dataset were assessed using the target gene library compiled on Enrichr: ENCODE and ChEA Consensus TFs from ChIP-X library (https://maayanlab.cloud/Enrichr/ accessed on 31.10.23). Transcription factor targets were compared against DEGs for MDMs and monocytes separately and the percentage of DEGs that were also target genes was measured. This method of assessing putative targets in the DEGs was also completed for GSE111382 and GSE100905 datasets. Finally, the Cardiogenics Consortium transcriptomic study dataset was used to compare expression profiles between top and bottom quartiles of donor MYC expression. Gene expression analysis was carried out between top and bottom quartiles of MYC expression in the MDM samples and genes with significant fold change were selected (FDR < 0.05). The direction of comparison for this was low expression - high expression. Gene set enrichment was then performed on webgestalt online tool (https://www.webgestalt.org/ accessed on 07.01.23) with community-contributed Hallmark50 database.

In order to assess MYC and USF1 expression in different monocyte subsets, GEO was searched for high-throughput datasets that directly compared classical and non-classical monocytes. Four datasets were found to do this (GSE25913, GSE18565, GSE16836 and GSE51997) and the GEO database was utilised to pull out raw expression values of MYC and USF1, as well as PUM1 that has been used throughout as a reference gene. Expression of MYC and USF1 was then normalised to PUM1 by dividing each donors MYC/USF1 expression by the expression of PUM1 in that monocyte subset. This method of normalisation was used as a way to compare each dataset without the need to consider batch effect.

5.2.2 Western blotting

Human MDMs were lysed in RIPA buffer (Sigma) with 1% protease inhibitor cocktail then added to Laemmeli buffer (9% (w/v) SDS, 9% (v/v) β-mercaptoethanol, 50% (v/v) glycerol in 375mM Tris-HCL, pH 6.8, 0.03% bromophenol blue) and heated to 99°C for 10 minutes. Protein was then resolved on a 4-12% bis-tris gel (Invitrogen) in MES buffer (Invitrogen) at 120V for 80 minutes. Proteins were then transferred onto a PVDF membrane (Immobilon Millipore) at 35V for 1 hour at room temperature. After blocking for 1 hour in 5% (w/v) milk in Tris buffered saline (1% (v/v) Tween, Sigma) (TBS-T) or 5% (w/v) BSA TBS-T, membranes were incubated with primary antibodies overnight at 4°C. Blots were washed 3 times in TBS-T with rolling then incubated for 1 hour with HRP-conjugated secondary antibodies at room temperature. Membranes were washed 3 more times in TBS-T then visualised by chemiluminescence using ECL-select (GE-Healthcare). Chemiluminescence was detected using Bio-Rad blot scanner and densitometry was determined using Image J software v2.9.0/1.53t. Membranes were stripped in ReBlot Plus Mild Antibody Stripping Solution (Sigma) at room temperature for 20 minutes, and re-probed for tubulin as a loading control. Band intensities were normalised against tubulin loading controls, presented as fold change.

5.3 Results

5.3.1 Human monocyte-derived macrophage function is reduced with age

Monocytes were isolated from healthy volunteer blood from two gender-balanced groups: aged 18-30 years (mean age 23.7 ± 1.2 years; young cohort) or > 50 years (mean age 60.5 ± 5.7 years; older cohort) and differentiated into monocyte-derived macrophages (MDMs). Further information on demographics of human donors can be found in Supplementary table 8.4.1.

Functional assays were then performed in both resting (M^0) MDMs and MDMs activated for 24 hours with LPS and IFNγ stimulation ($M^{LPS+IFN\gamma}$ MDMs) (Figure 5.1). The phagocytic capacity of MDMs was assessed by the uptake of opsonised fluorescent beads (Figure 5.1A-B) and measured as the mean fluorescence intensity, indicative of the number of beads taken up by each cell (Figure 5.1C).

Monocyte-derived macrophages from older humans had severely reduced phagocytic capacity compared to cells isolated from young donors (Figure 5.1A-C). Compared to the cells from the younger subjects, phagocytosis in M^0 MDMs from older subjects was reduced by 35% ($P < 0.05$) and by 42% ($P < 0.05$) in older $M^{LPS+IFN\gamma}$ MDMs. In young cells, $M^{LPS+IFN\gamma}$ MDMs showed 30% ($P < 0.05$) reduction in phagocytosis compared with M^0. Similarly, older $M^{LPS+IFN\gamma}$ MDMs also showed a slight reduction in phagocytosis compared with M^0 MDMs of the same age. However, this was not statistically significant, as mean fluorescence in older M^0 MDMs was already reduced to levels similar to young $M^{LPS+IFN\gamma}$ MDMs (mean ± SEM: 24.46±3.93 vs 26.23±2.08) (Figure 5.1C).

Similar to phagocytic capacity, cell migration in M^0 MDMs, as assessed in a scratch assay (Figure 5.1D-E), was significantly reduced in older donors compared to young (89% less migration in macrophages from older compared to young after 14 hours migration in a scratch wound assay; $P < 0.01$). Compared to the M^0 MDMs, migration of $M^{LPS+IFN\gamma}$ MDMs in the young group was much lower (98% reduction after 14 hours; $P = 0.0021$), but migration was comparable in both M^0 MDMs and $M^{LPS+IFN\gamma}$ MDMs in the older group (Supplementary figure 8.4.1).

To further assess chemotactic migration, we used the monocyte/macrophage chemoattractant MCP-1 in a transwell chemotaxis assay (Figure 5.1F). After three hours, chemotaxis of M^0 MDMs was 77% lower in the older subjects compared with the M^0 MDMs from the younger group (Figure 5.1G-H). As we observed above with migration, chemotaxis of the $M^{LPS+IFN\gamma}$ MDMs was significantly lower compared to the M^0 MDM population and there was no difference between the two age groups (Figure 5.1H).

Overall, these results show a reduction in M^0 MDM function with age.

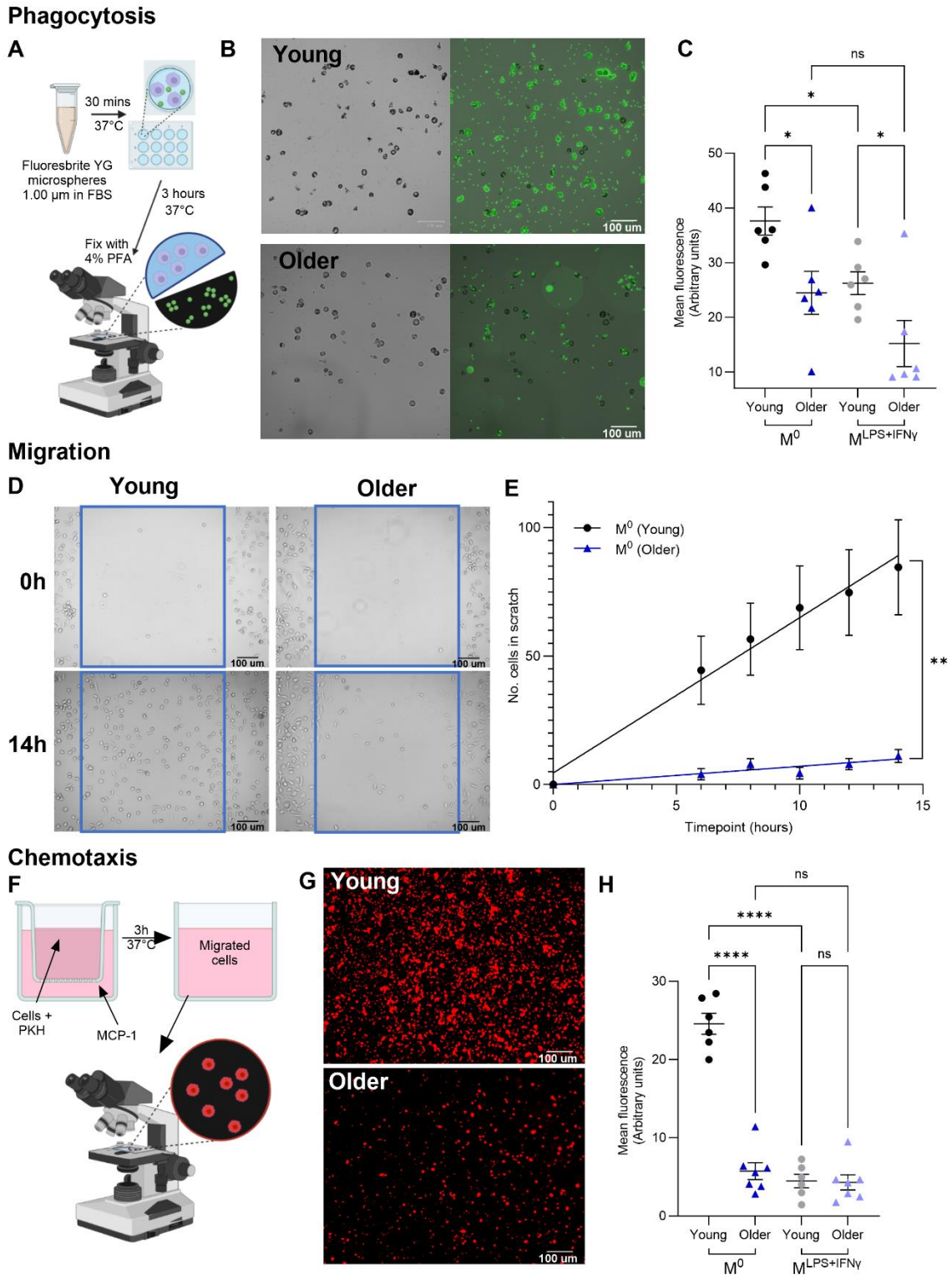


Figure 5.1 Human monocyte-derived macrophage phagocytosis and chemotaxis are reduced with age

A – Schematic diagram of phagocytosis assay design. Fluoresbrite YG microspheres 1.00 µm (Polysciences) were added to foetal bovine serum (FBS) and incubated at 37°C for 30 minutes before incubation for three hours with cultured monocyte-derived macrophages (MDMs). Cells were washed and fixed with 4% paraformaldehyde (PFA) before taking brightfield and green channel images. ImageJ "Analyze Particles" function was used to select cells in the brightfield image to produce regions of interest (ROIs). ROIs were overlayed onto the green channel image and mean fluorescence intensity was measured.

B – Representative images of fluorescent beads taken up by MDMs from young and older subjects showing brightfield and green channel overlayed image.

C – Bead uptake expressed as mean fluorescence intensity for MDMs from young and older subjects after 3 hours of incubation with fluorescent microspheres.

D – Representative images of MDMs from young and older subjects returning to the scratch after 14 hours of incubation. A box was drawn around the scratch at 0 hours and replicated onto the following timepoint images from the same well to count returning cells over time.

E – Number of MDMs returning to the scratch from young and older subjects over 14 hours, with data being collected at 0, 6, 8, 10, 12 and 14 hours after the scratch was drawn. Data are presented as mean ± SEM with each datapoint representing the mean of six donors, with three fields of view taken per donor for each condition.

F – Schematic diagram of migration assay design. Monocyte chemoattractant protein (MCP)-1 (5 nM) was added to the lower compartment of each well and PKH26-stained MDMs to the transwell and incubated at 37°C for 3 hours before imaging the lower well. Mean fluorescence was measured using ImageJ for each image.

G– Representative images of PKH26-stained MDMs that migrated through the transwell.

H – Migrated MDMs measured as mean fluorescence intensity from young and older subjects after 3 hours of incubation, with cells initially in the transwell and MCP-1 in the lower compartment.

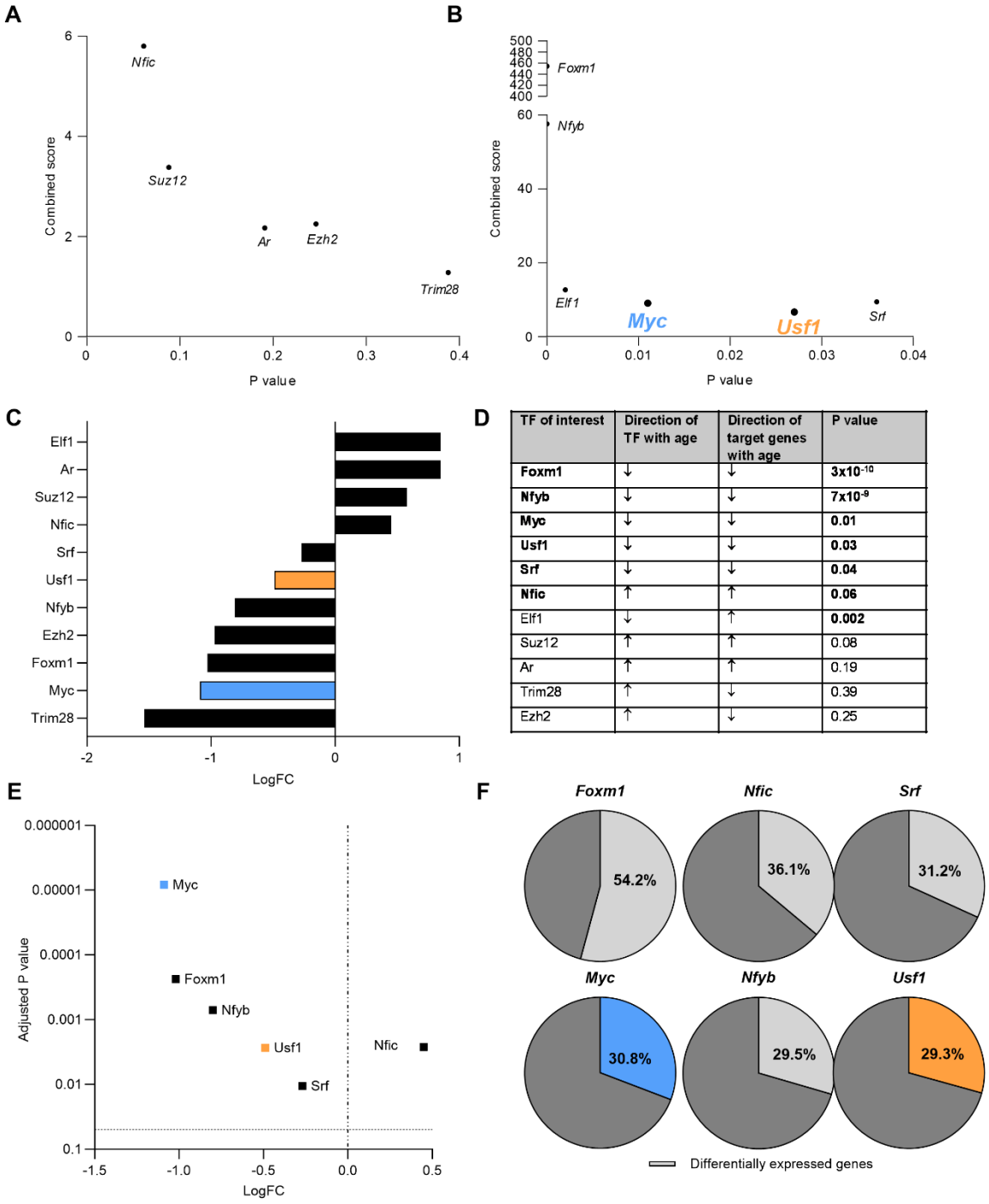
C,E,H. Two-way ANOVA with Tukey's multiple comparison, * $P < 0.05$, **** $P < 0.0001$.

Data are presented as mean ± SEM with each datapoint representing the mean of three fields of view taken per donor for each condition. MDMs were differentiated from human blood monocytes for 7 days in M-CSF and then either left at rest (M^0 MDM) or further polarised for 24 hours with lipopolysaccharide (LPS) and interferon (IFN)-γ ($M^{LPS+IFN\gamma}$ MDMs). Young (N=6, 22-25 years), older (N=6, 54-71 years).

5.3.2 Six transcription factors govern age-related transcriptomic alterations in murine alveolar macrophages

Having established a clear phenotypic difference in older human M^0 MDMs (Figure 5.1), we set out to uncover the transcriptional networks that may drive these changes, with a focus on those that may be conserved across human and murine cells, an important *in vivo* mammalian model of ageing. First, we analysed a published microarray dataset of alveolar macrophages isolated from young (2-4 months) and old (22-24 months) C57BL/6J mice [63], hypothesising that transcription factors with many target genes differentially expressed with age would likely be governing the age-related changes seen in macrophage function. Although alveolar macrophages are tissue resident cells with little monocyte infiltration during homeostasis, they primarily function in immune surveillance, making this dataset relevant to our work [453,464].

Differentially expressed genes with age included 213 upregulated genes (LogFC > 1.5, FDR < 0.05) and 122 downregulated genes (LogFC < -1.5, FDR < 0.05; Supplementary figure 8.4.2). Enrichment analysis of these genes using "ENCODE_and_ChEA_Consensus_TFs_from_ChIP-X" library in Enrichr was performed to identify the transcription factors responsible for differentially expressed transcripts (Figure 5.2A-B, Supplementary figure 8.4.2). Whilst five transcription factors showed weak associations with upregulated genes (none of which reaching statistical significance, Figure 5.2A), six transcription factors (*Myc*, *Elf1*, *Foxm1*, *Nfyb*, *Usf1* and *Srf*) were significantly associated with the downregulated gene group (P < 0.05) (Figure 5.2B). Assessing expression of these transcription factors themselves between the young and aged alveolar macrophages (with focus on the transcription factors differentially expressed in the same direction as their target genes), showed *Myc*, *Foxm1*, *Nfyb*, *Usf1* and *Srf* were significantly downregulated and *Nfic* was upregulated with age, making them the most likely regulators to be driving age-related differential gene expression (Figure 5.2C-E). In line with this, we found 29.3-54.2% of the macrophage-expressed putative target genes of these transcription factors to be dysregulated with age (Figure 5.2F).



TF of interest	Direction of TF with age	Direction of target genes with age	P value
Foxm1	↓	↓	**$3x10^{-10}$**
Nfyb	↓	↓	**$7x10^{-9}$**
Myc	↓	↓	**0.01**
Usf1	↓	↓	**0.03**
Srf	↓	↓	**0.04**
Nfic	↑	↑	**0.06**
Elf1	↓	↑	**0.002**
Suz12	↑	↑	0.08
Ar	↑	↑	0.19
Trim28	↑	↓	0.39
Ezh2	↑	↓	0.25

Figure 5.2 Transcription factors associated with age-related differentially expressed genes in murine alveolar macrophages

A – Transcription factors associated with genes upregulated with age in murine alveolar macrophages. Combined score measures a combination of P value and z score to enable comparison of rankings. P value is calculated using Fisher's exact test.

B – Transcription factors associated with genes downregulated with age in murine alveolar macrophages. Combined score measures a combination of P value and z score to enable comparison of rankings. P value is calculated using Fisher's exact test.

C – Direction and magnitude of differential expression of transcription factors associated with target gene list in aged *vs* young murine alveolar macrophages.

D – Ranking of transcription factors, differentially expressed with age in murine alveolar macrophages: differentially expressed in the same direction as the target gene list, significant association with target gene list ($P < 0.05$).

E – Fold change (FC) of transcription factor expression with age and P value adjusted for multiple-testing correction. Adjusted P value is calculated using Benjamini and Hochberg false discovery rate, ($FDR < 0.05$).

F – Percentage of transcription factor target genes expressed in murine alveolar macrophages that are dysregulated with age. Foxm1 – 45 differentially expressed/ of 83 targets, Nfic – 73/202, Srf – 82/ 263, Myc – 412/ 1336, Nfyb – 886/ 3004, Usf1 – 352/1200.

Differentially expressed genes with age ($LogFC > 1.5$) were identified in GSE84901 microarray dataset comparing alveolar macrophages from young (2-4 months) and aged (22-24 months) C57BL/6J mice.

5.3.3 *MYC* and *USF1* are downregulated with age in human and murine macrophages and their allelic variants are associated with monocyte percentage in human blood

To validate the six identified transcription factors as potential regulators of the observed ageing human macrophage phenotype, we next compared mouse BMDMs isolated from young (2-4 months) and aged (22-24 months) C57BL/6J mice and MDMs from our human cohort (Figure 5.3A-B).

MYC and *USF1* showed consistent downregulation with age in both murine BMDMs and human MDMs, occurring in M^0 cells for *MYC* (while the inflammatory $M^{LPS+IFN\gamma}$ phenotype had a complete lack of expression) and in both M^0 and inflammatory stimulated $M^{LPS+IFN\gamma}$ for *USF1*, although M^0 had better overall *USF1* expression in the young human MDM population. These two transcription factors were therefore analysed further to mechanistically assess their contribution to the ageing macrophage phenotypes. Although some consistency was seen in the other assessed transcription factors compared to the above analysed murine alveolar macrophage data (*NFIC* maintained upregulation and *SRF* downregulation with age in human $M^{LPS+IFN\gamma}$ MDMs, *NFYB* and *FOXM1* were both downregulated with age in human M^0 MDMs), lack of consistency across all assessed murine and human macrophage types meant these were not taken forward.

Publicly available transcriptomic data suitable for the analysis of age-related changes in human macrophages is very limited. However, the Cardiogenics Consortium transcriptomic study dataset includes MDM and monocyte expression profiles and genotype information from 596 human donors of varying ages (42-65 years) [457–459]. MDMs in this dataset have been cultured in a way consistent with those in the present study [457–459] so that we can consider them equivalent to our M^0 MDMs. To identify allelic variants of *MYC* and *USF1*, which are associated with RNA levels for these transcription factors, we analysed top and bottom age quartiles of this dataset to identify eQTLs. A total of 491 single nucleotide polymorphisms (SNPs) were associated with *USF1* and 1 SNP was associated with *MYC* in M^0 MDMs. A further 569 and 36 SNPs were associated with *USF1* and *MYC* in monocytes, respectively (FDR < 0.05). All of these were cis-eQTLs and for *USF1* represented three discrete association signals (Figure 5.3C). Exploration of other published datasets within Phenoscanner

revealed a further 7 signals (Supplementary table 8.4.2). Sentinels for all 10 *USF1* associations were assessed for association with human traits and disease in GWAS catalogues and databases, including Phenoscanner and Open Target. The most notable genome-wide associations were found to be with blood cell traits, including blood monocyte percentage (Supplementary table 8.4.3).

Whilst data on age-related changes in blood monocyte counts is sparse and there is currently no consensus on whether this is altered in aged individuals, it is widely accepted that the ratio of classical ($CD14^{+}CD16^{-}$) vs non-classical ($CD14^{dim}CD16^{+}$) monocytes is altered, with the non-classical monocyte subset expanding during ageing. Therefore, we assessed *MYC* and *USF1* expression between classical and non-classical monocyte subsets through a combined analysis of four published datasets, equating to 15 individual samples per monocyte subset [465–468]. RNA levels of *MYC* and *USF1* were normalised to the expression of *PUM1* in each dataset, a housekeeping gene found to be well conserved in our polarised and older cell populations, so that data from the individual datasets could be pooled and analysed together. We found *MYC* expression to be downregulated in $CD14^{dim}CD16^{+}$ monocytes compared with $CD14^{+}CD16^{-}$ population (Figure 5.3D-E), suggesting that the age-related reduction in *MYC* RNA levels could, at least in part, be due to the expansion of the low *MYC*-expressing non-classical monocyte subset. Further, we tested whether expression of *MYC* and *USF1* target genes were altered with age in the Cardiogenic Consortium dataset by comparing top and bottom age quartiles, comprising 201 donors in each age group. Among the differentially expressed genes with age (FDR < 0.05) in these monocytes and M^0 MDMs, more than 20% were putative *MYC* and *USF1* targets (Figure 5.3F). Those with LogFC > 0.1 are shown in Figure 5.3G. The significance of MYC target gene expression in these macrophages was further assessed by GSEA hallmarks, where MYC targets v1 and v2 were significantly enriched (FDR < 2.2e-16) between the top and bottom quartiles of *MYC* expression.

Putative MYC and USF1 targets were also assessed in other published datasets that related to this research, including GSE155992 assessing peritoneal macrophages isolated from young (3-4 months)

and aged (20-22 months) mice, where *MYC* itself was downregulated with age (adjusted P = 0.0034), alongside 16% of MYC targets and 7% of USF1 targets being dysregulated. Additionally, GSE111382 assessing peritoneal macrophages isolated from young (3 months) and aged (18 months) mice and GSE100905 assessing bone marrow cells from young and older healthy male human donors were found to have more than 10% of differentially expressed genes with age (FDR < 0.05) in MYC targets and 5% in USF1 targets.

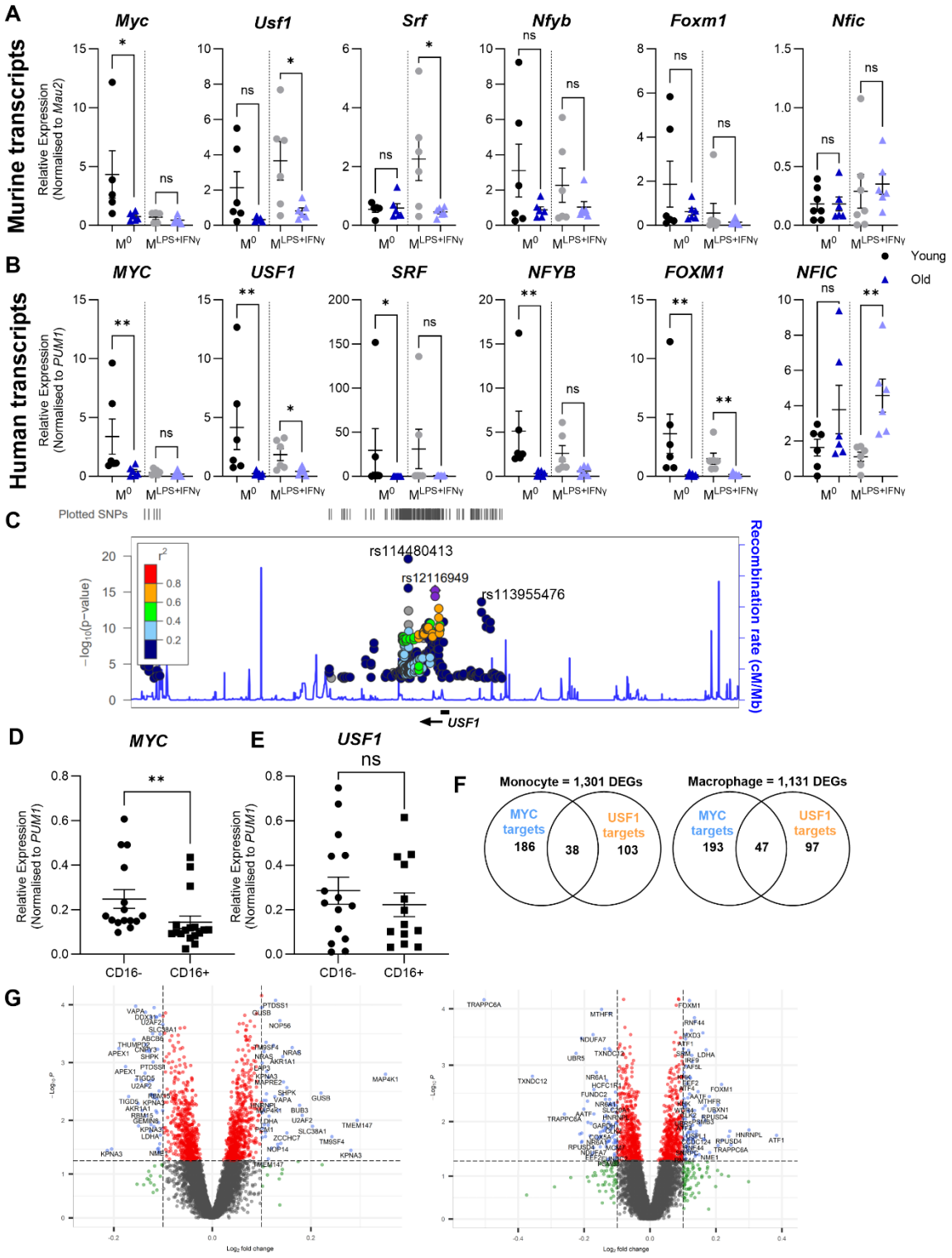


Figure 5.3 Transcription factor expression with age in murine bone marrow-derived macrophages and human monocyte-derived macrophages and genetic associations

A – Age-related changes in expression of transcription factors in bone marrow-derived macrophages isolated from young (2-5 months) and aged (22-24 months) C57BL/6J mice. *Mau2* expression was used as a housekeeping control.

B – Age-related changes in expression of transcription factors in human monocyte-derived macrophages (MDMs) isolated from young (22-25 years) and older (54-71 years) healthy donors. *PUM1* expression was used as a housekeeping control.

A,B. M^0 – cells left unstimulated, $M^{LPS+IFN\gamma}$ – cells stimulated with lipopolysaccharide (LPS) and interferon (IFN)-γ for 24 hours. Mann-Whitney test, N=6, * $P < 0.05$.

C – Manhattan plot of discrete signals of expression quantitative trait loci (eQTLs) associated with USF1 in Cardiogenic Consortium transcriptomic study monocytes. Conducted by Veryan Codd.

D – Relative expression of *MYC* in monocyte subsets from published datasets. *PUM1* expression was used as a control to normalise all samples. Mann-Whitney test, N=15, ** $P < 0.01$.

E – Relative expression of *USF1* in monocyte subsets from published datasets. *PUM1* expression was used as a control to normalise all samples. Mann-Whitney test, N=15, ** $P < 0.01$.

F – Association between MYC and USF1 targets and differentially expressed genes with age in Cardiogenics Consortium transcriptomic study monocytes (left) and MDMs (right).

G – Volcano plots of MYC and USF1 targets in differentially expressed genes with age in Cardiogenic Consortium transcriptomic study monocytes (left) and MDMs (right). Log2 fold change thresholds are illustrative and not used for differential expression analysis of genes

5.3.4 Loss of *MYC* or *USF1* in young human MDMs is sufficient to recapitulate an ageing macrophage phenotype

As older human macrophages displayed reduced function and both *MYC* and *USF1* were consistently downregulated with age across murine and human macrophage populations, we next investigated whether *MYC* or *USF1* expression levels are causatively linked to macrophage function. We tested this by a transient knockdown of *MYC* or *USF1* in young human MDMs and explored whether this could model the ageing macrophage phenotypes.

As shown in Figure 5.3, both *MYC* and *USF1* were well expressed in young M^0 populations and downregulated with age. Whereas *MYC* expression was minimal in the $M^{LPS+IFN\gamma}$ phenotype, *USF1* was ubiquitously expressed in this polarised macrophage phenotype and was also downregulated with age. In contrast, expression of *MYC* and *USF1* showed no consistency in age-related dysregulation in IL-4-stimulated ($M^{IL\text{-}4}$) macrophages, tested for completeness due to *MYC* expression being well documented in this macrophage phenotype (Supplementary figure 8.4.3 – Supplementary figure 8.4.4). Therefore, to test whether loss of *MYC* or *USF1* is sufficient to cause an old macrophage phenotype, we used siRNA gene silencing. The efficiency of *MYC* gene silencing was 97% (86.4-99.9) and of *USF1* gene silencing was 95% (81.3-99.9) in M^0 MDMs and 83% (46.9-99.4) in $M^{LPS+IFN\gamma}$ MDMs, determined by quantification of *MYC* or *USF1* mRNA levels (Figure 5.4A); downregulation of MYC protein was assessed by western blotting (Supplementary figure 8.4.5). Consistent with the aged MDM phenotype, phagocytosis was reduced in both si*MYC* M^0 MDMs and si*USF1* M^0 MDMs compared with control M^0 MDMs. There was no further reduction in phagocytosis in si*USF1* $M^{LPS+IFN\gamma}$ MDMs compared with $M^{LPS+IFN\gamma}$ MDM controls (Figure 5.4B). Similarly, migration and MCP-1 chemotaxis were reduced in both si*MYC* M^0 MDMs and si*USF1* M^0 MDMs, compared with M^0 MDM controls (Figure 5.4C-D).

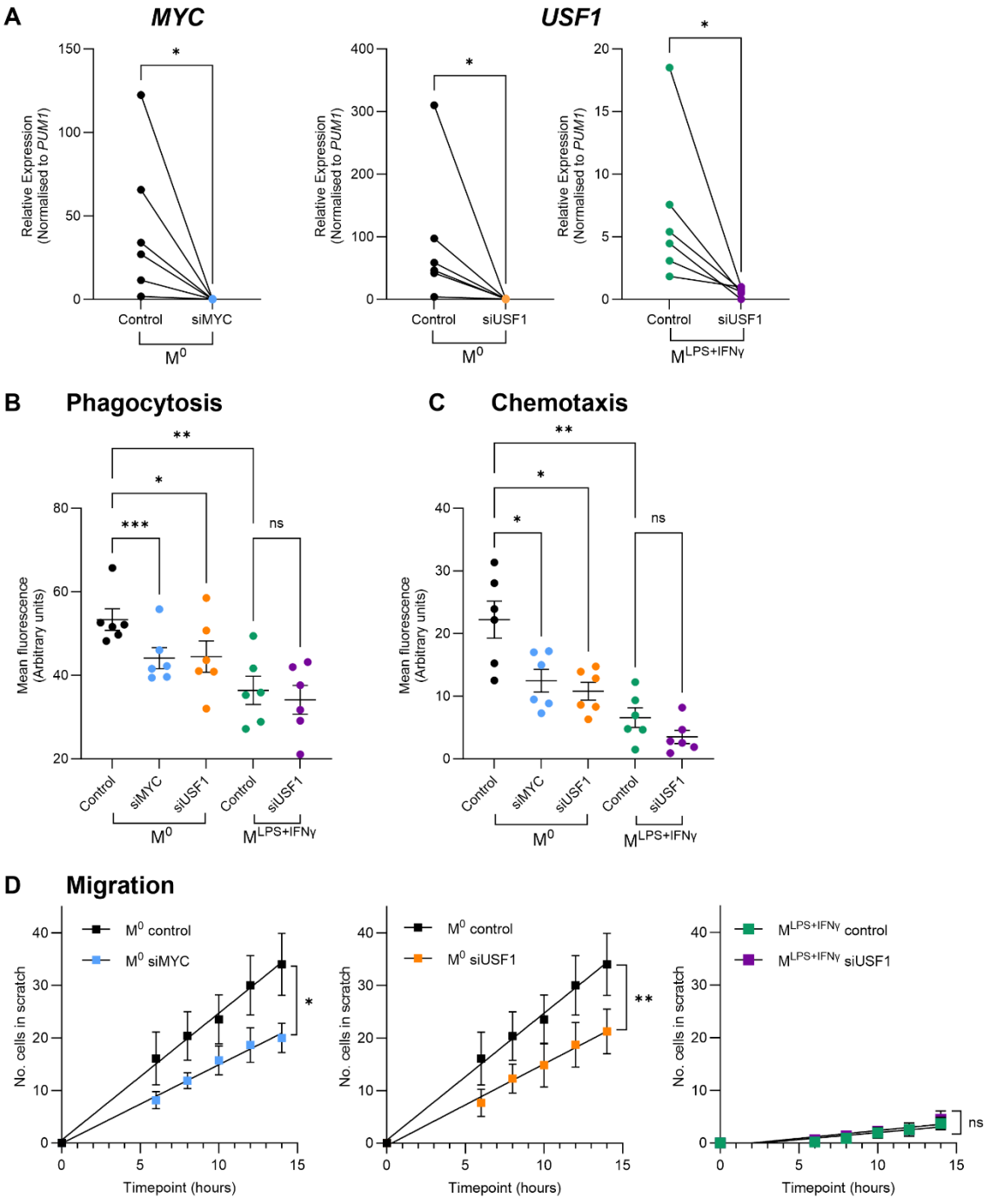


Figure 5.4 Loss of *MYC* or *USF1* in young human primary macrophages reduced phagocytosis and chemotaxis

A – Relative mRNA expression of *MYC* or *USF1* in respective siRNA knockdowns in monocyte-derived macrophages (MDMs) isolated from young donors. Each datapoint represents an individual donor. *PUM1* expression was used as an internal control. N = 6, Mann-Whitney test, * $P < 0.05$.

B – Bead uptake expressed as mean fluorescence intensity from young human MDMs with loss of *MYC* or *USF1* after 3 hours of incubation with fluorescent microspheres.

C – Migrated MDMs measured as mean fluorescence intensity from young human MDMs with loss of *MYC* or *USF1* after 3 hours, with cells initially in the transwell and monocyte chemoattractant protein 1 in the lower compartment.

B,C. Data are presented as mean ± SEM with each datapoint representing the mean of three field of view taken per donor for each condition. N=6, repeated measures one-way ANOVA with Tukey's multiple comparison, * P < 0.05, ** P < 0.01, *** P < 0.001.

D – Number of MDMs returning to the scratch from young human MDMs with loss of *MYC* or *USF1*. Data are presented as mean ± SEM with each datapoint representing the mean of six donors, with three fields of view taken per donor for each condition. Two-way ANOVA with Tukey's multiple comparison, * P < 0.05.

MDMs were differentiated from human blood monocytes isolated from young donors for 7 days with M-CSF, before addition of siRNA and stimulating agents. Control – non-targeting siRNA control, siMYC – MYC-targeting siRNA, siUSF1 – USF1-targeting siRNA, M^0 – unstimulated, $M^{LPS+IFN\gamma}$ – LPS and IFNγ stimulated for 24 hours after differentiation and knockdown.

5.3.5 Loss of *MYC* or *USF1* in young human MDMs reproduces the transcriptomic signature of older macrophages

To further evidence the causative role of *MYC* and *USF1* loss in the older macrophage phenotypes we had identified, we compared the transcriptomic signature of gene silenced *versus* unmodified MDMs from the younger subjects (Figure 5.5). Principal component analysis of transcripts that were dysregulated showed a significant clustering of the knockdown conditions which extended to 5,872 DEGs ($P < 0.05$) between si*MYC* M^0 MDMs and M^0 MDM control (Figure 5.5A), 6,148 DEGs between si*USF1* M^0 MDMs and M^0 MDM control (Figure 5.5B) and 6,350 DEGs between si*USF1* $M^{LPS+IFN\gamma}$ MDMs and $M^{LPS+IFN\gamma}$ MDM control (Figure 5.5C). An overall principal component analysis of all samples also showed clustering of males and females (Supplementary figure 8.4.6).

Among the DEGs between si*MYC* M^0 MDMs and M^0 MDM control (FDR < 0.05), associated hallmark gene sets [469] included MYC targets, MTORC1 signalling and interferon response (Supplementary table 8.4.4). Enriched biological processes included cell-cell adhesion, sensory development and extracellular matrix (ECM) formation (Figure 5.5A). Between si*USF1* M^0 MDMs and M^0 MDM control, cell-cell adhesion, development, cell differentiation and apoptosis processes were enriched (Figure 5.5B). DEGs were also linked to hallmark gene sets including MYC targets, glycolysis and hypoxia (Supplementary table 8.4.5). DEGs between si*USF1* $M^{LPS+IFN\gamma}$ MDMs and $M^{LPS+IFN\gamma}$ MDM control were linked to hallmark gene sets such as hypoxia, angiogenesis and heme metabolism (Supplementary table 8.4.6). Enriched biological processes included cell-cell adhesion and ion transport (Figure 5.5C).

To complement the analysis of human macrophages, we returned to our mouse transcriptional network dataset and performed gene set enrichment analysis (GSEA) on the DEGs from the published microarray dataset comparing young and aged alveolar mouse macrophages, this time including all significant genes (FDR < 0.05) without filtering for fold change [63]. Cell-cell adhesion and ECM components were also enriched biological processes, alongside cell cycle and DNA repair (Supplementary table 8.4.7). Hallmarks associated with DEGs included MYC targets, G2M checkpoint and E2F targets (Supplementary table 8.4.8).

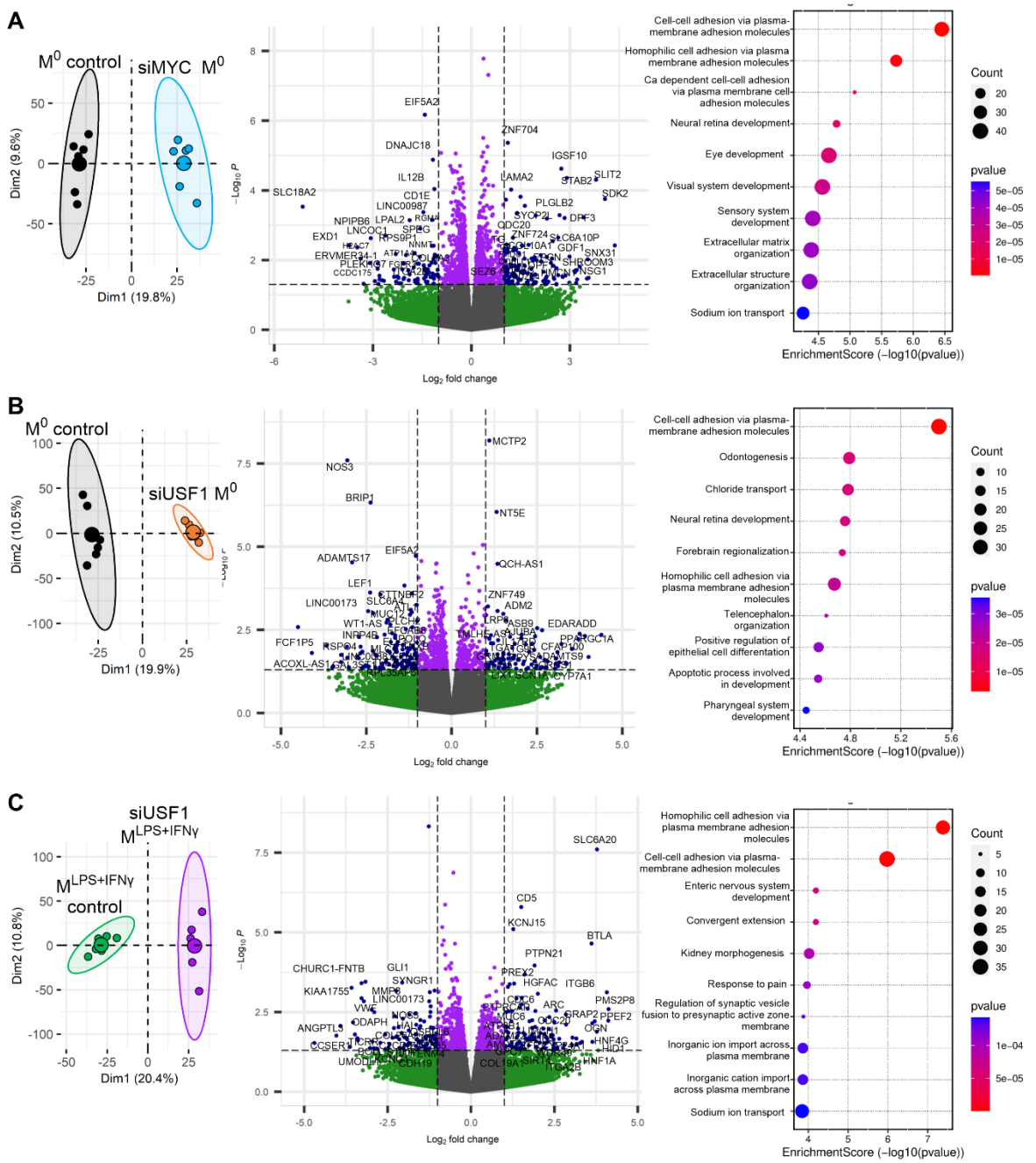


Figure 5.5 Loss of *MYC* or *USF1* produces changes in transcriptional signature associated with macrophage function

Principal component analysis of transcripts dysregulated in different conditions showing the first two dimensions (Dim) (left), volcano plot showing differentially expressed genes (Log_2 fold change (FC) > 1, P value < 0.05), with LogFC thresholding added for illustrative purposes only and not used for differential expression analysis of genes (centre) and dot plot showing associated enriched biological processes with count indicating the number of genes dysregulated in the pathway (right) in young monocyte-derived macrophages for si*MYC* M^0 *vs* M^0 control (A), si*USF1* M^0 *vs* M^0 control (B) and si*USF1* $M^{LPS+IFN\gamma}$ *vs* $M^{LPS+IFN\gamma}$ control (C).

As many DEGs were associated with similar hallmarks and biological processes between the *MYC* and *USF1* knockdowns and controls in the MDMs as well as in aged *versus* young macrophages in the mouse, we next assessed whether the same set of DEGs were also dysregulated in older human MDMs. GSEA results were used to rank DEGs by the number of times they appeared in different enriched gene sets. Leading edge analysis was then performed to sort the number of appearances of each gene with its LogFC and P value (Figure 5.6A-C). Genes with LogFC > 1.3 and appearance in at least 5 enriched gene sets ($P < 0.05$), and that showed relevance to macrophage function through literature search were then analysed by RT-qPCR in human MDMs. Expression of a total of 22 genes were assessed (Figure 5.6D) and nine of these had reproducible differential expression in older *versus* young MDMs as was found between transcription factor knockdown *versus* control (Figure 5.6E-F, Supplementary figure 8.4.7). Finally, specific analysis of *CCR2* (the MCP-1 receptor) expression, demonstrated reduced expression, providing a possible mechanism for dysregulation in MCP-1 chemotaxis [470], between si*MYC* M^0 MDMs compared with M^0 MDM control and between old and young murine M^0 BMDMs (Supplementary figure 8.4.8).

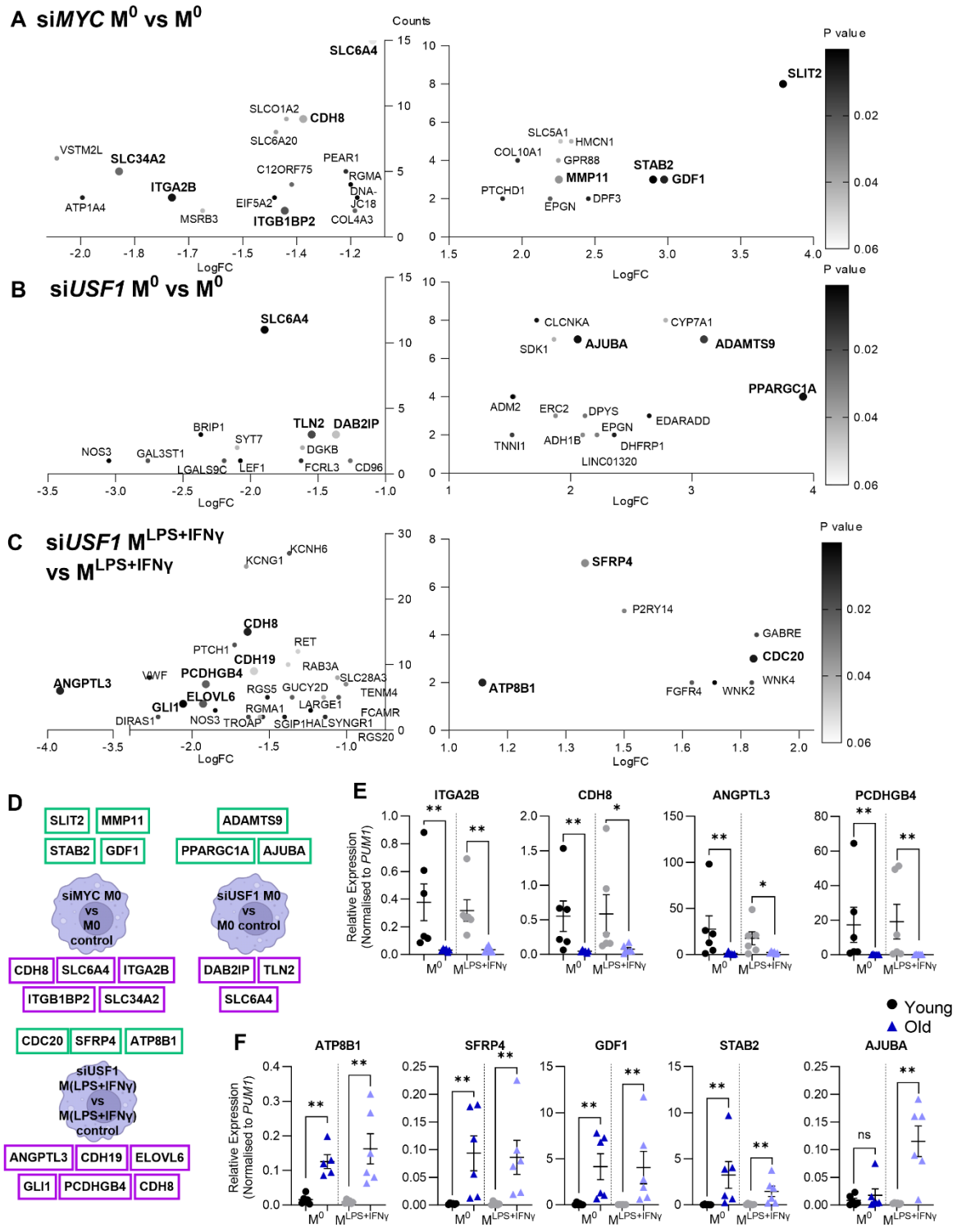


Figure 5.6 Age-related changes in gene expression are mimicked by loss of *MYC* or *USF1* in young monocyte-derived macrophages

A-C – Leading edge analysis of genes differentially expressed in si*MYC* M^0 *vs* M^0 control (A), si*USF1* M^0 *vs* M^0 control (B) or si*USF1* $M^{LPS+IFN\gamma}$ *vs* $M^{LPS+IFN\gamma}$ control (C), showing genes downregulated (left) and genes upregulated (right) plotted as number of enriched gene sets that the gene is associated with (y-axis) against the fold change of the gene (x-axis). Colour gradient of the dot corresponds to P value and dots of larger size were taken forward due to scoring highly in each field or association with macrophage function in literature search.

D – Selected genes from each comparison taken forward for assessment in young and older human MDMs. Green: upregulated genes, purple: downregulated genes.

E-F – Age-related changes in expression of selected genes in human monocyte-derived macrophages isolated from young (22-25 years) and older (54-71 years) healthy donors that corresponded to genes upregulated (E) or downregulated (F) in transcription factor knockdown RNA sequencing analysis. M^0 – cells left unstimulated, $M^{LPS+IFN\gamma}$ – cells stimulated with lipopolysaccharide (LPS) and interferon (IFN)-γ for 24 hours. *PUM1* expression was used as an internal control. N = 6, Mann-Whitney test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. The qPCRs were conducted by myself and Martha Clements under my supervision.

5.3.6 Age and loss of *MYC* or *USF1* in young human MDMs alter cytoskeletal structure

Having established the sufficiency of *MYC* and *USF1* loss in recapitulating the old macrophage phenotype, transcriptomic signature, and downregulation of *CCR2*, we used the above analysis of biological processes (Figure 5.5) to predict further mechanistic factors in the ageing macrophage phenotype. As we found genes associated with cell-cell adhesion, ECM components and the cytoskeleton enriched in our analysis, we hypothesised that cytoskeletal and cell morphological changes would be altered in older macrophages and those with loss of *MYC* or *USF1*. It has previously been established that F-actin content is a strong determinant of a cells ability to survey the local environment, move through the tissue and produce the phagosome required for phagocytosis [435,471,472]. Additionally it is known that the circularity of the cell is strongly associated with motility, with a more elongated phenotype being preferential for this function [436]. From our phenotypic and transcriptional data, we predicted that older macrophages and those with loss of *MYC* or *USF1* would exhibit a more spread, adhesive appearance and reduced capability for actin motility. To test our prediction we measured cell size, circularity and F-actin (Figure 5.7, Supplementary figure 8.4.9) and found that older M^0 MDMs were larger in size, less circular and had less F-actin content compared with young counterparts. Older $M^{LPS+IFN\gamma}$ MDMs were also more elongated than young counterparts. Comparing young MDMs, M^0s were smaller and had less F-actin content than $M^{LPS+IFN\gamma}$ MDMs. Finally, older M^0 MDMs were larger with less F-actin content than older $M^{LPS+IFN\gamma}$ MDMs (Figure 5.7B-D). Similar findings were seen with loss of *MYC* or *USF1* in young MDMs. Compared with M^0 MDM control, si*MYC* M^0 MDMs had increased size and elongation, while si*USF1* M^0 MDMs had increased elongation as well as reduced F-actin content. Compared with $M^{LPS+IFN\gamma}$ MDM control, si*USF1* $M^{LPS+IFN\gamma}$ MDMs had an increase in elongation (Figure 5.7E-G).

Thus, having identified an old macrophage phenotype in human MDMs, we have uncovered a novel *MYC*/*USF1* transcriptomic regulation of macrophage chemotaxis and phagocytosis, paired with cytoskeletal and receptor changes that can be recapitulated in young macrophages by reducing *MYC* or *USF1* RNA levels.

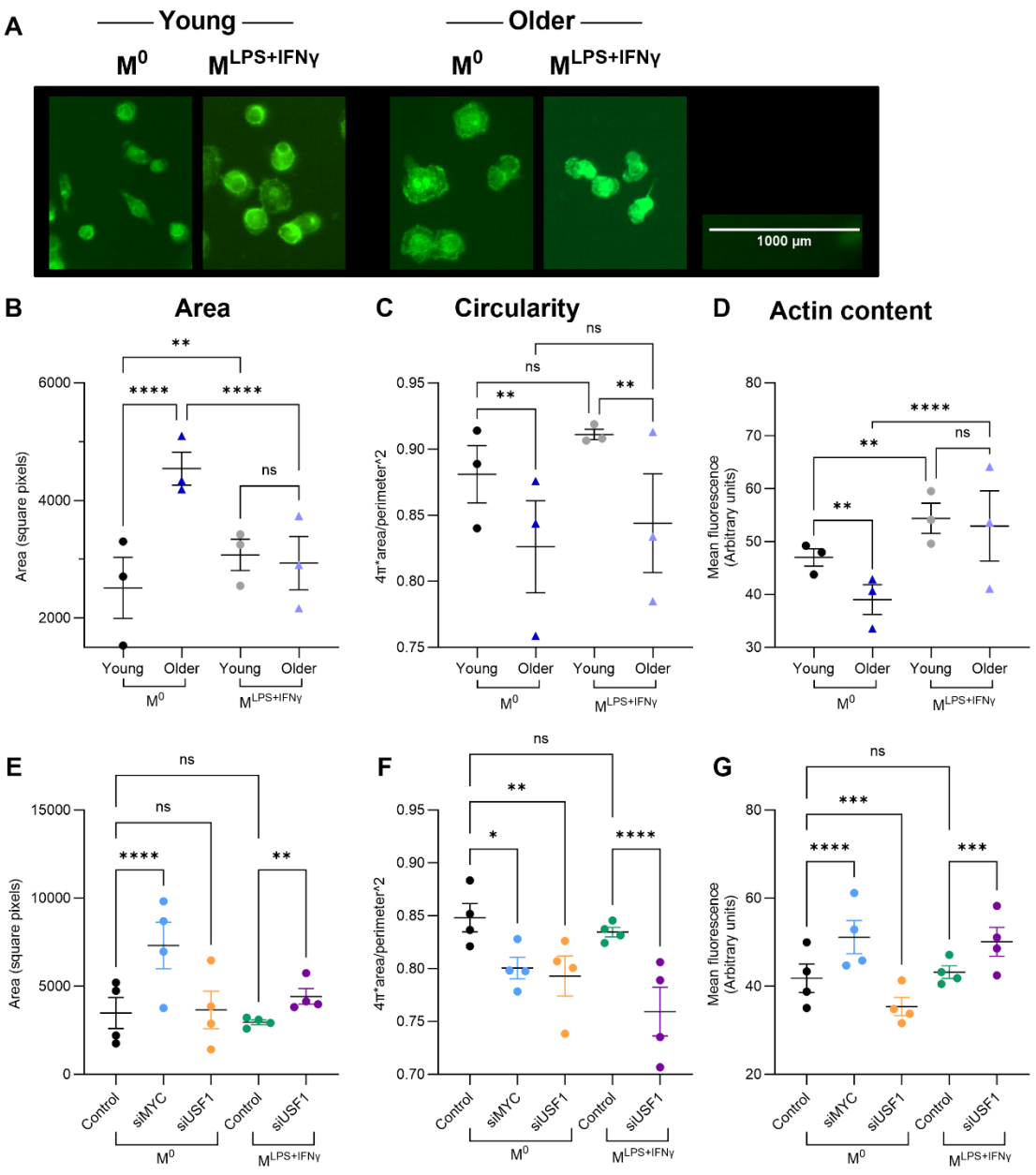


Figure 5.7 Monocyte-derived macrophage morphology and actin content change with age and loss of *MYC* or *USF1*

A – Representative images of young and older monocyte-derived macrophages (MDMs) stained with Fluorescein Isothiocyanate labelled Phalloidin. Staining and microscopy was conducted by Simon Johnston.

B-D – Morphological changes between young (N=3, 23-25 years) and older (N=3, 58-71 years) MDMs. Two-way ANOVA with Sidak's multiple comparison, * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001

F-H – Morphological changes with loss of MYC or USF1 compared with control in young MDMs (22-25 years). N=4, repeated measures one-way ANOVA with Tukey's multiple comparison, * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

B,E – Area by square pixel of MDMs in young and older (B) and knockdown (E) conditions. Each datapoint represents the median of four images taken per donor.

C,F – Circularity (where 1 is a perfect circle and approaching 0.0 is increasingly elongated) of MDMs in young and older (C) and knockdown (F) conditions. Each data point represents the median of four images taken per donor.

D,G – Mean fluorescence intensity of MDMs stained with phalloidin to measure actin content in young and older (D) and knockdown (G) conditions. Each datapoint represents the geometric mean of four images taken per donor.

A minimum of 100 cells per condition were analysed across the four images. Graphs are represented as the mean ± SEM.

5.4 Discussion

In this study, we revealed a key role of *MYC* and *USF1* transcriptional networks in age-related macrophage functional decline. We found that ageing led to a decrease in macrophage phagocytosis, migration and chemotaxis, as well as increased cell size and elongation. Loss of either *MYC* or *USF1* in young macrophages resulted in comparable functional decline and morphology, modelling the changes seen with age. We identified *MYC* and *USF1* transcripts to be downregulated with age in both murine and human macrophage populations, alongside dysregulation of transcriptional targets that may be driving age-dependent changes in function and morphology in older human macrophages.

Macrophages are key contributors to inflammaging and age-related immune decline. To date most of our understanding of macrophage ageing has been reported from mouse tissue populations, and there is a gap in functional and mechanistic understanding in human cells. Age-related changes are less well defined in monocyte-derived macrophage populations, which play a key role in age-related diseases including atherosclerosis, diabetes and fibrosis. We therefore assessed macrophage dysfunction with age in human monocyte-derived macrophages, showing both phagocytosis and chemotaxis to be reduced with age in these macrophages that were isolated as monocytes from human blood and differentiated for 7 days in the presence of M-CSF. It is worth noting that monocyte counts between the young and older cohorts were comparable and not likely influencing these changes (10.27 ± 3.13 million vs 9.36 ± 2.9 million cells isolated from 80 mL blood, respectively). While research has set out to assess changes in macrophage numbers with age, no real consensus has been drawn, with large tissue- and stimulus-specific variation, although it has been suggested that pro-inflammatory populations increase with age [365]. Interestingly, pro-inflammatory polarisation for 24 hours with LPS and IFNγ caused a slight but statistically significant reduction in phagocytosis compared with M^0, both in the young and older cohorts, contradicting published literature suggesting phagocytosis to be a principally inflammatory immune response [473]. $M^{LPS+IFN\gamma}$ MDMs isolated from the older cohort showed the lowest phagocytic ability, with older M^0 MDMs being

similar to young $M^{LPS+IFN\gamma}$ MDMs. Peritoneal macrophages that had been polarised with M1-like or M2-like stimuli had comparable phagocytic activity between young and old following M2 polarisation, but reduced phagocytosis with age in M1-like macrophages, similar to our findings [280]. Fix *et al.,* also found an impairment in phagocytosis with age in pro-inflammatory BMDMs [278]. In contrast to our findings in human MDMs, IFN/LPS stimulation has been found to increase phagocytosis in both young and old BMDMs compared with M^0 [288]. In our findings, LPS and IFNγ stimulation of M^0 MDMs from young human subjects led to a complete loss of chemotactic ability, while chemotaxis in the M^0 MDMs from older subjects was also comparable to that seen in the young $M^{LPS+IFN\gamma}$ MDMs, potentially demonstrating a chronically activated phenotype in the older M^0 MDMs and fitting with our current understanding of inflammaging [55,442]. Both phagocytosis and chemotaxis functions have been associated with improved murine lifespan. Where phagocytosis has been found to be decreased with age, long-lived (122 weeks) mice had increased phagocytosis compared to old (72 weeks) [474]. Additionally in mice, a positive correlation has been found between phagocytic ability and chemotactic ability and achieved lifespan [283]. Chemotaxis accounted for 50% of variance in lifespan and chemotaxis and phagocytosis together were found to predict almost 70% of final achieved lifespan, again with lower function being associated with shorter lifespan [475].

Systematic gene enrichment analysis of mouse alveolar transcripts identified specific transcription factors *Myc*, *Foxm1*, *Nfyb*, *Usf1* and *Srf* were downregulated and Nfic was upregulated with age. Consistent with this, we identified downregulated *Myc* and *Usf1* in old mouse BMDMs and in human MDMs from older donors, which led us to assess whether knockdown of *MYC* and *USF1* in young human MDMs could recapitulate an ageing phenotype. Loss of *MYC* and *USF1* significantly reduced both phagocytosis and chemotaxis in young macrophages. *MYC* is well established to play a key role in regulating the cell cycle, including proliferation, differentiation and apoptosis, as well as cell metabolism, tissue remodelling and pro- and anti-inflammatory cytokine production [167,476]. It is known to be a central marker for alternative macrophage activation, controlling expression of nearly half of the genes associated with macrophage alternative activation, partly through upregulation of

signal transducer and activator of transcription-6 and peroxisome proliferator-activated receptor γ signalling [165,166]. MYC is also implicated in the growth and progression of many cancer types [477], shown to be the most common abnormality in malignant tumours [153]. Yamanaka *et al.,* discovered that the introduction of MYC alongside three other transcription factors can reprogram differentiated cells back to a stem cell-like state [408]; short term cyclical expression of these factors can ameliorate hallmarks of ageing and prolong lifespan [478], the first indication of *MYC*'s potential involvement in the ageing of cells. MYC has since been implicated in ageing, with its expression having a key role in proper mitochondrial function [479,480], and in regulation of double-stranded DNA breaks, extensively reviewed elsewhere [481]. Mitochondrial dysfunction and genomic instability are two of the hallmarks of ageing [59]. The downregulation in Foxm1 with age that we found in the mouse alveolar macrophages and BMDMs is also intriguing, given the association between this transcription factor and senescence. Foxm1 has previously been shown to decline with age in human dermal fibroblasts [482], which is thought to be as a result of activation of the p53-p21-DREAM pathway, commonly found in senescent cells to prevent expression of early cell cycle genes [483]. This could therefore be indicative of a more senescent phenotype within these macrophage populations.

Transcription factor *USF1* has been shown to drive dysregulated cardiometabolic health [184] and cancers such as adenocarcinoma, where *USF1* expression may activate the *MYC* pathway [484], highlighting established links between these transcription factors. *USF1* responds to dietary components such as glucose, and recognises the E box CACGTG motif promoter region of target genes, many of which are involved in lipid metabolism [179]. Many studied polymorphisms in *USF1* SNPs have strong associations with plasma lipid levels, apolipoprotein E gene expression and familial hyperlipidaemia [185,485]. The rs3737787 and rs2073658 genetic variants of *USF1* have significant associations with familial hyperlipidaemia, a risk factor for premature cardiovascular disease, affecting pro-inflammatory cytokine secretion and serum lipid levels [186]. Further, *USF1* SNPs such as rs10908821 have an age-related association with brain lesion development in Alzheimer's Disease [486]. This study has identified that *USF1* polymorphisms in macrophages and monocytes transcript

from the Cardiogenic Consortium transcriptomic study and other published studies within Phenoscanner database indicate a further contribution to this ageing phenotype, since these polymorphisms are associated with decreased monocyte percentage, a known age-related change. Monocyte numbers are known to become dysregulated with age, with non-classical pro-inflammatory monocytes becoming the central population of the aged immune system [487,488]. Our analysis showed that *MYC* is downregulated in the non-classical compared with classical monocytes, which fits with this existing data. There are clear genetic associations linked to *USF1* and age-related disease. Further, the presence of many *MYC* and *USF1* downstream targets in the differentially expressed genes of MDMs and monocytes from the Cardiogenic Consortium transcriptomic study cannot be understated. It has previously been shown that assessing a single gene, the transcription factor itself, is an unreliable estimation of transcription factor binding activity, especially when effect size is small, such as that of the relatively limited age range analysed in this dataset [455,489]. By instead using downstream targets of the transcription factor, the challenge posed is overcome and a more reliable estimation of transcription factor activity is achieved. This therefore further adds to our hypothesis that *MYC* and *USF1* are driving transcriptional and therefore functional changes with age.

We found that human macrophages show a profound reduction in phagocytosis, migration and chemotaxis in the older age group. We investigated the mechanistic basis for these functional changes by assessing cytoskeletal rearrangement required for coordinated phagocytosis and migration [490]. Macrophage cytoskeleton is composed of cortical filamentous actin (F-actin). Resting macrophages morphology shows large actin-containing ruffles which function to survey the local environment, that are essentially precursors of phagosomes [491]. We found reduced F-actin content in older human macrophages and in young macrophages with reduced *USF1* expression, suggesting a decline in cytoskeletal function with age regulated by *USF1* which may therefore be contributing to reduced phagocytic capacity. Further, macrophage elongation has been shown to be essential for motility [492]. This correlates with our finding that the young M^0 MDMs are more motile and elongated than the $M^{LPS+IFN\gamma}$ MDMs, however both phenotypes of the older MDMs had increased elongation,

making this an unlikely mechanism for the reduced motility seen. Similar to our findings that older MDMs were larger than younger counterparts, older human cochlea macrophages were found to have a reduced number of processes projecting and increased cytoplasmic volume around the nucleus compared with young [306]. Interestingly, ageing-impaired actin function linked to Rac1 has been shown to reduce alveolar macrophage phagocytosis of bacteria [274].

In parallel to our findings, microglia have also been found to have altered morphology and functions with age. Similar to the human MDMs we tested, aged microglia have larger cell bodies [344] and are less mobile and phagocytic than young microglia cells [307,493]. Aged microglia have been associated with an increased state of inflammation [494] and display an amoeboid-like structure that was not seen in young that may be linked to their reduced motility [309].

As well as changes in morphology, migration requires complex adhesion interactions with other cells and the underlying extracellular matrix, a biological process that we found to be enriched by transcription factor knockdown in human MDMs and with age in murine alveolar macrophages, with integrins and cadherins making up key dysregulated genes in human MDM ageing. Adherence has previously been assessed in mice with consistent findings. Peritoneal macrophages show an increase in adherence capacity with age to smooth plastic meant to resemble tissue [286], fibronectin and type 1 collagen [495]. Cell adhesion pathways were also disturbed with ageing in transcriptomic analysis of microglia [496]. This was comparable to the analysis performed here with six shared dysregulated genes associated with cell adhesion (PCDHGA2, AJUBA, CDH2, CXADR, TENM4, CLDN1).

Transcriptional targets of *MYC* and *USF1* that we found to be dysregulated with age suggest several further mechanisms for the ageing macrophage phenotype. *ANGPTL3* regulates lipoprotein lipase activity shown to be involved in age-related diseases such as atherosclerosis, already identified as a potential druggable target to treat this disease [497]. Targeting *STAB2* ameliorates atherosclerosis by dampening inflammation [498]. *ATP8B1* is associated with macrophage efferocytosis and resolution of inflammation and may be a potential therapeutic target in chronic pancreatitis [499]. *SFRP4* is involved

in Wnt signalling and has been implicated in skin ageing through promoting SASP, while its knockdown suppressed SASP [500]. *SFRP4* is upregulated in ageing, correlating with our data, suggesting a role in macrophage chronic inflammation and ageing. Finally, *GDF1* is associated with cellular migration through Smad signalling in macrophages [501]. We also considered loss of MCP-1 receptor, CCR2, as a possible mechanism for reduced migration. It has been shown that changes in CCL2-CCR2 interaction may affect macrophage polarisation towards M1, with lower CCR2 expression contributing to increased M1 polarisation and higher pro-inflammatory cytokine release [470]. Disruption in this interaction may therefore be pushing the older resting macrophage towards a more pro-inflammatory state. Although *CCR2* mRNA levels were downregulated with loss of *MYC* in human M^0 MDMs compared with control and with age in murine BMDMs, human MDMs showed no significant difference with age in *CCR2* expression.

Mechanisms underlying downregulation of *MYC* and *USF1* in macrophages with age remain to be explored. Dysregulated metabolism and redox balance have been linked to downregulation of *Myc* in aged mice where macrophages are finely tuned to alter metabolism according to their environment [173]. It will be interesting to assess epigenetic changes that may lead to downregulation of *MYC* and *USF1* in macrophages with age in the future.

5.5 Conclusion

In summary, we have highlighted specific functions and key regulatory factors of *MYC* and *USF1* that decline with age in human macrophages. Importantly, the older age group, showing markedly reduced phagocytosis, migration and chemotactic migration, were from healthy individuals over 50 years old with a mean age of 61 years. This average age is more than 20 years below the average life expectancy in countries with modern healthcare. The clear phenotypical changes seen in the relatively young age of our older cohort shows that innate immune functional decline may represent the starting point of ageing, with a culmination of factors prompting age-related disease and frailty. The underlying mechanisms of human MDM ageing are far from completely understood; the

downstream functionally relevant targets of *MYC* and *USF1* that are also dysregulated with age will be a crucial starting point for future investigations.

Limitations of the Study:

Our studies using human monocyte-derived macrophages were from two gender-balanced healthy volunteer age groups: 18-30 years (mean age 24 ± 1.2 SD years; young cohort) or > 50 years (mean age 61 ± 5.7 SD years; older cohort). The healthy ageing donors are without comorbidities and frailty seen in the ageing human population, which will form more complex interesting future studies beyond the scope of this work. Human macrophage function was determined *in vitro*, since live phagocytosis and migration detection would not have been possible *in vivo*. Assessing function and gene expression under resting and specific stimulation conditions offers a simplified model compared to macrophages *in vivo*. Mouse macrophages were from male animals but these were extrapolated to human macrophage assessment from gender-balanced groups.

Chapter 6. Restoration of ageing macrophage function using senolytics

This results chapter assesses restorability of macrophage function with age, identifying ATP8B1 as a key gene in phagocytosis and senolytics as restoring transcription.

Declaration of Contribution

This results chapter is intended to be published in part, along with additional data from collaborators not presented here. The section on monocytes was published as supplementary to the publication in Chapter 5, while the Fisetin work will form part of a future publication. Martha Clements helped to perform the ATP8B1 knockdown experiments for some donors under my supervision. These data were analysed, interpreted and formulated by myself. This chapter was written by myself, without contribution from others.

Summary

Upstream stimulatory factor (*USF1*) and *MYC* are transcription factors that are downregulated with age in both human and murine macrophage populations, including human monocyte-derived macrophages (MDMs). In human MDMs, they have a role in age-related macrophage dysfunction, including reduced phagocytosis and chemotaxis, as knockdown of *USF1* or *MYC* in MDMs isolated from young individuals leads to a similar degree of dysfunction as that seen from older counterparts. It has not yet been established whether these age-related changes are present in monocyte precursors or whether they occur during macrophage differentiation. Downstream targets of *USF1* are also dysregulated with age, including an upregulation in expression of ATPase phospholipid transporting 8B1 (*ATP8B1*). However, whether modulation of *USF1* target genes in MDMs isolated from older individuals would have a positive effect on function has not been previously investigated. Additionally, senolytic treatments, that have been shown to cause an overall improvement in age-related morbidity and mortality, have not previously been assessed for any role in improving primary macrophage function with age, despite the extensive evidence of the role of macrophages in the progression of age-related diseases such as atherosclerosis.

In this study, we assessed expression of *MYC* and *USF1* in freshly isolated human monocytes from young and older individuals as well as their capacity for chemotaxis and phagocytosis. We performed *ATP8B1* knockdown in MDMs isolated from older individuals to investigate whether age-related macrophage dysfunction could be restored through this pathway. Further, we assessed changes in expression of *Myc* and *Usf1*, that are downregulated with age in bone marrow-derived macrophages (BMDMs), following Fisetin or Zoledronate treatment in BMDMs isolated from old mice. Our results showed that chemotaxis and phagocytosis were reduced in monocytes freshly isolated from older individuals compared with young. Expression of *MYC* but not *USF1* was downregulated with age in these monocytes. Treatment of old mice with Fisetin or Zoledronate partially restored *Myc* but not *Usf1* expression alongside other key dysregulated genes in BMDMs. Finally, *ATP8B1* knockdown in older MDMs increased phagocytosis when compared with control. In summary, these results

demonstrate that there may be different mechanisms of macrophage dysfunction via *USF1* or *MYC*, with *MYC* downregulation occurring at the monocyte precursor level. *ATP8B1* downstream of *USF1* could be targeted to improve macrophage function in ageing, while senolytic treatments could improve macrophage function via modulation of *MYC* expression. More work is needed to understand how this might affect macrophage function.

6.1 Introduction

The number of people over the age of 65 years is increasing worldwide. This comes with an increasing burden on healthcare systems, as people are living longer with diseases and many diseases have age as a major risk factor [1–3]. In addition, multimorbidity, referring to the presence of two or more chronic age-related diseases, occurs in approximately 40% of the global adult population [502]. Because of this, the fundamental mechanism of ageing are being increasingly researched as it is believed that pharmacologically targeting these may increase healthspan and delay onset or decrease severity of age-related disease and multimorbidity [503,504].

The increase with age in cellular senescence is one such mechanism, whereby proliferating and terminally differentiated cells enter a state of cell cycle arrest and apoptotic resistance, leading to an increase in expression of p21 and p16(Ink4a), cell cycle inhibitors that are commonly used as markers of senescence [505–507]. Additionally, cellular senescence is associated with secretion of pro-inflammatory cytokines and chemokines and extracellular matrix-degrading proteins, more commonly referred to as the senescence-associated secretory phenotype (SASP) [508,509]. Senescent cells therefore increase inflammation in the tissues, with SASP having a paracrine senescence-inducing effect on other cells [509] and further perpetuating age-related dysfunction in many different tissues [77,510]. However, their original discovery as drivers of ageing was through the benefits seen from therapeutically removing p16(Ink4a)-positive senescent cells [77]. This discovery, alongside that injection of senescent cells into healthy young tissues could drive frailty and mortality [511,512], has led to the development and repurposing of therapeutics able to selectively kill senescent cells, that we

refer to as senolytics, as well as those able to inhibit SASP, referred to as senomorphics [513]. Different tissues and cell types undergo senescence in differing ways, including expression of different markers, secretion of SASP components and activation of pathways to resist cell death [514], thus removal of senescent cells is not a one treatment fits all approach. However, a number of senotherapeutics are already being considered.

Fisetin is a naturally occurring bioactive flavonoid found in many fruits and vegetables [515]. It initially gained interest due to its antioxidant and anti-inflammatory activities [516]. It also has anti-proliferative capacity that can be utilised in the context of cancer. Its beneficial effects may be due to its ability to reduce angiogenesis and therefore tumour progression as well as inhibition of matrix metalloproteinase 1 that degrades and remodels extracellular matrix in many cancers [516]. Additionally, Fisetin is able to cause apoptosis in cancer cells more readily than healthy cells, which may relate to the different activated pathways between these cell types [516–518]. The ability of Fisetin to cause apoptosis in damaged cells is the main reason for it being assessed in the context of ageing. It is now a well-known senolytic that has been shown previously to reduce markers of senescence and SASP in multiple tissues [505]. Quercetin is an additional flavonoid senolytic that has already been assessed in human subjects with senescence-driven diseases. This has been shown alongside Dasatinib, a tyrosine kinase inhibitor, to reduce senescent cell number and SASP factors [519], likely paving the way for future clinical trials in this therapeutic class, including for Fisetin.

Zoledronate or zoledronic acid is a bisphosphonate currently used to treat osteoporosis due to its ability to kill cells that otherwise break down the bone, shifting to bone formation and increasing bone mass and density [520]. It has been safely used as a bisphosphonate therapeutic for over 20 years [521] and through clinical trials it has been established that those taking Zoledronate have a reduced risk of mortality [522] and incidence of cardiovascular diseases [521]. Zoledronate has since been assessed as a senotherapeutic, with *in vivo* work showing a reduction in a panel of SASP markers and *in vitro* work showing that it has a high selectivity for killing senescent cells [523].

Senescence is also a normal cellular response to oncogene activation or loss of tumour suppressor signals to limit the expansion of early tumorigenic cells [508,509,524]. Additionally, SASP is critical for immune infiltration after tissue injury, to allow rapid clearance of senescent cells and promote tissue repair [525]. In these responses, senescence and macrophages are thought to be interlinked: it is predominantly the responsibility of macrophages to clear senescent cells. For example, work by Yun *et al*. (2015), demonstrated that macrophages are essential for efficient clearance of senescent cells in salamander limb regeneration [99]. It is thought that macrophage senescent cell clearance is reduced with age and age-related diseases, perpetuating their age-related accumulation [526].

Macrophages are part of the mononuclear phagocyte system, comprising monocytes that circulate the blood, migrate into tissues and differentiate into monocyte-derived macrophages and tissue resident macrophage populations. The local tissue environment of these different populations largely determines the phenotype of the macrophage [6,21,24]. This then dictates the functions of the cell, which spans phagocytosis, antigen presentation and adaptive immune activation, cytokine production and secretion of growth factors for tissue repair [31,33]. Much work has already gone into assessing how these functions change with age; it is well established that macrophage dysfunction is associated with age-related disease, reduced responses to therapeutics and poor prognosis [527]. In diabetes, for example, there is an accumulation of inflammatory macrophages in tissues, leaving the tissue vulnerable to diabetic injury [528] and atherosclerosis is a principally inflammatory disease where macrophages play a large role in its pathology [128,529].

The mechanisms behind macrophage age-related functional decline are still not well defined. However, we have previously shown in human monocyte-derived macrophages (MDMs) that functions including phagocytosis and chemotaxis are reduced with age, and linked this to a downregulation in expression of transcription factors *MYC* and *USF1* (Chapter 5). Although these changes have been found in MDMs, it has not been assessed whether they are present in the monocyte precursors or occur as a result of the differentiation process, especially important because

the culture process itself can produce epigenetic changes and effect cellular function [530]. Additionally, it has not been established whether there is capacity to restore these functions. This includes their ability to clear senescent cells, as to date no work has established whether senolytics have any positive effects on macrophage function to allow them to better clear senescent cells.

Macrophage heterogeneity and plasticity has so far limited our ability to therapeutically target them, so an approach able to re-educate them could aid without effecting other essential functions. In addition, transcription factors themselves are rarely used as therapeutic targets due to the potential for off-target effects. For example the transcription factors that we have previously linked to age-related macrophage dysfunction have thousands of downstream targets that could potentially be affected by their modulation. This is also particularly relevant for MYC which is upregulated in cancer, suggesting a caution in selecting this as a target for induction [159].

We have previously assessed downstream targets of both MYC and USF1 and found a number of genes altered both with increasing age and transcription factor knockdown that would be better candidates for modulation. One example is ATPase phospholipid transporting 8B1 (ATP8B1), where expression is normally dampened by USF1, meaning increasing age and USF1 knockdown lead to its upregulation. ATP8B1 is a catalytic flippase that aids in the transport of phospholipids within the plasma membrane [531]. It has an important role in macrophage efferocytosis, a non-inflammatory clearance of dying cells, where it promotes M2 macrophage polarisation, dampens inflammation and allows efferocytosis to occur [499]. It therefore posed an interesting target for modulation. Further, we have previously identified four additional transcription factors: NFYB, SRF, FOXM1 and NFIC that are dysregulated in different macrophage populations and may give further insight into the mechanisms behind age-related macrophage dysfunction.

Overall, current knowledge indicates a major role of transcription factors MYC and USF1 in the mechanisms behind MDM functional decline with age, including their downregulation leading to a reduction in phagocytosis and chemotaxis. However, there is lack of evidence as to what stage in

MDM differentiation this dysfunction occurs and whether there is capacity for function to be restored. Additionally, senolytic treatments such as Fisetin and Zoledronate have not previously been assessed in the context of macrophage ageing, including in expression of key genes and function. We therefore assessed the expression levels of *MYC* and *USF1* and functional capacity of freshly isolated human monocytes from young and older donors in order to investigate where age-related dysfunction arises. We also assessed whether the ageing phenotype could be restored in MDMs isolated from older individuals and mouse BMDMs through modulation of downstream targets and treatment with senolytics, respectively to better understand the mechanisms underpinning macrophage ageing.

6.1.1 Hypothesis and aims

It has been established that monocyte-derived macrophages have a reduced function linked to transcription factors MYC and USF1. However, it is not known whether these changes are present at the monocyte precursor level or occur during macrophage differentiation. Macrophages have a major role in the progression of many age-related diseases and therapeutics targeting the underlying mechanisms of ageing are gaining ground. Understanding whether these therapeutics benefit macrophage function through MYC and USF1 pathways would aid in our understanding of the mechanisms driving macrophage age-related dysfunction. We hypothesise that age-related changes in monocyte-derived macrophage function are also present in monocytes and driven by MYC and USF1. Additionally, we hypothesise that this dysfunction is reversible through modulation of downstream targets in macrophages isolated from older individuals and that senolytic treatments would have a positive effect on macrophage transcription to contribute to restoration of function and overall improvements with these treatments. We addressed these hypotheses with the following aims:

1. Assess chemotaxis, phagocytosis and *MYC* and *USF1* expression in monocytes freshly isolated from young and older individuals

2. Assess phagocytosis in human MDMs isolated from older individuals following modulation of *ATP8B1* expression
3. Assess the effects of Fisetin and Zoledronate treatment on transcription in BMDMs isolated from old mice

6.2 Methods

Human monocytes and monocyte-derived macrophages and murine bone marrow-derived macrophages were isolated, differentiated, cultured and polarised as described in Section 2.3, that also gives details of transient transfection of differentiated human MDMs with small interfering RNAs targeting *ATP8B1* and *USF1*. The treatments for mice are described in Table 2.1, with further information on Fisetin and Zoledronate treatment protocols found in Supplementary figure 8.5.1, Supplementary figure 8.5.2 and Supplementary figure 8.5.3. For human monocytes and MDMs, phagocytosis and chemotactic migration assays were performed as described in Section 2.4. RNA isolation, cDNA synthesis and real-time qPCR were performed as described in Section 2.5.

6.3 Results

6.3.1 Age-related changes in monocyte-derived macrophages are present in freshly isolated monocyte precursors

We found previously that monocyte-derived macrophages isolated from older human donors have reduced chemotaxis and phagocytosis compared with young counterparts and this has been linked to a decrease in expression of transcription factors *MYC* and *USF1* (Chapter 5). These MDMs are differentiated for 7 days, this culture period potentially inducing epigenetic changes that are not physiological [530]. In order to minimise any effect of *in vitro* culture and differentiation on the age-related changes found, we first analysed the expression levels of *MYC* and *USF1* alongside chemotaxis and phagocytosis in freshly isolated monocyte precursors.

Monocytes were isolated from young (22-26 years) and older (50-71 years) groups of human donors that were gender-balanced. Expression of both *MYC* and *USF1* was measured by RT-qPCR. We found

that *MYC* but not *USF1* was downregulated with age in freshly isolated monocytes (Figure 6.1), with a 60.6% decline in expression of *MYC* in the older subjects compared with young (mean ± SEM young: 2.03 ± 0.52, older: 0.80 ± 0.10).

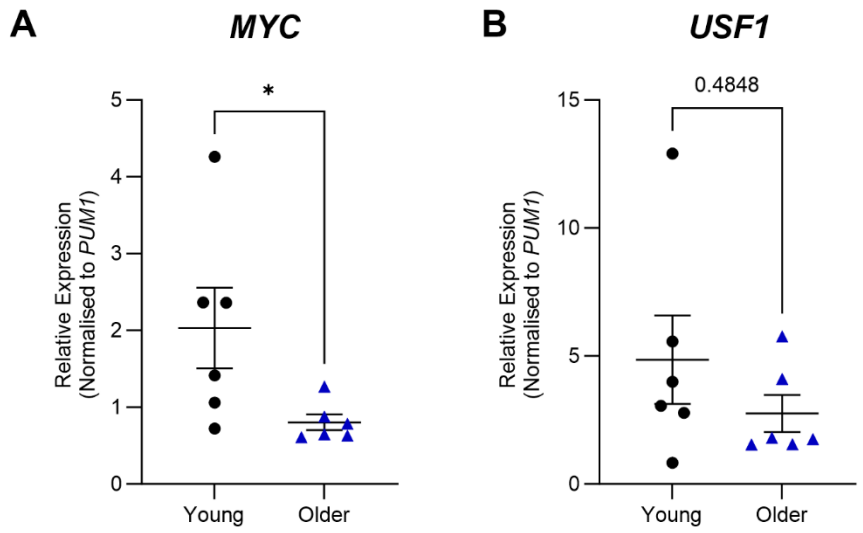


Figure 6.1 Monocytes isolated from older individuals have dysregulated *MYC* but not *USF1* expression compared with young counterparts

RT-qPCR analysis of *MYC* (A) and *USF1* (B) expression in human freshly isolated monocytes from young (22-26 years) and older (50-71 years) individuals. *PUM1* was used as a housekeeping control. Data are presented as mean ± SEM with each datapoint representing the mean of an individual donor performed in triplicate. N=6, Mann-Whitney test, * $P < 0.05$.

In order to determine the functional capacity of these monocytes, chemotaxis and phagocytosis were assessed. As with the work on human MDMs, chemotactic capacity was measured using an MCP-1-directed transwell assay. Chemotaxis was significantly reduced in the monocytes isolated from older individuals compared with young (Figure 6.2), with an overall 69.7% decline (mean ± SEM young: 4.02 ± 0.64, older: 1.22 ± 0.19), as measured by overall mean fluorescence of the bottom well compartment.

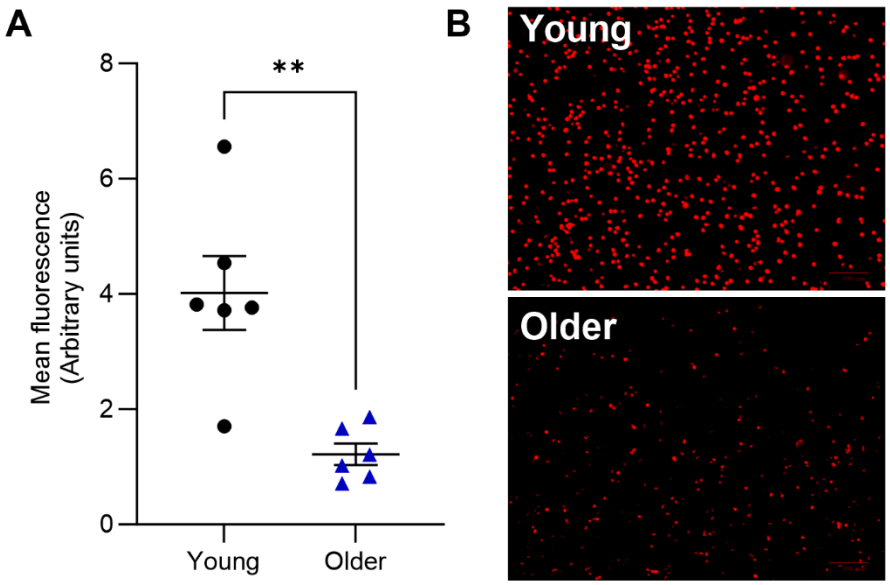


Figure 6.2 Chemotaxis is reduced in human freshly isolated monocytes from older compared with young individuals

A – Migrated freshly isolated monocytes measured as mean fluorescence intensity from young (22-26 years) and older (50-71 years) human subjects. Monocyte chemoattractant protein 1 (5 nM) or media only was added to the lower compartment of each well and PKH26-stained monocytes to the transwell. These were incubated at 37°C for 3 hours before imaging the lower well. Mean fluorescence intensity for each image was measured. Media only was used as a control and subtracted from the overall fluorescence. Data are presented as mean ± SEM with each datapoint representing the mean of three fields of view taken per donor normalised to media-only control. N=6, Mann-Whitney test, ** $P < 0.01$.

B – Representative images of PKH-stained monocytes that migrated through the transwell.

Phagocytic capacity was assessed by measuring the uptake of opsonised fluorescent beads in each cell as a direct comparator to the work on MDMs. Additionally, the uptake of fluorescent *Staphylococcus aureus* by monocytes was measured to give further insight into a more biologically relevant target of phagocytosis. Phagocytosis of opsonised beads was significantly reduced with age (Figure 6.3), with a decline of 28.4% (young: 15.95 ± 1.51, older: 11.42 ± 0.89). In addition, phagocytosis of *S. aureus*, measured by mean fluorescence of each cell in the field of view, was reduced by 30.3% in monocytes isolated from older compared with young individuals (young: 14.71 ± 0.66, older: 10.26 ± 1.38).

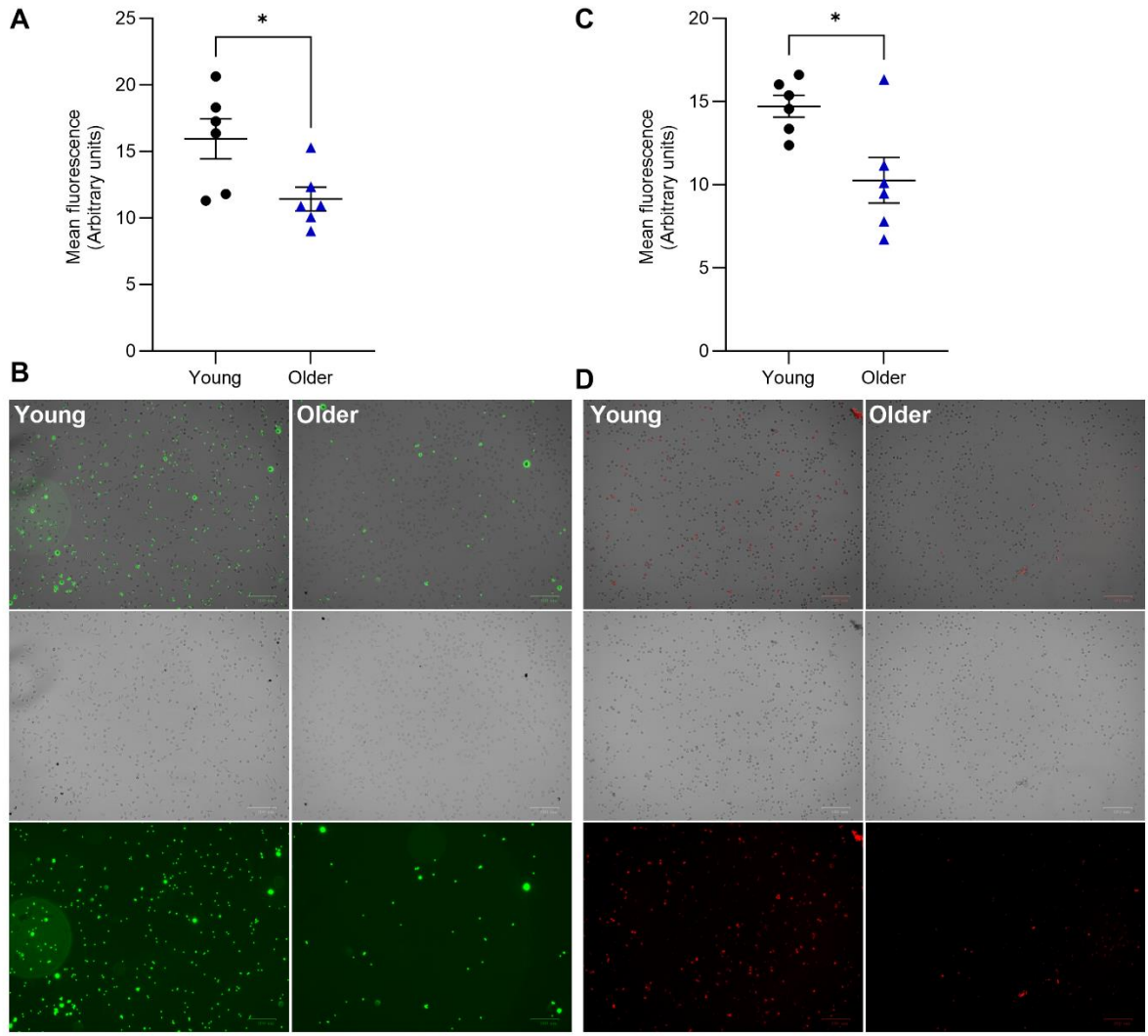


Figure 6.3 Phagocytosis of fluorescent beads and *Staphylococcus aureus* is reduced in human freshly isolated monocytes from older compared with young individuals

A – Bead uptake expressed as mean fluorescence intensity of monocytes from young and older subjects. Fluoresbrite YG microspheres 1.00 µm (Polysciences) were added to foetal bovine serum and incubated at 37°C for 30 minutes before incubation for three hours with freshly isolated monocytes at 37°C or 4°C (control).

B – Representative images of fluorescent beads taken up by monocytes from young and older subjects showing brightfield and green channel images, overlayed into a single image.

C – *Staphylococcus aureus* uptake expressed as mean fluorescence intensity of monocytes from young and older subjects. mCherry-stained *S. aureus* was added at a multiplicity of infection of 10 and incubated with freshly isolated monocytes at 37°C or 4°C (control) for three hours.

D – Representative images of mCherry-stained *S. aureus* and monocytes from young and older subjects showing brightfield and red channel images, overlayed into a single image.

Cells were washed and fixed with 4% PFA before taking brightfield and green or red channel images. ImageJ "Analyze Particles" function was used to select cells in brightfield image to produce regions of interest (ROIs). ROIs were overlayed onto the colour image and mean fluorescence intensity of each cell was measured. Data are presented as mean ± SEM with each datapoint representing the mean of three fields of view taken per donor normalised to 4°C and particle-free control. Young (22-26 years), older (50-71 years). N=6, Mann-Whitney test, * $P < 0.05$.

Overall, these results show a downregulation in expression of *MYC* and corresponding reduction in chemotaxis and phagocytosis in monocytes isolated from older compared with young donors. It was further assessed as to whether the level of *USF1* expression affected either function. However, no correlation between either chemotaxis or phagocytosis and *USF1* expression was identified (Supplementary figure 8.5.4).

6.3.2 Loss of *ATP8B1* in monocyte-derived macrophages isolated from older individuals restores phagocytosis

With the confirmation that the age-related changes in function and *MYC* expression found in MDMs were not the result of *in vitro* culture, we next assessed whether function could be restored in MDMs isolated from older individuals. Modulation of *MYC* or *USF1* directly could potentially have many downstream effects, as these are transcription factors with a reported 1,336 or 1,201 downstream targets expressed in macrophage populations, respectively. It was found previously that *ATP8B1* was upregulated with age-related downregulation of *USF1* and *USF1* knockdown in both M^0 and $M^{LPS+IFN\gamma}$ MDM phenotypes. We therefore assessed whether knockdown of *ATP8B1* in MDMs isolated from older individuals could rescue phagocytosis in these phenotypes.

To test the sufficiency of loss of *ATP8B1* to rescue phagocytosis in older MDMs, we used siRNA gene silencing. This was done in both young and older MDMs to ensure any changes seen in the older MDMs were not as a result of the reagents. *ATP8B1* is not well expressed in young MDMs, therefore knockdown was not possible (Figure 6.4A-B). In older MDMs, the efficiency of *ATP8B1* gene silencing was 93.9% (86.1 – 99.9) in M^0 MDMs and 91.9% (75.6 – 99.4) in $M^{LPS+IFN\gamma}$ MDMs (Figure 6.4C-D).

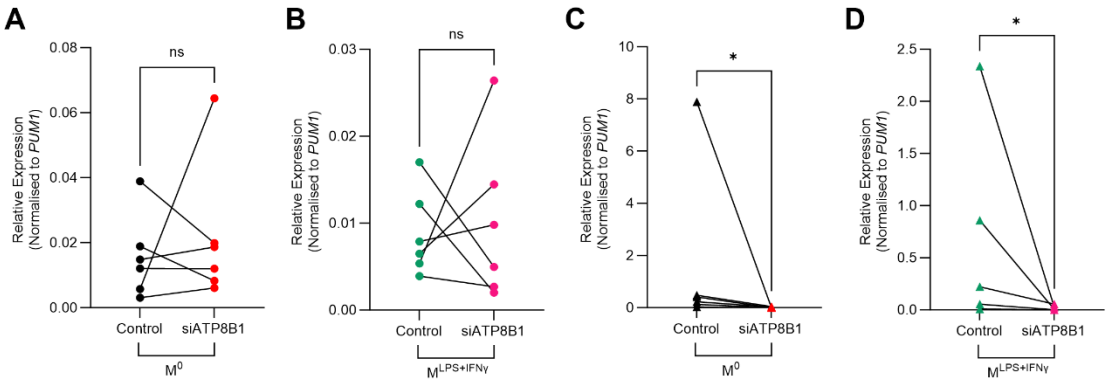


Figure 6.4 *ATP8B1* knockdown in human monocyte-derived macrophages isolated from older but not young individuals

RT-qPCR analysis of *ATP8B1* expression in young M^0 (A), young $M^{LPS+IFN\gamma}$ (B), older M^0 (C) and older $M^{LPS+IFN\gamma}$ (D) monocyte-derived macrophages (MDMs) in respective siRNA knockdowns. MDMs were differentiated from human blood monocytes isolated from young (22-25 years) and older (54-71 years) donors for 7 days in M-CSF and then incubated with siRNA for 72 hours. Cells were then either left untreated (A,C) or treated with LPS and IFNγ for 24 hours (B,D). Each datapoint represents the mean of an individual donor performed in triplicate. *PUM1* expression was used as a housekeeping control. N=6, Mann-Whitney test, * $P < 0.05$. ATP8B1 knockdown was performed by myself and Martha Clements under my supervision.

Phagocytosis was assessed by measuring the uptake of opsonised fluorescent beads in each cell (Figure 6.5). In M^0 MDMs isolated from older individuals, phagocytosis increased by 13.4% with loss of *ATP8B1* (Figure 6.5B). In the young M^0 MDMs, there was no change in *ATP8B1* expression and concordantly, no change in phagocytosis of beads. This was also compared with knockdown of *USF1*, where phagocytosis was significantly reduced in the young but unchanged in the older M^0 MDMs (Supplementary figure 8.5.5).

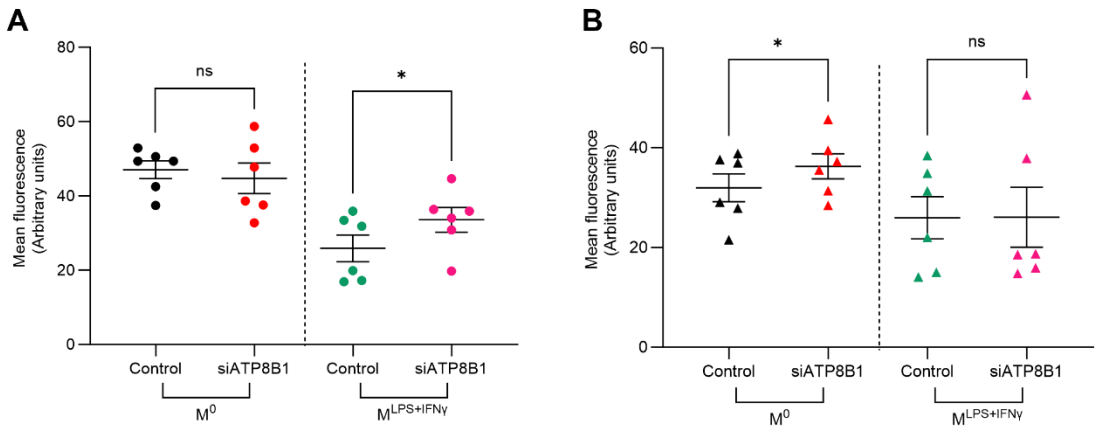


Figure 6.5 Loss of *ATP8B1* in monocyte-derived macrophages isolated from older individuals improves phagocytosis

Bead uptake expressed as mean fluorescence intensity for human monocyte-derived macrophages (MDMs) from young (22-25 years) (A) and older (54-71 years) (B) subjects with loss of *ATP8B1*. MDMs were either untreated (left of dotted line) or treated with lipopolysaccharide (LPS) and interferon (IFN)-γ for 24 hours (right of dotted line). Fluoresbrite YG microspheres 1.00 µm (Polysciences) were added to foetal bovine serum and incubated at 37°C for 30 minutes before incubation for three hours with MDMs at 37°C or 4°C (control). Cells were washed and fixed with 4% paraformaldehyde before taking brightfield and green channel images. ImageJ "Analyze Particles" function was used to select cells in brightfield image to produce regions of interest (ROIs). ROIs were overlayed onto the green channel image and mean fluorescence intensity of each cell was measured. Data are presented as mean ± SEM with each datapoint representing the mean of three fields of view taken per donor normalised to 4°C control. N=6, Wilcoxon test, * $P < 0.05$. ATP8B1 knockdown was performed by myself and Martha Clements under my supervision.

6.3.3 Senolytic treatments restore *MYC* expression in macrophages isolated from older individuals

With confirmation that there was capacity to restore macrophage function in MDMs isolated from older individuals, we assessed whether any existing treatments associated with longevity had any effects on macrophage function. Fisetin is a naturally occurring senolytic that has been shown to be beneficial in reversing a number of the negative effects associated with ageing and age-related disease. We assessed the effect of *in vivo* Fisetin treatment on macrophage function using an atherosclerotic mouse model. A PCSK9 overexpression model was used by injection of AAV8/mPCSK9 at 17 months of age. Mice were then fed a western diet for 12 weeks to induce atherosclerosis with half also receiving Fisetin in their food for 6 weeks in 2 week blocks. After 12 weeks the mice were culled and bone marrow harvested for differentiation to bone marrow-derived macrophages (Supplementary figure 8.5.2). *Myc* and *Usf1* expression were assessed as a measure of macrophage

function in atherosclerotic old (20 months) and Fisetin-treated, atherosclerotic old (20 months) mouse BMDMs that were left untreated (M^0) or polarised towards an inflammatory phenotype ($M^{LPS+IFN\gamma}$).

Fisetin treatment restored expression of *Myc* in the M^0 BMDMs when compared with the control mice ($P < 0.01$, Figure 6.6D). Although there was a trend towards an upregulation in expression of *Usf1* in both M^0 and $M^{LPS+IFN\gamma}$ phenotypes, Fisetin treatment did not significantly alter expression of *Usf1* in either phenotype compared with untreated controls (Figure 6.6E-F).

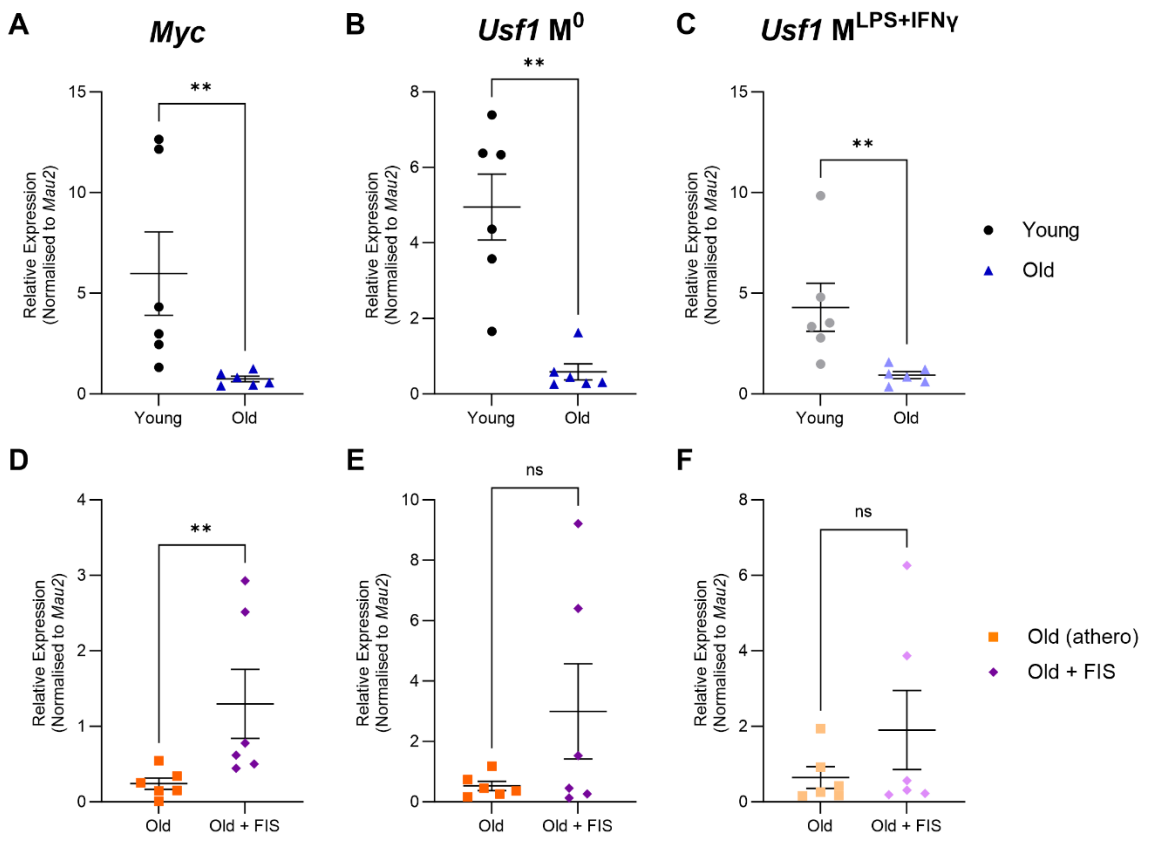


Figure 6.6 Bone marrow-derived macrophages isolated from old mice treated *in vivo* with Fisetin have improved *Myc* but not *Usf1* expression

RT-qPCR analysis of *Myc* (A,D) and *Usf1* (B-C,E-F) expression in bone marrow-derived macrophages (BMDMs) isolated from young (2-5 months) and old (20-22 months) (A-C) or old and old Fisetin-treated (20 months) (D-F) C57BL6/J mice. BMDMs were either untreated (A-B,D-E) or treated with lipopolysaccharide (LPS) and interferon (IFN)-γ for 24 hours (C,F). *Mau2* expression was used as a housekeeping control. Data are presented as mean ± SEM with each datapoint representing the mean of an individual mouse performed in triplicate. N=6, Mann-Whitney test, ** $P < 0.01$.

Zoledronate is another potential lifespan-extending therapeutic that was also tested through *in vivo* treatment of healthy old mice. This was given to mice at the age of 14 months twice a week for 6 months before mice were culled and bone marrow isolated and differentiated to BMDMs (Supplementary figure 8.5.3). Compared with old control mice, Zoledronate treatment significantly induced *Myc* expression ($P < 0.01$, Figure 6.7A), with an overall increase of more than 250% (mean ± SEM control: 0.74 ± 0.14, ZOL-treated: 2.62 ± 0.56). Again, *Usf1* expression had a trend towards upregulation following Zoledronate treatment but this was not significant in either the M^0 or $M^{LPS+IFN\gamma}$ phenotype (Figure 6.7B-C).

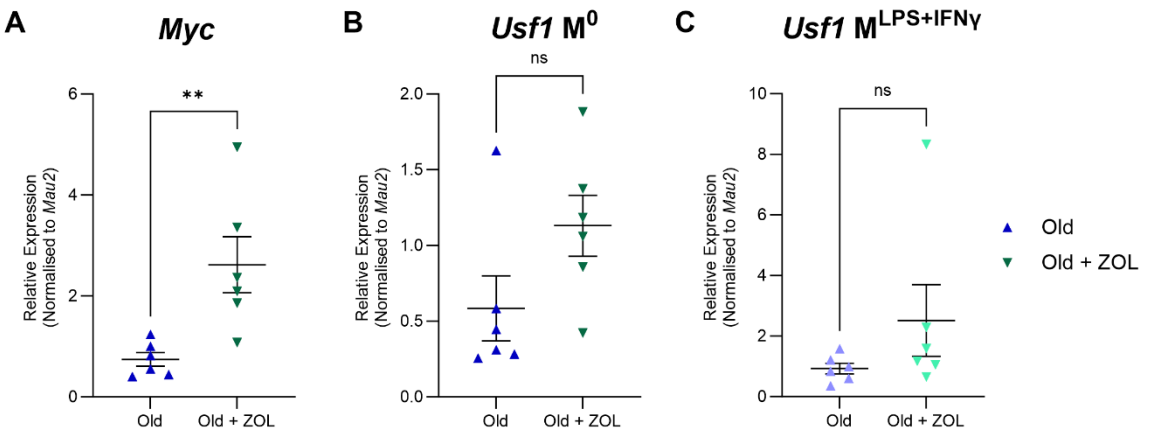


Figure 6.7 Bone marrow-derived macrophages isolated from old mice treated *in vivo* with Zoledronate have restored *Myc* but not *Usf1* expression

RT-qPCR analysis of *Myc* (A) and *Usf1* (B-C) expression in bone marrow-derived macrophages (BMDMs) isolated from old (20-22 months) and old Zoledronate-treated (20-22 months) C57BL6/J mice. BMDMs were either untreated (A-B) or treated with lipopolysaccharide (LPS) and interferon (IFN)-γ for 24 hours (C). *Mau2* expression was used as a housekeeping control. Data are presented as mean ± SEM with each datapoint representing the mean of an individual mouse performed in triplicate. N=6, Mann-Whitney test, ** $P < 0.01$.

As inflammatory LPS and IFNγ-treated macrophages were found to have decreased phagocytic capacity and *Usf1* expression with age, we also tested the expression of inflammatory marker genes to uncover further potential mechanisms of inflammatory macrophage dysfunction. *Calhm6* has previously been found to have inducibility in young BMDMs polarised towards an $M^{LPS+IFN\gamma}$ phenotype [532]. We tested the expression of *Calhm6* in BMDMs isolated from old and old Fisetin- or Zoledronate-treated mice.

Inducibility of *Calhm6* was maintained in all populations assessed (Supplementary figure 8.5.6). However, there was a significant reduction in expression in the BMDMs isolated from the old mice compared with young (Figure 6.8A). Further, Fisetin treatment significantly increased *Calhm6* expression when compared with atherosclerosis model only (Figure 6.8B). Treatment of older mice with Zoledronate did not effect *Calhm6* expression (Figure 6.8C).

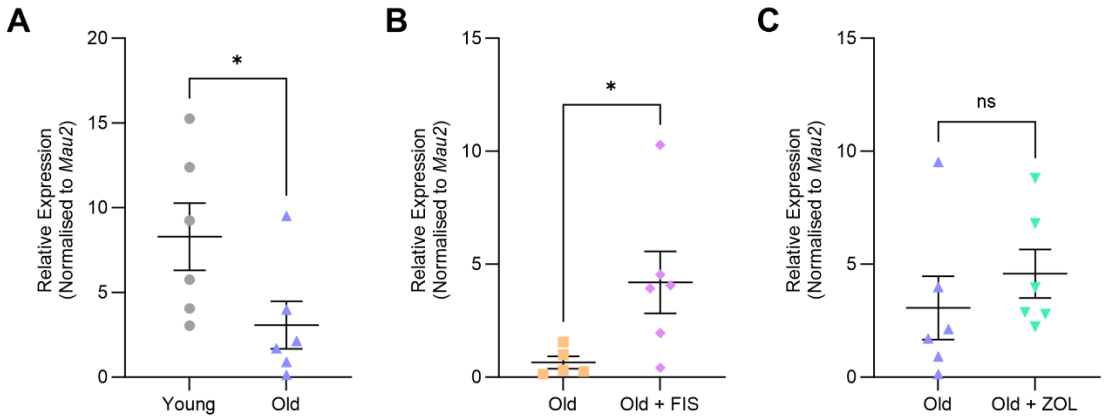


Figure 6.8 Bone marrow-derived macrophages isolated from old mice have restored *Calhm6* expression when treated *in vivo* with Fisetin

RT-qPCR analysis of *Calhm6* expression in bone marrow-derived macrophages (BMDMs) isolated from young (2-5 months) and old (20-22 months) (A), old and old Fisetin-treated (20 months) (B) and old and old Zoledronate-treated (20-22 months) (C) C57BL6/J mice. BMDMs were treated with lipopolysaccharide (LPS) and interferon (IFN)-γ for 24 hours. *Mau2* expression was used as a housekeeping control. Data are presented as mean ± SEM with each datapoint representing the mean of an individual mouse performed in triplicate. N=6, Mann-Whitney test, * $P < 0.05$.

We previously found an additional four transcription factors to Myc and Usf1 that may be linked to age-related differential expression of genes in the macrophage. In murine alveolar macrophages, *Srf*, *Nfyb* and *Foxm1* were found to be downregulated with age, while *Nfic* was upregulated (Figure 5.2). There was also some level of concordance in murine BMDMs and human MDMs (Figure 5.3). We assessed expression of these genes in murine BMDMs in response to either Fisetin or Zoledronate treatment *in vivo* to identify any potential further mechanisms by which these treatments may improve macrophage function.

While Fisetin had no effect on the expression of these four transcription factors, Zoledronate treatment was able to increase expression of *Srf*, *Nfyb* and *Foxm1* in M^0 BMDMs compared with old controls (Figure 6.9A-C). Additionally, *Srf* was significantly upregulated in Zoledronate-treated $M^{LPS+IFN\gamma}$ BMDMs compared with control counterparts (Figure 6.9A). *Nfic* showed no significant change in expression with either treatment.

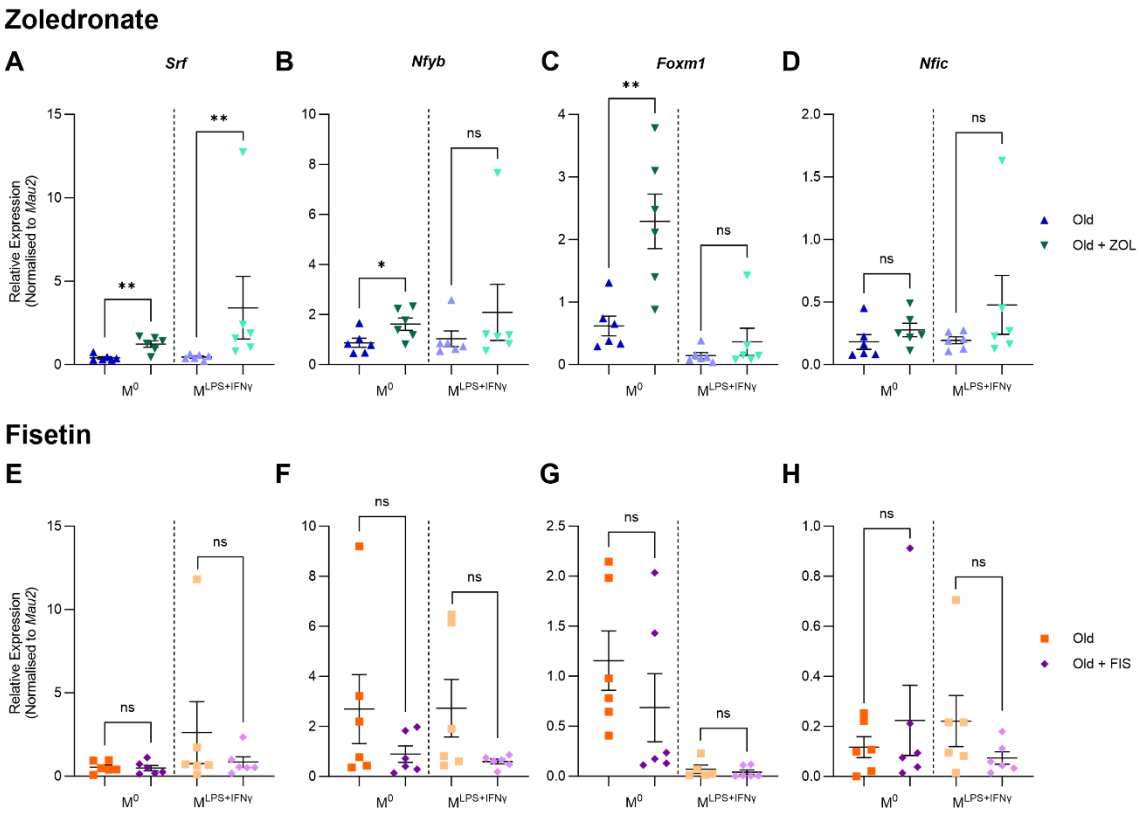


Figure 6.9 Bone marrow-derived macrophages isolated from old mice treated *in vivo* with Zoledronate have restored *Srf*, *Nfyb* and *Foxm1* expression

RT-qPCR analysis of *Srf* (A,E), *Nfyb* (B,F), *Foxm1* (C,G) and *Nfic* (D,H) expression in bone marrow-derived macrophages (BMDMs) isolated from old and old Zoledronate-treated (20-22 months) (A-D) and old and old Fisetin-treated (20 months) (E-H) C57BL6/J mice. BMDMs were either untreated (left of dotted line) or treated with lipopolysaccharide (LPS) and interferon (IFN)-γ for 24 hours (right of dotted line). *Mau2* expression was used as a housekeeping control. Data are presented as mean ± SEM with each datapoint representing the mean of an individual mouse performed in triplicate. N=6, Mann-Whitney test, * $P < 0.05$.

In all, we have uncovered a downregulation in *MYC* and resultant reduction in chemotaxis and phagocytosis in freshly isolated human monocytes that form the precursors of human MDMs. We have partially restored phagocytosis in MDMs isolated from older individuals through modulation of *ATP8B1*, a gene regulated by *USF1*. Additionally we have shown that *in vivo* senolytic treatments Fisetin and Zoledronate upregulate *MYC* expression in BMDMs isolated from old mice. Further, we have identified additional genes modulated by these treatments, including *Calhm6* and *Srf* in inflammatory and *Srf*, *Nfyb* and *Foxm1* in resting BMDMs that are otherwise dysregulated with age in different macrophage populations.

6.4 Discussion

In this study, we showed that the reduction in MYC with age seen in human monocyte-derived macrophages can be seen at the monocyte precursor level and found that both chemotaxis and phagocytosis were also reduced with age in these cells. We found that downregulating ATP8B1 expression in older MDMs restored phagocytosis, highlighting a potential targetable mechanism of macrophage ageing. Additionally, we identified a potential mechanism of action of senolytic treatments Fisetin and Zoledronate to improve healthspan via upregulation of MYC expression to restore macrophage function. Finally we have identified additional potential mechanisms dysregulated in ageing macrophages that senolytics are able to restore, potentially driving some of the improvements in immune function seen with these treatments.

Human MDMs are essential for proper immune function and we have previously reported that they have reduced phagocytosis and chemotaxis with older age. Transcription factors MYC and USF1 were found to be key regulators of this functional decline, with their downregulation in expression with age being linked to reduced function. However, the mechanisms behind this are still not well defined and where these changes occur in the MDM differentiation process or whether these expression and functional changes can be restored have not previously been assessed. We therefore measured expression of *MYC* and *USF1* alongside these functions in monocyte precursors that were freshly isolated from human donors, showing that *MYC* expression was reduced with age, as were phagocytosis and chemotaxis. It has previously been shown that around 10% of epigenetic changes seen in cells *in vitro* are a direct result of the culture process [530]. Assessing the monocyte precursors enabled us to remove this potential confounding effect, with the monocytes being utilised as soon as they had been isolated from fresh donor blood. The finding that *MYC* was indeed downregulated with age in the monocyte precursors therefore suggests that loss of *MYC* with age in human MDMs could be occurring at the epigenetic level. Alternatively, the downregulation in *USF1* with age could occur later in the macrophage differentiation process, with downregulation of both transcription factors having compounding effects on macrophage function via different mechanisms.

Ageing is known to give rise to epigenetic changes, including DNA methylation, histone modification and chromatin remodelling and it is thought that these are strong predictors of biological age [533]. Modifications at the epigenetic level are also good targets therapeutically, with much work having gone into this intervention strategy [533]. However, epigenetic changes can occur as a result of the absence of normal *in vivo* conditions, such as signalling from other immune cells that would not be present in *in vitro* culture and these changes could give rise to cellular dysfunction [530]. It was therefore important to rule out the contribution of *in vitro* differentiation to the downregulation of *MYC* expression and reduction in function with age in human MDMs. DNA methylation involves the transfer of a methyl group to a cytosine residue to form a 5-methylcytosine. Gene repression then occurs through recruitment of proteins involved in gene repression or inhibition of transcription factor binding [534]. Alternatively, histone methylation, particularly H3K9, H3K27 and H4K20 where methyl groups are added to arginine and lysine residues, can also occur and lead to repression of genes and is a mechanism for transcriptional silencing following DNA damage [535,536]. These are potential contributors to the downregulation in expression of *MYC* with age in both MDMs and monocyte precursors and further assessment is needed to confirm whether *MYC* downregulation can be attributed to epigenetic changes.

We were also able to successfully measure phagocytosis of *Staphylococcus aureus* in monocytes isolated from young and older individuals, presenting a more biologically relevant target of phagocytosis than opsonised beads used in previous works (Chapter 5). We found phagocytosis to be reduced in older monocytes to a very similar degree as that found with the opsonised beads, suggesting that our previous findings of reduced phagocytosis in older human MDMs would also occur for biologically relevant targets.

We found that modulation of *ATP8B1* in human MDMs isolated from older individuals significantly improved phagocytosis in the resting macrophage phenotype, a function that we have previously shown to be profoundly reduced in this cell type with age. Macrophage phagocytosis has been

shown to be a key predictor of lifespan [283], so it was important to assess whether this function could be restored. ATP8B1 is regulated by USF1, allowing expression to increase when USF1 is lost with age. It has previously been associated with efferocytosis and resolution of inflammation so its change in expression with age likely effects both of these functions [499], making it a good candidate gene to modulate in order to restore macrophage function. While Yang *et al*. (2022), has previously associated increased *ATP8B1* expression with efferocytosis and M2 macrophage polarisation, the increase in *ATP8B1* expression with age that we found was in resting and inflammatory macrophage phenotypes, which may indicate that it is inhibitory to proper immune functioning, such as inflammatory polarisation in response to infection. Downregulating *ATP8B1* expression would therefore be beneficial in improving macrophage phagocytosis and overall immune function. Careful modulation of *ATP8B1* in macrophages to improve the ageing phenotype would likely therefore be required to ensure resolution still occurs. However, the finding that phagocytosis can indeed be restored in these macrophages isolated from older individuals could be key in improving macrophage phagocytosis and the early response to infection in this population.

Bolstering the inflammatory phenotype in macrophages isolated from older individuals may be important to improve immune function, as we have found previously that USF1 expression and phagocytosis undergo an age-related decline in human $M^{LPS+IFN\gamma}$ MDMs. Senescent cells and SASP components also increase chronic inflammation with no benefit to macrophage polarisation, without which inflammation cannot be resolved [509]. There is still much conflicting evidence surrounding the full effect that senescent cells have on macrophages, including much debate surrounding whether macrophages themselves undergo senescence [95,96,100] or if indeed the older cells become worse at clearing senescent cells [98,99,526]. We addressed this in part through analysis of BMDMs following *in vivo* senolytic treatment of old mice, specifically measuring expression of key transcriptional targets dysregulated with age. We assessed the expression of transcription factors *Myc* and *Usf1* as we have associated these with age-related functional decline. In addition, we assessed *Srf*, *Nfyb*, *Foxm1* and *Nfic* as we have also found these to be dysregulated with age in different macrophage populations:

Srf was found to be downregulated in murine alveolar macrophages, murine BMDMs and human MDMs, *Nfyb* and *Foxm1* were found to be downregulated in alveolar macrophages and human MDMs, and *Nfic* was found to be upregulated with age in the same populations. Finally, we assessed *Calhm6* expression as we and others have previously found this to be a good marker of inflammatory macrophage polarisation [537,538].

While the inflammatory polarised macrophages that we assessed previously had reduced *USF1* expression and reduced phagocytosis, these were not interlinked as *USF1* knockdown in the young inflammatory macrophages had no further effect on this function. We therefore looked to identify further mechanisms underlying inflammatory macrophage function and found a potential mechanism of action of senolytic treatments in improving inflammatory macrophage polarisation via *Calhm6* (Fisetin) and *Srf* (Zoledronate) upregulation. CALHM6 is a membrane ion channel in the calcium homeostasis modulator family that has been found to be important in the early immune response. It has previously been shown to be induced in activated macrophages [537], particularly when primed with IFN-γ and then exposed to pathogen derived signals [538]. CALHM6 has also been shown to be crucially involved in the activation of NK cells by macrophages to elicit an IFN-γ response [538]. We were also able to strongly induce *Calhm6* expression in response to polarisation with LPS and IFNγ for 24 hours in BMDMs isolated from young and old mice. However, importantly we found this inducibility to be significantly reduced with age, indicative of a failure of these old BMDMs to fully activate and respond to these stimuli. This could therefore be a crucial mechanism effecting the dysfunction seen in these cells with age. While Danielli *et al*. (2023), did indeed assess the effect of *Calhm6* knockout on phagocytosis, finding no dysfunction compared with control [538], its downregulation with age could very well be linked to the broader functional decline with age that we have shown and warrants further investigation. SRF or serum response factor is a transcription factor crucial for cardiac development [539]. It regulates many genes involved in cell migration, cytoskeletal organisation and metabolism and has in fact been found to be essential in orchestrating the actin cytoskeleton and the dynamic changes required for cell functions including migration and

phagocytosis [540]. This includes regulation of the expression of many cytoskeleton genes in macrophages [541]. Therefore, downregulation in SRF with age could well be effecting macrophage function through cytoskeletal mechanisms. Both CALHM6 and SRF could provide further insights into the mechanisms behind age-related macrophage dysfunction as they are both involved in key macrophage functioning and downregulated with age. That they are both upregulated following senolytic treatment provides an insight into the mechanisms behind how these therapeutics might be working to improve macrophage function and further investigation is required to better understand whether and to what extent these macrophage changes effect overall immune function and therefore healthspan in the older population.

Fisetin and Zoledronate treatment may also be improving healthspan through upregulating *Myc* expression in resting macrophages to restore overall macrophage functional capacity. We found *Myc* to be upregulated following these treatments in murine BMDMs from old mice. Additionally, we found a trend towards an upregulation in expression of *Usf1* following Zoledronate treatment in murine BMDMs and this analysis may in fact be underpowered due to one old mouse sample having high baseline *Usf1* expression. Although the mechanisms surrounding how MYC downregulation occurs to cause dysfunction in the macrophage are not yet understood, the role of MYC in ageing is being established. A recent study involving a post-natal knockout of MYC in mice found altered tissues and cellular functions corresponding with premature ageing, including a decline in cell metabolism and enriched transcriptional programs such as DNA damage response, senescence and oxidative stress [173]. Interestingly, the study by Wang et al. (2023), also identified these mice to have an increased lifespan compared to wild-type controls attributed to a reduction in cancer incidence [173], further highlighting the complexity of the role of MYC and the need for further study.

M^0 BMDMs from Zoledronate treated mice also showed statistically significant improvements in expression of *Srf*, *Nfyb* and *Foxm1* that could also be working to improve macrophage function and need further investigation. FOXM1 has a known association with senescence and has previously been

found to be reduced with age as a mechanism by which cell cycle arrest occurs [482,483]. It therefore makes sense that senolytics would increase *Foxm1* expression, should a reduction in senescent cell number be occurring. It would be interesting to further unpick whether these senolytics are working to remove the senescent cells or improve the functionality of them (i.e. switching them back on) as either could result in an increase in expression of *Foxm1*. Senolytics therefore appear to improve both inflammatory macrophage polarisation and resting macrophage transcription that should improve macrophage function including pathogen and senescent cell clearance.

While Zoledronate was assessed in healthy old mice, Fisetin was assessed in BMDMs isolated from atherosclerotic old mice, modelling an age-related disease. This enabled a broader picture of the effects of senolytics in both health and disease, giving further insights into how age-related disease might affect gene expression to make senolytics more or less effective. In this instance, the atherosclerotic mice had altered expression of *Srf*, *Nfyb* and F*oxm1*, so while Zoledronate treatment could restore the effect of age on the expression of these genes, Fisetin treatment had no effect on the altered expression caused by atherosclerosis. Contrastingly, atherosclerosis further downregulated the expression of *Calhm6* in LPS and IFNγ treated BMDMs compared with the older healthy mouse expression, which Fisetin treatment was able to restore. Further, Fisetin and Zoledronate treatment restored expression of *Myc* in the corresponding BMDMs. This could mean that *Myc* downregulation is a shared mechanism to reduce macrophage function in ageing and atherosclerosis, that senolytic treatments are able to restore, highlighting MYC as a key player in macrophage dysfunction in ageing and age-related disease that warrants further investigation.

6.5 Conclusion

In conclusion, we have uncovered some of the potential mechanisms underpinning macrophage dysfunction with age, identifying *ATP8B1* as a key target that could be modulated to restore phagocytosis. We have provided further insight into the role of transcription factors MYC and USF1 in macrophage age-related functional decline, highlighting that downregulation in *MYC* begins at the

monocyte precursor stage and that this potential epigenetic change results in reduced phagocytosis and chemotaxis. MYC downregulation is also restorable in macrophages isolated from older subjects through senolytic treatment. The functional roles of MYC and USF1 in the macrophage are still not fully understood. How senolytics act to restore MYC expression and whether this restoration will lead to improved function in these cells is not yet known. Overall, this research further suggests MYC as a key player in mechanisms underpinning macrophage age-related functional decline.

Chapter 7. General discussion and conclusions

The discussion to my thesis brings together the key findings of the whole thesis and highlights potential future work opportunities.

Declaration of Contribution

This chapter was written by myself, without contribution from others.

7.1 Key findings

The overall aim of this PhD thesis was to uncover specific transcriptional networks contributing to macrophage ageing, with a particular focus on human primary macrophage ageing and the early immune response.

In our systematic review and meta-analysis of the existing literature, presented in Chapter 3, we showed a great amount of heterogeneity pertaining to macrophage ageing, with large discrepancies resulting from different species, tissue of origin or stimulus. We highlighted a lack of work addressing how human primary macrophage populations are affected by ageing as well as that addressing the interplay between macrophages and senescence. Finally, we collated a list of well-studied genes, proteins and functional changes in macrophage ageing that would help to better phenotype these cells in future studies, with the suggestion to maintain a more standardised approach in the field to enable biomarker development.

In Chapter 4, we produced a viable pipeline for bioinformatic analysis of microarray datasets in order to assess published dataset GSE84901 as a starting point for studying primary macrophage ageing. This assessed alveolar macrophages isolated from young and aged mice, and we were able to pull out key transcriptional regulators of age-related differentially expressed genes, including *Nfyb*, *Srf*, *Foxm1*, *Usf1*, *Nfic* and *Myc*. In order to undertake gene expression analysis in primary macrophages, including murine BMDMs and human MDMs, we optimised and validated *Mau2* and *PUM1* as housekeeping genes for RT-qPCR, respectively and showed that they maintained expression in these cell types with age and *in vitro* activation with pro- and anti-inflammatory stimuli.

Our work in Chapter 5 further assessed expression of these transcription factors, demonstrating that both *MYC* and *USF1* have consistent downregulation in expression with age in murine BMDMs and human MDMs. We assessed functional changes with age in human MDMs and found that phagocytosis, migration and chemotaxis were reduced with age in the resting phenotype. These changes were also attributed to transcription factors *MYC* and *USF1* through knocking them down in

human MDMs isolated from young individuals and reproducing the functional changes seen with age. Furthermore, we found key morphological changes including reduced actin content in the MDMs isolated from older humans and highlighted downstream targets of MYC and USF1 from our RNAseq experiment that were also dysregulated with age.

Finally, in Chapter 6 we looked to further understand the mechanisms by which MYC and USF1 are driving age-related macrophage functional decline, first through assessing monocyte precursors. We found that *MYC* expression, phagocytosis and chemotaxis were all reduced with age in this cell type, highlighting a potential epigenetic cause of human MDM age-related dysfunction. We were also able to partially restore phagocytosis in the human MDMs isolated from older individuals through modulation of *ATP8B1*, a gene downstream of USF1 that was dysregulated with age. Finally, we analysed the expression of the transcription factors presented throughout this thesis in BMDMs isolated from old mice following *in vivo* senolytic treatment, alongside *Calhm6* which we have established as a marker of inflammatory macrophage polarisation. We found that old mice had significant improvements in BMDM expression of *Myc*, *Nfyb*, *Srf*, *Foxm1* and *Calhm6* following senolytic treatment that we believe may improve macrophage function and the overall immune response in older individuals.

7.2 Broader context and future work

7.2.1 The relevance of monocyte-derived macrophages in the overall immune system

In the early immune response, monocytes migrate to the injured or infected tissue, where they differentiate into macrophages, phagocytose “the insult” and release pro-inflammatory mediators to encourage further immune cell infiltration [542]. The data presented in this thesis provide evidence that in older human subjects, while macrophage number remains unchanged, macrophage dysfunction is apparent at every stage, including a reduction in chemotactic capacity of monocytes that is also present in MDMs and a reduction in phagocytic capacity of both monocytes and MDMs. Further, we showed that *Calhm6*, a key marker of the inflammatory macrophage phenotype [537,538], had reduced

expression in the older inflammatory-polarised MDMs compared with young, indicating a reduced capacity to respond to inflammatory cues. These data correlate with existing literature assessing macrophage ageing in mice, including reduced phagocytosis in alveolar macrophages [274,277], BMDMs [278] and peritoneal macrophages [279–286]. Reduced chemotaxis was found to occur in peritoneal macrophages [297,543] and white adipose tissue macrophages [296]. Overall, there is good correlation between the functional decline in these murine models and the human cells we studied, producing strong evidence that age-related monocyte-derived macrophage dysfunction may contribute to a reduced ability to mount an immune response and respond to infection.

While tissue macrophages have a role in the early immune response, particularly in those tissues that have limited monocyte infiltration such as the central nervous system [544], monocyte expansion and infiltration is key to the proper response in many tissues, including peritoneal cavity [14], liver [545] and the heart [546,547]. Therefore, we considered that assessing the age-related changes in human monocytes and MDMs would give key insights into the ageing phenotype and progression of diseases associated with these tissues, and could allow for further studies for therapeutic intervention.

The data presented on functional changes to macrophages with age were assessed only in healthy humans and could therefore be indicative of macrophage dysfunction being a critical trigger for the development and/or exacerbation of age-related diseases. However, it would be interesting to phenotype these macrophages in such diseases to further clarify whether monocyte and derived macrophage dysfunction could be used as a prognostic tool to predict onset of morbidity. Macrophage phagocytosis and chemotaxis have previously been positively correlated with achieved lifespan in a number of murine models [283,474,475]. Of course, the data presented in this thesis come from an *in vitro* setting, where environment for macrophage differentiation is tightly controlled and regulated. The strong phenotypical differences in our model system provide strong evidence that

dysfunction is occurring *in vivo*, particularly as we have also tested freshly isolated monocytes that are not being influenced by culture conditions.

Overall, the age-related phenotypical changes seen in the monocytes and MDMs from our healthy cohort suggest that the tissues that they infiltrate would see a negative effect with age, contributing to the age-associated reduction in immune response to infectious stimuli that is known to occur and exacerbating age-related diseases at the tissue level. This should be assessed further as a potential prognostic tool to predict onset of unhealthy ageing.

7.2.2 The role of transcription factors in macrophage ageing

The mechanisms behind age-related macrophage dysfunction are far from understood, particularly in the context of human primary macrophage ageing. The data presented in this thesis characterised age-related transcriptional changes for the first time in human monocyte-derived macrophages and correlated these with murine BMDM and alveolar macrophage populations. We highlight the essential role of transcription factors MYC and USF1 in proper macrophage functioning and attribute their downregulation to a reduction in phagocytosis and chemotactic migration that is seen with ageing. Further, we found *MYC* downregulation with age but not *USF1* to occur in freshly isolated monocyte precursors, highlighting potential different mechanisms underpinning the downregulation in these transcription factors. We also presented, through analysis of published datasets, that *MYC* expression is downregulated in non-classical monocytes compared with the classical population. It is known that this non-classical population expands with age, suggesting a role of this cell population expansion in the reduction of *MYC* expression seen with age, although this does not account for the age-related reduction in *USF1* expression seen in the differentiated MDMs. It would form an interesting study to profile the differentiation process of MDMs from young and older donors, including the monocyte population that is undergoing differentiation, to understand where changes in USF1 expression arise and the corresponding functional consequences. We found clear phenotypical differences in the monocytes with age that could be driving transcriptional changes

during MDM differentiation and have also highlighted an established link between MYC and USF1 transcriptional programmes [484]. Therefore, the change in expression of *MYC* found at the monocyte level could also be contributing to the downregulation in *USF1* expression seen later in the MDMs.

MYC has strong associations with ageing, particularly in the context of cancer, where MYC deregulation is attributed to the initiation and progression of numerous malignancies [161,162,481]. It has previously been shown that MYC knockout mice have reduced incidence of cancer and age prematurely [173], fitting in with our findings that MYC expression goes down with age and contributes to functional decline in monocyte and macrophage populations. It is a well-considered phenomenon that cancer and ageing mechanisms are linked, often becoming deregulated in a divergent manner [548]. MYC overexpression in cancer has been linked to suppression of senescence pathways [169,171], cell cycle progression [549,550] and increased energy metabolism [551–553], all of which are opposingly deregulated mechanisms, established to occur in ageing [548]. That MYC is downregulated with age in macrophages therefore appears to be inevitable and it would be interesting to assess whether overexpression of MYC in MDMs isolated from older individuals would improve their function or eventually push these cells towards a tumorigenic phenotype.

We have considered the likelihood of epigenetic regulation in contributing to MYC deregulation with age in the macrophage. A growing body of evidence is suggesting that epigenetic mechanisms contribute to proper activation of the macrophage [554–556] as well as trained immunity in these cells [557,558], and it is well established that changes to the epigenome occur with increasing age, referred to as epigenetic drift [59,200]. MYC has also previously been associated with epigenetic changes, again in the context of cancer, where MYC hyperexpression could be as a result of MYC promoter undergoing epigenetic modifications including DNA hypomethylation [559]. In addition, histone modifications have been associated with MYC activity; MYC has a very short half-life, and is normally degraded through ubiquitination [160]. This process is hijacked in certain tumours to stabilise MYC expression [173]. Whether these mechanisms are inversely deregulated in ageing has not yet been studied. Although ageing,

MYC expression and epigenetic regulation have not been assessed in conjunction in the macrophage, this would form an interesting future study to better understand how MYC is epigenetically regulated and the mechanisms underpinning MYC downregulation with age in the macrophage.

USF1 also has known associations with cancer, aiding in cell growth and glycolysis, a principally inflammatory function in the macrophage [187–190,560], as well as age-related diseases of a metabolic nature, such as atherosclerosis where it is known that USF1 contributes to deregulation of lipid metabolism [178,182–184]. It has also been found that USF1 silencing in macrophages alleviates atherosclerotic burden through reduced secretion of pro-inflammatory cytokines [191]. That we found USF1 to be downregulated with age in our LPS and IFN-γ-activated MDMs could therefore be indicative of a reduced ability to mount an inflammatory response, also fitting with the reduction in inflammatory marker *Calhm6* that we found with age. Further, MYC has also been shown to be required for glycolytic flux in the initiation of inflammatory macrophage polarisation [561] and the lack of its expression in the resting MDMs from older individuals may be further perpetuating this. It would be interesting to look at the metabolic characteristics and supernatant of these cells to assess their capacity to uptake and breakdown glucose, and secrete pro-inflammatory cytokines following activation.

These data therefore shed more light on the roles of MYC and USF1 in ageing and newly attribute both transcription factors to the ageing macrophage phenotype. Although their known functions give some explanation as to why MYC and USF1 expression become dysregulated with age to cause macrophage dysfunction, much work is needed to understand the full transcriptional networks underpinning age-related macrophage functional decline. It would be interesting to assess the differentiation process of MDMs from monocytes to better understand the stage at which transcriptional networks are affected, as well as the epigenome for age-associated changes to marks in the promoters of MYC, USF1 and their target genes. Finally, future studies could focus on overexpression of *MYC* and *USF1* and their downstream targets in macrophages isolated from older

subjects to further understand the contribution of these transcriptional networks to macrophage function.

7.2.3 Opportunities for therapeutic interventions targeting the negative effects of macrophage ageing

The data presented in this thesis provide a strong platform for exploring interventions to rejuvenate the macrophage phenotype. Through identification of downstream targets of USF1 with age-related deregulated expression, we were able to uncover a role of ATP8B1 in macrophage phagocytosis, finding that removing the age-related upregulation in *ATP8B1* expression was sufficient to improve phagocytosis in resting MDMs isolated from older human subjects. This was not the case in the MDMs polarised towards an inflammatory phenotype, where *ATP8B1* was also sufficiently silenced but no improvement in phagocytosis was seen. Of course, the young inflammatory-primed MDMs also had a reduction in phagocytic ability and we postulate that the macrophages isolated from older individuals display a more pro-inflammatory phenotype already, through the reduction in functional activity in the resting cell population to a degree similar to that seen in our young inflammatory-polarised macrophage population. However, that these inflammatory-polarised macrophages have reduced phagocytosis compared with the resting population is also intriguing. Much of the published literature posits that phagocytosis is primarily a function of inflammatory-primed macrophages [542] and this leads us to believe that, like M2 populations, there may be a spectrum of M1 phenotypes. It would be interesting to further assess this in future studies, perhaps focusing on the phenotype directly after phagocytic stimuli are added.

That we were able to restore phagocytosis through modulation of gene expression in MDMs isolated from older individuals is an exciting prospect. Therapeutically targeting specific genes is a method already used in the treatment of cancers [562]. With strong interlinks between cancer and ageing [548], there is potential that these therapeutic mechanisms could be utilised to improve ageing phenotypes and prevent or delay onset of age-related diseases. Additionally, there are also cancer therapies able to specifically target tumour-associated macrophages, including causing their depletion,

reprogramming them away from M2-like tumour suppressor state and towards an M1 phagocytic state or preventing their recruitment [110]. It would be interesting to further assess these in the context of ageing, as to whether these mechanisms could be exploited to target the phenotypically older macrophages that we have uncovered.

MYC has long been an attractive target in cancer therapeutics, with up to 70% of cancers involving deregulated MYC expression [563]. However, this has so far been challenging as MYC cannot be targeted with antibodies due to it being found mainly in the nucleus and has no specific active site for targeting by small molecules [151,564]. Should MYC undergo epigenetic modifications as a result of ageing (as described above), this could be an alternative mechanism by which MYC could be therapeutically targeted. In addition, MYC also has established links with mammalian target of rapamycin (mTOR), whereby mTOR regulates MYC activity [565,566]. The contribution of mTOR to the ageing phenotype is well known, with inhibition of mTOR signalling increasing lifespan through diminishing its negative effects on nutrient sensing [567]. We further established this link between mTOR and MYC in our MYC knockdown MDMs, where mTOR signalling was found to be significantly enriched in these cells compared to control MDMs, indicative that this pathway undergoes MYC-driven deregulation with age. This may provide some explanation as to the mechanism behind our finding that senolytic treatments selectively restored MYC expression in older murine BMDMs.

The data that we presented assessed the effect of senolytic treatment on transcription for the first time in primary murine macrophages and showed that these treatments were collectively able to restore expression of many age-related deregulated genes, namely *Myc*, *Calhm6*, *Srf*, *Nfyb* and *Foxm1*. However, it has not yet been possible to ascertain whether these improvements in gene expression would correspond with increased macrophage function. In addition, while it is known that macrophages have a key role in clearance of senescent cells [99], which increase in number with age [77,510,511], it has not yet been established whether and to what extent this function is reduced with age in these primary macrophage populations. Senolytics are able to selectively kill senescent cells

[505,519,523] and a mechanism of action in doing this could be in improving the function of macrophages, so it will be important to explore this in future studies.

Overall, the improvement in phagocytosis that we have shown through modulation of specific genes, and the increase in expression of MYC and other key transcriptional targets following senolytic treatment, gives strong evidence for therapeutic opportunities to improve the ageing phenotype of macrophages. The existence of senolytic therapies that target ageing mechanisms, as well as cancer therapies that specifically target macrophages and these key transcriptional targets, should form a starting point for future work in this field. Overall, the aim of these therapies should be to target and improve function of primary macrophages, therefore improving the immune response to infection and the tissue environments that these macrophages infiltrate, so that older individuals can live longer in health, free from age-related diseases.

7.3 Concluding remarks

The features of unhealthy ageing can promote the development of many age-related diseases, alongside a decreased ability to fight infection and repair damage. Macrophages have a well-established involvement in the initiation of the immune response to infection, as well as the progression of many of these disease processes. It is apparent from the data presented in this thesis that primary human macrophages, such as monocyte-derived macrophages, undergo a marked reduction in function with age. Moreover, there is a clear role of transcription factors MYC and USF1 in age-related macrophage dysfunction. We have attempted to restore macrophage function in older individuals through modulation of dysregulated target genes and senolytic treatments, and while improvements have been found in MYC expression and phagocytic capacity, further studies are required to truly understand the mechanisms behind primary macrophage dysfunction with age and make the targeting of primary macrophages to improve overall immune function with age a more viable therapy against unhealthy ageing. In summary, these data show that transcription factors MYC and USF1 are key regulators in age-related macrophage functional decline, potentially contributing to

an overall decline in immune function and progression of age-related diseases. These aged cells could be viable targets for future therapeutics in the field, and future work should focus on these key regulators and restoration of function in this cell type.

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Chapter 8. Supporting information

The supporting information chapter to my thesis contains supplementary information relevant to each chapter, including that which formed part of the two publications.

Declaration of contributions

Supplementary figures 8.1.1-8.1.2 were produced by ethics leads. Supplementary tables 8.2.1-8.2.6 and 8.2.9-8.2.10 are output files generated from running MAIC code by Hew Phipps. Supplementary tables 8.4.2-8.4.3 were produced by Veryan Codd. Supplementary figure 8.4.5 was produced by Joshua V. Kimble. Martha Clements assisted in running aPCRs to generate the data in Supplementary figure 8.4.7 under my supervision. All additional data included were obtained, analysed and interpreted by myself, without contribution from others.

8.1 Supplementary material for Chapter 2 “General Materials and Methods”

Supplementary figure 8.1.1 Participant information sheet

Participant Information Sheet

1. Research Project Title:

Studies of the function of platelets and leukocytes in blood and plasma from healthy volunteers.

2. Invitation paragraph

You are being asked to provide a sample of blood for research being conducted by members of the Department of Infection, Immunity and Cardiovascular Disease on behalf of the University of Sheffield. Before you decide it is important for you to understand why the research is being done, what it will involve and the possible benefits, risks and discomforts. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

3. What is the project’s purpose?

Cardiovascular disease is known to involve injury to the wall of blood vessels. Inflammation and clot formation play a key role in the development of these diseases; cells in the blood known as platelets, white blood cells and stem cells play a crucial role in these processes and this research aims to improve our understanding of their function and to study the influences of substances on this, in order to help the development of new treatments. In order to investigate this we use cells and platelets isolated from human blood and study the effects of different drugs on the way they act in response to stimuli such as those involved in inflammation and clotting. We also investigate the role of these cells in the development of heart disease using experiments to look at how inflammatory these cells are, what substances can make them move and how one cell type can influence the function of another. It is important to investigate these things using blood from people who do not have active heart disease so that we can compare these results to those we get from patients.

4. Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep (and be asked to sign a consent form) and you can still withdraw at any time without any negative consequences. You do not have to give a reason. If you wish to withdraw from the research, please contact the Principle Investigator, Dr Victoria Ridger.

5. What will happen to me if I take part? What do I have to do?

We will check that you are suitable for this study and have not had any recent serious illnesses or anaemia, or received any medication recently that is likely to affect the function of platelets or leukocytes, including anti-inflammatory painkillers. A trained phlebotomist will take a blood sample from a vein in your arm (a maximum of 90 mL or about 5 tablespoonfuls) using a syringe and needle. The blood will only be used for studying platelet, leukocyte and stem cell function. You should not donate more than 200 mL blood in any one-month period so you should tell us if you have donated blood in the last month. The blood testing is not designed to detect any blood abnormalities but if we think there *might* be a problem with your blood cell counts we will suggest you see your general practitioner for a formal blood cell count. All computers where data is stored will be password protected and mobile devices (including laptops) will be encrypted. Anonymisation procedures will not be used, as the data will not be sensitive in nature. However the participants' log and the data will be held separately and only the investigators and their associated staff will analyse and have access to the data. The participants' log will be held for a period of up to 10 years.

6. What are the possible disadvantages and risks of taking part?

Taking blood with a syringe and needle from a vein in the arm can cause some pain and bruising.

7. What are the possible benefits of taking part?

You will not obtain any benefit from taking part in this research. We hope that the information obtained from this research will be of benefit to patients in the future.

8. Will my taking part in this project be kept confidential?

All the information that we collect about you (name, age, medication and sex) during the course of the research will be kept strictly confidential and will only be accessible to members of the research team. You will not be able to be identified in any reports or publications unless you have given your explicit consent for this. If you agree to us sharing the information you provide with other researchers (e.g. by making it available in a data archive) then your personal details will not be included unless you explicitly request this.

9. What is the legal basis for processing my personal data?

According to data protection legislation, we are required to inform you that the legal basis we are applying in order to process your personal data is that 'processing is necessary for the performance of a task carried out in the public interest' (Article 6(1)(e)). Further information can be found in the University's Privacy Notice https://www.sheffield.ac.uk/govern/data-protection/privacy/general.

10. What will happen to the data collected, and the results of the research project?

The results of the research may be published in the scientific literature but your identity will not be revealed. Due to the nature of this research it is very likely that other researchers may find the data collected to be useful in answering future research questions. We will ask for your explicit consent for your data to be shared in this way.

11. Who is organising and funding the research?

This research is funded by both commercial and charitable institutions. None of the staff involved in this research study will be paid for including you in the study.

12. Who is the Data Controller?

The University of Sheffield will act as the Data Controller for this study. This means that the University is responsible for looking after your information and using it properly.

13. Who has ethically reviewed the project?

This project has been ethically approved via the University of Sheffield's Ethics Review Procedure, as administered by The Medical School. The University's Research Ethics Committee monitors the application and delivery of the University's Ethics Review Procedure across the University.

14. What if something goes wrong and I wish to complain about the research?

If you have any complaints about the project in the first instance you can contact the Principle Investigator (contact details below). If you feel your complaint has not been handled to your satisfaction you can contact the Head of Department for Infection, Immunity and Cardiovascular Disease, Professor Allan Pacey, who will then escalate the complaint through the appropriate channels. If the complaint relates to how your personal data has been handled, information about how to raise a complaint can be found in the University's Privacy Notice: https://www.sheffield.ac.uk/govern/data-protection/privacy/general.

15. Contact for further information

Principle Investigator:
Dr Victoria Ridger
Senior Lecturer in Vascular Biology
Department of Infection, Immunity & Cardiovascular Disease
University of Sheffield Medical School
Beech Hill Road
Sheffield S10 2RX
Tel: 01142159549

Co-Investigator:
Dr Heather Wilson
Department of Infection, Immunity & Cardiovascular Disease
University of Sheffield Medical School
Beech Hill Road
Sheffield S10 2RX
Tel: 01142159560

Alternative Contact:
Dr Sam McCaughran
Department of Infection, Immunity & Cardiovascular Disease
University of Sheffield Medical School
Beech Hill Road
Sheffield S10 2RX
Tel: 01142159581

If you decide to take part in this research, you will be given a copy of this Information Sheet to keep.

Thank you for your help with this research.

Supplementary figure 8.1.2 Participant consent for

Studies of the Function of Platelets and Leukocytes in Blood and Plasma from Healthy Volunteers

Consent Form

Please add your initials to the appropriate boxes	**Initials**
Taking part in the project	
I have read and understood the project information sheet dated January 2023 or the project has been fully explained to me. (If you will answer No to this question please do not proceed with this consent form until you are fully aware of what your participation in the project will mean.)	
I have been given the opportunity to ask questions about the project.	
I agree to take part in the project. I understand that taking part in the project will include having blood taken.	
I understand that my taking part is voluntary and that I can withdraw from the study at any time; I do not have to give any reasons for why I no longer want to take part and there will be no adverse consequences if I choose to withdraw.	
How my information will be used during and after the project	
I understand my name, age, gender and current medication are the only personal details that will be recorded. These will be confidentially and securely stored by the investigators and will be shared anonymously with researchers working within the project.	
I understand and agree that other authorised researchers will have access to this anonymised data only if they agree to preserve the confidentiality of the information as requested in this form.	
I understand and agree that other authorised researchers may use my anonymised data in publications, reports, web pages, and other research outputs, only if they agree to preserve the confidentiality of the information as requested in this form.	
So that the information you provide can be used legally by the researchers	
I agree to assign the copyright I hold in any materials generated as part of this project to The University of Sheffield.	

Name of participant [printed] Signature Date

Name of researcher [printed] Signature Date

Project contact details for further information:
Principle Investigator: Dr Victoria Ridger, IICD, University of Sheffield Tel: 01142159549

Co-Investigator: Dr Heather Wilson, IICD, University of Sheffield. Tel: 01142159560.

Head of Department: Professor Allan Pacey

Save 2 copies of the consent form: 1 paper copy for the participant, 1 copy for the research data file

Supplementary table 8.1.1 Oligonucleotide sequences for RT-qPCR of human samples

Target gene	Forward primer (5'-to-3')	Reverse primer (5'-to-3')
PUM1	GCATTTGGACAAGGTCTGGCAG	GCTACAAGTCGAACAGGAGCTC
MYC	AGAGTTTCATCTGCGACCCG	GAAGCCGCTCCACATACAGT
USF1	GCTCTATGGAGAGCACCAAGTC	AGACAAGCGGTGGTTACTCTGC
NFYB	GGAATTGGTGGAGCAGTCACAG	CCGTCTGTGGTTATTAAGCCAGC
SRF	TCACCTACCAGGTGTCGGAGTC	GTGCTGTTTGGATGGTGGAGGT
FOXM1	TCTGCCAATGGCAAGGTCTCCT	CTGGATTCGGTCGTTTCTGCTG
NFIC	TGGCGGCGATTACTACACTTCG	GGCTGTTGAATGGTGACTTGTCC
ATP8B1	CTTCTTGCTCGCAGTTTGCCAC	GCCAAAGTTCCTGGCAGCGTTT
SFRP4	CTATGACCGTGGCGTGTGCATT	GCTTAGGCGTTTACAGTCAACATC
GDF1	GTCACCCTGCAACCGTGCCAC	AGGTCGAAGACGACTGTCCACT
STAB2	ACTGGCTCCTTACCAAACCTGC	GAGCAAACACTGTGTAGGCATCG
MMP8	CAACCTACTGGACCAAGCACAC	TGTAGCTGAGGATGCCTTCTCC
PLOD2	GACAGCGTTCTCTTCGTCCTCA	CTCCAGCCTTTTCGTGGTGACT
ITGB6	TCTCCTGCGTGAGACACAAAGG	GAGCACTCCATCTTCAGAGACG
ITGA2B	CTGTCCAGCTACTGGTGCAAGA	ATGTTGTGCCCAGTGGCTCCAA
CDH8	AACGCTGGCAACACCACTTGAC	GCGTTGTCATTGACATCCAGCAC
ADAMTS9	CCATTCAGAGGTGCAGTGAGTTC	ACCAGACCTGGCGGTGCTTATG
AJUBA	AGCCACCAGGTCCTTTCGTTCC	GGCATTGCTCTGCCCATAGATG
CDH19	ATTGGTCAGCCAGGAGCGTTGT	GCAGATTCAGAGACAGTCAAGCG
ANGPTL3	CCTGAAACTCCAGAACACCCAG	TTCCACGGTCTGGAGAAGGTCT
PCDHGB4	CAACACGGACTGGCGTTTCTCT	GATCATGGCTTGCAGCATCTCTG
DAB2IP	TCATCGCCAAGGTCACCCAGAA	CGCTGCATGTTGGTCCACTCAT
GLI1	AGCCTTCAGCAATGCCAGTGAC	GTCAGGACCATGCACTGTCTTG
PPARGC1A	CCAAAGGATGCGCTCTCGTTCA	CGGTGTCTGTAGTGGCTTGACT
SLC6A4	TCACAGTGCTCGGTTACATGGC	GAAAGTGGACGCTGGCATGTTG
SLIT2	CAGAGCTTCAGCAACATGACCC	GAAAGCACCTTCAGGCACAACAG
SLC34A2	CGTGTGTGCATGGGTCAAAG	CAATCTTGCTGCACGGCTAC
ELOVL6	CCATCCAATGGATGCAGGAAAAC	CCAGAGCACTAATGGCTTCCTC
CDC20	CGGAAGACCTGCCGTTACATTC	CAGAGCTTGCACTCCACAGGTA
MMP11	GAGAAGACGGACCTCACCTACA	CTCAGTAAAGGTGAGTGGCGTC
MMP13	GCACTTCCCACAGTGCCTAT	AGTTCTTCCCTTGATGGCCG
TLN2	CAAGGAAGTCGCCAACAGCACT	TTGAGGCGAACGCTGTCAGGTT
ITGB1BP2	GACCACACTGTGCTGAGAAGCT	AGCAGCTTCAGAGGCAACTCTG
WNT11	CTGTGAAGGACTCGGAACTCGT	AGCTGTCGCTTCCGTTGGATGT
GPR32	CTGGGGCCCTTAGCAATCAT	AGATGGACCAACAGCACCAC
CCR2	GGGATGACTCACTGCTGCAT	TGCTTTCGGAAGAACACCGA

Supplementary table 8.1.2 Oligonucleotide sequences for RT-qPCR of mouse samples

Target gene	Forward primer (5'-to-3')	Reverse primer (5'-to-3')
Mau2	TGGTTACCTGGAGAAGGCACAG	ATGCTCCAGCAGGATCACTTGG
Myc	TTGAAGGCTGGATTTCCTTTGGGC	TCGTCGCAGATGAAATAGGGCTGT
Usf1	CGTCTTCCGAACTGAGAATGGG	CTGGGTCATAGACTGAGTGGCA
Nfyb	ACCAAACAGCCGATTGGAGA	CTAGCTGGGAGGCATCTGTG
Srf	CACCTACCAGGTGTCGGAAT	GTCTGGATTGTGGAGGTGGT
Foxm1	GTCTCCTTCTGGACCATTCACC	GCTCAGGATTGGGTCGTTTCTG
Nfic	TGACTCAGTAAGTTCGGCGG	GTTGAACCAGGTGTAGGCGA
Ccr2	GCTGTGTTTGCCTCTCTACCAG	CAAGTAGAGGCAGGATCAGGCT

Supplementary table 8.1.3 R packages used in microarray analysis pipeline

Package	Description	Functions	Reference
GEOquery	A bridge between GEO and BioConductor packages	'getGEO()'	[374]
dplyr	A set of verbs to manipulate data and data frames	'select()' 'rename()' 'mutate()' 'filter()'	[375]
pheatmap	Allows the construction of clustered heatmaps	'pheatmap()'	[376]
ggplot2	A system of mapping variables to aesthetics for plotting graphs	'ggplot()' 'aes()' 'geom_point()' 'ggtitle()'	[377]
ggrepel	Repels overlapping text and labels for "ggplot2" package	'geom_text_repel()'	[378]
ggfortify	Unified plotting tool for commonly used statistics using "ggplot2"	'autoplot()'	[379,380]
readr	Enables parsing of flat files into data frames, reading of rectangular data and writing of rectangular data to a specific path	'write_csv()'	[381]
limma	Enables analysis of gene expression microarray data	'model.matrix()' 'lmFit()' 'makeContrasts()' 'contrasts.fit()' 'eBayes()' 'arrayWeights()'	[382]
viridis	A series of colour maps to improve graph readability	'plasma()'	[383]
enrichR	An interface to the Enrichr database for gene list enrichment analysis	'enrichr()' 'plotEnrich()'	[244–246,384]

8.2 Supplementary material for Chapter 3 “Markers of the ageing macrophage: a systematic review and meta-analysis”

Supplementary table 8.2.1 Input table for MAIC

Method	Publication	Entity type	Entity names
qPCR	[1a]	Gene	CD206, FIZZ1, TFGB, TNF, IL6, NOS2, FOXO3
Western Blot	[1b]	Protein	FOXO3
RNAseq	[9a]	Gene	IL4RA, IL13RA1, ARG1, RETNLA, IL13RA2, NOS1, NOS2
Flow Cytometry	[9b]	Protein	MHCII, Ki67, FIZZ1
Flow Cytometry	[11a]	Protein	CD11b, CD80
ELISA	[11b]	Protein	IL1B, IL6, TNF, IL10
qPCR	[11c]	Gene	ARG1, MRC1, MSR1, P53, P21, P16
ELISA	[15a]	Protein	TNF, IL1, IL12
ELISA	[15b]	Protein	TNF, IL1, IL12
ELISA	[15c]	Protein	TNF, IL1, IL12
qPCR	[24]	Gene	ARG1, NOS2, SLC7A2
qPCR	[32]	Gene	TNF, NOS2, ARG1, CD206, IL1RA
Flow Cytometry	[33a]	Protein	CD36
qPCR	[33b]	Gene	Fads2, Fdft1, Fdps, Scd1, Cyp11a1, Cyp27a1, Hadhb, Nr0b2, Nr1h3, Lta4h, Alox5ap, Alka1, Apof, Slc16a6, Slc27a1, Slc27a3, Stard4, Cxcl16, Olr1, Pcsk9, Stab2, p16, Fads3, Hmgcs2, Prkaa2, Cyp7b1, Lpl, Tbxas1, Adfp, Alox15, Fabp4
Transcriptomic	[33c]	Gene	Scd2, Dhcr24, Acsl3, Ceacam19, Idi1, Clec5a, Mir15a, Sgle, Cyp51, Csprs, Gimap4, Cd79a, Ly6d, Ms4a1, Cd79b, Mzb1, Cxcl13, Plac8, Ly6a
Mass Spec	[41]	Protein	QPCT, Granulin, ATP6AP1, LAMP1, PPT1, IL-41, CTSZ, GGH, CTSL1, CCR9, C1QA, TCN2, C1QB, LRP1, B2M, CREG1, CSF1R, CTSB, H2KW28, C1QC, SDF4, STAB1, SGP1A, PAFAH, HEXA, CST3, LGALS3BP, PRRG2, FBN1, COL1A1, POSTN, PAI1, COL1A2
ELISA	[41]	Protein	LRP1
Northern Blot	[55]	Gene	NOS2
Assay	[61]	Protein	PGE2, COX
Assay	[71]	Protein	PGE2, COX
Assay	[85]	Protein	TNF
Assay	[96]	Protein	TNF, ASGM1, IA
ELISA	[99]	Protein	IL1, TNF
Flow Cytometry	[110a]	Protein	CCR7, CD163
Assay	[110b]	Protein	ARG1, NOS2
ELISA	[110c]	Protein	IL1B, IL6, TGFB, IL10
qPCR	[127a]	Gene	CCL2, IFNB, TNF, IL12p40, IL10, MCSF, CD11b, COX2, MHCIIAa, CIITA, IRF1, IFNg, TLR1, IL1B, GMCSF, CD11c, CD206, TLR2, TLR4, TLR6
Flow Cytometry	[127b]	Protein	CD11b, CD11c, CD206, CD64, MHCIIA
Flow Cytometry	[129a]	Protein	CD14, TLR4
ELISA	[129b]	Protein	TNF, IL10
Assay	[129c]	Protein	ARG1, iNOS
Assay	[133a]	Protein	NFKB, p65, PGE2, COX2, CREB, AP-1, IKBa

Western Blot	[133b]	Gene	COX2, NOS2
Assay	[134]	Protein	ERK, JNK, P38, PGE2, COX2
Western Blot	[137]	Protein	STAT1B, STAT1A, STAT1Bp, STAT1Ap
qPCR	[137]	Gene	STAT1
Northern Blot	[147]	Gene	hsp70
ELISA	[149]	Protein	TNF, IL6
Western Blot	[150a]	Protein	TNF, RACK1, CD14, PKCA, PKCBII
qPCR	[150b]	Gene	TNF
ELISA	[156a]	Protein	TNF, IL1RA, IL6, IL10, CCL2, CCL3, CCL4, CCL5, CXCL1, CXCL2, CXCL10, TIMP1, CXCL13, GCSF, GMCSF, CCL1, CD54, IFNY, IL1A, IL1B, IL16, IL17, IL23, IL27, IP10, MCSF, CXCL12, TREM1, IL2, IL3, IL5, IL7, IL13, CXCL11, CCL1, CXCL9, CCL17, C5A
RNAseq	[156b]	Gene	Gm42427, Wdfy1, Tmem181bp, Dynlt1b, Cd59a, Sel1l3, Tmem181bp, Gm42031, Gm43305, Xlr, Rab4a, Nbea, AC157515.1, Gm42742, C2, Cfap69, Steap2, Stc1, Vegfc, Steap1, Fgf23, Hist1h4n, Gm10359, Saa1, Slc6a14, 1700028P14P, Lhb, mt-Nd4l, Gm14176, Gm5776, Emx2, Gm7609, Gm15446, Igkc, Gm16867, Hist1h4m, Ighg2b, Igkv10-96, Gm27177, Gm43802, Ifi208, AC125149.1, AC174776.1, Igkv12-46, Ighv1-18, Ighg2c, Ifi208, Ifnb1, Gm11189, Cdv3-ps
RNAseq	[157]	Gene	Vsig4, Cxcl13, Cxcl5, Fcna, Marco, Tmem132e, Saa3, C6, H2-M9, Ccl8, Lif, Cd209f, Cd209d, Tacr3, Gm16548, Csmd1, Cd209g, Inhba, Gpr176, Cxcl1, Cxcl2, Il6, Ccl2
qPCR	[158a]	Gene	Cox2, RXRa, Abca1, Abcg1, LXRa, LXRb
Western Blot	[158b]	Protein	COX2, RXRa
ELISA	[158c]	Protein	PGE2
qPCR	[167]	Gene	Cd11b, Cd206, TGFB
Proteomic	[183]	Protein	MYO1F, RARS, DPYS11, PIK3AP1, HUWE1, TPP1, NAPRT, PPAT, SF3B2, MARS, PGAM2, ACTG1, F1NC, CAPG, TUBA1A, ACTA1, YWHAZ, CAP1, ANXA5, F1NA, CTSD, H2-D1, HSC70, TIP-B1, FGH, GM2A, GPX1, ANXA6, CTSB, PRX, MDH2, ANXA2, UBA1, PGK, LCP1, ANXA1, HSP90AB1, ALDH-2, GRP78, HA1, LYZ, MOM2, VAT-1, RP14, ACTC1, CCT3, MELA, HSP70-1B, ARG1, DESMOYOKIN, GSTP1, H2BA, EUK, SET, SLC25A5, PSMD3, UBE2Q1, KRT28, EEF2
Immuno Cytochemistry	[186a]	Protein	iNOS
qPCR	[186b]	Gene	Nos2
qPCR	[188a]	Gene	TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9
Flow Cytometry	[188b]	Protein	TLR4
ELISA	[188c]	Protein	TNF, IL6
qPCR	[189a]	Gene	TNF, Nos2, CD40, CD11b, MHCII, Arg1, Mrc1
Flow Cytometry	[189b]	Protein	IFNyR1, CD11b, CD45
ELISA	[189c]	Protein	TNF
qPCR	[190a]	Gene	FoxO3, Cd206, TgfB, Fizz1, Nos2, Tnf, Il6
Western Blot	[190b]	Protein	FOXO3
Flow Cytometry	[194a]	Protein	IA
Western Blot	[194b]	Protein	IA
Northern Blot	[194c]	Gene	IaB, IeB, CIITA

ELISA	[196a]	Protein	TNF, IL6, P38, JNK
Western Blot	[196b]	Protein	P38, JNK
Flow Cytometry	[196c]	Protein	TLR4
Flow Cytometry	[197]	Protein	TNF, IFNy, IL12, IL6, CCL2, IL10
ELISA	[198a]	Protein	TNF
Assay	[198b]	Protein	PGI2
qPCR	[198c]	Gene	TNF
ELISA	[201a]	Protein	TNF, IL1B, IL6, IL12
Flow Cytometry	[201b]	Protein	TLR4, IL-6Ra
RNAseq	[206a]	Gene	Gm16867, Hist1h4m, 1700112E06Rik, Gm15446, Wdfy1, Tmem181b-ps, Dynlt1b, Marco, Csf2, Smo, Efcab7, Ptch1
Western Blot	[206b]	Protein	WDFY1
ELISA	[206c]	Protein	CXCL13, C5a, G-CSF, GM-CSF, CCL1, IFNy, IL1a, IL1B, IL3, IL4, IL7, IL13, IL18, IL17, CXCL10, CXCL11, CXCL9, CCL3, CXCL2, CXCL12, TIMP1, TREM1, CCL4, CD54, CCL5, TNF, IL6, IL1ra, MCSF, CCL4, IL23, IL27
RNAseq	[207]	Gene	Vsig4, Cxcl13, Cxcl5, Fcna, Marco, Tmem132e, Saa3, C6, H2-M9, Ccl8, Lif, Cd209f, Cd209d, Tacr3, Gm16548, Csmd1, Cd209g, Inhba, Gpr176, Cxcl1, Cxcl2, Il6, Ccl2
Assay	[211a]	Protein	iNOS, ARG1
ELISA	[211b]	Protein	IL1a, IL10, TNF
Assay	[212]	Protein	TNF
qPCR	[216]	Gene	Reverba
ELISA	[226]	Protein	IL6
Flow Cytometry	[230a]	Protein	TLR4, Marco
qPCR	[230b]	Gene	P16inka
Immunostaining	[230c]	Protein	BGalactosidase
Assay	[233a]	Protein	ARG1, iNOS
Flow Cytometry	[233b]	Protein	CD86, CD40, CD80, CD206
ELISA	[245a]	Protein	TNF, IL1, IL6
Assay	[245b]	Protein	TNF, IL1, IL6
ELISA	[249a]	Protein	TNF, IL6, IL10
Flow Cytometry	[249b]	Protein	TLR2, TLR4
Western Blot	[249c]	Protein	p38, MAPK-APK-2
RNAseq	[253]	Gene	Gm10260, Lilr4b, Hmox1, Oser1, Cd274, Ccl5, Tiparp, Clec4d, Gdf15, Cxcl2, H2-Eb1, Gm10036, Ly6e, Coro1a
Flow Cytometry	[254a]	Protein	Marco, TLR4
qPCR	[254b]	Gene	Rac1, N-WASP
Western Blot	[254c]	Protein	RAC1, N-WASP
Western Blot	[260a]	Protein	p62, ATG5, LC3-II
ELISA	[260b]	Protein	TNF, IL1B, IL6
Immuno flourescence	[260c]	Protein	iNOS, CD206
qPCR	[260d]	Gene	Tnf, Il1b, Il6, iNOS, Mcp1, Mrc1, Arg1
Microarray	[261]	Gene	miR-181c, miR-3068, miR-330, miR-5135, miR-10a, miR720, miR-29c, miR-714, miR-150, miR-195, miR-3273, miR-145, let-7d, miR-1247
Immuno flourescence	[264]	Protein	TNF

ELISA	[268]	Protein	IL1B, IL6, TNF
qPCR	[274a]	Gene	miR-142-3p
ELISA	[274b]	Protein	IL6
qPCR	[277a]	Gene	Cox2, miR-101b, miR26b
ELISA	[277b]	Protein	PGE2
Western Blot	[280]	Protein	A20, CYLD
Western Blot	[281]	Protein	COX2, PGE2, COX1
qPCR	[290a]	Gene	ABCA1, ABCG1, TNF, IL1B, PTGS2, CCL2, MMP9, TGFB, IL10, CD163, miR-33
Western Blot	[290b]	Protein	ABCA1, ABCG1
qPCR	[291]	Gene	TIMD4, Stabilin2, IL10, F4/80, CD31, CD36, ARG1, TNF, IL6, IL1B, IL4, CD68
RNAseq	[301a]	Gene	miR10a, miR10b, miR130a, miR142, miR19a, miR19b-1, miR19b-2, miR210, miR29b-1, miR301, miR301b, miR340, miR106a, miR125a, miR125b-1, miR125b-2, miR199b, miR24-1, miR24-2, miR30a, miR30d, miR30e, miR331, miR339, miR99b, miR103-1, miR107, miR126, miR140, miR148a, miR150, miR15b, miR181a-1, miR181b-2, miR185, miR191, miR192, miR1944, miR23a, miR25, miR26b, miR532, miR92-2, miR92b, miRlet7i, miR100, miR139, miR181a-2, miR222, miR23b, miR26a-1, miR26a-2, miR27a, miR27b, miR30b, miR30c-1, miR30c-2, miR324, miR484, miR744, miR99a, miR101a, miR101b, miR103-2, miR16-1, miR16-2, miR34a, miR425, miR451, miR7-1, miR7-2, miR7b, miR93, miRete.bSep07, miRlet7c-1, miRlet7d, miR155, miR1839, miR199a-1, miR199b, miR20a, miR218-1, miR221, miR31, miR322, miRlet7a-1, miRlet7a-2, miRlet7c-2, miRlet7e, miRlet7f-1, mIrlet7f-2, miRlet7g, miR29a, miR146a, miR18a, miR146b, miR22, miR423, miR15a
qPCR	[301b]	Gene	miR146b, miR22, miR423, miR15a, miR29a, miR146a, miR18a
Microarray	[302]	Gene	miR143, miR145, miR150, miR195, miR720
Immuno flourescence	[306]	Protein	FcuR, Fcy2AR, Fcy2BR, FceR, Fcy1r, FcaR, Ia, MAC1
qPCR	[308]	Gene	Tnf, Il1b, Il6, Il12/p40, TgfB, Il10
qPCR	[310]	Gene	miR350-3p, Il6
Flow Cytometry	[327]	Protein	IL10, CD40, CX3CR1, TGFB, IFNy, IL4, TNF
qPCR	[328]	Gene	Stat5a, Stat5b
qPCR	[329]	Gene	iNOS, Il1B, Il6, MHCII, CD40, TNF
Immuno flourescence	[333]	Protein	C5b, C3bi, IgG1, C3b, IgG2a, IgG2b, IgG3, IgM, IgA, IgE
Flow Cytometry	[334a]	Protein	ED2
ELISA	[334b]	Protein	TNF
RNAseq	[335]	Gene	Ifitm2, Tnfaip8, Ctsd, Ctss, Atf3, Gpx3, Dnajb4, Hspb1, Hspa1B, Dnajc10, Marco, Igkv3-2, Pla2g2d, Igha, Jchain, Ighv1-53, Zbtb7c, Igkc, AC133103.1, Ighm, Has1, Ighv1-55, Col4a2, Rasef, H2-M2, Zmat4, Srgap1, Ly6i, Vcam1, Dact2, Plin4, Abcb1b, Col4a1, Enpp5, Lcn2, Gm4951, Esr1, Esrrg, Cldn3, Aldh1a2, B630019K06Rik, Cd4, Alppl2, Ccdc7a, Hc, Map2, Mustn1, Kcnj15, Egfl6, Fam20c, Mlc1, Hmcn1, Palmd, Lrp2, AA467197, Lamp3, Cxcl3, AC168977.1, Sftpd, Itih4, Dpysl3, Egfem1, Ovol2, Adora3, Ikzf4, Inka1, Mterf2, Nr1d1, Zfp979, Penk, Itga6, A430106G13Rik, Btla, Esco2, Cenpk,

			Fam221a, Zbtb32, Clec4a1, Cenpw, Cbfa2t3, Ska1, Mir142b, Haao, Id3, E2f7, Cenpm, Rmi2, Fcho1, Ica1, Fam78b, Cdca7, Cd93, Fignl1, Cbx2, Nid2, Ptp4a3, Pclaf, Ccna2, Depdc1a, Myrip, Lrrc75a, Abi3, Fam83d, Fam92a, Uhrf1, C1qtnf12, Rad54b, Chek1, Sapcd2, Psat1, Deldc1b, Ifg1, Pdgfa, C3, Egfr, Rab27B, Cd36, Rab25, P2ry1, Sod3, Ccnt1, Csf1, Siglec1, Rab12, Hsf4, Csf2ra, Csf2rb, Csf1r, Il3ra, Jak2
ELISA	[338a]	Protein	IL10, IL1B, IL6, IL12, TNF
Flow Cytometry	[338b]	Protein	MAC1, B7.2, TLR4, CD14
Flow Cytometry	[339a]	Protein	TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TNF, IL6
Assay	[339b]	Protein	TNF, IL6, IL1B, IFNy, IL10
RNAseq	[341]	Gene	Ncan, Vcan, Cd44, Cspg4, Fibronectin

Number followed by letter in publication indicates the publication number and different method used

Supplementary table 8.2.2 Input table for genes upregulated MAIC analysis

Method	Publications	Gene names
qPCR	[1]	Tnf, Nos2, Il6
qPCR	[11]	Arg1, Mrc1, p53, p21, p16
qPCR	[32]	Tnf
Transcriptomic	[33a]	Csprs, Gimap4, Cd79a, Ly6d, Ms4a1, Cd79b, Mzb1, Cxcl13, Plac8, Ly6a
qPCR	[33b]	p16, Fads3, Hmgcs2, Prkaa2, Cyp7b1, Lpl, Tbxas1, Adfp, Alox14, Fabp4
qPCR	[127]	Ccl2, IfnB, Tnf, Il12p40, Il10, Mcsf, Cd11b, Cox2, MHCII, CIITa, Irf1, IfnG, Tlr1
Western Blot	[133]	Nos2, Cox2
RNAseq	[156]	Gm7609, Gm15446, Igkc, Gm16867, Hist1h4m, Ighg2b, Igkv10-96, Gm27177, Gm43802, Ifi208, AC125149.1, AC174776.1, Igkv12-46, Ighv1-18, Ighg2c, Ifi208, Ifnb1, Gm11189, Cdv3-ps
RNAseq	[157]	Vsig4, Cxcl13, Cxcl5, Fcna, Marco, Tmem132e, Saa3, C6, H2-M9, Ccl8, Lif, Cd209f, Cd209d, Tacr3, Gm16548, Csmd1, Cd209g, Inhba, Gpr176, Cxcl1, Cxcl2, Il6, Ccl2
qPCR	[158]	Cox2
qPCR	[186]	Nos2
qPCR	[189]	Tnf, Nos2, Cd40, Cd11b, MHCII, Arg1
qPCR	[190]	Nos2, Tnf, Il6
qPCR	[198]	Tnf
RNAseq	[206]	Hist1h4m
RNAseq	[207]	Vsig4, Cxcl13, Cxcl5, Fcna, Marco, Tmem132e, Saa3, C6, H2-M9, Ccl8, Lif, Cd209f, Cd209d, Tacr3, Gm16548, Csmd1, Cd209g, Inhba, Gpr176, Cxcl1, Cxcl2, Il6, Ccl2
qPCR	[230]	P16inka
RNAseq	[253]	Lilr4b, Hmox1, Oser1, Cd274, Ccl5, Tiparp, Clec4d, Gdf15, Cxcl2, H2-Eb1, Coro1a
qPCR	[260]	Tnf, Il1b, Il6, Nos2, Mcp1
Microarray	[261]	miR-714, miR-150, miR-195, miR-3273, miR-145, Let-7d, miR-1247
qPCR	[277]	Cox2, miR-101b, miR26b
qPCR	[290]	Il10, Cd163, miR-33
qPCR	[291]	TimD4, Stabilin2, Il10, F4/80, Cd31, Cd36, Arg1
Microarray	[302]	miR143, miR145, miR150, miR195
qPCR	[308]	Tnf, Il1b, Il6, Il12p40, TgfB, Il10
qPCR	[310]	miR350-3p, Il6
qPCR	[329]	Nos2, Il1b, Il6, MHCII, Cd40
RNAseq	[335]	Ifitm2, Tnfaip8, Ctsd, Ctss, Atf3, Gpx3, Dnajb4, Hspb1, Hspa1B, Dnajc10, Marco, Igkv3-2, Pla2g2d, Igha, Jchain, Ighv1-53, Zbtb7c, Igkc, AC133103.1, Ighm, Has1, Ighv1-55, Col4a2, Rasef, H2-M2, Zmat4, Srgap1, Ly6i, Vcam1, Dact2, Plin4, Abcb1b, Col4a1, Enpp5, Lcn2, Gm4951, Esr1, Esrrg, Cldn3, Aldh1a2, B630019K06Rik, Cd4, Alppl2, Ccdc7a, Hc, Map2, Mustn1, Kcnj15, Egfl6, Fam20c, Mlc1, Hmcn1, Palmd, Lrp2, AA467197, Lamp3, Cxcl3, AC168977.1, Sftpd, Itih4, Dpysl3
RNAseq	[341]	Ncan, Vcan, Cd44, Cspg4, Fibronectin

Number followed by letter in publication indicates the publication number and different method used

Supplementary table 8.2.3 Input table for genes downregulated MAIC analysis

Method	Publication	Gene names
qPCR	[1]	Cd206, Fizz1, TgfB
qPCR	[24]	Arg1, Nos2, Slc7a2
qPCR	[32]	Nos2, Arg1, Cd206
Transcriptomic	[33a]	Scd2, Dhcr24, Acsl3, Ceacam19, Idi1, Clec5a, Mir15a, Sgle, Cyp51
qPCR	[33b]	Fads2, Fdft1, Fdps, Scd1, Cyp11a1, Cyp27a1, Hadhb, Nr0b2, Nr1h3, Lta4h, Alox5ap, Alka1, Apof, Slc16a6, Slc27a1, Slc27a3, Stard4, Cxcl16, Olr1, Pcsk9, Stab2
Northern Blot	[55]	Nos2
qPCR	[127]	Il1B, Tnf
qPCR	[137]	Stat1
Northern Blot	[147]	hsp70
qPCR	[150]	Tnf
RNAseq	[156]	Gm42427, Wdfy1, Tmem181bp, Dynlt1b, Cd59a, Sel1l3, Tmem181bp, Gm42031, Gm43305, Xlr, Rab4a, Nbea, AC157515.1, Gm42742, C2, Cfap69, Steap2, Stc1, Vegfc, Steap1, Fgf23, Hist1h4n, Gm10359, Saa1, Slc6a14, 1700028P14P, Lhb, mt-Nd4l, Gm14176, Gm5776, Emx2
qPCR	[158]	RXRa, Abca1, Abcg1
qPCR	[167]	Cd11b, Cd206, TgfB
qPCR	[188]	Tlr1, Tlr2, Tlr3, Tlr4, Tlr5, Tlr6, Tlr7, Tlr8, Tlr9
qPCR	[189]	Mrc1
qPCR	[190]	FoxO3, Cd206, TgfB, Fizz1
Northern Blot	[194]	IaB, IeB
RNAseq	[206]	Wdfy1, Marco, Csf2, Dynlt1b, Smo, Efcab7, Ptch1
qPCR	[216]	Reverba
RNAseq	[253]	Gm10260, Gm10036, Ly6e
qPCR	[254]	Rac1
qPCR	[260]	Arg1, Mrc1
Microarray	[261]	miR-181c, miR-3068, miR-330, miR-5135, miR-10a, miR720, miR-29c
qPCR	[274]	miR-142-3p
qPCR	[290]	Abca1, Abcg1, Tnf, Il1B, Ptgs2, Ccl2, Mmp9, TgfB
qPCR	[291]	Tnf, Il6, Il1B
qPCR	[301]	miR146b, miR22, miR423, miR15a
Microarray	[302]	miR720
qPCR	[310]	miR350-3p
qPCR	[329]	Tnf
RNAseq	[335]	Egfem1, Ovol2, Adora3, Ikzf4, Inka1, Mterf2, Nr1d1, Zfp979, Penk, Itga6, A430106G13Rik, Btla, Esco2, Cenpk, Fam221a, Zbtb32, Clec4a1, Cenpw, Cbfa2t3, Ska1, Mir142b, Haao, Id3, E2f7, Cenpm, Rmi2, Fcho1, Ica1, Fam78b, Cdca7, Cd93, Fignl1, Cbx2, Nid2, Ptp4a3, Pclaf, Ccna2, Depdc1a, Myrip, Lrrc75a, Abi3, Fam83d, Fam92a, Uhrf1, C1qtnf12, Rad54b, Chek1, Sapcd2, Psat1, Deldc1b Ifg1, Pdgfa, C3, Egfr, Rab27B, Cd36, Rab25, P2ry1, Sod3, Ccnt1, Csf1, Siglec1, Rab12, Hsf4

Number followed by letter in publication indicates the publication number and different method used

Supplementary table 8.2.4 Input table for protein upregulated MAIC analysis

Method	Publication	Protein names
Flow Cytometry	[9]	FIZZ1
ELISA	[11]	IL1B, IL6, TNF, IL10
ELISA	[15]	TNF, IL1
FACS	[32]	iNOS, CD206
Mass Spec	[41]	PRRG2, FBN1, COL1A1, POSTN, PAI1, COL1A2
Assay	[61]	PGE2, COX
Assay	[71]	PGE2, COX
Assay	[96]	IA
Flow Cytometry	[110a]	CCR7, CD163
Assay	[110b]	ARG1
ELISA	[110c]	IL1B, IL6, IL10
Flow Cytometry	[127]	CD11b, CD11c, CD206, CD64, MHCIIA
Flow Cytometry	[129a]	CD14, TLR4
ELISA	[129b]	TNF, IL10
Assay	[129c]	ARG1, Inos
Assay	[133]	NFKB, p65, PGE2, COX2
ELISA	[149]	IL6
Western Blot	[150]	CD14
ELISA	[156]	CXCL2, CXCL1, IL1RA, IL10, CCL3, TIMP1, TNF, CXCL10, CCL4, CCL5, IL6, CCL2, CXCL13, MCSF, IFNY, CCL1, GCSF, CXCL11, IL17, CXCL12, TREM1, IL1B, C5a, IL1a, IL13, GMCSF, IL16, IL7, IL4, CXCL9, IL3, IL5
Proteomic	[183]	MYO1F, RARS, DPYS11, PIK3AP1, HUWE1, TPP1, NAPRT, PPAT, SF3B2, MARS, PGAM2, ACTG1, F1NC, CAPG, TUBA1A, ACTA1, YWHAZ, CAP1, ANXA5, F1NA, CTSD, H2-D1, HSC70, TIP-B1, FGH, GM2A, GPX1, ANXA6, CTSB, PRX, MDH2, ANXA2, UBA1, PGK, LCP1, ANXA1, HSP90AB1, ALDH-2, GRP78, HA1, LYZ, MOM2, VAT-1, RP14
Immuno Cytochemistry	[186]	iNOS
Flow Cytometry	[189a]	IFNyR1, CD11b, CD45
ELISA	[189b]	TNF
Flow Cytometry	[197]	TNF, IL10, CCL2
ELISA	[198a]	TNF
Assay	[198b]	PGI2
ELISA	[206]	CXCL13, C5a, G-CSF, GMCSF, CCL1, IFNy, IL1a, IL1B, IL3, IL4, IL7, IL13, IL18, IL17, CXCL10, CXCL11, CXCL9, CCL3, CXCL2, CXCL12, TIMP1, TREM1, CCL4, CD54, CCL5, TNF, IL6, IL1ra, MCSF, CCL4, IL23, IL27
Assay	[211a]	iNOS, ARG1
ELISA	[211b]	IL1a, IL10, TNF
Assay	[212]	TNF
ELISA	[226]	IL6
Flow Cytometry	[230a]	Marco

Immuno staining	[230b]	BGalactosidase
Assay	[245]	TNF, IL1, IL6
ELISA	[249]	IL10
Flow Cytometry	[254]	Marco
Western Blot	[260a]	p62
ELISA	[260b]	TNF, IL1B, IL6
Immuno flourescence	[260c]	iNOS
ELISA	[274]	IL6
ELISA	[277]	PGE2
Western Blot	[280]	A20
Western Blot	[281]	COX2, PGE2
Immuno flourescence	[306]	Fcy1R, FcyAR, IA, MAC1
Flow Cytometry	[327]	IL10, CD40, CX3CR1, TGFB
Immuno flourescence	[333]	C3b, IgG2a, IgG2b, IgG3, IgM, IgA, IgE
ELISA	[338a]	IL10
Flow Cytometry	[338b]	MAC1, B7.2, TLR4
Assay	[339a]	IL6
Flow Cytometry	[339b]	TLR2, TLR4

Number followed by letter in publication indicates the publication number and different method used

Supplementary table 8.2.5 Input table for protein downregulated MAIC analysis

Method	Publication	Protein names
Western Blot	[1]	FOXO3
Flow Cytometry	[9]	MHCII, KI67
Flow Cytometry	[11]	CD11b
ELISA	[15a]	TNF, IL1, IL12
ELISA	[15b]	IL12
ELISA	[15c]	TNF, IL1, IL12
ELISA	[32]	IL1RA, TNF
Mass Spec	[41a]	QPCT, Granulin, ATP6AP1, LAMP1, PPT1, IL-41, CTSZ, GGH, CTSL1, CCR9, C1QA, TCN2, C1QB, LRP1, B2M, CREG1, CSF1R, CTSB, H2KW28, C1QC, SDF4, STAB1, SGP1A, PAFAH, HEXA, CST3, LGALS3BP
ELISA	[41b]	LRP1
Assay	[85]	TNF
Assay	[96]	TNF, ASGM1
ELISA	[99]	IL1, TNF
ELISA	[110]	TGFB, IL10, IL6, IL1B
Flow Cytometry	[129a]	CD14
ELISA	[129b]	TNF, IL10
Assay	[129c]	ARG1, iNOS
Western Blot	[137]	STAT1B, STAT1A
ELISA	[149]	TNF
Western Blot	[150]	TNF, RACK1
Proteomic	[183]	ACTC1, CCT3, MELA, HSP70-1B, ARG1, DESMOYOKIN, GSTP1, H2BA, EUK, SET, SLC25A5, PSMD3, UBE2Q1, KRT28, EEF2
Flow Cytometry	[188a]	TLR4
ELISA	[188b]	TNF, IL6
Western Blot	[190]	FOXO3
Flow Cytometry	[194a]	IA
Western Blot	[194b]	IA
ELISA	[196a]	TNF, IL6, P38, JNK
Western Blot	[196b]	P38, JNK
Flow Cytometry	[197]	TNF, IFNy, IL12, IL6, CCL2
ELISA	[201]	TNF, IL1B, IL6, IL12
Western Blot	[206]	WDFY1
Assay	[211a]	iNOS, ARG1
ELISA	[211b]	IL10, TNF
Flow Cytometry	[230]	TLR4
Assay	[233]	ARG1
ELISA	[245a]	TNF, IL1, IL6
Assay	[245b]	TNF, IL1, IL6
ELISA	[249a]	TNF, IL6

Western Blot	[249b]	p38
Western Blot	[254]	RAC1
Western Blot	[260a]	ATG5, LC3-II
Immuno flourescence	[260b]	CD206
Immuno flourescence	[264]	TNF
ELISA	[268]	IL1B, IL6, TNF
Western Blot	[280]	CYLD
Western Blot	[290]	ABCA1, ABCG1
Immuno flourescence	[306]	FcuR, Fcy2AR, Fcy2BR, FceR
Flow Cytometry	[327]	IFNy
Immuno flourescence	[333]	C5b, C3bi, IgG1
Flow Cytometry	[334a]	ED2
ELISA	[334b]	TNF
ELISA	[338a]	IL1B, IL6, IL12, TNF
Flow Cytometry	[338b]	CD14
Assay	[339]	TNF, IFNy

Number followed by letter in publication indicates the publication number and different method used

Supplementary table 8.2.6 MAIC publications

Publication number	Citation
1	[568]
2	[570]
3	[572]
4	[575]
6	[340]
9	[579]
11	[305]
13	[288]
15	[584]
20	[586]
24	[589]
26	[592]
29	[595]
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225	[645]
226	[300]
228	[295]
230	[649]
231	[652]
233	[302]
237	[657]
238	[660]
239	[663]

Publication number	Citation
85	[569]
89	[571]
94	[573]
95	[576]
96	[277]
99	[580]
100	[581]
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274	[658]
277	[661]
278	[664]

Publication number	Citation
159	[338]
160	[474]
166	[574]
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178	[336]
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253	686
254	274
256	292
258	692
260	332
261	697
264	333

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281	670
282	673
283	676
284	677
285	678
286	680
290	682
291	449
292	687
293	689
296	691
297	693
301	695
302	698
304	644

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322	671
324	674
325	331
327	329
328	679
329	334
333	683
334	685
335	688
337	690
338	322
339	694
341	696
342	699
344	700

Supplementary table 8.2.7 Changes in macrophage numbers as a result of increasing age

Macrophage subtype	Direction of change with age	Additional factors	Citation
F4/80+ CD11b+	Decrease	Peritoneum	[279]
	Decrease	Liver	[701]
	Increase	Liver	[293]
	Increase	Aorta and mesentery	[702]
F4/80+CD11b+ CD64+	Decrease	Skin	[703]
Iba1+	Increase	Osseous spiral lamina	[704]
	Increase	Retina	[344]
	No change	Apical turn	[306]
Peritoneal	Decrease	Percentage live	[454]
	No change	Control	[327]
CD68+/DAPI+ CD206+/CD68+/DAPI+ CD206−/CD68+/DAPI+	No change No change Decrease	Skeletal muscle	[328]
CD11b+F4/80+Ly6Gneg	Increase	Spleen	[705]
CD68+	Increase	Prostate	[706]
	Increase	Vaginal wound	[340]
	Increase	Liver	[707]
	Increase	Kidneys	[693]
	Decrease	Prostate	[708]
	No change	Muscle	[601]
	No change	Peritoneum	[284]
F4/80+	Increase	Ovarian stroma	[692]
	Decrease	Peritoneum	[312]
	Decrease	White adipose tissue	[296]
	Decrease	RM-9 tumour	[709]
	Decrease	Glomeruli	[616]
	Decrease	Bone marrow-derived	[288]
	Decrease	Peritoneum	[694]
	Decrease	Peritoneum	[301]
	No change	Fracture callus	[575]
	No change	Carotid artery	[710]
	No change	Spleen	[625]
Arg1Hi	Increase	Skin wounds	[711]
Monocyte-derived	Increase	Spinal cord injury	[582]
Cardiac resident	No change	Steady-state control	[712]
CD11b+ F4/80+ Ly-6C+	Increase	Muscle	[713]
M2	Increase	Muscle injury	[608]
	Increase	Spleen and bone marrow	[329]
M1 (CD86+) M2 (CD163+)	Increase Decrease	Liver	[336]
	Decrease	Stroma	[697]
CD11b+ CD206+	Decrease	Intervertebral disc	[577]
	Increase	Peritoneum	[280]
CD45+F4/80+ Ly6E+Ly6Chi	Decrease	Muscle	[660]
HO-1+/iNOS+ and HO-1+/CD163+	Increase	Peritoneum	[714]
CD45+Ly6G-Siglec-F-NK1.1-CD11b+Ly6C-CD64+	Decrease	Skeletal muscle	[715]
M2 (RELM-α+) M1 (MHCII+)	Increase Decrease	Ovary	[579]

CD68+CD163+DAPI+	Decrease	Soleus	[572]
CD11c+ CD11b+	Increase	Alveolar	[598]
F4/80+ CD206+	Decrease	Kidney injury	[326]
CD68+iNOS+ CD68+IL-10+	Increase Decrease	Myocardial infarction	[335]
NG2+	Increase	Microglia	[696]
CD68+CD11b+	Decrease	Skeletal muscle	[716]
CD11b+ CD45high	Increase Increase	Brain Brain	[717] [599]
CD11b+MHCII+F4/80+	Increase	Bone marrow-derived	[449]
F4/80+GHS-R+	Increase	Peritoneum	[678]
F4/80+CD206− F4/80+CD206+	Increase Decrease	Cardiac	[586]
ED2high	Decrease	Peritoneum	[685]
F4/80+IL4R+	Decrease	Bone marrow-derived	[594]
CD11bhigh F4/80high	Decrease	Peritoneum	[581]
CD26+	Increase	Peritoneum	[718]
CD68+CD163+ CD11b+	Decrease Decrease	Skeletal muscle	[719]

Supplementary table 8.2.8 Findings from publications assessing changes in soluble mediator release with age

Soluble mediator	Direction of change with age	Cell environment	Additional factors	Citation
TNF, IFN-γ, IL-12, and MCP-1	Decrease	LPS-stimulated in vitro	Bone marrow or peritoneal	[615]
TNF, IL-6, IL-1β and IL-12	Decrease	LPS-stimulated	Splenic	[625]
IL-1	Decrease	LPS-stimulated	Splenic	[720]
TNF	Decrease	IFN-primed, LPS-stimulated	Alveolar	[277]
TNF, IL-1, IL-6	Decrease	LPS-stimulated	Peritoneal	[675]
IL-1, TNF	Decrease	LPS+IFNγ-stimulated	Peritoneal	[580]
TNF	Increase	Basal	Peritoneal	[638]
TNF	Decrease	LPS-stimulated	Alveolar	[632]
IL-1β	Increase	Control and Con A-stimulated	Peritoneal	[286]
TNF, NO, IL-12, IL-1β	Increase	LPS and IFN-γ stimulated	Alveolar	[584]
IL-10	Increase	LPS-stimulated	Splenic	[322]
TNF and IL-6	Decrease	TLR 2 and 4 ligand stimulation	Splenic	[357]
TNF and IL-6	Decrease	Basal	Peritoneal	[279]
IL-6 and TNF	Increased	Steady state and after LPS stimulation	Renal	[337]
IL-1β, IL-6, and TNF	Decreased	Salmonella extract and LPS-stimulated	Peritoneal	[280]
IL-6	Increase	LPS or S. pneumoniae stimulated	BMDMs	[300]
TNF Check IL-1ra	Increase	M1 (LPS-stimulated)	BMDMs	[597]
TNF, IL-1B, IP-10	Increase	Stimulated with soluble brain extract from aged mice	BMDMs	[334]
TNF	Increase	IFNγ-stimulated	BMDMs	[599]
IL-6 and TNF	Decrease	LPS+IFNγ-stimulated	Peritoneal and splenic	[366]
IL-10 IL-12	Increase Decrease	CpG-ODN+IFNγ	Peritoneal	[581]
TNF and IL-6	Decrease	Pg-challenged	BMDM	[680]
TNF, IFN-γ, and IL-6	Decrease	C albicans antigen-challenged	Dermal	[333]
TNF	Increase	Uf-Ni and Uf-Co stimulation	Alveolar	[593]
IL-2 IL-4	Decrease No change	Co-culture of macrophages and T cells	Peritoneal	[721]
TNF	Decrease	LPS, poly(I:C), CpG ODN, S. aureus and zymosan A	Splenic	[596]
TNF and IL-6, IL-1B and IL-12	Decrease	LPS-stimulated	Splenic	[625]
IL-1B, TNF, IL-6, IL-3, IFN-γ	Increase	Basal	Peritoneal	[607]
IL-6, TGFB IL-1β, IL-4	Decreased No change	LPS-stimulated	Peritoneal	[284]
VEGF, IL-10 IL-6	Increase Decrease	sFasL and LPS stimulation	BMDMs	[295]
IL-6 TNF PGE2	No change Increase	Basal and LPS-stimulated	Peritoneal	[590]
PGE2	Increase	Resting and LPS-stimulated	Peritoneal	[641]

TNF	Decrease	LPS	Peritoneal	[685]
TNF, IL-6, IL-1β MIP-2 IL-10	Decrease No change	Zymosan, yeasts cells, and hyphae C. albicans and hyphae	Peritoneal	[301]

Soluble mediator	**Direction of change with age**	**Cell environment**	**Additional factors**	**Citation**
ROS	Decrease	Proliferating and senescent cell media	Peritoneal	[305]
ROS	Increase	Spinal cord injury lesion epicentre, vehicle treated	Microglia	[722]
Nitric oxide	No change	IFNγ/LPS	BMDMs	[288]
XO , ROS and O2	Increase	Basal	Peritoneal	[474]
ROS	Increase	Spinal cord injury	Microglia	[723]
mtROS	Increase	Control and bleomycin-treated	BMDMs	[652]
NO	Increase No change Increase	Basal LPS-stimulated Basal LPS-stimulated	DA rats, peritoneal AO rats, peritoneal	[313]
ROS	Decrease Increase	Basal	Peritoneal Splenic	[285]
NO	No change Decrease	Basal LPS	Peritoneal and splenic	[366]
NO	Increase	LPS	Peritoneal, first two hours of exposure	[330]
NO	Increase	CpG-ODN + IFN-γ	Peritoneal	[581]
NO	Decrease	Pg 381	BMM	[680]
NO	No change	Classically activated	BMDM	[302]
NO	Increase Decrease	LPS and IFN-γ	Alveolar Peritoneal	[584]
NO	Increase	LPS	Peritoneal	[639]
O2	No change	HMP-glycol stimulation	Peritoneal	[724]
FOR	Increase	Basal and stress	Peritoneal and alveolar	[324]
NO	Decrease	Basal and exercise	Peritoneal	[725]
NO2	Decrease	LPS	Splenic	[619]
H2O2	Decrease	Basal and PMA stimulation	Tumour-activated	[290]
O – 2 anions	Increase	Zymosan	Peritoneal	[726]
H2O2	Increase	Basal	Peritoneal	[304]
NO	Increase	IFN	Peritoneal	[727]
NO	No change	Basal	Peritoneal	[618]
NO	Increase	IFN and LPS	Hepatic	[293]
FOR	Increase	Basal	Peritoneal and alveolar	[324]

Supplementary table 8.2.9 Mean MAIC scores for all genes

Gene	Downregulated	Upregulated
AA467197	0	1.003342
Abca1	-1.009128	0
Abcb1b	0	1.003342
Abcg1	-1.009128	0
AC125149.1	0	1.067736
AC133103.1	0	1.003342
AC168977.1	0	1.003342
AC174776.1	0	1.067736
Adfp	0	1.004692
Aldh1a2	0	1.003342
Alox15	0	1.004692
Alppl2	0	1.003342
Arg1	-1.415746	1.379062
Atf3	0	1.003342
B630019K06Rik	0	1.003342
C6	0	1.153306
Ccdc7a	0	1.003342
Ccl2	-1.009128	2.471590
Ccl5	0	1.012183
Ccl8	0	1.153306
Cd11b	-1.094324	1.379062
Cd163	0	1.073837
Cd206	-1.415746	0
Cd209d	0	1.153306
Cd209f	0	1.153306
Cd209g	0	1.153306
Cd274	0	1.012183
Cd31	0	1.071658
Cd36	0	1.071658
Cd4	0	1.003342
Cd40	0	1.505031
Cd79a	0	1.103789
Cd79b	0	1.103789
Cdv3-ps	0	1.067736
CIITa	0	1.318284
Cldn3	0	1.003342
Clec4d	0	1.012183
Col4a1	0	1.003342
Col4a2	0	1.003342
Coro1a	0	1.012183
Cox2	0	3.989184
Csmd1	0	1.153306
Csprs	0	1.103789
Ctsd	0	1.003342
Ctss	0	1.003342
Cxcl1	0	1.153306
Cxcl13	0	2.257096
Cxcl2	0	1.153306
Cxcl3	0	1.003342
Cxcl5	0	1.153306
Cyp7b1	0	1.004692
Dact2	0	1.003342

Dnajb4	0	1.003342
Dnajc10	0	1.003342
Dpysl3	0	1.003342
Egfl6	0	1.003342
Enpp5	0	1.003342
Esr1	0	1.003342
Esrrg	0	1.003342
F4/80	0	1.071658
Fabp4	0	1.004692
Fads3	0	1.004692
Fam20c	0	1.003342
Fcna	0	1.153306
Fizz1	-1.094324	0
FoxO3	-1.080482	0
Gdf15	0	1.012183
Gimap4	0	1.103789
Gm11189	0	1.067736
Gm15446	0	1.067736
Gm16548	0	1.153306
Gm16867	0	1.067736
Gm27177	0	1.067736
Gm43802	0	1.067736
Gm4951	0	1.003342
Gm7609	0	1.067736
Gpr176	0	1.153306
Gpx3	0	1.003342
H2-Eb1	0	1.012183
H2-M2	0	1.003342
H2-M9	0	1.153306
Has1	0	1.003342
Hc	0	1.003342
Hist1h4m	0	1.067736
Hmcn1	0	1.003342
Hmgcs2	0	1.004692
Hmox1	0	1.012183
Hspa1B	0	1.003342
Hspb1	0	1.003342
Ifi208	0	1.067736
Ifitm2	0	1.003342
Ifnb	0	2.386020
IfnG	0	1.318284
Igha	0	1.003342
Ighg2b	0	1.067736
Ighg2c	0	1.067736
Ighm	0	1.003342
Ighv1-18	0	1.067736
Ighv1-53	0	1.003342
Ighv1-55	0	1.003342
Igkc	0	1.067736
Igkv10-96	0	1.067736
Igkv12-46	0	1.067736
Igkv3-2	0	1.003342
Il10	0	1.318284
Il12p40	0	1.318284

Il1b	-1.009128	1.517531
Il6	-1.003042	2.835213
Inhba	0	1.153306
Irf1	0	1.318284
Itih4	0	1.003342
Jchain	0	1.003342
Kcnj15	0	1.003342
Lamp3	0	1.003342
Lcn2	0	1.003342
Lif	0	1.153306
Lilr4b	0	1.012183
Lpl	0	1.004692
Lrp2	0	1.003342
Ly6a	0	1.103789
Ly6d	0	1.103789
Ly6i	0	1.003342
Map2	0	1.003342
Marco	0	1.153306
Mcp1	0	1.517531
Mcsf	0	1.318284
MHCII	0	1.505031
miR-101b	0	1.531035
miR-33	0	1.073837
miR26b	0	1.531035
miR350-3p	0	1.465001
Mlc1	0	1.003342
Mmp9	-1.009128	0
Mrc1	-1.125250	1.058439
Ms4a1	0	1.103789
Mustn1	0	1.003342
Mzb1	0	1.103789
Nos2	-3.202137	3.989184
Oser1	0	1.012183
p16	0	1.058439
p21	0	1.058439
p53	0	1.058439
Palmd	0	1.003342
Pla2g2d	0	1.003342
Plac8	0	1.103789
Plin4	0	1.003342
Prkaa2	0	1.004692
Ptgs2	-1.009128	0
Rasef	0	1.003342
RXRa	-1.003042	0
Saa3	0	1.153306
Sftpd	0	1.003342
Slc7a2	-1.415746	0
Srgap1	0	1.003342
Stabilin2	0	1.071658
Tacr3	0	1.153306
Tbxas1	0	1.004692
TgfB	-1.094324	1.286951
TimD4	0	1.071658
Tiparp	0	1.012183

Tlr1	0	1.318284
Tmem132e	0	1.153306
Tnf	-1.009128	1.681907
Tnfaip8	0	1.003342
Vcam1	0	1.003342
Vsig4	0	1.153306
Zbtb7c	0	1.003342
Zmat4	0	1.003342

Supplementary table 8.2.10 Mean MAIC scores for all proteins

Protein	Downregulated	Upregulated
ACTC1	-1.100502	0
ARG1	-2.762423	2.800089
ASGM1	-3.402046	0
ATP6AP1	-1.057452	0
B2M	-1.057452	0
B7.2	0	1.404013
C1QA	-1.057452	0
C1QB	-1.057452	0
C1QC	-1.057452	0
C5a	0	1.389373
CCL1	0	1.389373
CCL2	-3.041867	3.742451
CCL3	0	1.389373
CCL4	0	1.389373
CCL5	0	1.389373
CCR9	-1.057452	0
CCT3	-1.100502	0
CD11b	0	1.412643
CD11c	0	1.412643
CD14	0	3.436139
CD206	0	4.347373
CD40	0	1.540760
CD45	0	1.094798
CD54	0	1.323200
CD64	0	1.412643
COX	0	2.172427
COX2	0	4.387966
CREG1	-1.057452	0
CSF1R	-1.057452	0
CST3	-1.057452	0
CTSB	-1.057452	0
CTSL1	-1.057452	0
CTSZ	-1.057452	0
CX3CR1	0	1.540760
CXCL1	0	1.389373
CXCL10	0	1.389373
CXCL11	0	1.389373
CXCL12	0	1.389373
CXCL13	0	1.389373
CXCL2	0	1.389373
CXCL9	0	1.389373
DESMOYOKIN	-1.100502	0
EEF2	-1.100502	0
EUK	-1.100502	0
Fcy1R	0	1.528066
FcyAR	0	1.528066
GCSF	0	1.389373
GGH	-1.057452	0
GMCSF	0	1.389373
Granulin	-1.057452	0
GSTP1	-1.100502	0
H2BA	-1.100502	0

H2KW28	-1.057452	0
HEXA	-1.057452	0
HSP70-1B	-1.100502	0
IA	-3.997293	3.360359
IFNy	-6.676268	1.389373
IFNyR1	0	1.094798
IL1	-7.200325	4.966004
IL10	-3.402046	4.890307
IL12	-6.373862	0
IL13	0	1.389373
IL16	0	1.389373
IL17	0	1.389373
IL18	0	1.323200
IL1a	0	2.244680
IL1B	-3.343294	2.225058
IL1Ra	-3.402046	1.389373
IL23	0	1.323200
IL27	0	1.323200
IL3	0	1.389373
IL4	0	1.389373
IL41	-1.057452	0
IL5	0	1.389373
IL6	-10.457448	4.646402
IL7	0	1.389373
iNOS	-1.451405	12.914487
JNK	-5.573576	0
KRT28	-1.100502	0
LAMP1	-1.057452	0
LGALS3BP	-1.057452	0
LRP1	-2.700771	0
MAC1	0	2.932079
MCSF	0	1.389373
MELA	-1.100502	0
MHCIIA	0	1.412643
NFKB	0	1.974389
P38	-5.573576	0
p65	0	1.974389
PAFAH	-1.057452	0
PGE2	0	7.282315
PPT1	-1.057452	0
PSMD3	-1.100502	0
QPCT	-1.057452	0
RACK1	-3.402046	0
SDF4	-1.057452	0
SET	-1.100502	0
SGP1A	-1.057452	0
SLC25A5	-1.100502	0
STAB1	-1.057452	0
TCN2	-1.057452	0
TGFB	-2.143216	1.540760
TIMP1	0	1.389373
TLR2	0	1.173465
TLR4	0	1.583411
TNF	-19.776293	8.007603

TREM1	0	1.389373
UBE2Q1	-1.100502	0

8.3 Supplementary material for Chapter 4 “Using bioinformatic analysis to study macrophage ageing”

Supplementary table 8.3.1 Full output of most conserved transcripts in human macrophages from HRT Atlas

Rank	Ensembl ID	Gene Symbol	Norm. Exp.	SD	MFC	Chr	Start position	End position
1	ENST00000335508	SF3B1	46.921	0.321	1.310	2	197389784	197435091
2	ENST00000339438	CSDE1	90.470	0.363	1.430	1	114716913	114757983
3	ENST00000342374	SERINC3	33.897	0.352	1.376	20	44497431	44522109
4	ENST00000265462	PRDX5	82.085	0.440	1.429	11	64318088	64321811
5	ENST00000295770	STT3B	45.514	0.407	1.416	3	31532790	31637622
6	ENST00000237654	CCNI	77.767	0.436	1.448	4	77047158	77076005
7	ENST00000338639	PARK7	80.410	0.476	1.435	1	7961663	7985279
8	ENST00000378720	ESD	57.143	0.414	1.570	13	46771256	46797232
9	ENST00000354725	SND1	51.860	0.428	1.495	7	127652180	128092607
10	ENST00000314797	COPG1	49.554	0.445	1.452	3	129249606	129277773
11	ENST00000366923	EPRS	30.393	0.373	1.508	1	219968601	220046658
12	ENST00000253108	EIF3G	62.938	0.483	1.461	19	10115017	10119902
13	ENST00000334660	CHP1	31.486	0.380	1.506	15	41231143	41281890
14	ENST00000354488	DDRGK1	43.862	0.411	1.514	20	3190350	3204685
15	ENST00000368521	C1orf43	70.407	0.450	1.726	1	154206706	154220606
16	ENST00000244711	MEA1	41.707	0.444	1.452	6	43012094	43013968
17	ENST00000234301	COX7A2L	48.908	0.437	1.639	2	42350507	42361216
18	ENST00000233468	SF3B6	58.441	0.466	1.750	2	24067584	24076443
19	ENST00000349048	PFKL	58.375	0.468	1.684	21	44300051	44327376
20	ENST00000322535	SF3B2	51.246	0.478	1.639	11	66052336	66068911
21	ENST00000373795	SRSF4	30.736	0.427	1.766	1	29147743	29181987
22	ENST00000369799	AHCYL1	36.921	0.476	1.595	1	109984686	110023735
23	ENST00000274712	ZMAT2	34.776	0.445	1.703	5	140700334	140706676
24	ENST00000322428	MAF1	42.877	0.460	1.708	8	144104499	144107611
25	ENST00000422840	GLG1	32.034	0.459	1.667	16	74451959	74607094
26	ENST00000297338	RAD21	40.014	0.489	1.601	8	116845935	116874866
27	ENST00000372422	YIPF3	42.176	0.491	1.637	6	43511827	43516990
28	ENST00000332118	SRPRA	42.970	0.467	1.797	11	126262919	126268959
29	ENST00000301764	DDB1	44.641	0.490	1.923	11	61299451	61333366
30	ENST00000337859	ZC3H15	30.130	0.481	1.687	2	186486156	186509363

Norm. Exp.: normalised expression as reads per kilobase million (RPKM); SD: standard deviation of log2 of RPKM; MFC: Maximum fold change, a ration of maximum RPKM and mean RPKM value observed for each transcript. To be considered stable this should be less than 2; Chr: Chromosome.

Supplementary table 8.3.2 Full output of most conserved transcripts in mouse macrophages from HRT Atlas

Rank	Ensembl ID	Gene Symbol	Norm. Exp.	SD	MFC	Chr	Start position	End position
1	ENSMUST00000031598	Ddx54	239.464	0.143	1.065	5	120612739	120628592
2	ENSMUST00000169860	Morf4l1	1375.58	0.207	1.071	9	90091665	90114774
3	ENSMUST00000041587	Gga1	73.052	0.118	1.074	15	78877190	78894585
4	ENSMUST00000022458	Bap1	113.115	0.135	1.079	14	31251450	31259944
5	ENSMUST00000021314	Nmt1	136.190	0.142	1.080	11	103028346	103068912
6	ENSMUST00000100484	Eif3d	243.793	0.156	1.080	15	77958998	77970813
7	ENSMUST00000030769	Psmc2	213.022	0.154	1.085	5	21785283	21803787
8	ENSMUST00000003912	Calr	5265.40	0.324	1.098	8	84841850	84846934
9	ENSMUST00000058438	Dcaf7	289.035	0.167	1.085	11	106036872	106059324
10	ENSMUST00000028238	Rab14	451.479	0.176	1.093	2	35180205	35201120
11	ENSMUST00000048657	Sec24c	354.249	0.158	1.095	14	20674308	20694852
12	ENSMUST00000030164	Vcp	497.455	0.186	1.093	4	42979963	43000507
13	ENSMUST00000018470	Ywhab	380.066	0.175	1.095	2	163994960	164018588
14	ENSMUST00000101087	Srp72	558.121	0.221	1.105	5	76974683	76999937
15	ENSMUST00000020238	Hsp90b1	2355.17	0.364	1.120	10	86690840	86705509
16	ENSMUST00000057934	Tcf25	126.178	0.169	1.090	8	123373824	123401892
17	ENSMUST00000100206	Larp4	417.249	0.241	1.092	15	99972780	100016358
18	ENSMUST00000053872	Cdc42se1	290.653	0.200	1.095	3	95228732	95236409
19	ENSMUST00000024739	Hsp90ab1	579.066	0.259	1.099	17	45567775	45573271
20	ENSMUST00000116514	Wbp11	256.562	0.221	1.089	6	136813654	136828233
21	ENSMUST00000028617	Api5	241.373	0.178	1.104	2	94411682	94438136
22	ENSMUST00000028691	Arfgap2	79.646	0.164	1.091	2	91265293	91276931
23	ENSMUST00000022842	Cct5	608.993	0.239	1.120	15	31590800	31601804
24	ENSMUST00000096639	Rnf121	62.202	0.146	1.109	7	102019137	102065158
25	ENSMUST00000033012	Copb1	348.271	0.218	1.107	7	114215559	114254711
26	ENSMUST00000201388	Sf1	270.677	0.201	1.105		6363922	6377196
27	ENSMUST00000001834	Rtcb	170.402	0.177	1.104	10	85938637	85957823
28	ENSMUST00000020420	Ap3d1	133.086	0.189	1.092	10	80706956	80742264
29	ENSMUST00000034960	Dpp8	127.842	0.171	1.104	9	65032414	65082651
30	ENSMUST00000020637	Canx	417.346	0.276	1.094	11	50294467	50325673
31	ENSMUST00000047431	AU040320	78.718	0.156	1.112	4	126753770	126854336
32	ENSMUST00000028683	Pdia3	1204.77	0.344	1.131	2	121413775	121438687
33	ENSMUST00000040270	Actr1a	279.301	0.197	1.122	19	46376811	46395747
34	ENSMUST00000166123	Eif5	511.772	0.229	1.143	12	111538016	111546752
35	ENSMUST00000034947	Ppib	39.306	0.148	1.114	9	66060231	66066623
36	ENSMUST00000034966	Rpl4	887.296	0.322	1.135	9	64173375	64178666
37	ENSMUST00000025483	Nars	165.154	0.205	1.101	18	64499658	64516608
38	ENSMUST00000111817	Gmfb	716.628	0.293	1.136	14	46808149	46822242
39	ENSMUST00000028841	Usp8	241.030	0.237	1.100	2	126707343	126759297
40	ENSMUST00000130411	Srsf6	55.147	0.161	1.105	2	162931528	162937121
41	ENSMUST00000043269	Hnrnpk	613.948	0.267	1.149	13	58391239	58402502
42	ENSMUST00000067663	Psmc3	481.170	0.297	1.115	2	91054009	91059432
43	ENSMUST00000025667	Rtn3	310.577	0.233	1.122	19	7425901	7483281
44	ENSMUST00000043990	Edc3	94.825	0.163	1.127	9	57708540	57752499
45	ENSMUST00000021063	Psmd12	117.245	0.180	1.120	11	107479484	107498173
46	ENSMUST00000023467	Pak2	261.451	0.237	1.113	16	32016290	32079342
47	ENSMUST00000025679	Otub1	75.663	0.176	1.108	19	7198202	7206316
48	ENSMUST00000100171	Hspa5	1784.54	0.471	1.165	2	34772095	34776531
49	ENSMUST00000002445	Ranbp3	160.261	0.207	1.116	17	56673358	56711764
50	ENSMUST00000164099	Snx19	75.296	0.176	1.117	9	30427108	30466726

Norm. Exp.: normalised expression as reads per kilobase million (RPKM); SD: standard deviation of log2 of RPKM; MFC: Maximum fold change, a ration of maximum RPKM and mean RPKM value observed for each transcript. To be considered stable this should be less than 2; Chr: Chromosome.

Supplementary table 8.3.3 Full output of most conserved transcripts in mouse bone marrow bulk tissue from HRT Atlas

Rank	Ensembl ID	Gene Symbol	Norm. Exp.	SD	MFC	Start position	End position	Chr
1	ENSMUST00000067284	Cpsf3	623.367	0.369	1.193	21286358	21315056	12
2	ENSMUST00000101087	Srp72	746.995	0.364	1.224	76974683	76999937	5
3	ENSMUST00000099490	Nsd1	675.177	0.390	1.215	55209782	55318325	13
4	ENSMUST00000038627	Zfp91	201.408	0.284	1.194	12767020	12796126	19
5	ENSMUST00000107107	Plaa	75.062	0.247	1.206	94569167	94603244	4
6	ENSMUST00000028981	Mapre1	451.374	0.318	1.206	153741274	153773310	2
7	ENSMUST00000001419	Zmat2	135.099	0.262	1.221	36793876	36799666	18
8	ENSMUST00000028617	Api5	325.328	0.341	1.200	94411682	94438136	2
9	ENSMUST00000022927	Rad21	333.464	0.357	1.209	51962240	51991747	15
10	ENSMUST00000030117	Smu1	88.591	0.267	1.222	40736542	40757923	4
11	ENSMUST00000006774	Gtf2h1	31.463	0.230	1.252	46796103	46823800	7
12	ENSMUST00000035010	Stt3b	422.476	0.375	1.217	115242581	115310421	9
13	ENSMUST00000054014	Ddx17	262.107	0.311	1.232	79527736	79546741	15
14	ENSMUST00000043152	Utp6	176.056	0.299	1.223	79933953	79962390	11
15	ENSMUST00000048657	Sec24c	432.656	0.363	1.237	20674308	20694852	14
16	ENSMUST00000020637	Canx	486.889	0.408	1.232	50294467	50325673	11
17	ENSMUST00000111147	Caprin1	609.657	0.435	1.260	103765018	103797104	2
18	ENSMUST00000163242	Atxn10	220.183	0.352	1.222	85336245	85463212	15
19	ENSMUST00000050552	Bzw1	488.057	0.337	1.296	58392898	58407353	1
20	ENSMUST00000025774	Sf3b2	35.544	0.258	1.255	5273932	5295455	19
21	ENSMUST00000033866	Vps36	106.903	0.276	1.255	22192809	22220843	8
22	ENSMUST00000094578	Sec31a	554.340	0.455	1.242	100361649	100416234	5
23	ENSMUST00000033012	Copb1	355.596	0.368	1.245	114215559	114254711	7
24	ENSMUST00000020382	Yeats4	401.171	0.370	1.256	117215222	117224507	10
25	ENSMUST00000022380	Psmc6	205.777	0.327	1.244	45329788	45349705	14
26	ENSMUST00000030164	Vcp	569.219	0.444	1.274	42979963	43000507	4
27	ENSMUST00000094657	Dnajc8	131.737	0.302	1.251	132535550	132553742	4
28	ENSMUST00000032071	Dusp11	211.153	0.321	1.253	85942268	85961667	6
29	ENSMUST00000043160	Aqr	69.982	0.293	1.241	114101170	114175339	2
30	ENSMUST00000058550	Ccni	551.627	0.472	1.251	93181933	93206495	5
31	ENSMUST00000007993	Rbm28	48.005	0.267	1.255	29123576	29165006	6
32	ENSMUST00000018727	G3bp1	267.485	0.390	1.240	55469685	55504838	11
33	ENSMUST00000094053	Tnrc6a	216.498	0.374	1.235	123124181	123195296	7
34	ENSMUST00000109832	Ppp2r5c	97.026	0.280	1.280	110485739	110583062	12
35	ENSMUST00000014339	Dnajc7	135.544	0.339	1.237	100582818	100620168	11
36	ENSMUST00000032888	Arl6ip1	505.361	0.488	1.245	118118891	118129662	7
37	ENSMUST00000027432	Psmd1	273.332	0.421	1.233	86064619	86139151	1
38	ENSMUST00000120381	Stt3a	503.652	0.448	1.270	36729344	36767679	9
39	ENSMUST00000002473	Babam1	176.102	0.342	1.254785	71396861	71404619	8
40	ENSMUST00000111610	Hnrnpc	397.252	0.379	1.298242	52073377	52104028	14
41	ENSMUST00000043775	Kdm3b	291.343	0.456	1.231827	34777047	34838660	18
42	ENSMUST00000029090	Gid8	43.849	0.264	1.312707	180710117	180718733	2
43	ENSMUST00000041733	Taf2	73.060	0.299	1.273169	55015131	55072152	15
44	ENSMUST00000049263	Sltm	199.179	0.365	1.255205	70542870	70592232	9
45	ENSMUST00000021046	Ddx42	44.553	0.279	1.291953	106216926	106249139	11
46	ENSMUST00000102927	Pdpk1	189.442	0.341	1.282937	24073680	24141595	17
47	ENSMUST00000025918	Stip1	257.955	0.401	1.259	7020702	7039967	19
48	ENSMUST00000113360	Cab39	222.930	0.385	1.269	85793441	85851576	1
49	ENSMUST00000151287	Tcp1	378.062	0.429	1.284	12916382	12925067	17
50	ENSMUST00000080919	Thrap3	312.910	0.393	1.296	126164082	126202760	4

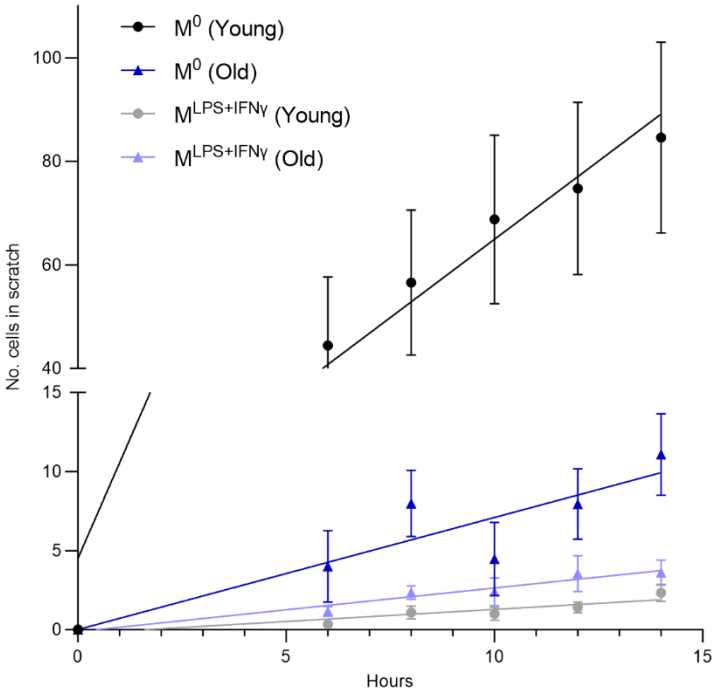
Norm. Exp.: normalised expression as reads per kilobase million (RPKM); SD: standard deviation of log2 of RPKM; MFC: Maximum fold change, a ration of maximum RPKM and mean RPKM value observed for each transcript. To be considered stable this should be less than 2; Chr: Chromosome.

8.4 Supplementary material for Chapter 5 “Ageing-related defects in macrophage function are driven by *MYC* and *USF1* transcriptional programmes”

Supplementary table 8.4.1 Demographics of human donors

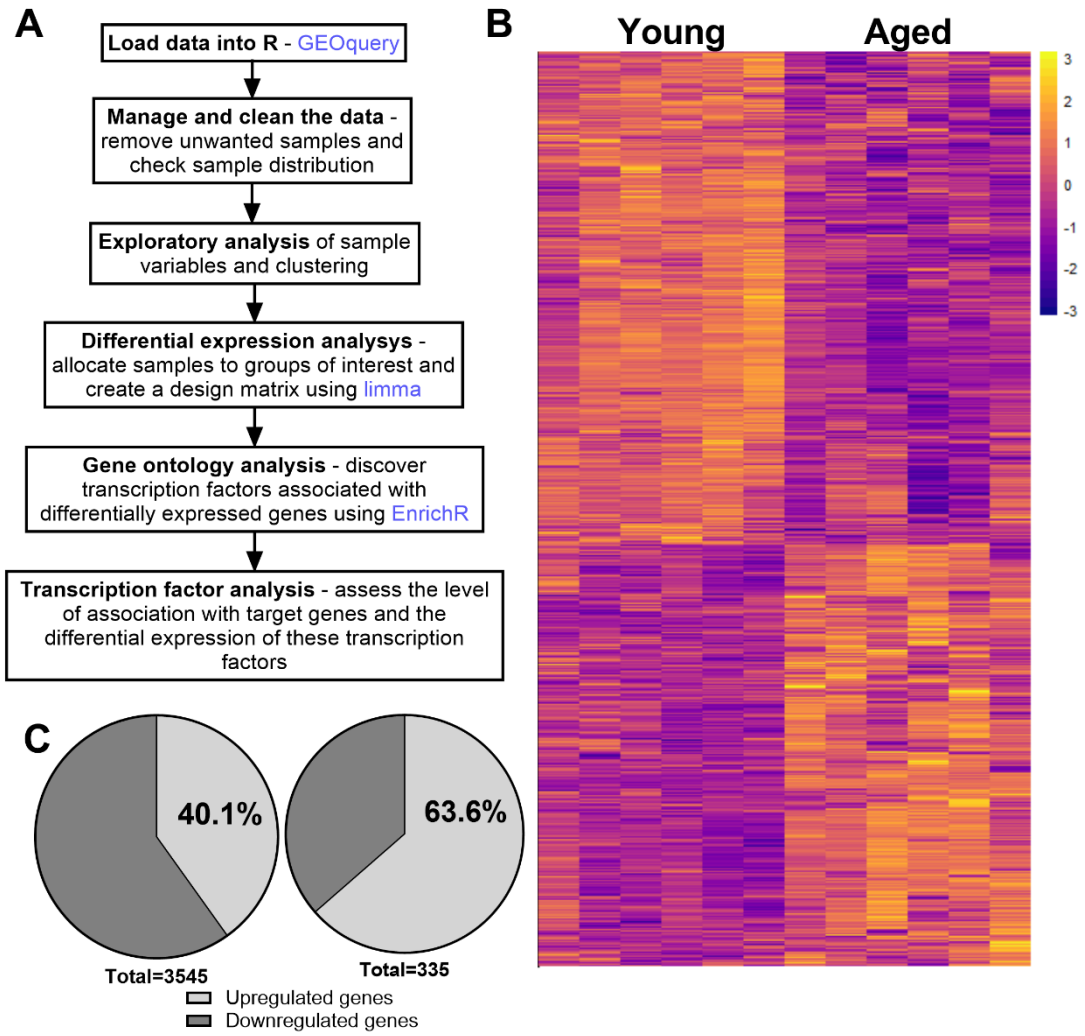
Age range	Mean age	Sex
18-30 years	23.7 ± 1.2 years	Female = 4, Male = 2
>50 years	60.5 ± 5.7 years	Female = 3, Male = 3

Age, sex and medication use were recorded. Ethnicity was not recorded as part of our ethics. Donors were excluded if they had an infection in the last 6 weeks, a vaccination in the past 2 weeks or if they were on medication altering immune function.



Supplementary figure 8.4.1 Motility of $M^{LPS+IFN\gamma}$ polarised human monocyte-derived macrophages

Number of MDMs returning to the scratch from young (22-25 years) and older (54-71 years) subjects over 14 hours, with data being collected at 0, 6, 8, 10, 12 and 14 hours after the scratch was drawn. Data are presented as mean ± SEM with each datapoint representing the mean of six donors, with three fields of view taken per donor for each condition. MDMs were differentiated from human blood for 7 days in M-CSF and then either left unstimulated (M^0) or further polarised for 24 hours with lipopolysaccharide (LPS) and interferon (IFN)-γ ($M^{LPS+IFN\gamma}$).



Supplementary figure 8.4.2 Age-related differentially expressed genes in mouse alveolar macrophages

A – Main steps in the pipeline for published microarray dataset GSE84901 analysis. Boxes represent the overall objectives, blue text shows the packages used.

B – A total of 3,545 genes were differentially expressed with age LogFC > 1, p-value < 0.05.

C – Of these differentially expressed genes, 1,422 (40.1%) were upregulated and 2,123 (59.9%) were downregulated with age. Increasing the threshold of differential expression to LogFC > 1.5 and p-value < 0.05, 213 genes (63.3%) were upregulated and 122 genes (36.4%) were downregulated with age.

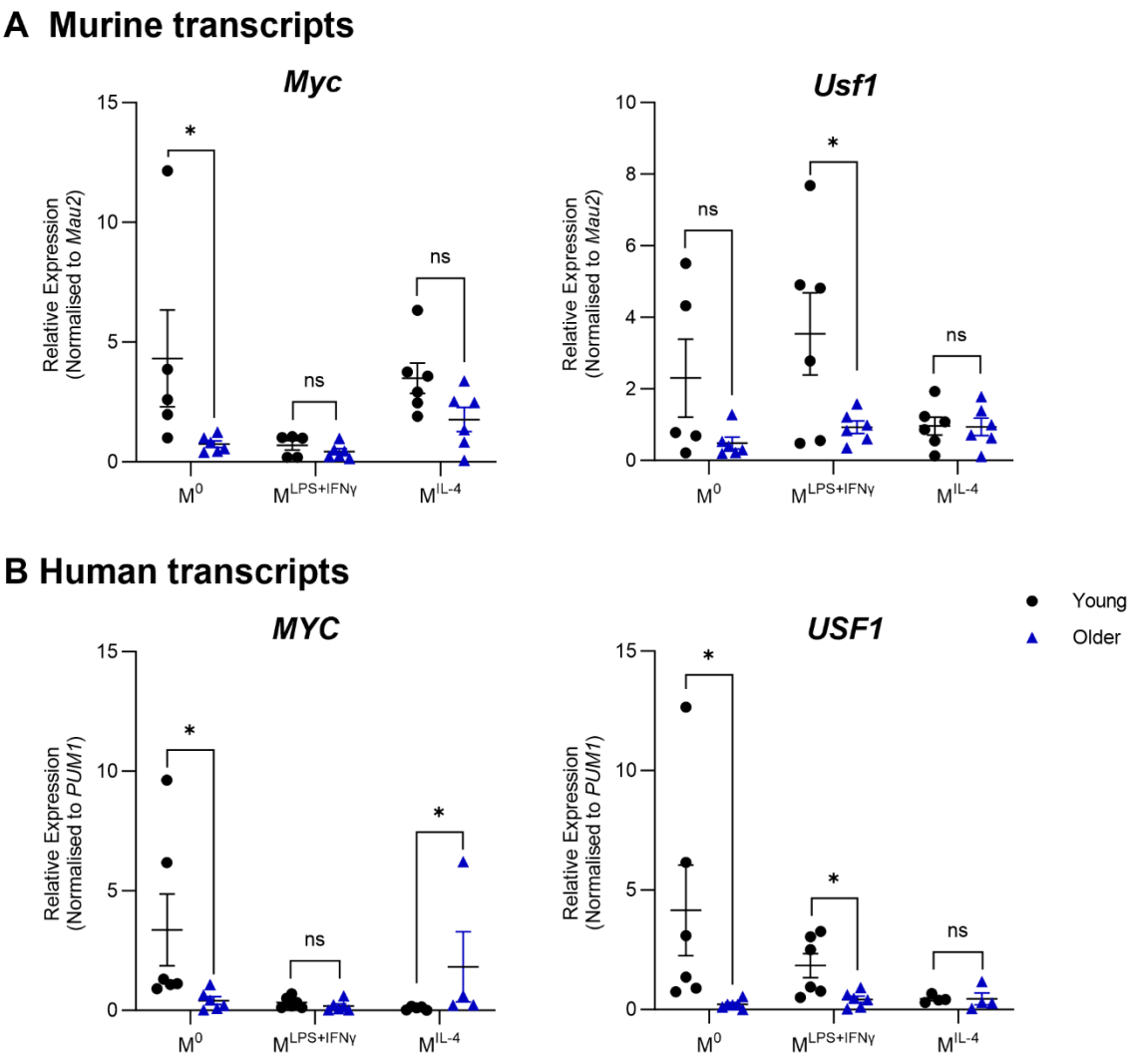
Supplementary table 8.4.2 *USF1* and *MYC* discrete signals from Phenoscanner

Data for 9 discrete (r^2<0.3) eQTL signals for *USF1* and 1 for *MYC* were extracted from Phenoscanner. All eQTL data is from whole blood. SNP marker (rsid), chromosome and position (hg38), effect allele (EA) and frequency (EAF, Eur) is shown alongside position in relation to the gene, source study, P-value and the direction of effect associated with the effect allele. Study: eQTLGen Consortium phase 1, N = 31,684; BIOSQTL, N = 2,116; Joehanes R, N = 5,257. Conducted by Veryan Codd.

Gene	Signal	SNP	Chromosome: position	EA	EAF	Position	Study	P	Direction	Tissue
USF1	1	rs147573079 rs75089506	chr1:161039365 chr1:161044519	A A	0.0129 0.0129	3_prime_UTR intron	eQTLGen eQTLGen	3.06E-11 2.99E-11	- -	Whole blood
USF1	2	rs3737787 rs2073658 rs2073656 rs2073655	chr1:161039733 chr1:161040972 chr1:161041565 chr1:161042800	A T C A	0.2942 0.2942 0.2942 0.2942	3_prime_UTR intron intron intron	BIOSQTL BIOSQTL BIOSQTL BIOSQTL	6.65E-46 5.35E-46 5.35E-46 5.35E-46	+ + + +	Whole blood
USF1	3	rs2516841 rs2774276 rs2073657 rs2516839 rs2774273 rs2516837	chr1:161040984 chr1:161041926 chr1:161041001 chr1:161043331 chr1:161044195 chr1:161044937	A C T T C A	0.2386 0.7614 0.6282 0.6292 0.6292 0.3708	intron intron intron 5_prime_UTR intron 5_prime_UTR	eQTLGen eQTLGen BIOSQTL BIOSQTL BIOSQTL BIOSQTL	2.1E-285 2.5E-286 1.1E-203 7.6E-207 7.6E-207 2.4E-207	- + + + + -	Whole blood
USF1	4	rs17221763 rs17175575	chr1:161041419 chr1:161041525	A T	0.0219 0.0229	splice_region intron	eQTLGen eQTLGen	5.17E-35 4.09E-36	- -	Whole blood
USF1	5	rs2516840	chr1:161041527	A	0.2694	intron	eQTLGen	4.9E-119	-	Whole blood
USF1	6	rs2073653 rs6686076 rs6427572 rs1556260 rs1556259	chr1:161042970 chr1:161043522 chr1:161043813 chr1:161044656 chr1:161044859	T T A T A	0.8678 0.8678 0.1332 0.1322 0.8678	intron intron intron intron intron	eQTLGen eQTLGen eQTLGen eQTLGen eQTLGen	1.1E-122 8.4E-123 1.1E-112 1.2E-122 9.6E-123	+ + - - +	Whole blood
USF1	7	rs149397699	chr1:161044634	T	0.9791	intron	eQTLGen	7.4E-26	+	Whole blood
MYC	1	rs2070583	chr8:127741008	A	0.9821	3_prime_UTR	Joehanes R	2.09E-08	-	Whole blood

Supplementary table 8.4.3 Genome-wide associations with sentinels for 10 *USF1* associations from OpenTargets

SNP	Trait	Trait Category	P-value	Beta	PMID	Author	N
rs12116949	Monocyte percentage of white blood cells	Measurement	9.80E-23	0.0228015	32888494	Vuckovic D	408112
	Monocyte count	Measurement	2.57E-16	0.016888	32888493	Chen MH	521594
	Monocyte percentage	Measurement	8.08E-11	0.0453607		UKB Neale v2	349861
	Mean platelet volume	Measurement	3.92E-10	-0.013118	32888493	Chen MH	460935
	Platelet distribution width	Measurement	7.21E-10	-0.00828198		UKB Neale v2	350470
	Mean platelet volume	Measurement	7.10E-09	-0.0129142	32888494	Vuckovic D	408112
	Platelet distribution width	Measurement	3.30E-08	-0.0129789	32888494	Vuckovic D	408112
rs2774276	Monocyte count	Measurement	4.40E-22	0.020875	32888493	Chen MH	521594
	Monocyte count	Measurement	2.70E-14	0.0167299	34226706	Barton AR	443529
	Lymphocyte counts	Measurement	2.16E-10	0.013872	32888493	Chen MH	524923
	Monocyte percentage of white blood cells	Measurement	1.50E-09	0.0148253	32888494	Vuckovic D	408112
	Lymphocyte counts	Measurement	5.00E-08	0.0133554	32888494	Vuckovic D	408112
rs2516840	Monocyte count	Measurement	4.00E-08	0.011392	32888493	Chen MH	521594



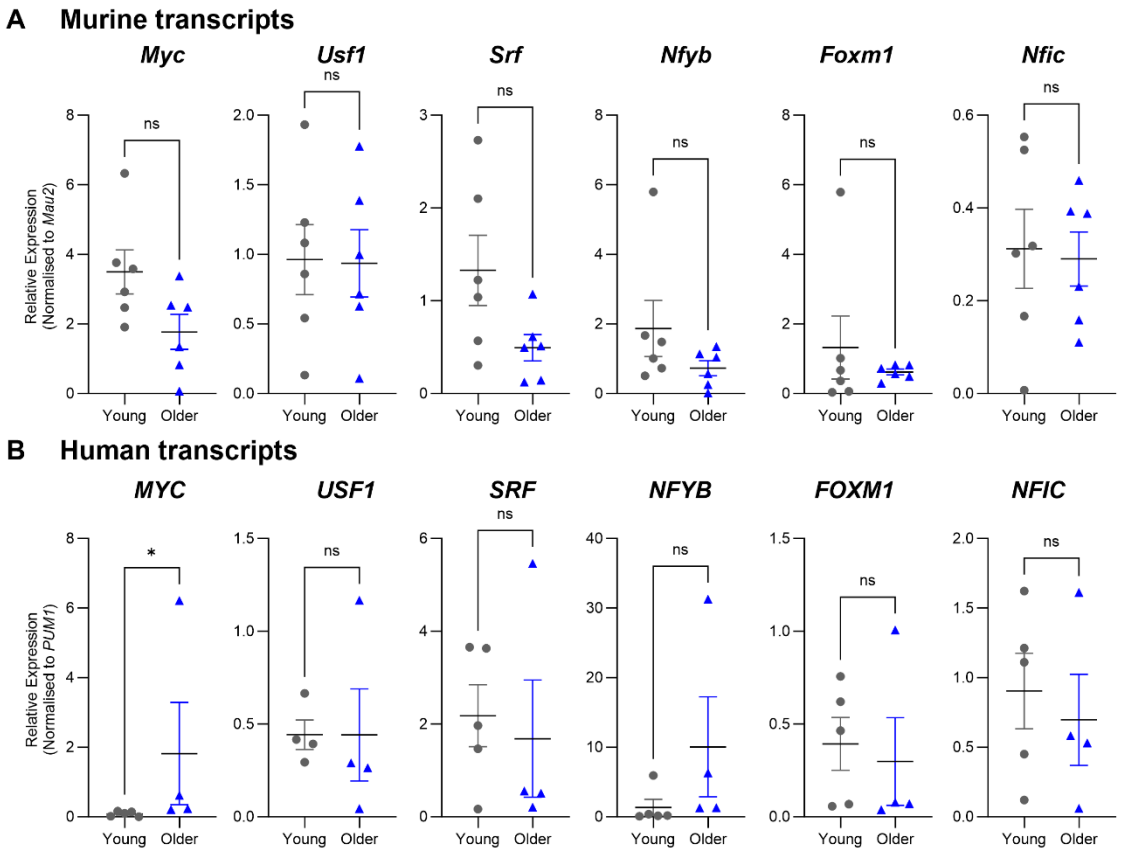
Supplementary figure 8.4.3 Expression of *MYC* and *USF1* with age in murine bone marrow-derived macrophages and human monocyte-derived macrophages polarised towards different phenotypes

A – Age-related changes in expression of *Myc* and *Usf1* in bone marrow-derived macrophages isolated from young (2-5 months) and aged (22-24 months) C57BL/6J mice. *Mau2* expression was used as an internal control.

B – Age-related changes in expression of *MYC* and *USF1* in monocyte-derived macrophages isolated from young (22-25 years) and older (54-71 years) healthy human donors. *PUM1* expression was used as an internal control.

Data are presented at mean ± SEM with each datapoint representing an individual mouse or human donor. N=6, Mann-Whitney test, * $P < 0.05$.

M^0 – cells left unstimulated, $M^{LPS+IFNγ}$ – cells stimulated with lipopolysaccharide (LPS) and interferon (IFN)-γ for 24 hours, M^{IL-4} – cells stimulated with interleukin (IL)-4 for 24 hours.

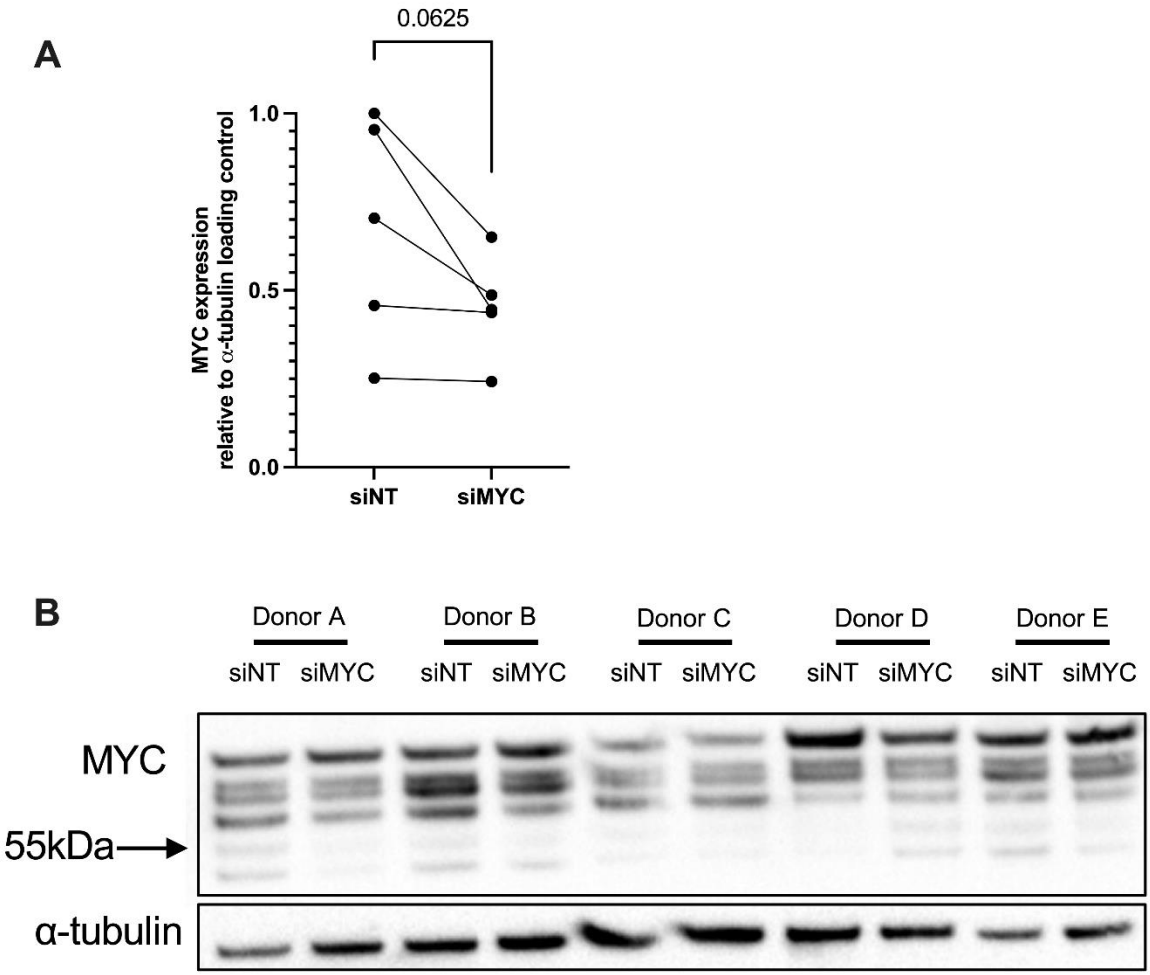


Supplementary figure 8.4.4 Transcription factor expression with age in IL-4-stimulated mouse bone marrow-derived macrophages and human monocyte-derived macrophages

A – Age-related changes in expression of transcription factors in bone marrow-derived macrophages isolated from young (2-5 months) and old (22-24 months) C57BL/6J mice. *Mau2* expression was used as a housekeeping control. N=6, Mann-Whitney test.

B – Age-related changes in expression of transcription factors in monocyte-derived macrophages isolated from young (22-25 years) and older (54-71 years) healthy human donors. *PUM1* expression was used as a housekeeping control. N=4-5, Mann-Whitney test, * $P < 0.05$.

Cells were stimulated with interleukin (IL)-4 for 24 hours following differentiation.

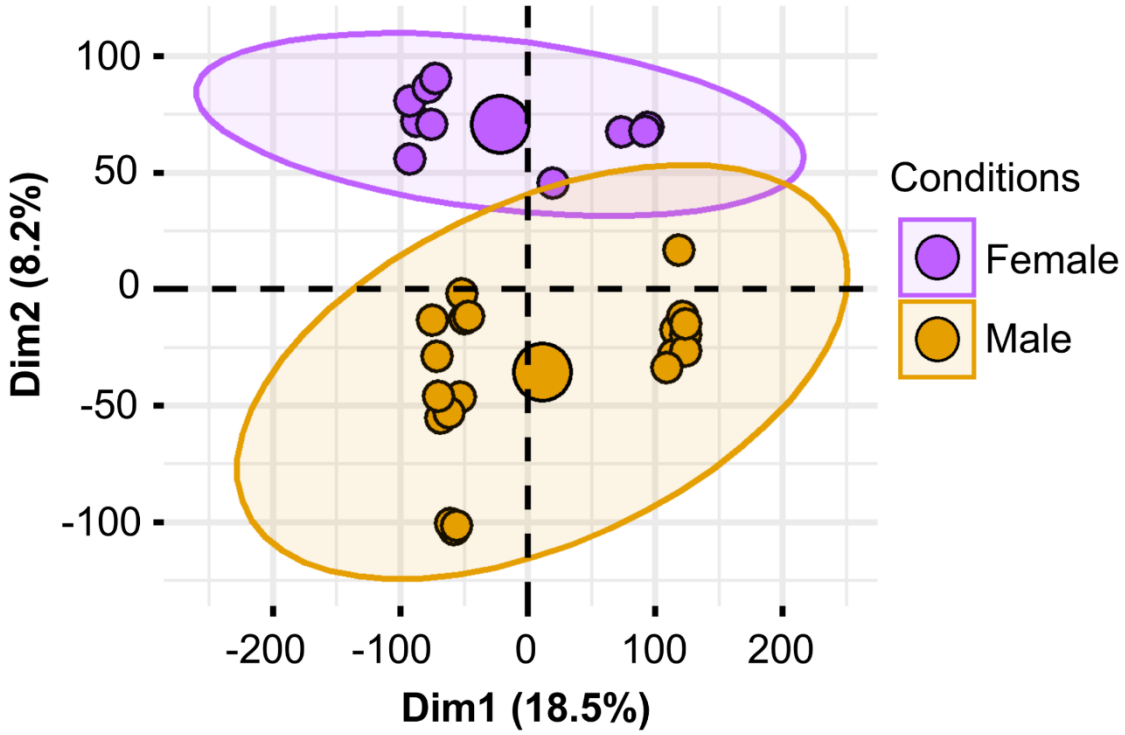


Supplementary figure 8.4.5 Immunoblotting for MYC in human MDMs isolated from 5 young donors with control (siNT) or MYC (siMYC) targeted siRNA knockdown

A – MYC expression quantified from total band density, normalised to α-tubulin loading control band density for each sample, using ImageJ. N=5, Wilcoxon matched-pairs signed rank test, P = 0.0625.

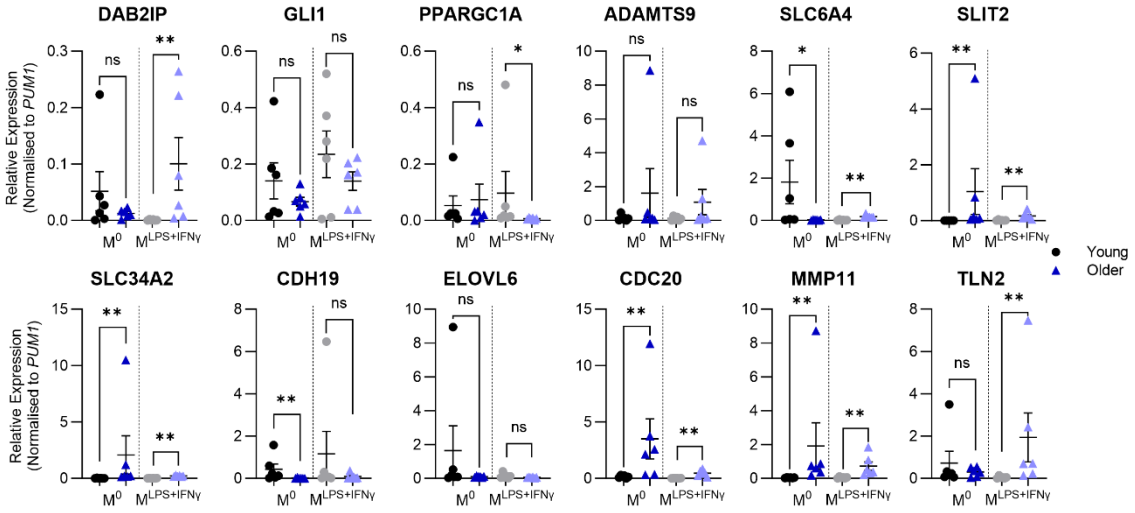
B – Immunoblots of human monocyte-derived macrophages from 5 separate young donors used for quantification in A. Upper blot is anti-human c-MYC, molecular weight 51kDa; all bands were used in quantification since c-MYC undergoes phosphorylation, ubiquitination, O-linked glycosylation, and acetylation. Lower blot is anti-α-tubulin used as loading control.

Conducted by Josh Kimble.



Supplementary figure 8.4.6 Macrophage phenotype and donor sex cause the largest variance in samples for RNA sequencing analysis

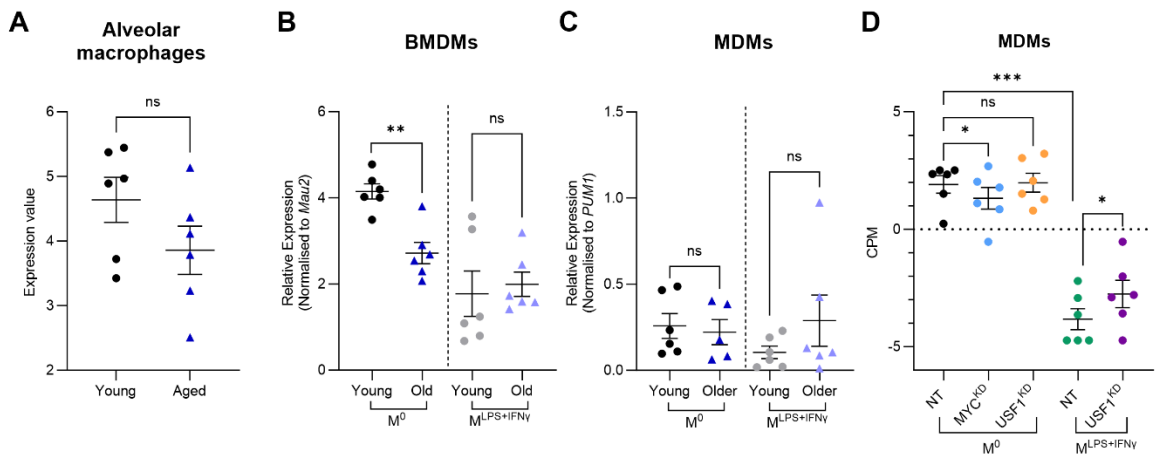
Principal component analysis of all samples highlighting the first two dimensions accounting for variability between samples. This was done using factoextra package in R.



Supplementary figure 8.4.7 Age-related changes in expression of genes of interest from RNA sequencing analysis of transcription factor knockdown

Age-related changes in expression of selected genes in monocyte-derived macrophages isolated from young (22-25 years) and old (54-71 years) healthy human donors. These genes were selected from transcription factor knockdown RNA sequencing comparisons. *PUM1* expression was used as an internal control. N = 6, Mann-Whitney test, * P < 0.05, ** P < 0.01. The qPCRs were conducted by myself and Martha Clements under my supervision.

M^0 – cells left unstimulated, $M^{LPS+IFN\gamma}$ – cells stimulated with lipopolysaccharide (LPS) and interferon (IFN)-γ for 24 hours.



Supplementary figure 8.4.8 *CCR2* expression in different murine and human macrophage populations

A – Age-related changes in *Ccr2* expression in murine alveolar macrophages isolated from young (2-4 months) and aged (22-24 months) C57BL/6 mice via microarray analysis. N=6, Mann-Whitney test.

B – Age-related changes in *Ccr2* expression in bone marrow-derived macrophages isolated from young (2-5 months) and old (20-22 months) C57BL/6 mice via RT-qPCR analysis. BMDMs were differentiated for 5 days with M-CSF and left unstimulated or activated with LPS and IFNγ for 24 hours. *Mau2* expression was used as an internal control. N=6, Mann-Whitney test, ** $P < 0.01$.

C – Age-related changes in *CCR2* expression in monocyte-derived macrophages isolated from young (22-25 years) and older (54-71 years) healthy human donors via RT-qPCR analysis. MDMs were differentiated for 7 days with M-CSF and left unstimulated or activated with LPS and IFNγ for 24 hours. *PUM1* expression was used as an internal control. N=6, Mann-Whitney test.

D – Relative expression of *CCR2* after *MYC* or *USF1* knockdown in young human monocyte-derived macrophages via RNA sequencing analysis. MDMs were differentiated for 7 days with M-CSF and stimulated with siRNA for 72 hours. They were then left unstimulated or activated with LPS and IFNγ for 24 hours. N=6, One-way ANOVA with Sidak's multiple comparison, * $P < 0.05$, *** $P < 0.001$.

Supplementary table 8.4.4 Hallmarks associated with differentially expressed genes in si*MYC* M^0 *vs* M^0 control by GSEA analysis

Hallmark gene set	Size	ES	Nominal P value	Genes
Interferon alpha response	56/95	-0.55	0.000	SP110, TDRD7, CMTR1, IFITM2, HLA-C, CCRL2, LGALS3BP, CASP8, SAMD9, IFI44, PARP14, B2M, DDX60, IL7, PARP12, PSMA3, MOV10, PSMB9, EIF2AK2, LAMP3, PARP9, EPSTI1, PSME2, BST2, MX1, PSMB8, CNP, HERC6, NUB1, IFITM3, PROCR, HELZ2, IFI35, TRIM21, CMPK2, IFIT3, RSAD2, ISG15, IFI44L, IFI27, IFIT2, LY6E, OASL, UBE2L6, PSME1, LAP3, TAP1, NMI, ISG20, USP18, TRAFD1, IFITM1, GMPR, IL15, CXCL11, GBP4
MTORC1 signalling	98/197	-0.48	0.000	HMGCR, SLC1A5, P4HA1, SLC7A11, PNP, PRDX1, ADD3, PSMA3, HSPD1, ATP5MC1, M6PR, ETF1, PPIA, PSAT1, CORO1A, HMGCS1, CDKN1A, RDH11, TM7SF2, TBK1, ARPC5L, ACTR3, PSMB5, EBP, DDX39A, G6PD, AURKA, SEC11A, ATP2A2, RPN1, HSPA9, SLC7A5, MAP2K3, ACLY, CCT6A, ACTR2, CALR, CFP, GLRX, GAPDH, RRM2, STARD4, PSMC2, GLA, PFKL, CACYBP, DHCR24, PPP1R15A, GBE1, CDC25A, SLC2A1, PPA1, PSME3, TOMM40, ENO1, RAB1A, HSPA5, DHCR7, FKBP2, LDLR, PSMC4, STIP1, HSP90B1, LTA4H, NFKBIB, TUBA4A, PSMD14, SQLE, TCEA1, MTHFD2, SERPINH1, IDI1, SLC9A3R1, RPA1, PSMA4, SC5D, ALDOA, ELOVL5, NUP205, PSPH, TUBG1, TES, NFIL3, SRD5A1, NUPR1, SYTL2, EIF2S2, ABCF2, HPRT1, SDF2L1, CYP51A1, MTHFD2L, HSPA4, ACSL3, STC1, POLR3G, ELOVL6, CTH
MYC target V1	100/195	-0.45	0.000	LDHA, MCM7, ODC1, RPL18, SF3B3, CCT2, SRPK1, NOLC1, EIF1AX, EIF4G2, RACK1, CDK4, RAD23B, SYNCRIP, PRDX3, EIF4E, UBE2E1, HNRNPA3, UBE2L3, HNRNPA2B1, HSPD1, PRPF31, PWP1, ETF1, PPIA, TOMM70, PSMA1, NDUFAB1, PRPS2, ILF2, YWHAQ, RPL22, CUL1, BUB3, RNPS1, AIMP2, RUVBL2, RPL14, SNRPA1, PSMA7, PSMD3, SRSF3, XRCC6, SNRPD2, CSTF2, PTGES3, TRIM28, HNRNPD, PCNA, PSMD8, SNRPB2, SET, SERBP1, MCM5, SSB, CBX3, PCBP1, HDGF, ERH, C1QBP, CCT5, PSMB3, SNRPD1, PSMD7, UBA2, NCBP2, CCNA2, CDK2, CCT3, PSMA6, HNRNPR, EIF2S1, GOT2, RANBP1, PSMC4, CYC1, COX5A, PSMD14, VDAC1, GLO1, KPNA2, MRPL23, CCT7, PSMD1, G3BP1, PSMA4, SNRPD3, HNRNPC, PRDX4, HSP90AB1, EXOSC7, PPM1G, EIF2S2, HPRT1, SSBP1, VBP1, TXNL4A, PSMB2, PSMA2, NCBP1
Oxidative phosphorylation	115/200	-0.44	0.000	COX7A2L, NQO2, MGST3, SLC25A5, ECH1, NDUFA3, VDAC3, DLAT, ATP5F1B, NDUFB7, UQCR10, ATP6V1D, SURF1, IMMT, ATP5MC3, COX8A, DECR1, SDHB, COX7A2, ATP5PD, COX6A1, NDUFB2, PDHB, SLC25A6, ACAA2, LDHA, PDHA1, COX5B, HTRA2, UQCRC1, ACAA1, SUPV3L1, NDUFC2, HCCS, SDHD, MRPS22, ACADM, MDH1, AIFM1, OGDH, GLUD1, PRDX3, ISCU, MPC1, ATP5MC1, UQCRB, TIMM50, COX7C, TOMM70, GRPEL1, NDUFS4, FXN, UQCRH, NDUFAB1, ATP5F1A, ATP1B1, LDHB, ETFB, HSPA9, ATP5F1D, MDH2, ATP6AP1, MRPS12, ATP5PF, OPA1, MRPS15, MTRR, ACAT1, SLC25A11, NDUFB4, ATP6V1G1, BAX, ATP6V0B, CYCS, GPX4, ACO2, COX4I1, ATP5F1E, MTX2, UQCRQ, FH, NDUFA9, UQCRFS1, MRPS11, TOMM22, ATP5F1C, HSD17B10, TIMM8B, GOT2, ATP6V1E1, CYC1, COX5A, VDAC2, NDUFB3, ATP6V0E1, NDUFS6, VDAC1, DLD, NDUFV2, CYB5R3, COX6C, ATP6V1F, COX17, TIMM17A, FDX1, COX11, TIMM10, CASP7, MRPL15, NNT, ATP6V0C, CYB5A, NDUFA8, COX6B1, ATP6V1H
TNF signaling via NFKB	107/199	-0.44	0.000	RHOB, LAMB3, KLF6, TGIF1, CCRL2, JUNB, TNIP2, JAG1, CD80, DNAJB4, IL15RA, ICAM1, EIF1, SLC16A6, TLR2, PNRC1, NFKBIA, CXCL2, CXCL3, DUSP5, G0S2, KLF4, TRIP10, PLEK, YRDC, EGR1, B4GALT5, TRAF1, CCL4, BIRC2, TNF, NINJ1, BIRC3, IL6, GCH1, BTG1, GEM, ACKR3, IER5, CDKN1A, CLCF1, BCL2A1, FOSL1, TRIB1, TNFAIP6, PLAUR, DUSP4, IER2, IER3, IL23A, MAP2K3, TNFAIP3, PMEPA1, GFPT2, KYNU, NFE2L2, CXCL1, B4GALT1, KLF9, NR4A3, MAFF, DUSP1, DRAM1, ATF3, PHLDA1, NFKB1, CD83, HES1, CSF2, PANX1, PPP1R15A, TSC22D1, FOS, EHD1, RCAN1, CCL2, ID2, TNFRSF9, TANK, IFIT2, LDLR, CCL20, CEBPB, DDX58, TAP1, MSC, IL12B, JUN, NR4A1, PTX3, SPHK1, SPSB1, FOSB, IFNGR2, SLC2A6, KLF2, TNFSF9, TUBB2A, NFKBIE, NFIL3, LIF, PHLDA2, RELB, BTG3, SDC4, CXCL11, PLK2
Interferon gamma response	87/198	-0.41	0.007	B2M, DDX60, ZBP1, IL7, PNP, PARP12, ZNFX1, CASP3, PSMA3, FAS, IL6, GCH1, BTG1, SOCS1, FCGR1A, CDKN1A, IRF4, PSMB10, CFB, TNFAIP6, PSMB9, PTPN6, EIF2AK2, CD274, TNFAIP3, OAS2, SLAMF7, EPSTI1, PSME2, GBP6, BPGM, BST2, MX1, CD40, MT2A, PSMB8, MVP, CCL7, HERC6, IFITM3, NFKB1, HELZ2, MYD88, LYSMD2, IFI35, STAT1, ITGB7, TRIM21, CMPK2, IFIT3, RSAD2, PML, VCAM1, ISG15, IDO1, IFI44L, CCL2, SRI, OAS3, AUTS2, IFI27, NLRC5, IFIT2, LY6E, OASL, IFIT1, UBE2L6, PSME1, DDX58, LAP3, TAP1, NUP93, NMI, MTHFD2, ISG20, USP18, TOR1B, TRAFD1, GPR18, CASP7, IL15, ISOC1, ARL4A, PSMB2, PSMA2, CXCL11, GBP4
Androgen response	41/99	-0.4	0.027	XRCC5, HOMER2, B2M, ANKH, FKBP5, HMGCR, IQGAP2, HMGCS1, MERTK, XRCC6, UBE2J1, PMEPA1, B4GALT1, ELK4, DHCR24, TSC22D1, ALDH1A3, SLC38A2, SMS, ACTN1, DBI, ELL2, PTPN21, CENPN, IDI1, MYL12A, SRP19, STK39, ADRM1, TPD52, ELOVL5, CDC14B, VAPA, KLK2, NKX3-1, MAP7, NGLY1, ACSL3, APPBP2, SPDEF, BMPR1B
G2M checkpoint	86/195	0.43	0.031	TRAIP, CDC20, SMAD3, PURA, ARID4A, KNL1, KIF20B, BUB1, CENPA, EGF, EXO1, PDS5B, NDC80, NUMA1, POLA2, CCNB2, RPS6KA5, SS18, STMN1, KIF23, SMC4, YTHDC1, MAD2L1, CDC7, ATRX, PLK4, SRSF10, MAP3K20, ORC6, RBM14, NUSAP1, RBL1, STAG1, CKS1B, TNPO2, CCNF, EZH2, CCNT1, POLE, ODF2, CDC6, PTTG3P, RASAL2, XPO1, NOTCH2, LBR, RACGAP1, KIF4A, KIF15, PBK, HMMR, E2F2, MTF2, CENPF, MKI67, TACC3, MCM3, CUL5, DBF4, MCM6, KIF11, PLK1, SFPQ, WRN, CDKN1B, CDC25B, INCENP, MYBL2, NUP50, DKC1, KIF22, PAFAH1B1, ESPL1, LIG3, SMARCC1, KMT5A, TROAP, EWSR1, CENPE, SAP30, TRA2B, PRPF4B, RAD54L, ABL1, ILF3, PTTG1

Supplementary table 8.4.5 Hallmarks associated with differentially expressed genes in si*USF1* M^0 vs M^0 control by GSEA analysis

Hallmark gene set	Size	ES	Nominal P value	Genes
Interferon alpha response	57/95	-0.6	0.000	CSF1, UBE2L6, IRF9, SELL, B2M, LAMP3, PARP9, LAP3, DDX60, ADAR, TRIM5, STAT2, PLSCR1, OAS1, PARP14, RTP4, SP110, DHX58, SAMD9L, CCRL2, GMPR, BST2, EIF2AK2, IFI44, GBP4, UBA7, IFITM2, EPSTI1, IFI30, IL7, PSMB9, LGALS3BP, TRIM25, TAP1, HERC6, MX1, IFI35, IFITM1, OASL, CXCL10, IFIT3, ISG15, IFIT2, IFITM3, LY6E, USP18, CMPK2, ISG20, TRAFD1, RSAD2, CD74, LPAR6, IFI44L, PARP12, IFI27, NCOA7, CXCL11
Interferon gamma response	89/198	-0.4	0.019	PML, RIPK1, SAMHD1, UBE2L6, NOD1, PTPN1, IRF9, B2M, APOL6, LAP3, XAF1, CD274, NFKB1, DDX60, ICAM1, ADAR, STAT2, CFB, PLSCR1, ARL4A, STAT1, PARP14, RTP4, SP110, DHX58, SAMD9L, IRF4, RNF213, METTL7B, KLRK1, BST2, IDO1, EIF2AK2, JAK2, MT2A, IFI44, CFH, GBP4, CSF2RB, TOR1B, GPR18, IFITM2, GBP6, EPSTI1, GZMA, DDX58, IFI30, OAS3, MX2, IL7, PSMB9, SLAMF7, LGALS3BP, HLA-DRB1, CMKLR1, TRIM25, OAS2, TAP1, IFNAR2, HERC6, MX1, IFI35, OASL, CXCL10, IFIT3, ISG15, TNFAIP2, IFIT2, IFITM3, LY6E, USP18, CIITA, HLA-DMA, IL18BP, CMPK2, HLA-DQA1, IFIT1, ISG20, TRAFD1, RSAD2, IRF5, ZBP1, CD74, IFI44L, BANK1, PARP12, PIM1, IFI27, CXCL11
MYC targets	100/195	0.53	0.000	DEK, PGK1, ORC2, CDC20, LDHA, GSPT1, PA2G4, ACP1, HDDC2, CTPS1, HDAC2, MCM5, COPS5, NHP2, CDC45, BUB3, SYNCRIP, IFRD1, MRPL9, SMARCC1, SRSF3, RAN, FBL, EIF2S1, TRA2B, U2AF1, PSMA4, MYC, HDGF, EIF3J, CCNA2, PSMA2, TYMS, HNRNPA3, CCT4, HPRT1, PSMC4, PSMD3, AP3S1, PSMA6, YWHAE, USP1, XPO1, HNRNPR, PRPS2, SET, PWP1, PPIA, LSM2, NME1, GNL3, POLD2, SNRPD2, RFC4, RAD23B, VDAC1, MCM6, TARDBP, SNRPD1, TCP1, APEX1, ETF1, HNRNPA1, RPS6, CCT7, RPS3, EIF3B, HNRNPA2B1, SSBP1, EIF1AX, SF3A1, EIF4H, PRDX3, ILF2, PSMC6, NPM1, SRM, RSL1D1, SNRPG, SRSF1, KPNA2, XRCC6, SNRPB2, RUVBL2, HSPE1, PSMA1, STARD7, CCT2, AIMP2, CDK2, MRPS18B, DDX21, PSMD7, FAM120A, EIF4E, CBX3, SERBP1, HSPD1, IMPDH2, CCT3
Glycolysis	103/198	0.5	0.000	PPFIA4, DEPDC1, SAP30, CENPA, PLOD2, EGFR, STC2, BIK, SPAG4, TFF3, FBP2, NT5E, PGK1, DCN, PGAM1, COL5A1, FUT8, PAXIP1, MIF, TPI1, PAM, EGLN3, EFNA3, KDELR3, LDHA, ERO1A, AK4, VLDLR, PFKP, CHST2, B4GALT7, ENO2, IRS2, VCAN, B3GNT3, RPE, AGL, PKM, HS2ST1, ENO1, SLC16A3, STC1, NDST3, ALDH7A1, PYGL, QSOX1, ANGPTL4, PLOD1, CASP6, LHPP, ANKZF1, GALK2, COPB2, PGM2, KIF2A, CHST12, DDIT4, ALDH9A1, PSMC4, ALDOA, FAM162A, ADORA2B, IGFBP3, LHX9, HMMR, XYLT2, PC, NOL3, CHST6, POLR3K, GLCE, GMPPA, KIF20A, PPIA, CHST4, GMPPB, GALK1, GFPT1, ME2, B4GALT4, ARTN, GALE, CLDN9, PFKFB1, ME1, TPST1, ALG1, VEGFA, CITED2, IER3, AGRN, P4HA1, P4HA2, GUSB, PGAM2, ARPP19, PHKA2, B4GALT2, NDUFV3, SLC37A4, MERTK, DLD, GYS1
Hypoxia	78/197	0.46	0.000	PPARGC1A, PPFIA4, CP, SAP30, AKAP12, PKP1, PCK1, INHA, EGFR, STC2, DTNA, LOX, ALDOC, PGK1, GCK, GPI, DCN, PFKFB3, COL5A1, KDM3A, BCAN, PGM1, MIF, ETS1, TPI1, PAM, BNIP3L, EFNA3, KDELR3, NR3C1, LDHA, ENO3, BGN, ERO1A, AK4, VLDLR, PRDX5, TMEM45A, PFKP, CHST2, SLC2A1, ENO2, PPP1R3C, IRS2, NDST1, TPD52, PDK1, B4GALNT2, KIF5A, SLC2A5, WSB1, PRKCA, ENO1, XPNPEP1, GCNT2, STC1, MYH9, ANGPTL4, BCL2, CASP6, SLC25A1, ANKZF1, ACKR3, PGM2, SIAH2, CA12, DDIT4, ALDOA, FAM162A, ADORA2B, IGFBP3, GAPDH, STBD1, NEDD4L, CAVIN1, TPST2, GBE1, SERPINE1
MTORC1 signaling	94/197	0.44	0.003	PHGDH, BUB1, PLOD2, PGK1, HMGCR, GPI, IGFBP5, FADS1, SORD, PGM1, PITPNB, LDLR, TPI1, UNG, EGLN3, LDHA, CCNG1, SRD5A1, ADIPOR2, ERO1A, AK4, VLDLR, ACACA, SLC2A1, CORO1A, UFM1, COPS5, PDK1, BCAT1, ENO1, IFRD1, PSMC2, GMPS, DHCR7, STC1, NAMPT, PSMA4, UCHL5, PSMG1, MLLT11, DDIT4, HPRT1, PSMC4, ALDOA, DHFR, GAPDH, CYB5B, ATP6V1D, CCT6A, TMEM97, XBP1, SLC1A4, GBE1, PSMB5, SSR1, ARPC5L, CCNF, EEF1E1, CACYBP, ACSL3, PPIA, SERPINH1, ACTR2, ITGB2, ELOVL5, STARD4, PNP, SERP1, SCD, ETF1, PNO1, SLC2A3, FADS2, CYP51A1, EDEM1, PFKL, SEC11A, PSMC6, ME1, FDXR, YKT6, PSMD12, P4HA1, ADD3, TES, ACTR3, ATP2A2, HSPE1, SLC37A4, TUBG1, CD9, TCEA1, TM7SF2, HSPD1
Unfolded protein response	57/110	0.44	0.020	STC2, DKC1, KDELR3, ERO1A, DDX10, CKS1B, NHP2, EIF2AK3, CNOT2, POP4, DCP2, SDAD1, EIF2S1, TTC37, MTREX, DDIT4, CXXC1, XBP1, SLC1A4, SSR1, EEF2, ERN1, EXOC2, BAG3, FUS, GEMIN4, CHAC1, DNAJB9, CNOT6, DNAJC3, SPCS3, ALDH18A1, SERP1, EIF4EBP1, LSM4, DCTN1, EDEM1, SEC11A, EXOSC5, NPM1, KIF5B, CNOT4, VEGFA, TUBB2A, EIF4A2, BANF1, NABP1, CEBPB, SHC1, PREB, TATDN2, EIF4E, LSM1, SRPRB, ZBTB17, PAIP1, NOLC1
Oxidative phosphorylation	105/200	0.42	0.013	ACADSB, GPI, SLC25A12, NDUFB1, SLC25A4, LDHA, ALDH6A1, COX17, TIMM50, NDUFB6, COX10, MRPL11, SUCLG1, PDHX, UQCRB, NDUFC1, ECHS1, DLST, NDUFA9, NDUFS1, GRPEL1, MTRF1, ECH1, ATP5MF, ATP5F1E, UQCR11, FXN, TOMM22, CS, COX7A2L, MRPS22, RETSAT, NDUFA1, ATP6V1D, HTRA2, ATP5PD, ATP5F1C, NDUFB8, ETFDH, NDUFA2, ISCA1, AFG3L2, ATP5ME, UQCRFS1, VDAC1, ALAS1, ATP5MC3, ISCU, VDAC2, ACAA1, GLUD1, MRPL35, NDUFB3, COX6B1, NDUFA4, NDUFC2, ATP5MG, MTRR, PRDX3, COX7A2, PDP1, BAX, ETFA, NDUFB2, POLR2F, BDH2, ATP5F1A, NDUFA6, PMPCA, SLC25A11, TIMM13, NDUFS3, SDHA, TIMM17A, FH, MTX2, ATP5PF, DLD, NDUFB5, MFN2, ETFB, SDHC, HCCS, NDUFA5, RHOT2, COX7C, TOMM70, IDH2, NDUFS6, NDUFS8, BCKDHA, ACADM, CYC1, MDH1, ATP5F1B, SDHD, SLC25A6, UQCRQ, LRPPRC, GOT2, IDH3A, MGST3, TIMM8B, OGDH, PDHB

Supplementary table 8.4.6 Hallmarks associated with differentially expressed genes in si*USF1* $M^{LPS+IFN\gamma}$ vs $M^{LPS+IFN\gamma}$ control by GSEA analysis

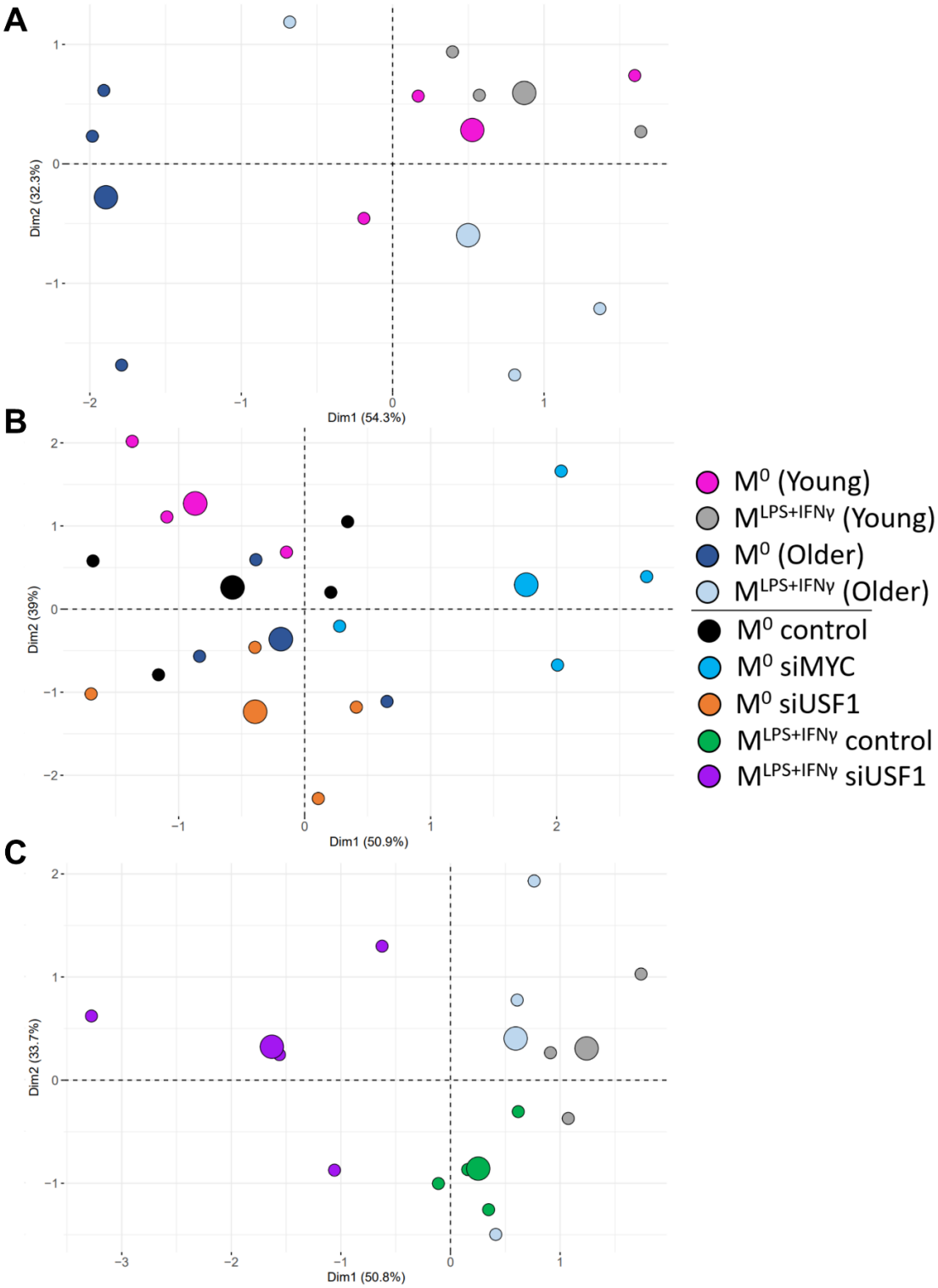
Hallmark gene set	Size	ES	Nominal P value	Genes
Hypoxia	91/197	-0.52	0.000	HEXA, PDK3, HAS1, VLDLR, VHL, SLC2A1, TPD52, CAVIN3, TPBG, CHST3, GPC3, TES, JUN, CA12, GBE1, SRPX, SIAH2, PLAUR, GPC4, PPFIA4, CDKN1C, SLC6A6, KDELR3, DPYSL4, PKLR, PGF, PDK1, CASP6, SDC3, IL6, CAVIN1, TGM2, KLHL24, PRKCA, SCARB1, TGFBI, SLC2A5, WSB1, ETS1, NDST1, MAP3K1, VEGFA, SLC2A3, HS3ST1, PHKG1, ERO1A, FBP1, ENO2, DDIT3, PGM1, ILVBL, STC2, FOSL2, GAPDHS, KLF7, ACKR3, ATP7A, EDN2, NFIL3, HK2, IRS2, FOXO3, LARGE1, LDHA, CITED2, ANKZF1, TPST2, BTG1, TKTL1, SDC2, LOX, KIF5A, CP, COL5A1, KDM3A, ERRFI1, P4HA1, PCK1, CDKN1B, MXI1, DCN, BNIP3L, EFNA3, CAV1, CXCR4, DDIT4, TMEM45A, PPARGC1A, CCNG2, STC1, BCAN
Angiogenesis	17/36	-0.63	0.012	TIMP1, JAG2, FGFR1, LRPAP1, ITGAV, VEGFA, LPL, NRP1, SPP1, APP, CXCL6, VCAN, TNFRSF21, FSTL1, OLR1, PF4, STC1
Heme metabolism	93/194	-0.48	0.000	SLC30A1, KHNYN, MPP1, HDGF, CTNS, MINPP1, SIDT2, SLC22A4, PPOX, TYR, PSMD9, HEBP1, DCAF11, FBXO34, ADIPOR1, CCND3, SLC2A1, CAST, DMTN, SDCBP, SMOX, MFHAS1, RBM5, TMCC2, TNS1, NFE2L1, RHD, FOXJ2, UROD, EPB41, IGSF3, FN3K, SYNJ1, HTRA2, ADD2, PICALM, ARL2BP, HTATIP2, HMBS, EIF2AK1, TCEA1, ACKR1, RNF123, MYL4, VEZF1, CA2, EPOR, GAPVD1, LMO2, LRP10, CLCN3, PC, LAMP2, NEK7, BMP2K, CTSB, SLC7A11, GDE1, EZH1, DAAM1, ABCG2, FOXO3, ALDH1L1, TFDP2, ASNS, FECH, CCDC28A, AGPAT4, TFRC, RANBP10, XK, ARHGEF12, ELL2, TRIM58, TRAK2, ALAD, ACSL6, KAT2B, YPEL5, DCUN1D1, HBQ1, CROCCP2, ALDH6A1, NUDT4, ERMAP, MXI1, BNIP3L, GATA1, MOSPD1, TSPAN5, CAT, SLC6A9, MBOAT2
Bile acid metabolism	49/112	-0.51	0.000	HSD17B4, GNMT, DHCR24, LONP2, CYP46A1, CYP8B1, PIPOX, AQP9, PEX13, ABCA6, HACL1, PXMP2, ABCD1, ATXN1, TFCP2L1, HSD17B6, PNPLA8, PECR, FADS2, HSD17B11, FADS1, PHYH, ABCD2, ALDH8A1, ALDH1A1, ABCA8, BMP6, HSD3B1, RXRA, SLC27A2, IDH2, IDH1, ABCA1, NR3C2, SLC29A1, SLC27A5, HAO1, NUDT12, AMACR, CYP7A1, PEX11G, ABCD3, PEX1, SLC23A1, ALDH9A1, PEX7, CAT, PEX19, ABCA4
Mitotic spindle	198	-0.45	0.000	NCK2, SMC1A, CLIP1, KIF1B, LATS1, RANBP9, FSCN1, CKAP5, FGD6, TBCD, ARHGEF2, SUN2, SPTAN1, SSH2, ARAP3, TPX2, CCDC88A, SMC3, EPB41, CCNB2, PCNT, DST, WASF2, CLIP2, STK38L, NIN, ROCK1, TRIO, RACGAP1, PALLD, NF1, TUBGCP3, GSN, TSC1, NCK1, PLK1, RABGAP1, DYNLL2, LLGL1, CENPF, PPP4R2, ALS2, ARHGEF3, ARFGEF1, ARHGAP29, BIRC5, KIF4A, MARK4, HOOK3, RHOF, ITSN1, RASAL2, DOCK2, VCL, FGD4, SPTBN1, CDK1, TUBA4A, EZR, ACTN4, SAC3D1, TLK1, NEDD9, SYNPO, ARHGEF12, WASF1, SOS1, WASL, GEMIN4, NDC80, PKD2, CNTRL, EPB41L2, CDC42BPA, APC, CD2AP, AURKA, NEK2, NUSAP1, MID1IP1, ABL1, ECT2, SASS6, SHROOM2
Apical junction	81/199	-0.44	0.000	RRAS,MMP2,ADAM23,FSCN1,ADRA1B,SIRPA,MPZL2,GNAI2,MPZL1,CNN2,TJP1,BAIAP2,CRB3,HRAS,NF2,CDSN,SYK,ITGA3,CRAT,SGCE,NF1,CDH4,ITGA2,LIMA1,SDC3,TSC1,ITGA9,TGFBI,PTPRC,CD99,COL17A1,INPPL1,CX3CL1,CLDN7,ALOX15B,JUP,MSN,CD34,NECTIN3,ITGB1,RHOF,NLGN2,CD86,GRB7,VCL,YWHAH,PDZD3,ACTN4,ADAM9,JAM3,B4GALT1,PCDH1,COL16A1,DSC3,PIK3CB,WASL,CERCAM,AKT3,VCAN,SLC30A3,GTF2F1,IRS1,MAPK14,TMEM8B,EPB41L2,CLDN6,EXOC4,PTEN,FBN1,PECAM1,MMP9,MDK,NECTIN1,AMIGO1,ARHGEF6,CADM3,PARVA,CDH8,ACTA1,SHROOM2,VWF
MTORC1 signaling	89/197	-0.42	0.010	TXNRD1, SKAP2, LGMN, ALDOA, MLLT11, ME1, ACTR2, STIP1, PSME3, IGFBP5, CACYBP, SC5D, VLDLR, SLC7A5, SLC2A1, DHCR24, SCD, TES, FGL2, ADIPOR2, GBE1, CDC25A, SLC1A5, CFP, MTHFD2, STARD4, CYP51A1, EIF2S2, SQLE, EBP, HMGCR, SLC6A6, RRM2, BCAT1, SSR1, ITGB2, HMBS, HMGCS1, CANX, PDK1, UNG, TCEA1, LDLR, CORO1A, PLOD2, ACLY, PLK1, DHFR, CTH, IFRD1, SLC2A3, CALR, RPA1, ERO1A, DDIT3, SLC7A11, PGM1, SLC9A3R1, LTA4H, FADS2, PHGDH, ATP2A2, SLC1A4, NAMPT, RDH11, FADS1, NFIL3, HK2, LDHA, TUBA4A, ASNS, CD9, TFRC, SORD, TRIB3, IDH1, PSPH, SHMT2, PSAT1, UCHL5, DHCR7, GOT1, AURKA, P4HA1, ELOVL6, CXCR4, DDIT4, ADD3, STC1
MYC targets V2	35/57	0.61	0.000	PPAN, NOP16, AIMP2, PES1, NOP2, RRP9, GNL3, SUPV3L1, HSPE1, RABEPK, MRTO4, IMP4, IPO4, PUS1, NPM1, NOLC1, MPHOSPH10, NOC4L, DUSP2, PPRC1, WDR43, PA2G4, WDR74, MYBBP1A, SRM, UTP20, RCL1, TBRG4, TCOF1, MCM4, HSPD1, NOP56, NDUFAF4, NIP7, CBX3
MYC targets V1	111/195	0.50	0.000	CDC20, PSMC6, SNRPA, NME1, NOP16, AIMP2, MRPL9, RRP9, GNL3, HSPE1, NHP2, ODC1, RPS10, EIF3B, SNRPD2, MRPL23, ABCE1, LSM7, DUT, TCP1, HDDC2, SNRPG, PSMB3, IMPDH2, FBL, C1QBP, CCT2, SNRPD1, NPM1, RANBP1, EEF1B2, RAN, SRSF7, ORC2, NOLC1, PSMA2, EIF3D, PRPS2, NDUFAB1, CCT3, PA2G4, CDC45, SRSF2, PTGES3, SRM, DHX15, TRA2B, RFC4, COX5A, PCNA, CSTF2, CTPS1, SNRPA1, TUFM, TOMM70, PSMD3, MCM4, HDAC2, RPS5, HSPD1, CUL1, CYC1, ILF2, SNRPB2, NOP56, TRIM28, PHB2, EIF4A1, PSMA1, ERH, RPS2, VBP1, APEX1, PSMA4, CCT5, CCT7, HNRNPA2B1, SNRPD3, CBX3, HNRNPC, CAD, HSP90AB1, RNPS1, EIF3J, TXNL4A, XRCC6, HNRNPR, RUVBL2, MYC, PPIA, HNRNPA1, PSMD14, RPL14, POLD2, LSM2, AP3S1, POLE3, DDX21, RPL6, SSB, RPL18, VDAC1, STARD7, PSMA7, PSMA6, SF3B3, UBE2E1, PSMD1, PSMD8, RPS6, SRSF3

Supplementary table 8.4.7 Enriched biological processes associated with differentially expressed genes between young (2-4 months) and old (22-24 months) alveolar macrophages isolated from C57BL/6J mice

Biological process	Gene count	Adjusted P value	Genes
Chromosome segregation	52/324	2.35E-10	Cpeb1; Ska1; Cdc20; Incenp; Anapc1; Dscc1; Birc5; Cenpq; Ube2c; Brca1; Ncapd2; Ect2; Ccnb1; Nusap1; Cenpt; Hjurp; Bub1b; Atrx; Plk1; Cenpn; Cdca8; Mis18a; Cenpk; Nipbl; Kif23; Nuf2; Uvrag; Top2a; Fbxo5; Usp9x; Prc1; Nsl1; Nup37; Chmp1a; Sirt7; Cenpf; Mad2l1; Bub1; Spag5; Fmn2; Chmp6; Aurkb; Dsn1; Ska3; Mms19; Cep192; Cenpw; Ncaph; Ncapg; Knstrn; Kif4; Oip5
Mitotic cell cycle phase transition	56/376	3.17E-10	Cdkn2a; Ube2e2; Ccnb2; Pdpn; Cdkn2b; Anapc1; Zfp36l1; Tcf19; Birc5; Cks1b; Tm4sf5; Plk2; Ube2c; Brca1; Ccnb1; Cdkn2c; Tjp3; Hinfp; Bub1b; Akt1; Cdc25c; Cdk4; Psme3; Pole; Plk1; Plrg1; Fhl1; Ptpn6; Cdk1; Mtbp; Pten; Ercc3; Chek1; Usp47; Fbxo5; FoxMLPS+IFNγ; Mepce; Brsk1; Sirt7; Cenpf; Mad2l1; Rfwd3; Bub1; Ticrr; Iqgap3; Aven; Cacul1; Dtl; Prmt2; Cdk5rap3; Aurkb; Cep192; Anxa1; Rdx; Cdk2; Cdc25a
Regulation of mitotic cell cycle phase transition	44/265	2.16E-09	Cdkn2a; Ube2e2; Pdpn; Cdkn2b; Zfp36l1; Birc5; Tm4sf5; Ube2c; Brca1; Ccnb1; Tjp3; Bub1b; Akt1; Cdc25c; Cdk4; Psme3; Plk1; Plrg1; Fhl1; Ptpn6; Cdk1; Mtbp; Pten; Ercc3; Chek1; Usp47; Fbxo5; Mepce; Brsk1; Cenpf; Mad2l1; Rfwd3; Bub1; Ticrr; Aven; Dtl; Prmt2; Cdk5rap3; Aurkb; Cep192; Anxa1; Rdx; Cdk2; Cdc25a
Mitotic sister chromatid segregation	32/151	2.39E-09	Cdc20; Incenp; Anapc1; Dscc1; Birc5; Ube2c; Ncapd2; Ccnb1; Nusap1; Bub1b; Atrx; Plk1; Cdca8; Cenpk; Nipbl; Kif23; Nuf2; Fbxo5; Prc1; Nsl1; Chmp1a; Mad2l1; Bub1; Spag5; Chmp6; Aurkb; Dsn1; Cep192; Ncaph; Ncapg; Knstrn; Kif4
Cell cycle phase transition	57/415	2.39E-09	Cdkn2a; Ube2e2; Ccnb2; Pdpn; Cdkn2b; Anapc1; Zfp36l1; Tcf19; Birc5; Cks1b; Tm4sf5; Plk2; Ube2c; Brca1; Ccnb1; Cdkn2c; Tjp3; Hinfp; Bub1b; Akt1; Cdc25c; Cdk4; Psme3; Pole; Plk1; Plrg1; Fhl1; Ptpn6; Cdk1; Mtbp; Pten; Ercc3; Chek1; Usp47; Fbxo5; FoxMLPS+IFNγ; Mepce; Brsk1; Sirt7; Cenpf; Mad2l1; Rfwd3; Bub1; Paf1; Ticrr; Iqgap3; Aven; Cacul1; Dtl; Prmt2; Cdk5rap3; Aurkb; Cep192; Anxa1; Rdx; Cdk2; Cdc25a
Mitotic nuclear division	43/268	6.31E-09	Igf1; Cdc20; Incenp; Pdgfb; Anapc1; Dscc1; Birc5; Ube2c; Ncapd2; Ccnb1; Nusap1; Il1b; Bub1b; Atrx; Plk1; Cdca8; Cenpk; Fbxw5; Nipbl; Mtbp; Kif11; Chek1; Kif23; Nuf2; Fbxo5; Prc1; Nsl1; Aaas; Chmp1a; Sirt7; Mad2l1; Bub1; Ereg; Spag5; Chmp6; Aurkb; Dsn1; Cep192; Ncaph; Ncapg; Knstrn; Kif4; Nme6
Regulation of cell cycle phase transition	45/297	1.31E-08	Cdkn2a; Ube2e2; Pdpn; Cdkn2b; Zfp36l1; Birc5; Tm4sf5; Ube2c; Brca1; Ccnb1; Tjp3; Bub1b; Akt1; Cdc25c; Cdk4; Psme3; Plk1; Plrg1; Fhl1; Ptpn6; Cdk1; Mtbp; Pten; Ercc3; Chek1; Usp47; Fbxo5; Mepce; Brsk1; Cenpf; Mad2l1; Rfwd3; Bub1; Paf1; Ticrr; Aven; Dtl; Prmt2; Cdk5rap3; Aurkb; Cep192; Anxa1; Rdx; Cdk2; Cdc25a
Sister chromatid segregation	33/181	4.14-08	Cdc20; Incenp; Anapc1; Dscc1; Birc5; Ube2c; Ncapd2; Ccnb1; Nusap1; Bub1b; Atrx; Plk1; Cdca8; Cenpk; Nipbl; Kif23; Nuf2; Top2a; Fbxo5; Prc1; Nsl1; Chmp1a; Mad2l1; Bub1; Spag5; Chmp6; Aurkb; Dsn1; Cep192; Ncaph; Ncapg; Knstrn; Kif4
Negative regulation of cell cycle	56/451	1.12E-07	Cdkn2a; Apbb2; Cdkn3; Cdkn2b; Pmp22; Zfp36l1; Birc5; H2-M3; Plk2; Sgsm3; Brca1; Myc; Ppp1r10; Ccnb1; Cdkn2c; Btg1; Kntc1; Hinfp; Dna2; Bub1b; Atrx; Cdk4; Cdk5rap1; Plk1; Nsun2; Fhl1; Cdk1; Cdk9; Mtbp; Pten; Trim35; Chek1; Wee1; Mdm4; Usp47; Top2a; Fbxo5; FoxMLPS+IFNγ; Chmp1a; Gadd45a; Brsk1; Mad2l1; Rfwd3; Bub1; Map2k1; Ticrr; Nabp2; Aven; Dtl; Prmt2; Cdk5rap3; Aurkb; Gas2l1; Cep192; Trrap; Nme6
Mitotic cytokinesis	19/69	2.83E-07	Kif20a; Snx18; Cenpa; Stmn1; Incenp; Birc5; Ckap2; Ect2; Nusap1; Plk1; Sptbn1; Unc119; Anln; Kif23; Prc1; Chmp1a; Chmp6; Kif4; Usp8
Nuclear chromosome segregation	39/262	3.15E-07	Cpeb1; Cdc20; Incenp; Anapc1; Dscc1; Birc5; Cenpq; Ube2c; Ncapd2; Ect2; Ccnb1; Nusap1; Bub1b; Atrx; Plk1; Cdca8; Cenpk; Nipbl; Kif23; Nuf2; Top2a; Fbxo5; Prc1; Nsl1; Chmp1a; Sirt7; Cenpf; Mad2l1; Bub1; Spag5; Fmn2; Chmp6; Aurkb; Dsn1; Cep192; Ncaph; Ncapg; Knstrn; Kif4
Negative regulation of mitotic cell cycle	36/236	6.65E-07	Cdkn2b; Zfp36l1; Birc5; Plk2; Brca1; Ppp1r10; Ccnb1; Btg1; Kntc1; Bub1b; Atrx; Plk1; Fhl1; Cdk1; Mtbp; Pten; Trim35; Chek1; Wee1; Usp47; Top2a; Fbxo5; Gadd45a; Brsk1; Mad2l1; Rfwd3; Bub1; Ticrr; Nabp2; Aven; Prmt2; Cdk5rap3; Aurkb; Cep192; Trrap; Nme6
Cytoskeleton-dependent cytokinesis	21/94	1.76E-06	Kif20a; Snx18; Cenpa; Stmn1; Incenp; Birc5; Ckap2; Ect2; Nusap1; Plk1; Sptbn1; Unc119; Anln; Kif23; Prc1; Chmp1a; Fmn2; Chmp6; Aurkb; Kif4; Usp8
Regulation of cell-cell adhesion	51/427	1.78E-06	Pla2g2d; Cdkn2a; Cd74; H2-Aa; Pdpn; Igf1; CeacaMLPS+IFNγ; H2-Ab1; Il6st; Itga6; Nfkbid; L1cam; H2-M3; Gm5150; Trpv4; Jak3; Malt1; Btn1a1; Il1b; Akt1; Icosl; Xbp1; Zfp608; Ptpn6; Dmtn; Tnfrsf21; Sh2b3; Cela2a; Cblb; Irak1; Rap1gap; Traf6; Ass1; Fut4; Bmi1; Fermt3; Ptafr; Erbb2; Nlrp3; Itch; Nr4a3; Map2k1; Anxa1; Cyld; Akna; Rdx; VcaMLPS+IFNγ; IcaMLPS+IFNγ; Specc1l; Ccl5; Nck2
DNA repair	55/481	1.99E-06	Zranb3; Mcm3; Nudt16l1; Terf2ip; Brca1; Recql; Ap5s1; Mcm5; Mms22l; Hinfp; Dna2; Actl6a; Mcm2; Parp2; Parp1; Atrx; Trim28; Fbxo6; Pole; Gins2; Huwe1; Cdk7; Pole2; Cdk9; Rad51; Nipbl; Ercc3; Chek1; Dmap1; Morf4l2; Usp47; Eid3; Uvrag; FoxMLPS+IFNγ; Ube2v1; Smug1; Exo1; Rfc3; Sirt7; Rad51ap1; Nfrkb; Rfwd3; Fmn2; Fancg; Ticrr; Gtf2h2; Nabp2; Dtl; Neil3; Wdr48; Mms19; Bod1l; Tdp2; Cdk2; Trrap

Supplementary table 8.4.8 Hallmarks associated with differentially expressed genes in old vs young C57BL/6J mouse alveolar macrophages

Hallmark gene sets	Size	ES	Nominal P value	Genes
MYC targets V1	78/126	-0.49	0.049	ODC1, TUFM, KARS1, CLNS1A, SNRPD1, CCT7, NME1, XRCC6, SNRPG, EIF4H, C1QBP, TFDP1, HDAC2, PHB1, SRSF7, PRDX3, CTPS1, SNRPD3, EIF2S1, PSMA6, RAN, KPNA2, DDX21, H2AZ1, GOT2, ORC2, HNRNPC, EIF4E, SERBP1, PCNA, CCT2, CYC1, PA2G4, PHB2, MCM7, RRP9, UBE2E1, MCM4, CSTF2, TXNL4A, MCM6, CNBP, TYMS, USP1, SLC25A3, RUVBL2, PSMC6, RACK1, PSMD1, GNL3, SNRPA1, RNPS1, NDUFAB1, SRSF3, TCP1, IARS1, NOP16, PRPS2, VBP1, KPNB1, IMPDH2, CCT3, CDK4, NCBP1, DUT, PPM1G, MYC, TOMM70, EXOSC7, PRPF31, LSM2, MRPS18B, MRPL9, CDK2, CDC45, TARDBP, AIMP2, MAD2L1
G2m checkpoint	51/116	-0.57	0.022	MKI67, HIRA, LMNB1, ARID4A, ORC6, UCK2, RACGAP1, E2F4, JPT1, TACC3, STMN1, AURKA, TROAP, KIF22, KPNB1, HMMR, HUS1, HMGB3, RASAL2, SMC2, CDK4, RBL1, TENT4A, MEIS1, PLK1, ODF2, POLE, CHAF1A, NEK2, MYC, NDC80, STIL, BRCA2, BIRC5, UBE2C, FBXO5, PLK4, EXO1, BUB1, NSD2, MCM3, CDC45, CDC6, CENPA, KIF23, TOP2A, NUSAP1, MAD2L1, KIF11, CDKN3, PBK
E2F targets	76/130	-0.57	0.014	DNMT1, POLE4, UBR7, STAG1, MCM7, TUBG1, PRIM2, MCM4, CIT, DONSON, MCM6, USP1, MYBL2, CENPE, MKI67, EXOSC8, TK1, LMNB1, RAD50, ORC6, TIPIN, NUDT21, ZW10, GINS4, RPA3, TRIP13, RACGAP1, PMS2, JPT1, TACC3, STMN1, CHEK2, AURKA, E2F8, KIF22, PPM1D, TIMELESS, HMMR, SNRPB, HUS1, CNOT9, UBE2T, CENPM, HMGB3, CDK4, DUT, MELK, PLK1, TCF19, CDCA3, TBRG4, POLE, NBN, CSE1L, MYC, CCP110, PRKDC, BRCA2, BUB1B, BIRC5, ANP32E, PLK4, RAD1, MCM3, PSIP1, SPAG5, SHMT1, TOP2A, MAD2L1, PSMC3IP, SLBP, WEE1, BRCA1, DSCC1, CDKN3, CDCA8
Spermatogenesis	25/51	-0.46	0.020	ZC3H14, STRBP, TCP11, ZPBP, PSMG1, TSN, GMCL1, PIAS2, SCG5, RAD17, PHF7, TNP2, AURKA, COIL, PRKAR2A, PCSK1N, POMC, NEK2, MTOR, RPL39L, MLF1, BUB1, NCAPH, PARP2, CDKN3
Epithelial mesenchymal transition	31/94	0.53	0.030	COL4A2, COL4A1, DPYSL3, SLC6A8, FSTL1, ECM1, GADD45A, GPC1, ANPEP, MYL9, ITGB3, DST, FGF2, AREG, CXCL12, VEGFA, ID2, MMP14, FAP, LAMC2, SDC4, SERPINE1, FLNA, SPP1, IGFBP4, SNTB1, ACTA2, CADM1, PVR, SAT1, LUM
Allograft rejection	30/110	0.47	0.035	CDKN2A, CXCL9, CSF1, EREG, PRKCB, CCL7, PF4, HLA-DQA1, ICOSLG, ITGAL, NLRP3, CXCR3, EIF4G3, HLA-DOA, IL13, ETS1, CD74, STAB1, GBP2, AKT1, HLA-DMB, HLA-G, CXCL13, IL10, CARTPT, CCL2, CCL4, SOCS1, FLNA, CD3E



Supplementary figure 8.4.9 PCA plots for cell morphology and actin staining

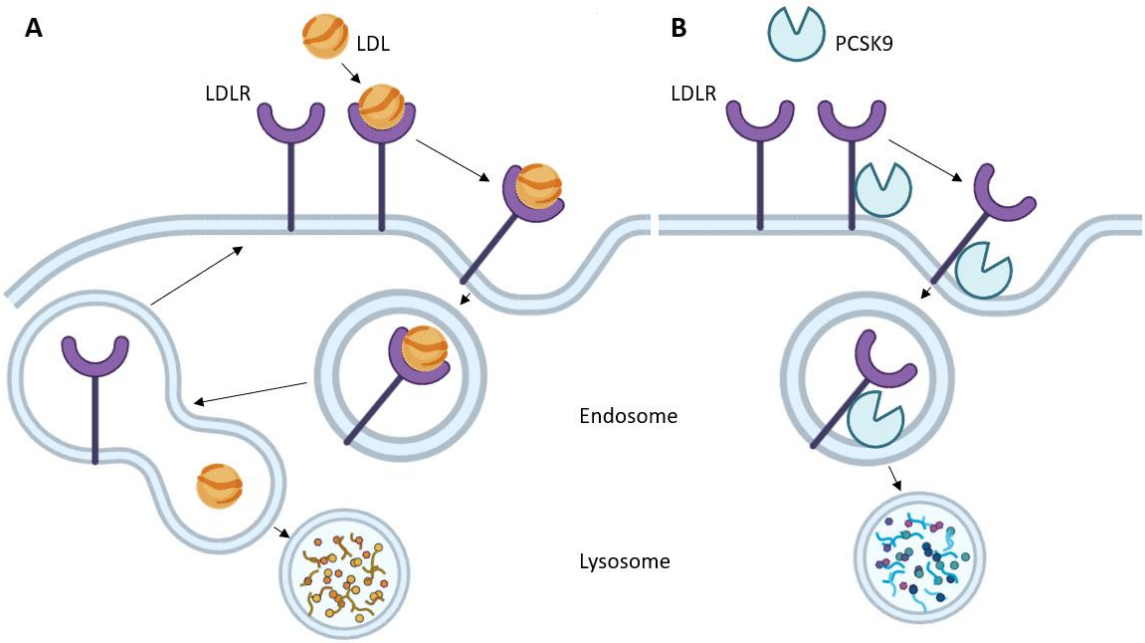
Principal component analysis clustering samples by cell size, circularity and mean fluorescence intensity using prcomp in R.

A – Young and old samples.

B – All M^0 samples.

C – All $M^{LPS+IFN\gamma}$ samples.

8.5 Supplementary material for Chapter 6 “Further insights into the role of *MYC* and *USF1* in macrophage ageing”

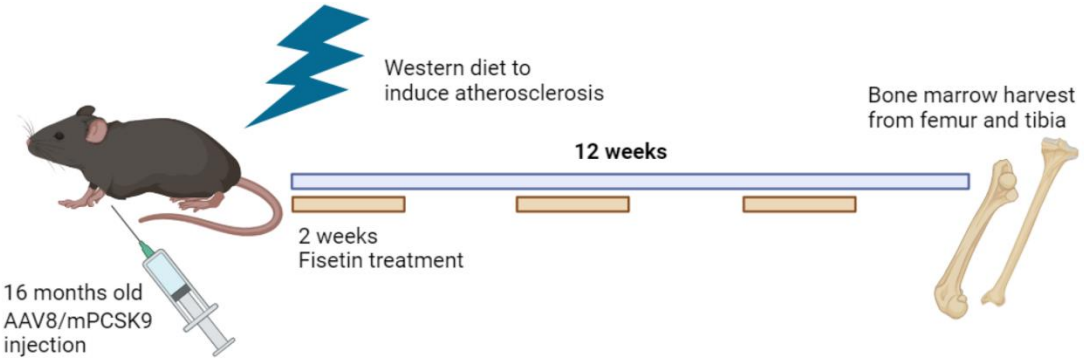


Supplementary figure 8.5.1 Mechanisms of PCSK9 on LDL receptor to initiate atherosclerosis

A – Normally, LDL binds to its receptor which is then internalised into an endosome. The LDL receptor splits from LDL and is recycled to the cell surface while LDL is degraded in a lysosome.

B – In the presence of PCSK9, PCSK9 binds the LDL receptor and prevents LDL binding. This complex is internalised and degraded so that the LDL receptor can no longer function. Adapted from [728,729]. Created in Biorender.

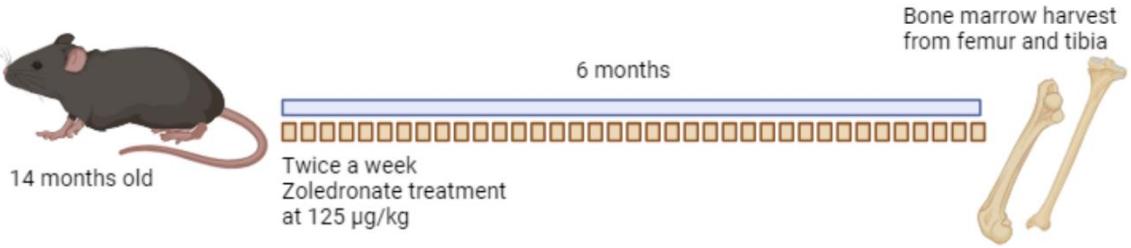
LDL: low density lipoprotein; LDLR: LDL receptor; PCSK9: proprotein convertase subtilisin/kexin type 9



Supplementary figure 8.5.2 Mouse Fisetin treatment design model

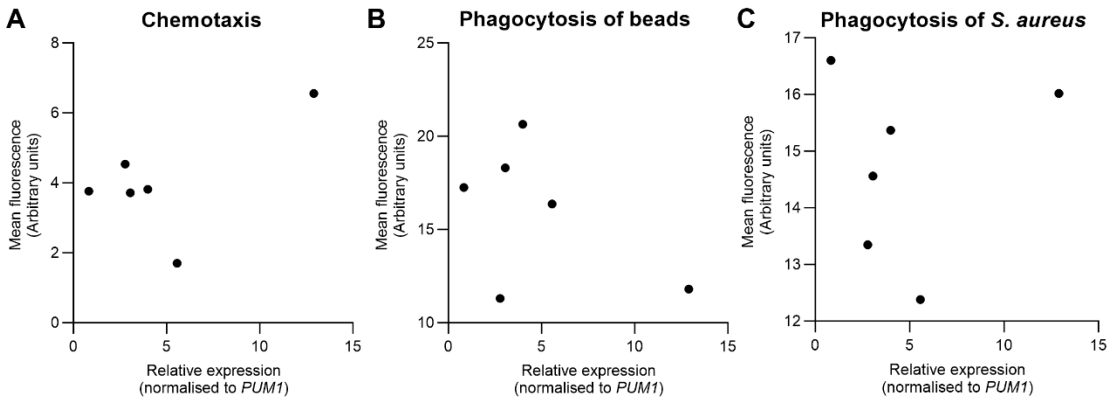
At 16 months of age, C57BL6/J male mice were injected with AAV8/mPCSK9 to induce atherosclerosis. They were then split into two groups, one receiving western diet alone for 12 weeks and the other receiving western diet with Fisetin treatment every 2 weeks for 2 weeks for a total of 12 weeks. Bone marrow was then extracted from femur and tibia bones and differentiated into bone marrow-derived macrophages.

PCSK9 – proprotein convertase subtilisin/kexin kinase type 9



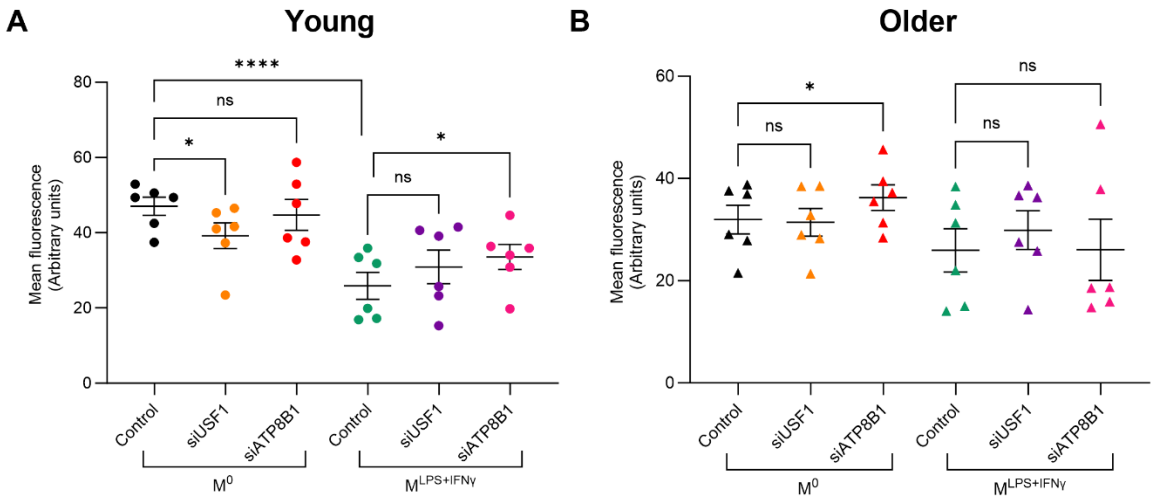
Supplementary figure 8.5.3 Mouse Zoledronate treatment design model

At 14 months of age, mice began treatment with Zoledronate at 125 µg/kg twice a week for 6 months. Bone marrow was extracted from femur and tibia bones and differentiated into bone marrow-derived macrophages.



Supplementary figure 8.5.4 Relative expression of *USF1* has no effect on chemotaxis or phagocytosis in monocytes isolated from young human donors

Comparison of RT-qPCR analysis of *USF1* expression plotted against chemotactic ability (A), phagocytosis of beads (B) and phagocytosis of *Staphylococcus aureus* in human freshly isolated monocytes from young (22-26 years) individuals. *PUM1* was used as a housekeeping control. Each datapoint represents an individual donor. N = 6. Statistical analysis was not performed.

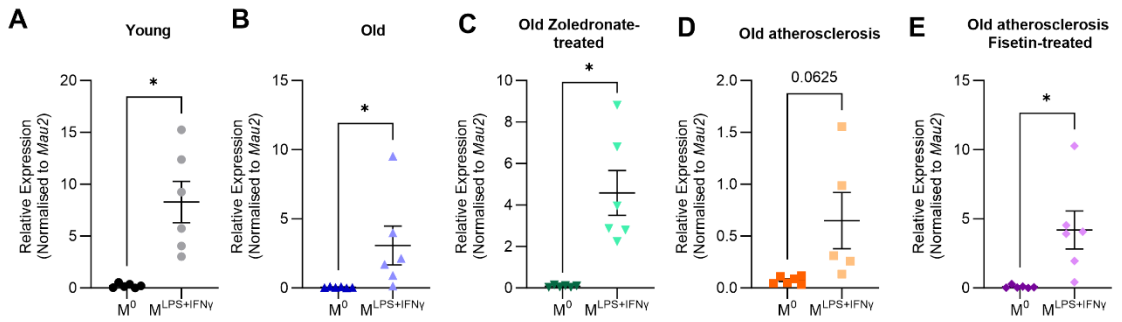


Supplementary figure 8.5.5 Effect of loss of *USF1* or *ATP8B1* on phagocytosis in human monocyte-derived macrophages

A – Bead uptake expressed as mean fluorescence intensity from young (22-25 years) human MDMs with loss of *USF1* or *ATP8B1*.

B – Bead uptake expressed as mean fluorescence intensity from older (54-71 years) human MDMs with loss of *USF1* or *ATP8B1*.

Data are presented as mean ± SEM with each datapoint representing the mean of three fields of view taken per donor, normalised to 4°C control. MDMs were differentiated for 7 days with M-CSF and left unstimulated or activated with lipopolysaccharide (LPS) and interferon (IFN)-γ for 24 hours. Data are presented as mean ± SEM with each datapoint representing the mean of an individual donor performed in triplicate. N=6, one-way ANOVA with Tukey's multiple comparison, * $P < 0.05$. **** $P < 0.0001$. ATP8B1 knockdown was performed by myself and Martha Clements under my supervision.



Supplementary figure 8.5.6 *Calhm6* expression in bone marrow-derived macrophages isolated from mice of different ages

RT-qPCR analysis of *Calhm6* expression in bone marrow-derived macrophages isolated from young (2-5 months) (A), old (20-22 months) (B), old Zoledronate-treated (20-22 months) (C), old atherosclerotic (20 months) (D) and old atherosclerotic Fisetin-treated (20 months) (E) C57BL6/J mice. BMDMs were either left untreated or treated with lipopolysaccharide (LPS) and interferon (IFN)-γ for 24 hours. *Mau2* expression was used as a housekeeping control. Data are presented as mean ± SEM with each datapoint representing the mean of an individual mouse performed in triplicate. N=6, Wilcoxon test, * $P < 0.01$.