The impact of ageing on monocytes and macrophages

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Abbreviations:

β-galactosidase	- β-gal
Cyclooxygenase 2	- COX2
C Reactive protein	- CRP
Dasatinib and Quercetin	- D+Q
Dendritic cells	- DCs
Interferon	- IFN
IFN regulatory transcription factor	- IRF8
Giant cell arteritis	- *GCA*
Lipopolysaccharide	- LPS
Micro RNA	- miR
Pattern recognition receptors	- PRRs
Polymyalgia rheumatica	- *PMR*
Prostaglandin 2	- PGE_2
Retinoic acid–inducible gene I	- RIG-I
Respiratory syncytial virus	- RSV
S-(2,3-bis(palmitoyloxy)-(2-RS)-propyl)-*N*-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys_4-	
OH,trihydrochloride	- Pam3Cys
Mammalian target of rapamycin complex 1	- TORC1
Tumor necrosis factor receptor–associated factor 3	- TRAF3
Toll-like receptors	- TLRs
Varicella-Zoster virus	- VZV

Abstract

Ageing is a global burden. Increasing age is associated with increased incidence of infections and cancer and decreased vaccine efficacy. This increased morbidity observed with age, is believed to be due in part to a decline in adaptive immunity, termed immunosenescence. However not all aspects of immunity decrease with age as ageing presents with systemic low grade chronic inflammation, characterised by elevated concentrations of mediators such as IL-6, TNFα and C Reactive protein (CRP). Inflammation is a strong predictor of morbidity and mortality, and chronic inflammation is known to be detrimental to a functioning immune system. Although the source of the inflammation is much discussed, the key cells which are believed to facilitate the inflammageing phenomenon are the monocytes and macrophages.

In this review we detail how macrophages and monocytes phenotype and function change with age. The impact of ageing on macrophages includes decreased phagocytosis and immune resolution, increased in senescent-associated markers, increase inflammatory cytokine production, and reduced autophagy and decrease in TLR expression. With monocytes there is an increase in circulating $CD16^{+}$ monocytes, decreased type I IFN production, and decreased efferocytosis. In conclusion, we believe that monocytes and macrophages contribute to immunosenescence and inflammageing and as a result have an important role in defective immunity with age.

Introduction

Ageing populations are becoming a global trend (1), however increasing lifespan is outstripping health-span. This results in people living longer with chronic health conditions, adversely impacting on quality of life. Older adults are at increased risk of hospitalisation and death from primary infections such as influenza (2), reactivation of latent infections such as shingles caused by Varicella-Zoster virus (VZV) (3), and are often living with chronic inflammatory diseases such as type 2 diabetes and rheumatoid arthritis. Although four vaccinations (Influenza, tetanus-reduced diphtheria-acellular pertussis [TdaP], Pneumococcal, and Herpes Zoster) are recommended for older individuals (>65 years) in the UK, vaccine efficacy decreases significantly with age (4-6).

All these age-related changes suggest that there are alterations in immunity which result in poorer antigen-specific immunity and worse vaccine efficacy. To date the majority of the research has focussed on the adaptive immune system which has been reviewed extensively (7, 8). Although T and B cell changes are important in ageing, there is clearly also a role for innate immune cells. In this review we discuss age-related inflammation and how monocytes and macrophages contribute to these inflammatory processes. We then focus on what defines monocytes and macrophages, then what changes occur in these cells with age, and how this underlies diseases commonly associated with ageing.

Inflammation and ageing

Ageing is arguably primarily characterised by the accumulation of cells which have undergone the process of permanent cell cycle arrest, termed senescence (9). Senescence can occur in all cells in the body, meaning that all tissues can contain senescent cells. Structural stromal cells, such as fibroblasts, show high levels of senescence with age. In the immune system, senescence has been shown in multiple cell types including macrophages and T cells (10-12). However there is evidence, certainly in T cells, that what has been defined as senescence can be reversed with the addition of p38 MAP kinase inhibitors, begging the question if this is proper senescence or indeed if senescence is not always a state of permanent cell cycle arrest (12). Senescence occurs as a result of irreparable cellular insults, such as excessive DNA damage, telomere erosion, or oxidative stress (13). Senescent stromal cells do not divide and are apoptosis resistant (14). They can be characterized by the expression of the CDK inhibitors p16INK4A and/or p21, telomere associated γH2AX foci and/or β-galactosidase expression. However, there is no one definitive marker of senescence and this subject has been reviewed extensively elsewhere (13).

Systemic increases in senescent cells are closely linked to age-related pathology and inflammageing, the chronic low-grade inflammation observed with age in humans (15). Senescent cells themselves secrete a raft of inflammatory mediators, termed the senescence associated secretory phenotype (SASP) (16). The SASP can drive paracrine senescence perpetuating an increasingly senescent and inflammatory tissue environment (17). Importantly, while the SASP is a profound source of inflammatory mediators, it does not encompass all mediators that are increased with age.

Inflammageing is characterised by an increase in circulating inflammatory mediators such as C Reactive protein (CRP), Interleukin (IL)-6 and Tumour Necrosis Factor (TNF)α (18). Although acute inflammation is important for clearance of infection or facilitating wound healing, it is becoming increasingly clear that chronic inflammation is detrimental to a functioning immune response. Indeed older people who have elevated circulating IL-6, CRP, TNFα, IL-1β or inflammasome-related genes have higher chance of all-cause mortality (19-21). Conversely, lower levels of inflammatory cytokines in the peripheral blood correlate with good health outcomes, longevity, and reduced risk of death of older adults (22). Not all older people age similarly - one such example is frailty, which is the individuals biological age rather than chronological, and is considered to be an excellent guide for establishing the health of the individual. Inflammation is a strong predictor for frailty, and those older individuals who are most frail have highest levels of circulating CRP, IL-6 and IL-8 (23). In addition, excessive inflammation has been shown to reduce vaccine efficacy (24, 25), antigen-specific immunity (26) and increased immunoregulatory mechanisms to combat the increased inflammation (27).

The source of the inflammatory cytokine production during ageing is believed to be multi-factorial. SASP is an obvious contributor to this, but additional mechanisms have been proposed. Geriatric mice have been shown to have increased gut permeability which results in bacterial lipopolysaccharide (LPS) leakage into the blood stream and activation of mononuclear phagocytes via binding to Toll-like receptor (TLR)4 (28, 29). Older adults exhibit increased visceral adiposity; visceral fat is an inflammatory site as infiltrating immune cells, including mononuclear phagocytes, secrete a raft of inflammatory mediators (30). Additionally, aged mice have elevated damage-associated molecular patterns (DAMPs), suggesting that human ageing may also lead to increased DAMP production (31). DAMPS bind to a range of pattern recognition receptors (PRRs) on innate cells leading to a cascade of inflammatory cytokine production. Finally, the most recent proposed mechanism for increased inflammation with age is a failure of inflammatory resolution in older adults. The onset of inflammation is a highly active process, involving multiple cell types and mediators. We now appreciate that switching off inflammation is an equally involved process with distinct signalling and effector

pathways all of which impact downstream immune responses (32). We recently showed that although the onset of inflammation is similar between old and young, the resolution of inflammation was defective in older people leading to a prolonged inflammatory response (33). Mononuclear phagocytes, consisting of monocytes and macrophages, were unable to engulf apoptotic immune cells following an inflammatory insult. This resulted in an accumulation of apoptotic cells, cellular debris, and mononuclear phagocytes that did not switch to a pro-resolution phenotype. Ultimately this kind of mechanism, of failed resolution, might underlie chronic inflammation such as that seen in aged people (33).

When Franceschi and colleagues coined the term inflammageing in 2000 (15), they suggested the root of age-related chronic inflammation was chronic activation of the macrophage. Whilst more recent data suggests that macrophages are not the sole source of inflammageing, it is clear that monocytes and macrophages are the central component in initiating the phenomenon. Although the effect of ageing on monocyte and macrophages has been studied and will be discussed in detail in this review, there are clearly facets of ageing in this context that are poorly understood. The focus of this review is an overview of the current knowledge of the impact of ageing on monocytes and macrophages, and how these cells can contribute to the inflammageing. In addition, this review will highlight areas of monocyte and macrophage biology where more research is required.

Macrophages

Macrophage phenotype and function

Macrophages are tissue resident cells known for phagocytosis, their name being derived from Greek meaning “big eaters”, first coined by Eli Metchnikoff in the late 19th century. He observed this population of cells in starfish larvae which had been pierced by tiny thorns going on to show that macrophages and the process of phagocytosis formed the “essence” of inflammation (34). However, even following Metchnikoff’s Nobel prize in 1908 (35), the macrophage had long been undervalued and underexplored in favour of work on the more high profile adaptive/humoral immune system. Only recently has research on macrophages increased in intensity and great strides have been made in understanding these complex cells (36). Macrophages express a broad range of PRRs, such as TLRs, that when triggered initiate an inflammatory signalling cascade. They are capable of ingesting a host of targets ranging from bacteria to apoptotic cells, thorns to tattoo ink, and grapple with helminths (37-39). This shows how important macrophages are in immune responses, both in terms of clearing infectious agents and subsequently cleaning up the debris caused by inflammation and infection.

Protean in their function, macrophages exhibit a high level of phenotypic plasticity with phenotypes historically being categorised based on *in vitro* models using discrete stimuli. Macrophages (and often monocyte-derived cells) exposed to the pro-inflammatory mediators interferon-γ and LPS are characterised as having the classically activated, pro-inflammatory M1 phenotype based on their release of cytokines such as TNFα, IL-1β, and IL-12 as well as their increased reactive oxygen species (ROS) production (40). Stimulation of macrophages using IL-4 or IL-10 results in alternative activation, or M2 type polarization, characterized by the release of anti-inflammatory and tissue repair molecules (41). *In vivo*, however, macrophages (and monocytes) display mixed phenotypes and are not completely polarised as described by the *in vitro* classification of M1/M2 (42, 43). Therefore, this nomenclature must be used with caution as it belies the complexity of macrophages phenotypes *in vivo*. As such this review will attempt to discuss macrophages in the context of their tissue environment and/or specific activating stimuli rather than describing them simply as M1 and M2 macrophages.

We now understand that macrophages are also key players in tissue homeostasis. Macrophages interact with the cells around them to maintain order and rapidly remove potentially hazardous debris (39). This mechanism is also used to bring about immune resolution and restore tissues to their homeostatic states while providing an environment conducive to immune memory (44, 45). Finally, different types of macrophages have very specific tissue functions, for example, microglia help orchestrate neuronal connectivity by pruning synapses (46), bone marrow macrophages enucleate erythroblasts during erythrocyte development (47) and splenic red pulp macrophages phagocytose and clear damaged erythrocytes (48).

The origins of tissue resident macrophages and their development have been extensively reviewed recently (36, 49-51). The original dogma proposed that monocytes were recruited to tissues where they subsequently differentiated into macrophages (52). This does indeed happen, for example in the gut or the dermis (53, 54). It has been shown in mice that embryonic precursors seeded in the intestine underwent *in situ* proliferation, during the neonatal period they were subsequently replaced by an influx of $Ly6C^+$ monocytes instructed by the local tissue environment and microbiota to differentiate into macrophages (53). However, other mouse studies have observed that in other tissues, such as in the brain, lung and epidermis, macrophages are exclusively embryonically derived (36). To add further complications to the field of macrophage ontogeny, murine studies have shown that there are situations where monocytes can temporarily replace embryonically derived macrophages when there is a deficit in the cell number due to an inflammatory event, to allow time for the

macrophage populations to proliferate (55). Unfortunately, data on whether this occurs in aged mice or humans are lacking.

Macrophage ontogeny is therefore tissue dependent for reasons that are not yet entirely clear. Each tissue imprints function upon the macrophage irrespective of whether it is embryonically derived or monocyte-derived, thus meaning that each tissue has unique macrophage populations (56). Furthermore, we have to acknowledge that macrophage heterogeneity, even within specific tissues, is greater than previously appreciated (36).

Macrophages and ageing

Here follows a discussion of what is known with regards to macrophages during ageing. Ageing results in a plethora of phenotypic and functional changes in macrophages, reliant upon tissue residency, metabolic state, senescence, and multiple other factors. These will be discussed in turn and are summarised in Figure 1.

Macrophage number and phenotype

Geriatric mice (24-28 months old) have elevated numbers of macrophages in the spleen and bone marrow as compared to young mice (57). This contradicts a study performed on human bone marrow, where no significant difference in the number of $CD68^+$ macrophages throughout adult life is observed. Interestingly, bone marrow in children and young adults (<19 years of age) contains significantly more macrophages as compared to adults (58). Further analysis of these macrophages has identified an increased frequency of CX_3CR1 expressing macrophages and conversely a reduction of $Ly6C^+$ macrophages in old mice compared to young (57). The alteration in these two markers suggests a skewing towards a more anti-inflammatory, pro-angiogenic phenotype of macrophage. Indeed, macrophages from old mice are more proangiogenic as compared to young macrophages (59). When adherent splenocytes (presumed to be myeloid cells) are removed from aged mice, they exhibit a reduced capacity to undergo classical *in vitro* polarization into M1- or M2-like macrophages. However, as mentioned previously, characterising macrophages as M1 and M2 is a little outdated and it has certainly become more apparent that macrophages are instructed on their function based on the tissue environment in which they are found. Further analysis suggests that it is not due to an inherent defect in the macrophages, but rather due to the 'old' environment in the mice which prevents the macrophage differentiation (60).

Changes in tissue environment with age impacting on macrophage function

Studies in aged humans have found that the composition of the microbiome changes over time. For example, the prevalence of *Bifidobacterium* and *Lactobacillus* species decreases with age, whereas the numbers and diversity of *Bacteroides, Clostridia* and *Fusobacteria* increased (61). It has been postulated that the reason for this change in microbiota is increased gut permeability and subsequently elevated levels of LPS found in the gut and plasma of aged mice (29). More recently it was shown that the gut does indeed become more permeable in aged mice, resulting in more circulating LPS. This ultimately leads to an increase in systemic, but low grade, inflammation in aged mice, akin to inflammageing seen in humans (29).

This LPS-driven chronic inflammation in geriatric mice is reflected in increases in circulating inflammatory cytokines such as TNFα. Indeed, TNFα deficiency or blockade protects from age-related inflammation and changes in the microbiota in mice (28). Elevated pro-inflammatory mediators have profound and negative effects on peritoneal and bone marrow-derived macrophages by reducing their capacity to ingest and clear bacteria further perpetuating an inflammatory phenotype (28). Interestingly mice that were kept in a germ-free environment did not develop age-dependent inflammation and had preserved macrophage function (28). It was recently shown that intestinal alkaline phosphatase activity declines in aged mice and humans. In mice, this decline resulted in increased liver dysfunction, increased portal vein TNFα levels, and a significant increase in circulating pro-inflammatory cytokine concentrations produced by bone marrow-derived macrophages (62). In humans, the increase in gut microbiome variability with age correlates with IL-6, IL-8 and CRP in the serum, implying that similar dysbiosis occurs in older adults (63).

In the murine lung, the resident alveolar macrophage population changes dramatically with age, with macrophage numbers declining and studies finding in excess of 3,000 genes being altered in young compared to aged mouse lungs (64). Amongst the affected genes were scavenging receptors such as CD204 (64), and macrophage receptor with collagenous structure (MARCO) (65), which impacts bacterial phagocytosis and efferocytosis of apoptotic neutrophils, adversely affecting inflammatory responses.

Candida albicans challenge in the skin of older adults results in reduced production of TNFα, IL-6 and IFN-γ by $CD163^{+}$ dermal macrophages, as compared to younger people (66). However, TLR1/2 and TLR 4 stimulation *in vitro* of isolated skin macrophages showed similar TNFα production between young and old adults (66). These data highlight that changes in the tissue environment with age can dictate macrophage function and studying macrophages in isolation is insufficient to give the whole picture.

Macrophage senescence

Many macrophage populations proliferate to maintain their numbers and as such have the potential to undergo cellular senescence. Microglia, one of the resident macrophage populations in the brain, are exclusively yolk sac-derived, capable of lifelong self-renewal have been reported to undergo senescence with age. Indeed, senescent macrophages have been proposed to contribute to age-dependent neurological dysfunction (67). Limited evidence of the SASP has been found by way of elevated IL-1β, IL-6 and TNFα in aged rat and murine brains (68-71). While clearly not seen in all models, this suggests chronic microglial activation as a result of sustained aberrant inflammasome formation occurring with age (72, 73). It should be noted that microglia priming is associated with peripheral immune challenge such as from surgical stress meaning events in the periphery could contribute to increased activation and senescence in the brain.

The peritoneum of aged mice contains macrophages expressing markers of senescence including p16 and β-galactosidase (β-gal). The increase in expression of these markers was due to bystander senescence from adjacent senescent stromal cells (74). However, the authors do not categorise these macrophages as senescent themselves. Indeed, β-gal^{+} foamy macrophages have been detected in atherosclerotic plaques in mice and p16-targeted depletion removed these cells, indicating atherosclerotic plaque macrophages exhibit signs of senescence (75). Other studies in aged mice show no effect of senolytic treatment of macrophage numbers in atherosclerotic plaques (76). Obesity-induced adipose tissue senescence results in monocyte recruitment and ultimately increased macrophage accumulation (77). Intriguingly this accumulation occurs in humans and can be reversed using senolytic treatment (Dasatinib and Quercetin, D+Q) (78). However, while D+Q treatment successfully reduced epidermal p16^{+} cell numbers, this decrease could not be attributed to Langerhans cell clearance, or recruitment of macrophages into the epidermis (78). Furthermore, D+Q treatment in aged mice did not affect CD68^{+} macrophage numbers in adipose tissue explants, regardless of their p16 expression (79). A potential confounder in this work is that β-gal is expressed in the lysosomes of macrophages when they are undergoing phagocytosis, meaning that β-gal is not an accurate marker of senescence in macrophages (80, 81).

P16 expression is seen in bone marrow-derived macrophages where it suppresses IL-6 (but not TNFα) production by degrading IL-1R-associated kinase 1 (82). This finding was directly contradicted in another model using bone marrow-derived macrophages where p16$^{-/-}$ macrophages secrete significantly less IL-6 compared to p16$^{+/+}$ cells, both basally and following LPS stimulation, instead resembling M2-like macrophages (83). Ablating p16^{+} cells

297 could therefore also target macrophages, which may be advantageous in the context of
298 atherosclerosis (75), but could be disadvantageous in other contexts.

299

300 *Inflammatory cytokine production:*

301 Most of the focus of macrophage work has been on their role in orchestrating inflammatory
302 responses. As discussed previously, there is increased TNFα and IL-6 produced from aged
303 mouse peritoneal macrophages in response to LPS and *S. pneumoniae (28)*. Aged microglia
304 also secrete more proinflammatory cytokines such as IL-6 and TNFα in response to TLR
305 stimulation as compared to young (84). This is in contrast to another study on splenic and
306 thioglycolate-elicited peritoneal macrophages which found that there was reduced TLR
307 expression in aged macrophages, which, as when stimulated with TLR stimuli they had
308 reduced proinflammatory cytokine production in old as compared to young (85). Perhaps the
309 differences observed between these studies could reflect the differences between different
310 tissue resident macrophages. One such study showed that there was elevated
311 Cyclooxygenase 2 (COX2) expression and subsequent Prostaglandin 2 (PGE_2) production
312 from aged macrophages as compared to young (11, 86), and that this expression of COX2
313 correlated with increased expression of inflammatory cytokines such as TNFα and IL-6. This
314 increase in COX2 is believe to be due to a higher rate of transcription, rather than transcript
315 stability (87). While pathways like COX-2 and p38 MAP kinase are implicated in altered
316 cytokine production in geriatric mice and older humans, there has not been much of a
317 concerted effort to explain why these pathways change with age. It is likely that, as with most
318 macrophage function, the tissue microenvironment will influence cytokine production.
319 However, it is equally possible that changes in macrophage metabolism and phagocytic ability
320 underlie these changes, and what we are seeing in terms of cytokine release is the result of
321 other issues.

322

323 *Phagocytosis*

324 In aged mice it has been observed that there is reduced wound healing compared to younger
325 mice, a difference attributed to reduced phagocytic capacity of macrophages collected from
326 old mice as compared to young (88-90). Defects in phagocytosis in older macrophages have
327 also been shown in other studies which demonstrated reduced clearance of apoptotic cells in
328 aged mice, which results in unresolved, chronic inflammatory responses (90, 91). This
329 efferocytic defect that leads to sustained inflammation has since also been observed in
330 humans (33). The tissue environment in which macrophages reside have been proposed to
331 be responsible for the reduced phagocytic activity in the old (92). A study which was carried

out in a rat model showed conversely that there was enhanced phagocytic activity in aged alveolar macrophages as compared to young, additionally the release of lipid mediators such as leukotrienes and prostaglandins was not altered with age (93). Overall, macrophage phagocytosis defects are prevalent in ageing research though we still do not fully appreciate how this comes about in different human tissues with age.

Metabolism

Immunometabolism, which is the study of metabolic pathway usage in an immune cell, is an emerging field of research and has been reviewed in detail previously (98). The metabolic pathways utilised by the cell have important implications for its phenotype. NAD^+ has been suggested to be a therapeutic target for ageing as its levels change significantly with age (94). In macrophages, NAD^+ synthesis is lower with age, much like during immune responses (95), affecting macrophage effector responses resulting in more pro-inflammatory function (96). Indeed, the question remains whether metabolic state causes macrophages to shift to a pro-inflammatory phenotype in aged tissues, or if macrophage activation causes a sustained metabolic switch. Evidence for the former consists of the fact that telomeric stress, such as that encountered with age, causes dysfunction in mitochondrial metabolism that results in increased ROS formation, inflammasome activation and IL-1β release (97). IL-1β is further seen as a result of age-related autophagy defects. An aged mouse study observed found that there was reduced autophagy flux in older mice, which has been proposed to be due to hypermethylation of the autophagy genes *Atg5* and *Lc3* (98). This subsequently results in an increase in the expression of IL-1β, hence it was proposed that a deficiency in autophagy could be a marker of senescence (99). While these findings are intriguing and could contribute to our understanding of senescence and ageing, these avenues of research are still in their infancy and will require more effort to be put into a relevant context.

Macrophage-driven age-related disease:

Some diseases are very strongly associated with increased age. Polymyalgia rheumatica (PMR) and giant cell arteritis (GCA) are two that essentially only occur in people over the age of fifty, often coexisting (100). Both diseases are characterised by IL-1β and IL-6 production by arterial macrophages and circulating monocytes, possibly contributing, or arising as a result of, inflammageing (101). Giant cell arteritis is an inflammatory disease of medium to large arteries characterised by the infiltration of T cells and macrophages. The eponymous giant cells, though present in only ~50% of cases, arise as a result of aberrant macrophage phagocytosis leading to cellular fusion (101). These giant cells are secretory (mainly producing

platelet derived growth Factor and Vascular endothelial growth factor) and it is likely that their cellular profile underlies disease heterogeneity. The subsets of macrophages involved in this disease are not entirely clear and it is possible they are at least in part monocyte-derived. Only a subset of the $CD68^{+}$ macrophages found in the artery tissue appear to contribute to the release of tissue-destructive proteases, and the pro-inflammatory cytokines IL-1β and IL-6 which cause the general symptoms associated with GCA (101, 102). PMR presents with aching and stiffness in muscles, mainly in the pelvic girdle, upper arms, shoulders and neck (103). Like GCA it comes with a significant component of systemic inflammation of acute phase proteins that are likely macrophage derived (100). This chronic inflammation and the strong age association of these diseases makes them likely candidates for over-exuberant inflammageing.

Cancer is known to increase in incidence with age. It has been proposed that aged macrophages are more permissive to tumour growth (57), as when macrophages from young and old mice were cultured with tumour cell-derived supernatants, macrophages from older mice secreted more IL-4. This increased IL-4 production from aged macrophages was shown to be immunosuppressive as it inhibited IFNγ production from T cells (57).

Atherosclerosis is an age-associated disease resulting from the accumulation of monocyte-derived macrophages (foamy cell macrophages that have ingested copious amounts of cellular debris, and apoptotic macrophages) and smooth muscle cells that occlude the blood vessels. TNFα, which is known to increase with and be pro-atherosclerotic, can induce CD47 expression on vascular cells, inhibiting their removal via macrophage-dependent efferocytosis (104). CD47 blockade using monoclonal antibodies can successfully initiate macrophage-dependent clearance of apoptotic vascular cells and protect against the development of atherosclerosis in mice (104).

Chronic obstructive pulmonary disease (COPD) , while often associated with smoking, is a disease most prevalent in older individuals and is strongly linked to inflammageing (105-107). It has previously been postulated that COPD may become fully chronic due to the involvement of DNA damage-induced cellular senescence and the SASP that follows (108). Indeed, increased ROS, such as seen with age, is linked to DNA damage in PBMCs and oxidative stress in the lung (109). Here, tissue-damaging proteins, such as elastase and MMPs, commonly seen as SASP mediators, are released by alveolar macrophages in COPD (110), through a NOX2-mediated mechanism (111). Much like with ageing in general, bacterial phagocytosis and efferocytosis are both impaired in alveolar and monocyte-derived lung macrophages from COPD patients, resulting in increased bacterial colonization and an elevated pro-inflammatory environment, including cytokines such as IL-8 and CCL2 (112).

Indeed, increased levels of serum IL-8 (and IL-6 and CRP) are strongly linked to the pathogenesis of COPD (113).

As exemplified, there is increasing evidence of age-related macrophage dysfunction that could be at the heart of many age-related pathologies we know today. However, the resident macrophage field is still relatively "young", particularly in human research. Much is currently being done surrounding macrophage ontogeny and tissue-dependent function, but more will be needed to understand how macrophages behave in ageing tissues and organisms. Moreover, we need to devote more time to the study of monocytes, blood-borne cells that can travel throughout the body and migrate into tissues where they are needed. In addition to their unique functions, monocytes are also capable of differentiating into macrophage-like cells. Therefore, it is important to know also how monocytes change with age as this will impact on macrophage function.

Monocytes

Here follows a discussion of what is known with regards to monocytes during ageing. Ageing results in a plethora of phenotypic and functional changes in monocyte populations. These will be discussed in turn and are summarised in Figure 2.

Monocyte phenotype and function

Monocytes historically were presumed to be precursor cells for macrophages and dendritic cells (DCs). Although this can be true, monocytes are recognised as established immune effector cells in their own right. Monocytes have various immune effector functions including pathogen recognition through TLRs and other PRRs and subsequent secretion of pro-inflammatory cytokines, antigen presentation, contribute to tissue remodelling and wound healing, and also can contribute to resolution of inflammation via efferocytosis and anti-inflammatory cytokine and lipid mediator production (33, 114-117).

Human monocytes are defined by their expression of the cell surface markers CD14 and CD16. The classical monocytes are defined as $CD14^{+}CD16^{-}$, the intermediate monocytes are $CD14^{+}CD16^{+}$ and then the non-classical monocytes are defined as $CD16^{+}CD14^{-}$ (118). In mice, the classical monocyte is $Ly6C^{++}CD43^{+}$, intermediate $Ly6C^{++}CD43^{++}$ and the non-classical is $Ly6C^{+}CD43^{++}$ (118). The relationship of one monocyte population to the other is often discussed, a clinical trial in 1994 in M-CSF treatment suggested that $CD14^{+}$ monocytes are precursors to $CD16^{+}$ monocytes (119). Further evidence that this was the case was

confirmed when transcriptomic analysis was performed on $CD16^-$ and $CD16^+$ monocytes, and it showed that all monocyte populations originated from a common precursor. Indeed it was observed that the $CD16^+$ expressing monocytes were transcriptionally more differentiated then the $CD16^-$ cells (120). A more recent study showed that in steady-state conditions $CD14^+$ monocytes originated from the bone marrow and then either migrated into the tissue or differentiated into $CD14^+CD16^+$ monocytes. $CD14^+CD16^+$ monocytes then terminally differentiate into $CD16^+CD14^-$ monocytes (121). Other cell surface markers which could be used to differentiate between the monocyte populations include CCR2 and CX_3CR1 which identify $CD14^+$ or $CD16^+$ monocytes respectively (114, 121).

These three different monocyte populations are proposed to have distinct effector functions. The classical monocytes, the majority population circulating in peripheral blood (80-90% of monocytes) (121), have the capability to migrate into tissues in homeostatic conditions. Once at the tissue site they can either transport antigen to the lymph nodes or repopulate the tissue resident macrophage population (53, 55, 122, 123). $CD14^+$ monocytes also have the ability secrete inflammatory cytokines such as IL-6 and chemokines such as IL-8, CCL2 and CCL3 in response to PAMPs or DAMPs, further recruiting inflammatory cells to the tissue site (114). For the $CD14^+$ monocytes, that do not migrate out of the blood, they differentiate into $CD14^+CD16^+$, intermediate monocytes. These intermediate monocytes can secrete large amounts of IL-1β and TNFα when stimulated with PAMPs such as LPS (114). The non-classical monocytes, $CD16^+CD14^-$, are known as 'patrolling' monocytes, as these monocytes are actively surveying the endothelium and removing debris (114, 124). CD16 is an Fc receptor for IgG antibodies, which means that CD16 expressing monocytes are efficient at antibody-dependent phagocytosis (125).

What is becoming increasingly clear is that monocytes have a distinct effector function of their own, unique from tissue resident macrophages. Indeed although monocyte-derived macrophages adopt many tissue resident macrophage characteristics, it is known that they maintain some monocyte identify and respond differently during inflammation (126). Therefore, it is imperative to understand how these cells change with increasing age.

Monocytes and ageing

Aged murine studies

The focus of many aged mouse studies has been on macrophages so the information on monocytes is rather limited. As discussed before aged murine studies have shown that older mice have more permeable intestine, which results in LPS leakage into the circulation (28, 29). This in turn leads to activation of circulating monocytes via LPS binding to TLR4 resulting

in inflammatory cytokine production including TNFα. Older mice have worse immunity to *Streptococcus pneumoniae*, and this is believed to be due to the direct effect of elevated TNFα, that is observed in older mice, on monocytes (127). Increased circulating TNFα promotes early monocyte egress from the bone marrow, which results in immature monocytes being recruited to sites of infection, and due to their immaturity they are unable to clear bacteria from the lung (127).

Phenotype of human monocytes:

Although it has been observed that there are no significant differences in the number of circulating monocytes in older adults as compared to younger adults (128), it has been proposed that the phenotype of these cells are different. Early studies identified that there was an increased frequency of $CD16^+$ monocytes in older adults as compared to young (129). It was found that both intermediate and non-classical monocyte compartments expand in older adults (129, 130). It has been proposed that the $CD16^+$ monocytes are in fact a senescent monocyte population, with shorter telomeres, increased inflammatory potential in line with SASP, and expression of a senescence-associated microRNA (miR) miR-146a (131, 132). Whether the non-classical monocytes are indeed senescent, with irreversible cell cycle arrest or just terminally differentiated still warrants further investigation as many of the markers of senescence are commonly expressed by mononuclear phagocytes, given their physiological role in inflammation. In addition, monocytes are relatively short-lived effector cells and it has been predicted that the $CD16^+CD14^-$ monocytes only live for an average of 7.4 days in the circulation (121). Therefore it is unlikely that the cells have accrued enough DNA damage to initiate senescence-associated pathways.

What factors drive the expansion of $CD16^+$ monocytes in older adults is unknown. It could be either due to a failure of clearance of old $CD16^+$ monocytes, or due to a defect in $CD14^+$ monocytes means they do not extravasate as efficiently and fail to leave the periphery and instead differentiate into CD16 expressing cells.

Inflammatory cytokine production:

Early studies on monocyte populations were either carried out in whole blood or PBMC cultures and a s a result there were conflicting results due to cell culture methods used and the non-specific way of measuring cytokine production by ELISA. LPS stimulation was found in some cases to have a similar effect on the age groups and in some cases older cultures produced less inflammatory cytokines (133, 134). Subsequent studies using intracellular

cytokine staining to specifically look at monocyte populations showed that there was no difference in the response to TLR4, 5 and 7 ligands between young and old monocytes (135). However, a small difference was observed in TLR4 expression, with higher expression on the young as compared to old (135). For TLR8 stimulation with Poly(U) there was a less IL-6, but not TNFα, produced when cells were stimulated with Poly(U) in old as compared to young (135).

A more recent study by Metcalf *et al*, which built upon earlier observations in PBMCs from the same lab (136), isolated the three monocyte populations and studied them individually, showing that at baseline, monocytes from young and old people were similar. However, upon stimulation with TLR4, TLR7/8 and retinoic acid–inducible gene I (RIG-I), aged monocytes produced less pro-inflammatory cytokines, such as IL-1β and IFNα (137). More recent analysis has shown that there is an impairment of primary and secondary RIG-I signalling in monocytes from older adults, due to decreased abundance of the adaptor protein tumour necrosis factor receptor–associated factor 3 (TRAF3) and IFN regulatory transcription factor (IRF8) respectively (138). This in turn results in reduced type I IFN production and is thought to be one of the reasons that older people are more susceptible to respiratory infections, as type I interferon is necessary to clear infection (138).

When monocytes are stimulated with TLR1/2 stimuli such as PamCy3 there reduced production of TNFα and IL-6 from aged monocytes as compared to young; this is believed to be due to a reduced expression of TLR1 on the monocytes of older individuals (129, 135). In fact, Nyugen *et al* suggest that there the defect in the TLR1/2 signalling is restricted to the monocytes that express CD16, as for the classical monocytes there was no difference (129).

Interestingly within the older adult population there are differences in monocyte number and function depending on the level of frailty. It has been observed that there is an increased overall number of monocytes in frail older adults as compared to those les frail older adults (139). Classical monocytes isolated from frail older adults had increased inflammatory associated genes in response to LPS as compared to non-frail older adults (140). However, further studies will be needed to confirm that change in mRNA level translated to change in protein expression.

Function:

There is currently limited data available about the effect of age on monocyte function. Recently we have shown that mononuclear phagocytes, presumed to be inflammatory monocytes, from older adults, recruited to a site of tissue damage fail to resolve inflammation as effectively as younger monocytes (33). This defect in resolution was due to lower expression of expression

of T cell immunoglobulin mucin receptor-4 (TIM-4), a receptor that recognizes apoptotic cells, and a subsequent failure to phagocytose apoptotic neutrophils as compared to younger monocytes (33). This led to sustained inflammation at the site of damage and a longer time to heal and is suggested to be a contributor to the aetiology of inflammageing. Interestingly this defect in resolution in the old could be reversed by pre-treatment with a p38-MAP Kinase inhibitor (Losmapimod), and thus identifies a therapeutic target for improving monocyte function in older people (33). In addition, we have shown that older people have an increased recruitment of monocytes to a site of needle challenge (air, saline or antigen) (26, 141). This increased non-specific inflammation negatively correlated with antigen-specific cutaneous immunity (26). It is observed that inflammatory monocytes inhibited antigen-specific immunity through increased PGE_2 production, and blockade of inflammation and PGE_2 production using Losmapimod significantly improved cutaneous immunity (141). However, this immunomodulatory property of monocytes may be a by-product of the increased inflammatory skin environment – as increased senescent stromal cells such as fibroblasts are present in older skin (10).

Metabolism:

Ageing results in the redistribution of body fat from subcutaneous to visceral fat – visceral fat is less efficient at storing fatty acids and as a result there is an increase in circulating free fatty acids in older adults (142). This has implications for circulating monocytes as certain free fatty acids such as palmitate promote an inflammatory phenotype and in turn may contribute to atherosclerosis pathology (143). It has been observed that respiratory capacity steadily declines with age in $CD14^+$ monocytes as a consequence of mitochondrial dysfunction (144). Mitochondria from aged classical monocytes have reduced membrane potential as thus do not work as well as mitochondria from young monocytes (145). Older $CD14^+$ monocytes also have reduced spare respiratory capacity as compared to younger monocytes (146). Immunometabolism is a new and active field of research, and more research is needed to fully understand the impact of age on metabolic pathway usage.

Future perspectives:

Although many studies have been performed to look at the effects of age on monocyte and macrophage function, there are still many unknowns within the field of ageing. Macrophage ontogeny experiments are carried out in young mouse models, so there is a lack of data on if the origin of macrophage populations changes as we reach advanced age. We do not know whether there is a change in the monocyte contribution to the macrophage pool with advanced

age. Also we do not know how age influences macrophage longevity and whether macrophages can be functionally senescent given a lifetime of proliferation, albeit at a supposedly low rate of turnover.

In the context of monocyte biology and the effect of age, current data is contradictory which is in in part due to differences in starting monocyte populations, *in vitro* stimulation and analysis of effector function. Non-classical monocytes have been neglected when it comes to studies about ageing, and certainly warrant further investigation as they are the population that increase in number with age. It will also be important to ensure that sex is taken into consideration when studying monocyte populations, as monocytes from males make considerably more inflammatory cytokines as compared to monocytes from females (147).

We believe that targeting inflammation caused by aged monocytes and macrophages has the potential to limit the detrimental effects of inflammageing and potentially boost immunity in older adults. We have shown that blocking monocyte-derived COX2-driven inflammation using the p38 MAP Kinase inhibitor, Losmapimod, could significantly reduce monocyte infiltration and downstream inflammatory processes (26, 141), as well as improve inflammatory resolution (33). These data pave the way for future studies where anti-inflammatory drugs such as Losmapimod or a COX2-specific inhibitor could be used to boost vaccine efficacy in older adults. Indeed, another anti-inflammatory that has been shown to improve efficacy of the flu vaccine in older adults is RAD001 which is a mammalian target of rapamycin complex 1 (TORC1) specific inhibitor (148). Although the authors note the beneficial effects of this inhibitor on adaptive immunity, there is every potential that it could also inhibit inflammation originating from aged mononuclear phagocytes.

In conclusion, monocytes and macrophages play a key role in ageing and age-related pathology, but further research is needed as the impact of age on macrophage ontogeny, monocyte contribution to macrophage numbers and the function of monocytes with age is still relatively unexplored. Indeed, we believe that monocytes and macrophages should not be looked at in isolation and should be considered together when investigating the impact of age on these cells.

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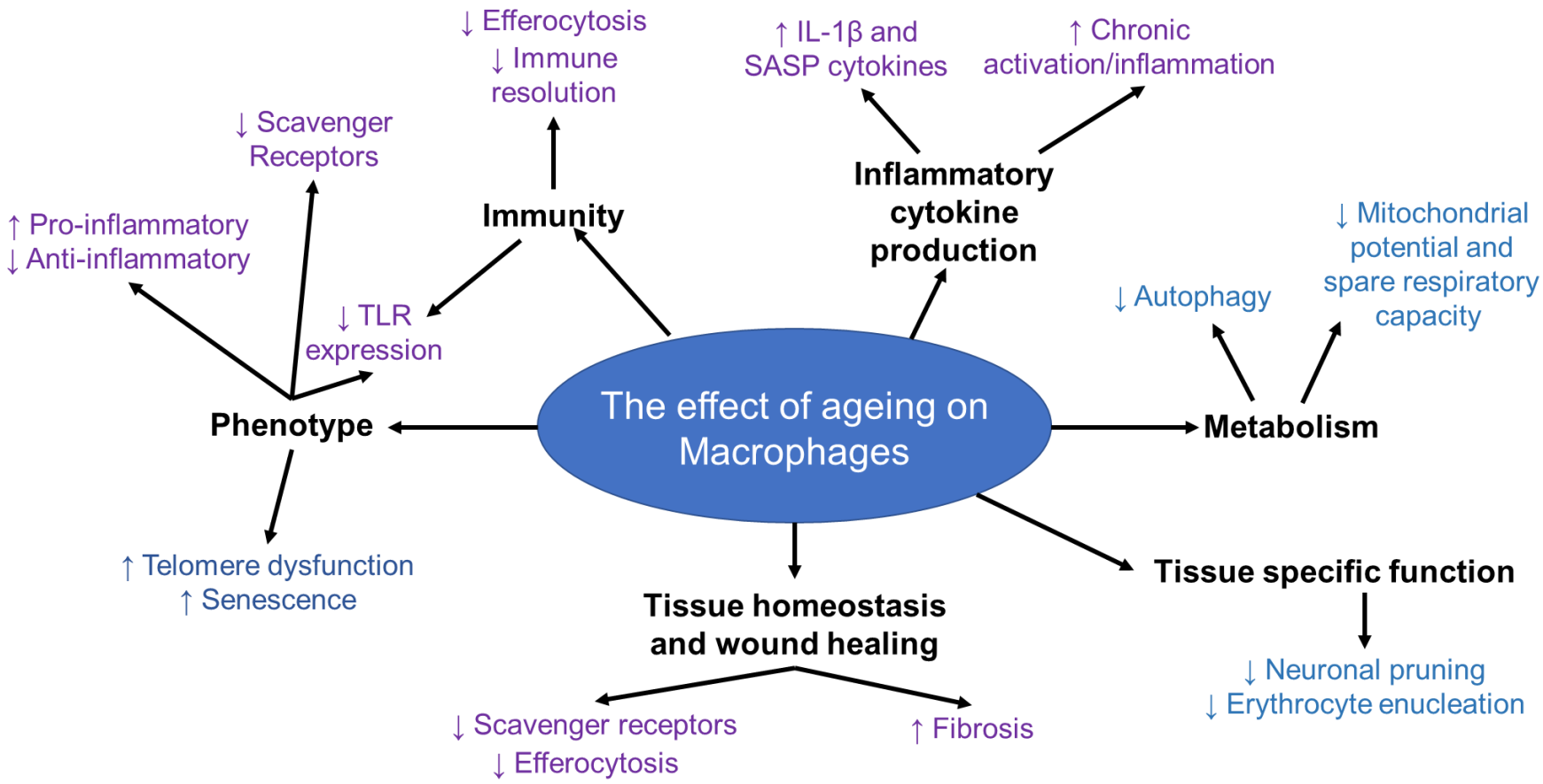

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Figure 1: How ageing alter macrophage phenotype and function

Schematic showing how macrophages change in mice (blue) and humans (purple) with increasing age.

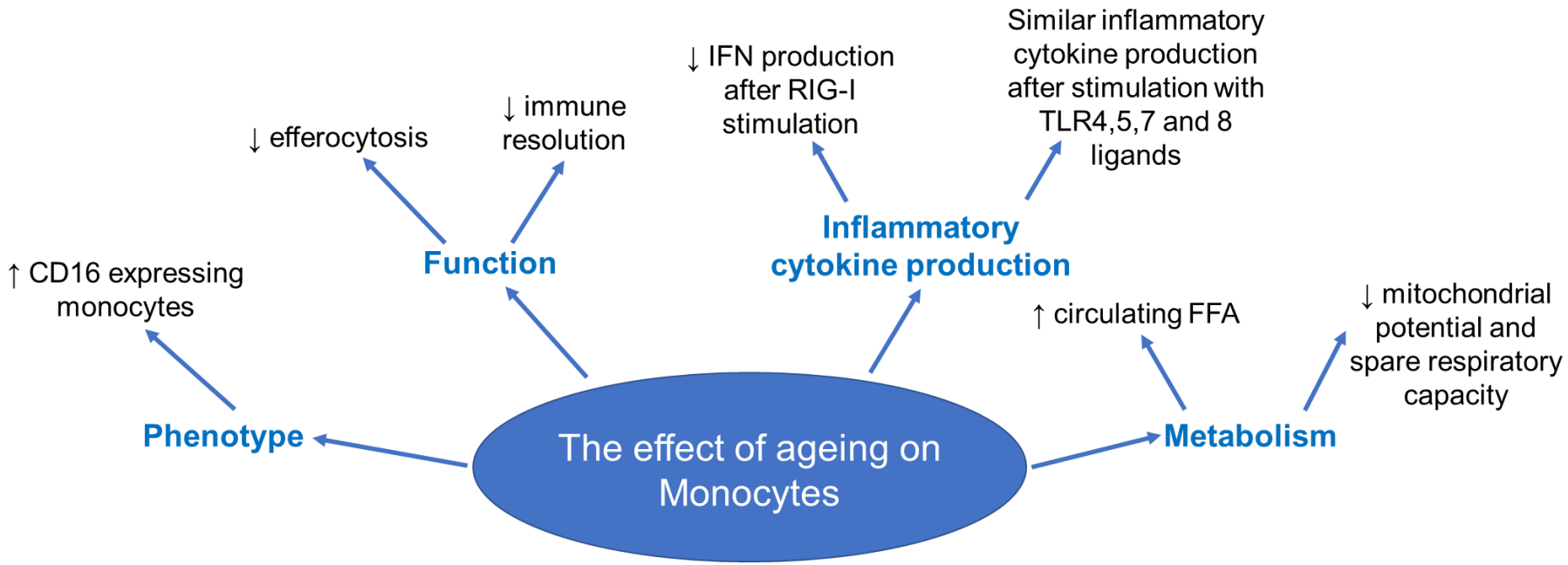

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Figure 1: How ageing alters human monocyte phenotype and function

Schematic showing how monocytes change with increasing age in humans.