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Metabolic Flexibility in Classical Monocytes is not Affected by Age

Johnathan Yarbro

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METABOLIC FLEXIBILITY IN CLASSICAL MONOCYTES IS NOT AFFECTED BY AGE

by

Johnathan Yarbro

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Abstract

Inflammaging is the chronic low-grade inflammation that occurs with age that contributes to the pathology of age-related diseases. Monocytes are innate immune cells that become dysregulated with age and which can contribute to inflammaging. Metabolism plays a key role in determining immune cell functions, with anti-inflammatory cells primarily relying on fatty acid oxidation and pro-inflammatory cells primarily relying on glycolysis. It was recently shown that lipopolysaccharide (LPS)-stimulated monocytes can compensate for a lack of glucose by utilizing fatty acid oxidation. Given that mitochondrial function decreases with age, we hypothesized that monocytes taken from aged individuals would have an impaired ability to upregulate oxidative metabolism and would have impaired effector functions. Aging did not impair LPS-induced oxygen consumption rate during glucose deprivation as measured on a Seahorse XFp system. Additionally, aged monocytes maintained inflammatory gene expression responses and phagocytic capacity during LPS stimulation in the absence of glucose. In conclusion, aged monocytes maintain effector and metabolic functions during glucose deprivation, at least in an ex vivo context.
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Introduction

Aging Demographics

The percentage of Americans over the age of 65 is expected to increase from 13.7% in 2012 to 21% in 2040, which is approximately 80 million people. This change will have significant effects on the economy, healthcare, and society in general. Currently, over two-thirds of the healthcare budget is spent on managing chronic diseases of the elderly. Aging is the highest risk factor for the majority of chronic diseases - including cardiovascular disease, diabetes, arthritis, and cancer, and nearly two-thirds of global deaths in 2008 were due to chronic diseases. While lifespan continues to rise, healthspan, the length of time someone is healthy, has increased more slowly and Americans are living longer with impaired health and disabilities. Due in large part to the growing prevalence of obesity and its associated diseases, morbidity is expected to increase over the forthcoming decades. While Americans aged 70+ have slightly better survival rates than people from other high-income countries, Americans 35-70 experience higher rates of mortality and morbidity, and the advantage 70+ year old Americans have is diminishing. Interventions are needed to improve the health and quality of life of the aging population, and studies show that the compression of morbidity is possible with lifestyle changes, pharmaceuticals, and continuous medical advances.

Inflammaging

Aging is associated with chronic, low-level, systemic inflammation (termed inflammingaging) that contributes to most, if not all, age-related diseases. Older adults have higher serum levels of several pro-inflammatory cytokines/proteins such as IL-6, IL-1β, CRP, and TNF-α. Elevated levels of these molecules in circulation, most notably IL-6, are correlated
with an increased risk of morbidity and mortality in elderly populations. Furthermore, they are associated with sarcopenia, malnutrition, reduced bone density, diabetes, arthritis, atherosclerosis, cognitive decline, and other diseases of aging. There is no consensus on the causes of inflammaging, though it’s likely due to a host of factors that become dysregulated with age. These “hallmarks of aging” include reduced autophagy and mitophagy, accumulation of DNA and mtDNA damage leading to genomic instability, epigenetic changes, telomere shortening, cellular and immune senescence, dysbiosis, chronic antigenic stress, diminished proteostasis, altered metabolic signaling, stem cell exhaustion, increased cellular garbage, and mitochondrial dysfunction. This review focuses on one of the potential mechanisms contributing to immunosenescence and inflammaging – dysregulation of monocyte function and phenotype with age.

**Monocytes**

Monocytes are circulating mononuclear phagocytes of the innate immune system that play an essential role in the defense against a variety of microbial pathogens. They have several functions including phagocytosis, antigen presentation, and the secretion of anti- and pro-inflammatory cytokines. Upon infection or tissue damage, they are extravasated into tissue and differentiate into macrophages or dendritic cells. They also serve as precursors to osteoclasts, fibrocytes, and other mononuclear phagocytic cells such as microglia and Langerhans cells in certain circumstances. It’s important to note that many tissue macrophages (and other mononuclear phagocytes) during steady-state are not derived from circulating monocytes. Many macrophage populations (including splenic, lung, peritoneal, and liver) are established prior to birth from embryonic tissues and are maintained throughout life without input from circulating monocytes. However, monocytes have been shown to replenish macrophage populations
during steady-state in some tissues which experience constant microbiota stimulation, such as the intestines and skin \(^{23,24}\), and are recruited to tissues with resident mononuclear phagocytes and differentiate into their progeny cells during inflammation. \(^{25}\)

Monocytes can be derived from two different types of myeloid precursors (which are derived from hematopoietic stem cells) called the monocyte/macrophage DC progenitor (MDP) or the granulocyte-monocyte progenitor (GMP), and display considerable heterogeneity depending on environmental cues, tissue type, and other factors. \(^{26,27}\) Monocytes have been divided into three subtypes based on their expression of the cell surface markers CD14 (a co-receptor with TLR4 for the detection of lipopolysaccharide (LPS)), and CD16 (a FCγIII receptor that mediates antibody-dependent cell-mediated cytotoxicity) \(^{28}\). Classical Monocytes (CD14+/CD16−) have high levels of CD14 on their surface, little to no expression of CD16, and are the most plentiful type (~80-90%). Non-classical (CD14dimCD16+) and intermediate monocytes (CD14+CD16+) both express CD16, although non-classical monocytes have very low expression of CD14 whereas intermediate monocytes have high expression of CD14 \(^{29}\). It’s clear from gene expression profiling that further monocyte heterogeneity exists and that monocytes form a continuum of more than just CD14 and CD16 expression. \(^{30,31,32}\) Within the currently defined categories, the intermediate subset is the most diverse. A recent study performing single-cell RNA sequencing found that ~62% of intermediate monocytes clustered with classical or non-classical monocytes and the remaining ~38% formed two separate clusters. \(^{30}\) Another study found intermediate monocytes could be divided into two functionally different subsets based off HLA-DR expression. \(^{33}\) Future research using gene expression profiling, flow cytometry, and other techniques will likely lead to further characterization of monocyte phenotypes.
There is inconsistent and contradictory information in the literature on the functions and expression levels of cytokines, chemokines, surface receptors, etc. of each monocyte subset. Some studies report classical \textsuperscript{34, 35} or non-classical monocytes \textsuperscript{29, 31} as being the most pro-inflammatory subset, while most report intermediate \textsuperscript{36, 37, 38, 39} monocytes as the most inflammatory subset. These disparate findings could be caused by differences in purification protocols, flow cytometry gating strategies, activating stimulus, time point of analysis, disease-context, or other factors. For instance, it’s been shown that some anti-CD14 mABs (monoclonal antibodies) can block responses to LPS, and Ficoll-Paque gradient separation increases the number of CD16+ monocytes. \textsuperscript{40, 41, 29}

In general, classical monocytes have been shown to be primarily involved in tissue repair, immune responses, migration to inflamed tissues, and phagocytosis. \textsuperscript{31, 29} They display the broadest range of cytokines in response to LPS, express the highest levels of genes involved in pattern recognition and phagocytosis (such as CD93, CD36, CD209, and CD163), exhibit high production of reactive oxygen species, and express high levels of chemotaxis genes CCR2, CXCR1, and CXCR2. \textsuperscript{35, 31, 42} During an inflammatory response (and to a lesser degree in the steady-state) CCL2(MCP-1), which binds and activates CCR2, is released from the bone marrow and promotes classical monocyte release into circulation by desensitizing CXCR4. \textsuperscript{43, 44} Once in circulation the classical monocytes make their way to the site of inflammation and release pro-inflammatory cytokines such as IL-6, TNF-a, and IL-1\textbeta. \textsuperscript{45} During steady-state they can display a pro- or anti-inflammatory phenotype depending on environmental cues. They may also differentiate into their progeny cells, and are the primary source of monocyte-derived dendritic cells \textsuperscript{45}, although this happens minimally in most tissues during steady-state. \textsuperscript{46} During steady-state, classical monocytes have a very short circulating lifespan (~1 day) and the ones that don’t
migrate out of circulation or die (~1%) transition into intermediate monocytes (~4 day lifespan), of which most eventually transition into non-classical monocytes (~7 day lifespan). During an inflammatory response (to intravenous LPS), acute monocytopenia occurs as monocytes are rapidly recruited and used. Within 4 hours classical monocytes repopulate circulation and CD16+ monocytes begin to appear at 24 hours. By day 7 monocytes return to normal values. It’s clear from this data that classical monocytes are the primary subset recruited into circulation both during an inflammatory response and during steady-state, and once in circulation can give rise to CD16+ monocytes.

Intermediate monocytes are thought to be a transitional population between the classical and non-classical subsets and there is a considerable amount of data to support this. Along with evidence of sequential transition of classical to intermediate to non-classical, there is a continuum of expression of some genetic and epigenetic markers. Dividing monocytes into 9 gates based off CD14 and CD16 expression showed gradual increases in CD86 expression as CD16 expression increased. Additionally, expression of ~40% of miRNAs gradually increased from classical to non-classical subsets. Due to their diversity, intermediate monocytes have a wide range of functions and can display characteristics of both classical and non-classical monocytes. They are primarily involved in antigen presentation, phagocytosis, and parasite recognition. Additionally, they have generally been shown to be the most pro-inflammatory subset, producing high amounts of TNF-α and IL-1β. Furthermore, they are proportionally increased with age and in many chronic diseases such as CVD, rheumatoid arthritis, Crohn’s disease, chronic kidney disease, atherosclerosis, and others.

Non-classical monocytes have high expression of the adhesion-related receptor CX3CR1 and display patrolling behavior within the vasculature, removing debris, rearranging the
cytoskeleton, preventing tumor metastasis, and resolving inflammation. They (and to a lesser extent the intermediate monocytes) selectively respond (via TLR7 & TLR8) to virally infected or damaged cells with the production of pro-inflammatory cytokines, though have limited ability to react to microbial pathogens (TLR1, TLR2, TLR4). They’re also involved in antigen presentation and wound healing, and can give rise to M2-like “anti-inflammatory” macrophages. Like intermediate monocytes they are also increased with age. Recently, they were shown to display more signs of senescence than intermediate or classical monocytes, as they express the highest levels of NF-κB, IL-1α, and miR-146a, and have the shortest telomeres, all of which are associated with a senescence-associated secretory phenotype.

Monocytes and Disease

Monocytosis, an increase in the number of circulating monocytes in the blood, occurs in a variety of different diseases and conditions including CVD, autoimmune diseases, some cancers, acute & chronic infections, and several systemic inflammatory disorders. It’s been most widely studied in the context of CVD and is involved throughout the pathogenesis of atherosclerosis. Atherosclerosis starts when oxidized lipoproteins, which can mimic pathogen or damage associated molecular patterns (PAMP/DAMP), enter the subendothelial space within arteries and trigger a low-level inflammatory response. Chemokine release from the activated endothelium attracts circulating monocytes to the area, which develop into pro-inflammatory foam macrophages. Further pro-inflammatory cytokine/chemokine release cause the recruitment of additional monocytes and other inflammatory leukocytes eventually leading to plaque formation and symptoms associated with atherosclerosis. All monocyte subsets are implicated in the disease, as atherosclerosis is effectively prevented in mice only when both classical(Ly6Hi)
and non-classical (Ly6Lo) monocyte entry is blocked, though the precise role each monocyte subset plays in humans during atherosclerosis is unknown. With age and in many diseases, especially inflammatory diseases, intermediate monocytes are proportionally increased relative to the other subsets. Classical monocyte proportions are often decreased, and non-classical proportions are often increased, though not to the degree of the intermediate subset. This has been observed in CVD, RA, liver diseases, Chron’s disease, obesity, asthma, tuberculosis, sepsis, and others. Additionally, increased intermediate monocyte levels are predictive of a number of adverse events including: mortality in critically ill patients, cardiovascular events, coronary stenosis in asymptomatic individuals, and development of type I DM in kids. It’s not clear if the expansion of intermediate monocytes in these diseases are causal or protective, but it’s likely they are both contributing to and affected by disease. During systemic lupus erythematosus, and undoubtedly in many other diseases, monocytes show dysregulated gene expression, including increased expression of pro-inflammatory cytokines. This is mediated by substantial epigenetic changes, especially within enhancer regions. 40% of monocyte enhancers and 28% of promotors showed changes in H3K4me3, a histone modification associated with transcriptional activation, resulting in overall increased gene expression and phenotypes vastly different than monocytes from healthy individuals. It’s been shown that β-glucans, LPS, and other bacterial ligands can alter epigenetic programming in monocytes leading to a “trained immunity”, and there is evidence that this is dependent on a metabolic shift towards glycolysis mediated by mTOR and HIF-1α activation. Furthermore, metabolic compounds such as (minimally-modified) LDL have been shown to promote a long-term pro-inflammatory phenotype in monocytes through epigenetic reprogramming. A recent
study found altered cholesterol levels positively correlated with pro-inflammatory cytokine production in monocytes across all subsets.  

While the intermediate monocytes were the most pro-inflammatory subset overall, the fact that all subsets were more pro-inflammatory implies that the inflammatory state could be a result of changes occurring in the myeloid precursors, likely through epigenetic changes caused by the altered pro-inflammatory environment. Alternatively, since the intermediate and nonclassical monocytes spend more time in circulation than do the classical monocytes, they may experience a greater degree of dysregulation in diseased contexts. Regardless, it seems that micro-environmental factors that are altered with disease and age can induce epigenetic reprogramming in monocytes leading to the pro-inflammatory phenotype seen in many diseases, further progressing the disease.

Why the intermediate subset is expanded relative to the others is still being investigated, though a recent paper suggests that subpopulations within the intermediate subset could be responsible. Connaughton et al. found that a subset of the intermediate monocytes (HLA-DR$_{mid}$) had the highest uptake and migration towards modified lipoproteins, and that this population (rather than HLA-DR$^{Hi}$ which wasn’t expanded) was responsible for the increased intermediate monocyte subpopulation in obesity.  

Noting that the classical subset was also decreased, they proposed that HLA-DR$_{mid}$ intermediate monocytes may represent a transitional state of classical monocytes that rise in response to increased metabolic or inflammatory stress. Alternatively, the decrease in classical monocytes could be due to migration out of circulation and into inflamed tissues. While this hasn’t yet been studied in the context of other diseases, it’s an exciting avenue for future research.
Monocytes and Aging

Although aging is multi-factorial, monocytes may very well play an essential role in aging pathology. Their heterogeneous and highly adaptable nature, ability to respond to pathogens and cellular garbage, communication with the adaptive immune system, and numerous defects with age make them a key contributor to inflammaging. Monocytes from older individuals have impaired migration and phagocytosis, altered receptor expression, cytokine production, and subset proportions, and a diminished ability to activate the adaptive immune system. While it’s not entirely clear if these changes are cell intrinsic or due to the inflammaging environment, evidence implies that it’s primarily due to the environment.

Older adults have higher circulating levels of mitochondrial DNA (mtDNA), IL-6, TNF-α, and other proinflammatory factors that dysregulate monocyte functions, which can in turn further progress inflammaging. For instance, mtDNA, which can act as a DAMP and activate monocytes via TLR-9, is increased with age and is positively correlated with TNF-α and IL-6 in the blood. 77 In vitro stimulation of monocytes from young adults with LPS and high mtDNA concentrations, similar to levels found in older adults, increased TNF-α production higher than by LPS alone. 77 Given that LPS is also elevated systemically with age, probably due to dysbiosis, 78 it’s easy to see how changes in the microenvironment, in this case increased circulating mtDNA and LPS, could dysregulate monocyte function and further contribute to the inflammaging environment.

Higher circulating TNF-α levels with age have been shown to cause multiple effects in monocyte phenotypes as well. TNF-α has been shown to induce the M1 (“pro-inflammatory”) phenotype in macrophages, and macrophage populations are skewed towards the M1 phenotype with age. 79 80 Additionally, TNF-α may skew monocytes towards a more pro-inflammatory
phenotype, as systemic TNF-α is positively correlated with intermediate monocyte frequency, and aged TNF-α knock-out mice had similar levels of circulating pro-inflammatory monocytes as younger mice. Higher TNF-α levels with age has also been shown to cause monocytes to leave the bone marrow prematurely, due to increased CCR2 expression mediated by TNF-α, and these immature monocytes have reduced pathogen clearance despite producing higher amounts of pro-inflammatory cytokines.

**Metabolic Flexibility in Monocytes and other Leukocytes**

In response to activation, immune cells regularly change their functions dramatically. For example, monocytes activated by LPS undergo vigorous cellular growth and an increased demand for the production of proteins, lipids, and DNA. Other changes include the production of cytokines and other effector molecules, chemotactic migrations, and generation of reactive oxygen species (ROS). This increased demand for biomolecules is associated with upregulation of proteins associated with glycolysis and downregulation of proteins associated with oxidative phosphorylation (OXPHOS), similar to the Warburg effect in cancer cells. Furthermore, fatty acid synthesis increases and concentrations of the TCA cycle and pentose phosphate pathway intermediates are amplified, as well as the downstream products of purine and pyrimidine metabolism.

So, regulated changes in metabolism in immune cells are vital to their function. A shift toward aerobic glycolysis is associated with pro-inflammatory phenotypes such as in macrophages and dendritic cells activated by LPS, M1 macrophages, and in T-helper lymphocytes that produce inflammatory cytokines. Oxidative metabolism (with limited rates of glycolysis) is associated with anti-inflammatory phenotypes such as in quiescent memory T-cells, regulatory T-cells and M2 macrophages. Additionally, aerobic glycolysis is distinctive
of short-lived immune cells, while OXPHOS is distinctive of long-lived immune cells \(^87\). The metabolic inflexibility of an immune cell can lead to increased inflammation. Macrophages are able to convert from one form to another and can switch their metabolism during inflammation from relying on glycolysis in the M1 state to relying on OXPHOS in the M2 state \(^88\). Inhibition of OXPHOS in macrophages inhibits the expression of the M2 anti-inflammatory phenotype \(^89\). Therefore, diminished mitochondrial function is presumed to affect the function and phenotype of certain immune cells.

Aging has been shown to cause decreased mitochondrial function with an estimated decline of 8% in ATP producing capacity per decade \(^90\). This decrease in function is thought to be largely caused by mitochondrial DNA (mtDNA) mutations, which occur at an estimated rate of 15x that of the nuclear genome \(^90\). Since mitochondrial dysfunction is impaired it seems cells in older individuals must produce more energy through non-oxidative metabolism. A recent study showed that aged mice had increased lactate and reduced glycolytic intermediates which suggest an increased reliance on anaerobic glycolysis \(^91\). Aging also alters monocyte phenotype and function \(^37\). As we age, circulating monocyte levels increase and they are more likely to enter circulation in an immature state \(^37\). Furthermore, monocytes from older individuals produce more pro-inflammatory cytokines (such as TNF and IL-6) in response to activation than monocytes from younger individuals \(^37\). Many studies have shown that older individuals have higher levels of TNF, IL-6 and C-reactive protein in their blood \(^92\) and that this chronic low-level systemic inflammation is associated with many diseases \(^93\).

Whether or not mitochondrial dysregulation is a primary cause of age-related monocyte dysfunction has yet to be determined; though, we have recently provided evidence that aging impairs maximal mitochondrial respiratory capacity in human classical monocytes \(^55\). In the
deprivation of glucose (as is often the case in tissues with inflammation), LPS activated monocytes are able to switch from aerobic glycolysis to OXPHOS (fueled by fatty acid oxidation) and continue to perform their functions (such as phagocytosis, cytokine production, and chemotactic migrations) \(^{81}\). This switch is regulated by AMP-activated protein kinase (AMPK) which stimulates catabolic pathways including fatty acid oxidation, glucose uptake, autophagy, and mitochondrial biogenesis \(^{81}\). The only effect this switch had on monocyte effector function was a decrease in NADPH oxidase-dependent respiratory burst as a result of AMPK activation \(^{81}\). Respiratory bursts generate ROS that are used to degrade pathogens, but which can also cause damage to the monocytes themselves \(^{94}\). Interestingly, ROS are associated with mtDNA mutations \(^{90}\), and may be involved in the expression of pro-inflammatory genes \(^{11}\), so increased ROS due to metabolic inflexibility may play a role in inflamm-aging as well. The switch to OXPHOS was also accompanied by increased levels of autophagy, presumably to compensate for nutrients generated by the pentose-phosphate pathway \(^{81}\). Among the many important functions of autophagy are cell-homeostasis \(^{95}\), balancing the beneficial and detrimental effects of immunity and inflammation \(^{95}\), promotion of longevity \(^{96}\), and monocyte to macrophage differentiation \(^{97}\). Decreased autphagic activity is also associated with age \(^{98}\) and decreased mitochondrial efficiency \(^{99}\) and may contribute to a number of age-associated pathologies \(^{96}\). So diminished autophagic activity may play a role in age-related monocyte dysfunction as well, although future studies need to confirm this.

In conclusion, metabolic flexibility is crucial to the function and phenotype of monocytes and other immune cells. In response to LPS, monocytes shift toward using aerobic glycolysis but are metabolically flexible enough to use OXPHOS in conditions with limited glucose availability. Furthermore, immune cells change their metabolism according to their function.
Anti-inflammatory phenotypes have been shown to be more associated with OXPHOS, while pro-inflammatory phenotypes are more associated with aerobic glycolysis. OXPHOS takes place in the mitochondria, so any impairment in mitochondria is presumed to effect processes involving OXPHOS. Aging has a detrimental effect on both mitochondria and monocyte function. The mitochondria’s ability to produce ATP is reduced, and the proportion of non-classical and intermediate monocyte phenotypes are increased (which produce inflammatory cytokines). It’s unclear if the diminished mitochondrial capacity seen with aging is a primary cause for altered monocyte phenotype and function. Although, it’s feasible that reduced metabolic flexibility in aged monocytes contributes to a more pro-inflammatory phenotype and is thus a possible contributor to immunosenescence and inflam-aging. An improved understanding of the role mitochondrial function and metabolic flexibility play in monocytes in age-related inflammation can lead to new discoveries that help improve the lives of the aging population.

**Hypothesis**

We hypothesized that classical monocytes taken from aged individuals would have reduced metabolic flexibility compared to young individuals due to diminished mitochondrial function. Specifically, we believed aged monocytes would have reduced ability to upregulate oxidative metabolism when glucose is unavailable. Furthermore, we expected aged monocytes to have impaired functions including diminished phagocytic activity and altered cytokine expression.
Methods

Participants

To determine the effects aging has on monocyte mitochondrial flexibility two different groups were recruited: aged and young. Males and females between the ages of 18-35 were recruited for the young group, which consisted of 11 subjects total. Males and females between the ages of 60-80 were recruited for the aged group, which consisted of 9 subjects total. Demographic and Anthropometric data of the subjects can be seen in Table 1. All subjects were recruited from the surrounding Memphis area via word-of-mouth, email, or flyers. Exclusion criteria included subjects who had diagnosed conditions that can affect metabolic or immune function. This includes obesity (BMI >30), cardiovascular disease, diabetes, hypertension, chronic fatigue syndrome, mitochondrial diseases, autoimmune diseases, etc. All subjects completed a questionnaire to determine eligibility, which asked for health history, list of medications and supplements, major illnesses or hospitalizations within the last two years, and exercise type/frequency. Subjects visited the lab in a fasted state, up to 6 times, and had 8-16 mL of blood taken by venipuncture into 1-2 EDTA vacutainer tubes per visit for monocyte isolation.

Monocyte Isolation and Metabolic Flexibility Assay

CD14+ monocytes were isolated from whole blood via magnetic sorting by negative selection using Stemcell Technologies’ EasySep Direct Human Monocyte Isolation Kit. CD16+ monocytes were excluded to prevent bias as monocyte subset proportions change with age. Therefore, in this study we only looked at classical monocytes. After isolation the monocytes were counted using EMD Millipore’s Scepter 2.0 cell counter for use in downstream assays. The Agilent Seahorse XFp Analyzer was used to test the metabolic flexibility of the isolated classical
monocytes. The Seahorse analyzer measures the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of the sample. OCR is an indicator of OXPHOS and ECAR is an indicator of glycolysis. Monocytes were seeded into the Seahorse plates at $1.5 \times 10^5$ cells per well (B-G) along with media (DMEM, pyruvate, glutamine), and either glucose or no glucose. 20µL of 100ng/mL LPS was added to the injection port of three wells containing glucose (B-D), three wells without glucose (E-G), and two wells (A, H) consisting of no cells, but media, which are used as blanks. The Seahorse plates were incubated for 60 minutes at 37ºC in a non-CO$_2$ incubator to de-gas the plate. The Seahorse machine was then run for 160 minutes to measure the acute response in OCR and ECAR of the monocytes activated by LPS in media with or without glucose. This data was used to compare the metabolic flexibility of isolated monocytes between the aged and young groups. Wells B-G of the Seahorse plate were imaged using a microscope at 10x magnification to confirm cell adherence and for use in cell number normalization when doing data analysis. After completion, wells B-G received 100µg of TRIzol, separated according to group (+ or – glucose) and were stored in a -80ºC freezer for later RNA quantification using Real-Time polymerase chain reaction (qPCR).

**Phagocytosis Assay**

To test potential differences in monocyte effector function between young and aged CD14+ CD16- monocytes a phagocytosis assay was used in the presence and absence of glucose. The assay uses latex beads coated with fluorescently labeled IgG to quantify phagocytosis in vitro and was measured using the Attune Nxt flow cytometer. Monocytes were isolated as previously mentioned and added to 2 2mL tubes at a concentration of $5 \times 10^5$ cells. The 2 groups consisted of media ((DMEM, pyruvate, glutamine) without glucose, and LPS + media without glucose. After 30 minutes of incubation at 37ºC with 5% CO$_2$, 1µL phagocytotic beads was
added. After another hour of incubation 20μL anti-CD14-PE antibody was added. After another 30 minutes of incubation 500μL of PBS was added, spun, and the supernatant aspirated. The cells were then resuspended in 400μL PBS and analyzed with the Attune Nxt flow cytometer to determine the mean fluorescence intensity (MFI) and percentage of beads (% gated) phagocytosed by CD14+CD16- monocytes.

**Cytokine Expression Quantification using qPCR**

To test differences in gene expression of 4 cytokines (IL-1β, IL-6, TNF-α, IL-10), qPCR was performed. Monocyte lysates dissolved in 100μg TRizol from previously performed Seahorse assays were thawed. RNA was isolated from CD14+CD16- monocyte lysates in TRizol according to the protocol (adapted from TRI Reagent protocol which uses BCP, ethanol, and isopropyl alcohol as reagents). After RNA isolation, RNA purity (A260/A280) and concentration (in ng/mL) were determined using the Thermo Scientific NanoDrop Lite Spectrophotometer. cDNA was then made from 0.5μg RNA using the Thermo Scientific High-Capacity cDNA reverse transcription kit on the Thermo Scientific SimpliAmp thermal cycler. qPCR was then performed using TaqMan Fast Advanced Master Mix with the Thermo Scientific QuantStudio 6 system. Relative expression levels were calculated using the comparative C_T method using β2 microglobulin (B2M) as a control. B2M was picked as a control as there is evidence suggesting it’s the most stable reference gene in LPS stimulated monocytes.
Statistical Analysis

Statistical analysis was performed using R software (R v. 3.5.1). Categorical demographic data was analyzed by chi-square test (sex, race). Continuous demographic and anthropometric data (age, height, weight, body mass index) were analyzed by independent-samples t-test between young and older subjects. For metabolic parameters all data followed a normal distribution according to Shapiro-Wilk tests. However, only difference in OCR (max-min) between groups had equal variances according to Levene’s test. Therefore independent-samples t-test was only used to calculate difference in OCR for metabolic parameters. Due to the unequal variances for the remaining parameters (max OCR, min OCR, area under the curve (AUC) kinetic OCR response) Mann-Whitney U tests were performed to test for significance between groups.

For cytokine gene expression data at least 1 group for all genes did not meet the criteria for approximating normal distribution according to Shapiro-Wilk test, although all genes displayed equal variance between groups according to Levene’s test. Therefore, Mann-Whitney U tests were performed to test for significance between groups. For phagocytosis, all data met criteria for normal distribution and equality of variances according to Shapiro-Wilk and Levene’s test. Between group differences were determined by 2x2 (Condition x Group) ANOVA. As is standard, a p value < 0.05 was considered significant. Reported results are mean ± SEM.
Results

Subject Characteristics

Subject demographics and anthropometric data are shown in Table 1. Medication use is shown in Table 2. There was a total of 9 subjects in the aged group and 11 subjects in the young group. Besides age, the two groups did not differ significantly on other demographic or anthropometric data.

Metabolic Flexibility Assay, Cytokine Expression, and Phagocytic Capacity

Aging had no significant main effects on any of the calculated measures for metabolic flexibility, cytokine expression, or phagocytic capacity in CD14+CD16- monocytes. OCR response to LPS in glucose-deprived monocytes was slightly higher across all timepoints in the aged group, though this difference wasn’t significant (Fig. 1a). ECAR Response to LPS in glucose deprived monocytes also showed no difference between groups (Fig. 1b). Aging had no effect on any of the calculated oxidative metabolic parameters (Fig. 1c & d) including maximum OCR (p = 0.3312), minimum OCR (p = 0.3702), the difference between them (p = 0.2206), and OCR kinetics as measured by AUC (p = 0.3312). Aging also had no significant effects on measures of cytokine expression in glucose-deprived classical monocytes in response to LPS (Fig. 1e) for IL-10 (p = 0.5675), IL-6 (p = 0.8421), TNF-α (p = .9048), or IL-1β (p = 0.7802).

Phagocytic capacity, as measured by percentage gated, was tested in glucose-deprived classical monocytes (Fig. 2). An example of the gating strategy used to isolate classical monocytes is shown in Fig. 2a, and an example of the gating strategy used to calculate percentage positive for beads phagocytosed is shown in Fig. 2b. Aging had no effect on the mean fluorescence intensity (MFI) in LPS-stimulated glucose-deprived classical monocytes (p =
.748, Fig. 2c), or in the MFI of unstimulated glucose-deprived monocytes (p = 0.1396, Fig. 2c).

Similarly, aging had no effect on percent of cells in the positive gate (Fig. 2d) in LPS-stimulated (p = 0.2340) or unstimulated (p = 0.6738) glucose-deprived classical monocytes. There was a significant main effect whereby LPS stimulation increased MFI compared to condition within-subjects (p = 0.00301, not shown). Likewise, LPS-stimulation caused a near significant increase in percent of cells in the positive gate compared to condition within-subjects (p = 0.0531, not shown).

Table 1: Demographic and Anthropometric Characteristics of Subjects
Yr, year; cm, centimeters; kg, kilograms; BMI, body mass index; N, number of subjects

<table>
<thead>
<tr>
<th>Age, yr. (range)</th>
<th>Aged (N = 9)</th>
<th>Young (N = 11)</th>
<th>Prob</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height, cm (range)</td>
<td>66.6 ± 1.4 (60–72)</td>
<td>27.5 ± 1.3 (20–34)</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Weight, kg (range)</td>
<td>173.8 ± 3.0 (163.0–191.0)</td>
<td>169.8 ± 3.2 (155.0–191.0)</td>
<td>p = 0.375</td>
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<tr>
<td>BMI, kg/m² (range)</td>
<td>77.3 ± 4.0 (54.4–99.8)</td>
<td>74.8 ± 7.5 (43.4–133.8)</td>
<td>p = 0.789</td>
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<td>Female, N (%)</td>
<td>4 (44)</td>
<td>4 (36)</td>
<td>p = 0.714</td>
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<td>White, N (%)</td>
<td>7 (78)</td>
<td>4 (36)</td>
<td></td>
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<td>Black, N (%)</td>
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<td>2 (18)</td>
<td>Race: p = 0.059</td>
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<td>Asian, N (%)</td>
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<td>5 (45)</td>
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</tr>
</tbody>
</table>

Table 2: Medication Use in Subjects
Numbers denote number of subjects reporting use of medications in the listed group

<table>
<thead>
<tr>
<th>Medication</th>
<th>Young</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td>Statins</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Metformin</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Reflux</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Thyroid</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Allergy</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Vitamin</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Birth Control</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Seizure</td>
<td>1</td>
<td>0</td>
</tr>
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</table>
Figure 1: Metabolic Responses to LPS in Glucose-Deprived CD14+CD16- Monocytes

Figure 2: Phagocytic Activity in CD14+CD16- Glucose-Deprived Monocytes

A) Example of Monocyte Gating Strategy. Top right graph shows percentage of CD14+ cells within gate. B) Example of percent positive for beads phagocytosed by CD14+ cells. C) Mean fluorescence intensity in LPS stimulated, or media only CD14+ monocytes in older and younger subjects. D) Percent positive for beads by LPS stimulated, or media only CD14+ monocytes in older and younger subjects.
Discussion

Monocytes switch from oxidative phosphorylation to aerobic glycolysis when activated by LPS to carry out their effector functions, which is dependent on the availability of glucose and glutamine. Monocytes routinely experience a variety of different microenvironments and must be metabolically flexible to retain their functions. In circulation glucose is readily available, but in areas with inflammation glucose is often drastically reduced, and monocytes must compensate through catabolic processes such as fatty acid oxidation and autophagy. AMPK is the critical regulator orchestrating this metabolic switch, which provides fatty acids to the mitochondria through downstream effects. Decreased mitochondrial function is known to occur in many cell types with age, and we have previously provided evidence for reduced mitochondrial function in classical monocytes with aging—namely that aging impairs mitochondrial maximal respiration and spare capacity.

We hypothesized that classical monocytes from aged individuals would have reduced ability to upregulate oxidative metabolism when glucose is unavailable - due to decreased mitochondrial function, and that this inflexibility would skew them towards a more pro-inflammatory state and hamper their ability to perform phagocytosis. In this study, we show that aging has no effect on the ability of glucose-deprived classical monocytes to upregulate oxidative metabolism, and has no effect on the expression of IL-10, IL-6, TNF-α, or IL-1β during acute LPS activation ex vivo. Additionally, we found aging has no effect on ex vivo phagocytic capacity in classical monocytes, regardless of the availability of glucose or whether they were activated by LPS. While these results are unexpected, they provide important information regarding the effects aging has on monocyte function. Aging in of itself, does not
appear to worsen mitochondrial function in classical monocytes ex vivo to a degree that worsens their ability to compensate for a lack of glucose and carry out some of their effector functions.

While there is an ever-growing body of evidence supporting age-related changes in monocyte and macrophage functions, it hasn’t been clear whether this is due to intrinsic changes in the monocytes themselves, or due to changes in circulating factors that alter gene expression. This study gives more credibility to the assumption that classical monocytes, at least in this specific context of glucose deprivation and LPS activation, are not experiencing intrinsic changes to a degree which effect their functions. If it turns out their metabolic flexibility is hindered in vivo, it’s likely a result of inflammaging and all the changes that accompany it.

There are several other potential reasons why we observed no differences in this study, which is likely not translatable to monocyte function in vivo. We previously corroborated evidence that alterations in monocyte subset proportions occur with age. CD14+CD16- classical monocytes are reduced, while CD14+CD16+ intermediate and CD14dimCD16+ nonclassical monocytes are increased with age. Our monocyte isolation technique removes CD16+ monocytes and thus only included the classical monocyte subset. This was done to prevent biased results, as CD16+ monocytes have generally shown to be more pro-inflammatory and are more prone to senescence. Furthermore, monocyte subsets have been shown to display varying responses to LPS. Isolating all monocyte subsets therefore may have yielded different results, and it would be interesting to see a similar experiment with respect to metabolic flexibility performed with CD16+ monocytes only, to see if there are intrinsic changes in CD16+ monocytes with age.

Monocytes also have varying responses to different types of pattern recognition receptors (PRR). Activation by Pam3CysSK4, a TLR2 agonist, shows significant differences in TCA
cycle, OXPHOS, and lipid metabolism compared to activation by LPS in glucose-fueled monocytes. Therefore, activation of PRR’s other than TLR4 may have given different results.

We recently showed aging has no effect on glycolytic metabolism ex vivo in LPS-stimulated classical monocytes, nor in acute cytokine expression. However, we showed there was a significant difference with age for IL-6 expression at 24 hours post-LPS stimulation, and near significant differences for IL-1β (p = 0.052) and IL-10 (p = 0.07), such that aged individuals had reduced expression of these genes. Similarly, if we had looked at later cytokine expression in this study we may have seen significant differences between groups for some calculated measures.

Limitations

Subjects from both cohorts reported using medications which could affect metabolic outcomes (Table 2). Most importantly, 2 subjects from the aged group reported using statins, and 1 person from the aged group, who hasn’t been diagnosed with diabetes, reported using both Metformin and thyroid medication. Statins have been shown to have immunosuppressive effects and may affect metabolic function. Metformin is a potent AMPK agonist, and has been shown to improve metabolic parameters in peripheral blood mononuclear cells. 2 subjects from both cohorts reported using allergy medications, which have immunomodulatory properties. We are underpowered to statistically detect differences due to medication use in this study, although these medications appeared to have little effect on our study parameters upon brief analysis. ~91% of adults aged 65+ take at least 1 prescription medication, and ~67% take 3 or more. Therefore, finding older adults who are not on some type of medication is challenging, and regardless we propose our subject pool is representative of the typical aged population. One of the biggest problems with using human subjects in general is the extreme
variability between subjects. Genetics and lifestyle factors can play a huge role in immune and metabolic function, and it is impossible to separate all the potential covariates. Confirming these results using isogenic rodents could address this limitation.

**Conclusions**

In summary, we showed that aging has no effect on the ability of ex vivo LPS-stimulated classical monocytes to compensate for a lack of glucose by upregulating oxidative metabolism. Furthermore, aging has no effect on cytokine expression in ex vivo LPS-stimulated glucose-deprived classical monocytes for IL-10, IL-6, TNF-α, or IL-1β. Aging also had no effect on the phagocytic ability of classical monocytes under various conditions.

**Future Directions**

As it appears that monocytes are influenced more so by their immediate environment than by intrinsic changes that occur with age, future studies should look at monocyte function in vivo, or in culture which contains conditioned media. Also, we only looked at a few factors related to monocyte function. Looking at other factors such as ROS production, chemotaxis, large-scale gene analysis, metabolites, etc. would give more of a complete picture of the changes that are occurring in glucose-deprived monocytes with age.
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