Classical monocytes from older adults maintain capacity for metabolic compensation during glucose deprivation and lipopolysaccharide stimulation

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ARTICLE INFO

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 classical monocytes taken from aged individuals would have an impaired ability to upregulate oxidative metabolism along with impaired effector functions. Aging did not impair LPS-induced oxygen consumption rate during glucose deprivation as measured on a Seahorse XFp system. Additionally, aged classical monocytes maintained inflammatory gene expression responses and phagocytic capacity during LPS stimulation in the absence of glucose. In conclusion, aged classical monocytes maintain effector and metabolic functions during glucose deprivation, at least in an ex vivo context.

1. Introduction

The number of Americans over the age of 65 is expected to increase by approximately 80 million people by 2040 (Colby and Ortman, 2015). 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 (Azhar and Wei, 2015). Aging is the highest risk factor for the majority of chronic diseases - including cardiovascular disease, diabetes, arthritis, and cancer (Kennedy et al., 2014). 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 (Chatterji et al., 2015). 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 (Kennedy et al., 2014; Jacob et al., 2016).

Aging is associated with chronic, low-level, systemic inflammation (termed inflammaging) that contributes to most, if not all, age-related diseases (Franceschi and Campisi, 2014). Older adults have higher serum levels of several pro-inflammatory cytokines/proteins such as IL-6, IL-1β, C-reactive protein (CRP), and TNFα (Salvioli et al., 2006). Elevated levels of these molecules in circulation, most notably IL-6, are correlated with an increased risk of morbidity and mortality in elderly populations (Franceschi et al., 2017). Furthermore, they are associated with sarcopenia, malnutrition, arthritis, atherosclerosis, cognitive decline, and other diseases of aging (Michaud et al., 2013). There is no consensus on the causes of inflammaging, though it is 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 (Lopez-Otin et al., 2013).

Monocytes are circulating mononuclear phagocytes of the innate immune system that have a diverse set of functions and play an essential role in the defense against a variety of pathogens (Serbina et al., 2008). Although aging is multi-factorial, monocytes may play an essential role in aging pathology. Their heterogenous and highly

Table 1
Demographic and anthropometric characteristics of subjects.

Characteristics	Aged (n = 9)	Young (n = 11)	Probability
Age, yr. (range)	66.6 ± 1.4 (60–72)	27.5 ± 1.3 (20–34)	p < 0.001
Height, cm (range)	173.8 ± 3.0 (163.0–191.0)	169.8 ± 3.2 (155.0–191.0)	p = 0.375
Weight, kg (range)	77.3 ± 4.0 (54.4–99.8)	74.8 ± 7.5 (43.4–133.8)	p = 0.789
BMI, kg/m^2 (range)	25.6 ± 1.2 (18.8–31.6)	25.7 ± 2.0 (17.4–36.7)	p = 0.980
Female, N (%)	4 (44)	4 (36)	p = 0.714
White, N (%)	7 (78)	4 (36)	
Black, N (%)	2 (22)	2 (18)	Race: p = 0.059
Asian, N (%)	0 (0)	5 (45)	

yr, year; cm, centimeters; kg, kilograms; BMI, body mass index; N, number of subjects.

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, diminished ability to activate the adaptive immune system, altered receptor expression, cytokine production, and subset proportions (Albright et al., 2016; Pence and Yarbro, 2018).

In response to activation, immune cells regularly change their functions dramatically. For example, monocytes activated by lipopolysaccharide (LPS) undergo vigorous cellular growth and an increased demand for the production of proteins, lipids, cytokines, and reactive oxygen species (ROS) (Raulien et al., 2017; Marsin et al., 2002). This increased demand for biomolecules is associated with upregulation of glycolysis and downregulation of oxidative phosphorylation (OXPHOS), similar to the Warburg effect in cancer cells (Rodriguez-Prados et al., 2010). A shift toward aerobic glycolysis is associated with pro-inflammatory phenotypes in immune cells such as dendritic cells activated by LPS (Krawczyk et al., 2010), M1 macrophages, and effector T cells (O'Neill et al., 2016). Oxidative metabolism (with limited rates of glycolysis) is associated with anti-inflammatory phenotypes as in quiescent memory T-cells, regulatory T-cells, and M2 macrophages (O'Neill et al., 2016). 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 (Ravi et al., 2014). Inhibition of OXPHOS in macrophages inhibits the expression of the M2 anti-inflammatory phenotype (Vats et al., 2006). 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 (Payne and Chinnery, 2015). 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 (Payne and Chinnery, 2015). Since mitochondrial dysfunction is impaired it is reasonable to assume that many cell types in older individuals must produce more energy through non-oxidative metabolism. Aged mice have been shown to have increased lactate and reduced glycolytic intermediates in muscle and liver tissue which suggest an increased reliance on anaerobic glycolysis (Houtkooper et al., 2011). Whether or not mitochondrial dysregulation is a primary cause of age-related monocyte dysfunction has yet to be determined, though we've recently provided evidence that aging impairs maximal and spare mitochondrial respiratory capacity in classical monocytes isolated from humans (Pence and Yarbro, 2018).

Raulien and colleagues recently showed that in the deprivation of glucose (as is often the case in tissues with inflammation) (Balbir-Gurman et al., 2006; Chow and Troy, 2014), LPS-stimulated monocytes are able to switch from aerobic glycolysis to OXPHOS while maintaining their effector functions (Raulien et al., 2017). This switch is regulated by AMP-activated protein kinase (AMPK) which stimulates

catabolic pathways including fatty acid oxidation, autophagy, and mitochondrial biogenesis (Raulien et al., 2017). Since we have previously demonstrated mitochondrial dysfunction in monocytes (Pence and Yarbro, 2018), we hypothesized that aging would result in a reduced ability to upregulate oxidative metabolism when glucose in unavailable following LPS-stimulation and that this would be associated with reduced inflammation and phagocytic capacity.

2. Methods

2.1. Participants

This study was approved by and conducted in accordance with the Institutional Review Board at the University of Memphis (protocol #4361). All subjects completed a written informed consent document. 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.

2.2. Monocyte isolation and metabolic flexibility assay

CD14 + CD16- classical monocytes were isolated from whole blood via magnetic sorting by negative selection using Stemcell Technologies' EasySep Direct Human Monocyte Isolation Kit. CD16 + intermediate and nonclassical monocytes were excluded to prevent bias as monocyte subset proportions change with age (Pence and Yarbro, 2018). After isolation the classical 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 freshly isolated classical monocytes. The Seahorse analyzer measures the oxygen consumption rate (OCR) and extra-cellular acidification rate (ECAR) of the sample. OCR is an indicator of OXPHOS and ECAR is an indicator of glycolysis. 50 μL of monocytes were seeded into the Seahorse plates at 1.5×10^5 cells per well (B–G) along with 130 μL XF media (DMEM, pyruvate, glutamine), and either glucose or no glucose. 20 μL of 100 ng/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 XF media, which are used as blanks. After injection, the final concentration of LPS was 10 ng/mL.

The Seahorse plates were incubated for 60 min at 37 °C in a non − CO_2 incubator to de-gas the plate. The Seahorse machine was then run for 150 min (30 min prior to LPS injection and 120 min post-LPS injection) to measure the acute response in OCR and ECAR of the monocytes activated by LPS in media with or without glucose. This protocol was adapted from a previous report (Raulien et al., 2017). The difference between pre- and maximal post-LPS metabolic response was compared between aged and young to determine whether aging affected the magnitude of the response. To determine differences in the kinetic response area under the curve (AUC) was calculated by the trapezoidal method. 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 100 μL of TRIzol, pooled by group (+ or - glucose) and were stored in a − 80 °C freezer for later RNA quantification using Real-Time polymerase chain reaction (qPCR). A detailed Seahorse protocol is available on protocols.io (Pence, 2018), which was modified in this case to omit glucose in the media.

2.3. Phagocytosis assay

To test potential differences in monocyte effector function between young and aged classical 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. Classical monocytes were isolated as above and added to 2 mL tubes at a concentration of 5×10^5 cells. The 2 groups consisted of XF media without glucose, and LPS + XF media without glucose. After 30 min of incubation at 37 °C with 5% CO_2, 1 μL phagocytotic beads was added. After another hour of incubation 20 μL anti-CD14-PE antibody was added. After another 30 min of incubation the cells were washed with PBS and resuspended in 400 μL PBS then analyzed with the Attune Nxt flow cytometer to determine the mean fluorescence intensity (MFI, the average amount of beads phagocytosed) and percentage of beads (% gated) phagocytosed by classical monocytes.

2.4. Cytokine expression quantification using qPCR

To test differences in gene expression of 4 cytokines (IL-1β, IL-6, TNFα, IL-10), qPCR was performed on monocyte lysates from the Seahorse assay as we have previously described (Pence and Yarbro, 2019). Relative expression levels were calculated using the comparative CT 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 (Piehler et al., 2010).

2.5. 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 Shaprio-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 Shaprio-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 2×2 (Condition x Group) ANOVA. As is standard, a p value < 0.05 was considered significant. Reported results are mean ± SEM.

3. Results

3.1. Data availability

The datasets and analytical scripts supporting the conclusions of this article are available in the FigShare repository (Pence, 2019).

3.2. Subject characteristics

Subject demographics and anthropometric data are shown in Table 1. 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. All subjects are the same individuals as those reported in our previous paper as cohort 2 (Pence and Yarbro, 2019).

3.3. Metabolic flexibility assay, cytokine expression, and phagocytic capacity

To determine the effect aging had on the ability to upregulate oxidative metabolism in glucose-deprived classical monocytes after acute (10 ng/mL) LPS-stimulation, we performed a Seahorse assay adapted from a previous report (Raulien et al., 2017). Aging had no significant effects on any of the calculated measures for metabolic flexibility, cytokine expression, or phagocytic capacity in CD14 + CD16- monocytes. The oxygen consumption rate (OCR) — an indicator of aerobic respiration through OXPHOS — response to LPS in glucose-deprived monocytes was slightly higher across all timepoints in the aged group, though this difference was not significant (Fig. 1a). The extracellular acidification rate (ECAR) — an indicator of glycolysis — response to LPS in glucose-deprived monocytes also showed no significant difference between groups (Fig. 1b). Aging had no significant 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 area under the curve (AUC) (p = 0.3312). For reference purposes for readers unfamiliar with Seahorse assays, we have also included representative results from an LPS response assay including glucose, demonstrating that OCR is decreased (Fig. 1e) and ECAR is increased (Fig. 1f) following LPS stimulation if glucose is present. Aging also had no significant effects on measures of cytokine expression in monocytes in response to LPS (Fig. 1g) for IL-10 (p = 0.5675), IL-6 (p = 0.8421), TNFα (p = 0.9048), or IL-1β (p = 0.7802) at 2 h post-stimulation.

Phagocytic capacity, as measured by percentage in the positive gate, was tested in glucose-deprived classical monocytes (Fig. 2). An example of the gating strategies used to isolate classical monocytes (Fig. 2a) and to calculate percentage positive for beads (Fig. 2b) are shown. Aging had no significant effect on the mean fluorescence intensity (MFI) in LPS-stimulated (p = 0.748, Fig. 2c) or unstimulated (p = 0.1396, Fig. 2c) glucose-deprived classical monocytes. Similarly, aging had no significant effect on percentage of cells in the positive gate (Fig. 2d) in LPS-stimulated (p = 0.234) or unstimulated (p = 0.674) glucose-deprived classical monocytes. There was a significant main effect whereby LPS stimulation increased MFI (p = 0.003). Likewise, there was a near-significant main effect of LPS stimulation on percentage of cells in the positive gate (p = 0.053).
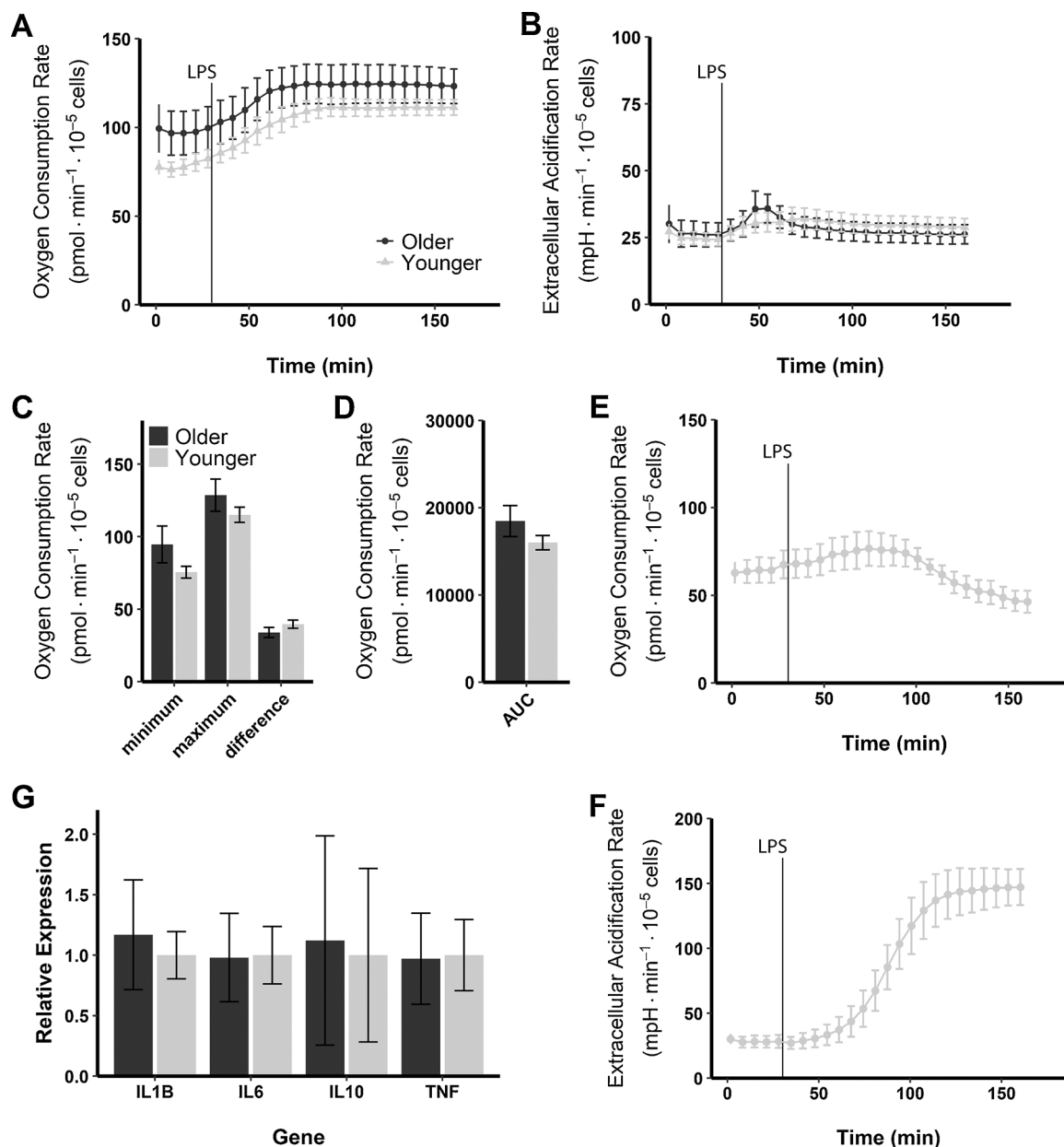
Fig. 1. Metabolic responses to acute (2 h, 10 ng/mL) LPS-stimulation in glucose-deprived CD14+CD16- classical monocytes. (A). Oxygen consumption response (OCR) to LPS in older and younger subjects. (B). Glycolytic response to LPS. (C). Calculated values for respiratory parameters. (D). Kinetic OCR response (AUC: area under the curve). (E) Representative Seahorse assay from one young subject, showing the OCR response to LPS if glucose is available. (F). Representative ECAR response to LPS if glucose is available. (G). Cytokine gene expression.

4. 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 (Raulien et al., 2017). AMPK is the critical regulator orchestrating this metabolic switch, which provides fatty acids to the mitochondria through downstream effects (Ravi et al., 2014). Decreased mitochondrial function is known to occur in many cell types with age (Payne and Chinnery, 2015), 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 (Pence and Yarbro, 2018).

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 impair their inflammatory responses and hamper their ability to perform phagocytosis. In this study, we report no significant differences between aged and young groups in any of the calculated effector or metabolic functions in LPS-stimulated glucose-deprived classical monocytes. There were no significant differences in the ability of glucose-deprived classical monocytes to upregulate oxidative metabolism, or in the expression of IL-10, IL-6, TNFα, or IL-1β during acute LPS activation ex vivo. Additionally, we found aging has no significant effect on ex vivo phagocytic capacity in classical monocytes, regardless of the availability of glucose or whether they were activated by LPS. The differences in mitochondrial respiration in aged
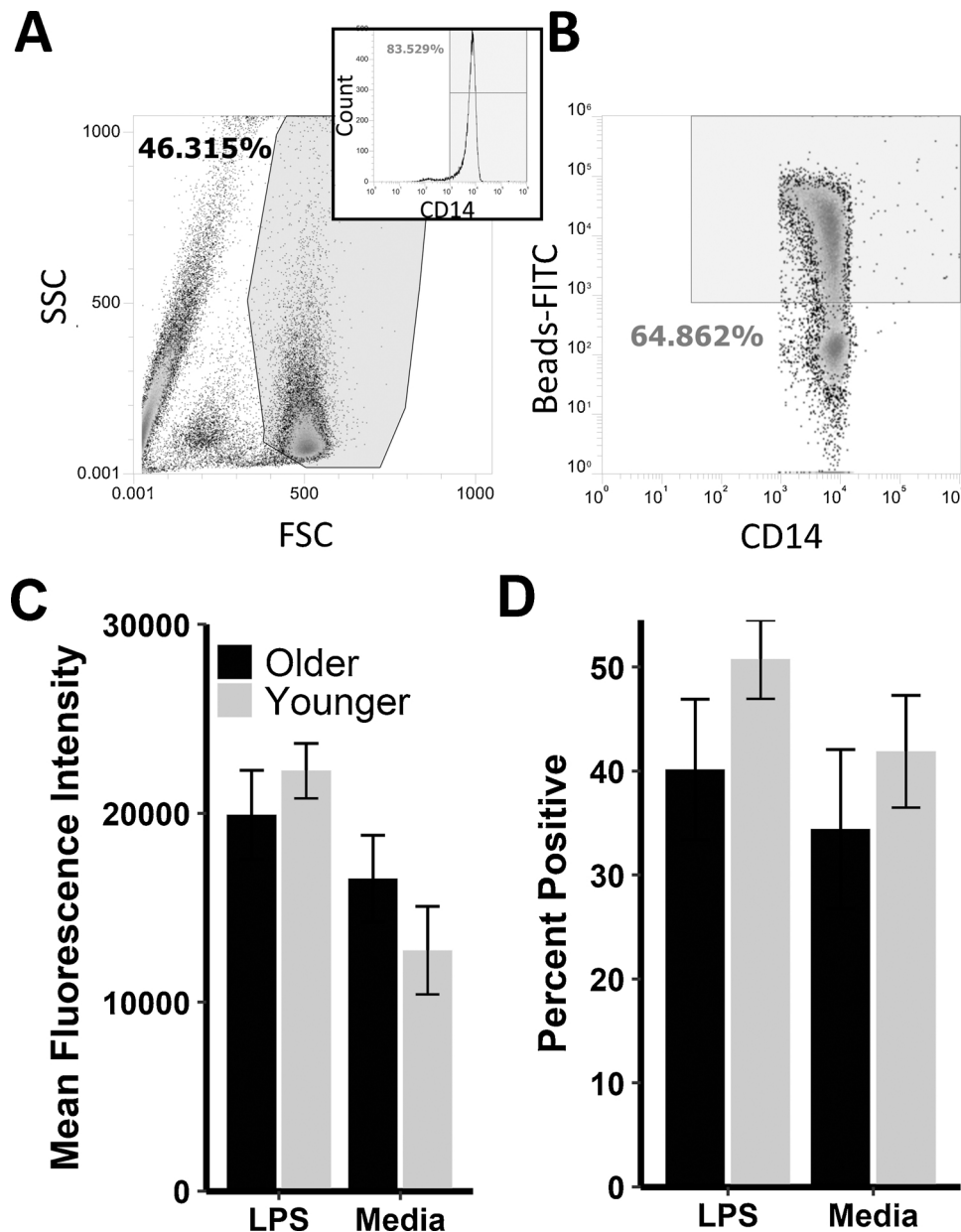
Fig. 2. Phagocytic activity in glucose-deprived CD14 + CD16- classical monocytes. (A). Example of monocyte gating strategy. Top right graph shows percentage of CD14 + cells within gate. (B). Example of percentage positive for beads phagocytosed by CD14 + cells. (C). Mean fluorescence intensity in acute (10 ng/ml) LPS-stimulated, or media only classical monocytes in older and younger subjects. (D). Percentage positive for beads by acute LPS-stimulated, or media only classical monocytes in older and younger subjects.

monocytes that we previously reported (Pence and Yarbro, 2018) were only under maximizing conditions (via stimulation by the OXPHOS uncoupler FCCP, which increases OCR to maximum), and thus normal conditions are likely not severe enough to cause any noticeable impairments in mitochondrial metabolism.

There are several potential reasons why we observed no differences in this study. We previously corroborated evidence that alterations in monocyte subset proportions occur with age (Pence and Yarbro, 2018). 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 (Ong et al., 2018). Furthermore, monocyte subsets have been shown to display varying responses to LPS (Aguilar-Ruiz et al., 2011).

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 (Lachmandas et al., 2016). Therefore, activation of PRRs other than TLR4 may have given different results. Additionally, in this study we assessed only gene expression at 2 h post-LPS stimulation. Gene expression at additional time points, or characterization of protein expression, may yield additional information. For the phagocytosis assay, FITC-conjugated latex beads may in some instances stick to the outside of the cells, giving a false positive reading even though the particle has not been phagocytosed. The use of pH sensitive fluorescent reporters

such as pHrodo bacterial particles may therefore give different results.

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

Funding

Availability of data and materials

The datasets and analytical scripts generated and analyzed during the current study are available in the FigShare repository, https://doi.org/10.6084/m9.figshare.c.4499219.

Author contributions statement

BP conceived and designed the study. BP and JY collected and analyzed the data. JY drafted the manuscript. BP and JY revised the manuscript drafts. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All study activities were approved by the Institutional Review Board at the University of Memphis (protocol #4361). Subjects provided informed consent and were free to withdraw at any time.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Acknowledgements

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